# Chondroitin sulfate GAG-replenishment therapy and its effect on restoring the barrier function of urothelium in an experimental model for BPS/IC.

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**Abstract**

Background and Purpose Bladder pain syndrome/interstitial cystitis (BPS/IC) has been clinically treated with glycosaminoglycan (GAG) replenishment therapy. [1, 2] This study was designed to further understand the physiological mechanism behind chondroitin sulfate (CS) treatment and to determine the effect of CS-therapy on recovery of urothelial barrier in an in-vitro chronic injury model. Experimental Approach In differentiated porcine urothelial cells the functional barrier was measured by transepithelial electrical resistance (TEER). A chronic urothelium was inflicted by multiple protamine instillations (3/day for 3 days), to approximate BPS/IC urothelium conditions. CS was instilled afterwards. Recovery of barrier function was followed in time. Additional analyses were performed for immunohistochemistry for barrier markers (tight junctions, GAG's, umbrella cells) and scanning electron microscopy. Statistics were described by means ± standard error, $\alpha = 0.05$. Key Results Barrier recovery (TEER) improved significantly with CS instillations compared to protamine only (T=7, 899.1 [.cm2] versus 589.6 [.cm2], p<0.001, 95% CI -394;-255). This recovery effect was seen on all three days and resulted in a significantly higher average TEER value in the CS group after 3 days (2606 .cm2 vs 750.5 .cm2). Immunohistochemistry and scanning electron microscopy showed decreased barrier markers after protamine treatment and enhanced recovery of urothelial GAG's and other barrier markers after therapeutic instillations. Conclusion and Implications GAG replenishment with CS can improve recovery of barrier function of chronically damaged urothelium in-vitro. This preclinical study supports the hypothesis behind the use of clinical GAG replenishment therapy for patients with a chronically impaired urothelium.

**Title** : Chondroitin sulfate GAG-replenishment therapy and its effect on restoring the barrier function of urothelium in an experimental model for BPS/IC.

**Short running title:** CS replenishment therapy in*in-vitro* model for BPS/IC.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

**Abstract**

**Background and Purpose** Bladder pain syndrome/interstitial cystitis (BPS/IC) has been clinically treated with glycosaminoglycan (GAG) replenishment therapy. [1, 2] This study was designed to further understand the physiological mechanism behind chondroitin sulfate (CS) treatment and to determine the effect of CS-therapy on recovery of urothelial barrier in an *in-vitro* chronic injury model.**Experimental Approach** In differentiated porcine urothelial cells the functional barrier was measured by transepithelial electrical resistance (TEER). A chronic urothelium was inflicted by multiple protamine instillations (3/day for 3 days), to approximate BPS/IC urothelium conditions. CS was instilled afterwards. Recovery of barrier function was followed in time. Additional analyses were performed for immunohistochemistry for barrier markers (tight junctions, GAG's, umbrella cells) and scanning electron microscopy. Statistics were described by means ± standard error, $\alpha = 0.05$.**Key Results** Barrier recovery (TEER) improved significantly with CS instillations compared to protamine only (T=7, 899.1 [.cm$^2$] versus 589.6 [.cm$^2$], p<0.001, 95% CI -394;-255). This recovery effect was seen on all three days and resulted in a significantly higher average TEER value in the CS group after 3 days (2606 .cm$^2$ vs 750.5 .cm$^2$). Immunohistochemistry and scanning electron microscopy showed decreased barrier markers after protamine treatment and enhanced recovery of urothelial GAG's and other barrier markers after therapeutic instillations.**Conclusion and Implications** GAG replenishment with CS can improve recovery of barrier function of chronically damaged urothelium*in-vitro* . This preclinical study supports the hypothesis behind the use of clinical GAG replenishment therapy for patients with a chronically impaired urothelium.

**Key words:** Bladder pain syndrome / Interstitial cystitis, GAG-therapy, *In-vitro* study

**Introduction**

The exact aetiology of BPS/IC is unknown or at least debated. Literature describes certain features that characterize BPS/IC in general. These include a deficient urothelial barrier function and bladder wall inflammation. The influx or presence of immune cells in the bladder wall is well documented, and the hypothesis of a leaky or impaired urothelium is referred to in the ESSIC consensus statement.[3] Normal healthy urothelium expresses barrier markers that contribute to the impermeability of the bladder wall to urine and its solutes.[4] In most patients with BPS/IC, histology shows a deficiency of these barrier markers such as uroplakins, GAGs, tight junctions and adherence junctions.[5, 6] This has formed the leaky epithelium hypothesis implying that bladder pain and inflammation is generated by increased permeability of the urothelium.

Of the deficient barrier markers, the glycosaminoglycans (GAGs) are of special interest because of their location in healthy urothelium, their barrier properties and as a target for treatment. The bladder con-

tains different GAGs including chondroitin sulfate(CS), heparan sulfate (HS), dermatan sulfate, heparin and hyaluronic acid. [7]

GAG therapy, both as instillations or oral treatments, are still used widely for the treatment of BPS-IC and other inflammatory bladder conditions, although with mixed levels of recommendations in guidelines. [1, 2] The primary aim of GAG therapy is to improve barrier function and recovery of the chronically damaged urothelium. [8] Currently clinical research still outweighs the efforts done to investigate the physiological mechanisms behind GAG therapy.

The exact location and barrier function of different GAGs in the urinary bladder have only been established in the last decade and currently there is still limited preclinical evidence of the physiological effects of oral medication such as pentosan polysulfate or intravesical GAG replenishment therapy on bladder barrier recovery.

CS is a GAG that is abundant in different areas in the bladder wall such as the lamina propria.[15] Most importantly, CS forms a layer covering the luminal side of the urothelium and thus forms the first line of defence against urinary solutes.[15] *In-vitro* studies have confirmed the barrier properties of the GAG-layer by transepithelial electrical resistance (TEER) before and after selective removal of CS using enzymatic digestion.[9] This enzymatic CS digestion model was later used in two in vivo studies that confirmed the role of CS in urothelial barrier. Offiah et al. also demonstrated that by removing CS and HS with enzymes in rats, it creates an inflammatory and pain pathway cascade that is comparable to what is observed in BPS-IC patients. [10, 11]

*In vitro* models can focus on isolated features of BPS/IC such as a deficient urothelial barrier, this allows for controlled conditions. Most of the known studies *in-vivo* and *in-vitro* , are performed using acute models. This implies mostly a single exposure of the urothelium to a harmful substance.[8] However, the chronic aspect of BPS/IC is one of its main features. This *in-vitro* study was aimed to 1) 1) to evaluate the therapeutic efficacy of CS replenishment therapy in a chronically damaged urothelial model and 2) establish the effects of chronic damage on the functional barrier and recovery of the bladder urothelium in a controlled environment.

## Materials and methods

Urothelial cells procured from porcine bladders were isolated and cultured according an adapted protocol as described by Fraser.[12] The cells were cultured in Primaria T75 flasks in keratinocyte serum-free medium (K-SFM) (Sigma Aldrich). The K-SFM was supplemented with 0.05 mg/mL bovine pituitary extract, 0.005 µg/mL recombinant epidermal growth factor (Gibco, Life Technologies), penicillin/streptomycin 1% (vol/vol) and 30 ng/mL cholera toxin. At the third passage the cells were seeded on slides for immunohistochemical staining and on inserts (360.000 cells/$cm^2$ , transwell Costar 24-wells plate; diameter 6.5mm; pore size 0.4 µm) with a porous PET membrane for functional barrier measurements. When confluency was reached, the medium was supplied with 5% fetal calf serum (FCS) and 2mM calcium chloride ($Ca^{2+}Cl$) to induce terminal differentiation. [8] Terminal differentiation was confirmed with TEER measurements and immunofluorescence analyses.

### *Histological effects*

Immunofluorescence (IF) staining was performed to evaluate the histological effects of protamine exposure (inflammation) and GAG-replenishment on urothelial barrier markers: chondroitin sulfate (CS), tight junctions and adherence junctions (Table 1). To induce mild-to-moderate inflammation, the cells were treated with protamine sulfate 1400 IE/mL for 4h at 37°C in 5% CO2 (LEO Pharma, Neu-Isenburg). [13] The cells were treated according to three different instillation protocols: 1) protamine sulfate (4h), 2) protamine sulfate (4h) & CS 0,2% (2mg/mL) (Gepan Instill, Pohl-Boskamp GmbH & Co., Hohenlockstedt) (1h) and 3) controls; instilled with Hank's balanced salt solution (Thermofisher, catalogusno. 88284). Samples were then treated with 3% paraformaldehyde in PBS and air-dried. After this, samples were rinsed with 0.2% triton in PBS and incubated with primary and secondary antibodies for respectively 60 and 30 min. A list

of antibodies is described in table 1. Slides were mounted using Dako mounting medium. Bright-field and epifluorescent binocular microscopy (Zeiss) and ImageJ (version 1.46) software were used for analysis.

*Scanning electron microscopy*

Three inserts were, after treatment according to the instillation protocol described above, fixed using glutaraldehyde 2.5% (EM Grade 1.06 Polysciences). This was followed by incubation with increasing concentrations of ethanol. After incubation in 100% ethanol, critical point drying (CPD) was performed. The dry inserts where then sputtered with gold particles as final preparation for scanning electron microscopy. The same protocol was followed as Janssen et al. [9]

*Functional measurements*

Urothelial barrier function was measured using transepithelial electrical resistance (TEER) (EVOM2 Epithelial Voltohmmeter (World Precision Measurements, Sarasota, Florida) on differentiated porcine urothelial cells. When the TEER reached a value of at least 500 .cm$^2$, which corresponds to a tight epithelium, samples were included for experiments. [14] A chronic inflammation model was created by exposing the samples to protamine treatments for three consecutive days. Samples (n=12) were treated with protamine sulfate 1400IE/mL (4h) followed by CS 0.2% treatment (1h) and other samples (n=12) with protamine sulfate only. After treatments, culture medium was placed in all samples. Baseline TEER was measured right after this (T=0) and at 7 hours (T=7). This protocol was repeated for three consecutive days. See figure 1.

**Results**

*Immunohistochemistry*

The positive IF staining for cytokeratin confirms the epithelial origin of the cells (figure 2a). In general, protamine treatment increases extracellular margins between adjacent cells and adherence junction formation is more disrupted (figure 2d-f). IF analyses showed a clear signal of occludin all along the cell borders in the controls (figure 2g). In both intervention groups this signal was reduced, and tight junctions were more interrupted across the cell borders, but still clearly visible. CS56 shows chondroitin sulfate is present in controls, but decreases after protamine treatment (figure 2j-k). Even after removing CS treatment by rinsing the inserts, IF analyses demonstrated an increased signal in the CS treated samples compared to controls and protamine alone samples (figure 2l).

*Scanning electron microscopy*

Images from the negative control and the protamine treated inserts are quite similar (figure 2m-n). The protamine treated samples showed more cracks in the urothelial cell surfaces. The samples that received protamine and CS treatments showed a distinct layer covering the urothelial cell membranes and cell borders, implying the luminal cells were coated with a mucous-like structure (figure 2o).

*Barrier measurements*

Mean TEER recorded before treatment was 2469 +- 753.6 [?]cm$^2$ and corresponds to a tight epithelium. The untreated samples remained stable during the experiment, with a mean TEER at T0 of 4051 +- 299 .cm$^2$ and at T7 of 3940 +- 372 .cm$^2$. In this chronic model, protamine treated samples had a significant lower start TEER in comparison with untreated samples ( 489.1+- 292 [[?]cm$^2$] vs 4051.7 +- 299.5 [[?]cm$^2$], $p < .001$). At the start of the experiment (right after protamine and CS treatment) the treated groups were homogenous in TEER, respectively 563.5 +- 20.4 [?]cm$^2$ and 589.6 +- 27.5[?]cm$^2$ (see table 2), therefore showing that CS solution does not give a direct TEER increase.

As seen in figure 3, CS treatment has a positive effect on recovery of TEER, this was observed on all 3 days and effects were more profound in time, leading to TEER values more than 3 times higher compared to non-CS treated samples (1598.5 +- 95.1 [?]cm$^2$ vs 544.8 +- 138.8 [?]cm$^2$, p=.001). The recovery rate (%) from baseline increased for both groups, but absolute values of TEER for the protamine alone group drop

behind. On day 2 and 3, TEER start and end values were significantly lower ($p<.001$), see table 2 and figure 3.

**Discussion**

This study shows the functional effect of exogenous CS instillations on urothelial barrier function after repeated damage with protamine*in-vitro* over the course of several days, to mimic the chronically impaired urothelium in patients suffering from BPS/IC. Protamine instillation impaired relevant barrier markers such as tight junction formation as was shown by immunohistochemistry.

Urothelial production of CS containing proteoglycans was confirmed with the IF assay and instillation of exogenous CS increased this signal clearly. The SEM analysis showed that CS instillation creates a mucous like covering layer on the urothelium, implying adherence of CS to the urothelium.

Although BPS/IC is a chronic condition, most known *in-vitro* and*in-vivo* studies focus on the acute effects of damage on the urothelium.[15][16][36][37] By using a chronic inflammatory *in-vitro* model, we were able to qualitatively investigate evident features of GAG therapy being barrier function enhancement and barrier recovery.

The culturing protocols and in vitro set-up of the experimental demonstrated a high degree of reproducibility.[15][16] TEER measurements can be conducted repeatedly in time without altering cell health and barrier function. To qualify as a representative urothelial barrier model, terminal differentiation is essential and this feature lacking in many preclinical studies who often use undifferentiated cell lines. TEER measurements demonstrate how much barrier properties vary between undifferentiated (100 $.cm^2$) and differentiated (>1000 $.cm^2$ ) urothelial layers and shows how tight a urothelial barrier is compared to other epithelia.[26]

Our in vitro model shows evident plasticity of urothelial cells with a remarkable recovery rate after multiple damaging events. Nonetheless, the TEER levels in the protamine alone treated samples remained approximately 5-6 times lower compared to pre-treatment throughout the experiments, corresponding to a chronic damaged barrier. This was also supported by the immunohistochemistry results that showed a decreased signal for adherence junctions, tight junctions and chondroitin sulfate which is comparable to the histology as is seen in BPS/IC.[6]

Experiments show that CS instillations increase the regeneration of urothelial barrier properties and thereby improves urothelial recovery after damage. CS treatment resulted in complete recovery of barrier after 3 days and even higher TEER. Results also show that CS treatment has a relatively larger effect on barrier repair after multiple protamine hits compared to controls. This could imply that it has a larger therapeutic effect in bladders with chronic urothelial damage. IF analyses showed the urothelium surface was covered with CS after treatment. This finding is in line with the animal study by Kyker et al. that showed adherence of CS to damaged urothelium, by installing labelled CS in damaged mouse bladders.[15] Hauser et al. also showed recovery of the barrier function after intravesical CS in a rat model of bladder damage as measured by permeability to a potassium ion mimetic.[16] Our results do not clarify what an optimal dosing scheme would be for clinical CS treatment.[17, 18]

The experimental data does not demonstrate a direct effect of CS on barrier (TEER). CS-treatment protects cells against the secondary harmful effects after a damaging event, possible hypothesis could be preventing exposure of cell membranes to hazardous ions or molecules. Or it could stimulate barrier regeneration via an undetermined signalling cascade. A recent publication by Rooney et all showed that HA+CS treatment stimulates the endogenous production of chondroitin sulfate in cultured urothelial cells. This effect on endogenous production of GAGs has not been investigated for CS separately thus far. [19] Inflammatory markers or growth factors were not evaluated in this study, but Stellavato et all also showed a reduction of interleukins (6 & 8) after GAG therapy.[20]

The *in-vitro* model offers the possibility to investigate the effects of therapy on the functional barrier of damaged urothelium that can be measured more precisely compared to in vivo models because it uses fixed surface areas and controlled cell densities. So far, this has only been conducted for CS, but other

intravesical compounds for BPS/IC such as heparin, hyaluronic acid and pentosan polysulfate could also be analysed and compared to each other.[21] Although a single randomized study showed an advantage for CS instillations compared to HA in BPS/IC.[22] To our knowledge, CS is the only sulphated GAG observed in the luminal layer of healthy urothelium, although presence of heparan sulfate was detected in deeper layers of the urothelium.[9] Especially the functional barrier properties of hyaluronic acid and pentosan polysulfate deserves more attention since these are also commonly used in daily clinical practice for BPS/IC treatment.

**Conclusion**

In chronically injured urothelium, GAG-replenishment therapy with CS has a beneficial effect on the recovery of barrier function after damage.

**Bullet point summary:**

*What is already known?*

GAG therapy are widely used for the treatment of BPS/IC and other inflammatory bladder conditions.

The aim of GAG-therapy is to improve barrier function and recover the chronically damaged urothelium.

*What this study adds:*

The effect of CS-replenishment therapy on barrier recovery in a chronic damaged setting.

*Clinical significance:*

BPS/IC is a chronic condition of the bladder, this study will add evidence for GAG-therapy.

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**Table 1. Antibodies**

| Antibody | Manufacturer | urothelial structure |
|---|---|---|
| RGE53 | Mubio | Cytokeratin18 |
| CS56 | Santa Cruz | Chondroitin sulfate |
| Anti-occludin | Invitrogen | Tight junctions |
| B-catenin | BD Biosciences | Adherence junctions |

**Table 2. Daily TEER measurement**

| Protamine TEER [.cm$^2$] N=12 | Protamine TEER [.cm$^2$] N=12 | Protamine TEER [.cm$^2$] N=12 | Protamine TEER [.cm$^2$ |
|---|---|---|---|
| | **Start** | **SD** | **end** |
| Day 1 | 563.5 | ±71 | 422.8 |
| Day 2 | 359* | ±85 | 352.3 |
| Day 3 | 544.8 | ±481 | 750.5 |

Average TEER (measured after protamine treatment) on three consecutive days at start and end with corresponding standard deviations (SD). * indicate a statistical significance of $p<0.001$ between groups and

# within groups.

**Figure 1 – daily timeline**

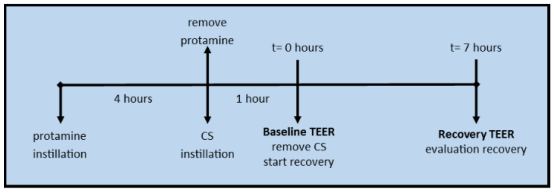


**Figure 2 – immunohistochemical staining and scanning electron microscopy**

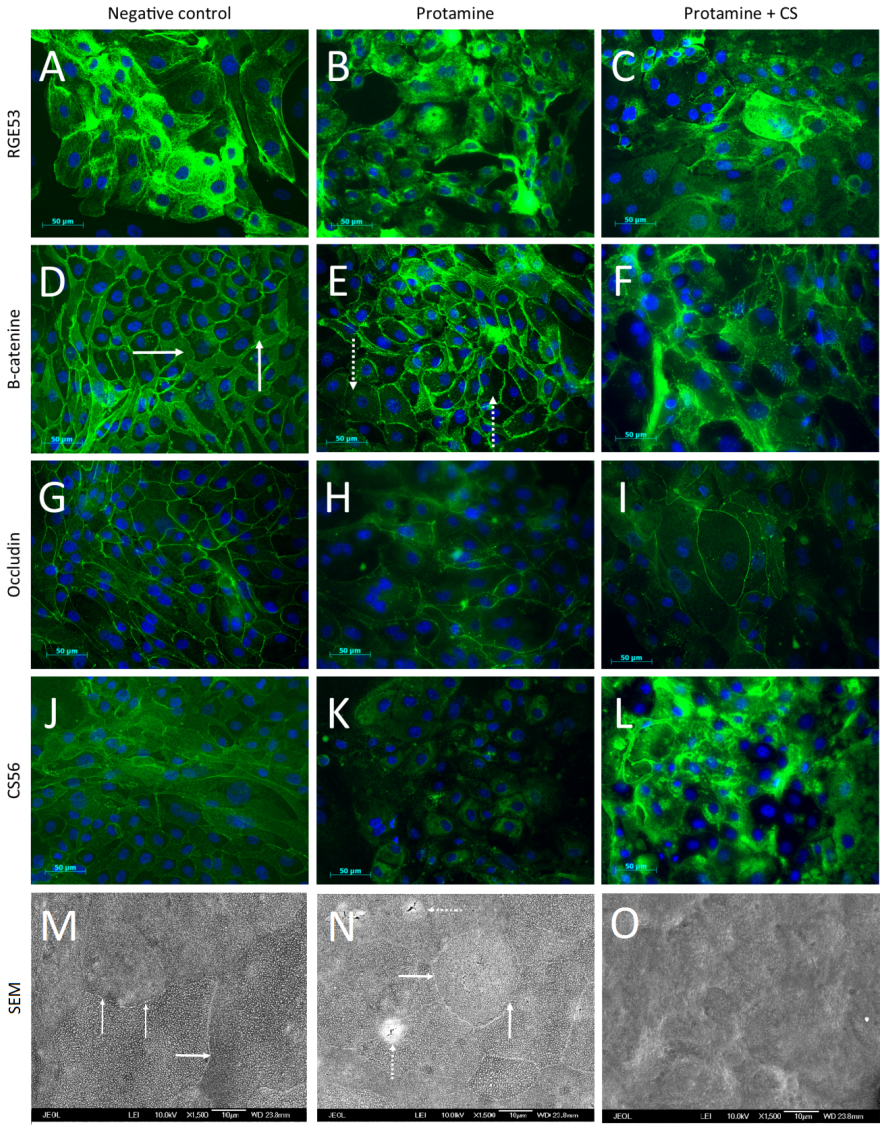


**Figure 3 TEER measurements day 1,2,3 for protamine- and protamine + CS group**

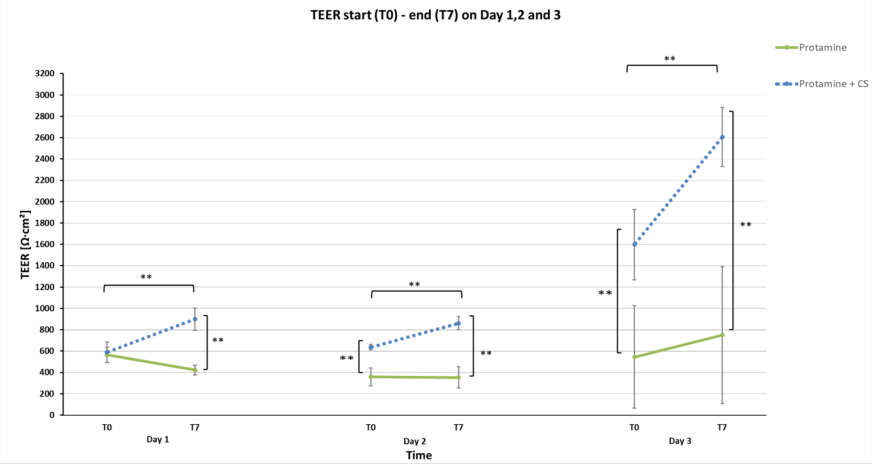


**Figure legends**

Figure 1.

Timeline: instillations with protamine and CS in the appointed study group was performed first over the course of 5 hours. The baseline for TEER measurements was started right afterwards at t=0 hours. At t=7 hours the daily endpoint for recovery was set and evaluated.

Figure 2.

Immunohistochemical staining and Scanning electron microscopy (SEM): Figure 2A-2C show staining for RGE 53 showing cytokeratin. Figure 2D-2F show staining for B-catenin which shows the adherence junctions. 2D: white arrows depicting columnar appearance of signal from the luminal side down to the basal side of the cell. 2e: dashed white arrow depicting merely the cell border signal. 2G-2I staining for occluding showing the tight junctions. 2J-2L: CS56 staining for chondroitin sulfate with a more diffuse signal with intense cell borders in 2J, less signal in 2K en more intense signal in 2L. In the negative control group (2M) cell borders are clearly visible with intact cells. In the protamine group (2N) the cell borders are still visible, but cracks start to show. In protamine+CS group (2O) there seems to be a layer covering the cells.

Figure 3.

TEER measurements on day 1,2,3. Showing a significant increase of TEER in the protamine + CS group on all 3 days, with also an absolute increasing value of TEER. In the protamine group, there is a significant increase of TEER on day 3, but in absolute values TEER resembles damaged urothelium.