FimH of uropathogenic Escherichia coli influenced the infection in prostate cells by the modulation of JAK/STAT signaling pathway

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Abstract

**Background:** The FimH expression may be a key factor affecting prostatitis caused by UPEC infection. Whether its regulation by the JAK/STAT pathway increases resistance to inflammation caused by infection with high FimH-expressing UPEC strains requires investigation.

**Methods:** The effect of FimH on the ability of knockout ΔFimH UPEC, FimH over-expressed UPEC, and wild-type strains to invade prostate cells and induce inflammation and the effects of different FimH levels on testosterone-treated UPEC and regulation of the JAK/STAT pathway were analyzed.

**Results:** Comparison of the three strains revealed the inhibitory effects of testosterone were more significant in the ΔFimH strain. Testosterone-pretreated ΔFimH UPEC showed weak inflammatory responses and JAK/STAT expression. FimH over UPEC better resisted the inhibitory effects of testosterone, which there was no significantly decreases except 20 μg/ml pretreated group in most JAK/STAT-related proteins. The effects of FimH showed a concentration-dependent response to testosterone, particularly to JAK1, STAT3, and pSTAT3, which also affected the subsequent expression of TLR4, IL-6, and IFN-γ. It has been suggested that regulation of the JAK1/STAT3 pathway may be associated with the effects of the FimH virulence factor on the inhibition of testosterone in UPEC infection.

**Conclusion:** The inhibitory effect of testosterone on UPEC infection in prostate epithelial cells was affected by the virulence factor FimH of UPEC, and reduced the production of inflammatory factors. The JAK/STAT pathway plays a key role in regulating UPEC infection and influences testosterone suppression responses in prostate cells. Our study provides a possible guideline for using testosterone to treat clinical recurrent UPEC infection and persistent prostatitis.

Background

Pathogenic *Escherichia coli* (UPEC) expresses a variety of adhesins that specifically attach to the sugar receptors of urothelial cells. [1] The adhesion of UPEC to invade and subsequently proliferate in host urothelial cells is critical to its pathogenicity in the urinary system. [2] The diversity of adhesins and cell surface receptors in different parts of the urinary tract causes different clinical UPEC infections. For example, P-fused UPEC causes pyelonephritis by binding to galactose-containing receptors in the kidney epithelium, whereas mannose-containing bacteria cause cystitis through the directional adhesion of type 1 pili to the surface of the bladder. [3] The filaments of UPEC flagella can firmly adhere to the urothelium to invade urinary tract cells. [4] *E. coli* strains with P-fimbriae cause more urinary tract infections and are more likely to cause bacteremia and pyelonephritis. [5]

FimH is a specific adhesin located at the tips of UPEC type 1 pili, and is an important UPEC virulence factor. UPEC uses FimH to bind to the urothelium by interacting with mannosylated urea receptors. [6] Studies have shown that ΔFimH *E. coli* have a decreased ability to adhere to and invade intestinal epithelial cells compared to wild-type strains. Similarly, ΔFimH strains have a decreased capacity to translocate to other organs such as the kidney, spleen, and lung. [7] FimH of type 1 pili can directly bind to Toll-Like Receptor 4 (TLR4) to initiate the innate immune response. In the case of natural infection, FimH is a strong inducer of
the innate antibacterial immune response, and is confirmed to be associated with the generation of type 1 IFN and the pathways of TLR4, MyD88, and IRF-3 transduction. [8, 9] FimH adhesion enables UPEC to invade bladder epithelial cells via TLR-4-dependent endocytosis and escape to the cytoplasm through a TRPML3-dependent mechanism, forming a biofilm-like intracellular bacterial community (IBC). [10] Cystitis infection experiments in mice showed that ΔFimH UPEC had significantly weaker infection ability compared to wild-type strains. [6, 11] Although UPEC strains with stronger FimH may have higher infectivity and toxicity to prostate cells, it remains unknown whether this affects regulation by TLR-4 or the downstream Janus kinase (JAK)/signal transducer and activator of transcription (STATs) pathway in host inflammation.

Testosterone and dihydrotestosterone bind to androgen receptors located in the brain, skin, muscle, kidney, liver, and bone. [12] In males, aging leads to lower testosterone concentrations, which is associated with metabolic syndrome, type 2 diabetes mellitus, carotid intima-media thickness, and aortic and lower extremity arterial disease. [13–15] In previous studies, we treated UPEC-infected or LPS-induced prostate cells PZ-HPV-7 with testosterone, and found that its anti-inflammatory effect was downregulated by JAK/STAT1 signaling pathways. [16] We also demonstrated that testosterone effectively inhibits UPEC infection and the size of IBCs in prostate cells by suppressing STAT3 expression, especially in the more virulent strain. [17] However, whether bacterial adhesins (such as FimH) in more virulent strains affect inhibition of UPEC-infected prostate cells by testosterone and the actual mechanism of action are rarely discussed.

The coordinated activation of inflammatory factors by IFN-γ and TLR-4 signaling is essential for the pathogenesis of the innate immune inflammatory response. [18] STATs are important mediators of biological responses to TLR-4 and are crucial for their regulatory mechanism contributing to inflammatory diseases. [19] One study showed that the expression levels of IFN-γ, TNF-α, and IL-1β are increased in the semen of patients with CP/CPPS. [20] Our previous study also confirmed that inhibiting the JAK and STAT1 signaling pathway can attenuate LPS-induced inflammation in prostate cells. [21] SOCS proteins play an important role in modulating inflammation by directly interacting with the JAK family and inhibiting their catalytic activity, or by acting on cellular receptors with phosphorylated tyrosine residues in the cytoplasm. [22, 23] In particular, SOCS1 and SOCS3 play an important role in regulating inflammation reactions. [24, 25] The transcription factor STAT3 is essential for the functioning of immune system several mediators, such as IL-6, IL-10, and IL-22. [26] IL-6 induces a large amount of SOCS3 protein, which can act as a negative regulator of IL-6 activation of STAT3. [27] Our previous research showed that the cooperative interaction of TLR-4 and the JAK/STAT1 signaling pathway is responsible for increased UPEC infection in urothelial cells in a high glucose environment. [28] Furthermore, IFN-γ-related signaling pathways, such as STAT-1, play an important role in the anti-prostatitis response in mice with experimental autoimmune prostatitis. [29] Therefore, understanding how UPEC binds to and subsequently regulates signal transduction, particularly the role played by FimH adhesin in UPEC infection, is key to reducing bacterial prostate infection.
JAK/STAT signaling pathway were analyzed. This study can provide guidelines for the treatment and prevention of bacterial prostatitis.

**Methods**

**Bacterial strain**

As a model, we used the UPEC strain CFT073 from the American Type Culture Collection (ATCC 700928) [30]. Bacterial strains were cultured in Luria Bertani (LB) broth supplemented with ampicillin (100 µg/mL) and incubated at 37°C for overnight. Bacterial growth was spectrophotometrically determined at an optical density of 600 nm (OD$_{600}$). For *in vitro* infections, bacteria were suspended in culture medium at a multiplicity of infection (MOI) of 1:10.

**ΔFimH CFT-073 and FimH$^{\text{over}}$ CFT-073 UPEC strains construction**

We constructed a *fimH*-deficiency mutant in *E. coli* CFT073, named ΔFimH CFT-073, using the lambda Red recombinase system [PMID: 10829079]. Two oligonucleotide primers (*fimH*$_{\text{H1}}$FRT20: 5'- AGGGAACCATTCCAGGCAGTGATTAGCATCACCTATACCTACA GCTGAACCCAAAGAGATGATTCCGGGGATCCGTCGACC-3' and *fimH*$_{\text{H2}}$FRT19: 5'-CAG GTTTTAGCTTCAGGTAATATGGCTACCTGATTACATGCCCTGTGATTTTTTATGTTAGCTGGGCTGCTTC-3') were designed for generating a *fimH* replacement cassette, which contained an apramycin resistance gene flanking with homologous sequences of *fimH*. Primer sequences in the first 60 base pair are homolog to either the upstream sequences from the start codon of *fimH* or the downstream from the stop codon. The 3’ end of primer sequences with underline indicate regions annealed to a template plasmid with apramycin resistance pIJ773 [PMID: 12563033]. After PCR amplification and purification, a *fimH* replacement cassette was electroporated into *E. coli* CFT073 harboring an expression plasmid, pKD46, which had been expressed lambda Red recombinase after arabinose induction. Transformants grown in LB agar containing apramycin were tested by PCR for replacement of *fimH* by apramycin resistance gene. Amplicon sizes of the parental strain and ΔFimH CFT-073 were 4.3Kb and 2.9Kb, respectively. To overexpress *fimH* in *E. coli* CFT073, a *fimH* expression plasmid under IPTG induction, named pQE31, was constructed. Plasmid pQE31-FimH was transformed into *E. coli* CFT073 and transformants were selectively grown on ampicillin-containing plate. Plasmids from the transformants were analyzed using restriction enzyme digestion. We applied Western blot against anti-FimH antibody to confirm high FimH production while the transformants were cultured in the presence of IPTG.

**Prostate cell culture, testosterone pretreatment, and UPEC infection**

The human normal prostate cell line RWPE-1 was cultured in Keratinocyte-serum free medium (GIBCO-BRL #17005-042) containing 5 ng/ml human recombinant epidermal growth factor and 50 ng/ml bovine pituitary extract at 37°C in a 5% CO2 incubator. The culture medium was replaced every 2–3 days. The cells were then treated with testosterone (MyBioSource, San Diego, CA, USA) at different concentrations (5, 10, and 20
µg/mL) for 24 h and infected with pGFP-UPEC at MOI 1:10 for 4 h. Untreated cells were used as the normal control group, and cells infected with pGFP-UPEC only were used as the positive control group. The other group of cells undergoing the same treatment were mixed with radioimmunoprecipitation assay (RIPA) buffer, and the cell protein was collected for subsequent protein expression determination.

**UPEC infection and quantification by colony-forming unit (CFU) counting**

After a 4 h UPEC incubation period, the infected monolayers were washed four times with phosphate-buffered saline (PBS) and incubated for 30 min in a growth medium containing gentamicin (100 µg/mL; Sigma-Aldrich, St. Louis, MO, USA). To measure bacterial invasion, the cells were lysed and harvested using 0.5% trypsin (Gibco) and 0.1% Triton X-100 (Amresco, Solon, OH, USA), and then plated onto nutrient broth (NB) medium containing ampicillin. The total CFUs were counted after 24 h of incubation to quantify bound bacteria.

**Cell protein extraction**

The treated cells were centrifuged at 500xg for 5 min at 4°C, and the supernatant was poured out and the cell pellet was placed in a protein lysis buffer (Pierce, Rockford, IL). The pellet was homogenized with a micro grinder and evenly mixed to collect the expressed proteins. After all the samples were collected, they were thawed on ice and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was collected to obtain total protein. The samples were stored in a refrigerator at −80°C for later use.

**Western blot analysis**

The proteins in the samples were harvested using RIPA lysis buffer (Millipore). The experimental procedure was conducted as per our previous publication [29]. The antibodies used were as follows: anti-JAK1 (BD Biosciences), anti-JAK2, anti-STAT1 (Cell Signaling, Beverly, MA); anti-pSTAT1, anti-STAT3 (Cell Signaling, Beverly, MA), anti-pSTAT3, anti-suppressor of cytokine signaling 3 (SOCS3, Abcam), anti-interferon (IFN)-γ (Abcam), anti-TLR-4 (Proteintech), anti-interleukin (IL)-6 (Bioworld Technology), and anti-β-actin (Santa Cruz) at room temperature (~25°C) for 1 h. After incubation with an appropriate secondary horseradish peroxidase-conjugated IgG antibody (R&D Systems) for 30 min at room temperature, the protein bands on the membrane were detected using an ECL-Plus Western Blot Detection system (GE Healthcare UK Ltd.) according to the manufacturer's instructions. All experiments were performed at least thrice. Graphical analysis of band density was performed using ImageJ software (version 1.41o) (National Institutes of Health, Bethesda, MD, USA) (http://rsb.info.nih.gov/ij/).

**Cytometric bead array (CBA) immunoassay**

The cell culture medium was centrifuged (13,000 × g, 20 min, 4°C), and the supernatant was assessed using a human inflammatory cytokine CBA (BD Biosciences, San Diego, USA) for the cytokines IL-8, IL-10, IL-1β, and IL-6. The cytokine capture bead, PE detection reagent, and recombinant standards or test samples were incubated for 3 h at room temperature. FACSCanto flow cytometer (BD Biosciences, San Diego, USA) was used to acquire the data, which were analyzed using the BD CBA analysis software to produce graphs [31, 32].
Statistical analysis

Data are expressed as mean ± standard deviation (SD). Analysis of variance was used to evaluate differences between various treatment groups and controls. Statistical differences between groups were determined using the Student’s t-test. Statistical significance was set at p < 0.05.

Results

UPEC CFT073 FimH overexpression and knockout ΔFimH strains

CFT073 FimH overexpression and knockout ΔFimH strains were produced by electroporation and gene transformation, respectively (Fig. 1A). Following antibiotic selection and PCR processing, the DNA of each strain was extracted and the FimH content of each strain was estimated by DNA colloid electrophoresis. Figure 1B shows that ΔFimH had the lowest FimH DNA fragment content of the three strains, while the FimH overexpression strain had significantly higher FimH DNA fragment content than the wild type (Fig. 1C). The concentration of the bacterial tubes shows that the strains established after electroporation are resistant to antibiotics, which is convenient for the culture of subsequent strains (Fig. 1D).

FimH levels of UPEC and testosterone inhibition in infected RWPE-1 cells

RWPE-1 cells were pretreated with different doses of testosterone (5, 10, and 20 µg/mL) for 24 h then infected with different UPEC strains (ΔFimH, wild-type, and FimH° CFT-073). The lysed cells were cultured on LB medium to count the intracellular bacteria. Figure 2 shows the effect of 5 µg/mL testosterone pretreatment on ΔFimH CFT-073 and wild-type strains was not significant; however, after increasing the doses to 10 and 20 µg/mL (p < 0.01 and 0.0001 in ΔFimH strain; p < 0.05 and 0.001 in wild-type strain), colony formation was inhibited by testosterone in a dose-dependent manner, and the inhibitory effect on ΔFimH strain was more noticeable. A large number of colonies were formed in the FimH° group even after testosterone pretreatment, but colony reduction occurred with the highest dose (20 µg/mL) (p < 0.01). The FimH° strain only showed a slight reduction compared to other strains at the same doses, except the 20 µg/ml group showed a similar reduction to the wild-type strain. This suggests that UPEC FimH is associated with UPEC resistance to testosterone treatment in UPEC-infected prostate cells.

Inflammatory cytokines in RWPE-1 cells infected with UPEC with different FimH levels after testosterone pretreatment

Following infection of RWPE-1 cells with UPEC with different FimH levels, we detected the cytokines in supernatants (Fig. 3) and found that the secretion of IL-6 and IL-8 increased significantly with increasing testosterone concentration in the ΔFimH and FimH° infection groups (p < 0.05). The wild type strain only exhibited cytokine secretion at the 20 µg/ml dose, but cytokine levels remained significantly lower than those
in the other groups (p < 0.001). The ΔFimH group exhibited the most significant IL-8 secretion, even higher than that of the FimH<sup>over</sup> infection group (p < 0.05); it increased rapidly at a testosterone dose of 5 µg/ml, peaked at 10 µg/ml, and then slightly decreased at 20 µg/ml. No significant difference in IL-10 secretion was observed among the testosterone concentrations. Additionally, IL-10 was largely absent in the ΔFimH group, but slightly decreased in the FimH<sup>over</sup> group at testosterone doses of 10 and 20 µg/ml. The secretion of IL-1<sub>β</sub> was not significantly different, suggesting that it may not be relevant.

**Inhibition of protein expression of JAK/STAT pathway-related factors in RWPE-1 cells infected with UPEC with different FimH levels after testosterone pretreatment**

RWPE-1 cells were pretreated with different doses of testosterone (5, 10, and 20 µg/mL) for 24 h, and then infected with different strains of UPEC (ΔFimH, wild-type, and FimH<sup>over</sup> CFT-073). Total protein from all groups was collected and detected by western blotting. Figure 4 shows that the expression levels of JAK1, STAT3, TLR-4, inflammatory IL-6, and IFN-γ in the FimH<sup>over</sup> groups were significantly higher than those in the wild-type groups, especially in the ΔFimH groups (p < 0.05). The effects of testosterone-induced inhibition were weak in the FimH<sup>over</sup> groups with increasing doses. However, in the ΔFimH group, the effect of testosterone on JAK/STAT-related proteins was more significant than that on inflammation-related factors (IL-6, TLR-4, and IFN-γ). Additionally, the expression levels of STAT1, phosphorylated STAT1, and STAT3 only differed between the FimH<sup>over</sup> and ΔFimH groups in the 10 and 20 µg/mL testosterone pretreated groups (p < 0.05).

As shown in Fig. 4B, the protein expression of the STAT antagonist SOCS3 showed the opposite trend to that of JAK and STAT expression after testosterone pretreatment of UPEC-infected cells. The SOCS3 expression in the FimH<sup>over</sup> group was lower than that in the other two groups (p < 0.05). Moreover, the effect of testosterone dose on JAK2 was not significant, and the difference between UPEC strains with different FimH levels was insignificant. The results suggested that the JAK/STAT pathway and inflammatory factors in UPEC-induced prostatitis reduced by testosterone pretreatment were indeed associated with FimH expression.

**JAK/STAT inhibitors influenced the effects of testosterone on RWPE-1 cells infected with UPEC with different FimH levels**

RWPE-1 cells were pretreated with testosterone (20 µg/mL) for 24 h, and JAK (25 µM) or STAT inhibitors (50 µM) were added for another 24 h. After infection with different UPEC strains (ΔFimH CFT-073, wild-type and FimH<sup>over</sup> CFT-073) (MOI = 1:10), the cells were lysed and cultured on LB medium to count intracellular bacteria and demonstrate the modulation of JAK/STAT pathway. As shown in Fig. 5, the inhibitory effects of testosterone on UPEC infection in RWPE-1 cells were significantly blocked by JAK inhibitors, especially in the ΔFimH UPEC group (p < 0.01). Additionally, colony formation in the FimH<sup>over</sup> group was higher than that in the ΔFimH group under testosterone pretreatment with or without STAT inhibitor co-culture and was also higher than that in the wild-type group under testosterone and JAK inhibitor pretreatment (p < 0.05). Notably, the colony numbers of STAT inhibitor-treated group were higher than the testosterone and STAT inhibitor co-cultured group only in the wild-type strain (p < 0.05).
JAK/STAT inhibitors interfered with the protein expression of JAK/STAT pathway-related factors in RWPE-1 cells infected with UPEC with different FimH levels under testosterone pretreatment

RWPE-1 cells were pretreated with testosterone (20 µg/mL) for 24 h, and JAK (25 µM) or STAT inhibitors (50 µM) were added for another 24 h. Subsequently, following infection with different UPEC strains (ΔFimH, Wild type and FimHover CFT-073) infection (MOI = 1:10), the total proteins of all cell groups were collected for detection of the expression of the JAK/STAT pathway.

Figure 6 shows that the inhibitory effect of testosterone on the expression of JAK1, STAT3, and TLR-4 proteins was significantly higher in both the FimHover and wild-type infection groups compared to the ΔFimH group, regardless of treatment with or without inhibitors (p < 0.05). However, STAT1, phosphorylated STAT1, and SOCS3 appeared not to be affected by UPEC FimH, which did not differ among the three groups. The expression of JAK2 and IFN-γ was significantly lower in the ΔFimH group than in the FimHover group when co-treated with testosterone and JAK/STAT inhibitors, but not when treated with the inhibitor alone. The difference in IL-6 appeared only with STAT inhibitor treatment (p < 0.05). The data suggest that the effect of FimH on testosterone suppression in UPEC prostatitis may be mainly related to regulation of the JAK1/STAT3 pathway and TLR-4.

Discussion

Our previous studies confirmed that UPEC infection affects the JAK/STAT signaling pathway in human urothelial cells and induces the expression of mediators involved in the inhibition of host cytokines. [29] The activation of adenosine A receptors inhibits STAT3 phosphorylation, thereby affecting the IL-8 response of UPEC-infected human urothelial cells. [33] We confirmed that testosterone inhibited the pathogenicity of UPEC in prostate cells, as well as induced inflammatory responses through the JAK/STAT1 pathway. [21] The structural composition of urothelial cells is affected in the UPEC-infected host, making it prone to chronic or recurrent infection, depending on differences in the virulence of different strains. [34] We previously revealed that prostate cells pretreated with testosterone showed a dose-dependent reduction in the number of UPEC colonizations and inhibited the size of the formed IBC, especially in the highly virulent strains. [17] However, the role of STAT proteins in the adhesion of UPEC FimH to host cells and regulation of host inflammatory responses during UTIs is not yet fully understood. Here, we demonstrated that testosterone can effectively inhibit UPEC infection in prostate cells through the JAK/STAT pathway, and the regulation of the JAK/STAT pathway and UPEC infection is related to the virulence of UPEC, especially FimH, which has a decisive influence.

Previous studies have shown that testosterone can prevent UPEC from forming a biofilm-like community in the urinary tract to reduce the incidence of renal abscess and prevent its progression, which has been observed in both female and male mice. [35] Comparison of the three strains (ΔFimH CFT-073, wild-type, and FimHover CFT-073) revealed that the inhibitory effects of testosterone were more substantial in the ΔFimH strain, indicating that the effectiveness of testosterone in treating prostate infections may be affected by the virulence of UPEC FimH. Although testosterone can enhance prostate protection against UPEC infection, this treatment against intractable or persistently recurrent UTI still depends on differential FimH virulence. Further
analysis of the effects of these three UPEC strains with different FimH expression on testosterone-induced secretion of inflammatory cytokines from prostate cells revealed that the ΔFimH UPEC resulted in the highest production of IL-6 and IL-8. The FimH$^{\text{over}}$ UPEC showed similar results and the wild type showed the lowest. No IL-10 expression was observed in the ΔFimH group, in contrast to that in the wild-type and FimH$^{\text{over}}$ strains. This suggests that the immunosuppression of IL-10 in prostate cells may be closely associated with the function of FimH in UPEC, such as bacterial attachment or invasion. H4, H1, and H7 flagella of E. coli are involved in motility, epithelial cell adhesion, and invasion; H4 flagella can enhance the induction of anti-inflammatory IL-10, a property that contributes to the adaptability of UPEC in UTI. [36]

Under testosterone pretreatment, the ΔFimH UPEC showed weak inflammatory responses and expression of the JAK/STAT pathway, whereas FimH$^{\text{over}}$ UPEC was better at resisting the inhibitive effects of testosterone, with no significant decreases except in the 20 µg/ml pretreated group in most JAK/STAT-related proteins. This interfering effect of FimH showed a concentration-dependent effect of testosterone, especially on JAK1, STAT3, and pSTAT3, which also affected the subsequent expression of TLR4, IL-6, and IFN-γ. STAT3 acts synergistically with pro-inflammatory pathways during Helicobacter pylori infection to regulate bacterial immune tolerance to persistent infection, leading to subsequent inflammatory and carcinogenic outcomes. [19, 37] We found that prostate cells co-incubated with testosterone and JAK/STAT inhibitors had the greatest effect on ΔFimH UPEC, especially the JAK inhibitor, which significantly weakened the inhibitive effect. This shows that the presence or absence of FimH is important for testosterone to regulate the JAK/STAT pathway for inhibiting inflammation and infection. Moreover, inhibitor experiments revealed that the inhibitory effect of testosterone differed significantly among the three UPEC strains, especially on JAK1, STAT3, pSTAT3, and IL-6. It has been suggested that regulation of the JAK1/STAT3 pathway may be closely associated with the effects of the FimH virulence factor on the inhibition of testosterone in UPEC infection. Additionally, the STAT inhibitor SOCS3 revealed significantly reversed differences among the three strains. To limit the production of inflammatory cytokines in the bladder mucosa, UPEC in persistent UTI causes SOCS3 upregulation and thus suppresses JAK/STAT pathway-mediated pro-inflammatory factors in urothelial cells. [38, 39] Utilization of sugar alcohol metabolites and maintaining resistance to ROS are important for the establishment and progression of UPEC IBCs in bladder epithelial cells, [6, 40] whereas STAT inhibition using testosterone may help interfere with IBC growth and attenuate UPEC virulence to urinary tract cells. [17] Therefore, UTI caused by UPEC strains with strong FimH expression may be more difficult to treat using testosterone and other hormones, likely due to the poor anti-inflammatory effect and downregulation of the JAK/STAT pathway of testosterone against strains with strong FimH virulence.

Stærk et al. demonstrated in pig infection experiments that type 1 pili is a key virulence factor in urinary tract infection and is critical for UPEC to overcome the initial bottleneck of infection. [41] The FimH and FliC proteins of UPEC were involved in the release of IL-6 and IL-8 in the co-culture model of HTB-5 and HMC-1 cells and promoted the adhesion of UPEC to cells. [42] In a previous study, we demonstrated that treating RWPE-1 cells with testosterone significantly decreased UPEC infection via regulation of the JAK/STAT pathway in a dose-dependent manner. [21] Testosterone inhibits the infection of different virulence UPEC strains differently; the effect is stronger on more virulent UPEC, and the JAK/STAT pathway-mediated anti-inflammatory effect is also more substantial. [17]
Conclusion

This study confirmed that the inhibitory effect of testosterone on UPEC infection in prostate epithelial cells was affected by the virulence factor FimH of UPEC, which reduced the production of inflammatory factors. The JAK/STAT pathway also plays a key role in promoting UPEC infection and is involved in testosterone suppression responses in prostate cells. UPEC strains interfere with the regulation and inhibition of inflammation, especially those closely associated with the FimH virulence factor. All the suppressive effects were distinctly observed in UPEC with weak FimH expression but were less effective in strains with strong FimH expression. Aggregate-forming pili (AFP) and type I fimbriae (TIF) have synergistic effects on the adhesion and colonization of intestinal and urinary epithelial cells; therefore, mixed *E. coli* strains possessing AFP and TIF may infect both the intestinal and urinary tracts and are therefore more difficult to treat. [43]

However, whether testosterone can be used as an anti-infective agent for defending UPEC to continuously infect prostate epithelial cells, especially against strains with strong virulence factors, such as FimH, remains to be further studied. In conclusion, our study indicates that testosterone can be used to treat clinical recurrent UPEC infection and persistent prostatitis. Future studies should explore the underlying molecular mechanism, including the regulation of the JAK/STAT pathway.

Declarations

Ethical Approval

We hereby declare that our research has not involved the use of human and/or animal related materials.

Competing interests

The authors do not have a commercial or other association that might pose a conflict of interest.

Authors' contributions

CHH, SPL and PCC established the experimental concept and designed the experimental framework, also provided the funding; CHH, TWH and PCC mainly performed the experiments; CHH and PCC wrote the main manuscript text and prepared figures. CKF assisted in obtaining experimental materials; All authors reviewed the manuscript.

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Availability of Data and Materials

Not applicable

References


Figures
**Figure 1**

**Preparation of UPEC strains with differing FimH expression**

**A.** The electroporation product was cultured and the replacement of the fragment of FimH was completed (FimH knock-out CFT073). E30 and E37 are electroporation products cultured at 30 and 37 °C respectively. The tubes cultured at 30 °C have less bacteria. C denotes the control in the middle and no bacterial growth occurred due to the antibiotics present. F denotes FimH-overexpressed strain, while W denotes wild-type CFT073. **B.** The ΔFimH (1), wild-type (2) and FimH overexpression (3) strains were prepared by PCR and the
difference in FimH content was estimated via gel electrophoresis. C. Comparison of the extracted plasmids of 
the wild-type (1) and FimH-overexpressed strain (2) to check whether the FimH plasmid was successfully 
implanted in the overexpressed strain. The No. 2 strain showed a 4.5 Kb plasmid. D. A few colony grew in LB 
agar containing apramycin showed that the ΔFimH CFT-073 transformants was successfully prepared and 
cultured.

Figure 2

Effect of testosterone on infection of three UPEC strains with different FimH expression.
RWPE-1 cells were pretreated with different doses of testosterone (5, 10, and 20 μg/mL) for 24 h, and subsequently infected by UPEC with differing FimH expression (∆FimH CFT-073, wild-type, and FimH-overexpressing CFT-073; MOI 1:10). UPEC infection in prostate cells was examined by plating cells on LB agar (A) and was determined using ImageJ software (B). In the ∆FimH CFT-073 strain and the wild-type group, 10 μg/ml testosterone had a significant inhibitory effect on colony formation, while low dose (5 μg/ml) testosterone did not. In the FimH\textsuperscript{over} CFT-073 group, substantial colony formation was observed even after pretreatment with 10 μg/ml testosterone. The FimH\textsuperscript{over} strain showed colony formation to a lesser extent only under a high dose (20 μg/ml). All three strains showed significant differences, except for the wild-type and overexpressed strain in the 20 μg/ml group. Colony-forming units (CFUs) were obtained after plating the lysed solutions of infected cells. Data are expressed as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared to positive control groups. #p<0.05, ##p<0.01, ###p<0.001 for comparison between the two groups.

Figure 3

Testosterone affects the secretion of inflammatory cytokines in different FimH-expressing UPEC-infected prostate cells.

RWPE-1 cells were pretreated with different doses of testosterone (5, 10, and 20 μg/mL) for 24 h, and then infected with UPEC (∆FimH CFT-073, wild-type, and FimH-overexpressing CFT-073) (MOI 1:10). The levels of
secreted cytokines IL-8, IL-10, IL-1β, and IL-6 from supernatants of all groups were collected for cytometric bead array (CBA) measurement. After UPEC infection, the expression of IL-6 increased with the concentration of testosterone, and IL-8 increased at 10 μg/ml of testosterone and then decreased slightly at 20 μg/ml. The expression of IL-6 and IL-8 in the wild-type strain was lower than that in the other two strains. IL-10 levels showed no significant difference among the groups, and the ΔFimH strain induced almost no cytokine secretion. IL-1β showed no significant difference among the groups. Cells infected with UPEC alone were used as positive controls. Data are expressed as the mean ± SD from three separate experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared with the respective control group. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001, for comparison between two groups.
Testosterone regulating the infection and inflammation in different FimH-expressed UPEC-infected prostate cells through JAK/STAT pathway.

RWPE-1 cells were pretreated with different doses of testosterone (5, 10, and 20 μg/mL) for 24 h, infected with UPEC (ΔFimH CFT-073, wild-type, and FimHover CFT-073), and total protein was detected in all cell groups. (A) Total protein expression of JAK1, JAK2, STAT1, STAT3, phosphorylated-STAT1/STAT3, and the inhibitors SOCS3, TLR-4, IL-6, and IFN-γ was analyzed by western blotting. (B) All data were normalized to the
internal reference, β-actin. The results were assessed using a densitometer and quantified using ImageJ software (NIH). Among the three genotypes of UPEC, ΔFimH had lower expression of related proteins than the wild-type and FimH-overexpressing strain. The results are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared with the respective control group. #p<0.05, ##p<0.01, ###p<0.001, for comparison between the two groups.

Figure 5
Differences in prostate cells infected with different FimH-expressing UPEC after testosterone pretreatment due to the modulation of JAK/STAT pathway.

RWPE-1 cells were pretreated with testosterone (20 μg/mL) for 24 h, and JAK inhibitors (25 μM) or STAT inhibitors (50 μM) were added for 24 h. Subsequently, UPEC (ΔFimH CFT-073, wild type and FimH overexpression CFT-073) was added for infection (MOI 1:10), and the cells were collected for plate culture. Cells infected with UPEC (ΔFimH CFT-073, wild type, and FimH overexpression CFT-073) alone were used as positive controls. Cells pretreated with testosterone (20 μg/mL) for 24 h followed by UPEC infection were used as the respective positive controls (T). Cells pretreated with 25 μM JAK inhibitor (Ji) or STAT inhibitor (Si) alone for 24 h, followed by UPEC infection, were used as controls. The image shown is representative of a typical result. Twenty-four hours post-infection, all infected cells were lyses and plated on LB agar and measured using ImageJ software to analyze UPEC colonization. B1 is the analysis result of each strain, while B2 presents the results of the integration and comparison of the three strains. Colony-forming units (CFUs) were obtained after plating infected cells with lysis. Data are expressed as the mean ± SD of three separate experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared with the respective control group. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001, for comparison between two groups.
Figure 6

Differences in modulation of JAK/STAT pathway during infection and inflammation after testosterone pretreatment of different FimH-expressing UPEC-infected prostate cells

RWPE-1 cells were pretreated with 20 μg/mL testosterone, 25 μM JAK inhibitor (TJ), or 50 μM STAT inhibitor (TS) for 24 h and then infected with UPEC as described above (MOI 1:10). Cells pretreated with 25 μM JAK inhibitor (J) or STAT inhibitor (S) alone for 24 h, followed by UPEC infection, were used as controls. Cells infected with UPEC (ΔFimH CFT-073, wild type, and FimH overexpression CFT-073) alone was used as a
positive control (U). Cells pretreated with testosterone (20 μg/mL) for 24 h followed by UPEC infection were used as the respective positive controls (T). Untreated cells were used as negative controls (C). The image shown is representative of a typical result. Total protein from all cell groups was collected for detection. (A) Total protein expression of JAK1, JAK2, STAT1, STAT3, phosphorylated-STAT1/STAT3, and the inhibitors SOCS3, TLR4, IL-6, and IFN-γ were analyzed using western blotting. (B) All data were normalized to the internal reference, β-actin. The results were assessed using a densitometer and quantified using ImageJ software (NIH). The results are presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared with the respective control group. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001, for comparison between two groups.

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