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BRIEF COMMUNICATION

Iran J Allergy Asthma Immunol
December 2019; 18(6):701-706.

Decreased Toll-like Receptor (TLR) 2 and 4 Expression in Spermatozoa in Couples with Unexplained Recurrent Spontaneous Abortion (URSA)

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Received: 23 January 2019; Received in revised form: 1 June 2019; Accepted: 10 June 2019

ABSTRACT

Studies have shown that toll-like receptors (TLRs) play some important roles in reproductive processes such as ovulation, spermatogenesis, sperm capacitation, fertilization, and pregnancy to the best of our knowledge, no study has evaluated the expression and role of these molecules and their impairment in spermatozoa; accompanied by pregnancy complications such as recurrent spontaneous abortion (RSA). Therefore, this study investigates the alteration of toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) expression in spermatozoa in men whose spouse have unexplained RSA.

Fifteen fertile couples and fifteen couples with unexplained recurrent spontaneous abortion (URSA) were included in this study. The level of TLR2 and TLR4 expression in untreated and lipopolysaccharide (LPS) or PAM3CYS in treated spermatozoa were examined by flow cytometry.

The results showed reduced expression of TLR4 in untreated spermatozoa and decreased LPS or PAM3CYS levels in treated spermatozoa in the URSA group compared to the control group. No significant differences were found in TLR2 expression of untreated spermatozoa in RSA and control groups. After the treatment of spermatozoa with LPS, the TLR2 expression was decreased in both groups. After the treatment of spermatozoa with PAM3CYS, the level of TLR2 expression was significantly increased in the URSA group; while no significant differences were shown in the control group in comparison to untreated spermatozoa.

We have concluded that decreased TLR4 expression and a differently increased TLR2 expression in response to ligand treatment in spermatozoa is associated with URSA.

Keywords: Recurrent miscarriage; Spermatozoa; Toll-like receptor 2; Toll-like receptor 4

INTRODUCTION

The presence of specific regulatory T cells (Treg) for spermatozoa antigens before and during pregnancy and the relation between the decreased number of these

cells and recurrent spontaneous abortion (RSA)¹ suggests that at the time of insemination, an appropriate and necessary immune response is induced for pregnancy. After exposure of the female reproductive tract (FRT) with semen, innate and adaptive immunities are induced against seminal and spermatozoa antigens.²⁻⁵ Therefore, any undesirable change in immunogenicity of semen or spermatozoa can lead to impaired immune response and consequently fertility

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complications such as infertility, preeclampsia, and RSA may occur.^{5,6} RSA is classically defined as the occurrence of three or more clinically detectable pregnancy losses before the 20th week of gestation.⁷ The etiology of RSA is varied and include genetic, anatomical, endocrine, placental anomalies, immunological and other factors.⁷ Approximately 50% of RSAs remain unexplained and unresolved, commonly defined as unexplained recurrent spontaneous abortion (URSA).^{8,9}

As mentioned above, spermatozoa antigens from men whose spouses suffer from RSA show reduced capacity to induce Treg cells.¹ It can be supposed that the cause of this reduced capacity can be the change of spermatozoa antigens and antigen changing may be the result of disturbed toll-like receptors (TLRs) expression or signaling in spermatozoa. Recently, investigations have shown that spermatozoa express TLR molecules^{10,11} and it has been suggested that these molecules play a role in the protection of spermatozoa from microbial pathogens.¹¹ The TLRs are a family of pattern-recognition receptors (PRRs) in mammals that recognize diverse classes of microbial products; known as the pathogen-associated molecular patterns (PAMPs), cause endogenous ligand release following cell injury or cellular death that is known as the danger-associated molecular patterns (DAMPs) and elicit immune responses.^{12,13} TLRs elicit intracellular signaling responses that ultimately result in some cell biological roles such as the production of cytokines and chemokines, the production of reactive oxygen species (ROS), induction of apoptosis, production of antimicrobial peptides, and increased expression of HLA (Human leukocyte antigen) class I & II.¹⁴⁻¹⁸ Almost all TLRs except TLR3 induces the activation of nuclear factor κ B (NF- κ B) and Activator protein 1 (AP-1). These transcription factors activate the transcription and translation of several genes. Considering these descriptions, it can be suggested that probably, the activation of TLRs in spermatozoa may lead to the expression of several proteins and the production of ROS. ROS is responsible for proteins tyrosine phosphorylation and lipid peroxidation¹⁸ and these events may change the spermatozoa antigenicity. Obviously, further studies must be performed to confirm this assumption. It is well known that spermatozoa in FRT encounter a large variety of microbiota¹⁹⁻²¹ but the result of this encountering remains to be identified. Nevertheless, there is a high

probability that the change in TLRs expression and their signaling pathway cause the change in spermatozoa function and consequent fertility complication. According to these descriptions, this study seeks to address the alteration of TLR2 and TLR4 expression (more known TLRs in reproduction) in spermatozoa before and after treatment with lipopolysaccharide (LPS), a ligand for TLR4, and PAM3CYS, a ligand for TLR2 in men whose spouse suffer from URSA in comparison with fertile couples.

MATERIALS AND METHODS

Subjects

Fifteen fertile couples with at least one child and 15 URSA couples with no live birth were included as control and case groups. Fertile couples were volunteers from family, university and laboratory personnel, students, and friends of the case group. The diagnosis of URSA was made after excluding definite causes such as abnormalities of the uterus or cervix, chromosomal abnormality, infection, endocrine and metabolic diseases, congenital thrombophilia, and autoimmune diseases. The husband of each woman in both groups had normal semen status, according to criteria from the World Health Organization released in 2010 (WHO). None of the male partners had any history of genital tract disorder such as a history of infection, undescended testis, inguinoscrotal surgery such as varicocele, genital trauma or testicular torsion. The protocol of this study was approved by the Ethics Committee of Isfahan University of Medical Sciences (Isfahan, Iran) (The ethics committee approval letter :IR.MUI.REC.1395.3.480). Informed consent was obtained from all couples who participated in this study.

Purification of Spermatozoa

Semen samples were collected by masturbation after 2-3 days of sexual abstinence. Sampling was performed in a sterile condition. After liquefaction, semen quality (macroscopic and microscopic properties) was assessed according to WHO standard guidelines (WHO, 2010). Couples were excluded from this study when the husband had abnormal semen. Two mL of AllGrad Wash (LifeGlobal Group, Canada) was added to the liquefied semen sample and centrifuged at 350 g for 10 minutes. The pellet was suspended in 1 mL of AllGrad Wash. In each tube, 1 mL of AllGrad 90%

gradient (LifeGlobal Group, Canada) was carefully added. The process was followed by adding 1 mL of AllGrad 45% gradient, and then 1 mL of the spermatozoa suspension. After centrifugation at 400 g for 18 minutes, the pellet was washed with AllGrad Wash and it was then re-suspended in Ham's F-10 medium (dacell, Iran) with 1% bovine serum albumin (BSA) (CMG, Iran). Ham's F-10 is one of the suitable media for spermatozoa because of having antioxidant properties.

Treatment of Spermatozoa

Spermatozoa were seeded at 6×10^6 cells/300 μ L Ham's F-10 medium with 1% BSA in 96-well plates in the presence of 100 ng/mL lipopolysaccharide (LPS) (Sigma, USA) or 10 μ g/mL of Pam3Cys (calbiochem, USA) and without any treatment (untreated spermatozoa). Cultures were incubated at 37°C, 5% CO₂ in the humidified atmosphere for 4 hours.

Flow Cytometric Assay

Untreated and treated spermatozoa with LPS and Pam3cys were stained with PE-mouse anti-human TLR4 (BD pharmingen, USA) and Fluorescein-5-isothiocyanate (FITC) mouse anti-human TLR2 (Southern Biotech). The cell density was as follows: 1×10^6 spermatozoa. After incubation for 30 minutes at room temperature and two washes with AllGrad Wash (400 g for 5 minutes), spermatozoa were run through the flow cytometer (BD FACS Calibur, USA). Data from at least 100000 events were collected; using forward scatter and side angle of light scatter (a logarithmic amplifier). Fluorescence data were obtained with the logarithmic amplifier. For isolation of dead spermatozoa from live spermatozoa, we compared the SSC and FSC properties of unpurified and purified spermatozoa (debris and dead spermatozoa effectively were removed with AllGrad). This comparison showed that the debris and dead spermatozoa have distinct SSC and FSC properties from live spermatozoa. Therefore, this technique allowed us to gate live spermatozoa. We used The *FlowJo* vx10 software for data analysis.

Statistical Analysis

One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare the differences between and within controls and URSA groups. Values were presented as mean \pm standard deviation (SD). p -value of <0.05 was considered to be

significant. All data analysis was performed; using IBM SPSS statistics 25 software.

RESULTS

Figure 1 shows flow cytometry dot plots in treated and untreated spermatozoa.

TLR2 Expression

The flow cytometry results showed no significant differences in the percentage of TLR2 expression in both the URSA and control groups. After the treatment of spermatozoa with LPS, the percentage of TLR2 expression decreased significantly in the URSA and control group (p -value >0.001).

In the treatment of spermatozoa by PAM3CYS, no difference was found in the percentage of TLR2 expression between untreated spermatozoa and PAM3CYS-treated spermatozoa in control group but in RSA group, the percentage of TLR2 expression increased significantly after treatment with PAM3CYS in comparison with untreated spermatozoa (p was 0.002). Accordingly, the percentage of TLR2 expression in PAM3CYS-treated spermatozoa from the RSA group was higher than the control group ($p=0.03$) (Figure 2, A). The mean \pm SD of untreated, LPS, and PAM3CYS-treated spermatozoa were 34.6 ± 0.98 , 31.58 ± 0.85 , and 35.6 ± 0.57 in the control group and 36.68 ± 0.76 , 30.66 ± 1.58 , and 6.84 ± 1.07 in RSA group, respectively.

TLR4 Expression

A significantly reduced percentage of TLR4 expression was detected in the URSA group compared with the control group. We also found that the percentage of TLR4 expression did not show any significant change in none of the URSA and control group in comparison with untreated spermatozoa after treating the spermatozoa by LPS and PAM3CYS.

Moreover, the percentage of TLR4 expression in LPS and PAM3CYS treated spermatozoa in the URSA group was lower than the control group (Figure 2, B).

All p -values were less than 0.0001. The respective mean \pm SD of untreated, LPS, and PAM3CYS-treated spermatozoa were 27.15 ± 4.04 , 27.21 ± 5.13 , and 25.78 ± 1.57 in the control group and 5.12 ± 1.04 , 5.56 ± 1.45 , and 40.2 ± 1.37 in the RSA group.

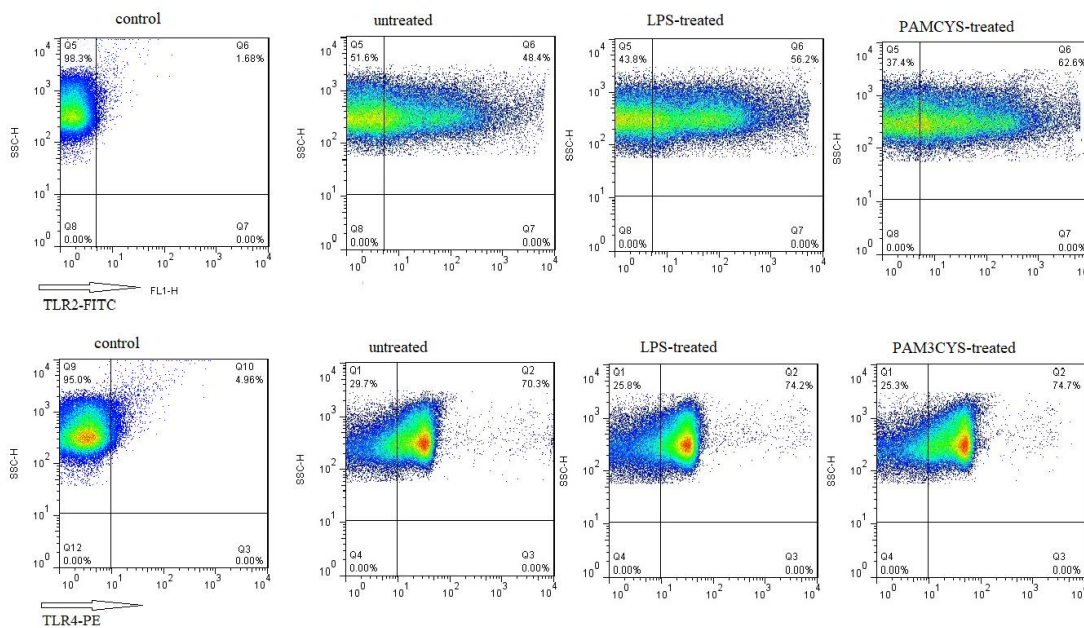


Figure 1. All p -value were less than 0.0001. The respective mean \pm SD of untreated, LPS, and PAM3CYS-treated spermatozoa were 27.15 \pm 4.04, 27.21 \pm 5.13, and 25.78 \pm 1.57 in the control group and 5.12 \pm 1.04, 5.56 \pm 1.45, and 40.2 \pm 1.37 in the RSA group.

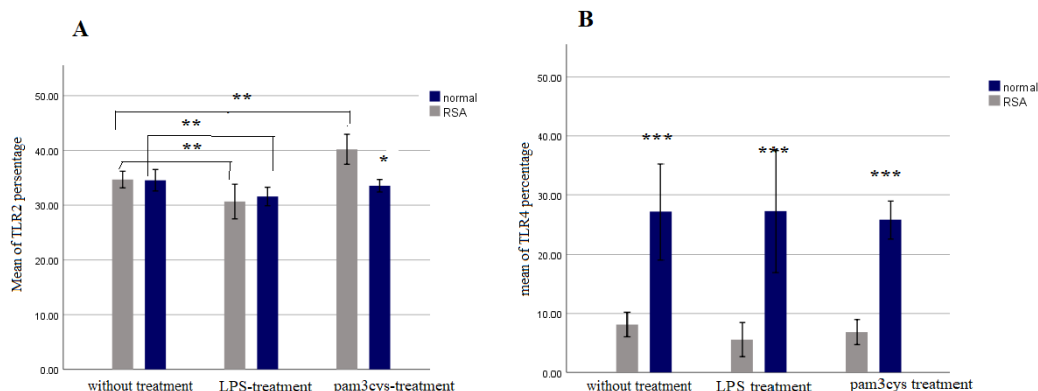


Figure 2. The effects of lipopolysaccharide (LPS) and or Pam3Cys on toll like receptor (TLR)2 (A) and TLR4 (B) expression on spermatozoa in control and recurrent spontaneous abortion (RSA) couples. Spermatozoa were stimulated with LPS (100 ng/ml) and PAM3CYS (10 μ g/mL) for 4 hours. * p <0.05, ** p <0.01 and *** p <0.001

DISCUSSION

This study was done to determine the alteration of TLR2 and TLR4 expression in spermatozoa in men whose spouses have URSA (URSA couples). The result of this study showed reduced expression of TLR4 in untreated and LPS- or PAM3CYS-treated spermatozoa in the URSA group compared to the control group. No significant differences were found in TLR2 expression in

URSA and control groups. After the treatment of spermatozoa with LPS, the TLR2 expression was decreased in both groups. After the treatment of spermatozoa with PAM3CYS, the level of TLR2 expression was significantly increased in the URSA group while no significant differences were shown in the control group in comparison to untreated spermatozoa. These results suggest impaired TLR2 and TLR4 expression and/or disturbed TLR2 and TLR4

ability for the response to microbial products in RSA couples. What causes lead to this impairment, need to be investigated.

As previously mentioned, less is known about the function of TLR molecule-expressed by spermatozoa. Youk Fujita *et al.* showed that TLR4 and TLR2 activation in spermatozoa resulted in the decrease of sperm motility and also the induction of apoptosis in these cells.²³ In another study, the activation of myeloid differentiation factor 88 (MYD88), phosphatidylinositol 3-kinase (PI3K), and glycogen synthase kinase (GSK)-3 α in TLR signaling pathway was introduced as a cause of decreased spermatozoa motility.²⁴ The activation of these molecules results in impaired mitochondria function and not providing the necessary adenosine triphosphate (ATP) for the motility of spermatozoa.²⁴ In the aforementioned study, it was suggested that TLR signaling controls spermatozoa motility and ATP level of mitochondria. Regarding the impaired expression of TLR2 and TLR4 in RSA couples, we supposed that TLRs may have some roles in other function of spermatozoa so that impairment in TLR expression leads to the deterioration of fertilization processes. Another possible role of TLRs in spermatozoa can be its function in spermcumulus-oocyte complex (COC) interaction. COC that is formed from the oocyte and surrounding cumulus cells have several endogenous ligands for TLRs including high mobility group box 1 (HMGB1),^{22,25-27} fatty acids, biglycan, defending 2, nucleic acids, heme, and heat shock protein family D (Hspd1).²⁸⁻³⁰ Nevertheless, the interaction of TLR in spermatozoa with TLR ligands in COC and the outcomes of these interactions have not been yet determined. Future studies on this context are therefore required in order to define this indeterminacy.

Another possible role for TLRs signaling in spermatozoa may be a role in sperm chemotaxis. Studies have displayed that spermatozoa express chemokine receptors such as C-C type chemokine receptor (CCR) 6, CCR5, and CCR3.³¹⁻³⁴ The interaction between these chemokine receptors on sperms with chemokines in FRT directed sperm to move in FRT and reach to oocyte.³¹⁻³⁴ It is assumed that TLRs in sperm may induce chemokine receptor gene expression in spermatozoa after interaction with their ligands in FRT. however, further work needs to be done to establish this hypothesis.

We are aware that our research has some limitations

such as low sample size, not checking the TLRs signaling pathway, and not assessing for other TLRs. We hope that we will be able to investigate these issues in future investigations.

We have demonstrated decreased TLR4 expression and a differently increased TLR2 expression in response to ligand treatment (PAM3CYS) in spermatozoa from URSA couples in comparison with control couples. The finding of this study suggests that disturbed TLR expression by spermatozoa can be related to URSA. This study has raised many questions about the role of TLR molecules expressed by spermatozoa in the function of these cells and the answer to these questions is a vital issue for our future researches.

ACKNOWLEDGEMENTS

The authors would like to thanks Mohadeseh Thogyani, Dr. Raziye Alipour, Dr. Mitra Rafiee and Dr. Mohammad Sadegh Hesamian for their valuable insights and recommendation and also for their contribution in conducting some of the experiments for the research.

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