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INCREASED SEMINAL PLASMA STLR-4 LEVELS IN RELEVANCE TO THE PRESENCE OF BACTERIOSPERMIA AMONG INFERTILE MEN

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ABSTRACT

Background: Soluble toll like receptor -4 protein level increased due to cellular activation during inflammatory process. **Aim:** This study aimed toquantitative evaluation of sTLR-4 in seminal plasma of bacteriospermic and none bacteriospermic infertile men. **Subjects**: A total of 170 semen samples were collected from infertile men attending High Institute for Infertility Diagnosis and Assisted Reproductive Technologies / AL-Nahrain University, Baghdad. Semen samples were processed according to WHO manual 2010 and seminal plasma were used for sTLR-4 measurement using sandwich ELISA kit. **Results**: The results showed that an elevated sTLR-4 was associated with reduction of grade A motility (r=-0.510) and increased grade D motility (r=0.680), round cell count (r=0.228). Interestingly, it was not related to the leukocyte number (r=0.126). **Conclusion**: sTLR-4 is sensitive and specific marker for Bacteriospermia in particular pattern of bacterial infection.

KEYWORDS: Toll-like Receptor 4, Bacteriospermia and infertility.

INTRODUCTION

Bacteriospermia is identified when bacteria in the ejaculate overdo 1000 cfu/ml.[1] It is generally the result of acute or chronic bacterial infections also is watched as a major health care difficult which has a negative effect on male fertility.[2,3] Definitely, it has been shown that about 15% of infertile men have significant amount of bacterial pathogens in the sperm (1). Several bacteria can affect different location (s) beside with genitourinary tract such as: the epididymis, the prostate, the testis and the urethra by different bacteria like: The most communal isolated pathogenic bacteria are Chlamydia trachomatis, Mycoplasma, Staphylococci, Klebsella, Escherichia Coli, and Enterococcus faecalis.[4]

Several pathophysiologic mechanisms have been examined to check the association of bacteriospermia with seminal fluid parameters, such as motility and vitality.[5] It is speculated that both the direct bacterial interaction and the participation of the immune competent cells impact spermatogenesis, weaken semen function and impede the urogenital tract.[6] Conversely, the definite impact of each pathogen on seminal fluid parameters still unknown.

Routinely, bacterial infections were dramatically problematic since its diagnosis and proper treatment increasing contributes in infertile couples, has turned scientific interest to the examination of the effect of bacteriospermia on male reproductive capacity.[7]

It is distinguished that bacterial infections are a significant cause of male issue infertility. Bacterial lipopolysaccharides (LPS) can initiate TLR-mediated signaling and prompt the appearance of TLR genes encoding many inflammatory cytokines and chemokines.[8,9] Therefore, changes in the expression of recognition molecule such as: TLR4 required for assembly of inflammation is essential to reflect the bacterial load that recognized in male genitalia.

Thus, its plausible reason to evaluate soluble TLR-4 as an indicator of bacterial existence in male genital tract in infertile men is not routinely practiced in Iraq.

This study aims to quantitative evaluation of sTLR-4 in seminal plasma of bacteriospermic and none bacteriospermic infertile men.

3. MATERIALS AND METHODS

3.1. Study design and setting

The study included 170 men; their ages were between 20- 45 years who attended the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies (ART's), Al-Nahrain University, Baghdad, Iraq. This prospective study was accomplished through the period from October 2018 till March 2019. Those patients were classified into different grades of leukocytospermia. They were convenient selected after diagnosis by consultant physician according to WHO criteria 2010.

Inclusion criteria: Infertile patients with all infertility subgroups who have abnormal seminal fluid analysis with or without leukocytospermia.

Exclusion criteria: Infertile male receiving immunosuppressive drugs.

3.2. Seminal fluid analysis

3.2.1. Semen Samples Collection

Every patient was asked to provide semen by masturbation after an abstinence period, ranging between 3-5 days and not exceeding 7 days. The patients were put in a private and quite room adjacent to the semen analysis laboratory. Semen was collected in a clean, sterile, dry, plastic and warm disposable Petri-dish, categorized with patient's name and the time of collection. Semen sample was then immediately transferred to the laboratory and was sited in an incubator at 37°C, waiting for liquefaction.

3.4. Endtz (Myeloperoxidase) test

The preparation of solution use in Endtz testinclude

A. Preparation of stock solution: a stock solution was prepared by mixing:

50 mL 96% ethanol, Dilute to 50 mL distal water, 0.125 g benzidine

B. Preparation of Working solution:

2 mL of stock solution and 25 µL of 3% H_2O_2 Protection the tube with aluminiumfoil.

Procedure

a. 20µl of liquefied semen sample was aspirated in a micro-centrifuge tube. Then, 20µL of working solution and 20 µL of working Endtz solution were added, then Vortex and incubated for 5 min. Neubauer counting chamber was loaded with 5µl of the prepared solution b.
b. Examination was done under 40 x bright field objective lens
c. The stained dark brown in color cells with round shape were considered leukocytes
d. Counting the cells in all RBC fields of Neubaur counting chamber was done. The total number was equal to 1 mm^3
e. A number of WBC was calculated by multiplying the whole number of cells by 4 to accurate it for a dilution factor. The whole number will be Nx10^6/mL semen. Significant leukocytospermia was defined as at least $1x10^6$ WBC/mL.

Human soluble Toll-Like Receptor 4 (sTLR4) ELISA Kit

This study involved 170 semen sample were processed for TLR-4 determination Catalog Number. CSB-E12954h intended for the quantitative determination of human toll-like receptor 4 (TLR4) concentrations in serum, plasma, tissue homogenates. With detection range of 0.156 ng/ml-10 ng/ml.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve was planned as the relative O.D.450 of all standard solution (Y) vs. the separate concentration of the standard solution (X). The TLR-4 concentration of the samples wereinserted from the standard curve.

Urinary Tract Infection Chromogenic Agar (UTIC)

Microbiological exam

The next results were found in the routine of the medium from type cultures after incubation at a temperature of 35 ± 2ºC and detected 18-24 hours.

Microorganisms	Growth	Colony color
Escherichia coli ATCC 25922	Good	Pink
Enterobacter aerogenes ATCC 13048	Good	Dark Blue
Klebsiella pneumonieae ATCC 13883	Good	Dark Blue
Proteus miriabilis ATCC 13315	Good	Light Brown
Staphylococcus aureus ATCC 25923	Good	(natural pigmentation] White Cream
Enterococcus faecalis ATCC 19433	Good	Light Blue
Pseudomonas aeruginosa ATCC 27853	Good	Amber

Statistical Analysis

Data were entered using Excel Microsoft program (2016) and analyzed by statistical package for social sciences (SPSS) type 21.Definite data designated as frequency and percentage while, numerical data defined means and standard deviation (SD). Chi square test was used to estimation the connotation between two uncompromising variables. While, independent sample t-test used for comparison of numerical data. Level of significance of ≤ 0.05 was reflected as significant.

RESULTS

This prospective cross-sectional study involved one hundred and seventy infertile men undergoing routine investigations for seminal fluid analysis as suspected male factor infertility and as male partner for female factor infertility. Their mean age was in the middle sexually active men 31.66±4.55 years old. Twenty-two (12.94%) of them were primary infertility and the majority of them 148 (87.06%) were secondary. Thirty-eight (22.35%) of them were had have leukocytospermia (>1 million of leukocyte per 1 ml) in their semen samples.

Regarding seminal fluid analysis of patients, their mean volume was 2.78±1.12, liquefaction time was 36.18±15.46, pH was 7.95±0.28, Sperm count was 45.56±29.98, normal morphology was 35.76±15.38, agglutination 6.97±2.94. Sperm motility was 15.91±7.89 was grade A, 18.29±9.77 was grade B, 22.28±11.35 was grade C and 33.42±19.51 were grade D. Round cells was 15.39±8.58 and leucocyte concentration according to Endtz test was 558818.18±427358.84 (Table 1).

Table 1: Seminal fluid analysis findings of study group.

		Value
Age (years)		31.66±4.55
Type of infertility	Primary	22 (12.94%)
	Secondary	148 (87.06%)
Volume of ejaculate (ml)		2.78±1.12
Liquefaction time (min)		36.18±15.46
pH		7.95±0.28
Sperm count (million/ml		45.56±29.98
Normal Morphology (%)		35.76±15.38
Agglutination		5.94±6.97
Grade A %		15.91±7.89
Grade B %		18.29±9.77
Grade C %		22.28±11.53
Grade D %		33.42±19.51
Round cell %		15.39±8.58
Endtz test	Leukocytospermia	38 (22.35%)
	Non leukocytospermia	132 (77.65)

Data expressed as mean±Standard deviation or frequency and percentage.

Soluble Toll-Like Receptor-4 is highly expressed in culture positive cases

In the current study, a new finding has been reported, soluble TLR-4 were measured in high concentration 1.28±0.88 ng/ml in comparison with culture negative group 0.25±0.22 ng/ml with high statistically significant difference p<0.001. This finding highlighted the role of TLR-4 as a recognition molecule for bacterial pathogens found in male genital tract. Furthermore, sTLR-4 were analyzed according to the pattern of culture results it have been noted that sTLR-4 were higher in *staphylococcus aureus*, *Enterococcusfaecalis*, *E. coli*, *K. pneumoneae*, *Proteus mirabilis* positive culture cases rather than negative for each isolated bacteria p<0.001, 0.001, 0.039, <0.001, and 0.003 respectively. While, in *Chlamydiatrachomatis* positive culture group were not different from negative group p=0.743 (Figure 1).

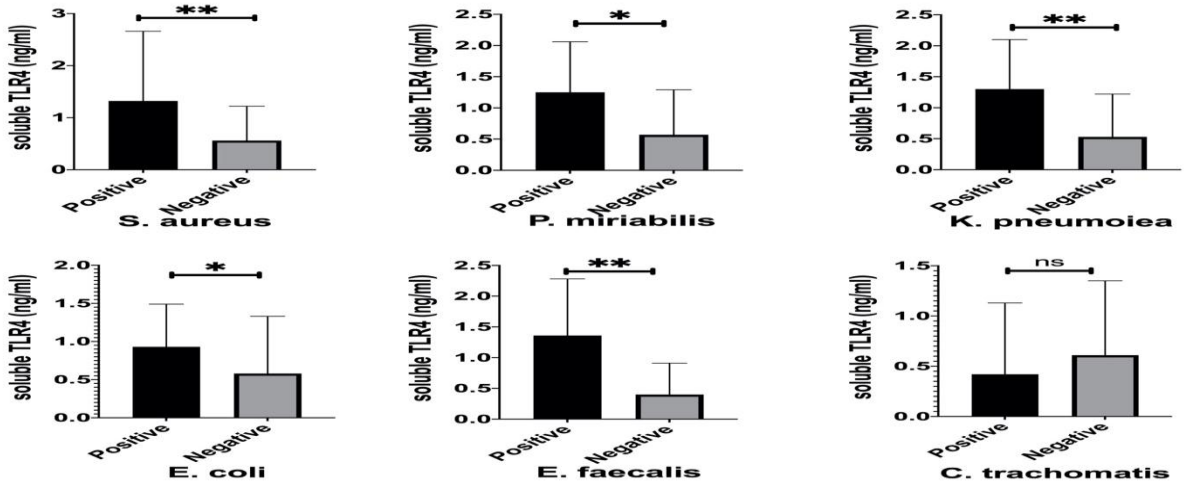

Figure 1: Comparison of seminal plasma sTLR-4 in according to the bacterial culture results. Data expressed as mean±Standard deviation. NS: none statistical significance (p>0.05). *: statistical significance difference (p<0.05). **: High statistical significance difference (p<0.001). Comparison made by using independent sample t-test.

Furthermore, using SPSS software program, the receiver operator curve was performed against the result of culture. The area under the curve was 0.943 with 0.02 standard error and the 95% confidence interval was (0.905-0.982) (Table 2). Then an appropriate cutoff value was selected as 0.45ng/ml with an acceptable sensitivity and specificity. Accordingly, Table 3 showed the performance of this cutoff value (0.45ng/ml) in comparison with culture results.

Table 2: Receiver operating curve analysis of sTLR-4.

Area	Std. Error	Asymptotic Significance	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.943	0.020	<0.001	0.905	0.982

Furthermore, the cutoff value was tested in comparison with the result of culture. Table 3 demonstrated that 53/60 were true positive and 7/60 were false negative, 11/110 were false positive and 99/110 were true negative. The tested cutoff showed 88.33% sensitivity, 90% specificity, 82.81% positive predictive value and 93.4% was negative predictive value.

Table 3: Diagnostic performance of sTLR-4 cutoff value 0.45 ng/ml in comparison with culture results.

	Growth		Total
sTLR-4	Positive	Negative	
Positive (≥0.45 ng/ml)	53	11	64
Negative (<0.45 ng/ml)	7	99	106
Total	60	110	170
Sensitivity	88.33 (77.82-94.23)		
Specificity	90 (82.98-94.32)		
Positive Predictive Value	82.81 (71.79-90.12)		
Negative Predictive Value	93.4 (86.99-96.76)		

DISCUSSION

Here in this study, we first time reported an evidence of sTLR-4 in the seminal plasma of infertile men. Furthermore, it has been found that an elevated concentration among patients with positive bacterial culture (Bacteriospermic >1000cfu) when compared with negative individuals. Looking for the basic function of TLR-4, it was belonged to a family of innate immune recognition molecules providing adequate signals responsible for inflammatory cascade.[10,11] Bacterial structures like lipopolysaccharides are the major ligands of the TLR-4,[8] Fujita, et al., 2011 was originally reported an evidence of TLR-2 and 4 expression by human sperms and its recognition of bacterial LPS.[12]

It's quite clear that sTLR-4 was associated with bacterial infection. This was explained by that direct interaction with bacterial components (LPS and peptidoglycans) were able to increase TLR-4 in experimental mice model.[12] Furthermore, it is recognized that TNF-a secreted from leukocytes in semen makes sperm apoptosis.[13] Consequently, as apoptosis can be stimulated in sperm together the TNF-a–TNFR1 and TLRs pathways, we propose that sperm react to bacterial infection in semen by direct TLR-regulated mechanisms and to leukocytes by a TNF-a-dependent pathway.

The essential role played by TLR4 in gram-negative infections arises from lessons on TLR4-mutated or TLR4-deficient mice.[14] It has existed experimental that TLR4-mutated strain C3H/HeJ is hyporesponsive to LPS and very susceptible to infection by gram-negative bacteria like Salmonella typhimurium and E.coli.[14,15] Instead, studies established that TLR - 4 mice were protected from endotoxin shock brought by E. coli, thus supportive TLR4 as a likely mark for therapeutic interference in sepsis.[16]

Specified the role of TLR4 in the stimulation of the proinflammatory response through infection, pharmacological methods targeting TLR4 have existed developed with the purpose to control host's injurious proinflammatory response called "cytokine storm" arising in the initial phase of sepsis. Inappropriately, the results of clinical trials with molecules targeting TLR4 were inacceptable,[17] suggesting that immune suppression, which shadows the "cytokine storm," represents the leading process in the evolution of sepsis.[18]

CONCLUSION

sTLR-4 is sensitive and specific marker for Bacteriospermia in particular pattern of bacterial infection.

Declarations

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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