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Research Article

Elevated Seminal Plasma TLR-2 Levels are Associated with Leukocytospermia

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Abstract

Introduction: Leukocytospermia is associated with male infertility, but its underlying mechanisms are not fully understood. This study aimed to investigate the association between seminal plasma toll-like receptor 2 (TLR-2) and prostaglandin E_2 (PGE_2) levels and leukocytospermia in infertile Iraqi men, and to evaluate their potential as differential biomarkers.

Methods: Eighty infertile men attending an infertility clinic in Iraq were enrolled. Semen analysis was performed according to WHO 2010 criteria. TLR-2 and PGE_2 levels in seminal plasma were quantified using ELISA. Participants were categorized based on leukocytospermia status, varicocele presence, and smoking habits. Statistical analyses included correlation tests and receiver operating characteristic (ROC) curve analysis.

Results: Seminal plasma TLR-2 levels were significantly higher in leukocytospermic patients compared to non-leukocytospermic men (15.14 ± 1.06 vs. 9.27 ± 1.42 ng/mL, $p < 0.05$). TLR-2 levels showed strong negative correlations with sperm concentration (r = -0.675), total sperm count (r = -0.673), progressive motility (r = -0.669), and normal morphology (r = -0.616) (all $p < 0.001$). Positive correlations were observed between TLR-2 and round cell concentration (r = 0.684) and white blood cell count (r = 0.668) (both $p < 0.001$). Smoking and varicocele did not significantly influence TLR-2 levels. ROC analysis revealed high diagnostic accuracy for TLR-2 in identifying leukocytospermia (AUC = 0.993, $p < 0.05$). In contrast, PGE_2 levels showed no significant differences or correlations with semen parameters.

Discussion: The elevated TLR-2 levels in leukocytospermic samples and strong correlations with semen parameters suggest a potential role for TLR-2 in inflammation-related male infertility. The persistence of this association regardless of smoking status or varicocele presence further supports TLR-2's specificity as a biomarker for leukocytospermia.

Conclusion: Elevated seminal plasma TLR-2 levels are associated with leukocytospermia and poor semen parameters in infertile Iraqi men. TLR-2 shows promise as a differential biomarker for male infertility, particularly in cases of leukocytospermia.

Keywords: biomarker, leukocytospermia, male infertility, semen parameters, toll-like receptor 2

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1. Introduction

The male reproductive tract's chronic inflammation is a common health issue significantly impacting male fertility, contributing to 6-10% of cases worldwide [1, 2]. Acute infectious inflammations of the male reproductive system often present with clear symptoms, whereas chronic non-infectious inflammatory processes frequently manifest subclinically or asymptomatically. Thus, this nature may lead to under-diagnosis and probably result in an underestimation of inflammation-mediated male infertility prevalence [3].

Toll-like receptors (TLRs) are essential mediators of innate immune activation, functioning as pattern recognition receptors that detect pathogen-associated molecular patterns (PAMPs) of microbial origin and damage-associated molecular patterns (DAMPs) released from cellular necrosis and tissue injury [4]. Of particular interest is TLR-2, which is important for recognizing both PAMPs and endogenous danger signals. Recent studies have demonstrated the expression of TLRs, including TLR-2, in various components of the male reproductive tract such as the testes, epididymis, and spermatozoa [5-8]. A study by Hagan et al. identified TLR-2, along with TLR-4, COX-2, and Nrf-2, as novel seminal biomarkers of inflammatory processes and oxidative stress in patients with leukocytospermia [9]. Their findings highlighted the potential importance of these markers in understanding the inflammatory processes associated with male infertility. However, despite this groundbreaking work, there remains a significant gap in our understanding of these biomarkers in different populations and geographical regions. Notably, to date, there have been no reports investigating the expression and role of TLR-2 in leukocytospermia patients in Iraq.

Prostaglandin E_2 (PGE_2) is another important mediator in the inflammatory process and has been implicated in male reproductive function [10, 11]. The PGE_2 is synthesized from arachidonic acid by cyclo-oxygenase enzymes and has diverse effects on sperm function, including modulation of sperm motility and acrosome reaction [10]. Yet, the interaction between TLR activation and PGE_2 production in the context of inflammatory processes in the male reproductive system and infertility needs further investigation.

Leukocytospermia, characterized by the presence of $\geq 1\times10^6$ leukocytes per milliliter of seminal fluid, is observed with a prevalence of approximately 10-20% among males with infertility [12, 13]. It is associated with elevated oxidative stress and the secretion of pro-inflammatory mediators, which may exert deleterious effects on spermatozoa quality and functional parameters [13, 14]. Varicocele, which affects 15-20% of males with reproductive issues [15], is linked to elevated oxidative stress and inflammation in the male reproductive tract [16]. Therefore, given the potential importance of TLR-2 and PGE_2 in mediating inflammatory responses in the male reproductive system, this study aimed to investigate the presence of TLR-2 and PGE_2 in seminal plasma and evaluate their association with leukocytospermia, semen parameters, and other clinical factors in infertile men as well as their differential value.

2. Materials and Methods

2.1. Study Setting and Participants

The current study was carried out at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies (Baghdad, Iraq), from March to August 2021. The study was registered and approved by the institute's Committee of Research Ethics. Eighty male participants aged between 28 and 44 years with primary or secondary infertility, were enrolled. All participants provided written informed consent. Exclusion criteria included oligozoospermia (sperm concentration $< 10 \times 10^6$ ml^{-1}) and confirmed spermatozoal infections.

2.2. Semen Fluid Analysis

Ejaculate samples were obtained via masturbation following a 3–5-day period of sexual abstinence. Specimens underwent liquefaction for 30 mins at 37°C before analysis. Seminal parameters were evaluated in accordance with the World Health Organization (WHO) 2010 guidelines, including volume, sperm concentration, motility, and morphology [12]. Leukocyte concentration was assessed using the peroxidase method [17]. Leukocytospermia was defined as $\geq 1 \times 10^6$ leukocytes/mL in semen.

2.3. TLR-2 and PGE_2 Measurement

Seminal plasma TLR-2 and PGE_2 concentrations were quantified using sandwich enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions. For TLR-2, an ELISA kit from Abbexa Ltd. (Cat no. abx256053) was used. Briefly, standards and samples were added to precoated wells and incubated. After washing, biotin-conjugated anti-TLR-2 antibody and HRP-conjugated Avidin were added sequentially. The reaction was visualized using a TMB substrate and stopped with an acidic solution. PGE_2 levels were determined using a competitive ELISA kit (Cayman Chemical, USA; Cat no. 514010). Samples and standards were incubated with PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) and PGE_2 monoclonal antibody. After washing, Ellman's reagent was added to develop the reaction. For both assays, absorbance was measured at 450nm using a microplate reader. TLR-2 and PGE_2 concentrations were calculated using their respective standard curves. All samples were analyzed in duplicate, and the average value was used for statistical analysis.

2.4. Statistical Analysis

Data was statistically evaluated by using SPSS version v. 27 (IBM Corp., USA) and GraphPad Prism v. 8.0 (USA) for data visualization. The normality distribution was assessed via the Shapiro-Wilk test.

Normally distributed variables were expressed as mean ± standard deviation (SD), while non-normally distributed variables were presented as median with interquartile range (IQR). Comparative analyses between the two groups employed independent samples t-tests for parametric data and Mann-Whitney U tests for nonparametric data. Kruskal-Wallis tests with Dunn's post-hoc analysis were utilized for non-parametric comparisons across multiple groups. Categorical variables were expressed as frequencies and percentages, with comparisons made using Chi-square or Fisher's exact tests as appropriate. Correlations were evaluated using Spearman's rank correlation coefficient. Receiver operating characteristic (ROC) curve analysis was performed to assess the discriminatory power of biomarkers. A p-value of ≤ 0.05 was considered statistically significant and all tests were two sided.

3. Results

Table **1** shows the basic characteristics of leukocytospermic (n = 30) and non-leukocytospermic (n = 50) infertile men. No statistically significant intergroup differences were observed in age, body mass index, infertility classification, tobacco use, varicocele presence, or alcohol consumption (all $p > 0.05$). However, significant differences were evident across all semen parameters between leukocytospermic and non-leukocytospermic groups (Table **1**). The leukocytospermic group showed significantly lower sperm concentration (21.00×10^6/mL vs. 53.00×10^6/mL, $p < 0.001$), total sperm count (52.50×10^6 vs. 185.5×10^6, $p < 0.001$), and progressive motility (35.00% vs. 55.00%, $p < 0.001$) when compared to the non-leukocytospermic group. Furthermore, leukocytospermic group also demonstrated significantly higher percentages of non-progressive motility (20.00% vs. 15.00%, $p < 0.001$) and immotile sperm (45.00% vs. 30.00%, $p < 0.001$). Total progressive motile sperm count was markedly lower in the leukocytospermic group (18.38×10^6 vs. 107.8×10^6, $p < 0.001$). Normal sperm morphology was substantially reduced in the leukocytospermic group (3.500% vs. 7.00%, $p < 0.001$). The leukocytospermic group showed significantly higher concentrations of round cells (1.750×10^6/mL vs. 0.600×10^6/mL, $p < 0.001$) and white blood cells (1.350×10^6/mL vs. 0.300×10^6/mL, $p < 0.001$) compared to the non-leukocytospermic group.

Table 1: Demographic and clinical characteristics of leukocytospermic and non-leukocytospermic infertile men.

Parameter	Leukocytospermic group (n = 30)	Non-leukocytospermic (n = 50)	p-value
Age (years)	33.00 (31.25–36.25)	32.00 (31.00–35.00)	0.213
BMI (kg/m^2)	25.69 ± 1.29	25.73 ± 1.16	0.875
Type of infertility			
Primary	20 (66.7)	39 (78)	0.301
Secondary	10 (33.3)	11 (22)	
Smoking status, n (%)			
Smoker	11 (37.0)	13 (26.0)	0.601
Ex-smoker	5 (17.0)	10 (20.0)	
Nonsmoker	14 (46.0)	27 (54.0)	

Table 1: Continued.

Parameter	Leukocytospermic group (n = 30)	Non-leukocytospermic (n = 50)	*p*-value
Varicocele			
Present	4	5	0.648
Absent	26	45	
Alcohol consumption			
Yes	2	6	0.441
No	28	44	
Sperm conc. ($\times 10^6$/mL)	21.00 (18.00–26.00)	53.00 (42.00–58.00)	< 0.001
Total sperm count ($\times 10^6$)	52.50 (45.00–65.00)	185.5 (147.0–203.0)	< 0.001
Progressive motility (%)	35.00 (30.00–40.00)	55.00 (50.00–60.00)	< 0.001
Non-progressive motility (%)	20.00 (20.00–25.00)	15.00 (10.00–15.00)	< 0.001
Immotile (%)	45.00 (40.00–45.00)	30.00 (30.00–35.00)	< 0.001
Total progressive ($\times 10^6$)	18.38 (13.50–26.00)	107.8 (72.85–115.5)	< 0.001
Normal morphology (%)	3.500 (3.000–4.000)	7.000 (6.000–8.000)	< 0.001
Round cell conc. ($\times 10^6$/mL)	1.750 (1.500–2.000)	0.600 (0.500–0.900)	< 0.001
WBCs ($\times 10^6$/mL)	1.350 (1.100–1.500)	0.300 (0.200–0.500)	< 0.001

Data are presented as median (interquartile range) for continuous variables, and n (%) for categorical variables. Mann-Whitney U test was used to compare all continuous variables including age and sperm parameters. BMI is presented as mean ± standard deviation and was compared using independent samples t-test. Fisher's exact test was used for all categorical data. BMI: Body mass index; WBCs: White blood cells.

The comparative analysis of TLR-2 and PGE_2 levels between leukocytospermic and non-leukocytospermic groups is illustrated in Figure **1**. As depicted in Figure **1A**, seminal plasma TLR-2 levels were significantly elevated in the leukocytospermic group compared to the non-leukocytospermic group (15.14 ± 1.06 ng/mL vs. 9.27 ± 1.42 ng/mL, $p < 0.05$). This marked increase in TLR-2 levels suggests a possible association between TLR-2 expression and the inflammatory state characteristic of leukocytospermia. In contrast, Figure **1B** shows that PGE_2 levels did not differ considerably between the two groups (1263 ± 56.83 pg/mL in leukocytospermic vs. 1247±60.46 pg/mL in non-leukocytospermic, $p > 0.05$). This indicates that while TLR-2 levels are distinctly altered in leukocytospermia, PGE_2 levels remain relatively stable, suggesting a more specific role for TLR-2 in the inflammatory processes associated with leukocytospermia.

Subgroup analyses were conducted to investigate the potential confounding effects of smoking status and varicocele presence on TLR-2 levels (Figure **2**). The Kruskal-Wallis test revealed no significant differences in TLR-2 levels among nonsmokers, ex-smokers, and smokers within either the leukocytospermic or non-leukocytospermic groups (Figure **2A**). However, when comparing leukocytospermic and non-leukocytospermic subgroups based on smoking status (Figure **2B**), significant differences were observed across all comparisons ($p < 0.05$, independent t-tests). Leukocytospermic individuals consistently showed higher TLR-2 levels regardless of smoking status (nonsmokers: 15.18 ± 1.11 vs. 9.35 ± 1.46; ex-smokers: 15.15 ± 0.80 vs. 9.06 ± 0.87; smokers: 15.09 ± 1.06 vs. 9.28 ± 1.59). The presence or absence of varicocele did not significantly affect TLR-2 levels within the leukocytospermic or non-leukocytospermic

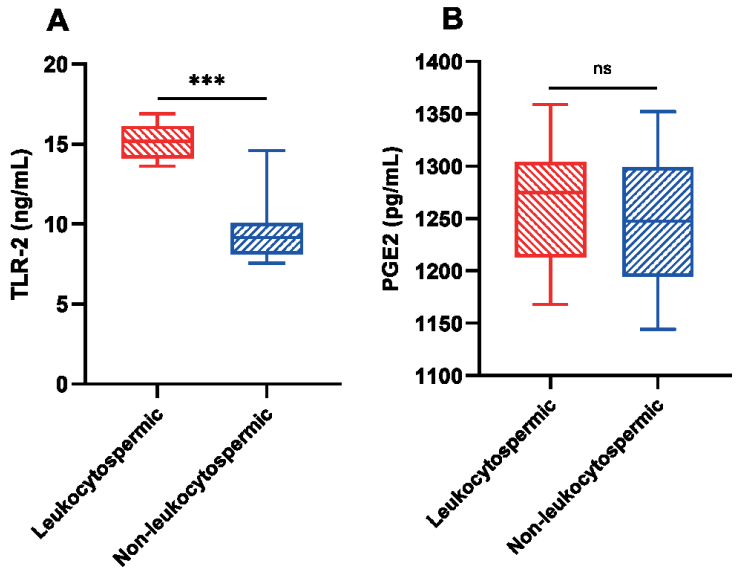

Figure 1: Comparison of TLR-2 and PGE_2 levels in seminal plasma of leukocytospermic and non-leukocytospermic infertile men: A) TLR-2 levels in seminal plasma and B) PGE_2 levels in seminal plasma. Statistical analysis was performed using independent samples t-test. *** indicate statistically significant differences ($p < 0.001$). TLR-2: Toll-like receptor 2, PGE_2: Prostaglandin E_2.

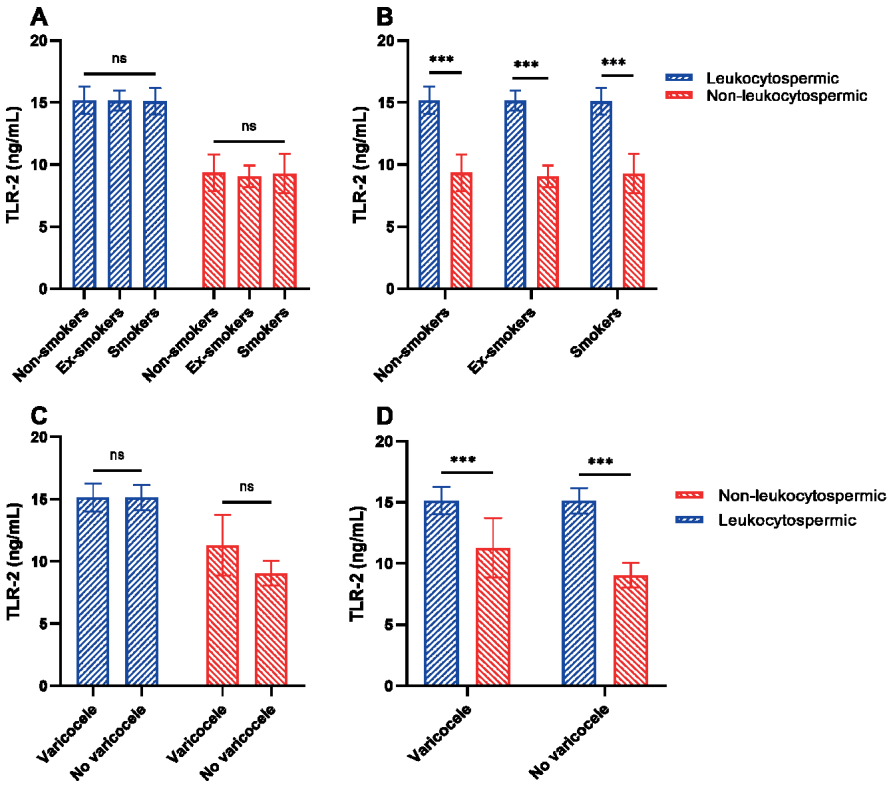

Figure 2: Subgroup analysis of TLR-2 levels based on smoking status and varicocele presence: A) Comparison of TLR-2 levels among nonsmokers, ex-smokers, and smokers within leukocytospermic and non-leukocytospermic groups (Kruskal-Wallis test). B) Comparison of TLR-2 levels between leukocytospermic and non-leukocytospermic subgroups based on smoking status (independent t-tests). C) Comparison of TLR-2 levels between subjects with and without varicocele within leukocytospermic and non-leukocytospermic groups (Kruskal-Wallis test). D) Comparison of TLR-2 levels between leukocytospermic and non-leukocytospermic subgroups based on varicocele presence (independent t-tests). TLR-2: Toll-like receptor 2, *** denotes statistical significance at p <0.001.

groups (Figure **2C**). However, significant differences were observed between leukocytospermic and non-leukocytospermic subgroups, regardless of varicocele status (Figure **2D**). Leukocytospermic men showed elevated TLR-2 levels in both varicocele (15.14 ± 1.12 vs. 11.29 ± 2.43) and nonvaricocele (15.14 ± 1.03 vs. 9.05 ± 1.01) subgroups ($p < 0.05$, independent t-tests). These findings suggest that the association between leukocytospermia and elevated TLR-2 levels persists independently of smoking status and varicocele presence, further confirming the nature of TLR-2 as a probable biomarker for leukocytospermia.

To elucidate the relationship between inflammatory markers and semen quality, correlation analyses were performed between TLR-2 and PGE_2 levels and various sperm parameters (Tables **2** and **3**). TLR-2 levels exhibited strong, statistically significant correlations with all examined sperm parameters (Table **2**). In particular, TLR-2 showed strong negative correlations with sperm concentration (r = -0.675, $p < 0.001$), total sperm count (r = -0.673, $p < 0.001$), progressive motility (r = -0.669, $p < 0.001$), total progressive motile sperm count (r = -0.762, $p < 0.001$), and normal morphology (r = -0.616, $p < 0.001$). On the other hand, TLR-2 levels demonstrated strong positive correlations with round cell concentration (r = 0.684, $p < 0.001$) and white blood cell count (r = 0.668, $p < 0.001$). These findings suggest that elevated TLR-2 levels are associated with deterioration in sperm quality across multiple parameters. In contrast, PGE_2 levels did not show significant correlations with any of the examined sperm parameters (Table **3**). All correlation coefficients for PGE_2 were weak (|r| <0.2) and statistically nonsignificant (all $p > 0.05$), indicating that PGE_2 levels do not have a strong association with sperm quality in our cohort.

Table 2: Statistical correlations between TLR-2 levels and sperm parameters in infertile men (n = 80).

Parameter	Correlation	
	r	*p*-value
Sperm conc.	-0.675	<0.001
Total sperm count	-0.673	<0.001
Progressive motility	-0.669	<0.001
Total progressive	-0.762	<0.001
Normal morphology	-0.616	<0.001
Round cell conc.	0.684	<0.001
WBCs	0.668	<0.001

r: Spearman's rank correlation coefficient. Sperm conc.: Sperm concentration; Total progressive: Total progressive motile sperm count; Round cell conc.: Round cell concentration; WBCs: White blood cells.

TLR-2 demonstrated superior differential efficacy, exhibiting an area under the = curve (AUC) of 0.993 ($p < 0.05$, Figure **3A**). At a cut-off value of >13.56 ng/mL, TLR-2 showed 100% sensitivity (95% CI: 88.4 - 100.0) and 98% specificity (95% CI: 89.4 - 99.9) for identifying leukocytospermia. While PGE_2 showed poor diagnostic performance with an AUC of 0.575 ($p > 0.05$, Figure **3B**). At a cut-off value of >1268 pg/mL, PGE_2 had a sensitivity of 53.3% (95% CI: 34.3 - 71.7) and specificity of 64% (95% CI: 49.2 - 77.1) for leukocytospermia detection.

Table 3: Statistical correlations between PGE_2 levels and sperm parameters in infertile men (n = 80).

Parameter	Correlation	
	r	*p*-value
Sperm conc.	0.030	0.743
Total sperm count	-0.023	0.756
Progressive motility	-0.135	0.396
Total progressive	-0.063	0.698
Normal morphology	0.035	0.712
Round cell conc.	0.019	0.878
WBCs	0.179	0.384

r: Spearman's rank correlation coefficient. Sperm conc.: Sperm concentration; Total progressive: Total progressive motile sperm count; Round cell conc.: Round cell concentration; WBCs: White blood cells.

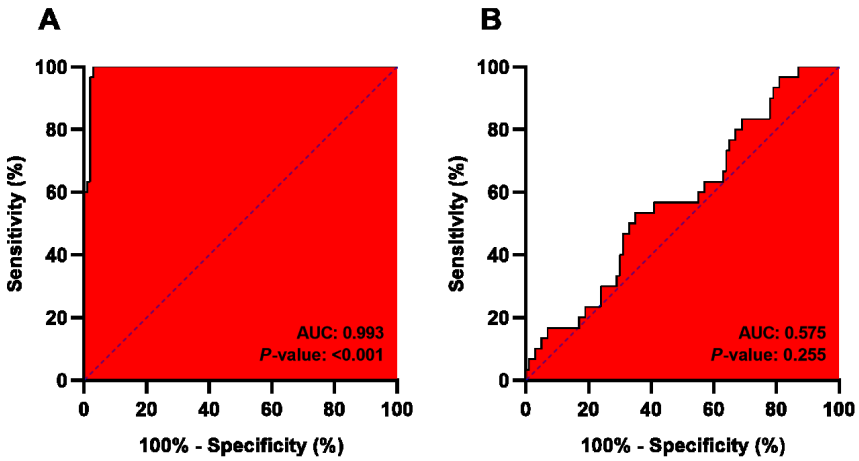

Figure 3: Receiver operating characteristic (ROC) curves for TLR-2 and PGE_2 in distinguishing leukocytospermic from non-leukocytospermic samples: A) ROC curve for TLR-2 [AUC = 0.993, $p < 0.001$, cut-off >13.56 ng/mL, sensitivity = 100% (95% CI: 88.4 - 100.0) and specificity = 98% (95% CI: 89.4 - 99.9)]. B) ROC curve for PGE_2 [AUC = 0.575, $p = 0.255$), cut-off >1268 pg/mL, sensitivity = 53.3% (95% CI: 34.3 - 71.7) and specificity = 64% (95% CI: 49.2 - 77.1)]. The diagonal line represents the line of no discrimination (AUC = 0.5). TLR-2: Toll-like receptor 2; PGE_2: Prostaglandin E_2; AUC: Area under the curve; CI: Confidence interval.

4. Discussion

This study investigated the association between seminal plasma TLR-2 and PGE_2 levels and leukocytospermia in infertile Iraqi men. The study's findings demonstrate significantly elevated TLR-2 levels in leukocytospermic patients compared to non-leukocytospermic individuals, along with strong correlations between TLR-2 and various semen parameters. In contrast, PGE_2 levels showed no significant differences or correlations.

The elevated TLR-2 levels observed in leukocytospermic samples align with previous research drawing attention to the probable involvement of TLRs in inflammatory processes of the male reproductive tract. Hagan et al., elucidated TLR-2, along with TLR-4, COX-2, and Nrf-2, as novel seminal biomarker of

inflammatory processes and oxidative stress in patients with leukocytospermia [9]. Our results extend these findings to an Iraqi population, confirming the probable usefulness of TLR-2 as a biomarker for leukocytospermia across different ethnic groups.

The strong negative correlations between TLR-2 concentrations and spermatozoal parameters, including concentration, motility, and normal morphology suggest that TLR-2 activation may be linked to impaired spermatogenesis and sperm function. This is consistent with studies showing that TLR activation in testicular cells can disrupt spermatogenesis [5, 6]. Wu et al., demonstrated that TLR activation in Sertoli cells leads to the production of inflammatory mediators that can impair spermatogenesis [5]. Relatedly, Shang et al., found that TLR activation in Leydig cells can suppress testosterone production, potentially affecting sperm development [6]. The positive correlation between TLR-2 levels and white blood cell count in semen further supports the link between TLR-2 activation and the inflammatory response in the male reproductive tract. This aligns with the known role of TLRs in initiating innate immune responses and recruiting inflammatory cells [4]. The presence of leukocytes in semen can lead to oxidative stress and sperm DNA damage, as demonstrated by Agarwal et al., [13] which may explain the negative impact on sperm parameters observed in our study.

Interestingly, the subgroup analyses revealed that the association between leukocytospermia and elevated TLR-2 levels persisted regardless of smoking status or varicocele presence. This suggests that TLR-2 activation may be a more direct indicator of reproductive tract inflammation than these other factors known to affect male fertility. It is noteworthy; however, that both tobacco use and varicocele have been implicated in the augmentation of oxidative stress and inflammatory processes within the male reproductive system [16], which could amplify the effects of TLR-2 activation.

The lack of significant findings related to PGE_2 levels was unexpected, given its known role in inflammation and sperm function [10, 11]. This may suggest that PGE_2 plays a less direct role in the inflammatory processes associated with leukocytospermia, or that its effects are more localized and not reflected in overall seminal plasma levels. For future investigations, more in-depth analysis is needed to clarify the role of PGE_2 in male reproductive tract inflammation.

The strong differential performance of TLR-2 in distinguishing leukocytospermic from non-leukocytospermic samples (AUC = 0.993) suggests its potential as a clinical biomarker. The high sensitivity and specificity could make TLR-2 a valuable tool for diagnosing leukocytospermia, particularly in cases where traditional methods may be inconclusive or in asymptomatic patients with idiopathic infertility. These findings contribute to the growing body of evidence implicating innate immune responses and TLR signaling in male infertility. The work of Nejsum et al., demonstrated the TLR-3 expression and apoptosis in testicular tissue following systemic inflammation [7]. The findings of Fujita et al., showed TLR-2 and TLR-4 mediation of bacterial endotoxin-induced apoptosis in human sperm [8] further emphasizing the complex interplay between innate immunity, inflammation, and male reproductive function.

Limitations of this study include its relatively small sample size, which preclude conclusions about causality. In addition, the study was conducted in a single center with a specific ethnic population, potentially limiting its generalizability. Further studies with larger and more diverse participants are needed to confirm these results and explore the potential of TLR-2 as a therapeutic target in male infertility treatment.

5. Conclusion

In conclusion, this investigation elucidates the possible utility of TLR-2 as a biomarker for leukocytospermia in male infertility within an Iraqi population. The significantly elevated TLR-2 levels observed in leukocytospermic patients, coupled with strong correlations between TLR-2 concentrations and various semen parameters, suggest that TLR-2 activation may play a fundamental role in the pathophysiology of inflammation-related male infertility. Importantly, the association between leukocytospermia and elevated TLR-2 levels persisted independently of smoking status and varicocele presence, further validating its specificity.

Acknowledgment

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Statement of Ethics

The current study was planned, conducted, and reported in accordance with the World Medical Association (WMA) Declaration of Helsinki.

Ethical Approval

The current study was approved by the High Institute's Ethics Committee (#0702-PF-2024R28 dated: March 5th, 2021). All the protocols followed were in accordance with the Declaration of Helsinki.

Informed Consent Statement

All included patients, in the current study, gave informed consent before enrollment.

Conflict of Interests

The author declares that there is no conflict of interest.

Artificial Intelligence (AI) Disclosure Statement

AI-unassisted Work.

Funding

No external fund was received.

Author Contribution

RSA: Conceived and designed the study, collected and analyzed the data, interpreted the results, wrote and revised the manuscript. In addition, she serves as the corresponding author, responsible for communication with the journal during the submission, peer-review, and publication process as well as takes full responsibility for the integrity of the work and the accuracy of the data analysis.

Data Sharing Statement

Date sets are not available publicly because of legal and privacy/policy reasons. However, it is available by request from the correspondence author.

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