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Adelmidrol + sodium hyaluronate in IC/BPS or conditions associated to chronic urothelial inflammation. A translational study

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ABSTRACT

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a chronic bladder condition characterized by frequent urination, bladder inflammation and pain. It is a particular challenging disease and a clear unmet medical need in terms of identifying new therapeutic strategies. The aim of study was to evaluate the anti-inflammatory effects of intravesical Vessilen® (a new formulation of 2% adelmidrol (the diethanolamide derivative of azelaic acid) + 0.1% sodium hyaluronate) administration in rodent models of IC/BPS and in IC/BPS patients or other bladder disorders.

Acute and chronic animal models of cystitis were induced by a single or repetitive intraperitoneal injections of cyclophosphamide (CYP); patients with IC/BPS or with bladder pain syndrome associated with symptoms of the lower urinary tract treated once weekly by bladder instillation of Vessilen® for 8 weeks.

CYP instillation caused macroscopic and histological bladder alterations, inflammatory infiltrates, increased mast cell numbers, bladder pain, increased expression of nitrotyrosine, decreased expression of endothelial tight junction zonula occludens-1. Intravesical Vessilen® treatment was able to ameliorate CYP induced bladder inflammation and pain by inhibiting nuclear factor-κB pathway and inflammatory mediator levels as well as reduced mechanical allodynia and nerve growth factor levels. A significant improvement in quality of life and symptom intensity were evident in patients with IC/BPS or other bladder disorders treated with Vessilen®. Vessilen® could be a new therapeutic approach for human cystitis.

1. Introduction

Interstitial cystitis/painful bladder syndrome (IC/BPS) is a chronic bladder condition characterized by frequent urination and pain, which seriously impacts the quality of life. Patients can be classified by the presence or absence of Hunner's ulcers in the bladder that normally contain inflammatory cell infiltrates in the epithelium or lamina propria; these frequently consist of T lymphocytes with or without mast cells (MCs) [1]. However, epithelial cell proliferation and gene expression are often anomalous in bladder tissue from IC/BPS patients

Abbreviations: CYP, cyclophosphamide; IC/BPS, interstitial cystitis/painful bladder syndrome; MCs, mast cells; iNOS, nitric oxide synthase; ZO-1, zonula occludens type 1; PEA, palmitoylethanolamide; MPO, Myeloperoxidase; (NF-κB) p65, nuclear factor-κB; (IL)-1β, interleukin; MCP-1, monocyte chemoattractant protein-1; NGF, nerve growth factor

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[1–3] with altered levels of proteins such as inducible nitric oxide synthase (iNOS), zonula occludens type 1 (ZO-1), and occludin [4]. The ineffectiveness of IC/BPS therapies in part results from a poor understanding of the underlying pathogenesis and etiology [5]. Cyclophosphamide (CYP) is recognized to cause cystitis in humans [6]. In rodents, intraperitoneal injection of CYP produces bladder edema and haemorrhage, leading to pain-related behaviors such as altered back posture, spontaneous licking and rapid respiration [7,8] and mechanical hypersensitivity [9,10]. Although there are several well-described cystitis animal models and clinical trials of candidate therapies [11], the use of diverse injection protocols and number or doses of injections renders difficult a clear comprehension of bladder pathophysiology. Due to the lack of fixed diagnostic measures and variety of clinical presentation, IC/BPS is challenging to treat [12].

Adelmidrol, a diethanolamide derivative of natural azelaic acid, has proven to be an effective topical treatment for human inflammatory skin disorders. The Adelmidrol exerts a physical effect, known as "entourage effect", which induces a significant endogenous increase, neither metabolic nor pharmacological, of local levels of palmitoylethanolamide (PEA), allowing the maintenance of mast cells normal reactivity [13]. Topical adelmidrol treatment improves MC granule density, proposing a decrease in their degranulation [14,15]. In addition, this molecule exhibited favorable effects in a pilot study on mild atopic dermatitis [16]. We also have demonstrated the beneficial effects of adelmidrol in acute and chronic inflammation [17], as well as the positive effects of adelmidrol in combination with sodium hyaluronate in a rat model of osteoarthritis [18].

Based on these findings, the aim of this study was two-fold: i) to evaluate the acute and chronic effects of Vessilen® bladder instillation (a new formulation of 2% adelmidrol + 0.1% sodium hyaluronate) in the treatment of animal models of CYP-induced cystitis; ii) to evaluate the effects of Vessilen® bladder instillation in patients with IC/BPS or with condition associated to local inflammation, urothelial lesions, voiding dysfunctions and pain in pelvis/perineal area to confirm drug efficacy in improving clinical outcome in human cystitis.

2. Materials and methods

2.1. Preclinical studies

2.1.1. Animals

Female Adult CD1 mice (25–30 g, Envigo, Italy) and female Sprague Dawley rats (200–250 g Envigo Italy) were housed in a controlled environment with free access to standard rodent chow and water. This study was approved by the University of Messina Review Board for the care of animals. Animal care conformed to Italian regulations on the use of animals for experimental and scientific purposes (D.M. 116,192) and EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.1.2. Acute and chronic experimental cystitis induction

Acute cystitis was induced in rats by a single intraperitoneal injection of the alkylating anti-neoplastic agent CYP (Sigma-Aldrich) at a dose of 200 mg/kg as previously described [19]. Control (sham) animals received an equivalent volume of saline. Rats were sacrificed 4 h after CYP injection. Chronic cystitis was induced in mice by repetitive intraperitoneal injections of CYP. CYP (100 mg/kg) or vehicle (saline) was administered every day for five days. All mice were sacrificed one week after the third CYP injection [20].

2.1.3. Experimental groups

The experimental design was divided in two steps. First, we evaluated the anti-inflammatory effects of Vessilen® in an acute model of CYP-induced cystitis in female rats. Rats were arbitrarily allocated to one of the following groups:

(i) CYP + saline: rats received a single injection of CYP and intravesical saline (N = 10).

(ii) CYP + Vessilen®: rats received a single injection of CYP and intravesical instillation with Vessilen® (300 µl Adelmidrol 2% + Sodium Hyaluronate 0,1%) concurrently and after 2 h (N = 10).

(iii) The sham-operated groups underwent the same procedures as the CYP group, except that drug or saline were administered instead of CYP (N = 10). The dose was chosen based on a preliminary dose-response assessment. Intravesical administration was performed by urinary bladder catheterization according to a previous report [21].

In the second step, based on results obtained in the acute phase of cystitis and to better understand the action of Vessilen® in the pathogenesis of cystitis and the possible pathways involved, the chronic anti-inflammatory effects of Vessilen® were evaluated in a female mouse chronic model of cystitis. Mice were arbitrarily allocated to one of the following groups:

i) CYP + saline: mice received CYP injections every day for five days and saline intravesical instillation daily for one week, starting from the third day of CYP injection (N = 10).

(ii) CYP + Vessilen®: mice were received CYP every day for five days and Vessilen® intravesical instillation (100 µl Adelmidrol 2% + Sodium Hyaluronate 0,1%) daily for one week, starting from the third day of CYP injection (N = 10).

iii) The sham-operated mice underwent the same equal surgical procedures as the CYP group, except that the drug or saline were administered instead of CYP (N = 10).

The dose was chosen based on a preliminary dose-response assessment. Intravesical instillation was performed by urinary bladder catheterization according to a previous report [21].

2.1.4. Macroscopic analysis of bladder damage

After completing observations the animals were sacrificed. The bladders were weighed and examined macroscopically for bleeding and edema formation. They were scored according to Gray et al [22]: 3-severe damage (fluid externally present in the bladder wall and evident bleeding), 2-moderate damage (fluid in the internal mucosa and little bleeding), 1-mild damage (little edema and no bleeding) and 0-no bladder effect [22].

2.1.5. Behavioral evaluation

Behavioral parameters were used as pain indexes and scored by an observer skilled in behavioral examination and blinded to the treatments. The time-course of observation was 30 to 240 min after CYP injection. Behavioral scoring scales were established previously [19]. A maximum value of 10 was considered for each of the 2 parameters observed, namely eye closure and abnormal posture (stretched posture and rounded back) with an extra 10 when rats also showed other behavioral changes such as licking the abdomen. Thus, the maximum result was 30 and the minimum score was 0. Abnormal posture was scored as 0-normal posture, 5-occasionally rounded back or stretching, 7-almost rounded back or stretched position, and 10-rounded back and stretching. Eye closure was scored as 0-completely open eyes, 5-half-closed eyes and 10-eyes completely closed. The total pain score derived from a composite of the scores from 7 measurements, each with a maximum score of 30, at 30-min intervals starting 30 min after CYP injection.

2.1.6. Assessment of mechanical hypersensitivity during chronic model

Mechanical hypersensitivity in mice was measured at different time points using an electronic von Frey anesthesiometer (IITC Life Science Inc., Woodland Hills, CA, USA) as previously described [23]. The stimulation was applied three times, and the mean value calculated as the mechanical threshold for each mouse.

2.1.7. Histological evaluation

For histopathological examination, bladder biopsies were taken 4 h following CYP injection during the acute model of cystitis and 7 days after the third CYP injection in the chronic model of cystitis. Tissue

segments were fixed for 24 h in 4% paraformaldehyde/0.1 M phosphate-buffered saline at room temperature, dehydrated through a graded series of ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (7 μm) were deparaffinized with xylene, stained with haematoxylin/eosin and evaluated by light microscopy connected to an Imaging system (AxioVision, Zeiss, Milan, Italy). The stained sections were scored by two investigators in a blind fashion, and the degree of inflammation was evaluated according to a score from 0 to 5, as follows: 0 = no inflammation, 1 = mild inflammation, 2 = mild/moderate inflammation, 3 = moderate inflammation, 4 = moderate/severe inflammation and 5 = severe inflammation. In the chronic model, sections were also stained by Masson's Trichrome method for collagen detection, and observed under light microscopy. The degree of fibrosis was evaluated as % fibrotic area (blue staining) and quantified using image analysis software (Image *J* 1.8.0).

2.1.8. Myeloperoxidase (MPO) activity

MPO, an enzyme released by neutrophils that represents a marker of neutrophil infiltration, was determined as previously described in acute and chronic models of cystitis [24]. The rate of absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 mM of peroxide within 1 min at 37 °C, and expressed in units per gram wet tissue.

2.1.9. Staining of MCs

Identification of MCs was performed in bladder sections by blue toluidine staining as described previously [25].

2.1.10. Immunofluorescence analysis

Immunofluorescence evaluation for MPO, nitrotyrosine and ZO-1 was carried out as previously described [26]. Sections were incubated with primary antibodies against MPO (1:50, Neomarkers DBA Italia srl, Milan Italy), nitrotyrosine (1:50, Millipore DBA Italia srl, Milan Italy) and ZO-1 (1:50, Thermo Fisher Scientific, USA) and then with FITC-conjugated anti-mouse Alexa Fluor-488 secondary antibody (1:2000 v/v Molecular Probes, UK) and with Texa Red-conjugated anti-rabbit Alexa Fluor-594 secondary antibody (1:1000 in phosphate-buffered saline, v/v Molecular Probes). After washing, nuclei were stained using 2 μg/ml 4′,6′-diamidino-2-phenylindole (Hoechst, Frankfurt; Germany) in phosphate-buffered saline. Sections were observed and photographed using a Leica DM2000 microscope (Leica).

2.1.11. Western blots for IKB-α, nuclear factor-κB (NF-κB) p65, interleukin (IL)-1β, monocyte chemoattractant protein-1 (MCP-1) and nerve growth factor (NGF)

Cytosolic and nuclear extracts were prepared as previously described [27]. The following primary antibodies were used: anti-IκB-α (1:1000; Santa Cruz Biotechnology, DBA Italia srl, Milan Italy), anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology, DBA Italia srl, Milan Italy), anti-iNOS (1:200; BD transduction DBA Italia srl, Milan Italy) anti-IL-1β (1:500, Santa Cruz Biotechnology, DBA Italia srl, Milan Italy), anti-MCP-1 (1:500, Santa Cruz Biotechnology, DBA Italia srl, Milan Italy) and anti-NGF (1:500, Santa Cruz Biotechnology, DBA Italia srl, Milan Italy) in 1x phosphate-buffered saline, 5% w/v non-fat dried milk, 0.1% Tween-20 at 4 °C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of lysate, they were also probed with anti-β-actin or anti-lamin A/C antibodies. Relative expression of protein bands (IkB-α, 37 kDa; NF-κB p65, 65 kDa; iNOS, 130 kDa, IL-1β, 33 kDa, MCP-1, 12 kDa, NGF, 13 kDa) was detected by enhanced chemiluminescence (Thermo, USA), visualized with Chemi Doc XRS (Bio-Rad, USA) and analyzed using Image Lab 3.0 software (Bio-Rad, USA).

2.1.12. Reagents

Vessilen® was provided by Epitech group S.p.A (Saccolongo, Italy). All other reagents were obtained from Sigma-Aldrich Co. Milan Italy and were of the highest grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl, Baxter Healthcare Ltd., Thetford, Norfolk, UK).

2.2. Clinical study

2.2.1. Assessment of vessilen® in patients with IC/BPS or other bladder disorders

A preliminary observational clinical investigation was carried out in patients with chronic IC/BPS or BPS to evaluate the effects of Adelmidrol + sodium hyaluronate (Vessilen®) bladder instillation according to Helsinki Declaration and to Good Clinical Practice. The research was performed between July 2016 – July 2017 involving 26 centers of urology across Italy (see footnote). All patients considered eligible for the study complained of a chronic pelvic pain associated with symptoms of the lower urinary tract including increased urinary frequency, urgency and unpleasant pressure and discomfort. Recruitment was limited to patients with symptoms lasting for at least 3–6 months. Patient clinical histories were analyzed to reveal concomitant pathological conditions characterized by chronic pain and thus identify those presenting an overlapping symptomatic profile. The IC/BPS diagnosis was performed by endoscopy, urodynamics, and histopathology of bladder biopsies. The differential diagnosis were performed in agreement with the National Institute of Diabetes and Digestive and Kidney Diseases (N.I.D.D.K)

Prior to treatment start, eligible patients were fully informed about the study's purpose and, after giving their written informed consent, were subjected to the following treatment scheme: once weekly bladder instillation Adelmidrol + sodium hyaluronate (Vessilen®) for 8 weeks.

At baseline and during the treatment period, all patients underwent weekly evaluations for pain, urgency and frequency symptoms using the Visual Analogue Scale (VAS). VAS is a tool for assessing intensity of symptoms perceived by the patient and is represented by a line, usually 10 cm long, where one end (0) indicates absence of the symptom, and the other end (10 cm) represents the worst imaginable symptom intensity. Evaluation of quality of life was carried out using the following two questionnaires: i) Pelvic Pain and Urgency / Frequency Patient Symptom Scale (PUF Questionnaire), which allows one to quantify how pain, urgency and frequency symptoms bother the patient; ii) a SF-12 questionnaire that allows for evaluation as to what extent a patient's physical activity and mental state can be conditioned by symptom intensity. These two questionnaires were self-administered by the patient, prior to the onset of treatment and after the 8th (final) bladder instillation. All patients were monitored to control for the occurrence of any adverse treatment effects.

2.2.2. Data analysis

For preclinical studies, all values (figures and text) are expressed as mean ± standard error of the mean (SEM) of *n* observations. For in vivo studies *n* represents the number of animals. In experiments involving immunofluorescence or histology, the figures shown are representative of at least three independent experiments. The results were analyzed by one-way or two-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. P-values less than 0.05 were considered as significant.

For clinical studies, patient data were subjected to intention-to-treat (ITT) analysis. Variations of mean values over time were evaluated using the Generalized Linear Mixed Model (GLMM) with SAS 9.2; this statistical model allows one to analyze missing values without exclusion of the patient and which includes variables such as age, sex, clinical center, diagnosis and onset as covariates.

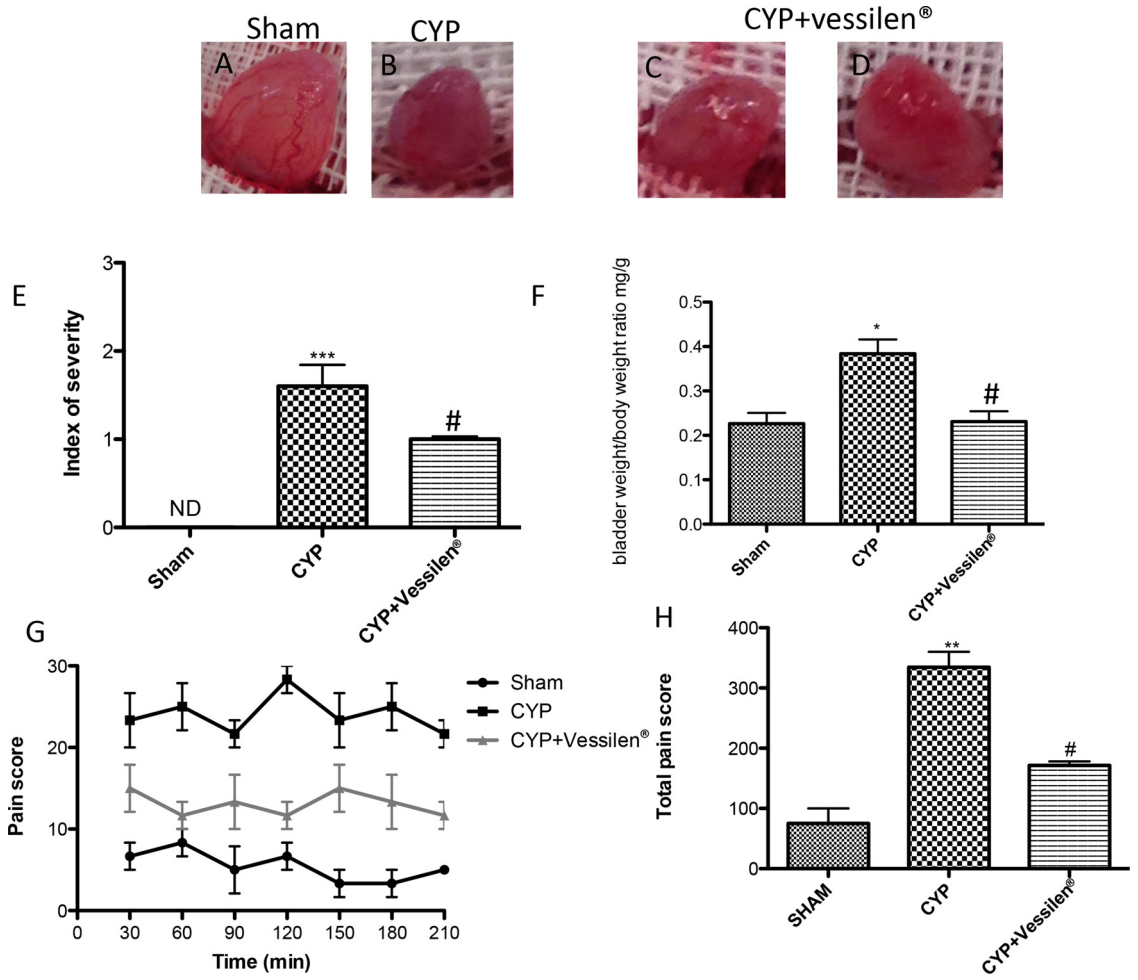

Fig. 1. Acute effects of Vessilen® on bladder damage and pain in CYP-injected rats.
Macroscopic damage in sham rats (A and E), CYP-injected rats (B, E) and CYP-injected rats treated with intravesical instillation of Vessilen® (C, D and E). Bladder weight/body weight ratio (F); pain score (G). Total pain score was a composite of scores derived from 7 measurements, each with a maximum of 30, at 30-min intervals starting 30 min after CYP injection (H). Values are means ± SEM of 10 animals for each group; *P < 0.05 vs sham; **P < 0.01 vs sham; ***P < 0.001 vs sham, #P < 0.01 vs CYP. ND: not detectable.

3. Results

3.1. Preclinical studies

3.1.1. Vessilen® acute effects on bladder damage and pain after a single CYP injection

Four hours after CYP injection edema formation and bleeding was evident in CYP-injected rats, in contrast to sham animals (Fig. 1A,B and see E). Intravesical Vessilen® treatment reduced bladder inflammation (Fig. 1C,D and E). Moreover, edema formation increased the weight of bladder tissues compared to sham (Fig.1F); Vessilen® decreased bladder weight evaluated as bladder weight/body weight ratio (Fig. 1F). In addition, behavioral parameters evaluated as index of pain after damage showed that CYP induced marked modifications in behaviors such as eye closures or abnormal postures (Fig. 1G). Over this time-course, Vessilen® treatment significantly decreased pain score (Fig. 1G, H).

3.1.2. Vessilen® acute effects on bladder histological injury after a single CYP injection

Bladder histological examination showed alterations after CYP injection (Fig. 2B, B1; see histological score D). Severe signs of cystitis, including sub mucosal edema and epithelial ulceration, were evident 4 h post-CYP injection (Fig. 2B, B1; see histological score D). In control mice, an intact urothelium and normal muscularis were evident with no signs of edema (Fig. 2A, A1; see histological score D). Vessilen® treatment significantly reduced histological damage (Fig. 2C, C1; see histological score D).

3.1.3. Vessilen® acute effects on bladder neutrophils, MCs infiltration and tight junctions after a single CYP injection

MPO activity and the presence of MCs were used to assess the degree of bladder inflammation. MPO levels measured by biochemical assay and immunofluorescence showed neutrophil-based inflammation as a consequence of CYP injection (Fig. 3B and D). Low MPO levels were found in sham groups (Fig. 3A and D). In addition, a significant increase in the number of MCs was observed 4 h after CYP injection (Fig. 4B and D) compared to controls (Fig. 4A and D). During acute inflammation neutrophils interact with endothelial cells, leading to endothelial tight junction disassembly and subsequent neutrophil extravasation. We also observed a decreased expression of ZO-1 in bladders of CYP-injected animals (Fig. 5B) compared to controls (Fig. 5A). Vessilen® treatment reduced neutrophil and MCs infiltration and increased ZO-1 expression (Figs. 3,4,5 C and see Figs. 3,4 D).

3.1.4. Vessilen® acute effects on bladder nitrotyrosine formation after a single CYP injection

CYP-induced cystitis results in oxidative stress that contributes to urinary bladder dysfunction. For this reason, we determined the

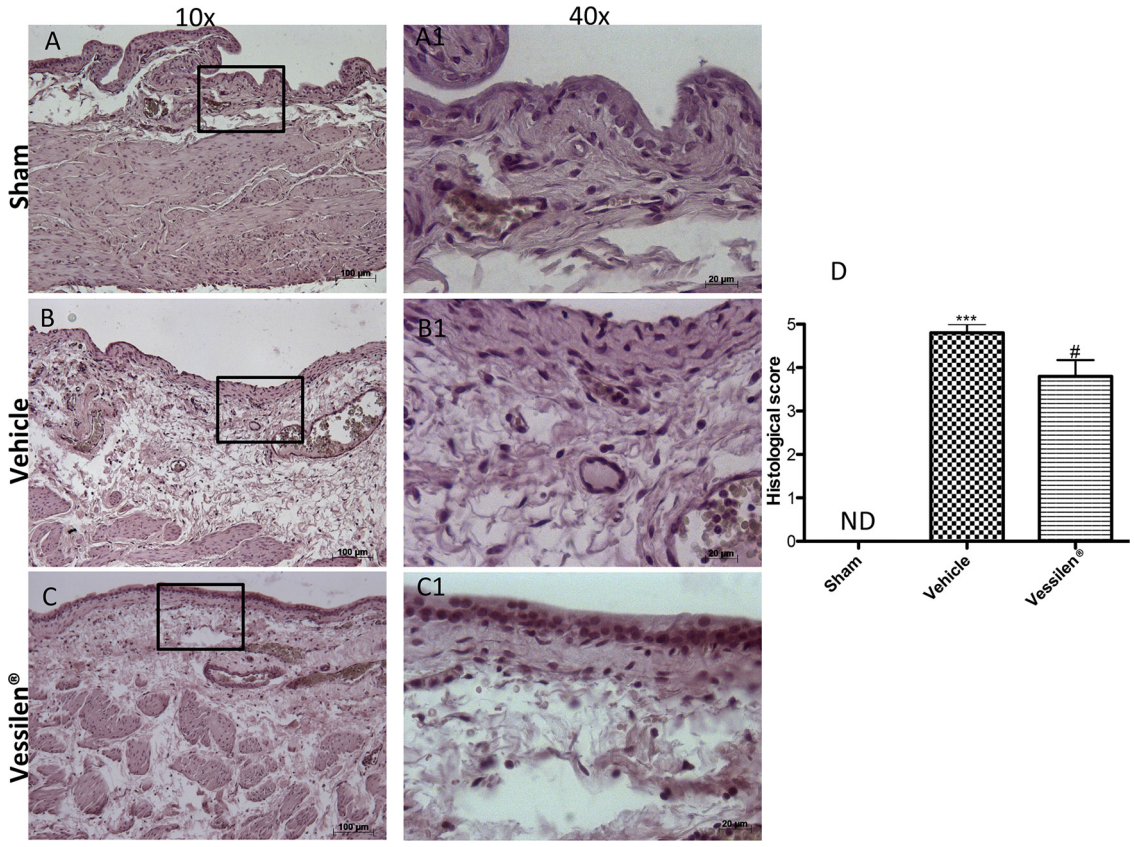

Fig. 2. Acute effects of Vessilen® on bladder histological alterations in CYP-injected rats.
Bladder histology examination in sham rats (A, A1 and D), CYP-injected rats (B, B1 and D) and CYP-injected rats treated with intravesical instillation of Vessilen® (C, C1 and D). (D) Histological score. Figures are representative of at least three separate experiments. ND: not detectable. Values are means ± SEM of 10animals for each group; ***P < 0.001 vs sham; #P < 0.05 vs CYP.

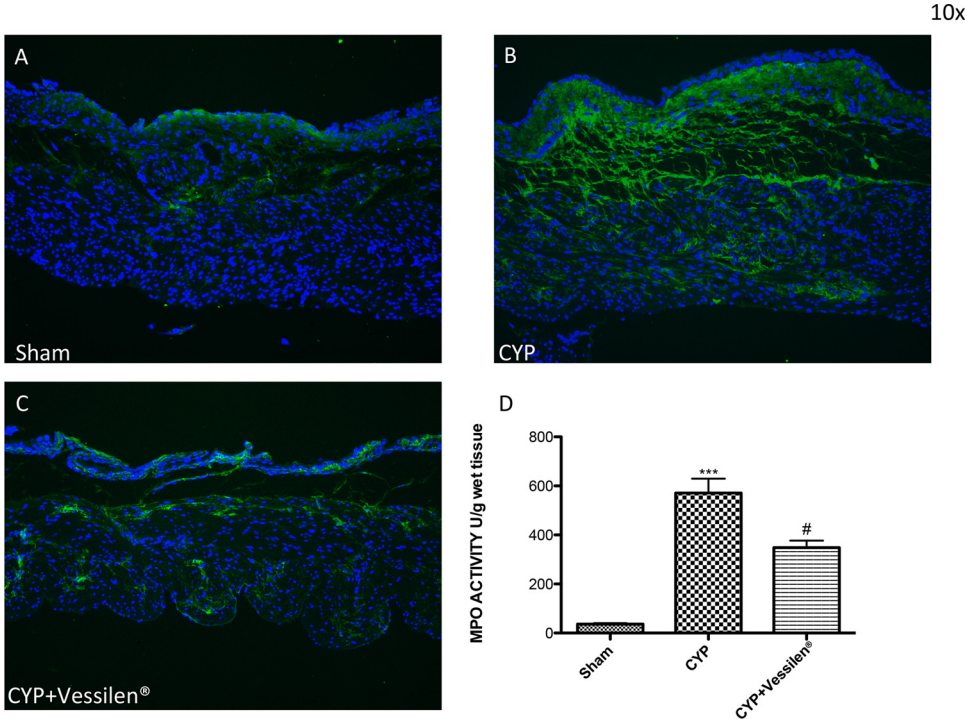

Fig. 3. Acute effects of Vessilen® on neutrophil infiltration in CYP-injected rats.
Immunofluorescence for MPO expression (green color) in sham rats (A), CYP-injected rats (B) and CYP-injected rats treated with intravesical instillation of Vessilen® (C). (D) MPO activity assay. Data are representative of at least three independent experiments. Images are representative of all animals in each group. Images were digitalized at a resolution of 8 bits into an array of 2048 × 2048 pixels. Pictures were captured at 10x magnification. Values are means ± SEM of 10 animals for each group; ***P < 0.001 vs sham; #P < 0.05 vs CYP.

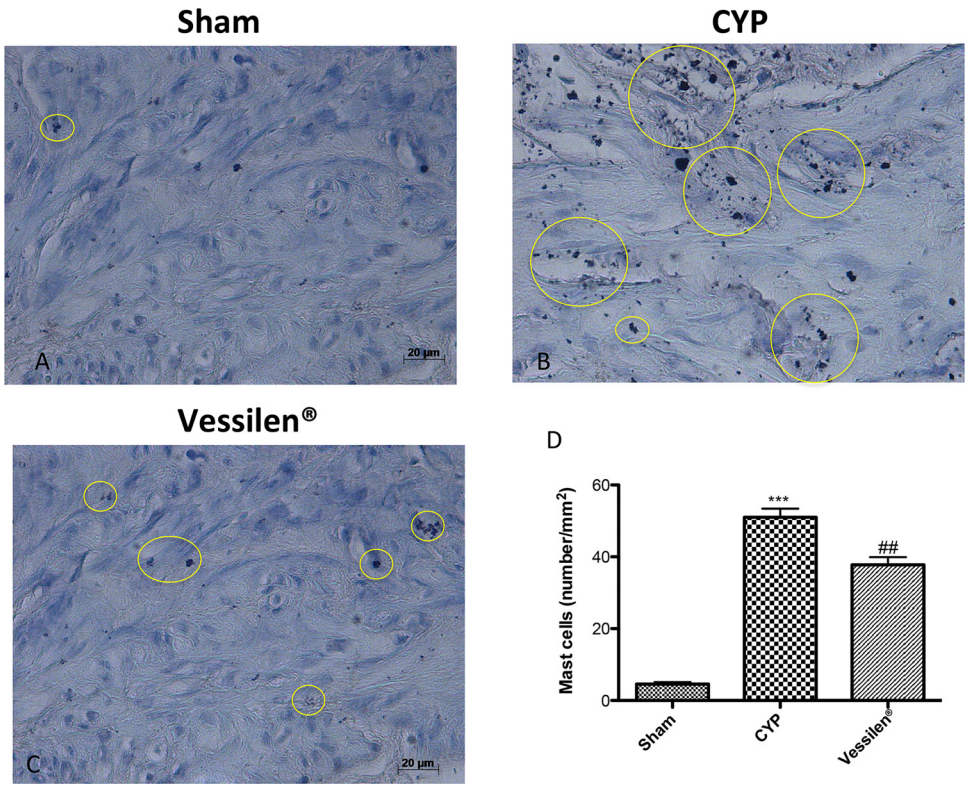

Fig. 4. Acute effects of Vessilen® on mast cell infiltration in CYP-injected rats.
Toluidine blue staining was used to identify MC infiltration (encircled), characterized by dark lilac blue granules: (A) sham group; (B) CYP + vehicle group; (C) CYP + Vessilen® group. (D) Mast cell number per unit area of tissue (mast cell density). Figures are representative of at least three separate experiments. Values are means ± SEM of 10 animals for each group. ***p < 0.001 vs sham, ##p < 0.01 vs CYP.

expression of oxidative stress markers such as nitrotyrosine. An increased expression of nitrotyrosine was found in CYP-injected rats (Fig. 5E) compared to sham animals (Fig. 5D). Treatment with Vessilen® significantly reduced nitrotyrosine formation (Fig. 5 F).

3.1.5. Chronic effects of vessilen® on bladder damage after repeated CYP injections

In mice, repeated CYP injections led to edema formation and bleeding, as compared to sham animals (Fig. 6B, and see D). Vessilen® intravesical instillation reduced this bladder damage (Fig. 6C and D).

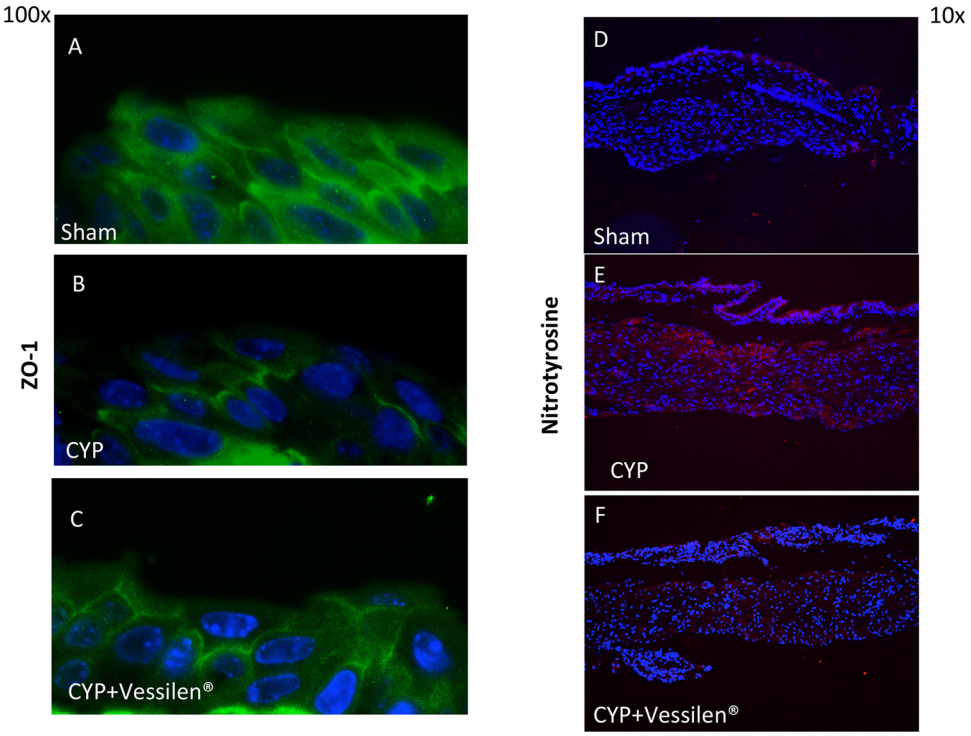

Fig. 5. Acute effects of Vessilen® on nitrotyrosine and ZO-1 expression in CYP-injected rats. Immunofluorescence for nitrotyrosine (red) and ZO-1 expression (green) in sham animals (A), CYP-injected animals (B) and CYP-injected rats treated with intravesical instillation of Vessilen® (C). Data are representative of at least three separate experiments. Images are representative of all animals in each group. All images were digitalized at a resolution of 8 bits into an array of 2048 × 2048 pixels. Pictures were captured at 10x (for nitrotyrosine) and 100x (for ZO-1) magnification. Values are means ± SEM of 10 animals for each group.

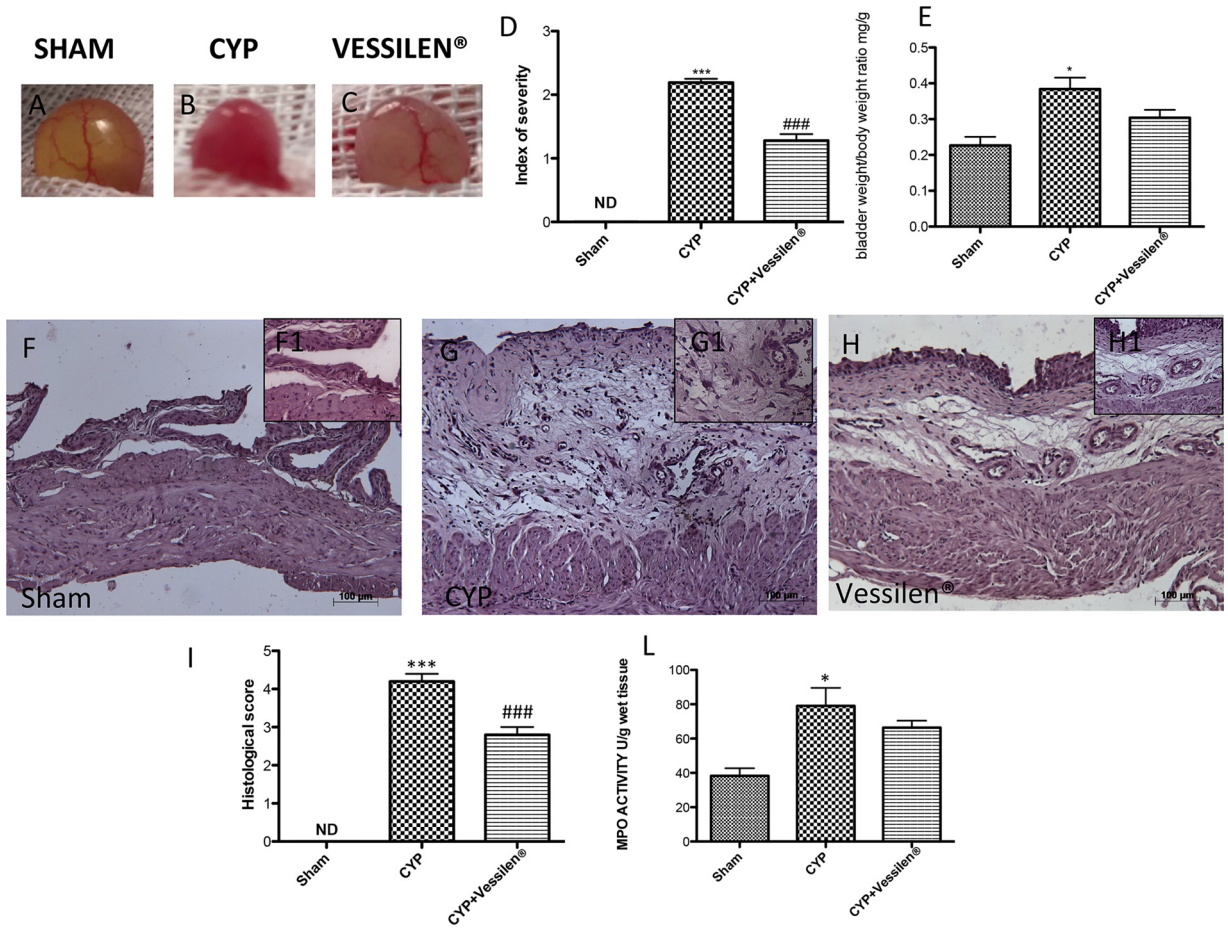

Fig. 6. Chronic effects of Vessilen® on bladder damage, histological score and MPO levels.
Macroscopic damage in sham mice (A and D), CYP-injected mice (B, D), and CYP-injected mice treated with intravesical instillation of Vessilen® (C and D). (E) Bladder weight/body weight ratio. Histological evaluation (hematoxylin/eosin staining): (F,F1) sham group, (G,G1) CYP + vehicle group, (H,H1) CYP + Vessilen® group, (I) histological score, (L) MPO levels. Figures are representative of at least three independent experiments. Values are means ± SEM of 10 animals for each group; *P < 0.05 vs sham; ***P < 0.001 vs sham, ###P < 0.001 vs CYP. ND: not detectable.

Moreover, swelling induced by CYP injections increased bladder weights compared to sham mice (Fig. 6E). Although Vessilen® treatment decreased bladder weight evaluated as bladder weight/body weight ratio the effect was not significant (Fig. 6E).

3.1.6. Chronic effects of vessilen® on bladder histological injury and fibrosis after repeated CYP injections

Mild edema of the submucosa and lamina propria was evident in mice given repeated injections of CYP (Fig. 6G,G1 and I) with neutrophil infiltrates (Fig. 6L), while no bladder damage was observed in sham animals (Fig. 6F, F1, and see I). Vessilen® treatment markedly reduced histological damage (Fig. 6H, H1; see histological score I), but was unable to effect a significant decrease in neutrophil infiltration (MPO activity) (Fig. 6L). Masson trichrome stain revealed a modest increase in fibrosis in inflamed bladders (Fig. 7B,B1 and D) in comparison to control (Fig. 7A,A1 and D). The degree of fibrosis (blue-stained fibrotic area) was larger in the CYP + Vessilen® group than in CYP + vehicle mice (Fig. 7C,C1 and D).

3.1.7. Effect of repeated injections of CYP on pain-related responses, MCs infiltration, and NGF levels

We next investigated the effects of Vessilen® on pain associated to chronic cystitis by the von Frey test. Repetitive injection of CYP gradually decreased each day the withdrawal threshold in response to abdominal stimulation, reaching the lowest level at around day 7 and persisting until day 10 (Fig. 8F). Vessilen® treatment resulted in a significant amelioration of pain-related responses compared to CYP + vehicle mice (Fig. 8F). MCs are important contributors to pain development, releasing vasoactive and inflammatory mediators upon activation, which then trigger inflammation and neuronal hyper excitability. Increased numbers and activation of bladder MCs were found in tissue sections of CYP + vehicle mice (Fig. 8B and D) compared to sham animals (Fig. 8A and D). Vessilen® markedly reduced MC numbers (Fig. 8C and D). Stem cell factor and NGF are major stimulators of MCs migration, maturation, proliferation and activation [5]. An increase in NGF was found in mice subjected to chronic cystitis (Fig. 8E), and Vessilen® treatment reduced NGF expression (Fig. 8E). These effects could, in part be attributable to a reduction in MC infiltration.

3.1.8. Chronic effects of vessilen® on iNOS, IL-1β, MCP-1, NF-κB and IKB-α

To characterize the inflammatory state and possible pathways involved in chronic cystitis, we used Western blotting to analyze the expression of key inflammatory mediators in whole bladders. Chronic CYP treatment resulted in a pronounced increase in iNOS and IL-1β expression compared to sham mice (Fig. 9 C,D). MCP-1 levels were also significantly increased, showing that CYP-treated bladders were subject to monocyte and neutrophil infiltration (Fig. 9E). Vessilen® treatment reduced the levels of these inflammatory mediators (Fig. 9 C,D,E). In addition, repeated CYP injections caused a significant increase in nuclear levels of NF-κBp65 in CYP + vehicle mice compared to sham animals (Fig. 9B). Treatment with Vessilen® prevented activation of NF-κB by reducing nuclear levels of NF- κB p65 (Fig. 9B). In addition, a

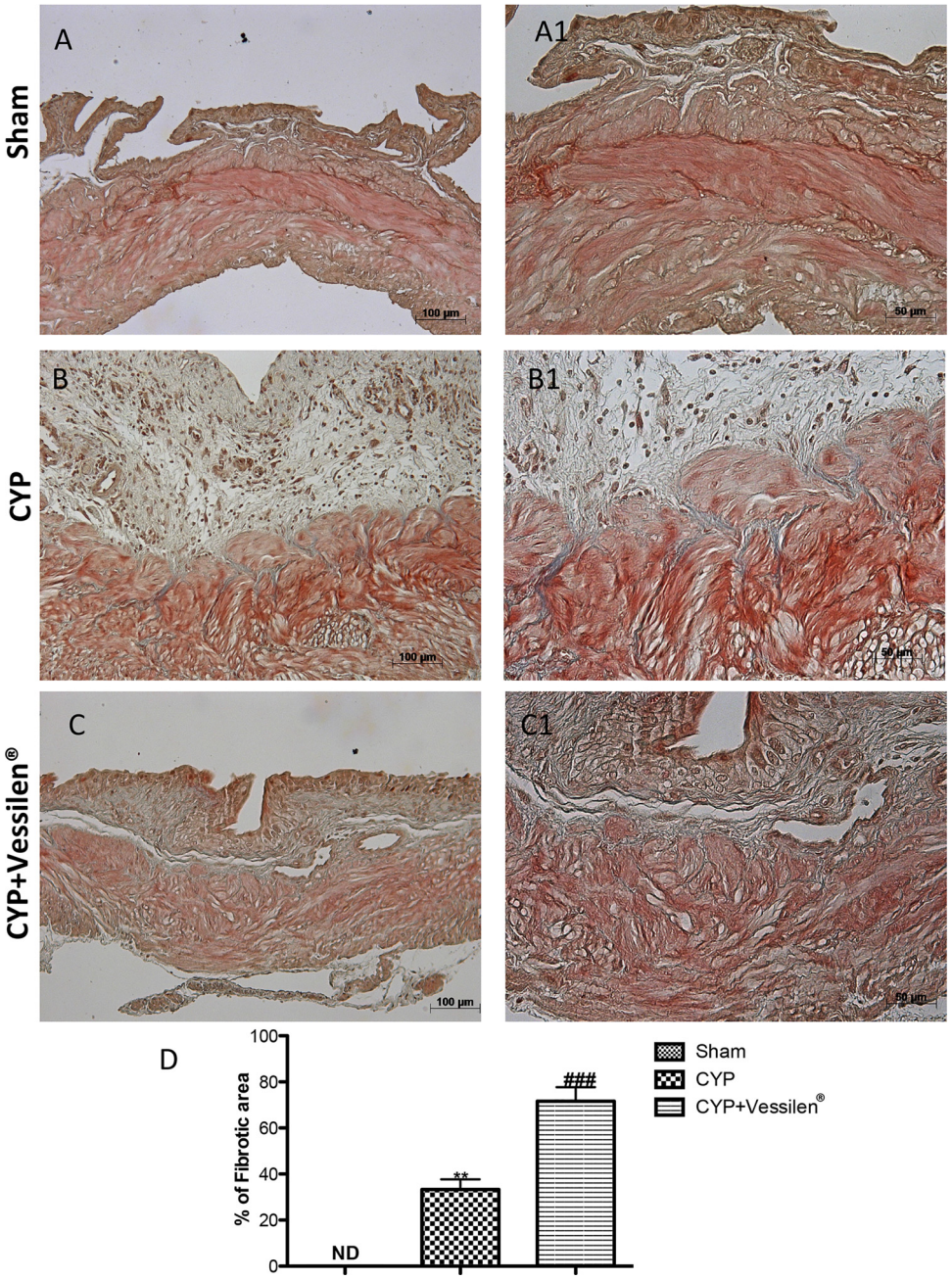

Fig. 7. Chronic effects of Vessilen® on fibrosis in CYP-injected mice.
The degree of fibrosis was evaluated by Masson trichrome staining (blue color). (A,A1) sham group, (B,B1) CYP + vehicle mice, (C,C1) CYP + intravesical instillation of Vessilen®. (D) Percent fibrotic area (blue staining). Figures are representative of at least three separate experiments. Values are means ± SEM of 10 animals for each group; **P < 0.01 vs sham, ###P < 0.001 vs CYP. ND: not detectable.

basal level of IκB-α was observed in bladder tissues from sham animals whereas IκB-α levels were substantially decreased in CYP mice (Fig. 9A). Intravesical instillation of Vessilen® prevented CYP-induced IκB-α degradation by increasing IκB-α levels compared to vehicle (Fig. 9A).

3.2. Clinical studies

Demographic and clinical data of the 128 enrolled patients (111 females and 17 males, aged 20–89 years) are reported in Table 1. Symptom onset was less than 1 year in 17 patients, 1–3 years in 28 patients, and lasting 3 or more years in the remaining 83 patients. Patients were divided into 4 groups, based on clinical history and diagnosis: 58 patients affected by IC (1st group), 9 patients with BPS (2nd group), 35 patients with cystitis (chronic, recurrent, bacterial, attinic,) (3rd group); 26 patients diagnosed with chronic pelvic pain associated with characteristic lower urinary tract symptoms due to concomitant uro-gynecological or prostate complications (abacterial prostatitis urinary incontinence, vulvovaginitis, previous surgical procedures for hysterectomy or prosthetic correction of Pelvic Organ Prolapse Suspension (4th group).

Table 2 shows the % distribution of symptoms presented by the patients at time of inclusion in the study. Sixteen of the initial 128 patients recruited prematurel dropped out of the study for the following reasons: 1 patient after the 1 st bladder instillation for pregnancy; 2 patients after the 2nd and 7th instillations, respectively, for constant

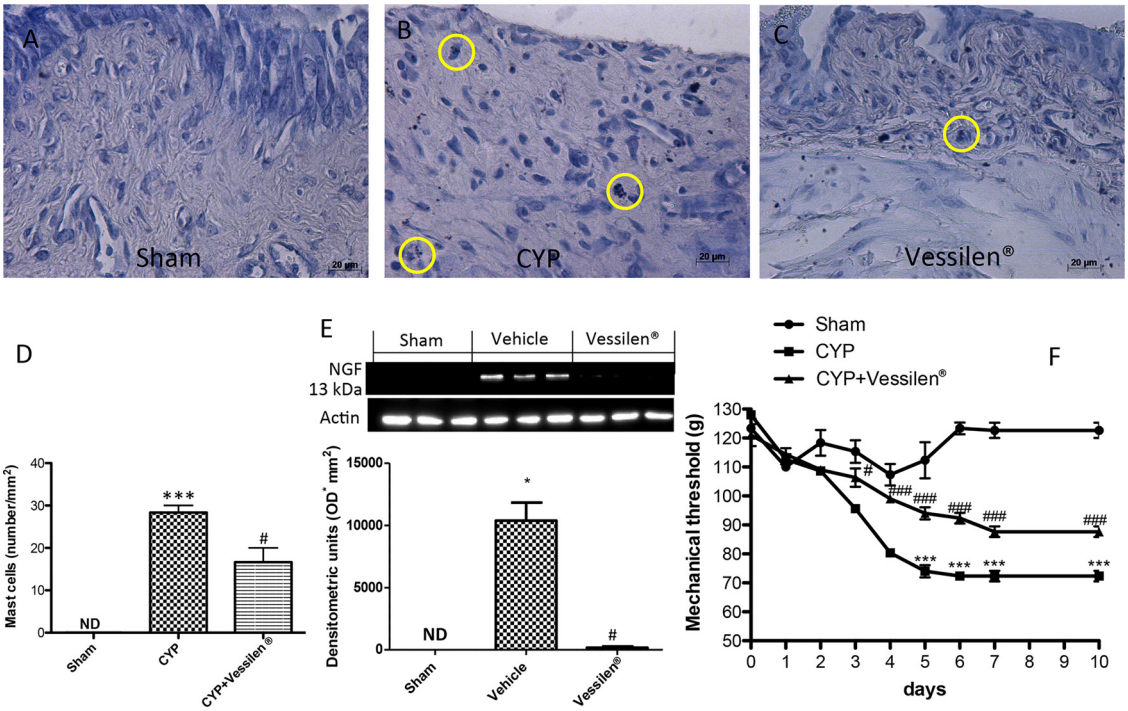

Fig. 8. Chronic effects of Vessilen® on mast cell activation, NGF levels, mechanical allodynia in CYP-injected mice.
Mast cell infiltration was evaluated by toluidine blue staining in the chronic cystitis model. Mast cells (encircled) were characterized by their dark lilac blue granules. (A) sham group, (B) CYP + vehicle group, (C), CYP + Vessilen® group. (D) Mast cell numbers per unit area of tissue (mast cell density). (E) NGF expression evaluated by Western blot in CYP + vehicle mice was significantly reduced by intravesical instillation of Vessilen® treatment. Shown is a representative blot of lysates from 10 animals/group together with the respective densitometric analysis.
(F) Effects of intravesical instillation of Vessilen® on mechanical allodynia evaluated by von Frey test. Figures are representative of at least three independent experiments. Values are means ± SEM of 10 animals for each group. *$p < 0.05$ vs sham, ***$p < 0.001$ vs sham, $^{\#}p < 0.05$ vs CYP, $^{\#\#\#}p < 0.001$ vs CYP.

burning; 1 patient after 4th instillation for hematuria; 1 patient after 5th instillation without giving any justification; 1 patient after 6th instillation for stationary symptoms; lastly 10 patients left the study prematurely (8 after the 4th instillation and 2 after the 6th) because they felt a noteworthy improvement with disappearance of symptoms. The remaining 112 patients completed the established treatment period.

At the end of the treatment period, intensity of pain, urgency, and frequency was significantly lower than baseline ($p < 0.0001$, $p < 0.0002$ and $p < 0.0001$, respectively) (Fig. 10A and Table 3). None of the covariates considered (age, sex, period of disease onset and diagnosis) had any significant influence on treatment efficacy. In particular, mean values, observed over time in the four patient groups divided by diagnosis (Fig. 10B, C and D) showed no significant difference between them. However, there was a significant disparity between the participating urology centers in terms of the scores attributed to the intensity of pain ($p < 0.0003$), urgency ($p < 0.0006$) and frequency ($p < 0.0002$).

A reduction in symptom intensity of 2 or more points on the VAS scale is considered a clinically significant improvement. On this basis, pain symptom improved in 73.2% of the patients, remained unchanged in 23.2% and worsened in 3.6%; urgency improved in 65.2% of patients, was unchanged in 33.9% and worsened in 0.9%. Lastly, frequency improved in 68.8% of patients, remained unchanged in 28.6% and worsened in 2.7%.

At treatment end, average values from self-administered questionnaires were indicative of a positive change in quality of life. The Pelvic Pain and Urgency / Frequency (PUF) patient symptom scale showed a significant improvement ($p < 0.0001$) of both symptoms and bothers. The total PUF scale score also improved significantly over time ($p < 0.0001$) from a mean baseline value of 22.1 ± 0.56 to 14.5 ± 0.65 at the end of treatment (Fig. 11A and Table 4). Even in PUF questionnaire there were no significant differences over time among the four patient groups divided by diagnosis (Fig. 11B, C and D).

Covariate age and diagnosis did not affect significantly the observed effect. PUF scale data appeared to be significantly influenced by the covariates: sex ($p < 0.0286$), and center ($p < 0.0.0484$). Males, compared to females, displayed higher values at all times (23.6 vs 21.9 at T0 and 17.8 vs 14.0 at T7, respectively), Finally, in this evaluation there remained a significant disparity between centers.

Significant improvement was also evident in the assessment of quality of life through the SF-12 questionnaire and in relation to both the mental ($p < 0.0001$) and physical ($p < 0.0001$) components, whose average values increased, respectively, from 34.7 ± 0.94 to 43.2 ± 0.94 and from 33.9 ± 0.80 to 41.4 ± 0.94 (Fig. 12A and Table 5). The mean scores found in these four groups divided by diagnosis appeared superimposable (Fig. 12B and C). Improvement in the mental component was influenced by onset time ($p < 0.0048$). In fact, patients with an onset time of 1 to 3 years had an average improvement of 15.1 points compared to an average improvement of 8.4 points for all other patients.

4. Discussion

IC/BPS is a chronic disorder characterized by frequent periods of pelvic pain or pressure and increased frequency of urination. Patients with IC/BPS or BPS have substantial impairment of mental and physical quality of life, depression, anxiety, and loss of social interactions [28]. The pathogenesis of IC/BPS is still unclear. Several causes have been suggested, including inflammation, MC activation, genetic predisposition, autoimmune mechanisms and neurogenic causes [29]. These circumstances make IC/BPS an especially challenging disease to study and a clear unmet medical need in search of new therapeutic approaches. Adelmidrol, the diethanolamide derivative of azelaic acid, has been

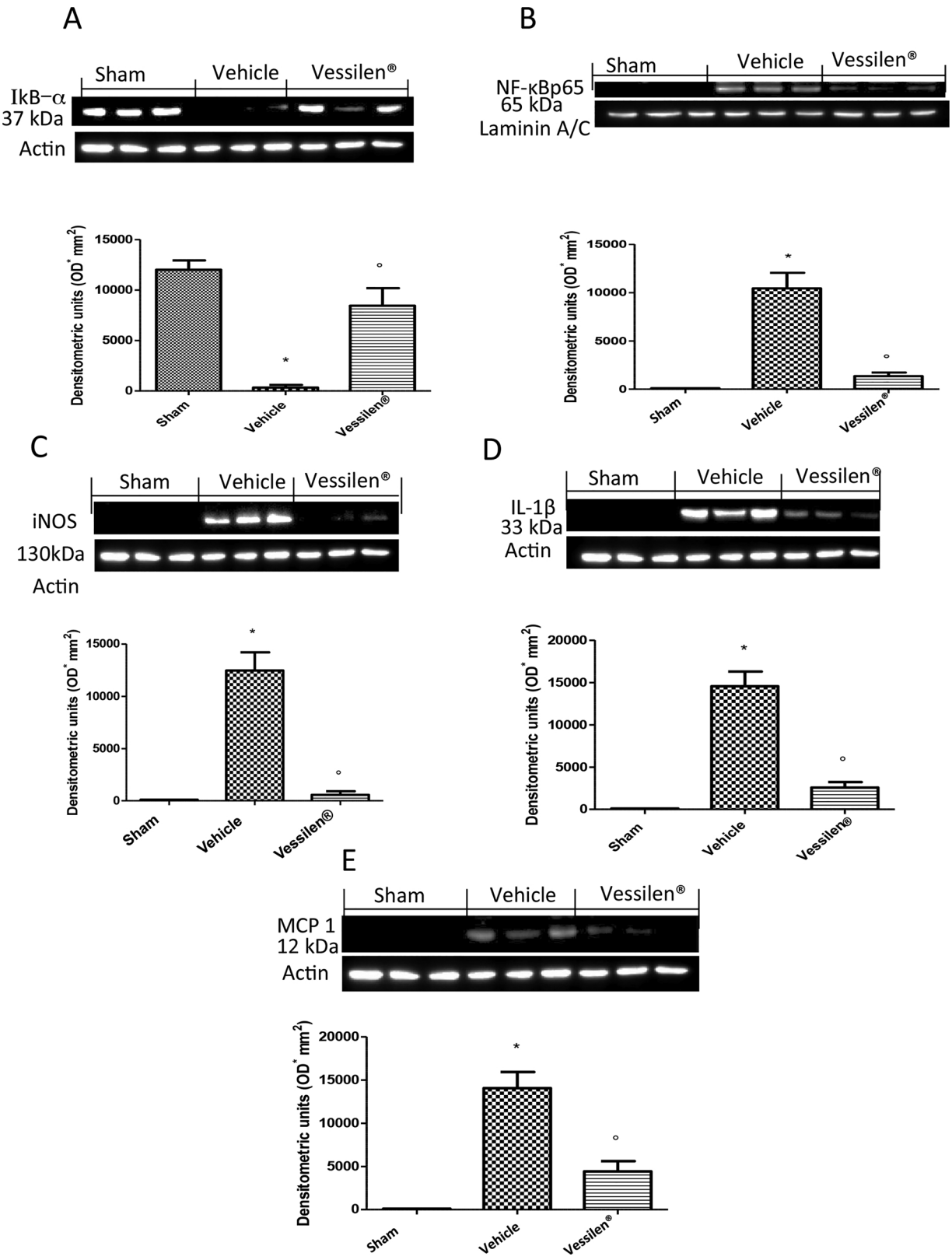

Fig. 9. Chronic effects of Vessilen® on the NF-κB pathway in CYP-injected mice.
Representative Western blots showing the effects of Vessilen® on: (A) IκB-α degradation, (B) NF-κB p65 translocation, (C) iNOS expression, (D) IL-1β expression, and (E) MCP-1 expression after chronic CYP injections. Intravesical instillation of Vessilen® reduced IκB-α degradation (A), NF-κB p65 translocation (B), and expression of iNOS (C), IL-1β (D) and MCP-1 (E). Shown is a representative blot of lysates from 10 animals/group, together with a densitometric analysis for all animals. The results in A, B C, D and E are expressed as means ± SEM of 10 animals for each group. *$P < 0.01$ vs. sham; °$P < 0.01$ vs. CYP.

Table 1
Demographic/clinical data of enrolled patients.

	Patients enrolled (128)	
Gender	F	111 (86.7%)
	M	17 (13.3%)
Age	Min e max	20 - 89
	Median ± SD	58.4 ± 14.1
Onset in years	≤ 1	17 (13.3%)
	1 - 3	28 (21.9%)
	≥ 3	83 (64.8%)
Diagnosis	Interstitial cystitis	58 (45.3%)
	BPS	9 (12.8%)
	cystitis*	35 (50.0%)
	other**	26 (37.1%)

*Chronic, recurrent, bacterial, attinic.
**Abacterial prostatitis, overactive bladder, vestibulitis, previous surgical procedures for hysterectomy or prosthetic correction of Pelvic Organ Prolapse.

Table 2
Distribution of symptoms noted by patients at baseline.

Symptom	Presence (%)
Pain	91.3%
Urgency	83.5%
Frequency	88.2%
Nicturia	70.1%
Hesitancy	14.2%
Weight sensation	44.9%
Tenesmus	35.4%
Dyspareunia	41.2%
Pubic pain	46.5%
Anal pain	22.0%
Other*	7.9%

*Dysuria, retention, Urinary Incontinence by Stress, urethrodynia, vulvodynia.

used successfully to treat human inflammatory disorders and its mechanism of action explored [30]. Adelmidrol belongs to the ALIAmide family [31] with comparable anti-inflammatory and anti-nociceptive proprieties of PEA [32,33] and able to control MCs hyper-reactivity in several pathophysiological conditions [34,35]. In addition, our previous results clearly demonstrated that the combination of hyaluronic acid and adelmidrol improved the signs of osteoarthritis induced by monosodium iodoacetate [18]. The present study was designed to better understand the acute and chronic protective effects by intravesical instillation of a new formulation of 2% adelmidrol + 0.1% sodium hyaluronate 0,1% (Vessilen®) in experimental models of CYP-induced cystitis, and to explore the potential for translatability of the findings to patients with chronic IC/BPS or other bladder inflammatory conditions.

Acute cystitis induced by a single CYP administration is a well-described model, and we further characterized a chronic cystitis model in female mice in the context of its importance to IC/BPS in man. In particular, the effects of Vessilen® on chronic bladder inflammatory conditions were investigated and the possible reactive oxygen species-induced signaling pathways involved.

Evidence from clinical and animal models of acute and chronic urinary bladder inflammation suggest a central role of MCs and sensory nerves [36]. Previous studies pointed to a contribution of sensory nerves and MCs in cystitis, e.g. increased number of MCs and morphological data of MC activation [13,37]. Following on from these earlier reports, we showed that acute and chronic CYP-induced cystitis caused macroscopic and histological bladder alterations, inflammatory cell infiltration, increased MC numbers as well as bladder pain-related behavior, all of which were significantly reduced by Vessilen® treatment.

The urinary bladder reacts to injury with an acute inflammatory response that may be followed by chronic inflammation, repair and fibrosis in patients with chronic cystitis [38]. We observed mild bladder damage and edema with an increased fibrotic area in chronic CYP-

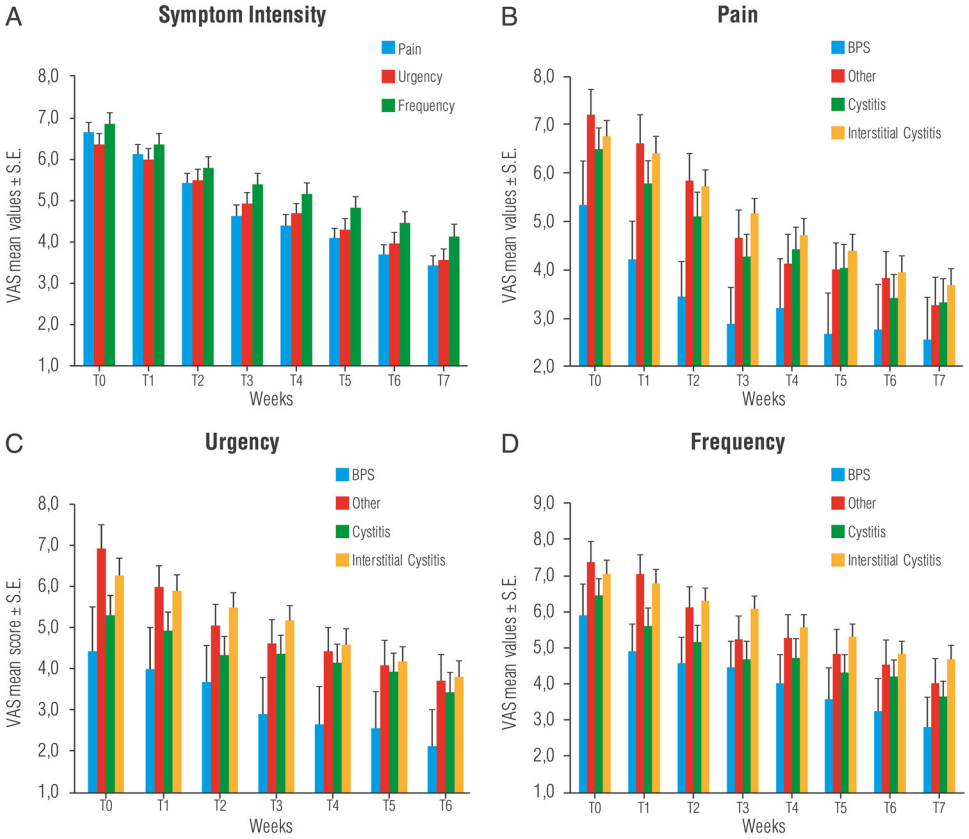

Fig. 10. VAS: Change over time of intensity of symptoms of pain, urgency and frequency. A): Intensity evaluation of each symptom in all patients; B): Pain intensity evaluated in the four patient groups subdivided by diagnosis; C): Urgency intensity evaluated in the four patient groups subdivided by diagnosis; D): Frequency intensity evaluated in the four patient groups subdivided by diagnosis.

Table 3
VAS: mean values (± S.E.) attributed to intensity of pain, urgency and frequency.

Evaluation period	T0	T1	T2	T3	T4	T5	T6	T7	
No Patients	128	127	126	126	117	115	113	112	p
Pain	6.7 ± 0.24	6.1 ± 0.25	5.4 ± 0.24	4.4 ± 0.25	4.1 ± 0.28	4.1 ± 0.25	3.7 ± 0.25	3.4 ± 0.25	< 0.0001
Urgency	6.4 ± 0.32	6.1 ± 0.25	5.5 ± 0.25	4.9 ± 0.26	4.7 ± 0.26	4.3 ± 0.26	4.0 ± 0.26	3.6 ± 0.26	< 0.0002
Frequency	6.9 ± 0.26	6.4 ± 0.27	5.8 ± 0.26	5.4 ± 0.27	5.2 ± 0.27	4.8 ± 0.27	4.5 ± 0.27	4.1 ± 0.27	< 0.0001

treated mice that was improved by intravesical instillation of Vessilen®, suggesting a possible repair-promoting effect. NGF levels are markedly elevated in inflamed tissues, and may contribute to chronic inflammation and damage [39]. An increase in NGF was also found in chronic CYP-induced cystitis with associated inflammatory response and mechanical hyperalgesia, effects that could, in part, be mediated by MCs activation. Vessilen® treatment reduced mechanical allodynia and NGF levels during chronic conditions. This last observation is in agreement with previous studies in which adelmidrol, alone or in combination, limited histological damage, neutrophil and MC infiltration while ameliorating pain-related responses during acute and chronic diseases [17,18,40].

Tight junctions in the urothelium have a principal role in the formation of the blood-urine barrier. Damage to tight junctions could disrupt barrier function and lead to urinary diseases [41]. In the present study immunoreactivity of ZO-1, a tight junction scaffolding protein was decreased in the bladder of CYP-injected mice. Oxidative stress can also contribute to bladder edema, inflammation and extravasation, indicating that reactive oxygen species may play a critical role in experimental models of CYP-induced cystitis [42]. Here, we observed increased nitrotyrosine formation and reduced ZO-1 expression 4 h after CYP injection; Vessilen® treatment significantly diminished nitrotyrosine and augmented ZO-1 expression. These findings posit that Vessilen® improvement of bladder injury could be due also to inhibition of bladder oxidative stress and inflammation.

Table 4
PUF mean values (± SE) attributed to symptoms and discomfort.

Evaluation	Basal (T0)	End of treatment (T7)	
No. Patients	127	118	p
Symptoms	12.3 ± 0.38	7.9 ± 0.38	< 0.0001
Bothers	9.8 ± 0.27	6.5 ± 0.31	< 0.0001
Total points	22.1 ± 0.56	14.5 ± 0.65	< 0.0001

Nitro-oxidative stress activates transcription factors such as NF-κB, which then induce inflammatory proteins (e.g. cyclooxygenase-2, iNOS, tumor necrosis factor-α (TNF-α), and IL-1β) expression during inflammation [43–46]. We observed that chronic cystitis was associated with activation of NF- κB and degradation of IKB-α. Some reports indicate that PEA inhibits activation of the NF-κB system in different experimental models [47,48]. Furthermore, adelmidrol was previously observed to reduce the levels of iNOS, TNF-α and nitric oxide [49]. This is consistent with a recent study in which we showed that adelmidrol

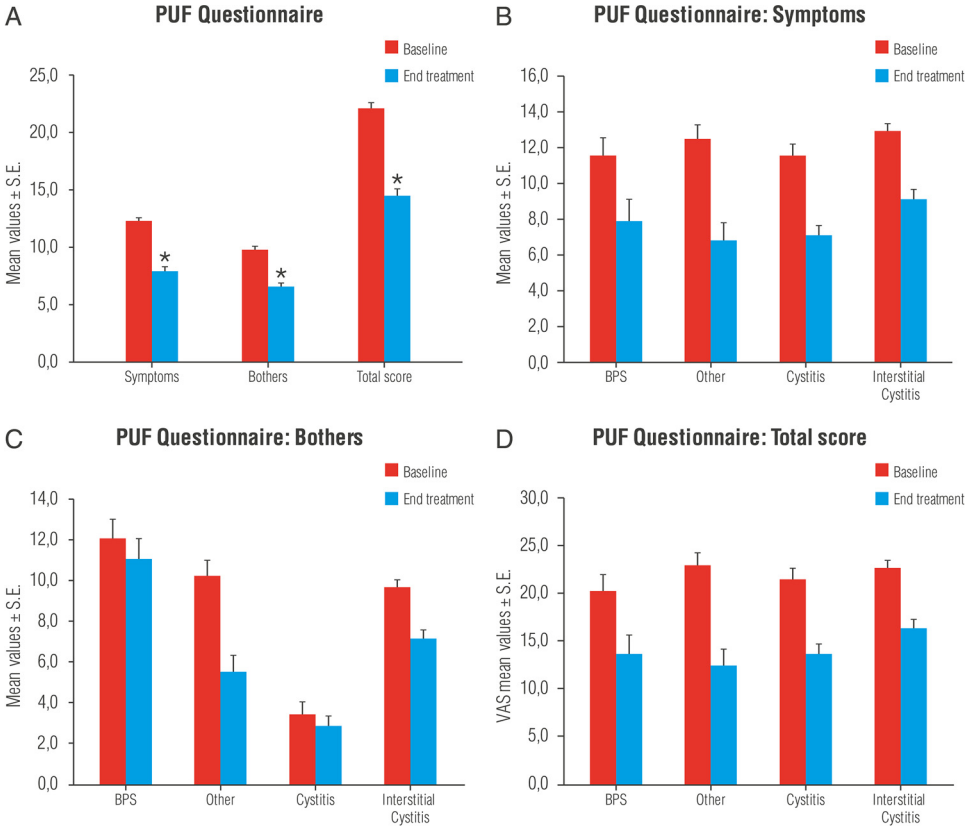

Fig. 11. PUF: Symptom and problem intensity encountered in the Questionnaire and total score.
A): Evaluation of symptom and bother intensity and of Questionnaire total score in all patients; B): Changes over time of symptoms frequency/urgency in the four patient groups subdivided by diagnosis; C): Changes over time of bothers in the four patient groups subdivided by diagnosis; D): Changes over time of Questionnaire total score in the four patient groups subdivided by diagnosis.

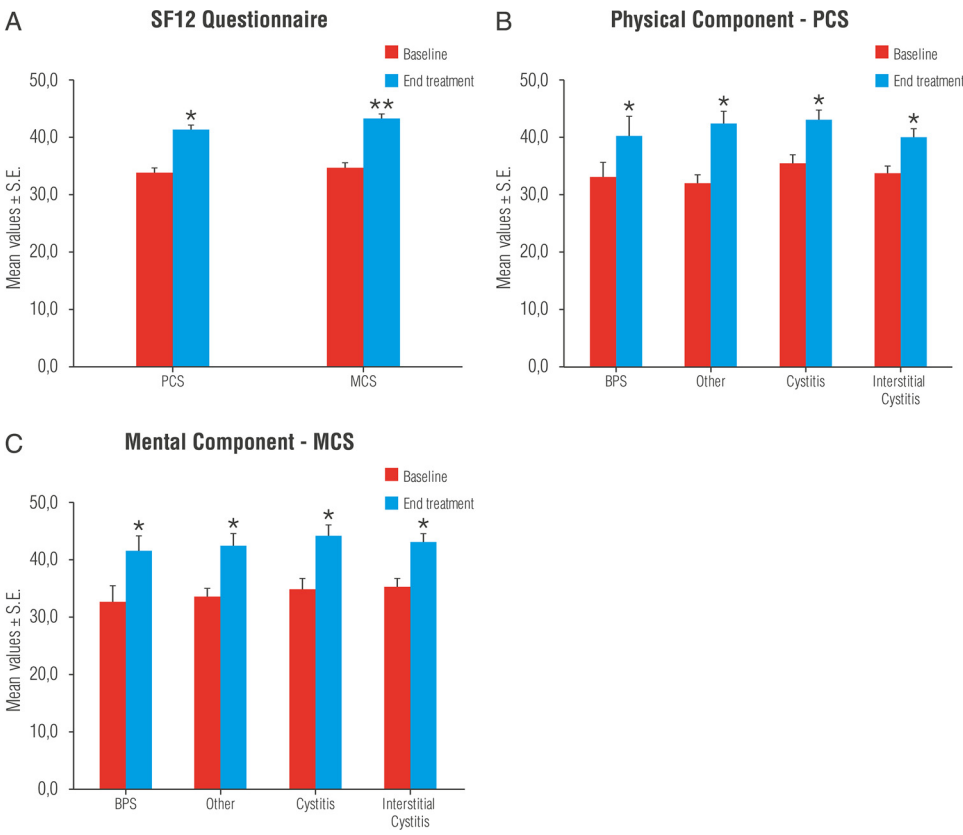

Fig. 12. SF-12: Scores in the physical and mental components of the Quality of Life Questionnaire.
A): Evaluation of the SF-12 and mental components in all patients at baseline and treatment end; B): Changes over time of median score in the SF12 physical components score at baseline and treatment end in the four patient groups subdivided by diagnosis. C): Changes over time of median score in the SF12 mental components score at baseline and treatment end in the four patient groups subdivided by diagnosis.

Table 5
Mean values ± S.E. for Physical Component (PCS) and Mental Component (MCS) of Questionnaire SF-12.

Evaluation	Basal (T0)	End of treatment (T7)	
No. Patients	126	109	p
SF12 - PCS	33.9 ± 0.80	41.4 ± 0.94	< 0.0001
SF12 - MCS	34.7 ± 0.94	43.2 ± 0.94	< 0.0001

reduced inflammatory mediator levels in a model of carrageenan-induced paw edema [17]. Here, Vessilen® was also able to inhibit the NF-κB pathway and decrease levels of TNF-α, IL-1β, iNOS and MCP-1 in the chronic model of cystitis.

An important aspect of research efforts on IC/BPS is being able to gain new knowledge on the underlying biological processes that allows for translatability to man and therapeutic intervention. The translatability of experimental studies on IC/BPS to the human IC/BPS remains a topic of much debate (for example, genetic differences, the absence of key features of the syndrome, large variation in clinical presentation) [11]. For this reason, the current report was designed to incorporate an observational study in patients suffering from pathologies related to an inflammatory, traumatic or iatrogenic alteration of the urothelium and relieved by Vessilen® bladder instillation. The significant mitigation of the pain, urgency and frequency intensity, characteristic symptoms of IC/BPS or of conditions associated to local inflammation, urothelial lesions, voiding dysfunctions and pelvic/perineal pain, observed after a weekly treatment for 8 weeks, confirms the accompaining preclinical data. Vessilen® by improving patient quality of life may, in turn, reduce pain-related disability. The sustained improvement of symptom intensity and tolerability of Vessilen® treatment encourages with further clinical studies evaluating its long - term effects in patients suffering from IC/BPS or other bladder diseases.

5. Conclusion

In conclusion we demonstrate, for the first time, that intravesical administration of adelmidrol in combination with sodium hyaluronate (Vessilen®) has an anti-inflammatory effect in acute and chronic animal models of cystitis inflammation. This action may be due, in part, to modulation of MCs activation and mediator release as well as to inhibition of reactive oxygen species-induced inflammatory pathways such as NF-κB. Moreover, these observations show the potential for translatability of a positive outcome in an animal cystitis model to patients suffering from IC/BPS or other bladder disorders.

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Appendix A

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