

Treatment of interstitial cystitis with intravesical instillation of recombinant human collagen type III

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Background: Interstitial cystitis (IC) is a chronic condition characterized by frequent urination, urgency, and pelvic pain. While its pathogenesis remains incompletely understood, the prevailing epithelial theory suggests that a defective glycosaminoglycan (GAG) layer increases bladder permeability, allowing urinary toxins to chronically stimulate the detrusor muscle and activate mast cells. Current therapies, such as intravesical hyaluronic acid instillation, have demonstrated limited efficacy. This study aimed to evaluate the feasibility of sterile recombinant human collagen type III (rHCIII) in mitigating bladder injury and its histological effects on urothelial integrity.

Method: An IC rat model was established using protamine sulfate and lipopolysaccharide. The model was subsequently treated with sterile rHCIII via bladder instillation. Bladder tissues were analyzed using Hematoxylin-eosin (H&E) staining for general histopathology, Masson and van Gieson staining for collagen fiber organization, and Toluidine blue (TB) staining for mast cell infiltration. Apoptosis was assessed

by Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling (TUNEL) staining and immunohistochemistry for Caspase-3. The localization of the perfused rHCIII was tracked using immunohistochemistry for a 6×Histidine (HIS) tag.

Result: Immunohistochemistry confirmed the presence of rHCIII in the bladder mucosal and submucosal layers. Treatment with rHCIII significantly reduced bladder cell apoptosis compared to the IC group ($p < 0.001$). Furthermore, the collagen fibers in the submucosal and muscular layers demonstrated a more compact and organized arrangement post-treatment. Mast cell infiltration was also markedly diminished following rHCIII instillation ($p < 0.001$).

Conclusion: Intravesical instillation of sterile rHCIII mitigates apoptosis of mucosal epithelial cells and reinforces the submucosal collagen structure. This intervention promotes a more organized tissue architecture, which may help maintain urothelial structural integrity. These findings provide preclinical evidence supporting the repair-oriented potential of rHCIII instillation for interstitial cystitis.

Key Words: interstitial cystitis, recombinant human collagen type 3, bladder perfusion, mast cells

Introduction

Interstitial cystitis (IC), commonly referred to as bladder pain syndrome (BPS), is a chronic condition defined by chronic pelvic pain and lower urinary tract symptoms such as urgency, frequency, and nocturia. It may also present with systemic manifestations,

suggesting a potential multi-organ involvement.^{1,2} IC demonstrates a striking female predominance, with a global incidence in women estimated at approximately 300 per 100,000 which is about tenfold higher than in men.³ Currently, the elusive etiology, pathogenesis, and pathophysiology of IC present significant challenges for its diagnosis and effective management.⁴

Among the prevailing etiological hypotheses, the osmotic epithelial theory is particularly prominent.^{5,6} It posits that a defective glycosaminoglycan (GAG) layer on the bladder surface increases permeability, allowing urinary solutes to chronically irritate the

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underlying tissues.⁷ However, the functional integrity of the bladder wall depends not only on the superficial epithelium but also on a healthy subepithelial extracellular matrix (ECM). The bladder ECM, a complex network of collagens and other macromolecules, provides critical structural support and mechanosensory properties. A well-organized collagen scaffold, predominantly composed of type I and III collagen, is essential for maintaining bladder wall compliance and function.^{8–10} Notably, alterations in collagen composition and architecture, such as a reduction in the more compliant type III collagen, have been associated with bladder dysfunction and impaired tissue repair in various pathological states.¹¹ Therefore, an approach targeting both epithelial integrity and stromal architecture may represent a rational direction for further investigation.

While exogenous GAG supplementation with agents like hyaluronic acid has been attempted to restore the surface barrier, its clinical benefits remain inconsistent.^{12,13} This underscores limitation of a surface-level approach and highlights the need for interventions capable of supporting tissue structural remodeling. Recombinant human collagen type III (rHCIII) represents such a potential candidate. Beyond serving as a structural scaffold, type III collagen is known mediate crucial cellular processes including adhesion, migration, and matrix remodeling through interactions with integrin receptors.^{14,15}

Herein, we investigate the therapeutic potential of intravesical instillation of sterile rHCIII in a rat model of IC, hypothesizing that it may improve bladder tissue architecture and modulate associated histopathological alterations.

Methods

Establishment of animal model

A total of thirty specific-pathogen-free (SPF) female Sprague-Dawley rats were used in this study. All procedures involving live animals (housing, modeling induction, and intravesical treatments) were performed at the accredited animal facility of Beijing Keweite Animal Technology Co., Ltd. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Beijing Keweite Animal Technology Co., Ltd. prior to study initiation (Approval No.: KWT-2025-02-03-01). All procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals, and every effort was made to minimize animal suffering. The experimental workflow is illustrated

in Figure 1. The rats were maintained in a controlled environment (temperature: 22°C ± 2°C, humidity: 60% ± 2%) with *ad libitum* access to standard chow and fresh water. Rats were randomly assigned to the PBS, IC model, and rHCIII treatment groups (n = 10 per group) using a computer-generated random sequence. To minimize bias, all samples were labeled with coded identifiers immediately after collection. Investigators responsible for tissue processing, image acquisition/quantification, and molecular assays were blinded to group allocation until all analyses were completed. Histological evaluation was independently performed by two experienced pathologists in a blinded manner. Prior to the procedures, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) to minimize suffering and ensure immobility during catheterization. Then, the rats were secured, and the urethral orifice was disinfected with 70% ethanol and iodine tincture. A sterile epidural catheter was then inserted into the bladder via the urethra. At the end of the experiment, all rats were euthanized under deep anesthesia. Euthanasia was performed by intraperitoneal injection of an overdose of pentobarbital sodium (150 mg/kg) until complete cessation of respiration and cardiac activity was confirmed. All procedures were conducted in accordance with established animal welfare guidelines and relevant ethical regulations.

PBS group: Rats received intravesical instillation of 1 mL phosphate-buffered saline (PBS) once daily for four consecutive days (Days 1–4).

IC model group: The IC group was induced by sequential instillation of 1 mL of 10 mg/mL protamine sulfate (PS) (retained for 45 min) followed by 1 mL of 750 µg/mL lipopolysaccharide (LPS) (retained for 30 min).¹⁶ The bladder was then rinsed three times with PBS. This PS/LPS induction protocol was performed once daily for four consecutive days (Days 1–4). Following induction, rats received intravesical instillation of PBS as vehicle control.

rHCIII group: Rats underwent the same PS/LPS induction protocol as described for the IC model group once daily for four consecutive days (Days 1–4). After the final PBS rinse at each induction, rats received intravesical instillation of 1 mL sterile 1% rHCIII solution, which was retained for 30 min.

On Day 5, all rats from all groups were euthanized simultaneously. Bladders were harvested, and each was divided into segments. Tissues designated for histology were fixed in 10% neutral buffered formalin, whereas tissues for molecular analysis were snap-frozen in liquid nitrogen. All samples were subsequently transferred to our laboratory at the

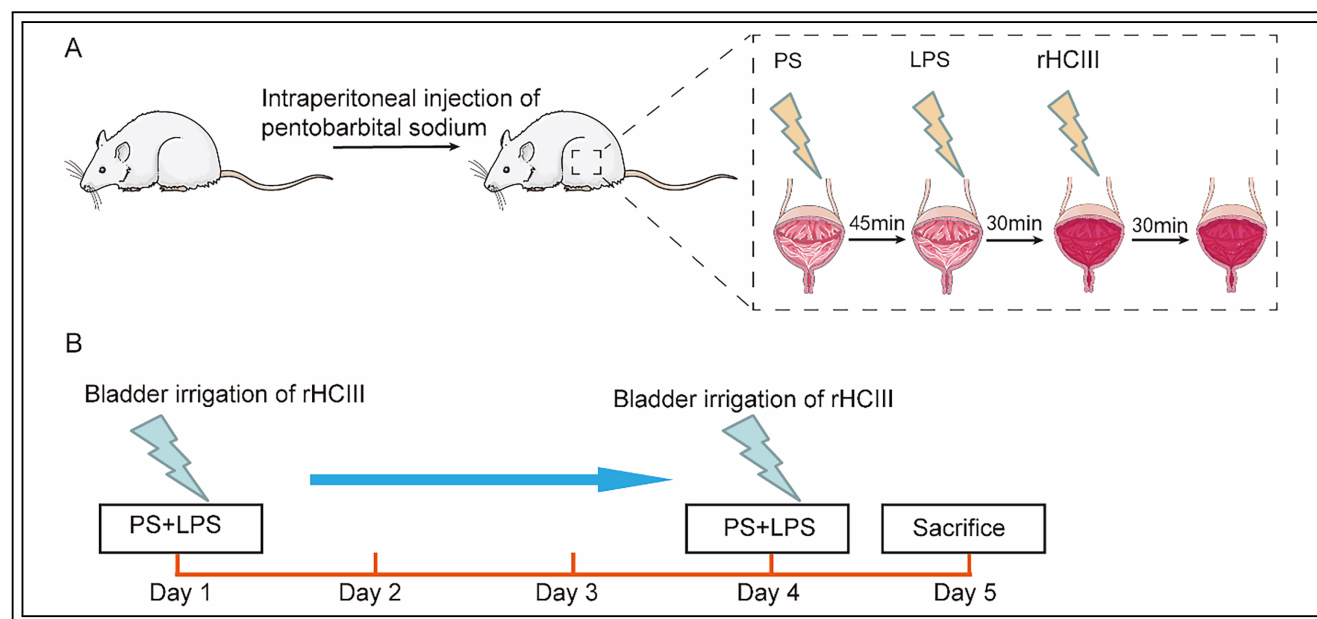


FIGURE 1. Experiment design for establishing and treating the rat IC model with rHCIII. (A): Schematic diagram of the intravesical procedure timeline; (B): Timeline of the experimental protocol. PS: Protamine Sulfate; LPS: Lipopolysaccharide; rHCIII: Recombinant Human Collagen Type III

Affiliated Changzhou Second People's Hospital of Nanjing Medical University for further analysis.

H&E staining

Bladder tissues were fixed in 10% neutral buffered formalin for over 24 h, dehydrated through a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, and embedded in paraffin. Sections of 5 μ m thickness were prepared, incubated at 60°C for 1 h, and then deparaffinized. The sections were stained with hematoxylin (H8070-5g; Solarbio, Beijing, China) for 4 min, differentiated in acid ethanol, blued in ammonia water, and counterstained with eosin (PT001; Bogoo, Shanghai, China). After final dehydration and clearing, sections were mounted with neutral resin. Pathological changes were examined under a light microscope (DMN-300D; Caikon, Shanghai, China).

Western blot

Bladder tissue proteins were extracted using RIPA lysis buffer (Beyotime, Shanghai, China, Cat. P0013B) supplemented with protease and phosphatase inhibitor tablets (Thermo Fisher Scientific, Massachusetts, USA, Cat. A32961). Protein concentrations were quantified using the BCA assay kit (Beyotime, Shanghai, China, Cat. P0012) according to the manufacturer's instructions. Protein concentrations were adjusted to 1 μ g/ μ L. A total

of 20 μ g of protein per sample was separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-IL6 (1:1000, ab233706; Abcam, Cambridge, UK), rabbit anti-IL17A (1:1000, ab267403; Abcam, Cambridge, UK), rabbit anti-TNF- α (1:50,000, ab32506; Abcam, Cambridge, UK), and rabbit anti-GAPDH (1:1000, ab9485; Abcam, Cambridge, UK). After washing, membranes were incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:2000, ab6721; Abcam, Cambridge, UK). All antibodies were from Abcam. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (WBKLS0500-2, Merck Millipore, MA, USA), and images were captured with a gel imaging system (Gel Doc XR; Bio-Rad, CA, USA). For Western blot analysis, each sample represented an independent biological replicate derived from an individual animal ($n = 10$ per group). Representative Western blot images are shown. Protein band intensities were quantified using ImageJ software and normalized to the corresponding GAPDH loading control. Samples intended for quantitative comparison were processed and analyzed within the same experimental run under identical experimental conditions to minimize inter-batch variability.

Immunohistochemistry (IHC)

IHC was performed using an Ultrasensitive Streptavidin Peroxidase Kit (KIT-9710, Maixin, Fujian, China) according to the manufacturer's instructions. For antigen retrieval, deparaffinized sections were immersed in 10 mM citrate buffer (pH 6.0) and heated in a microwave at 95°C for 20 min, followed by natural cooling to room temperature. Sections were incubated overnight at 4°C with primary antibodies: rabbit anti-Caspase-3 (1:100, ab32351; Abcam, Cambridge, UK) or rabbit anti-6×HIS tag (1:16,000, ab213204; Abcam, Cambridge, UK). Subsequently, biotinylated secondary antibodies and streptavidin-peroxidase were applied, each for 30 min. Diaminobenzidine (DAB) (DAB-2031; Maixin, Fujian, China) was used as the chromogen, followed counterstaining with hematoxylin and eosin. Stained sections were evaluated by two independent pathologists in a blinded manner.

TUNEL staining

TUNEL staining was performed using a commercial TUNEL assay kit (Beyotime, Beijing, China, C1091) to detect apoptotic cells. Paraffin-embedded sections were deparaffinized and rehydrated, followed by incubation with Proteinase K (Proteinase K:PBS = 1:9) at 37°C for 22 min for tissue permeabilization. After PBS washing, sections were treated with 0.1% Triton X-100 (Triton X-100: PBS = 1:1000) at room temperature for 20 min and washed again. Sections were then equilibrated for 10 min and incubated with the TUNEL reaction mixture (TdT enzyme:dUTP:buffer = 1:5:50) at 37°C for 2 h in a humidified chamber. Cell nuclei were counterstained with DAPI, and sections were mounted with an antifade mounting medium. Fluorescence images were acquired at 200× magnification, and three randomly selected non-overlapping fields per section were analyzed. TUNEL-positive cells were quantified using ImageJ software (National Institutes of Health, Maryland, USA), and all analyses were performed in a blinded manner.

Masson staining

Fixed bladder tissues were embedded in paraffin and sectioned. The sections were stained using a standard Masson's trichrome protocol to visualize collagen fibers (blue), muscle fibers (red), and nuclei (black). Images were acquired under a polarized light microscopy (XPT-480, Zhongheng, Shanghai, China), and collagen fiber density in the submucosal layer was quantified using Image-Pro Plus 5.1 software (Media Cybernetics, Maryland, USA).

Van Gieson (VG) staining

Tissue sections were stained with Weigert's hematoxylin for 15 min, rinsed, and then stained with VG solution for 5 min. Sections were briefly differentiated in 95% ethanol, dehydrated, cleared, and mounted. This staining highlights collagen fibers (red) and muscle fibers (yellow), allowing for the assessment of detrusor fibrosis.

Toluidine blue (TB) staining

Tissue sections were stained with 0.1% TB solution for 10 min, rinsed, and differentiated briefly in glacial acetic acid. After drying, sections were cleared in xylene and mounted. Mast cells were identified by their metachromatic purple-red granules. For quantitative analysis, three non-overlapping microscopic fields per section were randomly selected from the urothelial mucosa at 200× magnification. Random field selection was performed by scanning the entire section and choosing fields evenly distributed across the mucosal area while avoiding tissue edges and obvious artifacts. TB-positive mast cells were manually counted using ImageJ software (National Institutes of Health, Maryland, USA), and all analyses were conducted by two independent investigators blinded to group allocation.

Statistical analysis

For all histological, immunohistochemical, and staining-based quantitative analyses, one independent animal experiment was performed. One representative section was analyzed per animal. For each section, three non-overlapping microscopic fields were randomly selected for quantification. Measurements from multiple fields were first averaged to obtain a single value per animal, and these animal-level mean values ($n = 10$ per group) were used for subsequent statistical analyses.

All experimental data were analyzed using R software (Version 4.0.1). Bar plots were generated with ggplot2 (Version 3.5.2) and ggpubr (Version 0.6.2) packages. Data are presented as mean $\pm$ Standard Deviation (SD) (or median with interquartile range, as appropriate). Normality was assessed using the Shapiro-Wilk test. For comparisons among three groups, one-way ANOVA was applied for normally distributed data, followed by Tukey's post-hoc multiple-comparisons test for all multi-group comparisons. For non-normally distributed data, the Kruskal-Wallis test was used, followed by Dunn's post hoc test for multiple comparisons. A two-sided p value < 0.05 was considered statistically significant.

Results

Pathological alterations in bladder tissues of IC rats

Histological examination via H&E staining revealed distinct differences among the groups (Figure 2A–C). The PBS group exhibited intact bladder architecture with continuous mucosal epithelium, orderly stromal structure, and minimal inflammatory cell infiltration, confirming that the instillation procedure itself caused no significant damage (Figure 2A). In contrast, the IC group displayed severe pathological changes, including loss of epithelial continuity, pronounced submucosal edema, vascular extravasation, and a substantial increase in inflammatory cell infiltration, confirming the successful establishment of the IC model (Figure 2B). Notably, intravesical instillation of rHCIII resulted in noticeable attenuation of histopathological alterations. The rHCIII-treated group exhibited a more continuous urothelial layer

with markedly reduced submucosal edema and inflammatory infiltration compared to the IC group (Figure 2C).

rHCIII treatment modulates pro-inflammatory cytokine expression in IC rats

To quantitatively evaluate the inflammation-related molecular changes, we assessed the expression levels of key pro-inflammatory cytokines (IL6, TNF- α , and IL17A) by Western blot (Figure 2D). Densitometric analysis revealed that the expression levels of IL6, TNF- α , and IL17A were significantly upregulated in the IC group compared to the PBS group ($p < 0.01$). However, treatment with rHCIII was associated with a significant reduction in the expression of these pro-inflammatory cytokines. As illustrated in Figure 2E–G, rHCIII instillation markedly reduced the relative expression of IL-6 ($p < 0.05$), IL-17A ($p < 0.001$), and TNF- α ($p < 0.05$) compared to the IC group. These quantitative results indicate that rHCIII

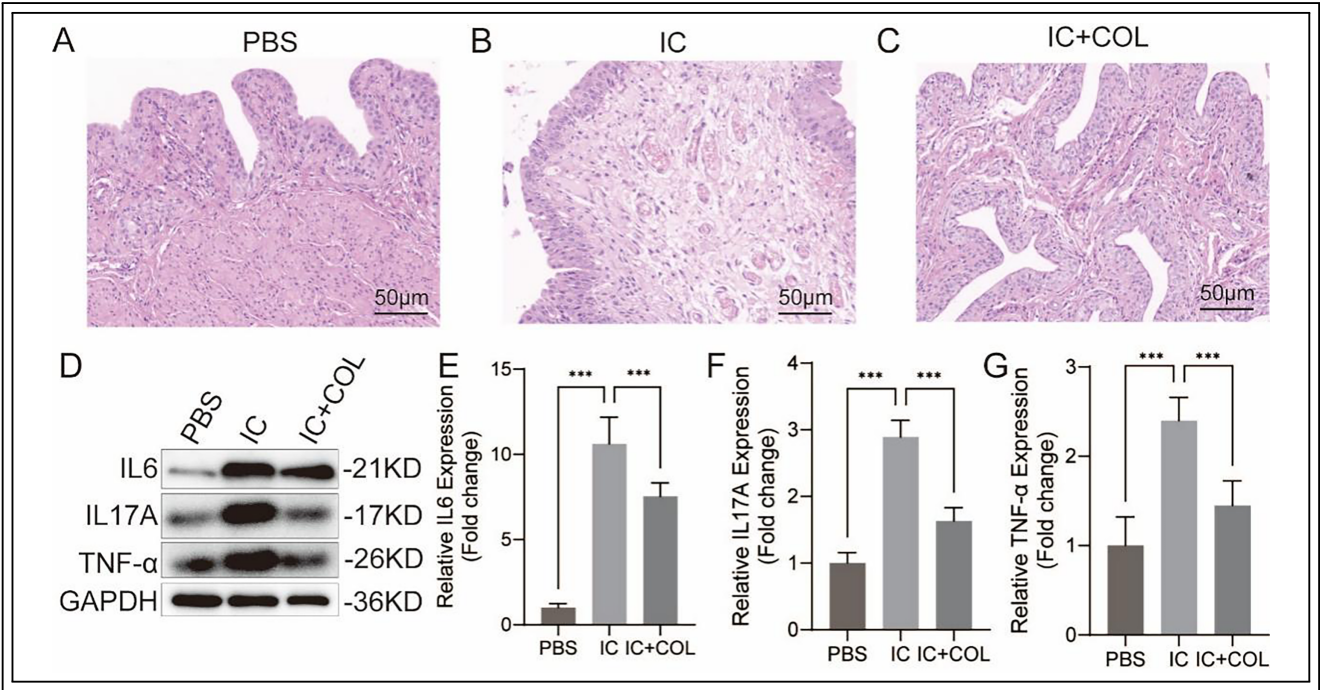


FIGURE 2. Histopathological and inflammatory cytokine expression analyses of bladder tissues in different groups. (A): H&E staining of bladder tissues in the PBS group; (B): H&E staining of bladder tissues in the IC group; (C): H&E staining of bladder tissues in the rHCIII group; (D): Western blot analysis of the expression levels of inflammatory cytokines in bladder tissues from the various treatment groups. (E–G): Quantitative densitometric analysis of the relative protein expression levels of (E) IL-6, (F) IL-17A, and (G) TNF- α . Band intensities were quantified using ImageJ software and normalized to GAPDH. Statistical significance was determined using one-way ANOVA. *** $p < 0.001$ vs. IC groups. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III; IL6: Interleukin-6; IL17A: Interleukin-17A; TNF- α : Tumor Necrosis Factor- α ; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

instillation is associated with reduced expression of inflammation-related cytokines in IC.

Effects of rHCIII on mast cell distribution in IC rats

TB staining demonstrated a conspicuous increase in mast cell infiltration in the IC group compared to the PBS group (Figure 3A,B). This infiltration was significantly reduced in the rHCIII group (Figure 3C). Quantitative analysis further confirmed that the density of mast cells was significantly lower in the rHCIII group compared to the IC group ($p < 0.001$, Table 1). These findings indicate that rHCIII instillation is associated with a reduced mast cell density in bladder tissues of IC rats.

Detection of 6×HIS-tagged rHCIII in bladder tissues

To track the localization of the instilled rHCIII, we performed IHC staining for the 6×HIS tag (Figure 4). The results showed that no positive 6×HIS staining was observed in the PBS or IC groups (Figure 4A,B), whereas clear positive staining was detected in both the mucosal and submucosal layers of the rHCIII group (Figure 4C). This indicates that the instilled rHCIII was detectable on the mucosal surface as well as within the submucosal region.

Effects of rHCIII on apoptosis in IC bladder tissues

To further evaluate apoptosis-related changes following rHCIII instillation, we performed TUNEL

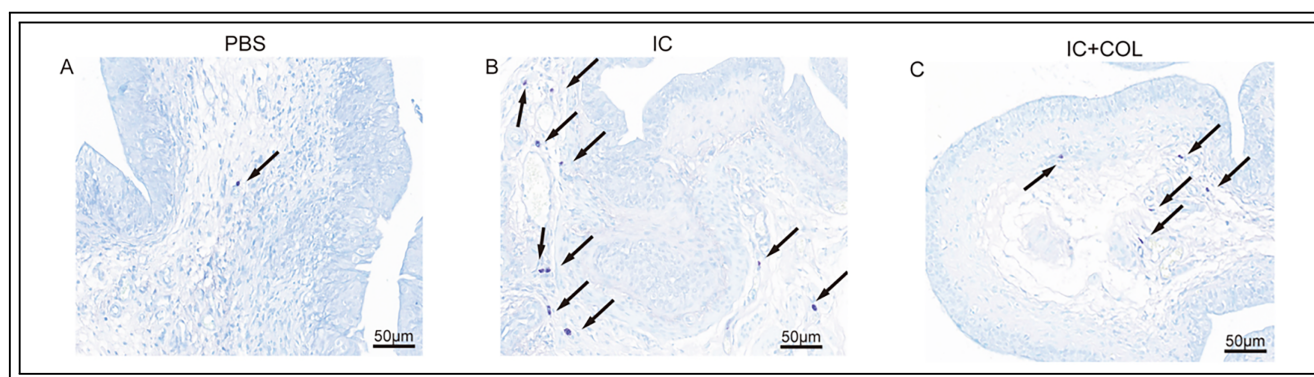


FIGURE 3. Toluidine blue (TB) staining for mast cell distribution in bladder tissues of different groups. (A): TB staining of bladder tissues in the PBS group; (B): TB staining of bladder tissues in the IC group; (C): TB staining of bladder tissues in the rHCIII group. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III; rHCIII: Recombinant Human Collagen Type III

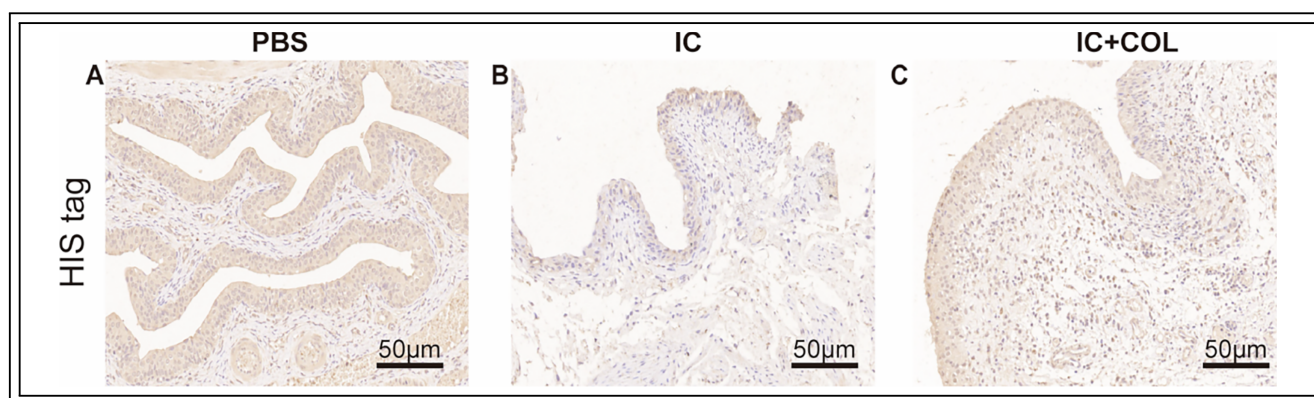


FIGURE 4. Immunohistochemical staining for 6×HIS tag in bladder tissues of different groups. (A): Immunohistochemical staining in the PBS group; (B): Immunohistochemical staining in the IC group; (C): Immunohistochemical staining in the rHCIII group. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III; HIS: 6×Histidine Tag

TABLE 1. Quantitative analysis of mast cell density in bladder tissues across different groups

Groups	PBS (n = 10)	IC (n = 10)	IC + COL (n = 10)	p-value (IC vs. PBS)	p-value (IC + COL vs. IC)
Mast cells	0.50 ± 0.76	11.63 ± 4.17	3.88 ± 1.25	2.83×10^{-10}	6.37×10^{-8}
Density (n/mm²)	1.02 ± 1.55	23.79 ± 8.54	7.93 ± 2.55	2.83×10^{-10}	6.37×10^{-8}

Note. Data are presented as mean ± SD. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III.

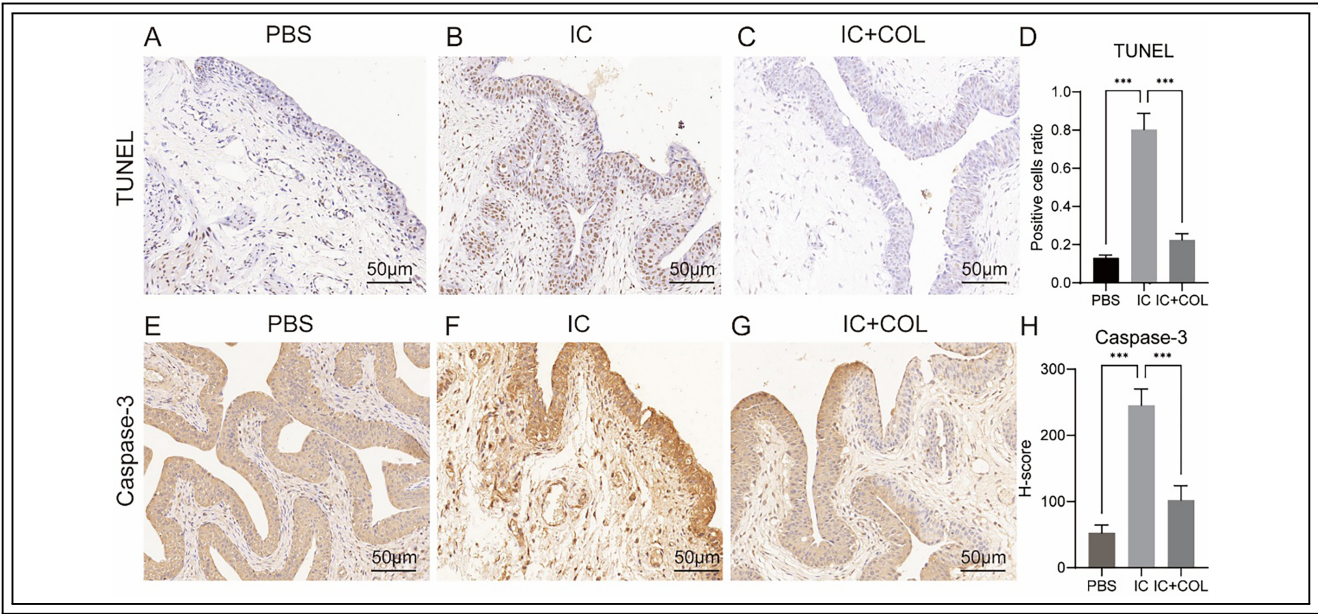


FIGURE 5. Evaluation of cell apoptosis in bladder tissues via TUNEL staining and Caspase-3 immunohistochemistry. (A–C): Representative images of TUNEL staining in the PBS (A), IC (B), and IC+COL (C) groups. Brown nuclei indicate apoptotic cells, while blue nuclei indicate normal cells. (D): Quantitative analysis of the TUNEL-positive cell ratio in each group. (E–G): Representative immunohistochemical images of Caspase-3 expression in the PBS (E), IC (F), and IC+COL (G) groups. (H): Quantitative analysis of Caspase-3 expression levels assessed by H-score. *** indicate $p < 0.001$. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III; TUNEL: Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick-End Labeling

staining and Caspase-3 immunohistochemistry. As shown in [Figure 5A,B](#), the IC group exhibited a widespread increase in TUNEL-positive apoptotic cells within the mucosal and submucosal layers compared to the PBS group. Notably, rHCIII instillation (IC+COL group) was associated with a markedly lower number of TUNEL-positive cells ([Figure 5C](#)). Quantitative analysis further confirmed that the apoptotic cell ratio was significantly elevated in the IC group but significantly decreased following rHCIII treatment ([Figure 5D](#)).

Consistently, immunohistochemical analysis of the apoptosis executioner protein, Caspase-3, revealed faint staining in the PBS group ([Figure 5E](#)),

whereas robust Caspase-3 expression was observed in the IC group ([Figure 5F](#)). In contrast, the IC+COL group displayed markedly lower Caspase-3 expression ([Figure 5G](#)), which was further corroborated by the quantitative H-score analysis ([Figure 5H](#)). Collectively, these data indicate that rHCIII treatment instillation is associated with reduced apoptosis in IC bladder tissues.

Effects of rHCIII on collagen fiber organization in IC bladder

The impact of rHCIII on collagen fiber architecture was assessed using Masson staining for the submucosal layer ([Figure 6A–D](#)) and Van Gieson

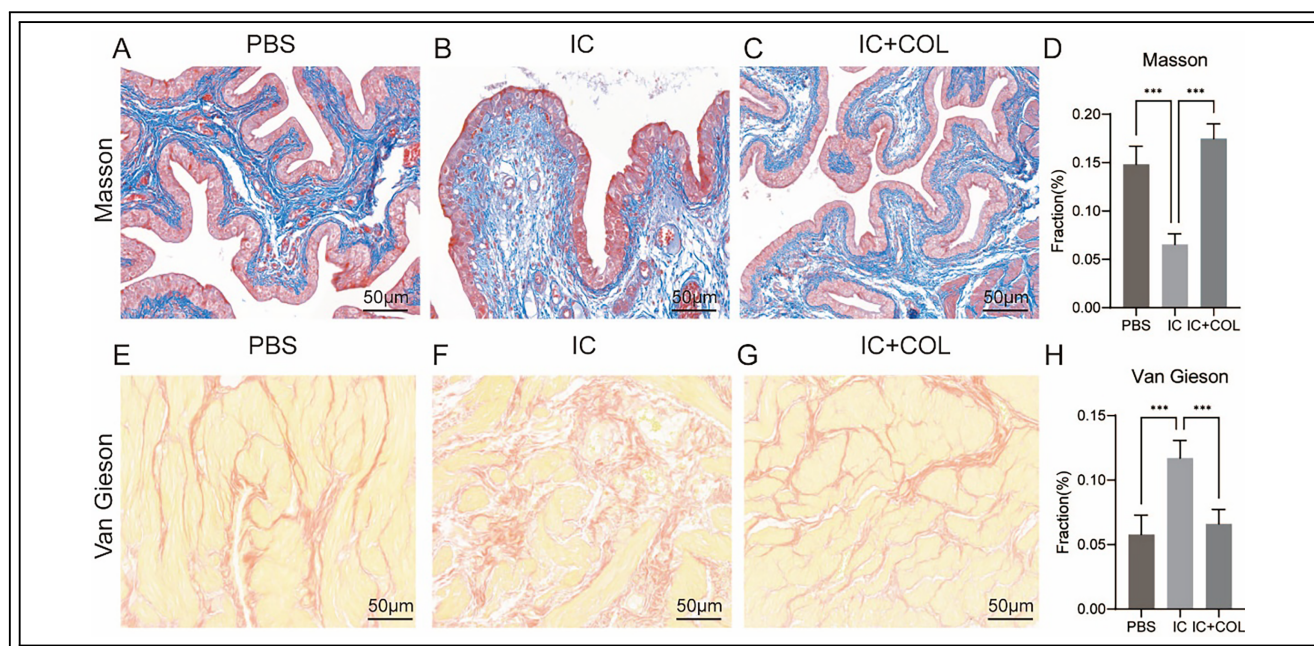


FIGURE 6. Analysis of collagen fiber architecture in bladder submucosal and muscular layers via Masson and Van Gieson staining. (A–C): Representative images of Masson's trichrome staining in the submucosal layer of the PBS (A), IC (B), and IC+COL (C) groups. Collagen fibers are stained blue, while muscle fibers and cytoplasm are stained red. (D): Quantitative analysis of the collagen fraction in the submucosal layer. (E–G): Representative images of Van Gieson (VG) staining in the muscular layer of the PBS (E), IC (F), and IC+COL (G) groups. Collagen fibers are stained red, and muscle fibers are stained yellow. (H): Quantitative analysis of the collagen fraction in the muscular layer. *** $p < 0.001$ vs. the indicated groups. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III

staining for the muscular layer (Figure 6E–H). In the submucosal layer, Masson staining revealed that the IC group exhibited loosely arranged, disrupted collagen fibers with a significantly reduced collagen fraction compared to the PBS group (Figure 6A,B). Following rHCIII treatment, the submucosal collagen density was markedly increased compared to the IC group, presenting a compact and organized architecture similar to that of the PBS group (Figure 6C). Quantitative analysis further confirmed that the collagen fraction was significantly decreased in the IC group, whereas rHCIII group showed significantly increased submucosal collagen content (Figure 6D).

In the muscular layer, Van Gieson staining revealed sparse and normally distributed collagen fibers in the PBS group (Figure 6E). In contrast, the IC group developed pronounced interstitial fibrosis, characterized by excessive collagen deposition between muscle bundles (Figure 6F). Following rHCIII treatment, collagen accumulation in the muscular layer was markedly reduced, suggesting reduced fibrotic features (Figure 6G). Consistent

with these observations, quantitative analysis demonstrated a significantly increased collagen fraction in the IC group, which was significantly lower than the IC group after rHCIII treatment (Figure 6H). These findings indicate that rHCIII instillation is associated with altered collagen fiber organization in both the submucosal and muscular layers.

All quantitative data corresponding to the experimental results presented in this study are compiled in Table A1 for comprehensive reference and comparison.

Discussion

Interstitial cystitis (IC) is a debilitating chronic condition that severely impairs quality of life, for which no curative treatment currently exists. Previous studies have indicated the therapeutic potential of rHCIII in various tissue repair contexts. Compelling evidence has demonstrated its ability to mitigate the inflammatory response and promote the healing process in

cutaneous wounds.^{17,18} Similarly, in the context of oral ulcer repair, rHClII facilitates healing by enhancing the proliferation, migration, and adhesion of human oral keratinocytes. This enhanced cellular activity contributes to accelerated wound closure, accompanied by a reduction in inflammatory mediators and effective alleviation of pain.¹⁹ Furthermore, rHClII has been shown to support endometrial regeneration and restore fertility in a murine model of thin endometrium, primarily by augmenting the proliferation, stemness maintenance, and migratory capacity of resident mesenchymal stem cells.²⁰ Its pleiotropic benefits also extend to vaginal wall repair, where it modulates fibroblast function, stimulates angiogenesis, and promotes the remodeling of the extracellular matrix (ECM).²¹

Despite these documented reparative and anti-inflammatory properties across diverse tissues, the application of rHClII for the treatment of IC had not been previously explored. When translating this regenerative strategy to intravesical therapy, the selection of an appropriate formulation and concentration is critical for evaluating its clinical applicability. The 1% rHClII formulation used in the present study represents a standardized product provided by the manufacturer and was selected to reflect its potential clinical use. In preliminary pilot experiments, this formulation exhibited optimal physicochemical properties for intravesical instillation, achieving sufficient adhesion to the urothelium without causing urethral obstruction, which may occur at higher viscosities, or rapid washout, which may occur at lower viscosities. Therefore, rather than performing a dose-response screening, the present study focused on validating the biological efficacy of this clinically relevant, standardized concentration. In addition, this study did not include a head-to-head comparison between rHClII and hyaluronic acid (HA), which is a commonly used intravesical agent for IC. Future studies directly comparing rHClII with HA will be necessary to determine their relative efficacy and translational advantages. Building on this rationale, our study demonstrates that intravesical instillation of rHClII was detectable within both mucosal and submucosal compartments, accompanied by reorganization of collagen fiber architecture. This structural remodeling may contribute to improved tissue organization within the bladder wall.

Although the present study primarily focused on histological outcomes, existing evidence supports several plausible molecular mechanisms that may underlie rHClII-mediated collagen reorganization and inflammation attenuation. Recombinant type III

collagen has been reported to be internalized by stromal cells, such as fibroblasts, where it can stimulate endogenous type III collagen synthesis and while relatively reducing type I collagen deposition, thereby shifting the type I/type III collagen ratio toward a more physiological composition.²² In the context of bladder injury, this rebalancing may contribute to the reorganization of extracellular matrix (ECM) homeostasis and improvement of tissue architecture.^{23,24} At the molecular level, type III collagen is a known ligand for $\beta 1$ -containing integrins (e.g., $\alpha 1\beta 1$ and $\alpha 2\beta 1$) expressed on urothelial cells and fibroblasts.^{25,26} Interaction of rHClII with these integrins may modulate downstream signaling pathways, including FAK/ERK and PI3K/Akt, which are known to regulate cell adhesion, survival, and ECM remodeling.^{27–30} Compared with type I collagen, type III collagen has been associated with a more restrained activation of Akt and ERK signaling, suggesting a potential role in maintaining tissue homeostasis rather than promoting fibrotic responses.³¹ In addition, integrin-dependent signaling is closely linked to epithelial structure and inflammatory regulation.³² Integrin $\beta 1$ has been shown to influence tight junction organization and epithelial permeability, while collagen-integrin interactions can modulate the production of pro-inflammatory cytokines.³³ Collectively, these observations suggest that rHClII may indirectly attenuate inflammation by influencing integrin-mediated signaling in urothelial cells, thereby contributing to promoting collagen reorganization and urothelial structural remodeling in the IC bladder.

Furthermore, a growing body of evidence underscores the pivotal role of pro-inflammatory cytokines in the pathogenesis of IC. Elevated levels of IL6 and IL17 are not only robustly correlated with clinical symptom severity but are also implicated in self-perpetuating inflammatory cycle, largely mediated through the aberrant activation of mast cells.^{34,35} This cytokine milieu fosters a pathological environment where mast cells release histamine and other algogenic substances, thereby stimulating sensory nerves, contributing to pelvic pain and amplifying local cytokine production. The clinical relevance of this inflammatory axis is highlighted by trials demonstrating that anti-TNF- α therapy can provide symptomatic relief in a subset of patients, affirming TNF- α as a key driver in the IC inflammatory network.^{36,37} In line with this established paradigm, our findings demonstrated reduced expression of IL6, TNF- α , and IL17 following rHClII instillation, suggesting modulation of inflammation-associated molecules. This potent anti-inflammatory effect,

evidenced by the downregulation of these critical mediators, suggests that rHCIII may exert effects beyond passive structural support but as an active modulator of underlying neuroimmune dysregulation in IC. By disrupting this key inflammatory cascade, rHCIII treatment may contribute to the interruption of chronic inflammatory signaling and its associated symptoms.

Mast cell infiltration is a well-established pathological hallmark of IC, and their activation is considered a central node in the disease's neuroinflammatory cascade.³⁸ Our data are consistent with this paradigm, demonstrating a significant increase in mast cell infiltration within the bladder tissue of the IC model group. Importantly, intravesical instillation of rHCIII was associated with a significantly lower mast cell density compared to the IC group. This finding suggests that rHCIII instillation may be associated with modulation of local inflammatory cytokine expression. The reduced mast cell density may partly explain the observed downregulation of key inflammatory cytokines and reflect inflammation-related molecular alterations, suggesting that rHCIII influences multiple histological and molecular features in IC.

Although functional outcomes such as pain behavior and urinary symptoms were not directly assessed in the present study, the observed histological and molecular improvements alterations may have structural relevance. Changes in urothelial organization and epithelial apoptosis may influence structural barrier components of the bladder wall. Such structural alterations may be relevant to the interaction between urinary solutes and suburothelial tissues,^{7,39–41} a process widely recognized as one of the contributors to pain generation in interstitial cystitis.^{42,43} In parallel, reorganization of submucosal collagen fibers and reduced fibrotic features may be associated with alterations in bladder wall structural properties.^{44–47} Reduced bladder compliance has been well documented to be associated with decreased bladder capacity and urinary symptoms.^{48,49} Moreover, the observed reductions in pro-inflammatory cytokine expression and mast cell density may reflect alterations in the local inflammatory microenvironment.^{50,51} Collectively, these findings highlight structural and molecular alterations associated with rHCIII instillation, warranting future studies incorporating behavioral assessments and urodynamic evaluation.

This study primarily aimed to assess the feasibility of intravesical rHCIII as a potential intervention approach for interstitial cystitis (IC). Several limitations should be acknowledged to better contextualize

the translational relevance of our findings. First, the PS/LPS-induced rat model represents an acute injury phase and may not fully recapitulate the chronic, idiopathic, and autoimmune features of human IC. Accordingly, future studies employing chronic or autoimmune IC models are warranted to further evaluate the biological and translational relevance of rHCIII. Second, the present study adopted an acute intervention protocol in which rHCIII was administered daily for four consecutive days concomitant with disease induction, and tissue analyses were performed immediately after the treatment period. As a result, the persistence of urothelial structural alterations and the potential recurrence of disease-related features following treatment cessation were not evaluated. Long-term follow-up studies will therefore be essential in future investigations. Third, only a single concentration of rHCIII (1%) was tested in this study, and a comprehensive dose–response analysis was not conducted. Future work will focus on optimizing the concentration and dosing frequency of rHCIII to optimize tissue responses and biological effects. In addition, although mast cell infiltration was examined as a key inflammatory feature, other inflammatory chemokines and cytokines were not systematically profiled, and the precise molecular mechanisms of rHCIII action remain to be fully elucidated. Moreover, due to the small size of rat bladders and the immediate formalin fixation performed to preserve epithelial integrity for histological analyses, gross morphological evaluation of the bladder mucosa was not feasible, precluding direct comparison with cystoscopic findings observed in clinical practice. Finally, functional outcomes such as pain related or behavioral parameters following rHCIII instillation were not assessed. These important questions warrant further investigation in future studies.

Conclusion

In summary, our findings demonstrate that intravesical instillation of sterile rHCIII is associated with histological and molecular alterations in a rat model of interstitial cystitis, characterized by reduced mucosal epithelial apoptosis and altered submucosal collagen organization. rHCIII instillation may influence urothelial and stromal structural components within the bladder wall and is associated with reduced inflammatory marker expression and mast cell density. These observations support its potential as a structural remodeling-oriented intervention approach warranting further investigation in IC.

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Author Contributions

The authors confirm contribution to the paper as follows: Li Zuo, conceptualization, supervision, writing—review and editing; Xiaokai Shi, experimental performance, data analysis, writing—original draft preparation. All authors reviewed and approved the final version of the manuscript.

Available of Data and Materials

The data that support the findings of this study are available from the corresponding author, Li Zuo, upon reasonable request.

Ethic Approval

All animal experiments were performed at the facilities of Beijing Keweite Animal Technology Co., Ltd. The experimental protocols were specifically

reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Beijing Keweite Animal Technology Co., Ltd. (Approval No.: KWT-2025-02-03-01), prior to the commencement of the study. All procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals.

Conflicts of Interest

The rHCIII material was provided by Taute Co., Ltd. (Jiangsu, China). The company had no role in the study design, data collection, analysis, or decision to publish.

Abbreviations

The following abbreviations are used in this manuscript.

IC	Interstitial Cystitis
rHCIII	Recombinant Human Collagen Type 3
H&E	Hematoxylin and Eosin
IHC	Immunohistochemistry
TB	Toluidine Blue
VG	Van Gieson
PS	Protamine Sulfate
LPS	Lipopolysaccharide
PBS	Phosphate-Buffered Saline
BCA	Bicinchoninic Acid
TBST	Tris-Buffered Saline with Tween-20

Appendix A

TABLE A1. Quantitative analysis of cytokine expression, apoptotic cells, and collagen fiber organization

Parameters	PBS Group (Mean $\pm$ SD)	IC Group (Mean $\pm$ SD)	IC + COL Group (Mean $\pm$ SD)	<i>p</i> -value (PBS vs. IC)	<i>p</i> -value (IC vs. IC + COL)
Inflammatory cytokines					
IL6 (Relative expression)	1.00 $\pm$ 0.25	10.62 $\pm$ 1.56	7.54 $\pm$ 0.80	$<4.22 \times 10^{-15}$	3.39×10^{-8}
TNF- α (Relative expression)	1.00 $\pm$ 0.32	2.39 $\pm$ 0.26	1.45 $\pm$ 0.27	1.98×10^{-11}	4.09×10^{-9}
IL17A (Relative expression)	1.00 $\pm$ 0.16	2.89 $\pm$ 0.25	1.63 $\pm$ 0.20	$<4.22 \times 10^{-15}$	2.84×10^{-14}
Apoptosis assessment					
TUNEL Positive Ratio (%)	13.02 $\pm$ 1.05	80.29 $\pm$ 8.50	22.57 $\pm$ 3.20	$<4.22 \times 10^{-15}$	$<4.22 \times 10^{-15}$
Caspase-3 (H-score)	52.67 $\pm$ 12.05	245.67 $\pm$ 24.38	102.00 $\pm$ 22.00	$<4.22 \times 10^{-15}$	$<4.22 \times 10^{-15}$
Collagen fiber analysis					
Masson (Submucosa, Area %)	14.82 $\pm$ 1.87	6.55 $\pm$ 1.10	17.47 $\pm$ 1.54	3.57×10^{-12}	3.18×10^{-14}
Van Gieson (Muscle, Area %)	5.78 $\pm$ 1.50	11.71 $\pm$ 1.36	6.60 $\pm$ 1.13	1.74×10^{-10}	3.97×10^{-10}

Note. PBS: Phosphate-Buffered Saline; IC: Interstitial Cystitis; COL: Collagen III; IL6: Interleukin-6; TNF- α : Tumor Necrosis Factor- α ; IL17A: Interleukin-17A; TUNEL: Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling; SD: Standard Deviation.

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