Role of Resistin and Follistatin hormones on oocyte quality and embryonic development of PCOS in samples of Iraqi women undergoing IVF/ICSI program

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Background: Infertility has increased significantly ,one of infertility causes is polycystic ovarian syndrome(PCOS) which is believed to be connected to a dysfunction in the maturation of follicle development that results in anovulation. Resistin and Follistatin are being investigated for its involvement in PCOS, though there is debate as to its direct role in this infertility disease.

Aim of study: To clarify the correlation between resistin and follistatin hormone levels with oocyte quality, and embryonic development in PCOS and non-PCOS women undergo IVF/ ICSI program and compare their outcome.

Patients and Methods: This was a prospective study that performed in the Higher Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. The duration of the study extended from November 2022 to April 2023. It included 80 infertile women were udergoing IVF/ICSI program. They were divided, based on the cause of infertility into a control (non-PCOS) group (40 women) and PCOS group(40 women) each group was categorized into two subgroups (20 for each) according to their BMI into normal and overweight / obese. Human serum follistatin and resistin concentrations were obtained and measured at day 2 of menstruation and after collection of follicular fluid and serum at day of oocyte pick up(OPU) using ELIZA method .The correlation between resistin and follistatin hormones with oocytes count and maturation ,embryonic development was accounted .

Results: In this study, 38.8% of study participants were got pregnant. Means of serum resistin and follistatin in day 2 and at day of OPU were significantly higher in patients with PCOS and normal BMI level compared to other study groups. No statistical significant correlation was recorded of resistin with oocyte count .No significant correction was found between resistin and follistatin with embryonic development and fertilization rate . There were positive correlations found between age and both of resistin follicular fluid. at OPU and serum follistatin level at OPU. Positive correlation was detected between LH and oocyte count with follicular fluid follistatin at day of OPU.

Conclusion: It is concluded from the data of present study that positive correlation was noticed between oocyte count and FF follistatin at day of OPU . As a result, resistin and follistatin may play important roles in the development of PCOS and might serve as a useful biomarkers for its treatment.

Keywords: IVF/ICSI, infertility, resistin, follistatin. Oocyte quality ,embryonic development

Introduction

Polycystic ovary syndrome (PCOS) is regarded to be as the most prevalent endocrinopathy in women of reproductive stage of life with an range of occurrence

from 8–13% [1]. The World Health Organization (WHO) data lead to the belief that about 116 million women (3.4%) are affected by PCOS globally [2].

Its pathogenesis consist of insulin resistance(IR) and hyperandrogenism (HA), which motivate the reproductive (menstrual dysfunction, infertility), metabolic (metabolic syndrome, diabetes, cardiovascular risk factors), and psychological (anxiety, depression, low quality of life) complexity, given its widespread and variable features throughout the lifespan, in addition to the high prevalence of obesity, which exacerbates its clinical features [2].

The pathogenesis is not well known, but evidence suggests that aberrant ovarian angiogenesis, caused by a mismatch between several angiogenic markers, take role in the etiology of PCOS [3].

Other than causing infertility, PCOS is related to obesity, IR , type-2 diabetes, dyslipidemia, cardiovascular diseases, hepatic steatosis, and endometrial cancer [4]. Nevertheless, IR is not necessary needed for PCOS diagnosis, as it is not always found in all PCOS women.

It was discovered that excess adiposity and increased adipokine production from adipocytes could be a link between IR and ovulation disturbances [5]. Resistin, a 12.5 kDa cysteine-rich protein from adipocytes, is thought to be a strong candidate for linking IR to an increase in adiposity. Circulating resistin levels in the blood are significantly higher in IR mice and genetically or diet-induced obese mice. Furthermore, dehydro-epiandrosterone has been shown to increase resistin expression, implying that resistin and androgen synthesis, a common condition in PCOS, may be correlated. [4].

Follistatin, a member of the transforming growth factor-b superfamily, is another type of adipokine that acts as an important regulator of follicular development and has been identified as a potential genetic marker for PCOS. Follistatin initially identified and isolated from follicular fluid due to its suppression of pituitary FSH secretion [6].

Lately, follistatin has been counted to have a role in the etiology of PCOS, as women diagnosed with this disorder presented with increased follistatin concentrations unlike healthy women , independently of body mass index (BMI) [7]. It had been observed that circulating levels of follistatin and resistin, whatever was the BMI of the woman , are higher in those with PCOS in comparison with non-PCOS women, showing that these adipokines may take a part in the pathology of PCOS [4]. The present study was designed to clarify the correlation between resistin and Follistatin hormone levels with oocyte quality, embryos development in PCOS and non-PCOS women undergo IVF/ ICSI program and compare their outcome.

Patients Materials and Methods : This study was performed for (80) infertile couples, who had attended the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University in Baghdad-Iraq. The duration of the study extended from November 2022 to April 2023.

3.1: Patients

The study was aimed to be a prospective study. The sample size was 80 infertile women undergoing intra-cytoplasmic sperm injection (ICSI). All females were assessed in the Females' Infertility Clinic. The women ages included were ranged from 18 to 40 years' old which complain of primary and secondary infertility for a period more than >1→16 years. The selected women were by design divided according to the cause of infertility into:

1. Control group which included 40 women which were with no signs and symptoms of PCOS, with regular cycles, and no endocrine abnormalities with primary or secondary infertility. They were undergoing ICSI cycle, and were divided into two subgroups (20 for each) according to their BMI into:
   - Normal weight (< or equal to 25 Kg/m ^{2})
   - Over weight / obese (>25 Kg/m ^{2}).
2. The PCOS group (no=40) represent women suffered the sign and symptoms of PCOS including hormonal, endocrine disturbance, classical picture of cystic ovarian changes in the U/S, with primary or secondary infertility, that group also subdivided into two subgroups (20 for each) in according to their(body mass index) BMI as mentioned above .

   All women undergoing ICSI cycles by antagonist protocol as it is shorter, less coast and patient with PCOS cannot have agonist protocol as it's possible complication with ovarian hyperstimulation syndrome (OHSS) as it involves the induction of both the endogenous LH surge and ovulation which leads to more mature follicles recruitment and more risk of OHSS, so we choose antagonist protocol to be standardized to all patients. The study was approved by the Medical Ethical Committee of the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, AL-Nahrain University. All subjects received a full clarification of the goal of the study and signed the consent form**.** The diagnosis of PCOS depends on attain at least two of three criteria, relayed on the Rotterdam ESHRE/ASRMS sponsored PCOS consensus workshop group[8]

Inclusion criteria:1-The women diagnosed with PCOS had at least two of Rotterdam criteria, based on Rotterdam consensus meeting (2003).
2-Women have written consent to participate in the study.3-Age: Women age of (18 - 40) years.4-Couple with mild male factor (e.g. Asthenospermia and oligospermia).

- Exclusion criteria:1-Endocrine pathology (Hyperprolactinemia, thyroid disease, DM, Cushing syndrome).2-Congenital malformation of female genital tract.3-Women whose follicular fluid was bloody during the oocyte retrieval because it effects on the quality.4-Cycles with no oocytes (empty follicles) were retrieved on the day of aspiration.5-Women age more than 40 years old.6-Women with serum FSH levels more than 12mIU/ml, as these patients with high.7-FSH may be highly consider poor reserve / responder**.** The couple underwent a complete history with physical examination in attempt to find the factors that may be the cause of infertility.

All women had been tested for basal serum FSH and LH levels in the second or third day of a spontaneous or induced menstrual cycle using Mini VIDAS apparatus .Day two serum Resistin and Follistatin measurement before the program of ovulation induction. In the day of oocyte retrieval serum and follicular fluid also were

assembled to measure the two markers. Hysterosalpingography was done to the patient or if she had it already to exclude tubal blockage or congenital malformations of the uterus like the septate uterus, the fibroids, and intrauterine polyps can reduce the implantation rate. Vaginal ultrasound scanning used for screening the morphological appearance of the ovaries with measurement of the ovarian volumes, and the antral follicle count (AFC).The uterine assessment for the presence of abnormalities, with the estimation of the endometrial thickness[9].

-Male evaluation

The male partner evaluation was performed by the urologist in charge at the Male Infertility Clinic. A part from history and physical examination, a complete semen analysis was done to measure sperm concentration, motility, and morphology as recommended by WHO (2021).

Controlled ovarian hyper stimulation protocol

Using GnRH antagonist protocol, it was started on menstruation cycle day (2-3) by Recombinant FSH 75IU in a solvent-prefilled syringe of Follitropin alpha as active ingredient injection, or human menopausal gonadotropin HMG corresponding to 75 IU LH+ 75 IU FSH (menogon®/ menopure®)with menotrophin as active ingredient presented as powder and solvent for injection .They administrated daily with a dose of (150-300)IU /day .In PCOS patients may decrease the FSH dose to 35IU in sever PCOS cases(even more than 20 follicle each ovary) , other patients normal or older than 35 may start with HMG dose 150 – 300. The GnRH antagonist as cetrotide®, or as Cetrolix Acetate 0.25mg injection to prevent premature LH surge with a short time and less cost in the stimulation protocol[10].

Oocyte retrieval

When at least three follicles are 18 mm with serum E2 measured (around 100-150 pg/ml for follicle), HCG was given to trigger and induce follicle maturation. Then after 34-36 hours of trigger administration, aspiration of oocytes was done by trans-vaginal ultrasound**.**

Intra cytoplasmic sperm injection (ICSI) procedure

Aspirated FF was handled to the embryologist to recognize the number and the quality of the retrieved cumulus-oocytes complexes. ICSI was done by the clinical embryologist 3-5 hours after oocyte aspiration in the laboratory. The denudation was performed by exposure to a buffered medium consisting of (40 IU/ml) hyaluronidase for enzymatic removal of corona cells and cumulus[11]. The oocyte maturation was evaluated by noticing the presence or absence of the germinal vesicle, and the first polar body. Next to ICSI procedure, the oocyte examined the next day to account the fertilization rate. The embryo quality and development was checked, according to[12].

Embryo transfer was done without anesthesia at day two (four cells embryos), day three (six-eight cell embryo), only G1-G2 embryos were transferred post-ICSI

depending on women's age, embryos quality, and number of embryos available. Embryo transfer was done according to the Malekkheili (2022)[13].

Progesterone therapy prescribed to all women for the luteal phase support, in form of Cyclogest (Actavis, Barnstable, UK) ® 200- 400 mg twice/day, or (Crinone, ® 8% progesterone gel, Merk) transvaginal, and IM, treatment started from the day of oocyte retrieval until pregnancy test was performed. The pregnancy test was performed after 14 days' post ET. Luteal phase support was continued up to 12 weeks of gestation. Vaginal ultrasound examination was done (6 - 7) week s, after the embryo transfer to confirm clinical pregnancy.

Blood sampling and hormones assays: From each woman participated in the study LH and FHS hormones levels were measured by] using mini VIDAS apparatus Hat day 2 or 3 of the cycle .Serum resistin and follistatin assayed by ELISA obtained from the collected samples .Again at the day of oocyte retrieval (oocyte pick up=OPU) both of the follicular fluid and blood serum were collected and measured both hormones .

Statistical analysis: Analyzed of data were done by SPSS version 26. The data presented as mean, standard deviation and ranges. Categorical data presented by frequencies and percentages. ANOVA, were used to compare the continuous variables accordingly for normally distributed data. Chi square test was used to determine the relationship between study groups and ICSI outcome, while the Fisher exact test was used when the expected frequency was less than 5.ROC curve analysis was used for determination follistatin level at day 2 for occurrence of pregnancy after ICSI. Pearson's correlation test (r) was used to assess correlation between continuous variables accordingly.

Results

1. Comparison of resistin and follistatin levels between the four study groups

Table -1 shows the comparison of resistin and follistatin levels between the four study groups. Means of serum resistin and follistatin in day 2 and at day of OPU were significantly higher (Resistin: P= 0.024 and P=0.027, follistatin: P=0.002 and P=0.003, respectively) in patients with PCOS and normal BMI level compared to other study groups.

At the same time the statistical analysis did not found a significant ($P \geq 0.05$) differences regarding the levels of resistin and follistatin in the follicular fluid between study groups.

Table 1: Comparison of resistin and follistatin levels between study groups

Hormonal Levels in FF and Serum	Study groups				P – Value
	A PCO + ↑ BMI	B PCO + ↔ BMI	C No PCO + ↑ BMI	D No PCO + ↔ BMI	
Resistin (pg/ml)	**Median**	**Median**	**Median**	**Median**	
At Day 2	1519.77	4145.69	3935.11	1375.4	**0.024 ***
FF (OPU)	2104.88	6485.92	2964.57	4044.63	**0.357 ***
Serum(OPU)	2141.43	5831.38	2582.32	1574.48	**0.027 ***
Follistatin (pg/ml)					
At Day 2	9.73 ± 7.0	15.12 ± 8.6	7.97 ± 4.0	6.22 ± 5.9	**0.002 ****
FF (OPU)	18.67 ± 9.2	23.86 ± 17.9	16.4 ± 12.7	13.83 ± 6.4	**0.126 ****
Serum OPU)	12.57 ± 7.5	19.29 ± 7.9	11.77 ± 7.8	8.76 ± 6.6	**0.003 ****

* Kruskal-Wallis test ,** One-way ANOVA test FF: follicular fluid

2.Correlation of resistin and follistatin levels with certain demographic characteristics

-Age and BMI: Correlation between resistin and follistatin levels with age and BMI is shown in table -2. Positive correlations were detected between age and both of resistin follicular fluid at OPU (r= 0.265, P= 0.022) and serum follistatin level at OPU (r= 0.285, P= 0.01). Whereas, no statistical significant ($P \geq 0.05$) correlations detected between all other parameters.

- Hormonal level: Correlation between resistin and follistatin levels with FSH and LH was shown in table-3. Positive correlation was detected between LH and serum follistatin level at OPU (r= 0.279, P= 0.03). However, there was, no statistical significant correlations detected between all other parameters ($P \geq 0.05$).

Hormonal aspects	Age (Year)		BMI (kg/m^2)	
	R	P - Value	r	P - Value
Resistin (pg/ml)				
At Day 2	0.095	**0.402**	0.17	**0.132**
At OPU- FF	0.265	**0.022**	- 0.134	**0.237**

At OPU-serum	0.009	**0.937**	- 0.173	**0.124**
Follistatin (pg/ml)				
At Day 2	0.172	**0.128**	0.015	**0.895**
At OPU- FF	0.065	**0.566**	0.114	**0.313**
At OPU-serum	0.285	**0.01**	- 0.014	**0.902**

Table 2: Correlation between resistin and follistatin levels with age and BMI

Table 3: Correlation between resistin and follistatin levels with FSH and LH

Hormonal aspects	**FSH (mIU/mL)**		**LH (mIU/mL)**	
	R	**P - Value**	**r**	**P - Value**
Resistin (pg/ml)				
At Day 2	- 0.044	**0.74**	- 0.123	**0.35**
At OPU- FF	- 0.159	**0.224**	0.09	**0.492**
At OPU-serum	- 0.013	**0.924**	- 0.029	**0.827**
Follistatin (pg/ml)				
At Day 2	0.173	**0.186**	0.237	**0.068**
At OPU- FF	- 0.017	**0.897**	0.104	**0.431**
At OPU-serum	0.09	**0.492**	0.279	**0.03**

3. Comparison between resistin and follistatin levels with oocyte characteristics, fertilization rate, and embryonic development.

The correlation between resistin and follistatin levels with oocyte characteristics, fertilization rate, and embryonic development was shown in Table -4. There was a positive correlation between oocyte count and follistatin in follicular fluid at OPU (r= 0.378, P= 0.004). Whereas, no statistically significant ($P \geq 0.05$) correlations were detected between all other parameters.

Table 4: Correlation between resistin and follistatin levels with oocyte characteristics and fertilization rate

Biomarker / Variable		Resistin (pg/ml)			Follistatin (pg/ml)		
		Day 2	FF (OPU)	serum (OPU)	Day 2	FF (OPU)	serum (OPU)
Oocyte	r	- 0.037	- 0.042	- 0.013	- 0.019	0.378	0.2
	P - Value	**0.785**	**0.756**	**0.925**	**0.887**	**0.004**	**0.136**
MII	r	- 0.087	0.017	0.026	- 0.016	- 0.082	- 0.042
	P - Value	**0.518**	**0.901**	**0.844**	**0.903**	**0.538**	**0.757**
Fertilization rate (%)	r	0.037	- 0.063	- 0.118	0.205	0.063	0.056
	P - Value	**0.779**	**0.638**	**0.373**	**0.119**	**0.634**	**0.673**
Embryonic development	r	-0.133	0.02	- 0.067	0.004	- 0.039	- 0.091
	P - Value	**0.338**	**0.885**	**0.63**	**0.987**	**0.78**	**0.513**

Discussion

1. Resistin and follistatin levels in study groups.

In this study (table-1), median of serum resistin on day 2 and at the day of OPU were significantly higher in patients with PCOS and normal BMI that's to say it is more significant, next is patients with non-PCOS with high BMI, then patients with PCOS high BMI and non-PCOS normal BM almost share the same level.

Regarding to resistin, it has been seen that morbidly obese people express more resistin in their adipocytes than lean people do, there may be a relation between insulin resistance and obesity[14, 15] In addition, in the adipocytes from women with PCOS, the mRNA level expressed for resistin is 2-fold higher than that in healthy women[16]. It has been found that Obese PCOS women with BMI > 25 kg/m2 compared with non-obese PCOS and non-obese healthy women have higher resistin levels. In mean while, no difference in resistin levels was reported between patients with PCOS and normal BMI. Furthermore, dehydroepiandrosterone has been shown

to increase resistin expression, suggesting that resistin and androgen synthesis, a common condition in PCOS, may be related [4]. The findings of current study suggest that resistin may play a role in PCOS and its clinical manifestations.

Mean of serum follistatin in day 2 and day of OPU were higher in PCOS with normal BMI group, and with high BMI respectively. This is understandable because activins stimulate pituitary FSH secretion, follicular development and function, as well as inhibit thecal cell androgen production[17], whereas follistatin is known to inhibit FSH secretion and to regulate folliculogenesis and ovarian steroidogenesis, mainly by modulating activin activity [18]. As a result, an increase in follistatin may halt follicular development while increasing ovarian androgen production, both of which are important in PCO patients.

It has been stated that Independent of obesity status, circulating levels of resistin and follistatin were higher in women with PCOS compared to controls, indicating that these adipokines may contribute to the pathology of PCOS[4].

-Correlation of resistin and follistatin levels with different factors

Regarding correlation with age and BMI, weak positive correlations was revealed between age and both serum follistatin and FF resistin (Table 1), while no significant correlations detected between all other parameters. In contrary, women enrolled with PCOS and had ICSI trail had no correlation between serum or follicular resistin levels and BMI [19]. Serum follistatin may act as a potential screening biomarker of PCOS regardless of age and/ or BMI[20].

Patients with PCOS may produce more ovarian androgen as a result of elevated resistin levels. No association between serum or follicular resistin levels and E2, LH, or testosterone has been proposed [21].

Although there was no evidence of a positive or a negative impact of resistin on pregnancy outcomes, it is possible that in PCOS women, the hormone may have a detrimental effect that is the same as the effect of LH. Additionally, it is unknown whether giving a GnRH analogue for ovulation induction lowers the levels of follicular resistin and whether aspirating the follicular fluid first would be preferable [21]. In fact, by blocking the ovum maturation inhibitors in the early phases of normal menstruation, LH is necessary for the structure and function of developing follicles. The LH peak may also stimulate the ovum to resume meiosis and reach final maturation [22].

About correlation between resistin and follistatin levels with oocyte characteristics and embryonic development (Table 3), it has been found a weak positive correlation between oocyte count and follistatin in follicular fluid.

It has been stated that resistin is not thought to be involved in the maturation and development of oocytes in PCOS, based on the fact that there is no correlation between serum or follicular resistin levels and fertility rate, pregnancy rate, or early miscarriage rate[21].

Whereas, Elevated follistatin levels in PCOS women's FF may inhibit granulosa cell proliferation, leading to failed cumulus expansion and subsequent ovulatory

dysfunction, as well as inhibition and glucose metabolism, resulting in abnormal glucose metabolism [23]. In turn, the present work results not recorded a significant difference in fertilization process and embryonic development in spite of a significant increase in oocytes count.

Interestingly, During the IVF/ICSI cycle, follistatin levels go up in the opposite direction of Activin-A. This is clear because most circulating follistatin binds to activin A with high affinity and irreversibly, neutralizing Activin activity. Therefore, the level of follistatin increased and activin A hormone decreased through luteal phase and following embryo transfer[24]. mechanism explained the reason of increase the follistatin in luteal phase of current study[25].

Conclusion: It is concluded from the data of the present study that no effects and correlation were found between resistin and oocyte quality and embryonic development while positive correlation was noticed between oocyte count and FF follistatin at day of OPU.

Conflict of interest: The authors state that they do not have any conflicts of interest.

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