Medical Devices: Evidence and Research

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ORIGINAL RESEARCH

Non Clinical Model to Assess the Mechanism of Action of a Combined Hyaluronic Acid, Chondroitin Sulfate and Calcium Chloride: HA +CS+$CaCl_2$ Solution on a 3D Human Reconstructed Bladder Epithelium

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Purpose: Medical Device Regulation (EU) 2017/745 requires the principal mode of action (MoA) to be demonstrated by experimental data. The MoA of Ialuril® Prefill (combined as HA+CS+$CaCl_2$: sodium hyaluronate 1.6%, sodium chondroitin sulphate 2% w/v and calcium chloride 0.87%) Class III medical device, indicated for intravesical instillation to reduce urinary tract infections, has been evaluated on a 3D reconstructed human bladder epithelium (HBE).

Methods: Three experimental designs; i) *E. coli* strain selection (DSM 103538, DSM 1103) to investigate the HA+CS+$CaCl_2$ properties in modifying bacterial growth in liquid broth (CFU 4h and 24h) at 80%, 50% and 25% concentrations; ii) evaluation of film forming properties on HBE after 15 min exposure by quantifying caffeine permeation across the epithelium; iii) capacity to counteract *E. coli* adhesion and biofilm formation on colonized HBE by viable counts and ultrastructural analysis by scanning electron microscopy (SEM) using ciprofloxacin as the reference antimicrobial molecule.

Results: No significant differences were observed in bacterial viability for both the *E. coli* strains. HA+CS+$CaCl_2$ reduced caffeine permeation of 51.7% and 38.1% at 1h and 2h, respectively and determined a significant decrease in caffeine permeation rate at both timepoints supporting HA+CS+$CaCl_2$ capacity to firmly adhere to the bladder epithelium creating a physical barrier on the surface. The viable counts in HBE treated tissues then infected with *E. coli* resulted not different from the negative control suggesting that the device did not inhibit *E. coli* growth. SEM images showed homogenous product distribution over the HBE surface and confirmed the capacity of HA+CS+$CaCl_2$ to adhere to the bladder epithelium, counteracting biofilm formation.

Conclusion: The results support the capacity of HA+CS+$CaCl_2$ to counteract bacterial invasion by using a physico-mechanical mode of action: this medical device represents a valid alternative to antibiotics in the treatment of recurrent UTIs.

Keywords: recurrent cystitis, urinary tract infections, *E. coli* UPEC, *E. coli* biofilm, hyaluronic acid, chondroitin sulphate, film forming properties, anti-bacterial adhesion, reconstructed 3D human bladder epithelium

Introduction

Intravesical instillations of glycosaminoglycans (GAGs) represent a valid therapeutical approach for different pathologies, such as bladder pain syndrome/interstitial cystitis (BPS/IC), chemical induced cystitis (including BCG therapy), radiation induced cystitis (irradiation of pelvic tumors) and recurrent urinary tract infections (UTIs). In particular, UTIs are one of the most prevalent infections both in the community (only second to respiratory tract's) and in hospital settings, affecting 50–60% of adult women in their lifetime.[1] Risk factors associated with UTIs include female gender, age, individual history of UTI, sexual activity, and medical comorbidities. UTIs are mainly caused by bacteria, although fungi and some viruses have also been implicated. Among bacteria, Gram-negative bacteria of the *Enterobacteriaceae*

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family, including *Escherichia coli* (*E. coli), Klebsiella, Enterobacter, Proteus* species, etc., are mostly involved. In particular, some *E. coli* strains with distinctive features have been identified as causing most UTIs: they have been designated uropathogenic *E. coli* (UPEC) and used as models to better understand host-pathogen interactions during urinary tract pathogenesis.[2–5] These strains possess diverse virulence-associated factors (VFs) that assist them in attaching to, invading, and injuring the host, and include adhesins, toxins, protectins, and biofilm production.[6,7] In particular adhesins, which appear as hair-like fibers called fimbriae (or pili), facilitate the colonization with *E. coli* in the urinary tract by attaching to host epithelial cells. This attachment promotes the persistence of the organism in the bladder and serves as a reservoir for ascending infection in the urinary tract.[8] However intracellular reservoirs of bladder epithelium have been discovered more recently.[9–11] Various adhesins have been identified and are classified mainly according to receptor specificity such as P-fimbriae, which, along with type 1 fimbriae, are particularly important in bladder colonization.[12] Antimicrobials are the first-line therapy for uncomplicated UTIs, aiming to alleviate symptoms and reduce recurrence.[13] However, the increase in antimicrobial resistance at clinical level is stimulating the use of alternative therapeutic approaches that can selectively treat and prevent UTIs as suggested by the European Association of Urology[14] (EAU) and the American Urological Association[15] (AUA), recommending the appropriate use of antimicrobials ensuring cost-effective therapy whilst minimizing microbial resistance. Alternative approaches for non-antibiotic prevention therapies, include glycomimetic antiadhesives, immunomodulatory therapies, and competition with probiotics.[1] The efficacy and the safety of these non-antibiotic approaches need to be demonstrated with appropriate randomized controlled trials, including the evidence of their mode of action for a proper product qualification as requested by the medical device regulatory framework.[16–18]

Ialuril® prefill (IBSA) is a class III medical device for intravesical instillation indicated to replenish the glycosaminoglycan (GAG) layer of the bladder epithelium in cases in which their loss causes frequent and recurring cystitis. It contains a 50 mL sterile solution of sodium hyaluronate (1.6% w/v – 800 mg/50 mL), sodium chondroitin sulphate (2% w/v – 1 g/50 mL) and calcium chloride (0.87% – 440 mg/50 mL). The intravesical instillation aids in the reduction of urinary tract infections (UTIs) incidence in patients with recurrent UTIs (rUTIs).

The aim of this research project was to investigate the physico-mechanical mode of action of a medical device (MD) that consists of a combined hyaluronic acid, chondroitin sulfate and calcium chloride HA+CS+$CaCl_2$ solution (Ialuril®) at bladder epithelium level by using an in vitro pre-clinical experimental approach.

A commercially available 3D reconstructed human bladder epithelium (HBE) has been applied as a biologically relevant test system to investigate HA+CS+$CaCl_2$ properties in forming a physical barrier on the epithelium, counteracting this mean *E. coli* adhesion and biofilm formation on the living tissue without showing a direct antimicrobial efficacy on *E. coli,* which is the mode of action of antibiotics.

Three different experimental designs have been defined with the following aims:

- To select the *E. coli* strain (DSM 103538, DSM 1103) and to investigate the HA+CS+$CaCl_2$ properties in modifying bacterial growth by a microdilution assay in liquid broth and resulting CFU (colony forming unit) considering three different concentrations of the product (80%, 50% and 25%).
- To evaluate the film forming properties of HA+CS+$CaCl_2$, applied topically as such on the bladder epithelium and its capacity to induce a temporary modification of caffeine permeation across the epithelium due to the product acting as a protective physical barrier ("film").
- To investigate the anti-bacterial adhesion properties of HA+CS+$CaCl_2$, applied as such, and the pili and biofilm formation prevention on *E. coli* DSM1103 colonized HBE for 4 h by performing viable counts at tissue level and ultrastructural analysis by scanning electron microscopy (SEM).

These protocols contribute to support product qualification and classification according to the Medical Device Regulation (EU) 2017/745 (MDR) and MDCG 2022–5 on borderline products by providing state-of-the-art evidence on the physico-mechanical mechanism of action exerted by HA+CS+$CaCl_2$ on the bladder epithelium, showing an interesting alternative product, mirroring the performance of antibiotics.

Materials and Methods

Test Items

HA+CS+$CaCl_2$ prefill (IBSA) contains sodium hyaluronate 1.6%, sodium chondroitin sulfate 2% and calcium chloride 0.87%. Ciprofloxacin (≥98) (Sigma-Aldrich) was used as a reference at the concentration of 0.312 µg/mL. White vaseline (Sella srl, Schio Italy) was used as a positive control in the film forming assay.

Test System

Human bladder epithelium (HBE) is produced by Episkin SA (Lyon, France): it is a 3D reconstructed human bladder epithelium originated from RT-112 urinary bladder transitional human carcinoma cells cultured on an inert polycarbonate filter with airlift technology, in a chemically defined medium. It is cultured for 5–7 days, reaching a mean thickness of 55–90 µm and it is viable for 1 week. The model is proposed for research use and it has been characterized by the manufacturer for its capacity to form an epithelial barrier confirming the expression of keratin 17, keratin 20 and CD44 as biomarkers of the organization of extracellular matrix and it has been further characterized for the expression and localization of Cadherin 1 and Claudin 4 (data not shown). The 3D bladder epithelium reproduces the inner mucosa of the bladder, a transition epithelium that covers the first tract of the ureters: this epithelium is impermeable to counteract urine re-absorption. The batch was tested for the absence of HIV, hepatitis B, hepatitis C and mycoplasma, and the maintenance medium was tested for sterility. The inserts containing the tissues at day 5 were placed at room temperature in a multi-well plate filled with an agarose nutrient solution in which they were embedded for shipment.

After arrival, the HBEs were removed from the agarose nutrient solution under a sterile airflow cabin. The inserts were rapidly transferred to 6-well plates previously filled with specific medium without antibiotics (1 mL/well) (Episkin SA, Lyon, France) at room temperature and incubated at 37 °C, 5% CO_2 and saturated humidity. The test was performed 2 days after the arrival, with tissues kept in maintenance medium till the end of the experiment.

Bacterial Strains

Escherichia coli is the most abundantly isolated, Gram-negative bacterium responsible for urinary tract infections (UTIs). It is able to produce several soluble, metabolically active and toxic molecules, such as pore forming toxins and proteases. Its adherence ability is mediated by biofilm production, which confers on *E. coli* a higher resistance to the antimicrobial substances access and improved adherence to host tissues.

In Table 1 the two uropathogenic strains used for the experiments provided by the German collection of microorganisms and cell cultures GmbH (DSMZ) are described.

Before the assay, each bacterial strain was inoculated on their medium (TSYE broth or agar) and incubated statically under the required growth conditions (at 37 °C in aerobic conditions for 24–48 h) to check their normal colony morphology and to use fresh cultures.

Chemicals for Microdilution

For bacterial culture: nutrient broth (code: 70149, Sigma-Aldrich), nutrient agar (code: PO5025A, Thermo Scientific), tryptone soy yeast extract broth (TSYEB, tryptic soy broth 30 g/L BD, yeast extract 3 g/L, Sigma-Aldrich), tryptone soy yeast extract agar (TSYEA, tryptic soy agar 30 g/L BD, yeast extract 3 g/L, Sigma-Aldrich) and sterile saline solution (sodium chloride 0.9%, EUROSPITAL) were used to resuspend bacterial culture.

Simulated urine (SU) was prepared with 7.1 g of urea, 1.5 g of creatinine, 1 g of ammonium citrate, 4 g of sodium chloride, 0.825 g of potassium chloride, 0.25 g of potassium bisulfate, 0.1 g of magnesium sulfate, 0.875 g of monobasic

Table 1 E. Coli Strains Characterization

Name	Strain	Growth Conditions
Escherichia coli strain CFT073 (pyelonephritis isolate)[5,19,20]	DSM 103538	TSYE broth or agar at 37 °C in aerobic conditions for 24–48h
Escherichia coli strain ATCC25922 (cystitis isolate)[21]	DSM 1103	Nutrient broth or agar at 37 °C in aerobic conditions for 24–48h

potassium phosphate and 0.25 g of potassium bicarbonate were dissolved in 250 mL of ultrapure water. All the compounds used were from Sigma-Aldrich. Ultrapure water was added to reach a final volume of 500 mL. The final pH of SU was 5.00. The SU was used within 24 hours from preparation.[22]

Microdilution Assay Method

The microdilution test was performed on the two *Escherichia coli* uropathogenic strains to investigate the mechanism of action of the product and to pick out the most appropriate strain to be used in the anti-adhesion and biofilm formation on HBE. HA+CS+$CaCl_2$ was tested at three different concentrations (25%, 50% and 80%).

Test Mixture Preparation

The bacterial cultures were resuspended in SU solution to simulate the physiological use conditions; their OD_{600nm} were checked by means of a spectrophotometer (UV/VIS ONDA TOUCH UV-21, ONDA SPECTROPHOTOMETER Italy) and the final concentration was adjusted to obtain a concentration of 10^7 CFU/mL.

Each test mixture was prepared in a tube, according to the following schemes and Table 2: mixing 8 mL of each bacterial suspension (10^7 CFU/mL) in culture medium (2x concentrated) with 32 mL of test item at 80%, mixing 20 mL of each microbial suspension (10^7 CFU/mL) in the culture medium (standard concentrated) with 20 mL of the test item at 50% or 25%.

Additional control mixtures were prepared adding the bacterial suspensions to their culture media and ciprofloxacin solution (0.0312 µg/mL), respectively as negative control and reference to validate the study.

All the obtained suspensions were then incubated at 37 °C in aerobic conditions and allowed to grow for up to 24 hours. The growth rate was checked, performing viable counts compared to the reference or negative control.

Bacterial Viable Counts

An aliquot from each mixture described in the paragraph before was taken after 4 h and 24 h for the viable counts performed by serial decimal dilutions in sterile saline solution. They were plated by spread method onto agar plates and incubated at bacterial growth conditions (37 °C, aerobic conditions).

The following formula was used to calculate the CFU/mL:

$$CFU/mL = \frac{n^{\circ}\ of\ colonies\ \ x\ dilution\ factor}{0,1mL} \quad (1)$$

Film Forming Protocol

The film forming protocol developed on epidermis[19] was adapted to HBE.

Table 2 Ialuril® Dilutions Preparations for Microdilution Test

Test Mixture	Bacterial Suspension (10^8 CFU/mL in SU)	Culture Medium	Culture Medium 2X	IALURIL®	IALURIL® Diluted 1:1 in Sterile Water
Negative Control	1 mL	39 mL	–	–	–
IALURIL 80%	1 mL	–	7 mL	32 mL	–
IALURIL 50%	1 mL	19 mL	–	20 mL	–
IALURIL 25%	1 mL	19 mL	–	–	20 mL

Bladder Epithelium Treatment

At arrival in the laboratory, culture inserts were placed in 6-well plates previously filled with 1 mL/well saline solution (basolateral compartment, receptor fluid). All the series were evaluated in triplicate. Fifty microliters of HA+CS+$CaCl_2$ and white vaseline, used as the positive control (PC), were applied directly on the bladder epithelium surface for an exposure of 15 min at room temperature. Negative control (NC) tissues were untreated.

After treatment with HA+CS+$CaCl_2$ and white vaseline, 100 µL of 0.5% w/v caffeine solution (1 mg caffeine/cm_2) was applied to the apical compartment. Caffeine penetration was monitored by collecting the receptor fluids (1 mL) from the basolateral compartment at 1 h and 2 h of exposure.

Analytical Method for Caffeine Quantification

The concentration of caffeine in the 0.5% reference solution and in the collected receptor fluid samples was quantified via UPLC method. Receptor fluid samples were stored at 4 °C before UPLC/MS analysis. The caffeine concentration was determined by using a 1290 Infinity II LC System (AGILENT Santa Clara, Ca. USA) equipped with a C18 reversed-phase column (ACQUITY UPLCBEH-C18, 1.7 µm, 100×2.1 mm, WATERS CORPORATION, Ma. USA) set at 25 °C. A 5 µL sample was injected for isocratic elution at 0.25 mL/min. The composition of the eluent was 80% water/20% methanol. The wavelength was set at 273 nm. Standard calibration curves for caffeine (0.1 and 1000 mg/L) were used.

The results are expressed in µg and % of the diffused caffeine in comparison to the applied dose as well as in comparison to the negative control.

$$\text{Caffeine } \% = \frac{\mu\text{g Caffeine applied}}{\mu\text{g Caffeine found}} \times 100 \quad (2)$$

Statistical Analysis

One-way ANOVA followed by a post hoc Tukey HSD test was performed using GraphPad Prism, version 9.2.0 (GraphPad Software, San Diego, Ca. USA). The product and the white vaseline were compared with the untreated negative control, and statistically significant results were reported as p-values.

Anti-Bacterial Adhesion on the 3D Bladder Epithelium

E. coli DSM1103 (cystitis isolate) was selected as the most relevant strain to assess the HA+CS+$CaCl_2$ properties to counteract its adhesion to the bladder epithelium.

The day of the colonization, *E. coli* DSM1103, previously grown in nutrient agar, was resuspended in SU solution to simulate the bladder epithelium microenvironment, and its concentration was checked by means of a spectrophotometer adjusting the inoculum level to 10^6–10^7 CFU/mL. The inoculum level was also checked, performing the viable counts with the appropriate 10-fold dilutions prepared in sterile saline solution and spreading them onto nutrient agar.

The day of the test, tissues (HBE) were pre-treated on the apical part (directly spread on tissue surface) with 50 µL of HA+CS+$CaCl_2$ or reference (ciprofloxacin, 0.312 µg/mL solution) during 4 h as reported in Figure 1. Not treated and not colonized HBE were used as negative control (NC) while non treated but colonized HBE served as positive control.

At the end of the exposure time (4 h) the residual volume of product or reference was removed and 30 µL of bacterial suspension were added on tissue apical surface and incubated at 37 °C with CO_2 5%. After 2 hours (*E. coli* DSM1103 replication time), the inoculum excess was removed from each apical surface and tissues were re-incubated for another 2 hours (4 h total).

The residual of *E. coli* DSM1103 viable rate was checked by viable counts by spread method onto agar plates (CFU/tissue) and SEM analysis compared to the reference.

E. Coli DSM1103 Viable Count on HBE

At the end of the colonization period (4 h), the tissues were collected for viable counts: Apical part: 0.3 mL of sterile saline solution was added to the apical compartment and then removed and collected in 2 mL vials. Homogenate: tissues were separated from the plastic insert with a scalpel, placed into a vial containing 0.5 mL of sterile saline solution and processed by a Minilysis homogenizer (3 cycles of 30 sec, lowest power) (VWR, USA).

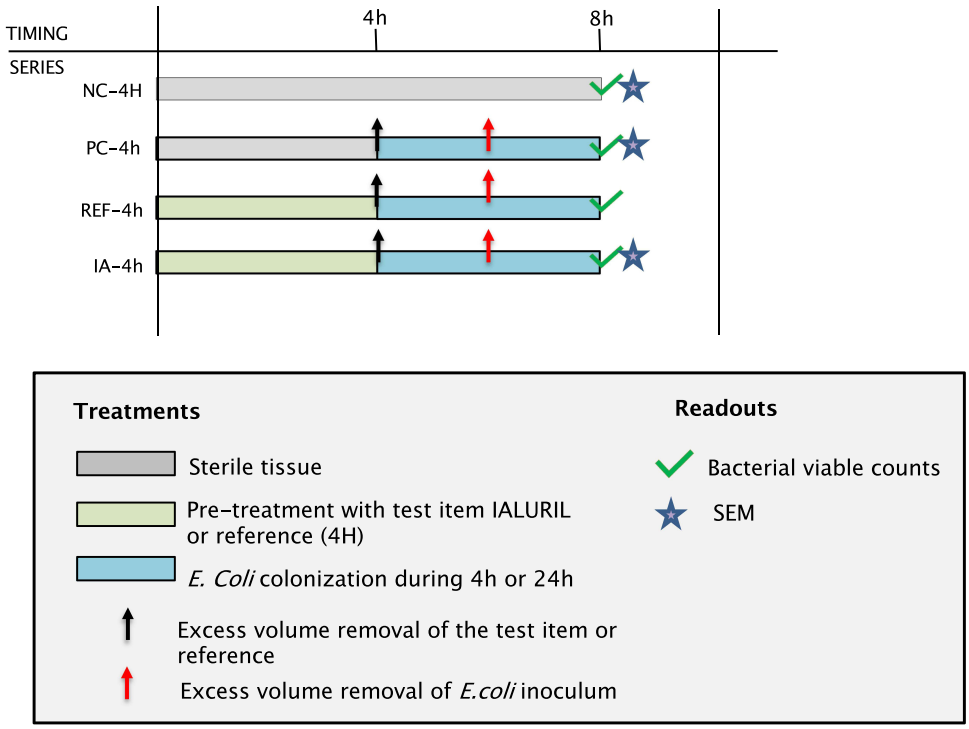

Figure 1 Scheme of the anti-bacterial adhesion assay on HBE.

The residual bacterial viable counts (CFU) were determined in the apical fraction (non-adherent) and on tissue homogenates (adherent) using the spread plate method onto agar plates.

Scanning Electron Microscopy (SEM)

SEM analyses were performed by Service Biotech srl (Naples, Italy). Samples for SEM were fixed by fixative buffer solution (proprietary composition Service Biotech srl). The fixed samples were washed in 0,1 M sodium cacodylate buffer, pH 7.4 and then carried out in 1% osmium tetraoxide (OsO_4) in the same buffer (2 h at RT). They were dehydrated in ascending grades of ethanol at room temperature and hexamethyldisilazane overnight.

The samples were placed on pins with carbon tabs coated with a layer of gold using the Polaron Equipment limited SEM coating unit E5100 and then transferred to the SEM electron microscope for viewing and photography. Thanks to a digital camera, the scanning electron microscopy analysis allows an examination of the epithelial surface ultrastructure that gives information in terms of bacterial phenotype, density, attachment to the epithelium, and formation of biofilm.

Results and Discussion

E. Coli Viable Counts on Microdilution Assay

In Table 3 are reported the results of the viable counts (as per formula 1) performed to assess HA+CS+$CaCl_2$ activity at three different concentrations on two *Escherichia coli* uropathogenic strains (DSM 103538, DSM 1103). The growth rates were compared to the negative control (ie culture media, in bold).

No significant differences were observed in bacterial viability for both the *E. coli* strains tested both at 4 h and 24 h. Due to the more representative origin of the uropathogenic *E. coli* strain ATCC25922, DSM1103 (cystitis isolate), this strain was selected for the anti-adhesion efficacy test on the bladder epithelium.

Film Forming on HBE

The results of caffeine quantification in the receptor compartment at 1 h and 2 h are presented in Figure 2. The results are expressed as the caffeine percentage compared to the amount of caffeine applied (510 µg) as a function of time. As

Table 3 Bacterial Viable Counts (Expressed as Log Values) at 4 h and 24 h

Series		Strain	Viable Counts 4h	Viable Counts 24h
Culture Media		*E. coli* DSM 103538	**8.25**	**8.01**
IALURIL	25%		7.93	8.16
	50%		7.94	8.05
	80%		7.73	8.19
Culture Media		*E. coli* DSM 1103	**8.10**	**7.99**
IALURIL	25%		8.10	7.82
	50%		7.89	7.71
	80%		7.48	8.04

Note: Bold values indicate *E. coli* control viable counts.

expected, the positive control has reduced caffeine passage through the bladder epithelium of 89.4% and 76.8% at 1 h and 2 h, respectively allowing us to validate the experimental run.

In the HA+CS+$CaCl_2$ treated tissues caffeine permeation after 1 h was significantly reduced ($p< 0.001$) compared to the untreated control: the percentage of permeated caffeine for HA+CS+$CaCl_2$ was found to be 15.6%, showing a reduction in caffeine penetration of 51.7%, compared to the untreated control for which a permeation of 32.3% was quantified. At 2 h, the percentage of permeated caffeine for HA+CS+$CaCl_2$ was found to be 32.2%, showing a reduction in caffeine penetration of 38.1%, compared to the untreated control.

The caffeine kinetics through the HBE at 1 h and 2 h is reported in Figure 3A and B, respectively. Compared to the negative control, HA+CS+$CaCl_2$ showed a significant decrease in the caffeine permeation rate at both timepoints ($p< 0.001$).

Anti-Bacterial Adhesion Efficacy on HBE

The results of the viable counts performed on the apical and homogenate fractions are reported in Figure 4.

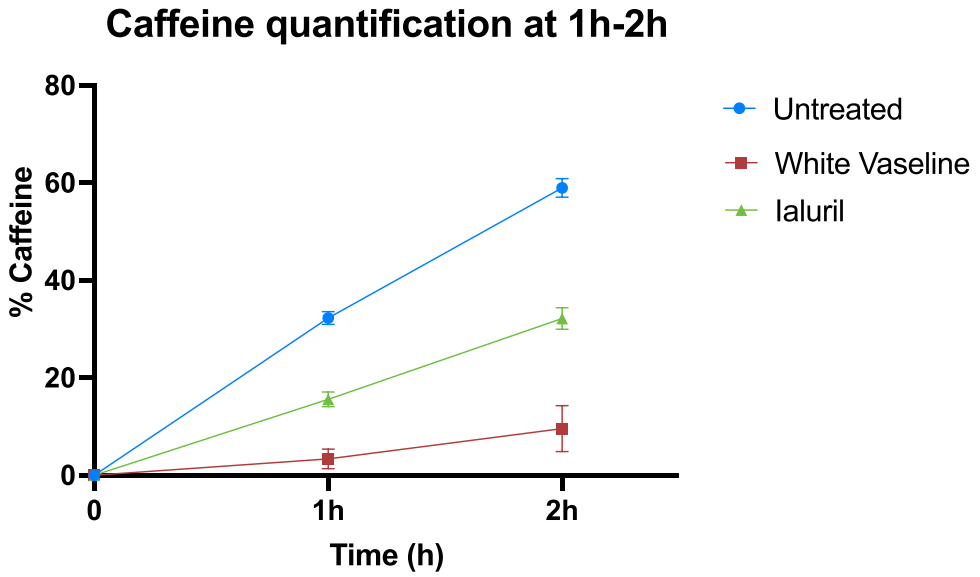

Figure 2 Caffeine quantification in the receptor compartment 1 h and 2 h after caffeine application, expressed as a percentage of applied caffeine. HA+CS+$CaCl_2$ significantly reduce the passage of caffeine compared to the untreated NC at both time points.

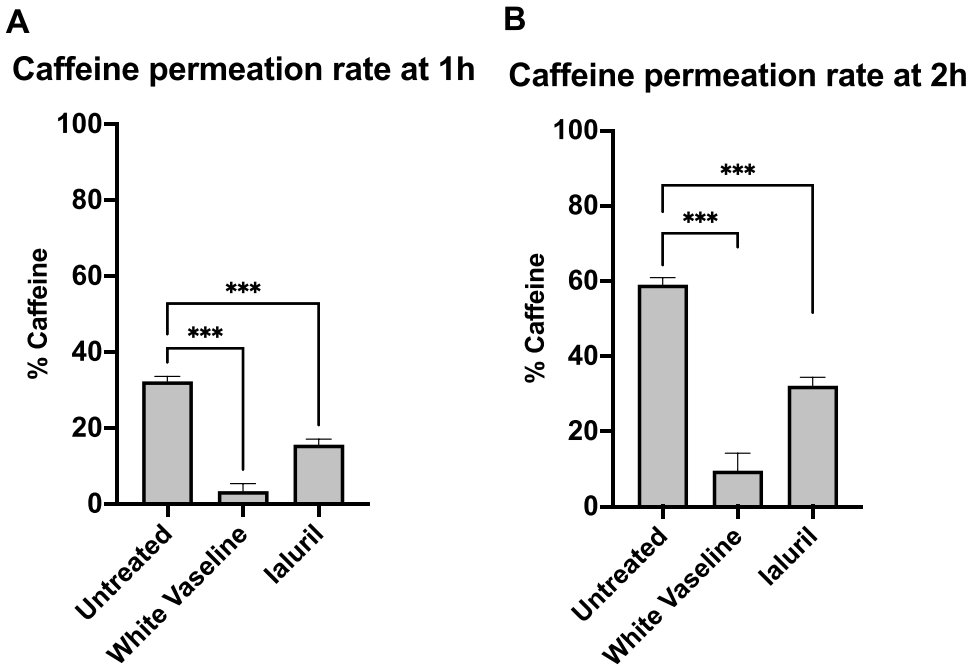

Figure 3 Statistic by "One-way ANOVA with post hoc Tukey HSD Test": ***p<0.001. Caffeine permeation rate at 1 h (**A**) and at 2 h (**B**) after caffeine application, expressed as the percentage of caffeine permeated at 1 h and 2 h with respect to the amount of caffeine present in the apical compartment at each timepoint. Triplicate RHBE tissues for each treatment were used.

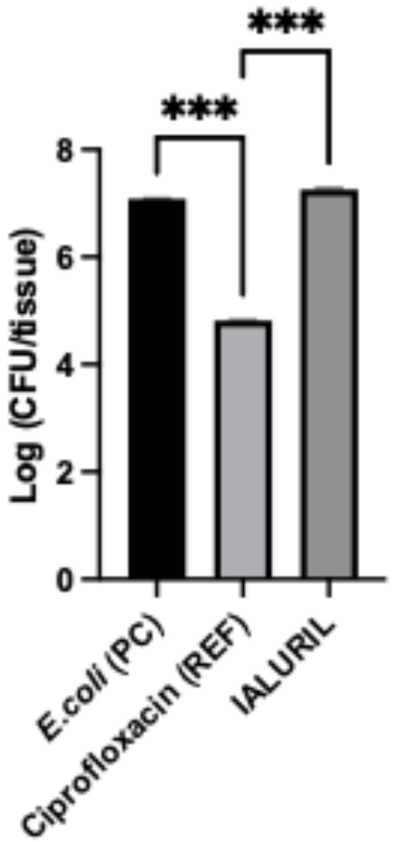

Figure 4 Statistic by "One-way ANOVA with post hoc Tukey HSD Test": ***p<0.001 on viable counts (as per formula 1) on HBE tissues expressed as Log values after 4 h HA+CS+$CaCl_2$ pre-treatment followed by 4 h colonization. Triplicate HBE tissues for each treatment were used.

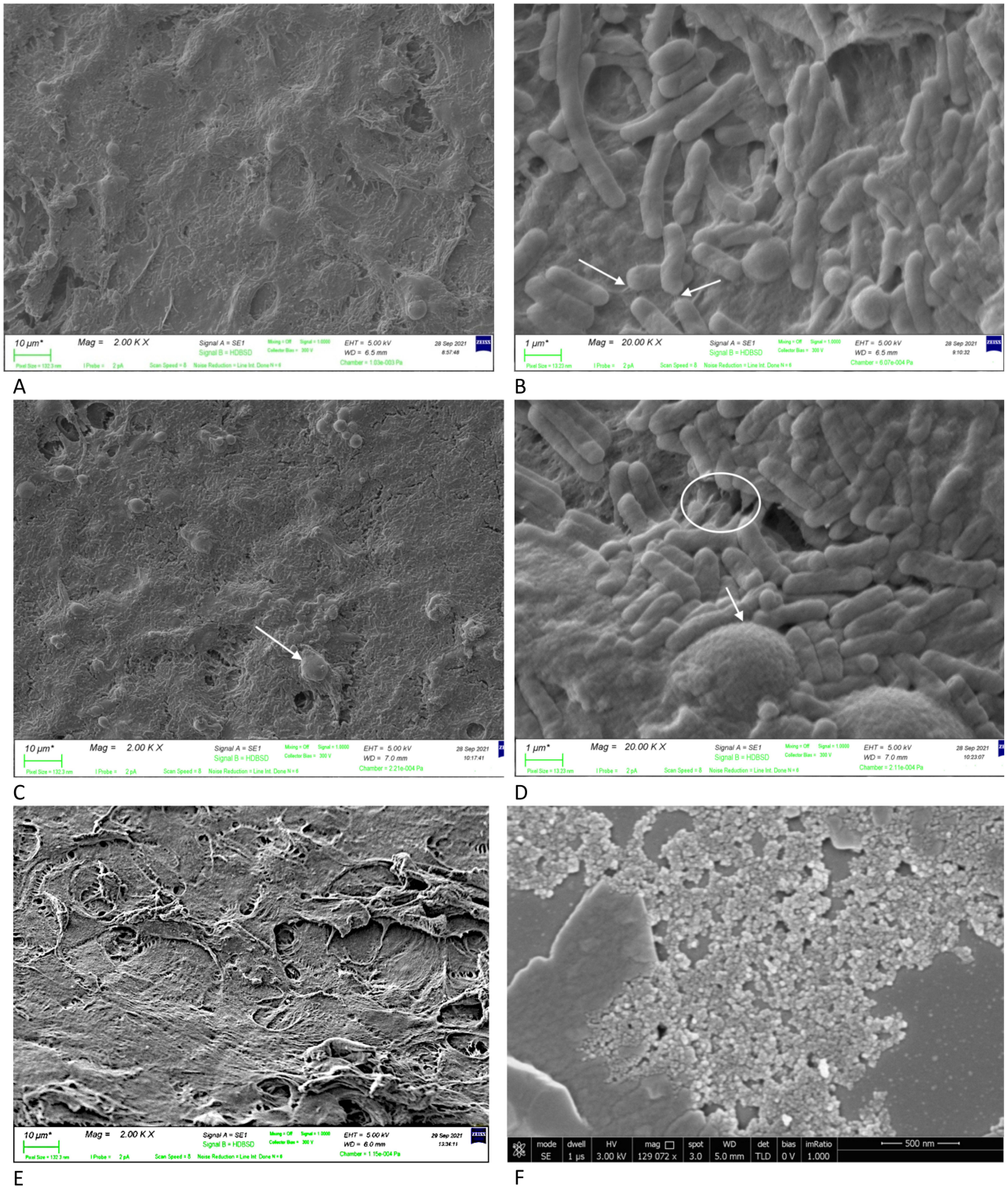

Figure 5 SEM analysis on colonized untreated 3D bladder epithelium (**A**): 2000x magnification and (**B**) 20,000x magnification, HA+CS+$CaCl_2$ pre-treated 3D bladder epithelium (4 h pre-treatment with HA+CS+$CaCl_2$ followed by 4 h colonization with E. coli DSM 1103 ((**C**): 2000x magnification and (**D**) 20,000x magnification, White arrows indicate bacterial pedestal structures and circles HA+CS+$CaCl_2$ product residues), not colonized untreated 3D bladder epithelium (negative control, (**E**) 2000x magnification) and laluril® (**F**): 129,072 magnification).

The results of the reference (ciprofloxacin) allowed a validation of the experimental run, demonstrating a significant inhibition (reduction >2Log, $p < 0.001$) of *E. coli* growth during 4 h and confirming its antimicrobial activity.

Based on the results of viable counts in HBEs treated during 4 h with HA+CS+$CaCl_2$ and then infected during 4 h with *E. coli*, no inhibition of *E. coli* growth was observed as the total viable count resulted in the same as the negative control (untreated).

Ultrastructural Analysis by SEM

The SEM analysis allowed an investigation, at ultrastructural level, of the bladder epithelium surface, giving information in terms of product distribution, bacterial phenotype, density, adhesion mechanism to the epithelium (pili formation) and biofilm.

In Figure 5 an efficient colonization and bacterial-epithelium interaction at 4 h is shown: pili (fimbriae) were visible in the *E. coli* DSM1103 wall (Figure 5A and B). *E. coli* appears organized in biofilm phenotype macro-aggregates that still show a replicative capacity.

In the samples pre-treated with HA+CS+$CaCl_2$ (Figure 5C and D) bacterial density seems no different compared to the colonized control and a thick film of amorphous electron dense material (corresponding to Ialuril®) is visible as a homogenous product distribution within the surface.

In the samples pre-treated with HA+CS+$CaCl_2$ it is possible to visualize the inhibition of *E. coli* pili formation (Figure 5D): pedestal structures are no more visible near the test item matrix. This evidence confirms HA+CS+$CaCl_2$ specific properties to selectively counteract *E. coli* adhesion mechanisms on bladder epithelium.

Conclusion

In vitro reconstructed human epithelia models are closer in term of morphology (multistratified epithelium), biochemical and physiological properties to in vivo human tissues and represent the most promising alternative to animals, ex vivo explants and submerged cell monolayers to access efficacy, mechanism of action and safety of topically applied products in different fields, including the medical device sector.[23–30]

The experimental approach adopted in this pre-clinical project was based on the use of a 3D reconstructed model of bladder epithelium chosen for its biological relevance as biological barrier it reproduces the inner mucosa of the bladder, a transition epithelium that covers the first tract of the ureters and is impermeable to counteract urine re-absorption. The main goal was to demonstrate that the test item was able to create an additional mechanical-physical barrier against *E. coli* adhesion without a direct interaction with the bacterium itself in terms of antimicrobial activity. It has been first demonstrated that the *E. coli* UPC cystitis isolated strain, DSM1103, in reconstituted urine was able to colonize the epithelium surface, mirroring a bladder microenvironment during 24 h (7.09 Log CFU/tissue). SEM ultrastructural analysis has been applied as a non quantitative readout to confirm experimental conditions it has been possible to visualize bacterial distribution on the epithelium surface and its specific adhesion mechanism, mainly based on the formation of fimbriae on cell walls and the establishment of a biofilm adherent to the epithelium.

This innovative test system (bladder epithelium colonized with *E. coli* UPC, DSM1103 in reconstituted urine) was applied to investigate the properties of HA+CS+$CaCl_2$ to form an additional mechanical-physical barrier counteracting *E. coli* overgrowth and to reduce its capacity to adhere to the bladder epithelium.

The mechanism of action demonstrated by the medical device HA+CS+$CaCl_2$ can be defined as physico-mechanical, based on the medical device's peculiar properties: it has been shown to firmly adhere to the bladder epithelium and to create a protective physical barrier ("film") inducing a temporary modification of tissue permeability (reduction of 51.7% and 38.1%, compared to the untreated control, respectively at 1 h and 2 h) to an hydrosoluble probe (caffeine) without showing a significant antibacterial efficacy (Log reduction calculated *versus* the NC were not significantly different) confirming no effect on bacterial growth and bacterial viability. On the contrary, treatment with ciprofloxacin induced a significant inhibition (reduction >2Log, $p<0.001$) of *E. coli* growth during 4 h confirming its antibiotic activity. SEM ultrastructural analysis has given qualitative information about the bacterial-epithelium interaction in the bladder epithelium previously treated during 4 h with Ialuril®: in the *E. coli* wall the formation of pili (fimbriae) appears inhibited as well as the biofilm formation.

The experimental data of the viable counts and the qualitative analysis based on SEM globally support the effective capacity of HA+CS+$CaCl_2$ to counteract the bacterial invasion by a physico-mechanical mode of action: this medical device represents an alternative to antibiotics in the treatment of recurrent UTIs.

Abbreviations

CFU, colony forming unit; HBE, human bladder epithelium; MDR, medical device regulation (EU) 2017/745; MD, medical device; NC, negative control; Ph.I.M, pharmacological, immunological or metabolic; PC, positive control; SEM, scanning electron microscopy; SBMDs, substance-based medical devices; UTIs, urinary tract infections.

Ethics

This in vitro study doesn't require ethics committee approval because it has been conducted on 3D human reconstructed bladder epithelium.

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Disclosure

VF is an employee of IBSA. The authors report no other conflicts of interest in this work.

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