Study of Some Biochemical and Physiological Parameters of Women with Polycystic Ovaries (PCOS) Before and After Treatment Undergoing the Intrauterine Injection Program (IUI)

Dhirgam Falih Al-Shimerty[1], Ali A. Sabi[2], Zainab W. Kermasha[3]
[1,2]Department of pharmacology and toxicology, Faculty of Pharmacy, University of Kufa, Iraq.
[3]Department of Optics Technologies, Al-Mustaqbal University College, Babel, Iraq.
Email: dhurghamf.hassen@uokufa.edu.iq

Abstract
This study was performed on a sample of 25 women who were subjected to intrauterine injection program. These women patients were suffering from polycystic ovaries and attending the Fertility Center at Al-Sadr Medical Hospital in Najaf Governorate, for the period between December 2020 and March 2021. Blood serum was withdrawn before and after the corresponding treatment to measure the study parameters including testosterone, prolactin, FSH, LH, cholesterol, triglycerides and interleukin-17 levels. Results showed a significant reduction in the levels of testosterone, prolactin, LH, cholesterol, triglyceride and interleukin-17 at $p < 0.05$ after intrauterine injection treatment in comparison with that recorded before treatment as well as in control group. Although FSH level was lower after the treatment, results were non-significant at $p < 0.05$.

1. Introduction

Infertility means the failure to achieve pregnancy during the first year of the marriage, and it is a widely spread disease with prevalence rate of around 15-20% 15-20 % (1).
Two types of infertility are recognized: primary infertility, when the pregnancy does not happen, and secondary infertility when the pregnancy occurs at least once, but it does not occur again (2). Epidemiological data demonstrated that infertility is present in 2-10% of recently married husbands in the first year of their marriage. About 40% of these cases are related to the wives, and about 40% are related to the husbands, while 10% of the cases are related to the couples together, and an unexplained infertility that accounts for 10 % (3).
Intracytoplasmic Sperm Injection (ICSI) is one of the most common methods used to treat infertility and it is done by injecting sperm into the oocyte to get the fertilized oocyte in vitro. Fertilizing oocyte placed in the culture medium several days before being transferred to the uterus of the patient. The first successful pregnancy by In vitro fertilization (IVF) was reported by Drs Robert Edwards and Patrick Steptoe in 1978. In the beginning, In vitro fertilization was considered primarily for patients with tubal worker infertility as it was increased the chance of sperm and oocytes to stay in close and fertilize outside the human body (4). In addition, the ICSI as well as the intrauterine insemination (IUI) represent the main techniques of assisted reproductive technology (ART) (5).
Polycystic ovary syndrome (PCOS) is one of the most common female endocrine disorders. PCOS is a complex, heterogeneous disorder of uncertain an etiology, but there is strong evidence that it can to a large degree be classified as a genetic disease (6). PCOS produces symptoms in approximately 5% to 10% of women of reproductive age (12–45 years old) and is thought to be one of the leading causes of female subfertility (7). And the most frequent endocrine problem in women of reproductive age, Women with polycystic ovary syndrome (PCOS) experience symptoms such as irregular menses, hirsutism, and acne, and are at heightened risk for developing obesity, metabolic syndrome, diabetes mellitus, infertility, and some cancers. Data also indicate an inverse correlation between PCOS and healthrelated quality-of-life indicators and self-image (8). The symptoms and severity of the syndrome vary greatly among affected women. It is characterized by oligo-anovulation leading to menstrual irregularity, androgen excess, disturbances in glucose metabolism, and polycystic ovaries (PCO) associated with changing in ovarian morphology (9). In the normal ovary there are a few follicles that are developing, and one of these eventually will develop into a mature follicle that will ovulate. In PCOS, the follicles that start to develop cannot do so, and they "stack up" at an immature phase of development. In essence, the excess of male hormones almost acts like a barrier preventing them from progressing. They continue to "stack up" until the ovaries look like the one seen in the previous slide. Many biochemical changes were associated with PCOS. The search for new parameters that affected by PCOS and vice versa is still an interesting field of research.
The description of the disorder and parameters changes will be discussed in the following paragraphs in details. It is difficult to view PCOS as

doi.org/10.31838/hiv22.02.384 Received: 18.04.22, Revised: 25.04.22, Accepted: 09.08.22

purely developmental or resulting only from intrauterine exposures or simply an adaptation gone astray (the cause of PCOS is not fully understood, but genetics may be a factor. If you have PCOS, your sisters and daughters have a 50% chance of developing PCOS (10). PCOS problems are caused by hormone changes (11) one hormone change triggers another, which changes another.

Aims of the study

The current study aims to demonstrate the effect of polycystic ovaries on some hormonal and biochemical parameters of women undergoing the intrauterine injection program, as well as the response of these women to treatment for the purpose of obtaining good pregnancy rates.

2. Materials and Methods

The present study was performed in the department of Biology, Faculty of Pharmacy, University of Kufa, and also and in the Fertility Center of the Al-Sadr Medical City in Al-Najaf governorate in Iraq during the period from January 2020 to March 2021. Serum specimens were collected from women which undergoing IUI that attended to fertility center women which suffering from polycystic ovaries.

Average of age for the women which undergoing IUI was 27.99±0.32 years, and the age range for patients are between16–33 years, the samples collected from women were tested during two period, the first period before treatment, the second period after treatment (The treatment period varies depending on the severity of the infection). The level of sex hormones (testosterone – prolactin – LH – FSH) was measured before and after treatment, as well as the level of cholesterol and triglycerides.

Sample Collection and Analysis

Blood Samples

About five ml of morning IV blood samples were drawn from women who undergo IUI during two periods, the first period before treatment, while the second period after treatment. The treatment period varies depending on the severity of the infection. The level of sex hormones, testosterone, prolactin, LH and FSH, IL-17 was measured before and after treatment, as well as the level of cholesterol and triglycerides. To complete blood clotting, about Five ml of blood left in gel tube for 10 minutes at room temperature, and then centrifuged at 3000 rpm for 5 minutes. Later, the separated serum was transferred into five tubes which stored at -40 °C.

Analysis

1- Testosterone Hormone Concentration

Testosterone levels were measured before and after treatment for women undergoing intrauterine insemination (IUI) with PCOS using enzyme-linked immunosorbent assay (ELISA) according to the procedure (See appendix 1).

2- Prolactin Hormone Concentration

Prolactin Hormone levels were measured before and after treatment for women undergoing intrauterine insemination (IUI) with PCOS using enzyme-linked immunosorbent assay (ELISA) according to the procedure (See appendix 2) .

3- LH Concentration

LH levels were measured before and after treatment for women undergoing intrauterine insemination (IUI) with PCOS using enzyme-linked immunosorbent assay (ELISA) according to the procedure (See appendix 3).

4- FSH Concentration

FSH levels were measured before and after treatment for women undergoing intrauterine insemination (IUI) with PCOS using enzyme-linked immunosorbent assay (ELISA) according to the procedure (See appendix 4).

5- Cholesterol Concentration

Cholesterol levels were measured before and after treatment for women undergoing intrauterine insemination (IUI) with PCOS using spectrophotometer according to procedure (See appendix 5).

6- Triglycerides Concentration

Triglycerides levels were measured before and after treatment for women undergoing intrauterine insemination (IUI) with PCOS using spectrophotometer according to procedure (See appendix 6).

7-Human Interleukin-17

According to the results of measurement of interleukin-17 concentration for three groups: implantation failure group, spontaneous miscarriage group and continuous pregnant group (control group) and studies effect interleukin-10 concentration by using enzyme-linked immunosorbent assay according to procedure (See appendix 7).

Statistical Analysis

The Graph Pad prism version 5 was utilized. The t-test and ANOVA (one-way analysis of variance) were utilized to compare the groups. The results were represented as (Mean ± Standerd Error). To evaluate the correlation between tags and parameters, correlation coefficients were then measured. Both the descriptive statistics and correlation coefficients were done using mega stat (Version 10.12) for excel 2010 software (12).

3. Results

Result of testosterone hormone concentration

The result showed a significant decrease ($p < 0.05$) in testosterone hormone concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 1).

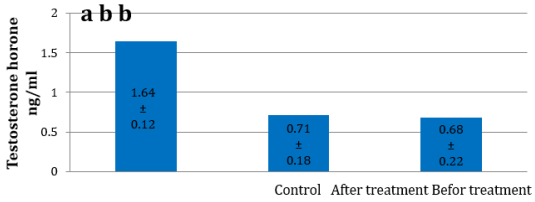

Figure (1): Results of testosterone hormone concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Different letters mean significant differences at ($p < 0.05$).
N=25.

Result of prolactin hormone concentration

The result showed a significant decrease ($p< 0.05$) in prolactin hormone concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 2).

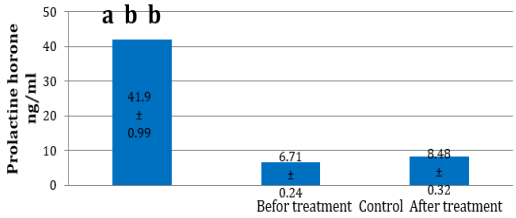

Figure (2): Results of prolactin hormone concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Different letters mean significant differences at ($p < 0.05$).
N=25.

Result of FSH hormone concentration

The result showed a non-significant decrease in FSH hormone concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 3).

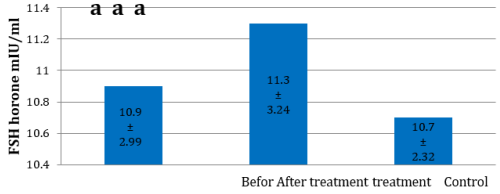

Figure (3): Results of FSH concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Non different letters mean non-significant differences at ($p < 0.05$).
N=25.

Result of LH hormone concentration

The result showed a significant decrease ($p < 0.05$) in LH hormone concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 4).

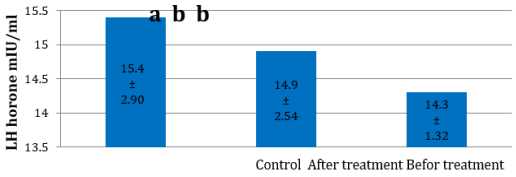

Figure (4): Results of LH concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Different letters mean significant differences at ($p< 0.05$).
N=25.

Result of triglycerides Concentration

The result showed a significant decrease ($p < 0.05$) in triglycerides concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 5).

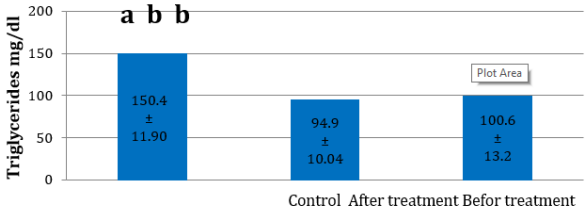

Figure (5): Results of triglycerides Concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Different letters mean significant differences at ($p < 0.05$).
N=25.

Result of Cholesterol Concentration

The result showed a significant decrease ($p< 0.05$) in Cholesterol concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 6).

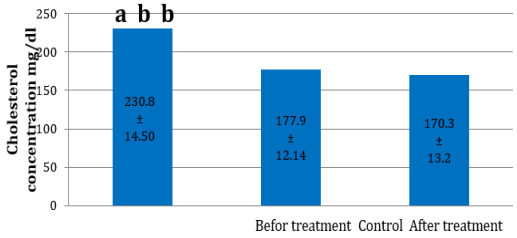

Figure (6): Results of Cholesterol Concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Different letters mean significant differences at ($p< 0.05$).
N=25.

Result of Interleukin-17 Concentration

The result showed a significant decrease ($p < 0.05$) in Interleukin-17 Concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI) (figure 7).

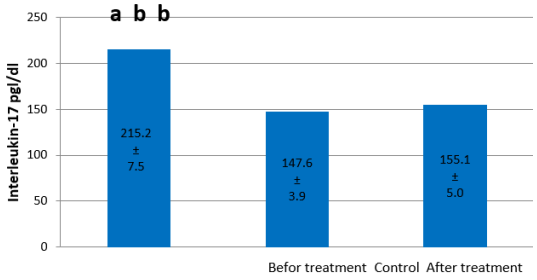

Figure (7): Results of Interleukin-17 Concentration in (control group) and (after treatment group) compared to (before treatment group) for women undergoing the intrauterine injection program (IUI).

Different letters mean significant differences at ($p < 0.05$).
N=25.

4. Discussion

The current study demonstrated an increase in the level of testosterone in women with polycystic ovaries significantly at $p < 0.05$, compared with the group of women after treatment and the control group, and perhaps the reason for this is that testosterone is produced mainly by the adrenal gland and from the ovary in general. Secondary and found in all women's bodies, but its levels rise in women with polycystic ovaries and this is due to the high level of the hormone LH and insulin, so skin cells and hair follicles are highly susceptible to simple increases in testosterone levels and this leads to increased hair growth in unusual places such as the face, chin, chest and thighs In a condition known as hirsutism, It may also lead to the appearance of pimples, as it may have other common symptoms for PCOS patients, as this study agrees with the findings (13). Where there was an increase in the level of the testosterone hormone in women with polycystic ovaries, and there was also an irregularity in the menstrual cycle for affected women, which may lead to infertility.

The current study also found a high level at the significance level ($p < 0.05$) in the level of the hormone prolactin for the samples of a group of women with polycystic ovaries, compared with the group of women after treatment and the control group, and perhaps the reason for this is that polycystic ovaries in women, including One or two ovaries lead to a hormonal imbalance in women, and as it is known that the hormone prolactin is linked to the sex hormones in women, and any imbalance in these hormones leads to an imbalance in the level of the hormone prolactin. This study agrees with the findings of (14). Where it was found that there is a close relationship between the problem of polycystic ovaries and the rise of the hormone prolactin, as the direct and main reason for the occurrence of high milk hormone in women is the presence of the problem of polycystic ovaries in one of the ovaries or in the ovaries, where the definition of polycystic ovaries is the appearance of these bags, usually small in size Its number ranges from (1-10) bags in most cases of infection, and it is linked to several diseases in women, including hormonal imbalance, leading to an elevated level of the prolactin hormone in women with polycystic ovaries, and its rise may also be caused by a genetic factor or due to the use of some treatments (Such as treatments used in the treatment of epilepsy) or a defect in the pituitary gland.

The findings of this study further exhibited an elevation at $p < 0.05$ in LH level for in women with polycystic ovaries in comparison with compared to the group after treatment and the control group, and perhaps the reason for this is the production of this hormone from the pituitary gland located at the base of the brain, which stimulates the ovaries to The production of eggs as well as the ovaries stimulates the production of testosterone as well, and most women with polycystic ovaries have. This study agrees with the findings of (15), where the high level of LH hormone was noted for women with polycystic ovaries compared with normal women.

The current study also showed an insignificant decrease ($p < 0.05$) in the level of FSH for the samples of a group of women with polycystic ovaries compared with the group after treatment and the control group. The reason for this may be due to a significant decrease in the level of the hormone inhibin B, which is responsible for keeping the level of FSH low and decreasing. Inhibin B hormone as a result of a decrease in the number of remaining follicles inside the ovary, so the body can only stimulate the pituitary to produce more hormone in order to mature the remaining follicles. This study agrees with the findings of (16), where a lower FSH level was found in women with polycystic ovaries compared to due to ovarian failure on The production of estrogen or inhibin B hormone thus leads to a high level of FSH and LH.

The findings of this study also showed a significant ($p < 0.05$) elevation in triglycerides and cholesterol levels in women with polycystic ovaries in comparison with that after treatment and in the control group, and perhaps the reason for this is that most women with polycystic ovaries suffer from insulin resistance. Thus, their bodies secrete more insulin hormone to overcome this, and insulin resistance also leads to weight gain and fat, which increases the symptoms of polycystic ovaries, because excess fat leads to the body secreting more insulin. This study agrees with the findings (11) Where it was proven that the increase in the proportion of insulin in the body due to insulin resistance leads to an increase in body fat, which also leads to weight gain. It was also found that high insulin levels lead to the appearance of dark spots on the skin behind the neck, under the armpits, between the thighs, and increased insulin leads to increase the secretion of certain hormones such as testosterone. PCOS may affect fertility in women in several ways, of these ways, ovulation problems which are usually the main cause of infertility in women with PCOS. Ovulation could not occur due to the excess in testosterone production or due to failure of

maturation of ovarian follicles. Even when ovulation does present, an imbalance in levels of hormones might inhibit the proper development of endometrium to allow the implantation of a mature ova.

Additionally, the findings of this research demonstrated a significant ($p < 0.05$) increment in the concentration of IL-17 in women with PCOS in comparison with that after treatment and the control group. Th reason is that PCOS women may suffer from infections, which may elevate the levels of leukocytes. Interleukin-17 is one of the cytokines that stimulate the occurrence of inflammation and is responsible for the initiation of the inflammatory process. The elevation in interleukin-10 level in women with polycystic ovaries indicates the role of interleukins which function to communicate between immune cells, and other cells who play a role in defense system of the body. Finally, many studies have demonstrated a significant correlation between the WBCs and interleukin levels in the body (17).

References

1- Poongothai, S., Rajendra, P\., Anbhazhagan, G. and Viswanathan M., (2009). "Prevalence of Depression in a Large Urban South Indian Population - The Chennai Urban Rural Epidemiology Study (Cures - 70)." *PLoS ONE* 4(9).

2- Swedloff, R.S. and Wong, C., (2004): The testis and male sexual function. In:Cecil textbook of medicen. Goldman, L. and Ausiello, D. (eds) 22nd ed. Philadelphia (PA): saunders; 1472-1483.

3- Isidori, A., Latini, M. and Romanelli, F., (2005). Treatment of male infertility. Contraception; 72:314-318.

4- Nazemian, Z., (2011). Infertility and Women's Age. A thesis of Master of Science Institute of Medical Science (IMS). University of Toronto; 118:63-70.

5- World, Health Organization (2010). Laboratory Manual for the semen and sperm cervical Mucus Interaction, 4th Ed, Cambridge University press, Cambridge.

6- Azziz R, Black V, Hines GA, Fox LM, Boots LR, (1998). Adrenal androgen excess in the polycystic ovary syndrome: sensitivity and responsivity of the hypothalamic-pituitaryadrenal axis. *J Clin Endocrinol Metab,* 83(7):2317-23.

7- Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO, (2004). The prevalence and features of the polycystic ovary syndrome in an unselected population. *J Clin Endoc Met*, 89(6):2745–9.

8- Crete J, Adamshick P, (2011). Managing polycystic ovary syndrome: what our patients are telling us. *J Holist Nurs*, 29(4):256-66.

9- Mulders, A.G., Laven, J.S., Eijkemans, M.J. *et al, (*2004). Changes in anti-Mullerian hormone serum concentrations over time suggest delayed ovarian ageing in normogonadotrophic anovulatory infertility. *Human Reproduction*, 19, 2036–42.

10- Barbieri RL, (2007). Polycystic ovary syndrome. In DC Dale, DD Federman, eds., ACP Medicine, section 16, chap. 5. New York: WebMD.

11- Al-fahham, A. and Al-Nowainy, H. Q. The role of FSH, LH, and Prolactin Hormones in Female Infertility. 6(5):110-118: 2016.

12- Motulsky, H.J., (2003). Graph pad prism 4.0 statistics Guide- statistical analyses for laboratory and clinical researchers. Graphpad Software, San Diego, CA.

13- Al-fahham, A. Correlation between oxidative stress and thyroid hormone levels in infertile women.inter. j. Sci. Res. Pub. 2015; 5(12):128-131.

14- Eskenazi, B.A.J.; Wyrobek, E.; Sloter, S.A.; Kidd, L.; Moore, S. and Youny, D. (2003) . The association of age and semen quality in healthy men. Human reproduction; 2: 447-454.

15- Majzoub, A., Cho, C. L., Agarwal, A., and Esteves, S. C., (2019). Conventional Semen Analysis and Specialized Sperm Function Tests in Patients with Varicocele. In Varicocele and Male Infertility (pp. 137-157). Springer, Cham.

16- Yuan, S., Liu, Y., Peng, H., Tang,C., Hennig, G.W., Wang, Z., Wang, L., Yu, T., Klukovich, R., Zhang, Y. and Zheng, H., (2019). Motile cilia of the male reproductive system require miR-34/miR-449 for development and function to generate luminal turbulence. Proceedings of the National Academy of Sciences, 116(9), 3584-3593.

17- Mukthayakka, G., Sajjan, A. G., Kashid, R. A., Thejaswini, H. S. and Vanitha, S., (2020). Elevated plasma levels of TNF-alpha, INF-gamna, IL-10 and TGF-beta in malaria patients from two malaria non-endemic regions in karnataka, India. International Journal of Medical and Biomedical Studies, 4(2).

Appendix 1

Measurement of testosterone hormone concentration according to Procedure
1. Twenty-Five microliters of the appropriate serum reference, control or specimen Pipetted into the assigned well.
2. Fifty microliters of the working Cortisol Enzyme Reagent was added to all wells.
3. The micro plate was swirled gently for 30 seconds to mix.
4. Fifty microliters of Cortisol Biotin Reagent was added to all wells.
5. The micro plate was swirled gently for 30 seconds to mix and then covered and incubated for 60 minutes at room temperature.
6. The contents of the micro plate were discarded by decantation and the plate dry was blotted with absorbent paper.
7. The contents of the micro plate were discarded by decantation. Then, 350µl of wash buffer was added and decanted (tap and blot). Two additional times were repeated for a total of three washes.
8. One hundred microliters of working substrate solution (A+B) were added to all wells.
9. The wells were incubated at room temperature for 15 minutes.
10. Fifty microliters of stop solution were added to each well and gently mixed for 20 seconds).
11. The absorbance was read in each well at 450nm by ELISA reader.

Appendix 2

Measurement of prolactin hormone concentration according to Procedure
Before proceeding with the assay, all reagents were brought, serum references and controls to room temperature (27°C).
1. Fifty microliters of the appropriate serum reference, control or specimen were pipette into the assigned well.
2. One hundred microliters of prl-enzyme reagent were added to all wells.
3. The micro plate was swirled gently for 30 seconds and incubated for 60 minutes at room temperature.
4. The contents of the micro plate were discarded by decantation and the plate was dried and blotted with absorbent paper.
5. Three hundred and fifty microliters of diluted wash buffer were added and then decanted (tap and blot). This step was repeated for three
Washes.
6. One hundred microliters of working substrate solution (A+B) were added to all wells, The wells were incubated at room temperature for 15 minutes, fifty microliters of stop solution were added to each well and gently mixed for 15 -20 seconds). The absorbance was read in each well at450nm by ELISA reader.

Appendix 3

Measurement of LH hormone concentration according to Procedure
Before proceeding with the assay, all reagents were brought, serum references and controls to room temperature (27°C).
1. Fifty microliters of the appropriate serum reference, control or specimen were pipette into the assigned well.
2. One hundred microliters of LH-enzyme reagent were added to all wells.
3. The micro plate was swirled gently for 30 seconds and incubated for 60 minutes at room temperature.
4. The contents of the micro plate were discarded by decantation and the plate was dried and blotted with absorbent paper.
5. Three hundred and fifty microliters of diluted wash buffer were added and then decanted (tap and blot). This step was repeated for three
Washes.
6. One hundred microliters of working substrate solution (A+B) were added to all wells, The wells were incubated at room temperature for 15 minutes, fifty microliters of stop solution were added to each well and gently mixed for 15 -20 seconds). The absorbance was read in each well at450nm by ELISA reader.

Appendix 4

Measurement of FSH concentration according to Procedure
Before proceeding with the assay, all reagents were brought, serum references and controls to room temperature (27°C).
1. Fifty microliters of the appropriate serum reference, control or specimen were pipette into the assigned well.
2. One hundred microliters of FSH-enzyme reagent were added to all wells.
3. The micro plate was swirled gently for 30 seconds and incubated for 60 minutes at room temperature.
4. The contents of the micro plate were discarded by decantation and the plate was dried and blotted with absorbent paper.

5. Three hundred and fifty microliters of diluted wash buffer were added and then decanted (tap and blot). This step was repeated for three washes.
6. One hundred microliters of working substrate solution (A+B) were added to all wells, The wells were incubated at room temperature for 15 minutes, fifty microliters of stop solution were added to each well and gently mixed for 15 -20 seconds). The absorbance was read in each well at450nm by ELISA reader.

Appendix 5

Measurement of cholesterol concentration according to Procedure
1 -Take 2 ml of reagent solution.
2- Put each 1 ml into a test tube.
3- We put in the first test tube 10 microns of a standard concentration of known (200 milligrams per deciliter).
4- We put in the second test tube 10 microns of the patient's serum.
5- Incubate for 5 minutes at 37°C.
6- Double absorbance for each tube with a 500 nm in spectrometer.
7- Apply the equation: serum absorbance/standard absorbance x standard concentration.

Appendix 6

Measurement of triglycerides concentration according to Procedure
1 -Take 2 ml of reagent solution.
2- Put each 1 ml into a test tube.
3- We put in the first test tube 10 microns of a standard concentration of known (200 milligrams per deciliter).
4- We put in the second test tube 10 microns of the patient's serum.
5- Incubate for 5 minutes at 37°C.
6- Double absorbance for each tube with a 500 nm in spectrometer.
7- Apply the equation: serum absorbance/standard absorbance x standard concentration.

Appendix 7

The assay procedure for measurement of Human Interleukin 17:
all reagents are allowed to come to room temperature prior to their use. Centrifuge the sample again after thawing before the assay. All the reagents that mixed thoroughly by gently swirling before pipetting.
Prepare all reagents, working standards, and samples as directed in the previous sections
Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells back into the pouch and store unused wells at 4°C.
Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
Remove the liquid of each well, don't wash.
Add 100µl of Biotin-antibody (1X) to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin-antibody (1X) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
Add 100µl of HRP-avidin (1X) to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
Repeat the aspiration/wash process five times as in step 6.
Add 90µl of TMB Substrate to each well. Incubate for 15-30 minutes at 37°C. Protect from light.
10-Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.
11-Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.