The role of heat shock protein 70 and dysglycemia in Iraqi Women with Polycystic Ovarian Syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is the most severe endocrine disease in females of reproductive age. Hormone imbalance due to increased levels of androgen (male hormone) , Symptoms including irregular menstrual periods, hirsutism, ovarian cysts can detected by an ultrasound. **Objective:-** The aims of the study were to assess the levels of HSP70 in PCOS patients' sera and compare them to those in the control group, as well as investigate their relationship with hormonal biomarkers and insulin resistance

Subjects and Method :- A case-control study involved 81 females in reproductive age [18-45 years old], 50 of them were diagnosed with polycystic ovarian syndrome, while another 31 were healthy controls. The samples were collected in the morning fasting state from November 2020 to May 2021, and HSP70 levels were analyzed using ELISA technique from Bioassay Lab company in China. Biochemical variables BMI calculated weight divided by square height in meter (Kg/m2) hormonal LH, FSH, Testosterone had been measured by fluorescence Immunoassay (Boditech – Korea), FBG level was measured by spectrophotometer (Liner-SPAIN), F insulin level was measured jn Cobas e 411 (Roche-Germany).

Result :- The mean HSP70 levels (ng/ml) in the patients group were significantly higher than in the controls group ($P<0.001$). in addition In comparison to healthy groups, hormonal biomarkers such as Testosterone, LH, LH/FSH Ratio, and metabolic biomarkers such as FBG, F Insulin, HOMA-IR, HbA1c, and BMI were shown to be significantly higher in patients. also found positive significant correlation between HSP70 and IR , F insulin in $P\leq0.05$, $p<0.001$ respectively.

conclusion :- HSP70 level is increase in PCOS patient and had positive correlation with IR, F insulin level. and a significant high level of the all hormonals and parameters including LH, total testosterone ,FBG , F insulin and HbA1c were found in women with PCOS as compared with the control, except the FSH values was decrease.The increase in HSP70 levels in sera of women with PCOS associated with increased risk of metabolic disorder and their complication in increase the infertility.

Keyword: - Heat shock protein 70, polycystic ovarian syndrome, dysglycemia

Introduction: -

Polycystic ovary syndrome (PCOS) is the most severe endocrine disease in females of reproductive age1 **(Mcewen and Hartmann, 2018)**. Hormone imbalance due to increased levels of androgen (male hormone) , Symptoms including irregular menstrual periods, hirsutism, abnormal hormones LH/FSH ratio is high, ovarian cysts can detected by an ultrasound2 **(Legro, 2008)**

many reasons for PCOS in women so we can classified depended it to four type of PCOS Insulin-resistant PCOS 3**(Marshall and Dunaif, 2012),**, inflammatory PCOS4 **(Borthakur,D Prabhu and Valsala Gopalakrishnan, 2020)**, adrenal PCOS5**(Halimova et al., 2019)**

and Thyroid dysfunction in women with polycystic ovarian syndrome 6 **(Kedar, Rewatkar and Akare, 2019)**. thats lead to disorder of clinical factors include reproductive (infertility, pregnancy-related problems) ,metabolic (obesity ,insulin resistance (IR),gestational and type II diabetes(DM2), and cardiovascular risk factors) as well as psychosocial (anxiety and depression,decreased quality of life, body image, and eating disorders)7**(Balen et al., 2016)**. Dysglycemia is a broad term that refers to an abnormality in blood sugar stability. This can include hypoglycemia (low blood sugar)or hyperglycemia (high blood sugar)8**(Crook,. 2013).** Blood sugar is regulated through insulin, It is a hormone produced in the pancreas. Insulin helps tissues in body to take glucose (sugar) to use for energy 8**(Crook,. 2013)**. In testes of PC0S patients were found dysglycemia hyperglycemia and may developed to prediabetes or type 2 diabetes. Persistently high blood sugar levels lead to organ and tissue damage, insulin resistance and increase oxidative stress thats causes to inflammation 9**(Sharif, 2019)** . One of the markers of inflammation and tissue damage is heat shock proteins (Hsps):- are family of highly homologous chaperone proteins which are induced in due to environmental, physical, and chemical stress which act as defensive moles limiting damage and facilitating cellular recovery10**(Hoter and Naim, 2019)** . They were first identified in relation to heat shock, but they are now known to manifest during a variety of other stresses, including exposure to cold11**(Matz et al., 1995)**, UV light, heavy metals, some medicines, dietary insufficiency, oxidative stress, acute or chronic inflammatory illnesses, and viral infections12**(Ikwegbue et al., 2017)**. "Heat-shock proteins are named according to their molecular weight. For example, Hsp60, Hsp70 and Hsp90 (the most widely studied HSPs) refer to families of heat shock proteins on the order of 60, 70 and 90 kilodaltons in size, respectively"13**(Buttacavoli et al., 2021)** . Hsp70 includes 641 amino acid residues with 70,052 Da in molecular weight 14**(Saibil, 2013)**. Heat shock protein70 (HSP70) acts in tandem with a group of co-chaperones and nucleotide exchange factors to ensure that proteins are of high quality, which helps cells survive by protecting them from environmental stresses 15**(Hartl, Bracher and Hayer-Hartl, 2011)**. As a result, it plays an important role in protein homeostasis and transport 16**(Breuninger and Erl, 2014)**. Hsp70 inhibits apoptosis directly and, as a result of its anti-apoptotic activity, acts as a "friend" of cancer development. Hence, researchers are working to improve cancer therapy methods by using the Hsp70 inhibitors 17 **(Hoter, Rizk and Naim, 2019) .**

On the other side, the presence of Hsp70 on the surface of cancer cells may make them more susceptible to natural killer (NK) cell cytotoxicity, which could aid in the development of effective immunotherapy 18**(Albakova et al., 2020)** . A high level of serum Hsp70 has also been associated to ovarian damage 19**(Narayansingh, Senchyna, Vijayan,& Carlson, 2004**)

Subject and material

This study was carried out by chemical and Biochemistry department, College of Medicine, University of Karbala and the cases individuals were obtained from Gynecological and Obstetric Hospital / Al-Batoul women's hospital / Wasit Health Iraq from the period November 2020 to May 2021 study included 50 women of PCOS (patients) and another 31 apparently healthy women (controls) with age range (18 - 45) years. Some of women were Exclusion involved some women were excluded they suffer from hyper-tension, pregnancy, diabetes mellitus, thyroid disease, women taking oral contraceptives Women with PCOS were diagnosed by using the Rotterdam criteria from2003 that involved Polycystic ovaries on (Oligo- or a ovulation), ultrasound, biochemical parameter and clinical signs of hyperandrogenism. Blood samples are collected in the morning at fasting state from vein puncture during the follicular phase of the menstrual cycle in all

cases. The samples were divided in to two parts the first part put in EDTA tubes for measuring glycated hemoglobin HbA1c. The second part of blood were transferred into gel tube and left for 15 minutes at room temp-erature to clotting then separated by centrifuge at 3000 rpm. for 10 min, and then measurement of serum hormones Testosterone, Follicle Stimulating Hormone(FSH), Luteinizing hormone (LH)were measured by fluorescence Immunoassay (FIA) (Boditech –Korea) based on the quantitative sandwich and fasting Insulin Fins was measured by immune-assay analysis Roche(Cobas e 411 – Germany), and parameter of fasting blood glucose was measurment by spectrophotometer at kit(Linear-Spain). HSP70 was tested by using ELISA method test based on the quantitative sandwich principle by (Bioassay lab-china) .

Results

The Statistical Package for the Social Sciences (SPSS) model 23 statistic program was used to examine the data of study. It was designed to make comparisons and use significant differences. It was considered to be significant when $p \leq 0.05$ presented as mean ±SD (standard deviation). Independent T-test statistics were applied for parameters to compared between patients and controls groups.

Table1:- Associations of biochemical parameters between PCOS women and healthy control

Parameters	PCOS n=50 Mean ± SD	Control n=31 Mean ± SD	P value
Age (years)	28 ± 6	30 ± 7	NS
BMI (kg/m^2)	30.8 ± 5.0	26. 7 ± 3.6	S
WC (cm^2)	43 ± 4.5	40.8 ± 1.6	S
LH (m IU/ml)	10.1 ± 3.7	4.3 ± 1.6	HS
FSH (m.IU/ml	5.47 ± 1.3	7.8 ± 1.85	HS
Testo	0.44± 0.25	0.21 ± 0.05	S
HbA1c %	5.5 ± 0.45	5.2 ± 0.3	S
FBG (mg/dl)	95.4 ± 9.2	87 ± 6	HS
Ins (m.IU/ml)	18.5 ± 2.1	16.3 ± 1.0	S
HOMA-IR	4.3 ± 0.6	3.5 ± 0.3	S
Ratio LH/FSH	1.8 ± 0.46	0.55 ± 0.1	HS
HSP70(ng/ml)	15.8 ± 4.7	9.7 ± 3.7	HS

SD: standard deviation,
BMI: Body mass index
WC Waist Circumference
LH: Luteinizing Hormone
FSH: follicle-stimulating hormone HbA1c: hemoglobin A1c
HOMA-IR stands for homeostatic model assessment of insulin resistance
FBS is for fasting blood sugar
ZAG: Zinc Alpha 2 Glycoprotein, HSP70: Heat Shock Protein 70

NS: t-test p- value ≥ 0.05
S: p- value ≤0.05,
HS: p- value ≤0.01

According to the presented data show mean of HSP70 levels (15.8 ± 4.7ng/ml) (9.7 ± 3.7ng/ml) in patient and control respectively, that significantly increased in patients group of PCOS in (P<0.001) compare to controls group. We show in table 1 our finding results that elevated high significant in biomarkers measured in hormonal LH, Ratio LH/FSH and metabolic biomarkers FBG, Fasting Ins , BMI, HOMA-IR in patients group compare to healthy groups, While decrease significant in FSH hormone. There was statistically non-significant changes in age mean values between the normal control group and the patients groups of PCOS.

According to the presented data which explain by Figure :1 showed the mean levels of HSP70 (ng /ml) increased in PCOS patient than controls group .

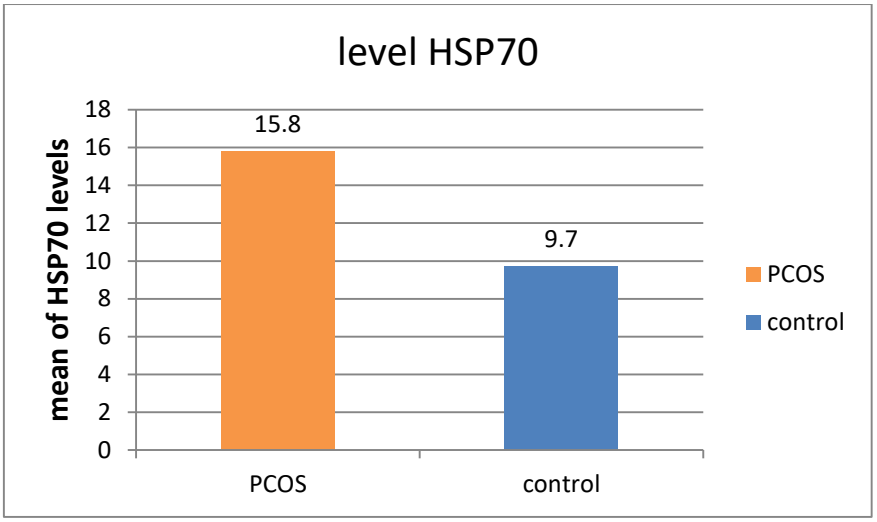

Figure 1 : Mean levels of Heat shock protein 70 (ng/ml) in patients group of PCOS compared to controls group.

In this study finding The percentage of hirsutism in group of patients women with PCOS was high reaching 68%while the percentage of patients without hirsutism was 32% as show in figure 2

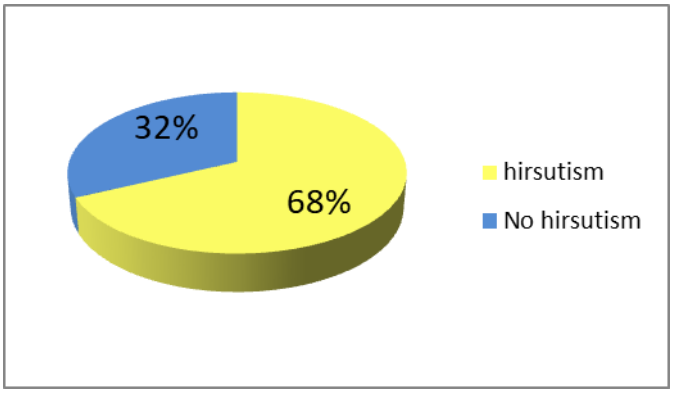

Figure 2:-The percent of hirsutism in patient group.

Body mass index :-We used Pie charts to described about the proportion of patients for each body mass index groups as normal weight 18-24.9 kg/m^2 has lowest percentage obtain 14% of patients groups, over weight (pre obesity) 25-29.9 kg/m^2 was 36% of patients and obesity over 30 kg/m^2 obtain higher percentage 50% of patient groups that's mean most of patient PCOS have high BMI as show in figure 3

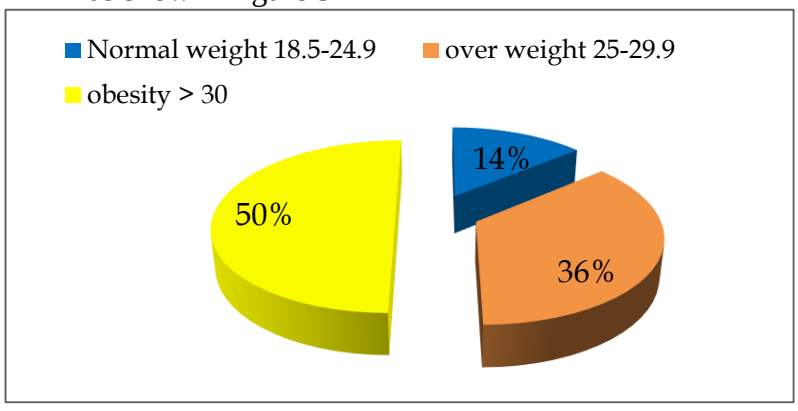

Figure 3 :- BMI proportion in patients groups.

one way ANOVA test. These groups include a group of primary infertility PCOS(n=32), secondary infertility PCOS(n=18) for patients groups and fertility (n=31)for contols groups. Table3.2 shows their testing parameters given the following results.

Comparison of groups by fertility:- The comparison is made between three groups, which were classified by the degree of fertility to Primary infertility, secondary infertility, and fertility for patients and controls respectively were studied using the

Table 2:- Comparison of groups by fertility for parameter and HSP70

parameters	PCOS		control	Sig
	Primary infertility n=32 G1	secondary infertility n=18 G2	fertilityn=31 G3	
Age (years)	29.3 ± 5	32.0 ± 6.4	30.9 ± 7.1	NS
BMI (k/m^2)	31.8 ± 4.3 b*	30.1 ± 5.4 b*	26.7 ± 3.6	S
LH	11.2 ± 3.3 b**	10.0 ± 4.3 b**	4.3 ± 1.6	HS
FSH	5.4 ± 1.4 b**	5.5 ± 1.1 b**	7.8 ± 1.8	HS
Testo	0.47 ±0.27 a*.b**	0.39 ± 0.20 b*	0.21 ± 0.05	S HS
FBG	95.7 ± 10.1 b**	94.8 ± 7.4 b**	87.7 ± 6.0	HS
HbA1c	5.47 ± 0.45 b*	5.40 ± 0.41	5.2 ± 0.37	S NS
FIns	18.6 ± 2.3 b*	18.4 ± 1.9 b*	16.3±1.6	S
HOMA-IR	4.25 ± 0.6 b*	4.0 ± 0.5 b*	3.5 ± 0.3	S
LH/FSH ratio	1.88 ± 0.44 b**	1.80 ±0.5 b**	0.55 ± 0.12	HS
HSP70(ng/ml)	16.3 + 4.9 b**	15.1 + 4.45 b**	9.7 + 3.75	HS

NS: t-test p- value ≥ 0.05, S: p-value ≤0.05, HS: p- value ≤0.01
a; Comparison between G1&G2 b: Comparison between G1&G3 or G2&G3
*--significant correlation p≤0.05 **-- highly significant p<0.001

In table 2 we noted the results of this comparison for HSP70 levels was (16.3 + 4.9 ng/ml) (15.1 + 4.45 ng/ml) (9.7 + 3.75 ng/ml) for primary infertility, secondary infertility and fertility respectively which increased HSP70 levels in group G1 than G2,G3 but no significant between primary G1and secondary infertilityG2 groups, while increase significant between both of them with fertility group G3 in p< 0.001 value as we note in figure 4. In other words the secondary infertility group was closer to control group than in G1primary infertility but didnot reach significance value. Figure4 explain HSP70 level in fertility groups

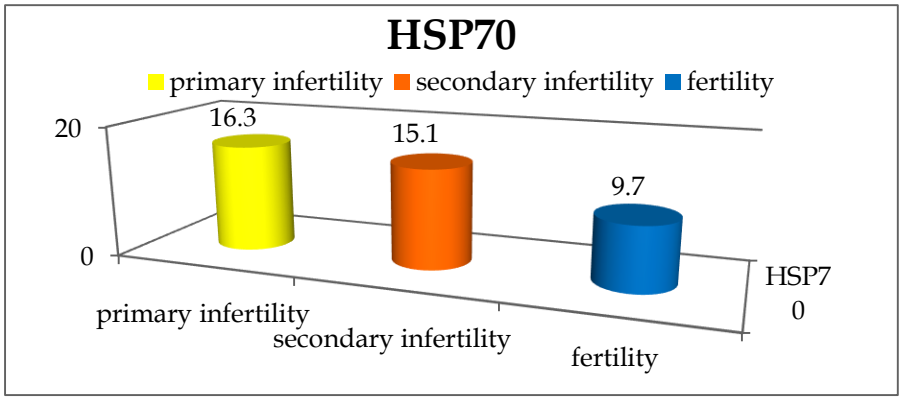

Figure 4 :- The mean levels of HSP70 within fertile groups

Also Table 2 shows that BMI, LH, Testo, HOMA-IR, and Fasting(Ins) hormone levels were higher in primary infertility than secondary infertility, while FSH hormone levels were lower in primary infertility than secondary infertility that's apparent in figure 5 . This means that the secondary infertility group had lower values showing decreases in PCOS Signs, but they were not of normal value.

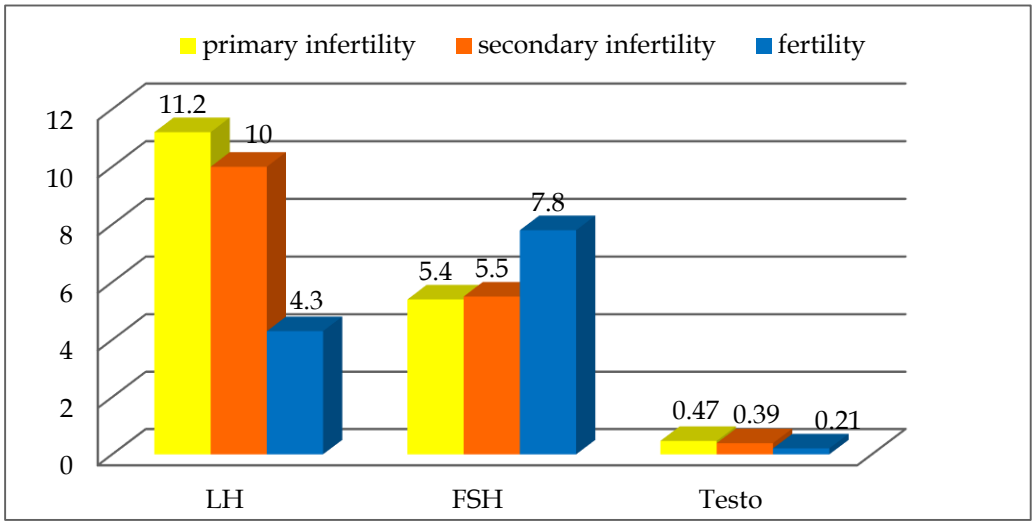

Figure 5:- The mean levels of LH,FSH and Testosterone hormones within groups of fertile.

Also found when classified the groups depended on fertility the highest BMI was in primary infertility group (31.8±4.3) k/m^2 than secondary infertility (30.1± 5.4) k/m^2 compare to fertility group (26.7 ± 3.6) k/m^2.
We discovered that the secondary infertility group had data that demonstrated statistically significant reductions in PCOS symptoms, but did not reach the normalize value.

Correlation Study

Correlations study between markers ZAG & HSP70 with all of the studied variables were estimated, by using Pearson's correlation coefficient (r) for the evaluation of data, the strength of Pearson correlation describe as

0.2-0.39 ------ low
0.4-0.69 ------- moderate
0.7-0.89 ---------high

shown in (Table 3). If the value of the correlation coefficient is negative sign, then this indicates the existence of an inversed relationship between the variables (negative correlation) While, the positive sign indicates direct proportion (positive correlation) **(Pearson's Correlation Coefficient, 2019).**

We found during this study that the correlation between HSP70 levels and IR that significant positive correlation with IR, insulin levels as shown below in table 3

Table 3:- The correlation between HSP70 and Parameters of groups.

Parameters	HSP 70	
	P-Value	**r**
BMI (K/m^2)	0.881	0.022
LH	0.771	0.042
FSH	0.66	-0.062
Testo	0.37	-0.128
HbA1c	0.85	0.03
FBG	0.354	0.134
FInsulin	0.003	0.414**
IR	0.021	0.326*

In these results found the concentration of HSP70 and FSH hormone were negatively in Pearson's correlation coefficient (r) was (-0.062) but not in significant p-value.
Result in the study was positive correlation HSP70 with IR and fasting insulin as shown in figures 6,7 respectivly .

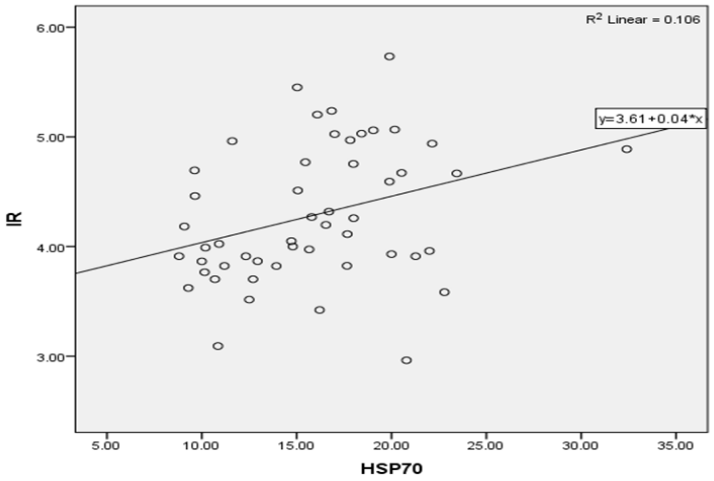

Figure **6:- Correlation between HSP70 level & IR in patient s group**

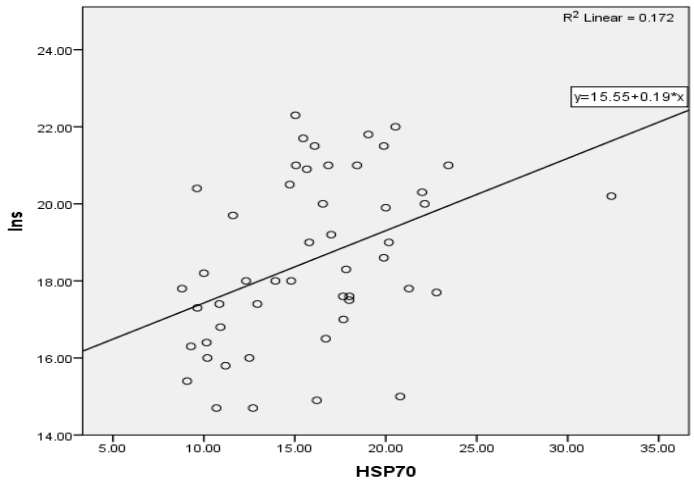

Figure 7:- Correlation between serum HSP70 & Insulin level in patients

Positive correlation between HSP70 levels with increased levels of other parameters but not significant value. Excepting with Insulin and IR . Finding also in the study No correlation between HSP70 and obesity.

Receiver Operator Characteristics (ROC) Curve

ROC analysis of serum HSP70

The ROC analysis for serum HSP70 show can detected the syndrome as in figure 3.17.The cut-off value of HSP70 concentration in PCOS with primary infertility was 12ng/mL with a sensitivity of 83.3% and a specificity of 80% and the area under the curve (AUC) was 0.876, while the cut-off value of HSP70 concentration in secondary infertility was 10.1ng/mL with a sensitivity of 77.8% and specificity 64% and the area under the curve 0.817 as shown in the table 3.6 and shows in figure 8 .

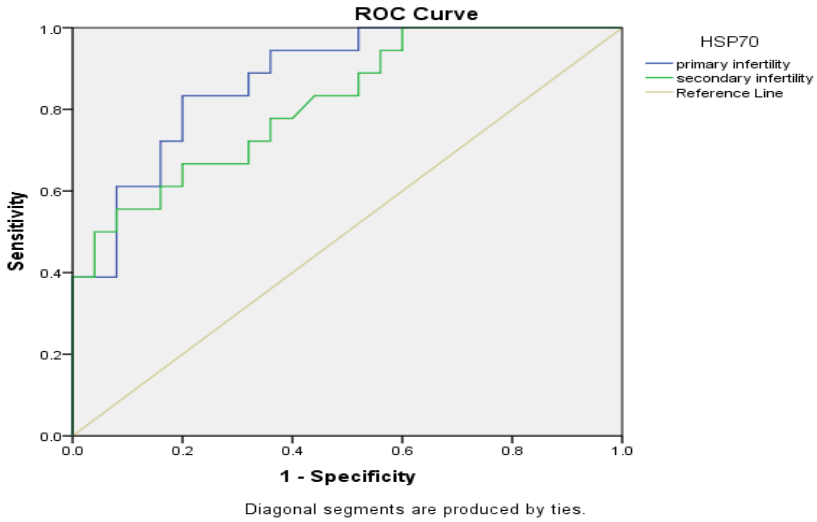

Figure 8:- ROC curve of HSP70 ng/ml in women within groups in infertility PCOS compared to control subject

Table 4:- Sensitivity and specificity of HSP70 ng/ml in women within groups of infertility PCOS compared to control subject

Infertility	Sensitivity	specificity	cutoff value	AUC
Primary infertility	83.3%	80%	12,0	0.876
Secondary infertility	77.8%	64%	10.1	0.817

Discussion

PCOS, or polycystic ovarian syndrome, is the most common cause of anovulatory infertility, affecting 6-15 percent of women of reproductive age **(Gao et al., 2013)**. The PCOS was diagnosed by Rotterdam criteria, which state that PCOS is present if any two out of the following three criteria are met: oligoovulation–anovulation, excess androgen activity or polycystic ovaries (deter-mined by gynaecologic ultrasound, and after exclusion of other androgen excess disorders.

According to the presented data in table 1 show mean of HSP70 levels (15.8± 4.77ng/ml) (9.7 ±3.7ng/ml) for patients and controls group respectively which significantly increased in patients group of PCOS in ($P<0.001$) and this results agreed with study Increased blood Hsp70 levels in PCOS patients and linked to a combination of IR, oxidative stress, and limited chronic inflammation in PCOS patients, indicating that Hsp70 may play a role in the pathophysiology of PCOS **(Gao et al., 2013)** while, Another study was Disagree with our results showed that HSP70 was significantly higher in ovarian tissues in PCOS rats than the controls, but significantly lower in serum **(Wu et al., 2019).**

our finding results that elevated high significant in biomarkers measured in hormonal LH, Ratio LH/FSH and testosterone level this agree with study (**Malini and Roy George, 2018)** that's PCOS and infertility are caused by an unusual increase in LH levels, as well as increased levels of ovarian testosterone and the LH/FSH ratio. And highly significant increase of metabolic biomarkers FBG, FIns , BMI, HOMA-IR in patients group compare to healthy group, This results were agreed with study **(DeUgarte, Bartolucci and Azziz, 2005)** that PCOS was defined as the presence of two or more of the following symptoms: oligo- or anovulation, and hyperandrogenemia, and polycystic ovarian cysts on ultrasound, according to the Rotterdam consensus workshop also obesity, insulin resistance, and hyperinsulin- emia are all common symptoms of PCOS.

Additionally some causes of increase LH level that, insulin was shown to clearly increase androgen production from theca cells resulting in an increase testosterone levels, an androgenic ovarian environment, and anovulation (resulting in low progesterone levels), Increased leptin levels due to an excess of adipose cells affect Gonadotropin-releasing hormone (GnRH) secretion and these correlated with fertilization rate **(The ObG Project, 2019).** Also finding high significant decrease in hormone FSH in patients and this result agreement with study **(Malini and Roy George, 2018)** which deficient FSH levels participate to impaired follicular development in PCOS.

In this study we finding The percentage of hirsutism in the group of patient women with PCOS was high, reaching 68%, while the percentage of patients without hirsutism was 32%. From patients in the study. Many studies were agreed with research **(P.R., M. and M., 2019)** that it's been PCOS is the most common reason of hirsutism, which is characterized by excessive hair growth in androgen-dependent areas PCOS is the most prevalent cause of androgen excess. Another

study was agreement with our results PCOS is the leading cause of hirsutism, with 72 to 82 % of cases **(Bode, Seehusen and Baird 2012) .**

Finding the proportion of patients for each body mass index category as normal weight has the lowest percentage 14 percent of patients groups, over weight (pre obesity) was 36 percent of patients, and obesity has the highest percentage 50 percent of patients groups Several investigations and researches have come to an agreement on discovering research outcomes, such as (**Diamanti-Kandarakis , 2007 ; Al-Tu'ma et al., 2015)]** That were covered to be a common problem feature on PCOS women which A high proportion of female with PCOS are obese and Obesity and abdominal fat accumulation worsen the physiological, hormonal, and metabolic symptoms of Polycystic ovary syndrome PCOS .

Another study that PCOS women with high levels of TT and LH had a metabolic defect, as obese and overweight PCOS women. PCOS women had a higher prevalence of overweight/obesity **(Liu et al., 2019).** One of the reasons why women with PCOS gain weight or have a hard time reducing weight is the inability of insulin to work correctly.

PCOS is the main cause of infertility due to anovulation. Therefore, the study groups were divided according to fertility, to observe the effect of the severity of the syndrome on the degree of fertility **(Bellver et al., 2017)]**.

When the groups were classified based on fertility, the highest BMI was found in the primary infertility group, which agreed with the study that a higher body mass index (BMI) is associated with a more complex treatment plan for fertility compared to normal-weight women with the syndrome, which is one of the leading causes of infertility **(Cena, Chiovato and Nappi, 2020).** Also Finding elevated testosterone levels in PCOS patients compared to the control group in p-value P<0.05 can worsen abdominal obesity and inflammation. Our findings agree with a research that found that patients with PCOS who had a high BMI are more likely to develop androgen excess functional problems **(Yuan et al., 2016).**

In this study after dividing the groups according fertility degree(primary infertility, secondary infertility, fertility) that the hormonal disorder in primary is higher than secondary infertility in patient groups and this was agreed with study done by AL Balen et al that increased luteinizing hormone levels (LH) in the blood have a negative effect on fertility **(Balen et al., 2016).**

The mean HSP70 levels was increased significantly in PCOS women groups of primary infertility and secondary infertility groups than control group(fertility group) in p< 0.001 value this results was agreed with study performed by [35]**(Sisti et al., 2015)** induction of heat shock protein production and activation When cells are under a variety of stresses-elevated temperature, hypoxia, inflammation, infection, oxidative stress, and nutrient deprivation.

Also Induction of Hsp70 in the ovary might explain why women with polycystic ovarian syndrome have lower fertility [35]**(Sisti et al., 2015)**. Result in the study was positive correlation HSP70 with IR and fasting insulin that's agree with study which increased blood HSP70 levels are linked to a combination of IR, oxidative stress, and limited chronic inflammation in PCOS patients [22]**(Gao et al., 2013).** Another study agreed with finding

There is a positive correlation between leptin and HSP70 in chronic inflammation, such as type 2 diabetes. It's possible that the link between serum HSP70 and leptin indicates a greater level of chronic inflammation [36]**(Nakhjavani et al., 2013).** There was a positive correlation between serum leptin, BMI, and insulin in PCOS. The mean BMI, LH, and LH: FSH ratios were all higher in PCOS women. PCOS women had

significantly higher levels of androgen as well as fasting insulin. LH hormone synthesis is stimulated by leptin [37]**(Chakrabarti,2013).** Finding also in the study No correlation between HSP70 and obesity this disagree with the study show HSP70 concentration inversely correlated with BMI, percentage body fat, waist circumference [38]**(Islam et al., 2014).**

Folate supplementation reduced plasma homocysteine while increasing glutathione levels, These changes indicate a reduction in oxidative stress, which decrease in serum Hsp70 in patient with type 2 DM **(Hunter-Lavin et al., 2004) .**the same mechanism is found in patient with PCOS use folate to decrease homocysteine level in blood **(Gholinezhad-Chari, Esmaeilzadeh andGhadimi,2017)**

These result of ROC analysis was agreed with study of **(Gao et al., 2013)** that explained the predictive value indicated by ROC curves research on Hsp70 level shows that it should be an important risk factor and may have clinical significance for PCOS.

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