Immunopathogenesis of Toll Like Receptors (2,4,9) among Patients with Male Infertility in Basrah Province

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Abstract

Infertility remains a global health challenge with devastating psycho-social consequences in many communities, and the underlying long-term risk of couple separation is also a major clinical and social problem. Infertility is defined as the inability of a couple to conceive naturally after one year of intercourse. The aim of the study was to determine the immunomolecular characterization of TLR genes. Toll-like receptors (TLRs) are an important family of receptors that constitute the first line of defense against pathogens. They can recognize both invading pathogens and endogenous danger molecules released from dying cells and damaged tissues and play a key role in linking innate and adaptive immunity. TLRs are widely distributed in both immune and other body cells.

A cross sectional case control study was carried out by ELISA technique, among male infertility patients who attended to the infertility and in vitro fertilization center (IVF) of Basrah province on September, 2021, and June 2022. A questionnaire paper was used to record special notes. Samples were collected from (176 patients and controls divided as 88 patients of male samples and 88 samples of the control group) including seminal fluid and blood.

The immunological study showed that there was a statistically significant difference in Toll-like receptor 2 (P.value <0.05) which increased in patients more than in control groups, where TLRs 4 and TLRs 9 have not significant statistical differences but TLR4 increased in patients more than in control groups, in addition, TLR2 concentration according to seminal fluid abnormalities was found to be statistically significant in patients with asthenozoospermia and necrozoospermia.

Keywords: - Male infertility, ELISA, Toll like receptors, DAMPs, PAMPs

1. Introduction

Infertility remains a major problem for couples throughout the globe. Clinically, it is referred to as the inability of a couple to conceive after one year of regular sex (Hamada, Esteves, Nizza, & Agarwal, 2012). 13-18% of couples suffer infertility, with the male component accounting for up to 50% of all cases (Havrylyuk, Chopyak, Boyko, Kril, & Kurpisz, 2015). Primary infertility is defined by the World Health Organization (WHO) as a woman who has never conceived, while secondary infertility It's the inability to become pregnant after at least one successful pregnancy (Benksim, Elkhoudri, Addi, Baali, & Cherkaoui, 2018). Primary infertility affects 67%-71% of patients, whereas secondary infertility affects 29%-33%. One in ten couple's experiences infertility for various reasons. Male infertility has several causes, More than 50% of infertile males have unknown (idiopathic) causes, which may be inherited or acquired (Poongothai, Gopenath, & Manonayaki, 2009).

Male infertility may be caused by medical (inherited or acquired), environmental (chemical substances, chemotherapeutic agents, radiation, pollution, and stress), and lifestyle variables (smoking, alcohol use, illegal recreational drug use) (Naz & Kamal, 2017). To evaluate male infertility, the urologist collects an assessment of the patient's medical history and a physical assessment that involves a semen test (Medicine, 2012). An infertile male's sperm examination may reveal the following conditions: (a) Oligozoospermia (low spermatozoa count), (b) Teratozoospermia (aberrant sperm), and (c) Asthenozoospermia (low sperm motility). This disorder is known as Oligoasthenoteratozoospermia syndrome when these anomalies are detected in sperm analysis (Jungwirth et al., 2012).

The immune system, comprising adaptive and innate immunological processes, offers the first line of protection against external threats by recognizing and responding quickly to infections and other immunogens, and by inducing inflammation. Innate immunity is key to male reproductive system infection responses (Hedger, 2015). Recent studies demonstrate that the immune cells are indeed mounting an antitumor response and that tumors develop mechanisms to combat an immune response (Jamel, Mahdi, & Alsaimary, 2022)

Pattern-recognition receptors, that identify certain motifs, or pathogen-associated molecular patterns (PAMPs), generated by bacteria, virus, fungi, and protozoan pathogens (Zhang & Liang, 2016) and damage-associated molecular patterns, are required for the trigger of the innate immune system (DAMP) (Yu & Feng, 2018).

Toll-like receptors, often known as TLRs, are one of the primary categories of pattern recognition receptors. These receptors identify the molecular patterns of infections, which helps the body's innate immune system

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detect foreign pathogens (Lakpour et al., 2017). Several TLRs react to distinct molecular patterns related to diseases, such as, lipopeptides (TLR1, 2, 6), lipopolysaccharide (TLR4), double-strand RNA viruses (TLR3, 7, 8) and CpG-rich unmethylated DNA (TLR9), bacterial flagella (TLR5) (Behzadi, García-Perdomo, & Karpiński, 2021). As a mediator, TLR not only plays a pivotal function in the induction of innate immunity However, it also serves as a bridge between innate and adaptive immune systems. TLRs are found on immune cells and cells that are not part of the immune system. These cells include B lymphocytes, dendritic cells, macrophages, natural killer (NK) cells, endothelial cells, fibroblasts, and epithelial cells (El-Zayat, Sibaii, & Mannaa, 2019). Furthermore, these receptors can dimerize on the cell membrane, in which case two identical proteins hemodimerize or two distinct TLRs heterodimerize. Specificity in these receptors has improved via heterodimerization (Lakpour et al., 2017). On the surface of cells, TLR1, 2, 4, 5, and 6 were shown to be connected with external microorganisms, whereas TLR3, 7, 8, and 9 were found on the membranes of cytoplasmic organelles, such as endosomes, to sense pathogen-related nucleic acids (Chang, 2010).

TLR induction signaling pathways in the host as a defense against attackers and to heal injured tissue (Wang, Song, Bai, & Vanhoutte, 2016), causing the secretion of several inflammatory cytokines and immune mediator (Wong et al., 2009). As a result of excessive TLR activation, persistent production of chemokines and pro-inflammatory cytokines impairs the immunological balance and hence leads to numerous illnesses (Yibo Wang et al., 2020).

In the male reproductive system, TLRs are few, although they have been demonstrated to be expressed all across the male reproductive system, involving the testis, vas deferens, epididymis, and accessory glands of male reproductive tissues (Nishimura & Naito, 2005). In men, TLRs seem to have a role in both normal and pathological testicular steroidogenesis and spermatogenesis (Girling & Hedger, 2007). invasion of the testis or other regions of the reproductive organs by pathogens activates innate immune responses and TLRs (Rodrigues et al., 2008). TNF-α and NO, inflammatory mediators produced by activated testicular macrophages via TLRs, may limit Leydig cell androgen synthesis and negatively impact sperm production if levels are elevated above normal (O'Bryan et al., 2000).

2. Materials and methods

Samples sources

This case control study was conducted between 1 September 2021 and 1 June 2022 in the province of Basrah. A questionnaire paper was used to record special note including no. of file, age, family history, varicocele, duration of marriage, infertility type, other disease, drugs, smoking, in addition to seminal fluid analysis, regarding all these individuals. Samples of blood have been collected from the male patients at Infertility and IVF center in Basrah province. Ethical approval was attempted according to acceptance from Research and Development center- Ministry of health and the approval of head master of each hospital was obtained, the objective of the study was explained to each participant.

Exclusion criteria: -

All patients who have atopic diseases.

All patients who have autoimmune diseases.

Patients who have an infectious disease, varicocele and reproductive organ surgery.

Determination of Toll Like Receptors concentrations by ELISA Technique:

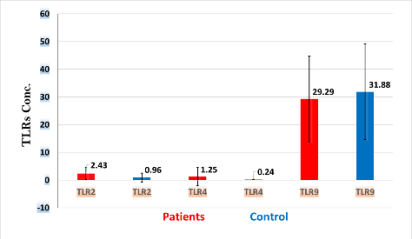
Five ml of blood was collected by venipuncture from all patients whose, clinical history, clinical examination, hormonal assay & seminal analysis revealed male factor infertility. The collected blood was divided into two tubes; 3ml of blood was added to gel tubes which placed for 20 minute at room temperature and then centrifugation to get serum which stored in Eppendorf tube at (-20) o C till be used, whereas the other 2ml of blood was added to EDTA tube for molecular studies. the ELISA kits of human Toll Like Receptors (2,4,9) from MybioSource company (USA) were used to analyses 176 samples (88 patients and 88 control).

Statistical analysis: - Statistical analysis was performed with SPSS (Standard Program for Social Science) statistical program version 23 and Microsoft Excel 2010. Numerical data were defined according to mean, standard deviation of mean. For comparison between different groups, logistic regression was used. The lowest accepted difference in statistical importance was 0.05 or less.

3. Results

Immunological study

Human toll like receptors levels in patients vs. controls



This Figure (1) illustrate different mean concentration of TLRs. The only toll like receptor level which showed significant statistical difference between patient and control groups was the TLR2 (P.value <0.05), where TLRs 4 and TLRs 9 have not significant statistical difference (P.value>0.05)

Figure (1): - Toll like receptors conc. among studied groups.

Toll like receptors levels according to seminal fluid abnormalities: -

This Figure (2) show different mean of TLRs concentration according to seminal fluid abnormalities. TLRs 4 and 9 was found statistically not significant in Azoospermia, Oligozoospermia, Asthenozoospermia, Necrozoospermia, Teratozoospermia and Leukocytospermia, where TLRs 2 was found statistically not significant, in patients with azoospermia, Oligozoospermia, Teratozoospermia, Leukocytospermia

except in patient with Asthenozoospermia and Necrozoospermia was found to be significantly statistically different (P. value <0.05).

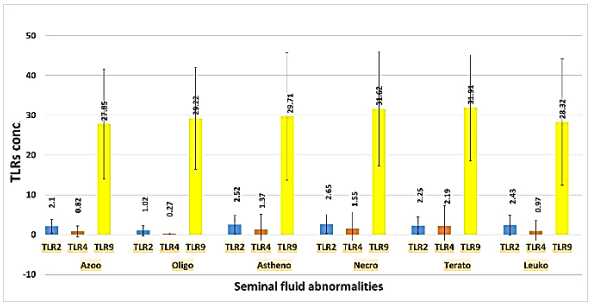


Figure (2) Toll like receptors levels according to seminal fluid abnormalities.

Toll like receptors levels according to patients age groups: -

This table (1) show the mean of TLRs concentration according to ages which was found statistical significant in TLR2 and not significant in TLR4 and TLR9 (P. value >0.05).

Table (1): - TLRs according to different age groups.

Age	Thirty Years or Younger (N=47) Mean±SD	From 31 To 40 (N=34) Mean±SD	Older Than 40 (N=7) Mean±SD	P. Value
TLR2	3.01±2.53	34.1±2.08	1.17±1.71	<0.001
TLR4	1.77±4.49	0.775±1.16	0.275±0.138	0.498
TLR9	30.1±16.7	28.1±14.8	30.1±12.0	0.805

Toll like receptors levels according to infertility type: -

This figure (3) illustrate TLRs concentrations according to type of infertility which was found statistically not significant when compared according to primary or secondary infertility (P. value>0.05).

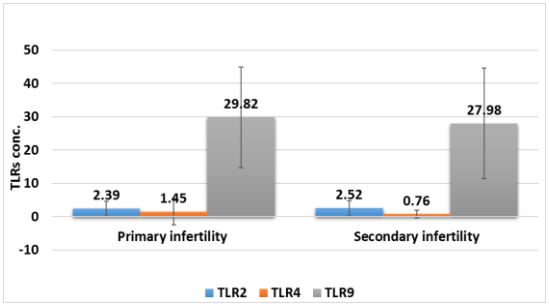


Figure (3): - Toll like receptors levels according to infertility type.

Toll like receptors levels according to smoking and residency:

This table (2) show the mean of TLRs concentration according to smoking and residence which was found no any significant statistical difference (P. value >0.05).

Table (2) Toll like receptors levels according to smoking, residency.

Smoking		Patients			Controls		
		TLR2	TLR4	TLR9	TLR2	TLR4	TLR9
No	NO.	44	44	44	33	33	33
	Mean	2.46397	1.24315	26.32790	1.79317	.24290	33.83783
	SD	2.473132	3.102536	14.247452	2.504033	.000000	19.132268
Yes	NO.	44	44	44	55	55	55
	Mean	2.38913	1.25477	32.25013	.45950	.23131	30.71190
	SD	1.920743	3.595896	16.250742	.324510	.036651	17.127852
P. value		0.741	0.828	0.082	0.064	0.439	0.744
Residency							
Central	NO.	43	43	43	44	44	44
	Mean	2.04063	.98926	31.33016	.58938	.22841	38.41650
	SD	2.070892	2.385197	11.911101	.378635	.040977	7.189375
Peripheral	NO.	45	45	45	44	44	44
	Mean	2.78834	1.49244	27.37544	1.32987	.24290	25.35175
	SD	2.280564	4.047229	18.138715	2.260586	.000000	22.240795
P. value		0.094	0.981	0.327	0.833	0.317	0.292

* Mann-Whitney U Test

Regression line analysis: -

These Figures show the simple linear regression equations between each two of the correlated variable.

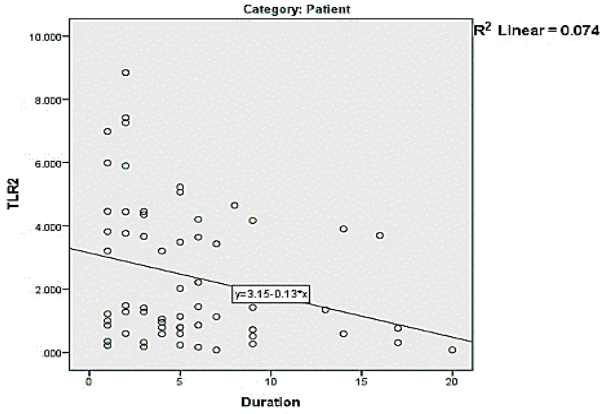


Figure (4): - Linear regression between age and TLR2 in patients and control.

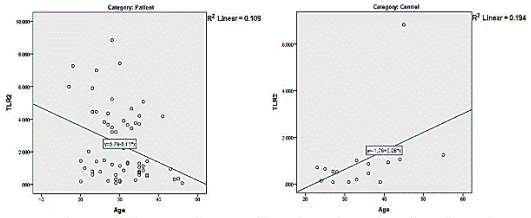


Figure (5): - Linear regression between disease duration and TLR2 in patients

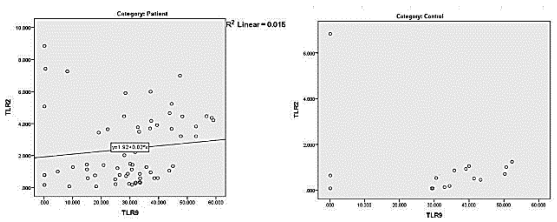


Figure (6): - Linear regression between TLR4 and TLR2 in patients and control.

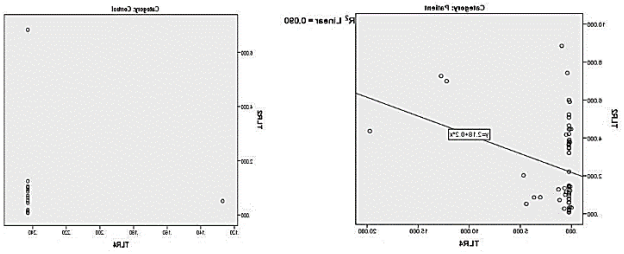


Figure (7): - Linear regression between TLR9 and TLR2 in patients and control.

4. Discussion

Toll Like Receptor 2 (TLR-2)

The concentration means of TLRs. TLR2 was the only toll like receptor was showed significant statistically difference between patients and control groups (P.value <0.05), there were no similar studies done by ELISA technique on blood serum but there were other studies done in sertoli cell of human testicular tissue by various technique such as western blotting analysis that showed the level of TLR3 protien of Azoospermic Patients expression was higher than TLR2 (Lakpour et al., 2017). Other Rt-PCR and western blot studies demonstrated the expression and localization of TLR2 in human sperm; immunofluorescence demonstrated that TLR2 was localised to the acrosomal and tail regions of human sperm (Fujita et al., 2011). Using commercial antibodies, another study found that the TLR 2 protein was present in human testicular peritubular cells as well as in tubular and interstitial compartments; this led researchers to conclude that TLR 2 is responsible for sterile inflammation and infertility in males (Mayer et al., 2016).

In addition, there was a research that investigated the role of TLRs during spermatogenesis, which analyses the relative expressions of TLRs 2, 3, and 4 in testicular tissues of TESE+ and TESE samples by Q-PCR. There was only a statistically significant difference in TLR2 mRNA expression in samples that included spermatozoa (TESE+) and those that did not (TESE) (Saeidi et al., 2014). This may indicate that TLR2 may play a function in spermatogenesis or that spermatogenesis-related factors might affect TLR2 gene expression. In fact, it is known that various phases of spermatogenesis release endogenous TLR ligands (DAMPs) by sertoli cells (Zetterström, Strand, & Söder, 2006). Furthermore, apoptotic markers such as caspases 1, 3, 8, and 9 and damaged DNA have been identified (Aitken, De Iuliis, Finnie, Hedges, & McLachlan, 2010). With the known function of TLR2 in apoptosis in other cell types and the fact that apoptosis is a natural outcome of spermatogenesis, it is sufficient to interpret why TLR2 gene expression is elevated in TESE+ samples (Saeidi et al., 2014).

Toll Like Receptor 4 (TLR-4)

TLR4 showed no significant statistical difference between patient and control groups, there were no similar studies done by ELISA technique on blood serum in male infertility patients, but there was other study done between leukocyte sperm LCS and non-leukocyte sperm patient in seminal plasma by western blot and immunofluorescence technique showed significant increase in TLR4 and TLR2 (P. Value <0.001) In LCS sperm sample compared with non LCS sample (Hagan et al., 2015). Other research using Rt-PCR and western blots demonstrated the expression and location of TLR4 in human sperm, while immunofluorescence demonstrated that TLR4 was localized to the acrosomal and tail regions of human sperm (Fujita et al., 2011). Other studies on TB patients revealed an increased concentration of TLR2,4,9, which was statistically significant (Jamel et al., 2022).

Toll Like Receptor 9 (TLR-9): -

TLR 9 showed no significant statistical difference between patient and control groups, there were no similar studies done by ELISA technique on blood serum in male infertility patients, but there was other study of TLRs localization on epididymis epithelial cells which consist of four region (initial segment , Caput ,Corpus , Cauda) immunohistochemical localization was used to determine which cell type in the epididymis express TLRs , immunoreactivity for TLR9 was intense in all cell of the epithelium from initial segment through cauda epididymis with fain staining of interstitial cells. The same study showed the localization of TLRs on spermatozoa by immunoblot and immunofluorescence, TLR9 was detected on cauda spermatozoa which coat the entire surface of spermatozoa (M. A. Palladino, Savarese, Chapman, Dughi, & Plaska, 2008). other study in the male rat reproductive system by immunoblot technique that showed detection of TLR9 in the testis, vas deferens and, epididymis (M. Palladino, Johnson, Gupta, Chapman, & Ojha, 2007).

In addition, TLRs concentration according to seminal fluid abnormalities, TLRs 4 and 9 was found statistically not significant azoo, oligo, astheno, necro, terato, leuko, whereas TLRs 2 was found statistically not significant, in patients with azoo, oligo, terato, leuko except in patient with astheno and necro was found to be significantly statistically different (P. value <0.05) there were no similar studies. On the other hand, TLRs concentrations according to type of infertility which was found statistically not significant when compared with primary or secondary infertility (P. value>0.05).

Conclusions: - TLRs are a novel field, and further research is required to reach conclusive conclusions. We recommend additional research into the underlying processes of TLRs in male reproductive biology and sperm function since this work is just preliminary in its implications.

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