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Original Article

Interleukin 17 and Growth Differentiation Factor 9 Influence in a Sample of Iraqi Infertile Males

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Abstract

Infertility is defined as the inability of couples to conceive after 1 year of regular unprotected intercourse, which affects 10-15% of couples. The present study aimed to investigate the influence of Interleukin-17 (IL-17) and growth differentiation factor 9 (GDF9) on three groups of infertile males, including control, azoospermia, and oligozoospermia. In total, this study was performed on 93 participants, consisting of 18, 65, and 10 subjects in the Azoospermia, oligozoospermia, and control groups, respectively. The mean plasma levels of IL-17 in the azoospermia and oligozoospermia groups were 21.317±3.605 and 15.101±2.416 ng/l, respectively, which were significantly higher than that in the control group (5.392±1.731 ng/l). Furthermore, the mean plasma levels of GDF9 in the azoospermia and oligozoospermia groups were 3.299±1.051 and 6.2603±2.621 ng/l, respectively, which was significantly higher than that in the control group (12.807±2.170 ng/l). One-way analysis of variance and least significant difference post-hoc test were performed to assess significant differences among means. R-squared measures how well the linear regression model fits the data. It can be interpreted as the proportion of variance of the outcome Y explained by the linear regression model. R-squared is a number between 0 and 1. In non-obstructive forms of severe oligozoospermia and azoospermia, like the case in the current study, intracytoplasmic sperm injection is suggested by using testicular biopsy for spermatozoa extraction, if viable spermatozoa are present.

Keywords: Interleukin 17, Growth, Factor 9, Infertile males

1. Introduction

Infertility is defined as the inability of couples to conceive after 1 year of regular unprotected intercourse, which affects 10-15% of couples (1, 2). According to the latest WHO statistics, about 50-80 million people worldwide are infertile (3, 4). Spermatogenesis is a long and complex process to produce male germ cells called spermatozoa (5). It is a complex and temporal event involving mitotic and meiotic divisions and extensive cellular remodeling. Spermatogenesis occurs in seminiferous tubules of the testis, a unique site that contains three types of cells, namely male germ cells, Sertoli cells, and myoid cells (6).

Interleukin-17 (IL-17) is a pro-inflammatory cytokine that appears to be involved in the maintenance of testicular immunity and spermatogenesis (7). Semen analysis has shown that semen samples with low-activity sperms have higher IL-17 levels, while IL-17 levels have no significant influence on sperm densities, morphologies, and pH values. The sperm mortality rate of low IL-17 level samples is remarkably lower than that of high IL-17 level samples. Correlation analysis indicated that seminal fluid IL-17, IL-6, IL-8, and TNF-α were positively correlated, and that seminal IL-17 level had no effects on bacteria colony counts and serum follicle-stimulating hormone, luteinizing

hormone, and testosterone levels. Results of previous research have indicated that IL-17 may play an essential role in male reproduction (8).

Growth differentiation factor 9 (GDF9) from the transforming growth factor-β (TGFβ) superfamily is a critical regulator of germ cell development. It acts as the extracellular ligand of the signal transduction pathways regulating proliferation, apoptosis, and other aspects of cell behavior. As a member of the TGF-β superfamily, GDF9 in males has been detected primarily in round spermatids and pachytene spermatocytes, but not in the SCs in adult rats, cats, alpacas, and humans (9).

2. Materials and Methods

2.1. Sample Collection

Serum samples were collected from infertile males and healthy controls in Kamal AL Samarrai Hospital, Baghdad, Iraq for infertility and *in vitro* fertilization. The samples were obtained from blood collected from a peripheral vein in a coagulant tube. After clotting the samples, the serum was isolated, stored at -80 °C, and centrifuged (200 g).

2.1.1. Enzyme-Linked Immunosorbent Assays

The IL-17 and GDF-9 were assayed using commercially available enzyme-linked immunosorbent assay kits, according to the instructions of the manufacturer (Fernhurst, USA). Wells were determined for diluted standard, blank, and sample dilutions. Each prepared standard, blank, and sample dilution (100 µL) was added into the appropriate well, covered with the plate sealer, and incubated for 1 h at 37 °C. Afterward, the liquid was removed from each well without washing it. Subsequently, detection reagent A working solution was added (100 µL) to each well, the wells were covered with the plate sealer and incubated for 1 h at 37 ºC.

The solution was aspirated and washed with the addition of 350 µL of 1× wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer, and the wells were left to sit for 1-2 min. The remaining liquid was obliterated from all wells by snapping the plate onto absorbent paper. The washing process was repeated three times after the last wash, and any remaining wash buffer was removed by aspiration or decantation. Afterward, the plate was inverted and blotted against absorbent paper. Detection Reagent B working solution (100 µL) was added to each well, and the wells were covered with the plate sealer and incubated for 30 min at 37 °C. The aspiration/wash process was repeated five times as in the previous step.

Afterward, 90 µL of Substrate Solution was added to each well, and they were covered with a new plate sealer. They were incubated for 10-20 min at 37 °C (do not exceed 30 min). It should be noted that the plate was protected from light. With the addition of the Substrate Solution, the liquid turned blue. Stop Solution (50 µL) was added to each well, which caused the liquid to turn yellow. The side of the plate was tapped gently to ensure thorough mixing. Any drop of water was removed, and a fingerprint was added to the bottom of the plate to confirm no bubbles on the surface of the liquid. Immediately, the microplate reader-run was used and the absorbance was measured at 450 nm.

2.2. Testicular Sperm Extraction (Biopsy)

Testicular biopsy from a male with azoospermia infertility was obtained at Al-Jadiriah Private Hospital, Baghdad, Iraq, using the testicular sperm extraction technique. For this purpose, a fragment of testicular parenchyma (2x2x2 mm) was removed from the testis and washed in buffered medium (Quinn's Advantages Medium with HEPES, SAGE, Cooper Surgical, Pasadena, USA) with 2.5% human serum albumin (Albutein, AlphaTherapeutic Milan, Italy), fixed in Bouin's solution (1 ml) and sent to the pathology laboratory.

The same pathologist performed all histological examinations based on the histopathological pattern. The testicular histology was classified into normal spermatogenesis, hypospermatogenesis (i.e., a reduction in the number of normal spermatogenetic cells), maturation arrest (i.e., an

absence of the later stages of spermatogenesis), and Sertoli cell only (i.e., the absence of germ cells) (10).

3. Results and Discussion

3.1. Plasma Level of Interleukins

3.1.1. Plasma Level of Interleukin-17

Cytokines are essential molecules in both normal and pathological conditions which communicate with different cells in the body. Many cytokines are secreted by Leydig and Sertoli cells and are involved in the regulation of testicular function. Inflammation and infection of the testicles may affect spermatogenesis and fertility due to increased pro-inflammatory cytokine secretion.

In the current study, plasma IL-17 level in infertile patients was significantly higher than that in healthy controls. This result agrees with those of a study carried out by Alamiri (11). According to table 1, the patients subjected to interleukin plasma level analysis were divided into three groups (groups one, two, and three involved azoospermia cases, asthenospermia cases, and controls, respectively).

Table 1. Interleukin-17 plasma level (ng/l) in patients and control groups

Groups	pg / ml
Group 1, Control (n=10)	5.392 ± 1.731 a
Group 2, Azoospermia (n=18)	21.317 ± 3.605 b
Group 3, Oligoasthenospermia (n=65)	15.101 ± 2.416 c

Means with a different letter in the same column are significantly different (P<0.05)

The mean plasma levels of IL-17 in groups two and three were 21.317±3.605 and 15.101±2.416 ng/l, respectively, which were significantly higher than that of the control group 5.392±1.731 ng/l. Investigation of the relationship between serum IL-17 level and parameters among the studied patients showed no significant correlation between plasma IL-17 level and sperm concentration. It should be mentioned that the azoospermic patients had the highest mean level (21.317±3.605 ng/l). Figure 1 illustrates the standard curve of the IL-17 level with R^2=0.98.

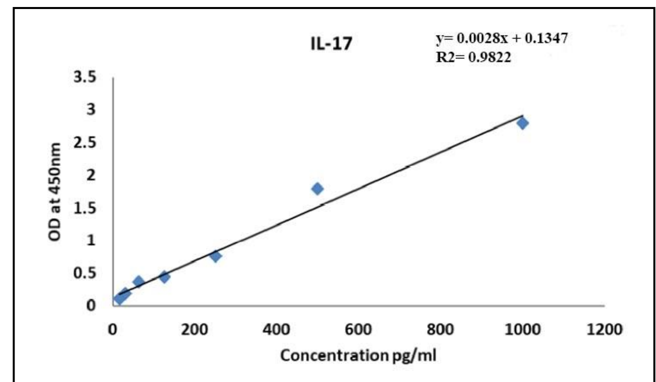

Figure 1. Standards curve of Interleukin-17

3.1.2. Plasma Level of Growth Differentiation Factor 9

Table 2 summarizes the significant differences between the GDF9 levels among groups. The mean plasma levels of GDF9 in groups two and three were 3.299±1.051 and 6.2603±2.621 ng/l, respectively, which were significantly higher than that in the control group (12.807±2.170 ng/l). This result indicates the role of GDF9 in male fertility. A study performed by Nicholls, Harrison (12) showed that GDF9 up-regulates the expression of inhibin subunit and stimulates the production of dimeric inhibin B protein as a germ cell-specific factor in the rat testis, which modulates essential Sertoli cell functions. Figure 2 shows the standard curve of the IL-17 level with R^2= 0.96.98.

Table 2. Growth Differentiation Factor 9 plasma level (ng/l) in patients and control groups

Groups	pg / ml
Group 1, Control (n=10)	12.807 ± 2.170 a
Group 2, Azoospermia (n=18)	3.299 ± 1.051 b
Group 3, Oligoasthenospermia (n=65)	6.2603 ± 2.621 c

Means with a different letter in the same column are significantly different (P<0.05)

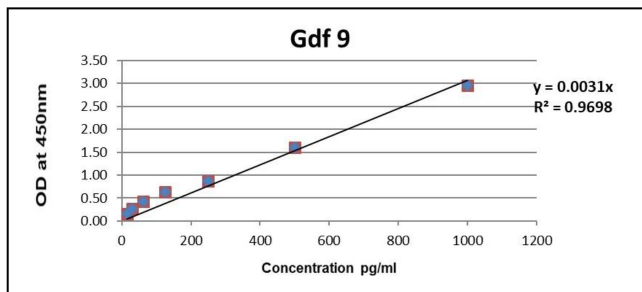

Figure 2. Standards curve of growth differentiation factor 9

3.2. Statistical Analysis

Statistical analysis of data was performed in SPSS software (version 26). Moreover, one-way analysis of variance and least significant differences post-hoc test were performed to assess significant differences among mean values. The R-squared measures how well a linear regression model fits the data. It can be interpreted as the proportion of variance of the outcome Y explained by the linear regression model. It is a number between 0 and 1 ($0 \leq R^2 \leq 1$). Values closer to 1 explain the higher variability of the model. Furthermore, $R^2=0$ means that the model cannot explain any variability in the outcome Y.

- **A value of r close to -1**: means that there is a negative correlation between the variables (when one increases, the other decreases, and vice versa).
- **A value of r close to 0**: indicates that the two variables are not correlated (no linear relationship exists between them).
- **A value of r close to 1**: indicates a positive linear relationship between the two variables (when one increases, the other does, too).

3.3. Selection of one Azoospermia Case for Histopathological and Cytological Examinations

3.3.1. Histopathology

The gross finding of the testicular biopsy specimen showed a single grayish piece of tissue measuring 0.3 cm (Figure 3). The microscopical finding section showed a single fragment of testicular tissue with an adequate number of seminiferous tubules with a severe degree of spermatogenic hypoplasia. Moreover, no mature sperms were observed, few tubules were arrested at primary spermatocytes, while most tubules were arrested at spermatogonia. In addition, some tubules were hyalinized, with many degenerated nuclei, very thick hyalinized basement membrane, stromal fibrosis, and focus of hemorrhage.

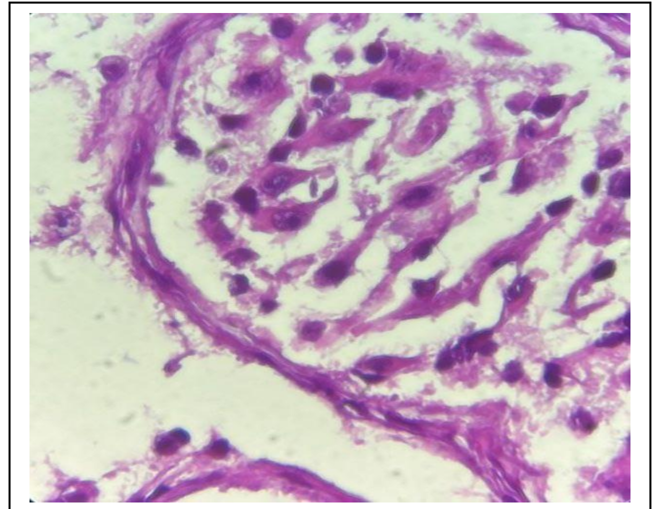

Figure 3. Testicular biopsy specimen

It must be mentioned that neither intratubular germ cell neoplasia nor malignancy was observed. In infertile men, the testicular biopsy may be one method of diagnosis, but it does not explain the real cause of infertility. It merely confirms the disturbance of spermatogenesis in males with low sperm concentrations and elevated follicle-stimulating hormone. Testicular biopsy plays a distinctive role in the diagnosis of obstructive azoospermia.

In most cases, surgical repair of the genital tract leads to spermatozoa in the semen, and spontaneous pregnancy occurs (13). In non-obstructive forms of severe oligozoospermia and azoospermia, like the case in the current study, intracytoplasmic sperm injection (ICSI) is suggested if viable spermatozoa are present by using testicular biopsy for spermatozoa extraction. Spermatozoa can be retrieved from the testes and used for ICSI in about 50% of males with non-obstructive azoospermia and in 100% of males with obstructive azoospermia (14)

Authors' Contribution

Study concept and design: D. S. A.

Acquisition of data: H. M. H.
Analysis and interpretation of data: Q. A. A.
Drafting of the manuscript: D. S. A.
Critical revision of the manuscript for important intellectual content: D. S. A.
Statistical analysis: H. M. H.
Administrative, technical, and material support: Q. A. A.

Ethics

The human study was approved by the ethics committee of the Baghdad University, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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