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Modulation of Inflammatory Responses and miRNA Expression in UPEC-Induced Orchitis: Therapeutic Potential of Ciprofloxacin and Doxycycline

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ABSTRACT

This study investigates the molecular mechanisms involved in uropathogenic *Escherichia coli* (UPEC)-induced orchitis in male Wistar rats, with a particular focus on the expression of specific miRNAs and their potential modulation by antibiotics, ciprofloxacin (CIP) and doxycycline (DOX). Forty-two male rats were divided into control and UPECI-induced groups, with subgroups treated at different time points post-infection (24 h, 72h, and one-week). UPEC infection was introduced via epididymal tail injections, and tissue samples were collected for gene expression analysis. The study measured the expression levels of miR-155, miR-138, miR-124, and key inflammatory markers such as SHIP1, SOCS1, TNF-α, and IL-6. Total RNA was extracted from testicular tissues, and quantitative real-time PCR (qRT-PCR) was performed to assess mRNA levels. Results revealed that UPEC infection significantly altered the expression of the studied miRNAs, with upregulation of miR-155 and downregulation of miR-138 and miR-124. Antibiotic treatment with CIP and DOX reduced the inflammatory response, as evidenced by decreased levels of miR-155, SHIP1, SOCS1, TNF-α, and IL-6, and restored the expression of miR-138 and miR-124, particularly in the CIP-treated groups. These findings suggest that CIP and DOX not only inhibit bacterial growth but also modulate immune responses, providing potential therapeutic strategies for UPEC-induced male infertility. This study underscores the role of miRNAs in regulating testicular inflammation and highlights the importance of timely antibiotic intervention in mitigating reproductive damage associated with UPEC infections. Further research is needed to optimize treatment protocols and explore long-term fertility outcomes.

Keywords: Uropathogenic E. coli (UPEC), Orchitis, miRNA expression, Ciprofloxacin, Doxycycline

1. INTRODUCTION

Bacterial infections of the male reproductive system have emerged as a critical contributor to male infertility, with approximately 10%-15% of male infertility cases being attributed to bacterial etiologies (Wang et al., 2021). Among these, Escherichia coli (E. coli), particularly uropathogenic E. coli (UPEC), stands out as the primary pathogen responsible for a range of genitourinary infections, including orchitis. Orchitis, defined as inflammation of the testes, can lead to irreversible damage to the male reproductive organs, impairing spermatogenesis and significantly affecting sperm quality and function. The resulting infertility, linked to UPEC-induced damage, presents a serious clinical challenge (Whelan et al., 2023). The pathogenicity of UPEC involves a multifaceted interplay of bacterial virulence factors, host immune responses, and cellular dysfunction, all of which contribute to the compromised function of the male reproductive system.

UPEC infection triggers significant histological changes in the testicular tissue, including the inflammation of seminiferous tubules and degeneration of spermatogenic cells (Lu et al., 2021). These structural alterations are associated with a range of sperm dysfunctions, such as reduced motility, mitochondrial impairment, and DNA fragmentation (Vahedi Raad et al., 2024). The blood-testis barrier (BTB), a crucial defense mechanism that maintains testicular immune privilege, is often disrupted during UPEC infection, which further exacerbates the inflammatory response (Santacroce et al., 2022). This disruption weakens the integrity of Sertoli cell junctions, promoting an inflammatory environment that elevates the production of anti-sperm antibodies and compromises testicular homeostasis (Justin Margret and Jain, 2024). Such pathological changes underscore the profound impact of UPEC infection on male fertility and the need for a deeper understanding of the molecular mechanisms involved.

A key mediator of inflammation in response to UPEC infection is the dysregulation of microRNAs (miRNAs), which are small non-coding RNAs that regulate gene expression by targeting the 3′-untranslated regions (UTRs) of mRNAs (O'Brien et al., 2018). MiRNAs play essential roles in cellular processes such as proliferation, apoptosis, and immune modulation, all of which are critical to the pathogenesis of inflammation and tissue damage (Azizan et al.,

2025). Several studies have shown that UPEC infection leads to altered miRNA expression profiles in the testes, with certain miRNAs contributing to both the inflammatory response and sperm dysfunction. Specifically, miR-155, a well-known pro-inflammatory miRNA, is upregulated following bacterial challenge, resulting in increased secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interlukine-6 (IL-6) (O'Connell et al, 2010). These cytokines not only exacerbate local inflammation but also activate systemic immune responses that further compromise spermatogenesis (Cabrera-Rivera et al., 2022).

In contrast, miRNAs such as miR-138 and miR-124, which exert anti-inflammatory effects (Blosse et al., 2019). These miRNAs negatively regulate the NF-κB signaling pathway, a key mediator of the inflammatory response, by targeting molecules such as IKKβ and p65 (Markopoulos et al., 2018). Thus, the downregulation of miR-138 and miR-124 can allow for the upregulation of pro-inflammatory cytokines, thereby amplifying the inflammatory response and contributing to testicular damage and infertility. This dysregulation of miRNA expression represents a critical factor in the pathogenesis of UPEC-induced orchitis and highlights the potential of miRNAs as biomarkers for assessing the severity of infection and inflammation (Das and Rao, 2022).

UPEC virulence is driven by a variety of factors, including lipopolysaccharides (LPS), α-hemolysin (HlyA), fimbriae, iron acquisition systems, and capsular polysaccharides (Whelan et al., 2023). LPS, in particular, is a potent immunogenic molecule that activates the host immune response through toll-like receptors (TLRs), triggering the NF-κB pathway and promoting the production of pro-inflammatory cytokines (Kumar et al., 2024). This inflammatory cascade not only contributes to tissue damage but also increases oxidative stress, further impairing testicular function and sperm quality (Esteves et al., 2023).

Given the significant impact of UPEC-induced orchitis on male fertility, it is crucial to investigate potential therapeutic strategies to mitigate the effects of bacterial infection and inflammation. Antibiotics such as ciprofloxacin (CIP) and doxycycline (DOX) have been proposed as promising agents due to their antimicrobial and anti-inflammatory properties (Miettinen et al., 1991). Both CIP and DOX have been shown to modulate the host immune response and reduce the inflammatory burden associated with bacterial infections (Clay et al., 2021). Therefore, considering all these subjects, the present study aims to explore the molecular mechanisms underlying UPEC-induced inflammation, focusing on miRNA (miR-155 and miR-138, and miR-124) expression profiles and inflammatory pathways (SHIP1 and SOCS1, TNF-α, and IL-6), and to assess the therapeutic efficacy of CIP and DOX in alleviating UPEC-induced testicular damage and infertility. In line with this issue, this study seeks to contribute to the growing body of knowledge on the pathophysiology of UPEC-induced orchitis and to evaluate the potential of CIP and DOX as adjunctive therapies for the management of bacterial-induced infertility.

2. METHODS AND MATERIALS:

Animals and grouping

To perform the current study, 42 mature male Wistar rats, weighing 200–220 grams, were obtained from the animal house of the Faculty of Veterinary Science, Urmia University, Iran. The rats were housed in a controlled environment with standard conditions (at 23 ± 2 °C, a 12-hour light/dark cycle), and were received rats standard pellet diet and water ad libitum. The study was conducted in accordance with the ethical guidelines of the Urmia University Ethical Committee, in accordance with the Principles of Laboratory Animal Care Ethics.

After adaptation period (one week), the rats were divided into two groups: a control group (no intervention, n=6) and an UPECI-induced group (n=36). The UPECI-induced group was further subdivided into four groups: 24 hours post-UPECI induction (n=6), 72 hours post-UPECI induction (n=6), one week post-UPECI induction (n=12), and two weeks post-UPECI induction (n=12). The rats in the 24-hour and 72-hour post-UPECI induction groups were euthanized after 24 and 72 hours, respectively. The remaining rats in the one-week and two-week post-UPECI induction groups were treated for an additional week with either 12.5 mg/kg of ciprofloxacin (CIP) or 10 mg/kg of doxycycline (DOX), with 6 rats in each treatment group.

Inducing UPEC infection model

The control group received no treatment throughout the experiment. In the experimental groups, UPEC (specific catalog number and details) was administered to induce infection. Anesthesia was achieved using a ketamine-xylazine cocktail (Alfasan, Woerden, Netherlands) to ensure adequate sedation of the animals. A small incision was then made in the inguinal region, located in the groin area, to provide surgical access to the target site. For the experimental procedure, a bacterial suspension containing 0.1 ml and 0.2 ml of UPEC (at a concentration of 3×108 CFU) was injected into the epididymal tail of both testes.

Total RNA extraction, cDNA synthesis, and qRT-PCR:

To extract miR-155, miR-138, miR-124, SHIP1, SOCS1, TNF-α, and IL-6 from testicular tissue, the tissue samples were immediately preserved in RNAlater solution (Millipore Merck, MDL N: MFCD03453003) to prevent RNA degradation. Then, the testicles were homogenized in TRIzol reagent (Thermo Fisher Scientific, Inc.) to lyse cells and release RNA, which was followed by phase separation using chloroform (Ibrahim and Salah-Eldin, 2019). Next, the aqueous phase of samples containing RNA was carefully collected. The isopropanol was used for RNA precipitation. Following centrifugation, the RNA pellet was washed with 75% ethanol, air-dried, and resuspended in RNase-free water. Purity and concentration of the extracted RNA were assessed using a spectrophotometer (MATEROGEN, Taiwan). Specific miRNAs were then reverse-transcribed using sequence-specific stem-loop primers designed for miR-155, miR-138, and miR-124, enabling quantitative analysis of miRNA expression levels (Table 1).

Table 1: Primer sequences

Gene	Forward	Reverse
miR-155	5′-GGAGGTTAATGCTAATTGTGATAG-3′	5′-GTGCAGGGTCCGA GGT-3′
miR-138	5′-GTATTGACTAGATTAATCACTGT-3′	5′-CTCGCTTCGGCAGCACA-3’
miR-124	5’-GCTAAGGCACGCGGTG-3’	5’-GTGCAGGGTCCGAGGT-3’
IL-6	5'-GCCCTTCAGGAACAGCTATGA-3'	5'-TGTCAACAACATCAGTCCCAAGA-3'
SOCS1	5'-ATGACCCCAAGGACCACT-3'	5'-GCGGTGACAAAGTCTGAC-3'
TNF-α	5'-AAATGGGCTCCCTCTCATCAGTTC-3'	5'-TCTGCTTGGTGGTTTGCTACGAC-3'
SHIP1	5'-CTTCAAGGCAACGCCATC-3'	5'-CTGACCCAGGCTCTCATT-3'
IKKβ	5'-TGAGTACCTGGACTTGCAGAACG-3'	5'-TGTAGATGCCTCTCCAAGGATGG-3'
p65	5'-CAGCCAGGAGGACAGTGT-3'	5'-CTTCGTCGCTGGTGCTG-3'
B-Actin	5’-CTCTTCCAGCCTTCCTTCCT-3’	5’-TCATCG TACTCCTGCTTGCT-3’
U6	5’-CTCGCTTCGGCAGCACATATACT-3’	5’-ACGCTTCACGAATTTGCGTGTC-3’

For reverse transcription, the RNA was then processed with M-MLV reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer’s instructions. PCR was conducted using an Pantamere, Germany cycler, with cycling conditions set as follows: denaturation at 95°C, annealing at 60°C, and extension at 70°C. The 20 µl PCR reaction mixture contained 2.0 µl of the RT product, 1 µl of each PCR primer, 10 µl of Premix Ex Taq (Takara Biotechnology Co., Ltd.), and 6 µl of deionized water. This mixture was incubated at 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Each reaction was performed in triplicate. The relative mRNA levels of SHIP1, SOCS1, TNF-α, IL-6, IKKβ, and p65 were normalized to the reference gene β-actin using SYBR-Green technology. For miR-155, miR-138, and miR-124, relative expression was determined using a SYBR PrimeScript miRNA RT-qPCR kit (Takara Biotechnology Co., Ltd.) per the manufacturer’s protocol, with U6 as an internal control. All data analysis was conducted using the $2^{-\Delta\Delta Cq}$ method.

3. STATISTICAL ANALYSES

Kolmogorov-Smirnov and Levene's tests were used to normalize the statistical results. Analyses of quantitative findings regarding histological, biochemical, and molecular findings were conducted using one-way ANOVA with appropriate post-hoc tests (Tukey's multiple comparison test). Data correlations and regressions were tested using SPSS software (version 11.00, California, USA). A p-value of 0.05 was considered a significant difference for all statistical analyses, and all data were presented as Mean±SD. The graphs were prepared using GraphPad Prism software.

4. RESULTS:

MiR-155, SHIP1, and SOCS1 expression at mRNA level:

Observations indicated a significant increase ($p<0.05$) in the mRNA levels of miR-155 at 24 and 72 hours post-UPECI induction. In contrast, administration of CIP and DOX significantly ($p<0.05$) reduced miR-155 levels at one and two weeks following UPECI induction, with the lowest levels observed in the CIP-treated group one-week post-induction. Regarding SHIP1 mRNA levels, there was a notable ($p<0.05$) increase at 24 and 72 hours post-UPECI induction compared to the control group. Similar to miR-155, rats treated with CIP and DOX showed a significant ($p<0.05$) reduction in SHIP1 mRNA levels relative to the UPECI-only group. Lastly, the UPECI-only group demonstrated a significant elevation ($p<0.05$) in SCOCS1 mRNA levels compared to controls, whereas CIP and DOX treatment significantly ($p<0.05$) decreased SCOCS1 mRNA levels at one and two weeks post-UPECI induction (Fig. 1A, 1B, 1C).

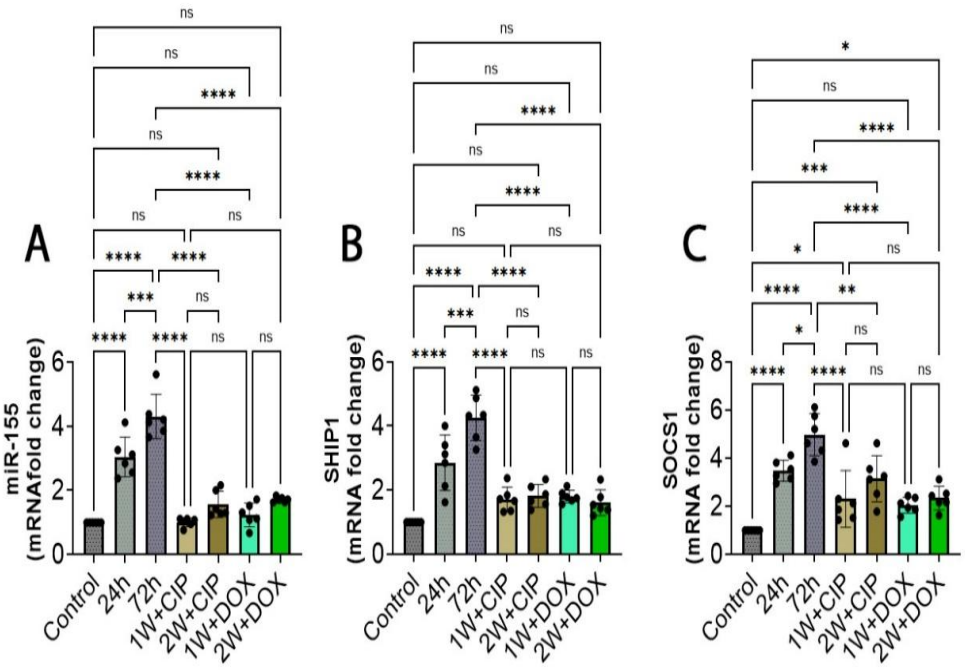

Fig. 1: Mean changes in the miR-155 level, and (B) SHIP1, and (C) SOCS1 mRNA levels in different groups. All data are presented in Mean±SD. Different letters are presenting significant differences between marked data (n=6/each group).

miR-138, IKKβ, and p65 expression at mRNA level:

Rats in the UPECI-only group exhibited a significant ($p<0.05$) decrease in miR-138 levels compared to controls, with levels notably increasing 72 hours post-UPECI induction. CIP- and DOX-treated rats demonstrated a significant ($p<0.05$) increase in miR-138 levels compared to the UPECI-only group. In contrast, IKKβ mRNA levels significantly increased ($p<0.05$) at both 24 and 72 hours post-UPECI induction relative to the control group, while CIP and DOX treatments led to a significant reduction in IKKβ mRNA levels compared to the UPECI-only group, with the lowest levels observed one week post-CIP administration. Finally, UPECI-infected rats showed a significant ($p<0.05$) increase in p65 mRNA levels at 24 and 72-hour post-UPECI induction, whereas CIP and DOX treatments significantly ($p<0.05$) reduced p65 mRNA levels compared to the UPECI-only group (Fig. 2A, 2B, 2C).

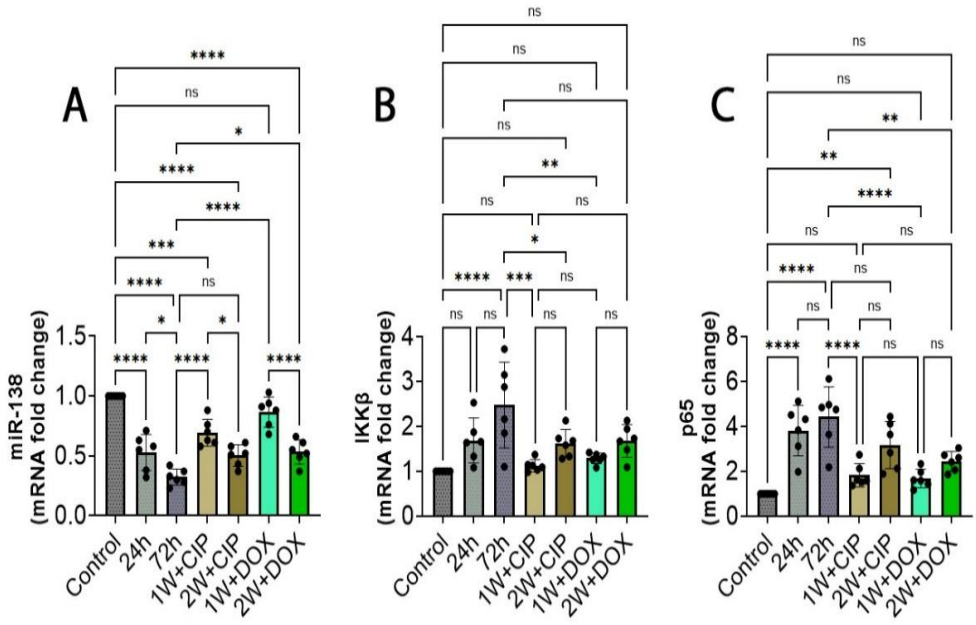

Fig. 2: Mean changes in the miR-138 level, and (B) IKKβ, and (C) p65 mRNA levels in different groups. All data are presented in Mean±SD. Different letters are presenting significant differences between marked data (n=6/each group).

miR-124, IL-6, and TNF-α expression at mRNA level

In UPECI-induced rats, a significant reduction ($p<0.05$) in miR-124 expression was observed at 24 and 72 hours post-UPECI induction compared to control rats. Conversely, miR-124 expression increased in the CIP- and DOX-treated groups, with this increase being most pronounced in the CIP-treated rats one-week post-induction. Additionally, IL-6 mRNA levels were assessed, revealing a significant increase ($p<0.05$) in UPECI-induced rat's relative to controls, with peak levels observed 72-hour post-induction. CIP and DOX treatments significantly reduced ($p<0.05$) IL-6 mRNA levels compared to untreated rats, with the lowest levels found in DOX-treated rats one-week post-induction. Finally, TNF-α mRNA levels were elevated in UPECI-induced rats at 24 and 72-hour post-induction. CIP and DOX treatments led to a significant decrease ($p<0.05$) in TNF-α mRNA levels compared to untreated rats, with the lowest levels observed in DOX-treated rats one week post-UPECI induction (Fig. 3A, 3B, 3C).

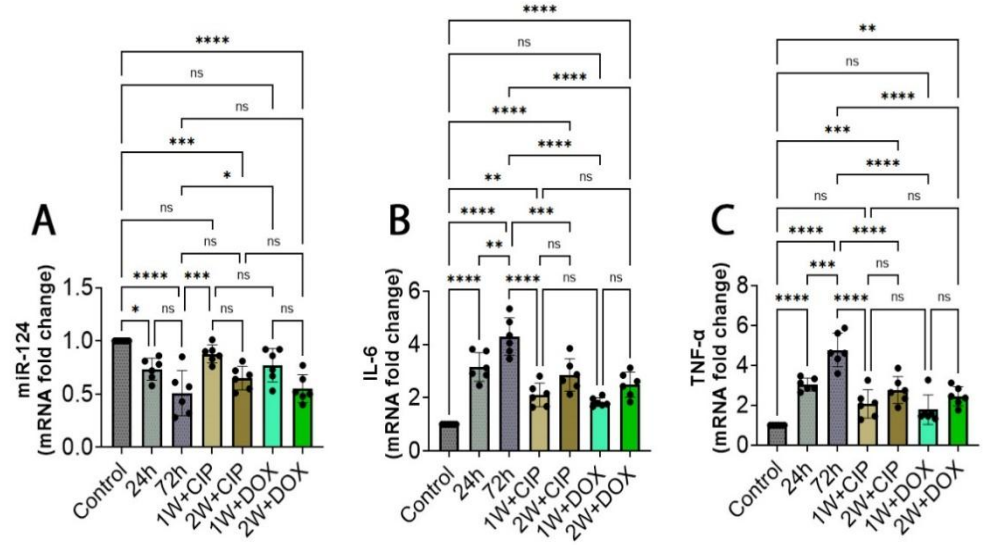

Fig. 3: Mean changes in the miR-124 level, and (B) IL-6, and (C) TNF-α mRNA levels in different groups. All data are presented in Mean±SD. Different letters are presenting significant differences between marked data (n=6/each group).

5. DISCUSSION

Bacterial infections of the male reproductive organs, particularly orchitis, are recognized as major contributors to male infertility, with uropathogenic E. coli (UPEC) emerging as a leading causative agent. The inflammatory response induced by UPEC infection is associated with substantial damage to the seminiferous tubules, severely compromising testicular function (Wang et al., 2021; Zagaglia et al., 2022). The primary objective of this study was to investigate the impact of UPECI on the expression of specific miRNAs within testicular tissue and evaluate the potential protective effects of antibiotic therapies, specifically CIP and DOX, on the inflammatory response. In particular, we focused on the expression levels of miR-155, miR-138, and miR-124, key regulators of immune and inflammatory pathways(Zagaglia et al .,2022).

Our findings revealed that UPECI is associated with a significant alteration in the expression of miR-155, miR-138, and miR-124, each of which plays a crucial role in modulating inflammatory responses. miR-155, known for its pro-inflammatory function, is upregulated during infections driven by lipopolysaccharide (LPS), a potent inducer of inflammation in E. coli infections (Simmonds, 2019; Yin et al., 2021; Zhou et al., 2022). We observed a marked increase in miR-155 expression within testicular tissue following UPECI, suggesting its role in promoting inflammation. This upregulation of miR-155 is likely responsible for enhancing pro-inflammatory cytokine production, such as TNF-α and IL-6, which are crucial mediators of the inflammatory response (Hu et al., 2022; Simmonds, 2019). Moreover, treatment with CIP and DOX led to a significant reduction in miR-155 expression, particularly at one-week post-infection, suggesting that both antibiotics possess immunomodulatory properties beyond their antimicrobial action. These results indicate that CIP and DOX may provide a dual benefit by not only inhibiting bacterial growth but also mitigating the inflammatory cascade initiated by UPEC infection.

The relationship between miR-155 and its target genes, SHIP1 and SOCS1, further elucidates the molecular mechanisms underlying the inflammatory response in UPECI. SHIP1 negatively regulates the Akt signaling pathway, which is essential for immune cell activation and proliferation (Pauls and Marshall, 2017), while SOCS1 is involved in the inhibition of the JAK-STAT signaling pathway, a key regulator of cytokine responses (Liau et al., 2018). Our data demonstrated that miR-155 upregulates these inflammatory pathways by suppressing the expression of SHIP1 and SOCS1, thereby enhancing NF-κB activation, a central transcription factor in inflammation (Chen et al., 2021). The observed elevation in SHIP1 and SOCS1 expression in the UPECI-only group suggests a compensatory mechanism in response to the inflammatory stimulus. However, treatment with CIP and DOX significantly reduced the expression of these markers, suggesting that antibiotic treatment may help normalize the inflammatory response, potentially preserving testicular function.

In contrast to miR-155, the expression of miR-138 was found to be downregulated in response to UPECI. miR-138 has been shown to exert anti-inflammatory effects by targeting and downregulating IKKβ, a key activator of the NF-κB signaling pathway (Yin et al., 2021). IKKβ phosphorylates IκB, leading to its degradation and the subsequent release and nuclear translocation of the NF-κB p65 subunit, which drives the expression of pro-inflammatory cytokines (Stephenson et al., 2023). We observed that UPECI significantly decreased miR-138 levels, which in turn contributed to the upregulation of IKKβ and p65, further amplifying the inflammatory response. Interestingly, treatment with CIP and DOX restored miR-138 expression, which effectively suppressed IKKβ and p65 levels, suggesting a modulation of the NF-κB pathway that promotes a balanced immune response. Notably, CIP was more effective than DOX in restoring miR-138 levels, underscoring the potential of CIP as a more potent immunomodulatory agent in this context (Yin et al ,2021)

The third miRNA of interest, miR-124, is known for its anti-inflammatory properties, particularly in inhibiting NF-κB signaling and reducing the expression of pro-inflammatory molecules such as TNF-α and MCP-1 (Das and Rao, 2022; Zhou et al., 2022). Our results demonstrated that UPECI significantly suppressed miR-124 expression, which in turn led to an increase in the levels of TNF-α and IL-6. The restoration of miR-124 expression following antibiotic treatment, particularly with CIP, suggests a critical role for this miRNA in counteracting the inflammatory cascade initiated by UPEC infection. The optimal time for restoring miR-124 expression was found to be one-week post-infection, after which the effect of antibiotic treatment diminishhed, highlighting the time-sensitive nature of therapeutic intervention in UPECI.

In agreement with the molecular findings, the mRNA expression levels of TNF-α and IL-6 were significantly elevated in the UPECI-only group, confirming the presence of a robust inflammatory response. Both CIP and DOX treatment led to significant reductions in the mRNA levels of these pro-inflammatory cytokines, with DOX showing the most pronounced effect at one-week post-infection. This reduction in TNF-α and IL-6 levels further supports the hypothesis that CIP and DOX may not only inhibit bacterial proliferation but also exert immunomodulatory effects that ameliorate the inflammatory damage associated with UPEC-induced orchitis.

6. CONCLUSION

This study provides valuable insights into the molecular mechanisms underlying the inflammatory response in UPEC-induced orchitis and highlights the potential of CIP and DOX as therapeutic agents that not only target the bacterial pathogen but also modulate the host's immune response. The restoration of miR-155, miR-138, and miR-124 expression by these antibiotics suggests a multifaceted role in controlling both bacterial growth and the inflammatory pathways that contribute to testicular damage. These findings support the use of CIP and DOX as potential therapeutic options for managing UPEC-

induced male infertility, with the optimal therapeutic window occurring within one-week post-infection. Further studies are needed to explore the long-term effects of antibiotic treatment on fertility outcomes and to refine the treatment protocols for improved therapeutic efficacy

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