


Reactive Oxygen Species Secreted by Leukocytes in Semen Induce Self-Expression of Interleukin-6 and Affect Sperm Quality

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Abstract

Reproductive tract inflammation is considered an important cause of male infertility. Increased leukocytes in semen can produce many reactive oxygen species (ROS), which affect sperm function. The aim of this study is to identify the main source of ROS in seminal plasma and to assess the effect of ROS on leukocytes. Semen samples ($n = 20$) with leukocyte concentration $>1 \times 10^6$ were collected from a male infertility clinic. This study mainly compares the sperm function parameters of the normal group and the semen white blood cell group $>1 \times 10^6$. The results identified that ROS in semen was closely related to sperm function parameters, and CD45⁺ leucocytes were the main source of ROS. Compared with the control group, the concentration of IL-2, IL-4, IL-6, IFN- γ , and TNF- α was higher in the experimental group. Leukocytes in semen may regulate the secretion of ROS through the mammalian target of rapamycin (mTOR) pathway. A considerable amount of ROS can upregulate the expression of IL-6 in leukocytes via the nuclear factor kappa-B (NF- κ B) pathway.

Keywords

Leukocytes, reactive oxygen species, mTOR, NF- κ B, IL-6

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Although semen analysis is used as a golden standard for determining male fertility, this test alone cannot accurately diagnose infertility, since 6%–27% of men with normal semen parameters are infertile (Irvine, 1998; Moghissi & Wallach, 1983). One possible explanation is that semen analysis cannot detect molecular-level lesions in infertile patients. Oxidative stress caused by excessive production of reactive oxygen species (ROS) may have a profound impact on the sperm plasma membrane and subsequent sperm functional integrity (Aitken et al., 2014; Mahfouz et al., 2010). ROS usually have a negative impact on somatic or germ cell lines, which is the main cause of cell damage. Examples of ROS include hydrogen peroxide (H₂O₂), superoxide anion ($\cdot\text{O}_2^-$), hydroxyl radical ($\cdot\text{OH}$), and the peroxy radical ($\cdot\text{HO}_2^-$). ROS is primarily produced by mitochondrial electron chain complexes I and III (Chen et al., 2009). Due to its extensive distribution and active chemical properties, ROS is usually used as

a marker of oxidative damage. ROS induces cell damage in a variety of ways, involving lipids, proteins, DNA, and so on (Agarwal, Mulgund, et al., 2014; Agarwal, Virk, et al., 2014; Du Plessis et al., 2015). Excessive ROS in male semen can lead to oxidative stress, which has potential toxic effects on the quality and function of sperm (Gosalvez et al., 2017). Studies

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have shown that the level of ROS in 25%–50% male infertility patients is higher, which may be related to sperm motility, membrane integrity, and DNA quality abnormalities (Ferramosca et al., 2013).

Leukocytosis is defined by the World Health Organization (WHO) as semen white blood cell (WBC) concentration $>1 \times 10^6/\text{mL}$. Although it is considered to be a possible marker of male reproductive tract infections (orchitis, epididymitis), high levels of sperm leukocytes cannot predict actual microbial infections (Castellini et al., 2019). A considerable number of men show a large number of sperm WBCs but no symptoms of genital tract infections (Wolff, 1995). Conflicting results may depend on different WBCs detection methods or simply relying on the total number of WBCs may not be enough. Subsets of WBCs should also be analyzed. Leukocytes in semen are mostly produced by the testis and epididymis (Anderson et al., 1991). Leukocytes in semen play a key role in immune surveillance and abnormal sperm phagocytosis (Muller & Hinkelmann, 1991; Tomlinson et al., 1993). Leukocytes in semen contain granulocytes (50%–60%), macrophages (20%–30%), and T lymphocytes (2%–5%; Aitken et al., 1995). Activated leukocytes secrete ROS, protease, and cytokines, which may lead to sperm damage through lipid peroxidation and DNA fragments (Agarwal, Mulgund, et al., 2014; Agarwal, Virk, et al., 2014; Henkel et al., 2005). The involvement of pro-inflammatory cytokines, including IL-6 and IL-8, have been reported as key factors in semen inflammation (Fraczek & Kurpisz, 2007). Studies have been conducted to assess the cutoff value of IL-6 to distinguish inflammatory and noninflammatory semen. It suggests that IL-6 is more suitable to evaluate semen inflammation than leukocytes. Depuydt et al. (1996) identified that the cutoff value of IL-6 is 14.8 pg/mL, which is the most accurate marker to identify inflammation in semen.

Numerous studies have confirmed that excessive ROS can significantly reduce sperm quality parameters. The effect of ROS on WBCs in semen has rarely been studied. The regulation mechanism of IL-6 secretion in seminal plasma needs further elucidation. Our study aims to clarify the origin of ROS and elucidate the regulation mechanism of ROS on leukocyte function.

Materials and Methods

Ethics Statement

This study was approved by the ethics committee of Nanjing Medical University (Nanjing, China, ethics committee approval number: K2014001), and informed consent was obtained from each volunteer. For the

patients included in the project, the purpose of the study and the use of samples were informed, and patient privacy was not disclosed. After oral consent was obtained from patients, their information was inputted into our management system. The samples involved in this study were all residual samples after clinical testing, which would not further inconvenience the patients

Semen Samples

Men were selected if they were between 25 and 47 years old, seeking fertility evaluation, and consented to donate a semen specimen for research. Participants were asked to practice abstinence for 2–7 days prior to semen collection. All participants used the same sperm extraction chamber, and the samples were sent to the andrological laboratory immediately after sperm extraction to ensure the quality of the semen specimens. Semen samples were collected by masturbation into sterile cups. Semen specimens were excluded if participants reported semen spillage or loss during collection. The study design was divided into two groups based on the concentration of leukocytes in semen: the control group ($n = 20$) consisted of patients with normal semen profiles and proven fertility, and the leukocytes group ($n = 20$) consisted of patients with high level of leukocytes ($>10^6/\text{mL}$). Patients with severe inflammatory diseases of the reproductive system such as symptomatic urethritis, orchitis, epididymitis, or prostatitis were excluded. All subjects had no history of chemotherapy, radiotherapy, chronic diseases, or drug treatment.

Analysis of Sperm Motility Parameters

The parameters of sperm quality—including sperm concentration, sperm motility, fast moving sperm rate, positive motility sperm rate, and nonexercise sperm rate—were evaluated by an automatic sperm quality analyzer (Beions 3-3).

Reactive Oxygen Species Assay

2',7'-dichlorofluorescein diacetate (DCFH-DA) was diluted to 10 $\mu\text{mol/L}$ with a serum-free medium (Beyotime biotechnology, China). After centrifugation, the semen specimens were suspended in diluted DCFH-DA at a concentration of 1×10^6 – 2×10^7 . They were then incubated in 37 °C cell incubators for 30 min, with mixing every 5 min. DCFH-DA entered the cell and was hydrolyzed by esterase to form DCFH. Intracellular ROS oxidized DCFH to produce fluorescent 2',7'-dichlorofluorescein (DCF). The detection of fluorescence intensity (Arbitrary Unit [A.U.]) represents the amount

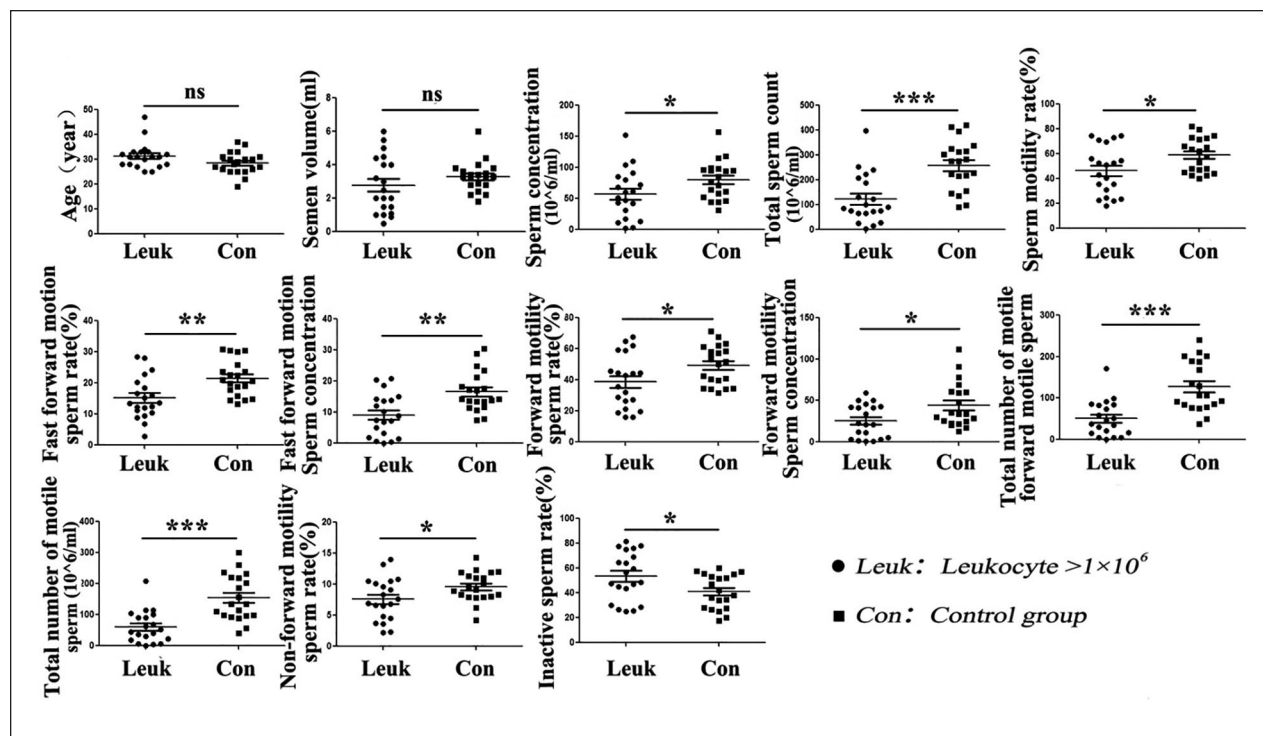


Figure 1. Sperm motility parameters decreased in the group with a high level of leucocytes. Note. The age, semen volume, sperm concentration, total sperm count, sperm motility rate, fast forward motility sperm rate, forward motility sperm rate, forward motility sperm total number, nonforward motility sperm rate, and inactive sperm rate of the two groups (Leuk. $n = 20$, Con. $n = 20$) were counted. * $p < .05$. ** $p < .01$. *** $p < .001$.

of ROS in the cell. Cell surface molecules and cytokines were detected by flow cytometry.

Semen samples were prepared as a single cell suspension and incubated with fluorescein-conjugated antibodies (CD45-percp, IL-6-FITC; Biolegend, San Diego, USA) for 20 min at 4 °C. The cells were washed twice with ice cold phosphate buffer saline (PBS). Cells were collected with a flow cytometer (Navios, Beckman Coulter, USA), and data were analyzed using the Kaluza software (Beckman Coulter, USA).

Cytokine Detection

The cytokines IL-4, IL-6, and IL-10 were detected by the immunomagnetic bead method (SaiJi biotechnology, China). Semen samples were centrifuged at 3500 rpm/min for 15 min to obtain semen. A 25-liter seminal plasma was mixed with 25-liter capture microspheres. The microspheres had different fluorescence intensities and were coated with antibodies against IL-2, IL-4, and IL-6. The second antibody labeled with P-phycoerythrin (PE) was added. By analyzing the fluorescence intensity of the double-antibody sandwich complex, the concentration (pg/mL) of cytokines in the sample was calculated according to the standard curve.

Statistical Analysis

Each experiment was performed at least thrice. Before the t test, the data were tested for homogeneity of variance. The Student's t test was used for parameter values. One-way analysis of variance (ANOVA) was used to determine significant differences among the groups (GraphPad Prism version 5.0), and a p value $< .05$ was considered statistically significant.

Results

Sperm Motility Parameters

We compared the functional parameters of sperm between the control group and the group with leucocytes $>1 \times 10^6$ /mL, including age, semen volume, total sperm count, sperm motility rate, fast forward motion sperm rate, forward motility sperm rate, nonforward motility sperm rate, and inactive sperm rate. There were no significant differences in age and semen volume between the control group and the leucocytes group (Figure 1). The rate of inactive sperm in the leucocytes group was significantly higher than that in control group. The sperm motility parameters in leucocytes group were significantly lower than those in control group (Figure 1), suggesting that

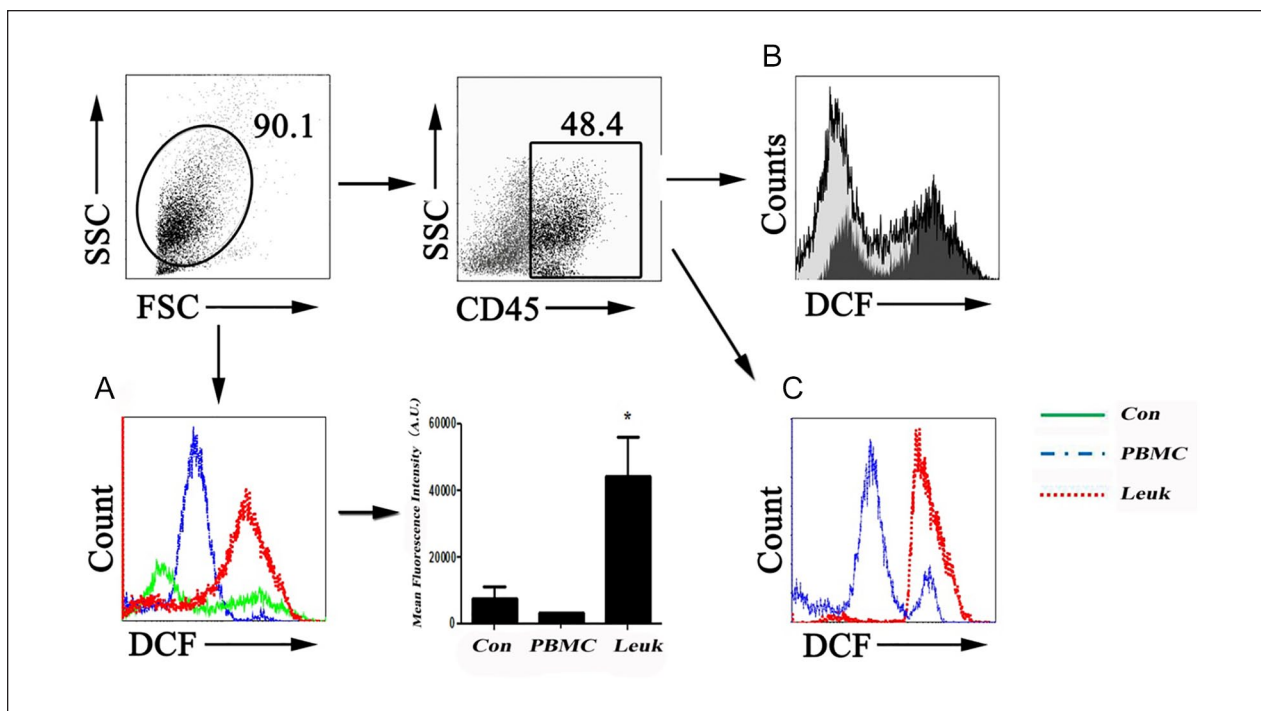


Figure 2. Leukocytes in semen are the main source of ROS. *Note.* Semen samples of the two groups (Leuk. $n = 12$, Con. $n = 12$) were washed with PBS, incubated with anti-human CD45 antibody for 20 min, and then tested for ROS. (A) ROS levels in all cells in semen, statistical analysis of average fluorescence intensity. (B) The dark part is the ROS level in CD45⁺ leukocytes and the light part is the ROS level in all cells. (C) Comparison of ROS levels between leukocytes in semen and leukocytes in peripheral blood. ROS = reactive oxygen species.

excessive leucocytes may have a negative impact on sperm function.

Main Sources of Reactive Oxygen Species in Seminal Plasma

In view of the significant deterioration of sperm motility in semen samples with a high leucocyte concentration, we speculate that it may be due to the high concentration of ROS in seminal plasma. We measured the production of ROS in all cells in semen and found that the production of ROS in the leucocyte group was not only significantly higher than that in the control group but also higher than that in the normal human peripheral blood mononuclear cells (PBMCs; Figure 2A). Most ROS are produced by CD45⁺ leucocytes (dark part represents CD45⁺ leucocytes; Figure 2B). Due to the low concentration of leucocytes in normal semen, we chose normal PBMCs as a control group. CD45⁺ leucocytes from semen produced more ROS than PBMCs isolated from peripheral blood (Figure 2C).

Rapamycin, a specific inhibitor of mammalian target of rapamycin complex 1 (mTORC1), plays an important role in the secretion of ROS by sperm cells. Our results showed that rapamycin significantly reduced ROS

levels not only in all cells in semen (Figure 3A) but also in CD45⁺ leucocytes (Figure 3B). These results suggest that the mammalian target of rapamycin (mTOR) pathway is at least partially involved in the formation of ROS in semen.

Since the presence of large amounts of leucocytes in semen can alter sperm function, we examined the differences in the expression of some cytokines (IL-2, IL-4, IL-6, IFN- γ , TNF- α) in the seminal plasma of the two groups to assess whether there were functional differences in leucocytes in the semen of the two groups. The results showed that the expression of IL-2, IL-4, IL-6, IFN- γ , and TNF- α in seminal plasma of the leucocyte group were significantly higher than that in the control group (Figure 4).

To further elucidate the reasons for the upregulation of cytokines secreted by leucocytes in semen, we treated washed cells in semen with 1% H₂O₂, which was used to simulate the high ROS environment of leucocytes. Then the expression of IL-6 in leucocytes was observed by flow cytometry. The results showed that the CD45⁺ leucocytes treated with H₂O₂ expressed higher levels of IL-6 (Figure 5). The nuclear factor kappa-B (NF- κ B) pathway is a classical inflammatory cytokine regulatory pathway. BAY11-7082 is a selective inhibitor of the NF- κ B

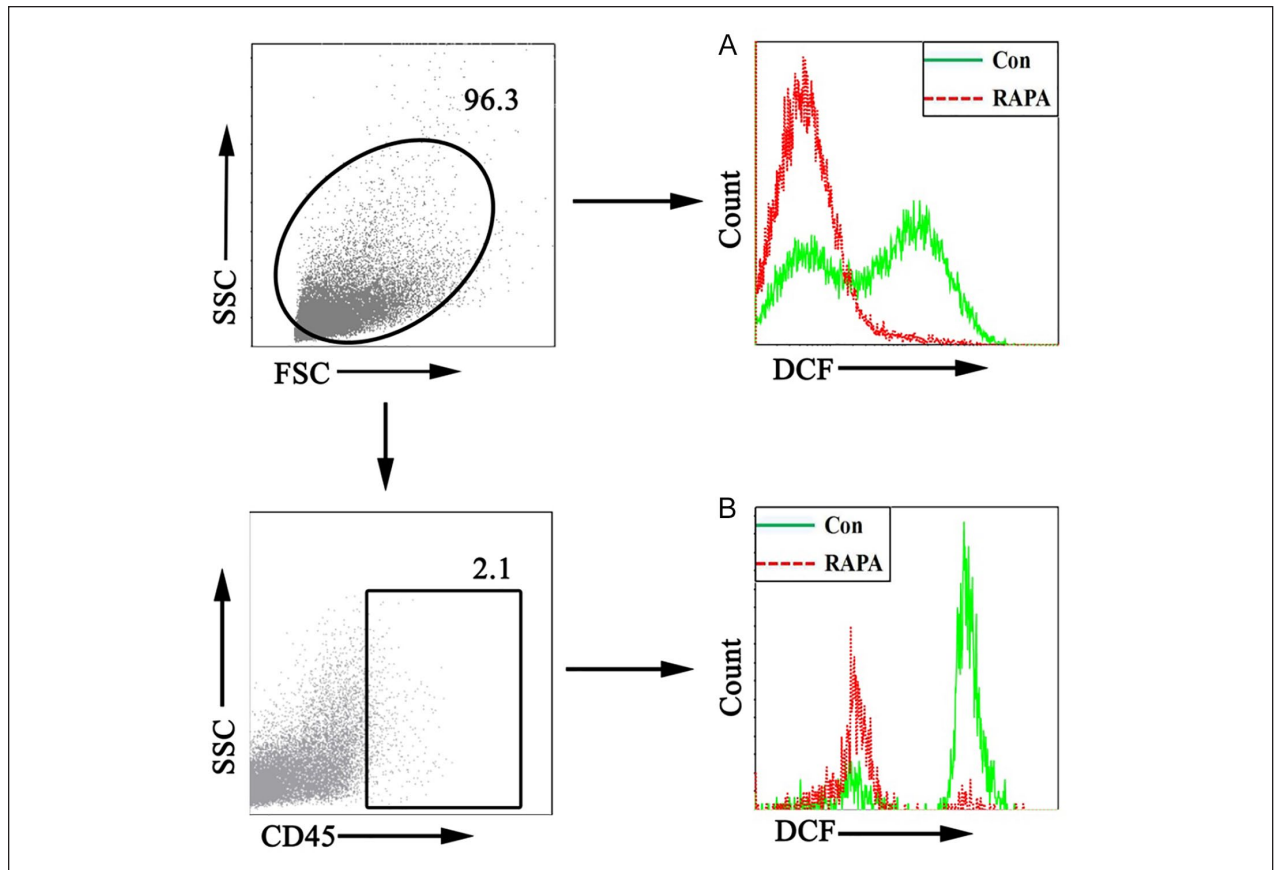


Figure 3. Rapamycin, an inhibitor of mTOR, inhibits the secretion of ROS by leucocytes in semen. *Note.* 500 ng/mL rapamycin was added to semen specimens and the content of ROS was detected by flow cytometry after incubation for 4 hr. (A) Levels of ROS in total cells in semen treated with rapamycin (Rapa. $n = 12$, Con. $n = 12$). (B) Levels of ROS in CD45⁺ leucocytes after rapamycin treatment. mTOR = mammalian target of rapamycin; ROS = reactive oxygen species.

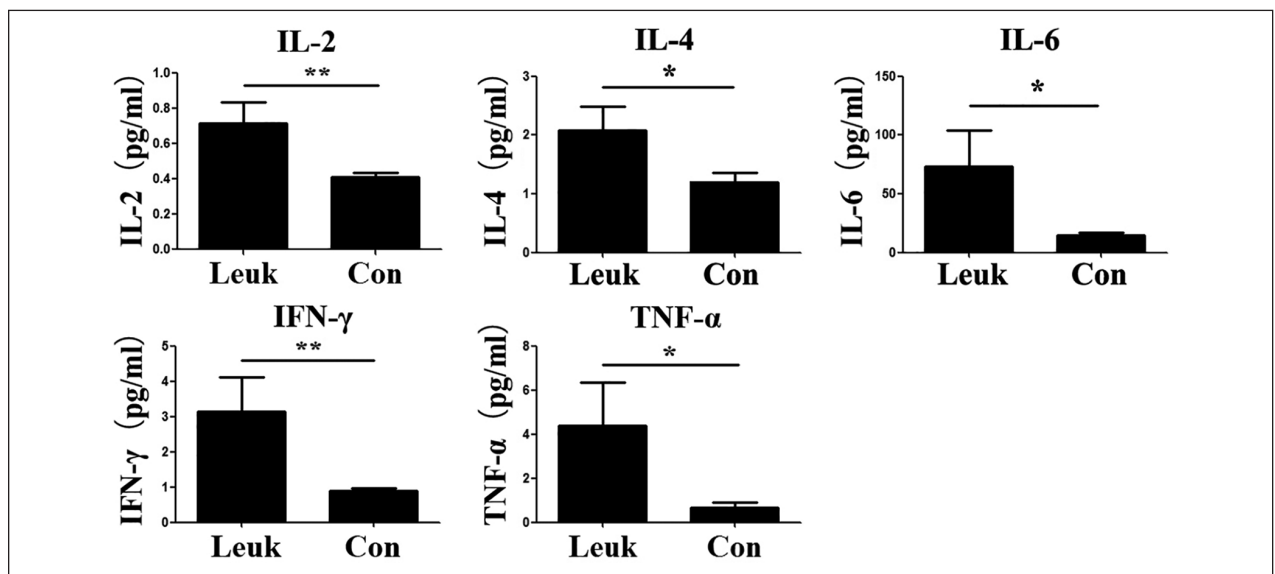


Figure 4. Increased levels of IL-2, IL-4, IL-6, IFN- γ , and TNF- α in seminal plasma with a high concentration of leucocytes. *Note.* The seminal plasma of the two groups (Leuk. $n = 12$, Con. $n = 19$) was collected, and the concentrations of IL-2, IL-4, IL-6, IFN- γ , and TNF- α were measured by the immuno-magnetic bead method. * $p < .05$. ** $p < .01$. *** $p < .001$.

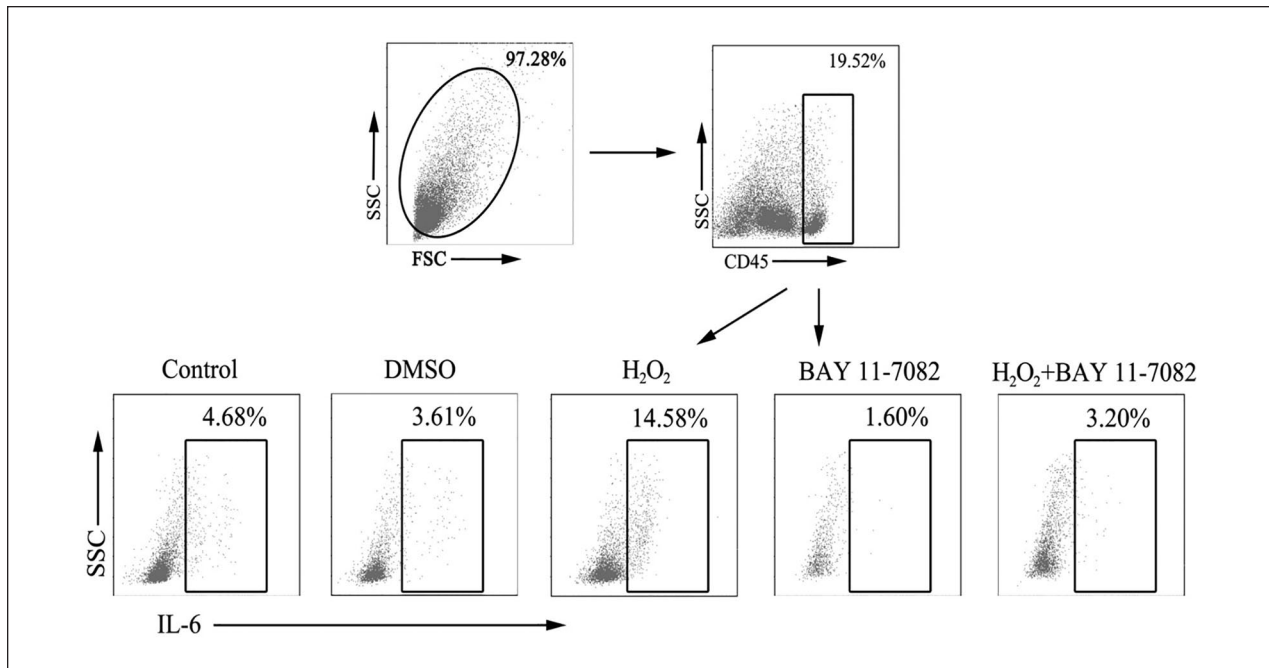


Figure 5. The increase of ROS promotes the upregulation of IL-6 expression in semen leucocytes, which is related to the NF- κ B pathway. *Note.* Leukocyte concentration $>1 \times 10^6$ /mL specimens were divided into five groups, each group contains 1×10^7 cells ($n = 12$): control group; Dimethyl sulfoxide (DMSO) solvent control group; H₂O₂ group (adding 1% H₂O₂); BAY 11-7082 group (1 mM, MCE, USA); and H₂O₂ + BAY 11-7082 group. After incubation for 2 hr, the expression of IL-6 in CD45⁺ leucocytes was detected by flow cytometry. H₂O₂ = hydrogen peroxide; NF- κ B = nuclear factor kappa-B; ROS = reactive oxygen species

pathway. The high expression of IL-6 in leukocytes induced by H₂O₂ can be partially reversed by BAY11-7082. These results suggest that high ROS can increase the secretion of IL-6 by leukocytes, and this process may be regulated by the NF- κ B pathway.

Discussion

The biological significance of leucocytes and their production of semen is a controversial issue. In a series of clinical studies, the increase of leucocytes in human semen was associated with impaired semen quality and poor results of assisted reproductive technology, while other studies showed conflicting results (Eggert-Kruse et al., 2007, 2009; Henkel, Kierspel, et al., 2003; Henkel, Maass, et al., 2003). Although leukocytosis has been widely accepted as a diagnostic feature of male genital tract infections, in our daily work we identified that semen with a high leucocyte count is not necessarily accompanied by low sperm function parameters. The results showed that a single detection of leucocyte concentration in semen had little significance in the diagnosis of genital tract infection. The possible reasons are as follows: (a) Leukocytes are a heterogeneous population and can be divided into multiple cell subsets with different functions (Micillo et al., 2016). (b) Only activated

leukocyte subsets can exert their biological functions. (c) The mechanism by which leucocytes impair sperm function is unclear. To sum up, the detection of functional molecules secreted by leukocytes may be more meaningful than the detection of leukocytes themselves. We found that the levels of ROS and inflammatory cytokines IL-2, IL-4, IL-6, IFN- γ , and TNF- α increased in semen with high leukocyte concentration. The regulatory effect of ROS on sperm and leucocytes themselves was further discussed in our research.

Consistent with many studies, we found that high ROS in semen significantly reduced sperm function, and leucocytes were the main source of ROS (Homa et al., 2015; Micillo et al., 2016). After being treated with mTOR inhibitors, the secretion of ROS in semen cells was markedly reduced. It shows that there is a closed relationship between mTOR and ROS secretion. In a study of maple syrup urine disease, it was reported that 10 mmol/L branched-chain amino acids (BCAAs) (similar to the concentration in patients with maple syrup urine disease) can activate PBMCs and induce ROS production (Zhenyukh et al., 2017). BCAAs in PBMCs promoted the phosphorylation of mTOR at Ser2448. This effect depends on protein kinase B (PKB) and is consistent with observations of other cell types (Gran & Cameron-Smith, 2011; Memmott & Dennis, 2009). Once

activated on Ser2448, mTOR binds to raptor and other proteins, forming the active enzyme complex, mTORC1, which signals through phosphorylation of downstream targets (Yang & Guan, 2007). Ultimately, ROS production increases. Another study identified that the activation of mTORC1 induced NADPH oxidase (NOX)-dependent increase in intracellular ROS formation. ROS further increased the activation of mTORC1 through a positive feedback loop, which is necessary for hypoxia-inducible factor 1 α (HIF1 α) accumulation. HIF1 α initiated the whole cell metabolic reprogramming required for inflammation (Sohrabi et al., 2018). Our study is the first to confirm that ROS secretion in semen is at least partially related to the activation of mTOR.

Importantly, in this paper, we have described the transcription factor NF- κ B in leukocytes activated by ROS for the first time. It is well known that ROS can activate or inhibit NF- κ B in a context-dependent manner (Nakajima & Kitamura, 2013). NF- κ B is a redox-sensitive transcription factor (Janssen-Heininger et al., 2000). The increase in ROS production is likely to promote the activation of NF- κ B in leukocytes. ROS can activate the TLR4/NF- κ B signaling pathway and induce the expression and release of inflammatory factors such as TNF- α and IL-6, thus leading to immune/inflammatory response (Kotas & Medzhitov, 2015). After TLR4 binds to its ligand, myeloid differentiation factor 88- (MyD88-)-dependent pathway was activated, after which NF- κ B was activated via MyD88 and interleukin-1 receptor associated kinase (IRAK) to make p65 into the nucleus, and the downstream inflammatory factors IL-6 and TNF- α were transcriptionally activated. Finally the immune inflammatory response was triggered (Zhang et al., 2018). Activation of NF- κ B further increased ROS through the pathways including cyclooxygenase activation, but did not increase the production of mitochondrial ROS (Jurk et al., 2014). In addition, high levels of ROS can lead to sperm DNA damage. Cytokines such as IL-6 and TNF- α are also toxic to sperm (Kopa et al., 2005; Moretti et al., 2009). It has been reported that IL-6, TNF- α , and other pro-inflammatory cytokines alter sperm function by inducing apoptosis (Perdichizzi et al., 2007). These data further demonstrate that the effects of leukocytes on sperm are complex and multifaceted.

This research is restricted in many ways. First, since our results are based on 20 patients, in order to reach a consensus on the relationship between leukocytes and sperm, further studies on larger and more representative semen samples are needed. Second, although this study confirms that the activation of the mTOR pathway in leukocytes leads to an increase in ROS secretion, the reason for the activation of mTOR remains to be elucidated. Third, detailed regulatory mechanisms of mTOR and

NF- κ B pathways in leukocytes have not been fully elucidated. This study provides a new perspective for elucidating the relationship between leukocytes and sperm in semen. The relationship between ROS, sperm, and white blood cells was initially revealed from the perspective of signal pathway. It provides a new research idea for the diagnosis and treatment of hyperleukocytic semen disease.

In conclusion, leukocyte semen samples showed a significant decrease in semen parameters and an obvious increase in ROS. Leukocytes in semen may regulate the secretion of ROS through the mTOR pathway. A considerable amount of ROS can upregulate the expression of IL-6 in leukocytes via the NF- κ B pathway.

Declaration of Conflicting Interests

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

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