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Altered Expression of Toll-Like Receptors and Key Signaling Genes in Sertoli Cells of Azoospermic Patients

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Abstract

Background: Azoospermia, the complete absence of sperm in the ejaculate, is a major cause of male infertility. Sertoli cells are essential for spermatogenesis, and disruptions in innate immune immune pathways, particularly Toll-like receptors (TLRs), may impair their function. This study investigated the expression of TLR1–10 and downstream signaling molecules (MYD88, NFKB, TRIF, IRF3, and TRAM) in Sertoli cells of azoospermic patients.

Methods: Testicular tissue were collected from 20 azoospermic men undergoing testicular sperm extraction (TESE). Patients were categorized into two TESE positive (sperm present, n=10) and TESE negative (sperm absent, n=10). Sertoli cells were isolated using enzyme digestion and purified via fluorescence-activated cell sorting (FACS). Gene expression of TLR1–10 and signaling molecules was quantified by RT-PCR. Data were analyzed using independent-samples T-test, with significance set at $p< 0.05$.

Results: Significant downregulation was detected in TLR10 (20.6-fold, $p<0.0001$), TLR9 (4.6-fold, $p<0.05$), TLR7 (4.8-fold, $p<0.01$), TLR6 (12.4-fold, $p<0.05$), TLR5 (13.5-fold, $p<0.001$), TLR4 (3.2-fold, $p<0.05$), and TLR3 (3.1-fold, $p<0.01$). Among signaling molecules, MYD88 (4.1-fold, $p<0.01$) and IRF3 (4.2-fold, $p<0.05$) showed significant reductions, indicating impaired immune signaling in Sertoli cells of TESE-negative men.

Conclusion: Altered expression of TLRs and associated signaling molecules in Sertoli cells of azoospermic men suggests innate immune dysregulation as a potential mechanism underlying defective spermatogenesis. These findings highlight immune privilege-associated pathways as possible targets for developing diagnostic biomarkers and novel therapeutic approaches for male infertility.

Keywords: Azoospermia, Male infertility, Signaling transduction, Sertoli cells, TESE, Toll-like receptors.
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Introduction

Infertility is a global health concern that deeply affects the physical, emotional, and social well-being of couples. It is estimated that about 1 in 6 couples worldwide, roughly 49 to 72 million individuals, struggle with infertility (1, 2). Among these cases, approximately 43.3% are

attributed to male factors, 32% to female factors, 12.5% to both partners, and 13.6% remain unexplained (3, 4). Among the causes of male infertility, immunological and infectious factors account for nearly 13–15% of cases, underscoring the significance of immune mechanisms in male reproductive health (5). The testes are specialized organs responsible for sperm production, and they possess several defense mechanisms to maintain a protective environment. These mechanisms include the immune-privileged status of the testis, the blood–testis barrier (BTB), tight temperature regulation, and the critical role of Sertoli cells in supporting germ cell development (6). Sertoli cells are large, irregularly shaped somatic cells located within the seminiferous tubules, where they play a central role in supporting and regulating spermatogenesis (7). They perform numerous essential functions, such as the production of anti-Müllerian hormone (AMH), which inhibits the development of female reproductive structures; the secretion of inhibin B to regulate follicle-stimulating hormone (FSH) levels; and the synthesis of androgen-binding protein (ABP), which concentrates testosterone within the seminiferous tubules (8, 9). Additionally, Sertoli cells nourish spermatogonia and modulate the spermatogenic process via FSH receptors, contributing to immune regulation within the testis (10). One key aspect of their immune function involves the expression of pattern recognition receptors (PRRs), which allow Sertoli cells to detect pathogens and cellular damage signals (11, 12). PRRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), forming a crucial link between innate and adaptive immunity (13).

Upon recognition of these molecular signatures, PRRs activate signaling cascades, such as the NFKB pathway, promoting Sertoli cells to release pro-inflammatory and antimicrobial cytokines like TNF-α, IL-1β, and IL-6 (14). Among the different PRR families, TLRs particularly detect conserved structural motifs on pathogens and activate primary immune responses (15, 16). TLRs have been found across a wide range of tissues, including the intestine (17), lungs (18), kidneys (19), airways (20), vascular smooth muscle cells (21), and components of the human reproductive system (22, 23). Within the male reproductive tract, previous studies have reported the expression of TLR1–TLR9 in testis, epididymis, and vas deferens. Specifically, TLR2 to TLR6 have been identified in cultured Sertoli cells, whereas TLR11exhibits weaker expression (24, 25). Notably, TLR4 has been linked to the onset of puberty and the development of spermatogenic cells (26).

Structurally, TLRs are composed of an extracellular leucine-rich repeat (LRR) domain (27) that detects microbial ligands such as proteins, sugars, lipids, and nucleic acids, and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain that initiates intracellular signaling (28). Upon activation, the TIR domain interacts with adaptor proteins such as MYD88, TRAM, TIRAP/Mal, and TRIF. These molecules activate downstream cascades like mitogen-activated protein kinases (MAPKs), eventually triggering NFKB and interferon regulatory factor (IRF) pathways to elicit inflammatory and antiviral responses (29). Building on previous studies in animal testis that investigated the role of TLRs in spermatogenesis (30, 31), the purpose of the current study was evaluation of their function in human testis. Given the pivotal role of TLR signaling in immune surveillance and its potential impact on testicular function, an attempt was made to investigate the expression levels of TLRs and associated signaling molecules in the Sertoli cells of azoospermic patients. Understanding these molecular changes could offer new insights into the immunological underpinnings of male infertility and open avenues for novel diagnostic and therapeutic strategies.

Methods

Sample collection and study groups: This study was conducted using human testicular tissues obtained from azoospermic patients who provided written informed consent. A total of 20 participants undergoing TESE at the Royan Institutes male operating room. For each patient, 2 to 4 small testicular fragments were collected during surgery. Following collection, the samples were washed in phosphate-buffered saline (PBS) containing penicillin, streptomycin, and gentamicin to minimize contamination, and were then transferred to DMEM/F-12 culture medium for further processing.

Patients were categorized into two groups based on the presence of spermatozoa in testicular biopsy samples: TESE positive, where sperm were detected, and TESE negative, in which no sperm were observed. Inclusion criteria consisted of patients diagnosed with azoospermia, whereas individuals with a history of chronic infections or

inflammatory disorders were excluded to avoid confounding immune-related variables. Therefore, patients diagnosed with azoospermia attributable to testicular causes were selected, excluding those with pre-testicular or post-testicular etiologies. Biopsy specimens weighing approximately 100–200 *mg* were immediately transferred to Ham's F10 medium and processed for cell isolation.

Isolation and cultivation of Sertoli cells: Sertoli cell isolation was performed using a two-step enzyme digestion protocol, based on previously established methods (32). In the first digestion step, testicular tissue samples were incubated at 37°*C* for 30 *min* in DMEM/F-12 medium containing a cocktail of enzymes: 1 *mg/ml* collagenase, 1 *mg/ml* hyaluronidase, 0.5 *mg/ml* trypsin, and 0.05 *mg/ml* DNase. The mixture was gently pipetted at regular intervals during incubation to ensure thorough dissociation of cells.

After enzymatic digestion, the cell-tissue suspension was centrifuged several times at 1100 *rpm* to remove interstitial cells and debris. The remaining seminiferous tubule fragments were then subjected to a second round of enzymatic digestion under similar conditions for 45 *min*. The dissociated cells were washed in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) and prepared for selective enrichment of Sertoli cells.

To enhance the purity of Sertoli cells, the digested cell suspension was seeded onto petri dishes coated with Datura stramonium agglutinin (DSA) lectin (Sigma, USA), which selectively binds to Sertoli cells. DSA was first dissolved in PBS at a concentration of 5 *µg/ml* and used to coat the dishes. These were then washed with PBS containing 0.5% bovine serum albumin (BSA) and air-dried before use. Cells were incubated on DSA-coated plates at 37°*C* in a humidified 5% CO_2 incubator for 2–3 *hr*. Non-adherent cells were removed by washing, while the adherent cell population, comprising mainly Sertoli cells and some fibroblasts, was retained for culture.

The attached cells were then cultured in fresh DMEM/F-12 medium and maintained for 3–4 days, with routine medium replacement to support attachment and proliferation. Once cells had reached adequate confluence, they were detached using a 0.25% trypsin-EDTA solution in PBS without calcium and magnesium at 37°*C* for 5 *min*. The cells were then resuspended in DMEM/F-12 containing 10% FBS and transferred to multi-well plates or new petri dishes for continued expansion.

Cell viability was assessed using 0.04% trypan blue exclusion, and viable cell counts were performed using a Neubauer hemocytometer under a light microscope. The final cell density was calculated and adjusted based on experimental requirements.

Purification of Sertoli cells by flow cytometry: While traditional approaches for Sertoli cell enrichment, particularly lectin-based selection techniques, have proven useful, they often fall short of required specificity, occasionally leading to contamination with mesenchymal or peritubular cells. To achieve a higher level of purity, FACS was employed in this study, utilizing the follicle-stimulating hormone receptor (FSHR) as a specific surface marker to identify and isolate mature Sertoli cells. Sertoli cells were first dissociated enzymatically from cultured testicular tissue using a mild solution of 0.02% trypsin-EDTA. Following incubation, cells were gently pipetted to achieve a single-cell suspension, then washed with PBS containing 1% FBS to block nonspecific binding. The resulting suspension was centrifuged at 1500 *rpm* for 5 *min* at 4°*C*. For immunolabeling, cells were incubated with rabbit polyclonal anti-FSH receptor primary antibody (Abcam, USA) at a concentration of 20 *µl* per 100 *µl* PBS for 45 *min* at 4°*C*. After primary incubation, cells were washed again with PBS containing 1% FBS and resuspended. The labeled cells were then incubated with a FITC-conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution, Abcam, USA) under the same conditions. A final wash was performed, and the cell pellet was resuspended in a sorting buffer consisting of PBS, 1% FBS, 1 *mM* EDTA, and 25 *mM* HEPES to preserve cell viability during sorting. Flow cytometry was carried out using a BD FACSAria II Cytometer (BD Biosciences, USA), and gating strategies were applied to isolate FSHR-positive cells, thereby enriching for Sertoli cells while excluding other testicular cell types. Negative control samples, including unstained and secondary antibody-only controls, were included to ensure specificity of signal detection. This strategy allowed us to obtain a highly purified population of Sertoli cells with minimal contamination, suitable for subsequent molecular and functional analyses. The use of FSHR as a positive marker provides an advantage over older techniques and ensures higher

fidelity in cell identification for studies focusing on immune signaling and spermatogenesis.

Enzyme-linked immunosorbent assay (ELISA): To quantify the secretion of AMH by Sertoli cells, a commercial ELISA kit (Ansh Labs, Germany) was used following the manufacturer's instructions. Culture media from Sertoli cell cultures were collected at defined time points, centrifuged to remove cellular debris, and stored at −80°*C* until analysis. Each sample was analyzed in duplicate, and absorbance was measured at 450 *nm* using a microplate reader. AMH concentrations were calculated using a standard curve generated from known concentrations provided with the assay kit. This hormone is a well-established marker of Sertoli cell function and developmental stage.

RNA isolation: Immediately following the culture period, Sertoli cell samples were carefully transferred into sterile cryovials. To preserve RNA integrity and prevent degradation during storage and handling, an appropriate volume of RNAlater stabilization solution (Sigma, UK) was added to each sample. The cryovials were then stored at –80°*C* until RNA extraction.

Total RNA was isolated from the cultured Sertoli cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions. To ensure complete removal of genomic DNA contamination, the extracted RNA was treated with DNase I (Fermentas, Germany), following the recommended protocol. Throughout the entire procedure, RNase-free pipette tips and tubes were used, and all steps were performed on ice to minimize any potential RNA degradation.

The purity and concentration of the isolated RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Samples with an A260/A280 absorbance ratio between 1.8 and 2.0 were considered acceptable for downstream applications, indicating high-quality RNA, free of protein or phenol contamination.

cDNA synthesis: Complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Oligo(dT) primers were utilized to selectively bind to the poly(A) tail of mRNA molecules, enabling reverse transcription of mature mRNA transcripts into cDNA. Each reaction was performed in a final volume of 20 μl containing 1 μg of total RNA, dNTPs, reverse transcriptase, and reaction buffer. The reverse transcription reactions were incubated at 42°*C* for 60 *min* to allow cDNA synthesis, followed by a heat inactivation step at 70°*C* for 5 *min* to terminate the reaction.

Quantitative real-time PCR (qRT-PCR): Quantitative real-time PCR (qRT-PCR) was carried out to determine the relative expression levels of target genes. The cDNA obtained from the reverse transcription step was used as a template in PCR reactions, prepared using a standard master mix. Each reaction was performed in triplicate to ensure technical accuracy and reproducibility.

PCR amplification was conducted in a final reaction volume of 20 μl using gene-specific primers (listed in table 1) on an Applied Biosystems Real-Time PCR system (Thermo Fisher Scientific, USA). The PCR protocol included 50 amplification cycles. GAPDH was selected as the housekeeping gene to normalize expression data.

Relative gene expression levels were calculated using the comparative threshold cycle (ΔΔCt) method, which allows for the assessment of fold changes in gene expression across experimental groups.

Statistical analysis: Statistical analyses were performed using SPSS software, version 27 (IBM, USA). Data were presented as mean±standard error of the mean (SEM). Comparisons between experimental groups were evaluated using the independent-samples T-test. A p-value of less than 0.05 was considered statistically significant.

Results

Morphological assessment of cultured Sertoli cells: Following their initial isolation, Sertoli cells underwent distinct morphological transformations during the culture period. As shown in figure 1, these changes were particularly evident in the first and third weeks of culture. In the first week (Figure 1A), Sertoli cells displayed a sparse distribution across the culture surface. Their morphology was marked by the loss of typical cytoplasmic projections and the adoption of irregular, flattened shapes. The cells appeared granular and uneven, likely due to intracellular lipid droplets, a known characteristic feature of Sertoli cells in early adaptation to in vitro conditions.

Additionally, cytoplasmic extensions began to elongate, with some cells initiating contact with neighboring cells, suggesting early stages of cellu-

Table 1. Sequence of primers used in this study

Gene name	Forward primer/Reverse primer
GAPDH	CTCATTTCCTGGTATGACAACGA CTTCCTCTTGTGCTCTTGCT
TLR1	GGGTCAGCTGGACTTCAGA AAAATCCAAATGCAGGAACG
TLR2	TCGGAGTTCTCCCAGTTCTCT TCCAGTGCTTCAACCCACAA
TLR3	GTATTGCCTGGTTTGTTAATTGG AAGAGTTCAAAGGGGGCACT
TLR4	TGATGTCTGCCTCGCGCCTG AACCACCTCCACGCAGGGCT
TLR5	CACCAAACCAGGGATGCTAT CCTGTGTATTGATGGGCAAA
TLR6	GCCACCATGCTGGTGTTGGCT CGCCGAGTCTGGGTCCACTG
TLR7	CCTTGAGGCCAACAACATCT GTAGGGACGGCTGTGACATT
TLR8	CTTCGATACCTAAACCTCTCTAGCAC AAGATCCAGCACCTTCAGATGA
TLR9	TTCCCTGTAGCTGCTGTCC ACAGCCAGTTGCAGTTCACC
TLR10	TGCCCACCACAATCTCTTCCATGA AGCAGCTCGAAGGTTTGCCCA
MYD88	GTCTCCTCCACATCCTCCCT TCCGCACGTTCAAGAACAGA
TRIF	CCTGCTGAAGAAGCCCAAGA GGTTGTCCTGTGAGGTAGGC
IRF3	CTTGGTGGAGGGCATGGATT GTTGAGGTGGTGGGGAACAG
TRAM	GAAAGGAACAGGACACCCGAG TGAGTAGGCTGCGTTCAGTG
NFKB	CTGGATCTGCTGGTGGACAG CTGTGGCTAGATGCAAGGCT

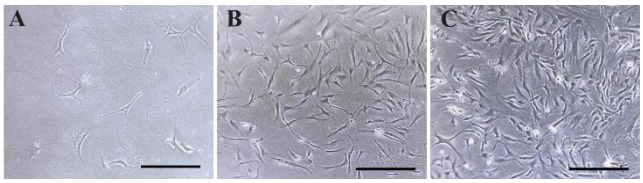

Figure 1. Morphological changes in Sertoli cells during in vitro culture. A) Sertoli cells after 1 week, sparse distribution with irregular morphology and granular appearance; B) Sertoli cells after 3 weeks, formation of a confluent monolayer with enhanced intercellular connections; and C) two months after culture. Scale bars in A-C = 200 μm

lar networking. By the third week (Figure1B), a dramatic shift in cell behavior was evident. Sertoli cells had proliferated and formed a confluent monolayer with a more organized and interconnected morphology. The cells demonstrated enhanced adherence, more defined boundaries, and prominent cytoplasmic extensions linking adjacent cells. These changes indicate successful adaptation to the culture environment and support the establishment of a stable Sertoli cell monolayer, an essential prerequisite for downstream experimental procedures. Interestingly, a noticeable challenge was observed in cultures derived from azoospermic men whose testicular biopsy samples were negative for spermatozoa (TESE negative). In these cases, the isolation and expansion of Sertoli cells were markedly more challenging. In several samples, Sertoli cells could not be successfully isolated. Even when present, these cells showed minimal detachment from the tissue matrix, poor adherence, and a markedly slow proliferation rate.

AMH concentration in Sertoli cell-conditioned medium: To evaluate the functional activity of Sertoli cells isolated from testicular biopsies, the concentration of AMH in the culture supernatants was measured using a commercially available ELISA kit. AMH, a glycoprotein hormone secreted by Sertoli cells, plays a critical role in male gonadal development and serves as a valuable biomarker for assessing Sertoli cell viability and secretory capacity *in vitro*. The analysis of the conditioned medium from 60 independent Sertoli cell cultures revealed a median AMH concentration of 4.59 *ng/ml*, with measured values ranging from 0.6 to 13.7 *ng/ml*. Interestingly, the mean concentration of AMH was markedly higher, calculated at 23.8 *ng/ml*, indicating a positively skewed distribution likely due to high AMH secretion in a subset of cultures.

Purification of Sertoli cells using FSHR-targeted FACS: To ensure the accuracy of downstream analyses, particularly gene expression profiling of Toll-like receptors, an essential step involved isolating a pure population of Sertoli cells from testicular cell suspensions. FACS was employed

Table 2. Comparison of qRT-PCR results and gene expression levels (fold change)

qRT-PCR results			
Gene symbol	**TESE positive**	**TESE negative**	**Fold change**
TLR1	0.97±0.28	0.89±0.1	1.09
TLR2	1.04±0.21	1.21±0.54	0.86
TLR3	3.14±0.28	1.02±0.12	3.09 **
TLR4	3.22±0.2	1.14±0.43	2.83 *
TLR5	13.53±0.9	1.01±0.08	13.45 ***
TLR6	12.42±2.76	1.1±0.35	11.28 *
TLR7	4.89±0.79	1.03±0.16	4.76 **
TLR8	1.16±0.09	0.16±0.009	7.34 ***
TLR9	4.62±0.41	1.04±0.18	4.45 *
TLR10	20.67±0.52	1.02±0.12	20.32 ****
TRIF	1.84±0.57	1.02±0.15	1.8
IRF3	4.21±0.63	1.14±0.34	3.68 *
NFKB	1.19±0.29	1.12±0.35	1.06
TRAM	3.46±0.92	1.11±0.33	3.11
MYD88	4.18±0.61	1.03±0.19	4.04 *

Data are presented as mean±SEM, with statistical significance indicated by: * (p<0.05), ** (p<0.01), *** (p<0.001), and **** (p<0.0001)

using a lectin-independent, receptor-specific strategy targeting FSHR, a well-established surface marker for mature, functionally active Sertoli cells. Prior to sorting, cells were incubated with a monoclonal antibody specific to FSHR, followed by staining with a fluorescent secondary antibody. Flow cytometry analysis of the unstained control sample confirmed negligible background fluorescence, with only 1.83±0.9% of events falling within the positive gate (Figure 2A). In contrast, the antibody-labeled test sample showed a clearly distinguishable population of FSHR-positive cells accounting for 30.25±14.1% of the total events, which were gated as population P3 (Figure 2B). These FSHR-expressing cells were interpreted as the target Sertoli cell population due to their specific receptor expression profile. Following FACS, the P3 gate was collected and re-analyzed to evaluate the enrichment of Sertoli cells. As shown in figure 2C, the sorted fraction demonstrated 91.6±5.1% purity, confirming that the applied antibody-based FACS method was effective in isolating a highly enriched Sertoli cell population suitable for subsequent molecular analyses. This step was especially critical given the initial heterogeneity of the testicular cell isolates, which included fibroblast-like mesenchymal cells and other non-Sertoli cell types. These findings not only validate the specificity of the FSHR antibody labeling approach but also reinforce the importance of cellular purification in transcriptomic and secretomic studies focused on male infertility mechanisms.

Altered expression of TLRs in Sertoli Cells from TESE positive and TESE negative azoospermic cases: To investigate the potential involvement of innate immune signaling in male infertility, the mRNA expression levels of TLR family members (TLR1–TLR10) were quantitatively assessed in Sertoli cells isolated from two groups of azoospermic cases: those with successful sperm retrieval (TESE positive) and those with failed sperm retrieval (TESE negative). Quantitative real-time PCR was performed using GAPDH as a reference gene to normalize expression levels across samples. The results are summarized in table 2. The analysis revealed distinct differences in the expression patterns of TLRs between the two groups. In the TESE negative group, TLR1 expression appeared lower in comparison to TESE positive group, though the difference did not reach statistical

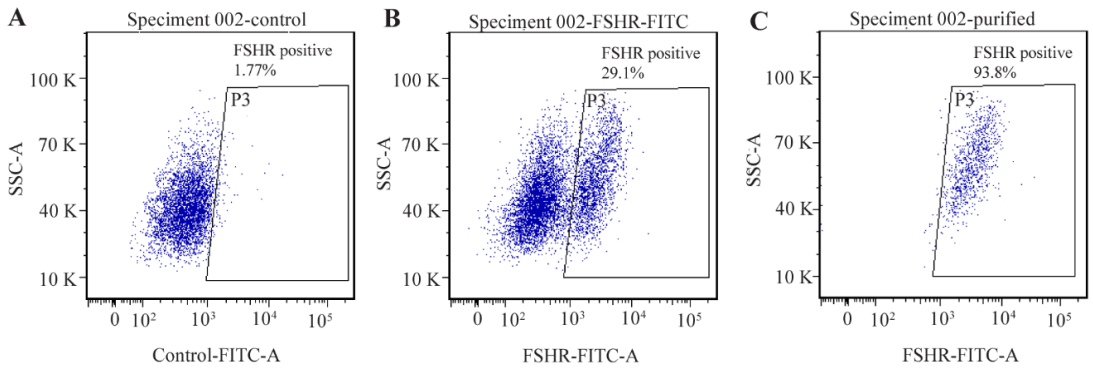

Figure 2. Flow cytometry analysis for Sertoli cell purification. A) unstained control showing minimal background fluorescence (1.77%), B) FSHR-positive population identified in the test sample (29.1%, gated as P3), and C) post-sorting analysis demonstrating 93.8% purity of the isolated Sertoli cell population

significance (p>0.05). Conversely, TLR2 showed a modest increase in the TESE negative group; however, this variation was also not statistically significant (p>0.05). More notably, a statistically significant downregulation (p<0.05) was observed in the expression levels of multiple key TLRs including TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 in Sertoli cells from the TESE negative group compared to the TESE positive group. This broad downregulation suggests a global impairment in TLR-mediated signaling pathways in the testes of men with more severe forms of spermatogenic failure.

Differential expression of downstream TLR signaling molecules in TESE positive and TESE negative Sertoli cell populations.

To further elucidate the mechanisms underlying altered Toll-like receptor signaling in Sertoli cells of azoospermic men, the expression of key adaptor and transcriptional regulator genes involved in the TLR signaling cascade, TRIF (TICAM1), IRF3, NFKB, TRAM, and MYD88, was examined in Sertoli cells derived from TESE positive and TESE negative individuals. The relative mRNA levels were quantified using RT-qPCR, with GAPDH used as a reference gene for normalization. The comparative results are illustrated in table 2. Although a downward trend in the expression of TRIF, TRAM, and NFKB was observed in the TESE negative group compared to TESE positive, the differences in these genes did not reach statistical significance (p>0.05). However, this subtle reduction may still indicate a general suppression in TRIF-dependent TLR signaling, especially considering that TRAM functions as a bridging adaptor for TRIF recruitment in the TLR4 pathway. More strikingly, the TESE negative group demonstrated a significant decrease in IRF3 expression (p<0.05) relative to the TESE positive group. Similarly, the expression of MYD88, the central adaptor protein in the MYD88-dependent TLR signaling pathway, was found to be significantly reduced in the TESE negative group (p<0.05).

Discussion

Immune privilege in the testis is largely maintained by Sertoli cells, through the formation of BTB and secretion of immunoregulatory factors. However, infections and inflammation can disrupt this privilege, impairing spermatogenesis and leading to infertility (33). TLRs expressed on Sertoli cells are pivotal in recognizing pathogens and initiating innate immune responses, thereby playing a critical role in regulating testicular immunity. Additionally, Sertoli cells produce anti-inflammatory cytokines and other molecules that modulate the local immune environment, further contributing to immune privilege (34, 35). It has been shown that Sertoli cells exhibit differential growth in these two tissues, and in samples lacking sperm, they are difficult to isolate and demonstrate markedly slower proliferation.

This study demonstrated that the expression levels of TLRs and signaling markers were different among study groups. Unlike TLR1 and TLR2, TLR3-TLR10 showed significant downregulation which could highlight their potential role in immune regulation and spermatogenesis. Additionally, the expression of TRAM, NFKB, TRIF, IRF3, and MYD88 was lower in the TESE negative group. The downregulation of IRF3 and MYD88 was statistically significant, suggesting their involvement in testicular immune dysfunction and male infertility. Previous studies in line with our data have shown the active role of TLR family genes in the male reproductive system. TLR1, TLR3, TLR5, and TLR11 are expressed throughout all stages of spermatogenesis, whereas TLR4 is active during early and intermediate stages, and TLR2, TLR7 are restricted to early stages (36). In rats, the expression of TLR1-TLR9 mRNA was identified in different regions including testis, epididymis, and vas deferens, whereas TLR10 and TLR11 were less frequently detected. Proteins such as TLR1, TLR2, TLR4, and TLR6 are distributed across the male reproductive tract, while TLR3, TLR5, and TLR9–TLR11 are expressed at lower levels (25). Our previous study confirmed the expression of TLR2 and TLR3 in TESE, also demonstrating significantly higher TLR3 transcript levels compared to TLR2 in Sertoli cells of azoospermic men (37).

Our results suggest that higher TLR expression in the TESE positive group may enhance Sertoli cell's function, contributing to an immune-protective and homeostatic testicular environment. Enhanced immune function in Sertoli cells may contribute to improved spermatogenesis. Prior research also indicates that TLR2, TLR5, and TLR6 stimulation in cultured Sertoli cells upregulates ICAM-1 expression and activates some inflammatory pathways, such as IL-1α, IL-6, and interferons, supporting spermatogenesis (31). Thus, the presence of TLRs in Sertoli cells may enhance their role in the elimination of microbial patho-

gens.

Additional findings of our study revealed decreased expression of TRAM, NFKB, TRIF, IRF3, and MYD88 in the TESE negative group as compared to the TESE positive group with IRF3 and MYD88 showing statistically significant reductions. Increased gene expression of their adaptor molecules in the cytoplasm of Sertoli cells indicates the close connection between these molecules. The results could suggest the possible role of these genes in the development of testicular immune dysfunction and male infertility.

Consistent with our findings, multiple studies have demonstrated the role of TLRs and their associated signaling pathways in testicular immunity and male infertility processes. For example, Nishimura and Naito in 2005 identified the presence of TLR1–TLR10 and associated factors, MYD88 and TIRAP, in the testis, emphasizing their role in local immunity and reproduction issues (38). The MYD88-dependent pathway, through the activation of TLR1, TLR2, and TLR6, can be one of the main inflammatory pathways triggered in response to sperm with high DNA fragmentation index (DFI) in men with recurrent implantation failure (RIF) (39).

Reduction in the expression of MYD88, TRIF, and TRAM and also TLRs in the TESE negative group, likely results in decreased NFKB and IRF3 activation, potentially leading to decreased production of pro-inflammatory cytokines. Collectively, diminished activity of TLRs pathways may lead to increased susceptibility to chronic infections or tissue damage, and finally disrupt spermatogenesis (40). This highlights the importance of balancing the activation and inhibition of TLR pathways to prevent inflammation and tissue damage (41). The downregulation of TLRs and their associated inflammatory mediators, such as NFKB and IRF3, in TESE negative samples suggests a compromised immune response in sperm-negative testicular tissue.

This study demonstrated a significant reduction in key components of TLR signaling pathways, including MYD88 and IRF3, in the TESE negative group, alongside decreased expression of TLRs. Impairment in immune signaling not only weakens the testicular immune response but also increases susceptibility to pathogens and disrupts spermatogenesis, highlighting a potential link between dysregulated immune pathways and male infertility.

Conclusion

This study lays a critical foundation for advancing our understanding of innate immunity in male reproductive health. By revealing specific disruptions in TLR signaling pathways within Sertoli cells, particularly in sperm-negative tissues (TESE negative), it highlights key molecular alterations that may impair spermatogenesis. This study offered valuable insights that could support the development of targeted diagnostic markers and therapeutic approaches for male infertility. However, further research is essential to fully elucidate how TLR signaling influences Sertoli cell function, proliferation rate and germ cell support, ultimately guiding future strategies to restore or enhance male fertility.

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Conflict of Interest

Authors declare no conflict of interest.

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