Development and Validation of NLRP3 Immunochromatography Assay as an Indicator for Inflammation and Bad Seminal Quality

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Abstract— **Estimation of seminal inflammation is highly necessary for management of Infertile patients, unfortunately peroxidase staining and leukocyte count is not indicative for true inflammation. This study aimed to develop a rapid NLRP3 immunochromotography (ICT) strip as an alternative tool for indication of inflammation rather than confusing Endtz test. Methodology: Immunochromotography cassette developed in the Department of Microbiology/ College of Medicine/ AL-Nahrain University. Polyclonal capture rabbit anti-NLRP3 (test line) and goat anti-rabbit IgG antibodies (control line) were applied on each nitrocellulose membrane. Gold-in-a-Box™ conjugation kit (40nm. 15OD) conjugated anti-NLRP3 were adsorbed in conjugate pad, then assembled in the test cassette. The developed kit was tested in vitro using ELISA kit depending on serial concentration of standard NLRP3 protein. Fifty infertile patients included in this study, seminal fluid analysis and Endtz test were done. Results: the developed NLRP3 ICT was sensitive at the level of 5 ng/ml. NLRP3 was detected in seminal fluid of 30 (60%) of infertile patients. ICT assay showed 92.86 sensitivity and 81.82 specificity in comparison with ELISA test. Conclusion: NLRP3 protein in seminal plasma may be associated with the increased leukocytospermia, bad sperm quality among infertile male with higher sensitivity than Endtz test. NLRP ICT kit could be used for diagnosis of inflammation in infertile men.**

Index Terms—**Leukocytospermia, Immunochromatography, Infertility, Inflammasome, .**

I. INTRODUCTION

Leukocytospermia defined as the presence of at least 1*10^6 leukocytes/mL in a semen sample (World Health Organization 2010). This could be found in both fertile and infertile men with or without genital tract infection (Aggarwal et al. 2015; Lackner et al. 2010). However, several methods have been used for estimation of inflammatory state in semen sample predicting its importance as a diagnostic tool for infection or correlate with seminal fluid analysis (Agarwal, Clinic, and Agarwal 2014; Aziz et al. 2004; Sandoval, Raburn, and Muasher 2013).

At least three methods used for detection of leukocytospermia in semen sample, Direct counting of round cells, Immunocytochemistry and peroxidase staining (Agarwal, Gupta, and Sharma 2016; Sandoval et al. 2013). Direct counting gives false positive results as its inaccurate to distinguish the white blood cells from immature germ cells (Riedel 1980), or by expensive method like flocytometry using specific antibody(Ricci et al. 2000) or by peroxidase staining (Agarwal et al. 2016).

NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome is a complex multiprotein that mediate maturation of inflammatory cytokines (interleukin 1ß and interleukin 18)(Elliott and Sutterwala 2015). NLRP3 inflammasome activation and its released cytokines have been shown to participate directly in inflammatory process of infectious diseases as a result of toll like receptor recognition of pathogen associated molecular patterns. Inflammasome also participate in several autoimmune diseases, Alzheimer's dementia, and others, which are well-reviewed elsewhere (Guo, Callaway, and Ting 2015).

It has shown that elevated inflammasome related cytokines in infertile men with spinal cord injury and none spinal cord injury were associated with bad seminal fluid analysis affecting sperm motility(Hayrabedyan et al. 2016; Ibrahim et al. 2013; Jurewicz et al. 2014; Travis et al. 2016). Furthermore, inflammasome activation and its associated cytokines could affect spermatogenesis and Sertoli cell function via induction of reactive oxygen species (ROS) induced sperm cell damage during or before ejaculation (Jiang et al. 2016; Omu et al. 1999).

The NLRP3 protein in seminal plasma may be associated with the increased leukocytospermia, bad sperm quality among infertile male and may interfere with pregnancy outcome after different methods of assisted reproductive technologies using intra-uterine insemination or in vitro fertilization or intra-cytoplasmic insemination.

This study aims to measure NLRP3 protein in seminal plasma in relation with grade of leukocytospermia and semen fluid analysis among infertile male. Determine the validity of NLRP3 immunochromotography assay as qualitative test as compared with ELISA method in seminal plasma.

DOI: http://doi.org/10.24086/biohs2022/paper.757

II. Materials and Methods

A. Infertile patients and sample processing:

The study included fifty infertile men attending the High Institute of Infertility Diagnosis and Assisted Reproductive Technology / Al-Nahrain University period from December 2016 till March 2017.

Seminal fluid analysis done according to WHO criteria 2010, grades of leukocytospermia were evaluated by using Endtz test according to standard method(Shekarriz et al. 1995).

Seminal plasma was separated by centrifugation of liquified semen at 1000 × g for 20 minutes then aliquots were made and stored at -50°C until assay.

B. Preparation of standards and controls

Rabbit anti-NLRP3 polyclonal antibody (orb156462) NLRP3 protein (orb374803) and Goat anti Rabbit IgG antibody (orb98806) were obtained from Biorbyt, UK.

The polyclonal antibody was conjugated of 40nm gold nanoparticle (Gold-in-a-Box kit, BioAssay Works [Ijamsville, MD, USA]) and fixed on conjugate pad, while capture antibody and anti-isotype antibody were fixed on nitrocellulose membrane as a test line and control lines respectively.

A serial dilution of the NLRP3 protein were performed as 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.781, 0.390 and 0.19 ng/ml prepared from NLRP3 protein (orb374803) Biorbyt UK. These standards were prepared and tested three times, in addition to NLRP3 free distilled water and bovine serum albumin as quality controls for the test.

C. Gold nanoparticle-antibody conjugation protocol:

After shaking of gold solution, 0.5 ml was added into each ten 1.5ml tubes. Fifteen microliters of anti-NLRP3 antibody (equivalent to 15 µg) added to each tube containing gold nanoparticle, vortexed at low speed mixed thoroughly for 2 - 3 seconds. Then allowed to stand (continue the conjugation process) for a total of 30 minutes. A slight purple or no change in color tube indicates sufficient gold nanoparticle – antibody conjugation. Blocking of gold nanoparticles achieved by adding Fifty microliters of Bovine Serum Albumin to each tube for (for prevention of binding with other molecules).

D. Reaction strip and cassette assembly:

The preparation of cassette parts (sample pad, nitrocellulose membrane, and conjugation pad) were done as previously described (Huang 2006) with modifications. Cellulose fiber (sample pad) was treated blocking buffer and then dried overnight at 37 °C. After blocking of the nitrocellulose membrane with phosphate buffered saline (PBS) buffer, anti-NLRP3 and goat anti-RABBIT IgG antibodies were doted by Hamilton syringe as a test and control lines with repeating dispenser consistently dispenses 0.5µl in each spot. The conjugation pad (glass fiber) was blocked with blocking buffer then, the membrane and conjugation pad were dried at 37 °C for 4 hours. Then 0.5 µL of prepared gold nanoparticle antibody conjugate was applied to a conjugate pad and completely dried at 37 °C for 3 h. the lateral flow strips were assembled on thick adhesive tape and cut in 0.6 cm width and inserted into a plastic cassette.

E. NLRP3 Lateral Flow Assay Procedure:

One hundred microliters from each of normal saline (negative control), NLRP3 Standards protein (positive control) at different concentrations (100, 50, 25, 12.5, 6.25 and 3.125 ng/ml) were applied to the sample pad to determine the sensitivity and specificity of ICT test. 100 µl of seminal plasma were diluted with equal volume of phosphate buffered saline (pH=7.4), then applied on the sample pad. Up to 30 minutes later reaction will be developed and interpreted.

Test strip sensitivity was assessed by different concentrations of positive control. Specificity was assessed by applying NLRP3 free serum (Bovine serum albumin) and normal saline to the sample pad.

F. Human NACHT, LRR and PYD domains-containing protein 3 (NLRP3) ELISA:

This sandwich ELISA kit was purchased from Abbexa company UK (abx516996) for quantitative detection of human NLRP3 with detection range is 0.156 ng/ml - 10 ng/ml with Sensitivity: 0.156 ng/ml. the measurement of plasma NLRP3 were done according to manufacturer instruction and optical density read at 450 channel. Linear standard curve was generated, NLRP3 concentration calculated according to the equation of curve.

G. Statistical analysis:

Data obtained from this study analyzed with statistical package for social sciences (SPSS) version 21. Data described as mean and standard deviation (SD). Validity and predictability of developed NLRP3 ICT assay was assessed in relation to gold standard test peroxidase by calculating sensitivity, specificity, predictive value of positive and negative test results. Correlation regression were calculated to determine the correlation among variables(Matthews and Farewell 2015).

III. Results

The mean age of infertile was 29.44 years old and their seminal fluid analysis summarized in table 1. Among them, 19 (38%) have leukocytospermia more than 1 million/ml with mean 2.16±5.41 leukocyte/ml.

Table 1: Patients demographic and laboratory results.

		Total (n=50)
Age (year)		29.44±5.00
Volume (ml)		2.55±0.65
pH		7.36±0.06
Sperm Concentration (million/ml)		39.26±23.92
Motility (%)	Grade A	8.80±11.05

DOI: http://doi.org/10.24086/biohs2022/paper.757

	Grade B	15.60±9.24
	Grade C	18.40±12.91
	Grade D	57.60±25.92
Morphology (%)		20.48±5.67
Round cell (%)		10.52±7.25
NLRP3 RADT Positive (%)		30 (60)
NLRP3 ELISA (ng/ml)		5.12±1.85
Leukocyte count (million/ml)		2.16±5.41

Data presented as mean± standard deviation.
Comparison made by independent sample t-test.

A. Analytical sensitivity and specificity of lateral flow test:

Upon optimization of NLRP3 antibody and its protein, the consistent results were obtained and the developed kit reported to be sensitive at the level of 5 ng/ml and none reactivity to NLRP3 free serum.

Fig. 1: results of standards in lateral flow test.

B. Accuracy of NLRP3 rapid antigen detection test:

The accuracy of the lateral flow assay was determined by a recovery test. The quantitative measuring of NLRP3 concentration in seminal plasma using ELISA kit was determined for each sample and standards of the kit, then the minimal detection limit of developed kit was 5 ng/ml was proposed as a cut off value. The results in table 2 showed an excellent sensitivity 92.86% and very good specificity 81.82% with positive predictive value 86.67% and negative predicative value 90%.

Table 2: Accuracy results of rapid NLRP3 antigen detection test.

NLRP3		ELISA	
		Positive (≥5 ng/ml)	Negative (<5 ng/ml)
RADT	Positive	26	4
	Negative	2	18
Total		28	22
Effect size		Value	
Sensitivity		92.86 (77.35-98.73)	
Specificity		81.82 (61.48-92.69)	
Positive Predictive Value		86.67 (70-32-94.69)	
Negative Predictive Value		90 (69.9-98.22)	
Likelihood Ratio		5.107	

C. Correlation of sample analysis using different methods:

A total of 50 infertile patients' semen samples was analyzed for indication of leukocytospermia using Endtz test, and seminal plasma NLRP3 protein using quantitative sandwich ELISA assay and the newly developed kit. The results of correlation analysis were presented in table 3. A positive correlation were obtained between RADT assay and both quantitative and qualitative NLRP3 ELISA test (r=0.578 and 0.510) respectively and leukocyte count and grade of leukocytospermia (0.357 and 0.384) respectively.

The results in table 4 showed that positive RADT and quantitative NLRP3 ELISA test were negatively correlated with Sperm concentration, grade B and C and positively correlated with grade D sperm motility indicating that both leukocytes and the presence of NLRP3 have adverse effect on seminal fluid analysis by reducing sperm motility, grade B and C sperm motility and higher rate of grade D motile sperm. Round cells count was positively related count and grade leucocyte indicating that higher rate of false positive round cell count.

Table 4: correlation among different methods and change in sperm motility after in vitro sperm activation.

			RADT	ELISA (ng/ml)
Volume		r	-0.159	-0.108
		p	0.27	0.454
Ph		r	-0.152	-0.076
		p	0.293	0.601
Concentration		r	-0.353*	-0.359*
		p	0.012	0.010
Change in sperm motility (%)	Grade A	r	-0.108	-0.226
		p	0.454	0.115
	Grade B	r	-0.348*	-0.346*
		p	0.013	0.014
	Grade C	r	-0.326*	-0.304*
		p	0.021	0.032
	Grade D	r	0.313*	0.357*
		p	0.027	0.011
Morphology		r	-0.185	-0.167
		p	0.199	0.248
Round cell		r	-0.043	0.037
		p	0.765	0.797
Endtz (million/ml)		r	0.357*	0.203
		p	0.011	0.157
leukocyte grade		r	0.384**	0.343*
		p	0.006	0.015
ELISA (cutoff 5ng/ml)		r	0.510**	0.597**
		p	≤0.001	≤0.001

DOI: http://doi.org/10.24086/biohs2022/paper.757

IV. Discussion

The association between inflammation of the male reproductive system has subsequently resulted in development of infertility. It's more likely described as cause and effect relationship. In particular, the quality of seminal fluid analysis could be reduced in case of inflammatory processes through impairment of secretory capacity of sexual glands, obstruction of the sperm, increased pro-inflammatory mediators, increased oxidative stress and dysregulated spermatogenesis(La Vignera et al. 2011, 2013). To the best of our knowledge, this is the first study that determine the seminal plasma level of NLRP3 protein among infertile men using two different methods.

The first one is quantitative method performed by Enzyme linked immunosorbent assay (ELISA), the study reported that the mean NLRP3 5.12±1.18 ng/ml and this might reflect an inflammatory state among infertile men. Based on the results of improvement in *in* vitro sperm activation the results showed that 36 infertile patients (72%) were improved in sperm motility and only 14 (28%) were not improved. Furthermore, based on this classification NLRP3 seminal plasma level found to be higher among patients whom not improved in their sperm motility than that improved ones 6.54±2.31 versus 3.77±1.31 respectively. In this study, the results of receiver operating curve showed that 4.5 (ng/ml) as a cutoff value discriminating those whom can improve their sperm motility or not and that 22 (44%) of the patients were positive with equal or more than 4.5 ng/ml and the remainder were negative 28 (56%).

The second one is qualitative immunochromatography method, the current developed kit is sensitive at the level of 5 ng/ml (according to reference standards). Clinically, 30 (60%) were positive and 20 (40%) considered as negative. Furthermore, positive infertile subjects have lower seminal fluid parameters including sperm concentration, grade B and grade C motility (Table 4.4), furthermore, the positive NLRP3 cases haven't ability to improve their sperm motility after *in vitro* activation (odd ratio=14.53).

This study highlighted the risk of increasing NLRP3 protein in seminal plasma was 8.33 much more likely to get un improved sperm motility after in vitro activation (Table 4.6). Basically, a lot off researchers highlighted the impact of inflammation on the seminal fluid analysis parameters (reviewed by (Azenabor, Ekun, and Akinloye 2015)). Several parameters have been applied as indicators for semen inflammation reviewed by La Vignera, et al., 2013. They depend on enzymes(Atig et al. 2012) and cytokines(Havrylyuk et al. 2015) that participate in inflammatory process of male genetalia(La Vignera et al. 2013).

Group of researchers highlighted the importance of inflammasome protein NLRP3 as a "physiological low level of inflammation" for fertility(Hayrabedyan et al. 2016). It had been suggested that low level of NLRP3 is essential for Sertoli cell production of low levels of IL1B to maintain IL1 alpha (Fettelschoss et al. 2011) but higher rate of inflammasome expression will results in higher level of inflammasome activation cytokines such as IL-1B and IL-18(Martinon, Burns, and Tschopp 2002) and served as a proinflammatory signals generating an inflammatory process in the testis such as leucocyte infiltration and cell death signaling and caspase 1 activation(Abal 2017; Inoue et al. 2012; Minutoli et al. 2015). Also, it might activate ASC adaptor protein in both sperm cell and leucocytes in semen resulting in inflammatory cell death(Miao, Rajan, and Aderem 2011).

The recent studies have been focused on inflammasome research (NLRP3 or its related cytokines), due to its direct PAMPs-TLR recognition and subsequent activation of inflammation in monocytes, macrophages, granulocytes, dendritic cells, epithelial cells and osteoblasts(Feldmann et al. 2002). NLRP3 expression in myeloid cells is highly inducible(Guarda et al. 2011) and other epithelial and Sertoli cells(Hayrabedyan et al. 2016). So, the leukocyte counting is not always giving a true inflammatory state, because of wide range of cells have the ability to respond and considered as a source of inflammatory mediators. This gives an advantage of measurement of NLRP3 as a biomarker for inflammatory processes and its targeting is crucial for improvement in sperm cell quantity and quality in infertile men(Ibrahim et al. 2014).

The developed NLRP3 ICT kit provides a direct qualitative detection of NLRP3 protein in seminal plasma. So, the detection of NLRP3 is not restricted by one cell type like: monocytes or other inflammatory cell and none inflammatory cells. Thus, it's concentration represents the true activation of innate immunity in male genitalia. Here in this study, the diagnostic performance of rapid antigen detection could vary across patients, this referred to as spectrum effect (spectrum bias)(Mulherin and Miller 2002; Willis 2008)including amount of sample on swab; expert and training of sample collectors; and room temperature (Cohen et al. 2012).

Conclusion

The developed kit is user friendly, simple to use and interpret, no antigen extraction procedure is needed. The sample (seminal plasma) was diluted 1:1 with buffer in order to dilute the sample and reducing its viscosity. The reduction of viscosity will facilitate sample absorption and flow across membranes.

Appendix

Appendixes, if needed, appear before the acknowledgment.

Acknowledgment

Authors greatly acknowledge Dr. Kh. Kadhem Hussain for his assistance in facilitating works and sample collections.

References

Abal, Miguel. 2017. "Characterizing the Contribution of Inflammasome-Derived Exosomes in the Activation of the Immune Response." *Annals of Translational Medicine* 5(7):172–172.

Agarwal, Ashok, Cleveland Clinic, and Ashok Agarwal. 2014. "The Role of Leukocytospermia in Male Infertility in Male Infertility." (January).

Agarwal, Ashok, Sajal Gupta, and Rakesh Sharma. 2016. "Leukocytospermia Quantitation (ENDTZ) Test." Pp. 69–72 in *Andrological Evaluation of Male Infertility*. Cham: Springer International Publishing.

Aggarwal, Richa, Manju Puri, Rima Dada, and Gyan Saurabh. 2015. "Correlation Between Leukocytospermia and Oxidative Stress in Male Partners of Infertile Couples With Leukocytospermia." *International Journal of Reproduction, Contraception, Obstetrics and Gynecology* 4(1):1.

Atig, Fatma, Monia Raffa, Ben Ali Habib, Abdelhamid Kerkeni, Ali Saad, and Mounir Ajina. 2012. "Impact of Seminal Trace Element and Glutathione Levels on Semen Quality of Tunisian Infertile Men." *BMC Urology* 12:2–9.

Azenabor, Alfred, Ayodele Oloruntoba Ekun, and Oluyemi Akinloye. 2015. "Impact of Inflammation on Male Reproductive Tract." *Journal of Reproduction and Infertility* 16(3):123–29.

Aziz, Nabil, Ashok Agarwal, Iwan Lewis-Jones, Rakesh K. Sharma, and Anthony J. Thomas. 2004. "Novel Associations between Specific Sperm Morphological Defects and Leukocytospermia." *Fertility and Sterility* 82(3):621–27.

Cohen, Jérémie F., Martin Chalumeau, Corinne Levy, Philippe Bidet, Franck Thollot, Alain Wollner, Edouard Bingen, and Robert Cohen. 2012. "Spectrum and Inoculum Size Effect of a Rapid Antigen Detection Test for Group A Streptococcus in Children with Pharyngitis." *PloS One* 7(6):e39085.

Elliott, Eric I., and Fayyaz S. Sutterwala. 2015. "Initiation and Perpetuation of NLRP3 Inflammasome Activation and Assembly." *Immunological Reviews* 265(1):35–52.

Feldmann, Jérôme, Anne-Marie Prieur, Pierre Quartier, Patrick Berquin, Stéphanie Certain, Elisabetta Cortis, Dominique Teillac-Hamel, Alain Fischer, and Geneviève de Saint Basile. 2002. "Chronic Infantile Neurological Cutaneous and Articular Syndrome Is Caused by Mutations in CIAS1, a Gene Highly Expressed in Polymorphonuclear Cells and Chondrocytes." *The American Journal of Human Genetics* 71(1):198–203.

Fettelschoss, A., M. Kistowska, S. LeibundGut-Landmann, H. D. Beer, P. Johansen, G. Senti, E. Contassot, M. F. Bachmann, L. E. French, A. Oxenius, and T. M. Kundig. 2011. "Inflammasome Activation and IL-1 Target IL-1 for Secretion as Opposed to Surface Expression." *Proceedings of the National Academy of Sciences* 108(44):18055–60.

Guarda, G., M. Zenger, A. S. Yazdi, K. Schroder, I. Ferrero, P. Menu, A. Tardivel, C. Mattmann, and J. Tschopp. 2011. "Differential Expression of NLRP3 among Hematopoietic Cells." *The Journal of Immunology* 186(4):2529–34.

Guo, Haitao, Justin B. Callaway, and Jenny P. Y. Ting. 2015. "Inflammasomes: Mechanism of Action, Role in Disease, and Therapeutics." *Nature Medicine* 21(7):677–87.

Havrylyuk, Anna, Valentyna Chopyak, Yaryna Boyko, Iryna Kril, and Maciej Kurpisz. 2015. "Cytokines in the Blood and Semen of Infertile Patients." *Central European Journal of Immunology* 40(3):337–44.

Hayrabedyan, Soren, Krassimira Todorova, Asma Jabeen, Gergana Metodieva, Stavri Toshkov, Metodi V. Metodiev, Milcho Mincheff, and Nelson Fernández. 2016. "Sertoli Cells Have a Functional NALP3 Inflammasome That Can Modulate Autophagy and Cytokine Production." *Scientific Reports* 6(1):18896.

Huang, Su Hua. 2006. "Gold Nanoparticle-Based Immunochromatographic Test for Identification of Staphylococcus Aureus from Clinical Specimens." *Clinica Chimica Acta* 373(1–2):139–43.

Ibrahim, E., S. M. Castle, T. C. Aballa, R. W. Keane, J. P. De Rivero Vaccari, C. M. Lynne, and N. L. Brackett. 2014. "Neutralization of ASC Improves Sperm Motility in Men with Spinal Cord Injury." *Human Reproduction* 29(11):2368–73.

Ibrahim, E., X. Zhang, T. Aballa, J. P. De Rivero Vaccari, R. Keane, W. D. Dietrich, C. Lynne, and N. Brackett. 2013. "INFLAMMASOME INACTIVATION IMPROVES SPERM MOTILITY IN MEN WITH SPINAL CORD INJURY." *JURO* 189(4):e773.

Inoue, Makoto, Kristi L. Williams, Michael D. Gunn, and Mari L. Shinohara. 2012. "NLRP3 Inflammasome Induces Chemotactic Immune Cell Migration to the CNS in Experimental Autoimmune Encephalomyelitis." *Proceedings of the National Academy of Sciences* 109(26):10480–85.

Jiang, Lu, Ting Zheng, Jun Huang, Jinhua Mo, Hua Zhou, Min Liu, Xingcheng Gao, and Bolan Yu. 2016. "Association of Semen Cytokines with Reactive Oxygen Species and Histone Transition Abnormalities." *Journal of Assisted Reproduction and Genetics* 33(9):1239–46.

Jurewicz, M., E. Ibrahim, G. Attia, S. Roberge, C. Lynne, and N. Brackett. 2014. "Inflammasome Activation in Men with Abnormal Semen Quality." *Fertility and Sterility* 102(3):e9.

Lackner, Jakob E., Ashok Agarwal, Reda Mahfouz, Stefan S. du Plessis, and Georg Schatzl. 2010. "The Association between Leukocytes and Sperm Quality Is Concentration Dependent." *Reproductive Biology and Endocrinology : RB&E* 8:12.

Martinon, Fabio, Kimberly Burns, and Jürg Tschopp. 2002. "The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of ProIL-β." *Molecular Cell* 10(2):417–26.

Matthews, D. E., and V. T. Farewell. 2015. *Using and Understanding Medical Statistics*. S. Karger AG.

Miao, Edward A., Jayant V Rajan, and Alan Aderem. 2011. "Caspase-1-induced Pyroptotic Cell Death." *Immunological Reviews* 243(1):206–14.

Minutoli, Letteria, Pietro Antonuccio, Natasha Irrera, Mariagrazia Rinaldi, Alessandra Bitto, Herbert Marini, Gabriele Pizzino, Carmelo Romeo, Antonina Pisani, Giuseppe Santoro, Domenico Puzzolo, Carlo Magno, Francesco Squadrito, Antonio Micali, and Domenica Altavilla. 2015. "NLRP3 Inflammasome Involvement in the Organ Damage and Impaired Spermatogenesis Induced by Testicular Ischemia and Reperfusion in Mice." *Journal of Pharmacology and Experimental Therapeutics* 355(3):370–80.

Mulherin, Stephanie A., and William C. Miller. 2002. "Spectrum Bias or Spectrum Effect? Subgroup Variation in Diagnostic Test Evaluation." *Annals of Internal Medicine* 137(7):598–602.

Omu, Alexander E., Fawzia Al-Qattan, Fahad M. Al-Abdul-Hadi, M. Tunde Fatinikun, and Sanjit Fernandes. 1999. "Seminal Immune Response in Infertile Men with Leukocytospermia: Effect on Antioxidant Activity." *European Journal of Obstetrics Gynecology and Reproductive Biology* 86(2):195–202.

Ricci, G., G. Presani, S. Guaschino, R. Simeone, and S. Perticarari. 2000. "Leukocyte Detection in Human Semen Using Flow Cytometry." *Human Reproduction (Oxford, England)* 15(6):1329–37.

Riedel, H. 1980. "Archives of Andrology Techniques for the Detection of Leukocytospermia in Human Semen in Human Semen." *Archives of Andrology* 5(3):287–93.

Sandoval, Juan Sebastian, Doug Raburn, and Suheil Muasher. 2013. "Leukocytospermia: Overview of Diagnosis, Implications, and Management of a Controversial Finding." *Middle East Fertility Society Journal* 18(3):129–34.

Shekarriz, M., R. K. Sharma, A. J. Thomas, and A. Agarwal. 1995. "Positive Myeloperoxidase Staining (Endtz Test) as an Indicator of Excessive Reactive Oxygen Species Formation in Semen." *Journal of Assisted Reproduction and Genetics* 12(2):70–74.

Travis, Alexander, Cristina Cardona, Melissa Moody, Alana Simpson, and G. Charles Ostermeier. 2016. "INDICATORS OF INFLAMMASOME ACTIVATION IN MEN WITH ABNORMAL SEMEN PARAMETERS." *The Journal of Urology* 195(4):e909.

La Vignera, S., E. Vicari, R. A. Condorelli, R. D'Agata, and A. E. Calogero. 2011. "Male Accessory Gland Infection and Sperm Parameters (Review)." *International Journal of Andrology* 34(5 PART 2).

La Vignera, Sandro, Rosita A. Condorelli, Enzo Vicari, Dario Tumino,

DOI: http://doi.org/10.24086/biohs2022/paper.757

Giuseppe Morgia, Vincenzo Favilla, Sebastiano Cimino, and Aldo E. Calogero. 2013. "Markers of Semen Inflammation: Supplementary Semen Analysis?" *Journal of Reproductive Immunology* 100(1):2–10.

Willis, Brian H. 2008. "Spectrum Bias - Why Clinicians Need to Be Cautious When Applying Diagnostic Test Studies." *Family Practice* 25(5):390–96.

World Health Organization. 2010. *Examination and Processing of Human Semen*. Vol. Edition, V.

DOI: http://doi.org/10.24086/biohs2022/paper.757