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Full Length Research Paper

## Evaluation of Serum Levels of Pro-inflammatory Cytokines (interleukins 2, 6, 8) in Fertile and Infertile Men

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*The concentration of interleukin (IL) -2, IL-6 and IL-8 were determined in human sera from fertile and infertile males with various types of infertility; Azoospermia, Oligozoospermia, Asthenozoospermia and Teratozoospermia in AL-Najaf AL-Ashraf governorate during March 2013 to August 2013. Oligozoospermia was predominant in the age group 19-29yrs (48)men, while azoospermia recorded the lowest number (6) men in group >40 yrs. Oligozoospermia had the highest record number of 117 men (36%) followed by asthenozoospermia 97 men (30%), teratozoospermia 73 men (22%) and azoospermia 41 men (12%) with statistical differences between various types of infertility  $P<0.05$ . The mean level of concentration of interleukin -2 elevated in serum of infertile men reached 159.63 pg/ml in comparison with fertile men (control) which reached 24.35 pg/ml. The mean of concentration of interleukin -6 elevated in the serum of infertile men reached 328.75 pg/ml in comparison with fertile men which reached 116.24 pg/ml. The mean level of concentration of interleukin -8 elevated in the serum of infertile men reached 575.68 pg/ml in comparison with fertile men which reached 141.51 pg/ml. The means of interleukins concentration among various types of infertility of infertile men and control (fertile men) were evaluated in the present study. High differences were found among the interleukin concentrations, the various types of infertility  $P<0.001$  and the concentration of interleukin in control the group  $P<0.01$ .*

**Keywords:** Cytokines, interleukins, fertile, infertile men.

### INTRODUCTION

Cytokines play an important role in intercellular communication. They are involved in numerous physiological and pathological processes, particularly in the mediation of inflammatory responses,<sup>1</sup> and have important functions in the reproductive physiology of women and men. Apart from their role in immune modulation, there is evidence that some of these polypeptides are directly involved in the regulation of testicular function, and also may be potent modulators of steroid release from the testes.<sup>2</sup> There are complex mechanisms for control of their action. Human semen contains a repertoire of cytokines whose effects on semen quality and sperm function, however, are subject to debate. Inflammatory

cytokines are produced by white blood cells (WBC), mainly by macrophages, in response to foreign antigens, pathogens (infection challenge) and also in chronic inflammation (immunological activation).<sup>3,4,5</sup> Acute and chronic infections may play a contributory role in male infertility. The clinical relevance of silent infection in asymptomatic patients is, however, not clear.<sup>6</sup> Moreover, the interpretation of the markers commonly used for its diagnosis is controversial; for example the role of seminal leukocytes and clinically significant thresholds.<sup>7</sup> Positive semen cultures, a frequent finding during extended infertility investigation in asymptomatic men, and with bacterial prevalence depending on the extent of the microbial

screening, are insufficient to diagnose male genital tract infection. The reproductive tracts of both men and women contain a myriad of immune response cells.<sup>8</sup> Activation of these cells, for example by microorganisms, stimulates them to secrete lymphokines and monokines.<sup>2,9</sup> These act, at least in part, locally to regulate immunological reactions, but also affect tissues outside the immune system. Interleukin (IL) -8 is a potent neutrophil chemotactic and activating factor. It is involved in angiogenesis and adhesion processes, and enhances the adherence of neutrophils to endothelial cells and subendothelial matrix proteins. It exerts its biological activities by binding to specific cell surface receptors.<sup>4,6,10</sup> IL-8 may be involved, within a network of other cytokines, in intratesticular signal transduction, and may also adversely affect sperm membrane properties.<sup>11</sup> Few investigations have been made concerning this cytokine with regard to reproduction. Interleukins are part of the local defence mechanism against infectious diseases, but they are also implicated as mediators of the pathology of these diseases.<sup>12,13,14,15</sup> IL-8 concentrations may fluctuate during certain pathological conditions.<sup>16</sup> There is little information about IL-8 interference with sperm production and semen quality, and whether it increases in parallel to leukocyte counts and other potential markers of subclinical infection/inflammation in the same ejaculates, potentially interrelated with other cytokines, e.g. IL-6. Also unknown is whether potentially pathogenic microorganisms in the semen of patients, who are without symptoms of genital tract infection, are associated with increased pro-inflammatory seminal cytokine concentrations, which might impair sperm functional capacity.<sup>17,18,19</sup> Therefore, in the present prospective study, the concentrations of IL-8, and additionally IL-6, were determined in the seminal plasma of a group of randomly chosen asymptomatic, subfertile males.<sup>11,20</sup> Cytokine concentrations were related to the multiple determinants of semen quality, for example the outcome of microscopic sperm analysis, local antisperm antibodies (Ab) to the evaluation of sperm function using different approaches, to seminal WBC and other potential markers of silent inflammation, and to the results of a broad microbial screening that also included patients' female partners.<sup>5,13,16,21</sup> During genital infection cytokines and various soluble receptors of immunoregulatory cytokines are expressed distinctly in seminal plasma.<sup>22</sup> These factors also may be involved in the regulation of sperm cell functions and thus may affect male fertility. Measuring the level of cytokines, both in seminal plasma and serum, does not only expand the diagnostic options, but also through the growing knowledge of immune processes, can give rise to new therapeutic methods of improving the quality of semen and increasing the chance to reproduce. In addition, some of these factors may affect physiologic events underlying male reproductive function. For example, elevated seminal plasma concentrations of several cytokines, including interleukin-1 (IL-1), IL-2, IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), have been associated with poor semen quality and male infertility.<sup>3,10,23,24,25</sup>

Interleukin 2 (IL-2) is an interleukin, a type of cytokine signaling molecule in the immune system. It is a protein that regulates the activities of white blood cells (leukocytes, often lymphocytes) that are responsible for immunity.<sup>26</sup> IL-2 is part of the body's natural response to microbial infection, and in discriminating between foreign ("non-self") and "self". IL-2 mediates its effects by binding to IL-2 receptors, which are expressed by lymphocytes. IL-2 is necessary for the growth, proliferation, and differentiation of thymic-derived lymphocytes (T cells) to become 'effector' T cells.<sup>27</sup> IL-2 is normally

produced by T cells during an immune response. Antigen binding to the T cell receptor (TCR) stimulates the secretion of IL-2, and the expression of IL-2 receptors IL-2R. The IL-2/IL-2R interaction then stimulates the growth, differentiation and survival of antigen-specific CD4+ T cells and CD8+ T cells.<sup>28</sup> As such, IL-2 is necessary for the development of T cell immunologic memory, which depends upon the expansion of the number and function of antigen-selected T cell clones. IL-2 is also necessary during T cell development in the thymus for the maturation of a subset of T cells that are termed regulatory T cells (T-regs).<sup>29</sup> After exiting from the thymus, T-Regs function to prevent other T cells from recognizing and reacting against self antigens, which could result in autoimmunity. T-Regs do so by preventing the responding cells from producing IL-2. Also, because T-Reg cells constitutively express IL-2 receptors, they bind, internalize, and degrade IL-2, thereby depriving neighboring effector T cells of IL-2. Thus, IL-2 is required to discriminate between self and non-self, one of the other hallmarks of the immune system.<sup>22,26</sup>

Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. In humans, it is encoded by the *IL6* gene. IL-6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation.<sup>30</sup> IL-6 also plays a role in fighting infection, as IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae*. In addition, osteoblasts secrete IL-6 to stimulate osteoclast formation. Smooth muscle cells in the tunica media of many blood vessels also produce IL-6 as a pro-inflammatory cytokine.<sup>31</sup> IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF- $\alpha$  and IL-1, and activation of IL-1ra and IL-10. IL-6 is an important mediator of fever and of the acute phase response.<sup>18,32</sup> It is capable of crossing the blood-brain barrier and initiating synthesis of PGE<sub>2</sub> in the hypothalamus, thereby changing the body's temperature setpoint.<sup>33</sup> In muscle and fatty tissue, IL-6 stimulates energy mobilization that leads to increased body temperature. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs bind to an important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs).<sup>34,35</sup> IL-6 is responsible for stimulating acute phase protein synthesis, as well as the production of neutrophils in the bone marrow. It supports the growth of B cells and is antagonistic to regulatory T cells.<sup>36,37,38</sup>

Interleukin 8 (IL-8) is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells.<sup>39</sup> Endothelial cells store IL-8 in their storage vesicles, the Weibel-Palade bodies.<sup>40,41</sup> In humans, the interleukin-8 protein is encoded by the *IL8* gene. IL-8, also known as a *neutrophil chemotactic factor*, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection.<sup>42</sup> IL-8 also induces phagocytosis once they have arrived. IL-8 is also known to be a potent promoter of angiogenesis.<sup>43</sup> In target cells, IL-8 induces a series of physiological responses required for migration and phagocytosis, such as increases in intracellular Ca<sup>2+</sup>, exocytosis (e.g. histamine release), and the respiratory burst. IL-8 can be secreted by any cells with toll-like receptors that are involved in the innate immune response.<sup>44,45,46</sup> Usually, it is the macrophages that see an

antigen first, and thus are the first cells to release IL-8 to recruit other cells.<sup>47</sup> Both monomer and homodimer forms of IL-8 have been reported to be potent inducers of the chemokine receptors CXCR1 and CXCR2. The homodimer is more potent, but methylation of Leu25 can block the activity of homodimers.<sup>48,49</sup> The purpose of this study was to document the prevalence and concentration of interleukins 2, 6 and 8 in fertile and infertile men, to provide further insight into mediators of immune defence and reproductive function in the normal male genital tract, and to establish reference values to support future studies on the role of these factors in pathologic conditions.

## PATIENTS AND METHODS

### Patients (Infertile men)

A total of 328 Seminal fluid and 65 serum samples were collected from patients (males) aged between 19-60 yrs who were attended to at the fertility center / AL-Sadder medical City in AL-Najaf AL-Ashraf governorate from March 2013 to August 2013. None of the patients had clinical signs of genital tract infection apart from their infertility problem, and were therefore considered asymptomatic of sexually transmitted disease, they were healthy individuals. During the time of the study, none of the patients was treated with antibiotics, corticosteroids or antiphlogistics.

\*All above samples collected through MSc study of Kais Khudhair A. ALhadrawi Submitted to the Council of Faculty of Education for girls- University of Kufa, 2013<sup>50</sup> with approval of the ethical committee under supervision of specialists.

### Fertile Controls

Semen and serum samples were analysed from 20 fertile men attended at the same above center. The control group is similar in age and demographic characters of the patients. Seminal fluid analysis and classification of infertility types into Azoospermia, Oligozoosperm, Asthenozoospermia and Teratozoospermia in comparison with normal fertility status (control) Normospermia were done according to (WHO, 1999)<sup>51</sup>. Detection and quantitation of the various cytokines (interleukins 2,6 and 8) were done for both fertile and infertile men and accomplished using commercially available enzyme-linked immunosorbent assay (ELISA) kits or the Boster Immunoleader, BOSTER BIOLOGICAL TECHNOLOGY Co., Ltd. Fremont, CA 94538, USA (www.bosterbio.com) following the manufacturers' protocols as follows.

### IL-2 Reagent Preparation and Storage

#### A. Reconstitution of the human IL-2 standard

IL-2 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of IL-2 standard (10ng per tube) are included in each kit. Use one tube for each experiment.

a. 10,000pg/ml of human IL-2 standard solution: Add 1ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.

b. 1000pg/ml of human IL-2 standard solution: Add 0.1ml of the above 10ng/ml IL-2 standard solution into 0.9ml sample diluent buffer and mix thoroughly.

c. 500pg/ml→15.6pg/ml of human IL-2 standard solutions: Label 6 Eppendorf tubes with 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 1000pg/ml IL-2 standard solution into the 1st tube and mix. Transfer 0.3ml from 1st tube to 2nd tube and mix. Transfer 0.3ml from 2nd tube to 3rd tube and mix, and so on.

**Note:** The standard solutions are best used within 2 hours. The 10ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

#### B. Preparation of biotinylated anti-human IL-2 antibody working solution

The solution should be prepared no more than 2 hours prior to the experiment.

a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)

b. Biotinylated anti-human IL-2 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human IL-2 antibody to 99µl antibody diluent buffer.)

#### C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution

The solution should be prepared no more than 1 hour prior to the experiment.

a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)

b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluent buffer.)

### Assay Procedure

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. The standard IL-2 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of IL-2 amount in samples.

1. Aliquot 0.1ml per well of the 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml human IL-2 standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of human cell culture supernates, serum or plasma (heparin, EDTA, citrate) to each empty well. It is recommended that each human IL-2 standard solution and each sample be measured in duplicate.

2. Seal the plate with the cover and incubate at 37°C for 90 min.

3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.

4. Add 0.1ml of biotinylated anti-human IL-2 antibody working solution into each well and incubate the plate at 37°C for 60 min.

5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let the washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. Plate Washing Method: Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)

6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.

7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let the washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).

8. Add 90µl of prepared TMB color developing agent into each well and incubate plate at 37°C in the dark for 25-30 min

**(Note:** For reference only, the optimal incubation time should be determined by the end user. And the shades of blue can be seen in the wells with the four most concentrated human IL-2 standard solutions; the other wells show no obvious color).

9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.

10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The human IL-2 concentration of the samples can be interpolated from the standard curve.

**Note:** If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

## IL-6 Reagent Preparation and Storage

### A. Reconstitution of the human IL-6 standard

IL-6 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of IL-6 standard (10ng per tube) are included in each kit. Use one tube for each experiment.

a. 10,000pg/ml of human IL-6 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.

b. 300pg/ml of human IL-6 standard solution: Add 0.03 ml of the above 10ng/ml IL-6 standard solution into 0.97 ml sample diluent buffer and mix thoroughly.

c. 150pg/ml→4.69pg/ml of human IL-6 standard solutions: Label 6 Eppendorf tubes with 150pg/ml, 75pg/ml, 37.5pg/ml,

18.75pg/ml, 9.38pg/ml, 4.69pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 300pg/ml IL-6 standard solution into the 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

**Note:** The standard solutions are best used within 2 hours. The 10ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

### B. Preparation of biotinylated anti-human IL-6 antibody working solution

The solution should be prepared no more than 2 hours prior to the experiment.

a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)

b. Biotinylated anti-human IL-6 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human IL-6 antibody to 99µl antibody diluent buffer.)

### C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution

The solution should be prepared no more than 1 hour prior to the experiment.

a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)

b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluent buffer.)

## Assay Procedure

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. The standard IL-6 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of IL-6 amount in samples.

1. Aliquot 0.1ml per well of the 300pg/ml, 150pg/ml, 75pg/ml, 37.5pg/ml, 18.75pg/ml, 9.38pg/ml, 4.69pg/ml human IL-6 standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of human cell culture supernates, serum or plasma (heparin, EDTA, citrate) to each empty well. We recommend that each human IL-6 standard solution and each sample is measured in duplicate.

2. Seal the plate with the cover and incubate at 37°C for 90 min.

3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.

4. Add 0.1ml of biotinylated anti-human IL-6 antibody working solution into each well and incubate the plate at 37°C for 60 min.

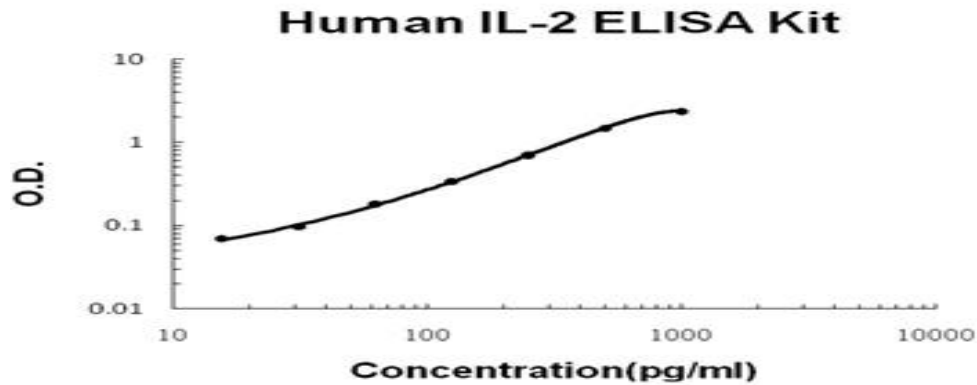


Figure 1: Standard curve of interleukin-2

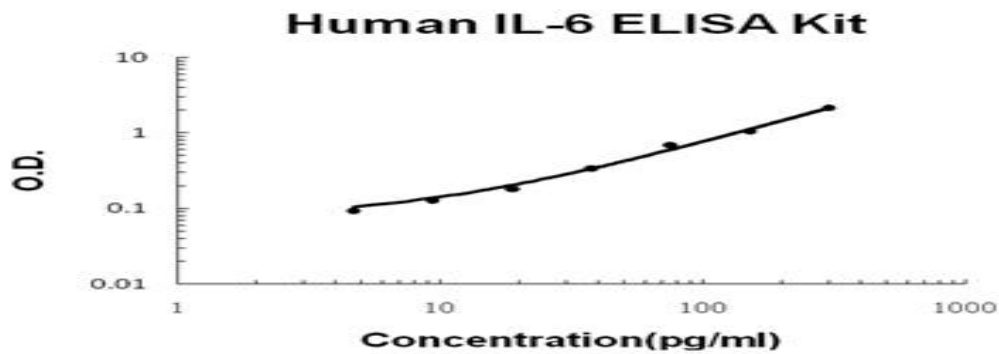


Figure 2: standard curve of interleukin-6

5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let the washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. Plate Washing Method: Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)

6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.

7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let the washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels

or other absorbent material. (See Step 5 for plate washing method).

8. Add 90µl of prepared TMB color developing agent into each well and incubate plate at 37°C in the dark for 25-30 min

**(Note:** For reference only, the optimal incubation time should be determined by the end user. And the shades of blue can be seen in the wells with the four most concentrated human IL-6 standard solutions; the other wells show no obvious color).

9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.

10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be

plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The human IL-6 concentration of the samples can be interpolated from the standard curve. **Note:** If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution (Figure 2).

## IL-8 Reagent Preparation and Storage

### A. Reconstitution of the human IL-8 standard

IL-8 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of IL-8 standard (10ng per tube) are included in each kit. Use one tube for each experiment.

a. 10,000pg/ml of human IL-8 standard solution: Add 1ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.

b. 1000pg/ml of human IL-8 standard solution: Add 0.1ml of the above 10ng/ml IL-8 standard solution into 0.9ml sample diluent buffer and mix thoroughly.

c. 500pg/ml→15.6pg/ml of human IL-8 standard solutions: Label 6 Eppendorf tubes with 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 1000pg/ml IL-8 standard solution into the 1st tube and mix. Transfer 0.3ml from 1st tube to 2nd tube and mix. Transfer 0.3ml from 2nd tube to 3rd tube and mix, and so on.

**Note:** The standard solutions are best used within 2 hours. The 10ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

### B. Preparation of biotinylated anti-human IL-8 antibody working solution

The solution should be prepared no more than 2 hours prior to the experiment.

a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)

b. Biotinylated anti-human IL-8 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human IL-8 antibody to 99µl antibody diluent buffer.)

### C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution

The solution should be prepared no more than 1 hour prior to the experiment.

a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)

b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluent buffer.)

## Assay Procedure

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard IL-8 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of IL-8 amount in samples.

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2. Seal the plate with the cover and incubate at 37°C for 90 min.

3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.

4. Add 0.1ml of biotinylated anti-human IL-8 antibody working solution into each well and incubate the plate at 37°C for 60 min.

5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. Plate Washing Method: Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)

6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.

7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let the washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).

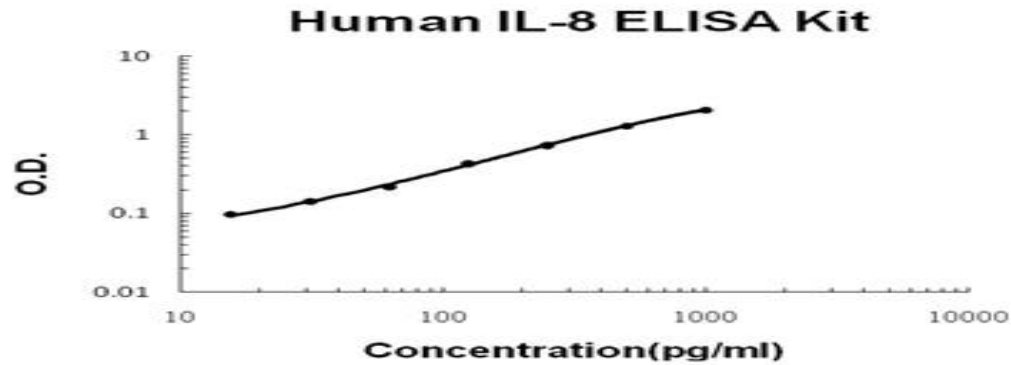
8. Add 90µl of prepared TMB color developing agent into each well and incubate plate at 37°C in the dark for 25-30 min

**(Note:** For reference only, the optimal incubation time should be determined by the end user. And the shades of blue can be seen in the wells with the four most concentrated human IL-8 standard solutions; the other wells show no obvious color).

9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.

10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X).



**Figure 3:** Standard curve of interleukin-8

**Table-1:** Numbers and percentages of studied infertile males and infertility types according to age stages.

Total	Azoo spermia	Teratozoospermia	Asthenozoospermia	Oligozoospermia	Age (Yrs)
(%44)143	21	29	45	48*	29 – 19
(%33)109	14	22	33	40	39 – 30
(%23)76	6	22	19	29	>40
(%100)328	41	73	97	117	Total

$p < 0.05$  \*

The human IL-8 concentration of the samples can be interpolated from the standard curve.

**Note:** if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution. figure 3.

## RESULT

Table.1 illustrated the numbers and percentages of the studied infertile male and infertility types according to age stages. Oligozoospermia was predominant in the age group 19-29yrs (48)men, while azoospermia recorded lowest number (6) men in group >40 yrs. There are significant differences between the various types of infertility  $p < 0.05$

Figure 4. Showed the various types of infertility. Oligozoospermia was found in the highest number of patients, 117 men (36%) followed by asthenozoospermia 97 men (30%), teratozoospermia 73 men (22%) and azoospermia 41 men (12%) with statistical differences between various types of infertility  $P < 0.05$

Mean level of concentration of interleukin -2 was elevated in serum of infertile men reached 159.63 pg/ml in comparison with fertile men (control) which reached 24.35 pg/ml, with very

highly statistical differences between two groups  $P < 0.001$ . Table 2.

The mean level of concentration of interleukin -6 was elevated in serum of infertile men reached 328.75 pg/ml in comparison with fertile men (control) which reached 116.24 pg/ml, with very highly statistical differences between two groups  $P < 0.001$ . table 3.

The mean level of concentration of interleukin -8 was elevated in serum of infertile men reached 575.68 pg/ml in comparison with fertile men (control) which reached 141.51 pg/ml, with very highly statistical differences between two groups  $P < 0.001$ . table 4.

Table 5 illustrated the determination means of interleukins concentration among various types of infertility of infertile men and control (fertile men). There are very high differences between interleukin concentrations, the various types of infertility  $P < 0.001$ . and the concentration of interleukins the in control group  $P < 0.01$ .

**Table-2: Concentration of interleukin-2 among fertile and infertile men.**

Studied groups	Conc. Of IL-2 (Pg/ml) $\pm$ SD	ANOVA Test	P-value
Fertile control men	24.35 $\pm$ 6.38	68.364	0.000
Infertile men	159.63 $\pm$ 17.41		

**Table-3: concentration of interleukin-6 among fertile and infertile men.**

Studied groups	Conc. Of IL-6 (Pg/ml) $\pm$ SD	ANOVA Test	P-value
Fertile control men	116.24 $\pm$ 14.22	80.861	0.000
Infertile men	328.75 $\pm$ 36.41		

**Table-4: concentration of interleukin-8 among fertile and infertile men.**

Studied groups	Conc. Of IL-8 (Pg/ml) $\pm$ SD	ANOVA Test	P-value
Fertile control men	142.51 $\pm$ 22.34	48.398	0.000
Infertile men	575.68 $\pm$ 48.937		

**Table-5: Means of interleukins concentration among various types of infertility of infertile men and control (fertile men).**

Studied groups	Infertility types	Means of interleukin concentration Pg/ml			P-value
		IL2	IL6	IL8	
Infertile men	Oligozoospermia	160.33	356.22	612.45	0.000
	Asthenozoo spermia	165.38	358.45	598.34	0.000
	Teratozoospemia	142.82	339.87	588.57	0.000
	Azoo spermia	153.52	345.21	547.61	0.000
Fertile men normospermia		24.35	116.24	142.51	0.00



## Discussion

Proinflammatory cytokines of humoral and cellular immune defence were detectable at normal (low) levels in fertile men in comparison with high level in infertile men. These data can serve as reference values for future studies on the role of these factors in male genital tract infection and infertility. Increased concentration of cytokines, as an increased production of some pro-inflammatory cytokines has also been reported during immune responses in major depression. Genetic factors also substantially influence the production of cytokines.<sup>52</sup> Also cytokine concentrations may more accurately indicate an early phase of infection/inflammation.<sup>53</sup> Cytokines are potent polypeptides that are released from inflammatory cells as part of the host response. Many cell types, including monocytes/macrophages, T cells and neutrophils, can produce IL-8 in response to a wide variety of signals frequently initiated by infection or injury.<sup>54</sup> Cytokines play a pivotal role as a mediator of numerous physiological and pathological processes, particularly in the initiation of the immunoinflammatory cascade.<sup>55,56,57,58</sup> Cytokines are also involved in allograft rejection. An increased production of some pro-inflammatory cytokines has also been reported during immune responses in major depression.<sup>59</sup> Genetic factors also substantially influence the production of cytokines.<sup>60</sup> In addition to their function in the immune system, cytokines may have a significant role in modulating the neurocrine control of reproduction. They probably play a physiological part as local mediators of the action of sex hormones, and are involved in the paracrine regulation of spermatogenesis.<sup>61,62,63</sup> Another mechanism of interference with sperm quality may be an adverse effect on sperm membrane properties, e.g. lipid peroxidation<sup>64</sup>. Increased oxidative stress may additionally modulate the concentration of these cytokines.<sup>65</sup> However, the role of reactive oxygen species (ROS) in human semen is complex and seminal plasma scavenging systems also have to be considered. This is also in agreement with previous studies. This cytokine promotes the development of B cells and the production of IgA and may play a role in humoral immune defence of the male genital tract. Furthermore, IL-2 receptors are expressed in the germ line of human testis and in ejaculated sperm, suggesting that IL-2 may also play a role in the physiology of the human testis. It would therefore be of interest to study levels of this cytokine in semen from men with infertility.<sup>66</sup> IL-6 and IL-8 have been associated with allergic inflammation and autoimmunity.<sup>67</sup> Prominent cytokines associated with immune cell function, including IL-2, IL-6, IL-8 were detected at low concentration in fertile men. This suggests that cellular immune activity is low in the genital tract of normal men. Elevated levels of some of these factors have been detected in semen of infertile men, indicating that cell-mediated immunity may be up-regulated by genital tract infections. The relation between proinflammatory cytokines, including IL-2, IL-6 and IL-8, has been reported previously in infertile men.<sup>22,34,36,57,68</sup> Variables that could affect levels of cytokines and other immunologic factors in semen from different ethnic and demographic groups include genetic polymorphisms, differences in diet, hygiene, sexual practices and drug use. In addition, genital flora and sexually transmitted infections (STIs) can influence seminal cytokine and antibody levels.<sup>65</sup> The men enrolled in the current study were at lower risk for STIs and subfertility, but more detailed testing would be required to conclusively rule out the influence of these variables in this study. Likewise, since the patients did not undergo an extensive physical examination, it is impossible to

conclusively exclude other reproductive conditions that could affect semen quality such as testicular maldescent or atrophy, and current varicocele.<sup>23,44,45</sup> The role of cell-mediated immunity in the aetiopathogenesis of male infertility is far from being defined. The cytochemokine interleukin-8 (IL-8) has a key role in T-cell mediated immune responses. We detected a significant difference of IL-8 in sera between fertile and infertile men, in accordance with previous studies.<sup>60,62,64</sup> The correlation between cytokines and infertility explained as cytokines may accumulate and activate leukocytes in the male genital tract, where activated leukocytes produce large amounts of elastase. It has also been shown that polymorphonuclear (PMN) elastase is an inhibitor of sperm motility, and that secretory leukocyte protease inhibitor (a potent inhibitor of leukocyte elastase) in seminal plasma reduces the extent of motility inhibition caused by elastase. Other scientists explained the effect of IL-8 on infertility, as the role of IL-8 elevations may represent part of a nonspecific acute-phase response, or they may be due to specific interactions between viruses (or other stimuli) and the immune system.<sup>44,45,46</sup> The subsequent interaction between virus and inflammatory cytokines could lead to a state of (silent) inflammation (possibly with the generation of reactive oxygen species) which could induce suppression of adequate spermatogenesis. Recently, it is reported that measuring the level of cytokines, in seminal plasma does not only expand the diagnostic options, but also, through the growing knowledge of immune processes, can give rise to new therapeutic methods of improving the quality of semen and increasing the chance to reproduce.<sup>5,8</sup> The results of the present study were approved the results of other studies which reported that certain kinds of cytokine in the sera and seminal plasma might play an important role in improving semen quality.<sup>16,18,19</sup> Although, the seminal plasma concentration of IL-8, an important mediator of inflammatory processes and is significantly associated with seminal leukocyte, Interleukin-6 is a multifunctional cytokine found in human sera that is produced by various types of cells in the genital tract. Levels of IL-6 correlate with the secretory activity of Sertoli cells<sup>56,57</sup>. Currently, little is known about IL-6 levels in seminal plasma of men characterized according to the etiological diagnosis of infertility. Significantly elevated IL-6 levels were seen in vasectomy reversal patients, compared with normal healthy men. However, high IL-6 levels have been associated with male infertility. These pro-inflammatory cytokines (IL-6) may modulate the pro-oxidant activities in male genital tract. The elevated levels of seminal ROS have been shown to decrease the fertilization rates *in vitro* by causing lipid peroxidation of sperm membrane.<sup>62,65,66</sup> The positive correlation observed in our study suggests a common underlying pathophysiology that causes the dysfunction of spermatozoa. Lack of significant correlation between the IL-6 and sperm parameters may be due to small sample size of the study.<sup>27</sup> The observed cytokine elevations may represent part of a nonspecific acute-phase response or may be due to specific interactions between CMV and the immune system. It has been proposed that the subsequent interaction between virus and inflammatory cytokines could lead to a state of inflammation and graft damage after solid-organ transplantation.<sup>34</sup> monitored IL-6 levels daily in 17 patients following lung transplantation and found that specific patterns of IL-6 release were predictive of acute rejection and infection, leading the authors to suggest that IL-6 monitoring may be beneficial. In this larger cohort of BMT patients, although IL-6 levels correlated with the development of CMV infection and disease, significant variations among patients and the

nonspecific inflammatory cytokine response could limit the clinical utility of such an approach.<sup>41,42,44,45</sup> The most significant finding of the present investigation was the levels of IL-2, 6 and 8. That detected in seminal plasma of fertile, infertile men, its levels were significantly lower in the sera of fertile men as compared to those of infertile men, with some infertile men having 2- to 4 fold higher values than those observed in sera of fertile men.<sup>69</sup> Interestingly, the higher levels of interleukins in the sera of infertile men seem to have clinical significance because they correlated significantly with the total sperm number, penetration rates, and some sperm motion parameters. A recent report demonstrated significantly higher levels of another interleukin, IL-8, in the seminal plasma of infertile men having leukospermia compared to those in the seminal plasma of fertile men.<sup>70</sup> IL-8 is a chemotactic factor for neutrophils; thus the higher IL-8 levels in the seminal plasma of infertile men than those of fertile men might explain the reason for the increased cell number of granulocytes in infertile semen. IL-6 is produced by a variety of cell types, including macrophages, endothelial cells, fibroblasts, and trophoblasts and has been demonstrated to influence the growth and differentiation of B cells.<sup>71</sup> Because the male genital tract is an immunologically dynamic system and a number of studies have indicated an increased number of leukocytes in the ejaculate of infertile men as compared to fertile men, the higher levels of IL-6 in seminal plasma of infertile and immunoinfertile men may be due to an increased number of leukocytes (secreting IL-6) in the sera of these patients. A recent study carried out in rats demonstrated that Sertoli cells, but not the spermatocytes, spermatids, and peritubular cells, secrete bioactive IL-6 when cultured *in vitro*.<sup>72</sup> Interestingly, it was further found that FSH augments Sertoli cell IL-6 secretion in a dose-dependent manner, indicating that IL-6 secretion may be regulated by a complex interplay of various hormonal factors. Thus hormonal (especially FSH) imbalance may also be contributing to defective levels of IL-6 in some of these infertile men, because increased IL-6 levels correlated inversely with the sperm number in the ejaculate, and FSH has been shown to have a predominant role in regulating spermatogenesis.<sup>73</sup> The results of this study show that the sera concentration of IL-8, an important mediator of inflammatory processes, is significantly associated with seminal leukocytes and with some clinically relevant parameters of semen quality. All patients enrolled in this prospective study were without symptoms of genital tract infection. The assays for cytokine determination were performed in aliquots from the same ejaculates that were used to examine other variables such as standard sperm analysis, ASA, sperm migration testing, SCMP, leukocyte counting and microbial evaluation. Cytokines rarely act in isolation, but rather in a network of other cytokines.<sup>12,22,33,34,35</sup> The absolute concentrations of IL-8 in seminal fluid were much higher compared with IL-6, making the use of a highly sensitive assay for the latter parameter necessary. Seminal plasma concentrations of IL-8 were markedly higher than serum concentrations reported elsewhere.<sup>70,73</sup> In the present study, IL-6 concentrations in semen were within the range reported for healthy men, but also much higher than in serum<sup>39,40,48</sup>, and were within a wide inter-individual range. In this study, the concentrations of IL-8 and IL-6 were significantly interrelated, which has also been shown for other cytokines, such as IL-6 and IL-2.<sup>7,9</sup> Results of this and other studies are limited to the one-point determination of these interleukins, and future investigations will need to show the intra-individual variation over a longer time period and potentially influencing factors. The populations of soluble receptors of the cytokines must also

be considered, though previous investigations have failed to show a significant correlation between IL-6 and its soluble receptor in either semen or serum.<sup>61,63</sup> When the relationship of sperm with semen quality of the same ejaculates was analysed in the present study, a particular association was noted with the total number of motile spermatozoa.<sup>32,38,41</sup> It has been suggested that sperm production rates are more suitable for estimating testicular function than the standard parameters of semen analysis, for example, sperm count per ml. The relationship of IL-8 with percentage progressive motility or 'classical' oligozoospermia was less obvious, and there was no association with standard morphology.<sup>51</sup> However, in this population, which was unselected for infertility factors of the involuntarily childless couples, severe male factor cases were relatively rare. The association might be more pronounced in a group of males selected on the basis of andrological disorders.<sup>49</sup> The relationship of pro-inflammatory cytokines (e.g. IL-6) with semen quality in other studies is controversial. In landmark studies, others showed a significant effect of soluble products of activated immune cells and of some lymphokines and tumour necrosis factor (in high concentrations) on sperm motility and on the outcome of the zona-free hamster egg test.<sup>55,57,59,60</sup> This contrasts with other reports which did not show a relationship of, for example, IL-6 with standard parameters of semen analysis, possibly due to differing population characteristics and assay methods.<sup>65,69,71</sup>

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