

**BRIEF COMMUNICATION**

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

Nasrin Sereshki<sup>1</sup>, Alireza Andalib<sup>1</sup>, Ataollah Ghahiri<sup>2</sup>, Ferdos Mehrabian<sup>2</sup>, Roya Sherkat<sup>2</sup>, and Abbas Rezaei<sup>1</sup>

<sup>1</sup> Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

<sup>2</sup> Department of Obstetrics and Gynecology, Al-Zahra Hospital, Isfahan University of Medical Sciences, Isfahan, Iran

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### **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)<sup>1</sup> 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.<sup>2-5</sup> Therefore, any undesirable change in immunogenicity of semen or spermatozoa can lead to impaired immune response and consequently fertility

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**Corresponding Author:** Abbas Rezaei, PhD;  
Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. Tell: (+98 913) 129 4310, E-mail: rezaei@mui.ac.ir

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

As mentioned above, spermatozoa antigens from men whose spouses suffer from RSA show reduced capacity to induce Treg cells.<sup>1</sup> 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<sup>10,11</sup> and it has been suggested that these molecules play a role in the protection of spermatozoa from microbial pathogens.<sup>11</sup> 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.<sup>12,13</sup> 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.<sup>14-18</sup> Almost all TLRs except TLR3 induces the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ 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 peroxidation<sup>18</sup> 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 microbiota<sup>19-21</sup> 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  $6 \times 10^6$  cells/300  $\mu$ 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  $\mu$ g/mL of Pam3Cys (calbiochem, USA) and without any treatment (untreated spermatozoa). Cultures were incubated at 37°C, 5% CO<sub>2</sub> 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 pharmlingen, USA) and Fluorescein-5-isothiocyanate (FITC) mouse anti-human TLR2 (Southern Biotech). The cell density was as follows:  $1 \times 10^6$  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  $34.6 \pm 0.98$ ,  $31.58 \pm 0.85$ , and  $35.6 \pm 0.57$  in the control group and  $36.68 \pm 0.76$ ,  $30.66 \pm 1.58$ , and  $6.84 \pm 1.07$  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  $27.15 \pm 4.04$ ,  $27.21 \pm 5.13$ , 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.

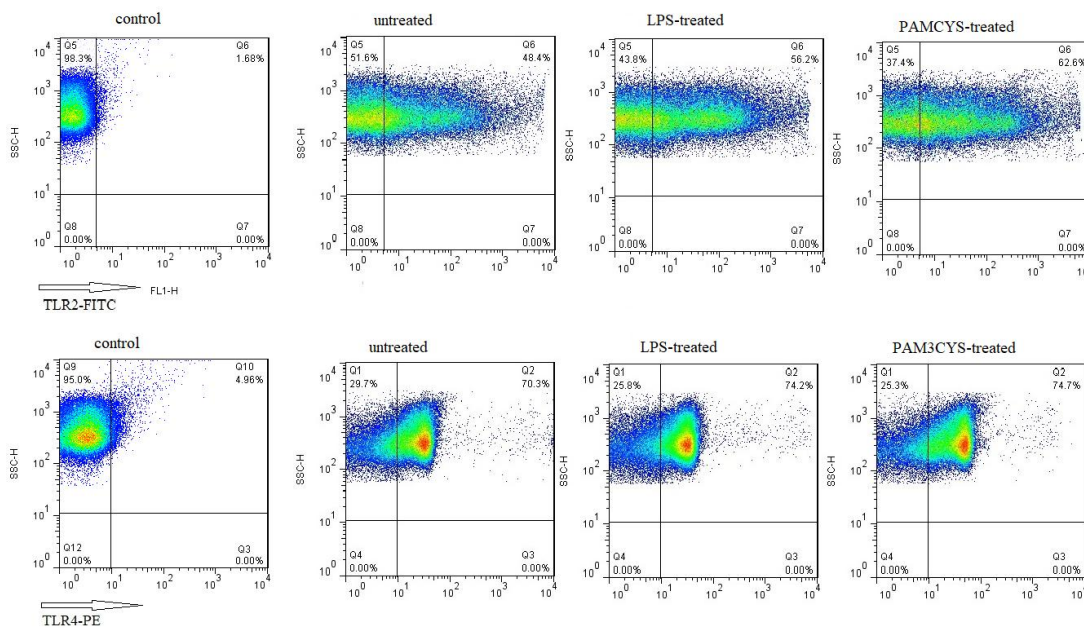


Figure 1. All  $p$ -value were less than 0.0001. The respective mean $\pm$ SD of untreated, LPS, and PAM3CYS-treated spermatozoa were 27.15 $\pm$ 4.04, 27.21 $\pm$ 5.13, and 25.78 $\pm$ 1.57 in the control group and 5.12 $\pm$ 1.04, 5.56 $\pm$ 1.45, and 40.2 $\pm$ 1.37 in the RSA group.

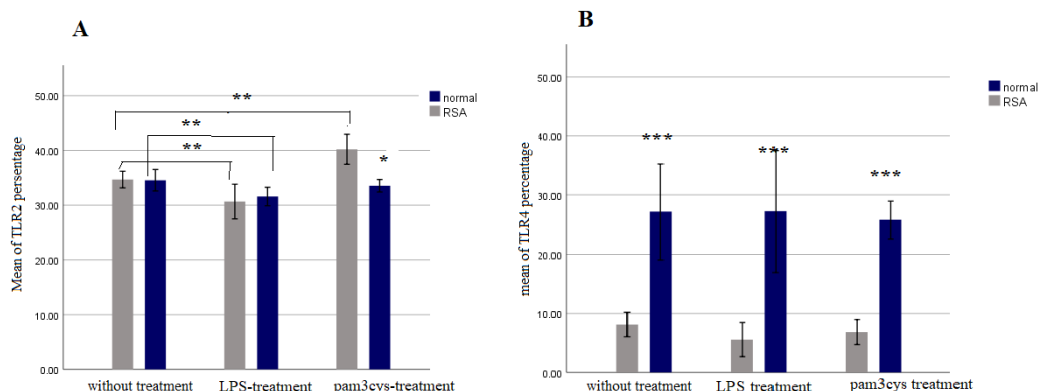


Figure 2. The effects of lipopolysaccharide (LPS) and or Pam3Cys on toll like receptor (TLR)2 (A) and TLR4 (B) expression on spermatozoa in control and recurrent spontaneous abortion (RSA) couples. Spermatozoa were stimulated with LPS (100 ng/ml) and PAM3CYS (10  $\mu$ g/mL) for 4 hours. \* $p$ <0.05, \*\* $p$ <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.<sup>23</sup> In another study, the activation of myeloid differentiation factor 88 (MYD88), phosphatidylinositol 3-kinase (PI3K), and glycogen synthase kinase (GSK)-3 $\alpha$  in TLR signaling pathway was introduced as a cause of decreased spermatozoa motility.<sup>24</sup> The activation of these molecules results in impaired mitochondria function and not providing the necessary adenosine triphosphate (ATP) for the motility of spermatozoa.<sup>24</sup> 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),<sup>22,25-27</sup> fatty acids, biglycan, defending 2, nucleic acids, heme, and heat shock protein family D (Hspd1).<sup>28-30</sup> 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.<sup>31-34</sup> The interaction between these chemokine receptors on sperms with chemokines in FRT directed sperm to move in FRT and reach to oocyte.<sup>31-34</sup> 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.

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#### REFERENCES

1. Liu C, Wang X-Z, Sun X-B. Assessment of sperm antigen specific T regulatory cells in women with recurrent miscarriage. *Early Hum Dev* 2013; 89(2):95-100.
2. Johansson M, Bromfield JJ, Jasper MJ, Robertson SA. Semen activates the female immune response during early pregnancy in mice. *Immunology* 2004; 112(2):290-300.
3. Robertson SA, Sharkey DJ. Seminal fluid and fertility in women. *Fertil Steril* 2016; 106(3):511-9.
4. Bromfield JJ. Seminal fluid and reproduction: much more than previously thought. *J Assist Reprod Genet* 2014; 31(6):627-36.
5. Schjenken JE, Robertson SA. Seminal Fluid Signalling in the Female Reproductive Tract: Implications for Reproductive Success and Offspring Health. *Adv Exp Med Biol* 2015;868:127-58
6. Kverka M, Ulcova-Gallova Z, Bartova J, Cibulka J, Bibkova K, Micanova Z, et al. Sperm Cells Induce Distinct Cytokine Response in Peripheral Mononuclear Cells from Infertile Women with Serum Anti-Sperm Antibodies. *Plos One* 2012; 7(8).
7. Sereshki N, Gharagozloo M, Ostadi V, Ghahiri A, Roghaei MA, Mehrabian F, et al. Variations in T-helper 17 and regulatory T cells during the menstrual cycle in

- peripheral blood of women with recurrent spontaneous abortion. *Int J Fertil Steril* 2014; 8(1):59-66.
8. Charles A, Omwandho HR, Tinneberg AG, Tumbo O, Timothy K, Falconer RJ. Recurrent pregnancy losses and the role of immunotherapy. *Arch Gynecol Obstet* 2000; 264(1):3-12.
  9. Pandey MK, Rani R, Agrawal S. An update in recurrent spontaneous abortion. *Arch Gynecol Obstet* 2005; 272(2):95-108.
  10. Palladino MA, Savarese MA, Chapman JL, Dughi MK, Plaska D. Localization of Toll-Like Receptors on Epididymal Epithelial Cells and Spermatozoa. *Am J Reprod Immunol* 2008; 60(6):541-55.
  11. Saeidi S, Shapouri F, Amirchaghmaghi E, Hoseinifar H, Sabbaghian M, Gilani MAS, et al. Sperm protection in the male reproductive tract by Toll-like receptors. *Andrologia* 2014; 46(7):784-90.
  12. Nardo D-D. Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine* 2015; 74: 181-9.
  13. Erridge C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukoc Biol* 2010; 87(6):989-99.
  14. Hedger MP. Toll-like receptors and signalling in spermatogenesis and testicular responses to inflammation-a perspective. *J Reprod Immunol* 2011; 88(2):130-41.
  15. Malm J, Nordahl EA, Bjartell A, Sørensen OE, Frohm B, Dentener MA, et al. Lipopolysaccharide-binding protein is produced in the epididymis and associated with spermatozoa and prostasomes. *J Reprod Immunol* 2005; 66(1):33-43.
  16. Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biol Pharm Bull* 2005; 28(5):886-92.
  17. McClure R, Massari P. TLR-dependent human mucosal epithelial cell responses to microbial pathogens. *Front Immunol* 2014; 5:386.
  18. Marcato L, Ferlini A, Bonfim R, Ramos-Jorge M, Ropert C, Afonso L, et al. The role of Toll-like receptors 2 and 4 on reactive oxygen species and nitric oxide production by macrophage cells stimulated with root canal pathogens. *Oral Microbiol Immunol* 2008; 23(5):353-9.
  19. Reid G, Brigidi P, Burton JP, Contractor N, Duncan S, Fargier E, et al. Microbes Central to Human Reproduction. *American Journal of Reproductive Immunology*. 2015;73(1):1-11.
  20. Schoenmakers S, Steegers-Theunissen R, Faas M. The matter of the reproductive microbiome. *Obstetric Medicine*. 2018.
  21. Smith SB, Ravel J. The vaginal microbiota, host defence and reproductive physiology. *Journal of Physiology*-London. 2017;595(2):451-63.
  22. Organization WH. WHO laboratory manual for the examination and processing of human semen. 2010.
  23. Fujita Y, Mihara T, Okazaki T, Shitanaka M, Kushino R, Ikeda C, et al. Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. *Hum Reprod* 2011; 26(10):2799-806.
  24. Zhu X, Shi D, Li X, Gong W, Wu F, Guo X, et al. TLR signalling affects sperm mitochondrial function and motility via phosphatidylinositol 3-kinase and glycogen synthase kinase-3 $\alpha$ . *Cell Signal* 2016; 28(3):148-56.
  25. Harris HE, Raucci A. Alarmin (g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep* 2006; 7(8):774-8.
  26. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol Rev* 2007; 220(1):47-59.
  27. Ibrahim ZA, Armour CL, Phipps S, Sukkar MB. RAGE and TLRs: relatives, friends or neighbours? *Mol Immunol* 2013; 56(4):739-44.
  28. Shimada M, Yanai Y, Okazaki T, Noma N, Kawashima I, Mori T, et al. Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. *Development* 2008; 135(11):2001-11.
  29. Richards JS, Liu Z, Shimada M. Immune-like mechanisms in ovulation. *Trends Endocrinol Metab* 2008; 19(6):191-6.
  30. Liu Z, Shimada M, Richards JS. The involvement of the Toll-like receptor family in ovulation. *J Assist Reprod Genet* 2008; 25(6):223-8.
  31. Barbonetti A, Vassallo MRC, Pelliccione F, D'Angeli A, Santucci R, Muciaccia B, et al. Beta-chemokine receptor CCR5 in human spermatozoa and its relationship with seminal parameters. *Hum Reprod* 2009;24(12):2979-87.
  32. Muciaccia B, Padula F, Vicini E, Gandini L, Lenzi A, Stefanini M. Beta-chemokine receptors 5 and 3 are expressed on the head region of human spermatozoon. *FASEB J* 2005; 19(11):2048-50.
  33. Isobe T, Minoura H, Tanaka K, Shibahara T, Hayashi N, Toyoda N. The effect of RANTES on human sperm chemotaxis. *Hum Reprod* 2002; 17(6):1441-6.
  34. Caballero-Campo P, Buffone MG, Benencia F, Conejo-García JR, Rinaudo PF, Gerton GL. A role for the chemokine receptor CCR6 in mammalian sperm motility and chemotaxis. *J Cell Physiol* 2014; 229(1):68-78.