Association of Iron Status in Follicular Fluid with Pregnancy Outcomes in Infertile Women Undergoing IVF/ICSI

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Abstract

Iron status may influence the outcome of infertile women under the intracytoplasmic sperm injection (ICSI) technique of *in vitro* fertilization (IVF). The aim of this study is to evaluate iron status and ceruloplasmin ferroxidase activity in the follicular fluid (FF) and their association with IVF outcomes. The study enrolled fertile women with male cause infertility (n=25), infertile women with polycystic ovary syndrome (PCOS; n=21), infertile women with low AMH level (n=26), and women with unexplained infertility (UI; n=27), all undergoing IVF/ICSI. On the day of oocyte suction, the selection of FF samples was accomplished. Iron, ferritin, and transferrin levels, as well as ceruloplasmin (CP) ferroxidase activity, were measured in the FF. In the PCOS group, iron showed significantly higher level (P<0.05) as compared to the control and UI groups. In the PCOS group, ferritin showed significantly higher level (P<0.05) compared with the control group. In the PCOS group, transferrin showed significantly higher level (P<0.05) when compared with the UI group. Also, Cp. ferroxidase activity in the PCOS group showed a lower level, but non-significant difference, compared with the other groups. In conclusion, the increased iron level in the follicular fluid of women with PCOS may lead to decrease pregnancy success after applying IVF protocol.

Keywords: Iron, Ferritin, Transferrin, Ceruloplasmin ferroxidase activity, follicular fluid, *in vitro* fertilization.

ارتباط حالة الحديد في السائل الجريبي مع مخرجات الحمل في النساء العقيمات اللاتي يجرين التلقيح الخارجي

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الخلاصة

قد تؤثر حالة الحديد على نتائج الإخصاب في النساء المصابات بالعقم اللواتي يخضعن للتلقيح الخارجي / الحقن المجهري. الهدف من هذه الدراسة هو تقييم حالة الحديد وفعالية سرولوبلازمين فيروكسيداز في السائل الجريبي, وارتباطهما بنتائج التلقيح الخارجي. تضمنت الدراسة نساء سليمات مع سبب عقم من الذكور (n = 25) ، والنساء المصابات بمتلازمة تكيس المبايض ؛ (21 = n) ، والنساء المصابات بالعقم مع انخفاض مستوى هرمونAMH (26 =n) ، والنساء غير المعروف سبب عقمهن ؛ (27 = n) الخاضعات للتلقيح الخارجي / الحقن المجهري. تم جمع عينات السائل الجريبي في يوم سحب البويضات. تم قياس مستويات الحديد والفرتين والترانسفرين ؛ وكذلك ، فعالية سرولوبلازمين فيروكسيداز في السائل الجريبي في مجموعة

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متلازمة تكيس المبايض . أظهر الحديد فرقاً معنوياً اعلى (0.05 > P) مقارنة بمجموعة النساء السليمات ومجموعة النساء غير المعروف سبب عقمهن. في مجموعة متلازمة تكيس المبايض ، أظهر مستوى الفرتين فرقاً معنوياً (0.05 > P) أعلى مقارنة مع مجموعة النساء السليمات. في مجموعة متلازمة تكيس المبايض ، أظهر الترانسفرين فرقاً معنويًا (0.05 > P) أعلى عند مقارنته بمجموعة غير المعروف سبب عقمهن. أظهر فعالية فيروكسيداز في مجموعة متلازمة تكيس المبايض مستوى أقل لكن لا يوجد فرق معنوي مقارنة بالمجموعات الأخرى. يمكن الاستنتاج ، قد يؤدي ارتفاع مستوى الحديد في متلازمة تكيس المبايض إلى انخفاض نجاح الحمل بعد الخضوع لعلاج التلقيح الخارجي في مجموعة متلازمة تكيس المبايض.

Introduction

Infertility results from the reproductive system diseases that impair the ability of the body basic reproductive function. It is a failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse [1]. Treatment and evaluation may be justified based on physical findings and medical history, as specified by the American Society for Reproductive Medicine (ASRM), after six months for women older than 35 [2]. Theca and granulosa cells from the secondary follicles secrete a fluid that accumulates in the antral cavity or "antrum", which eventually surrounds the oocyte [3]. Such fluid is the product of blood filtration through the theca and additional secretions from both the theca and the granulosa layers, and it is called follicular fluid (FF) [1, 3]. Cumulus cells surrounding the oocyte nurture it with essential compounds obtained from the FF. The communication between mural granulosa cells and cumulus cells is achieved through secretions of extracellular vesicles to the FF. The oocyte completely depends on its maturation and it is in continuous contact with the FF. Therefore, variations in FF composition may affect oocyte development directly or indirectly [2].

Iron is a potent pro-oxidant and its elevated level can be a cause of oxidative stress in the body, rising type 2 diabetes (T2DM) and cardiovascular disease (CVD) risks [4]. Whereas, relatively high body store is associated with glucose tolerance [5]. Glucose tolerance is caused by the progressive accumulation of iron in the pancreas, suggesting that iron overload at any degree is associated with hyperinsulinemia, insulin resistance, and pancreatic β-cell dysfunction [6], that may lead to increase the inflammatory status associated with T2DM [7]. In addition, elevated iron shells are proposed to be consistent with both insulin resistance and metabolic syndrome, which are essential aspects of polycystic ovary syndrome (PCOS) [8].

In granulosa cells and oocytes, transferrin and its receptor have been identified. The immunocytochemical and molecular data suggest that some granulosa cells can synthesize their own transferrin which is translocated to and across the membrane for endocytosis by the oocyte [9]. Ceruloplasmin is the most abundant Cu-dependent ferroxidase enzyme with a Cu-dependent oxidation activity. Copper deficiency may lead to osteoporosis or reduced iron absorption and red blood cell production, which leads to anemia, growth reduction, and infertility [10].

The aim of this study is to evaluate iron status and ceruloplasmin ferroxidase activity as well as to explore their association with IVF outcomes.

Materials and Methods

Subjects

A prospective case control study was conducted at the IVF center in Al-Qema hospital in Baghdad – Iraq from November 2019 to January 2020. The study protocol was approved by the scientific committee in the College of Science and authorized by the University of Baghdad. Oral consent was obtained from the patients participating in the research. The panel included ninety-nine married women who were first admitted for IVF / ICSI and were subdivided into: 21 PCOS infertile women (mean age 27.71 ±5.1 years), 26 women with low anti-Müllerian hormone (AMH) level (mean age 31.23±6.4 years), 25 women who represent the control group because the cause of infertility was due to man (mean age 30.2±7.3 years), and 27 women with unexplained cause of infertility (UI) (mean age 30.44±7.2 years). The diagnosis of PCOS was achieved by a gynecologist physician as defined by the European Society of Human Reproduction (ESHRE) / American Society for Reproductive Medicine (ASRM) and the standards of Rotterdam European Society of Embryology and Human Reproduction [11]. Infertile women with PCOS cause under treatment with metformin. Patients were defined as having unexplained infertility (UI) based on the recommendations of the American Society Practice Committee for Reproductive Medicine, which is focused on normal infertility tests [12]. These

measures included semen, ovulation, hysterosalpingogram, and laparoscopy and ovarian reserve examinations. If the test results were normal, the patients would be admitted as UI.

The percentage of primary infertility is more than that of secondary infertility in the entire samples of all women, as follows: primary=72%; secondary=28% in the control group, primary=62%; secondary=38% in the PCOS group, and primary=61%; secondary=39% in the low AMH level group. However, in the UI group, the primary infertility percentage was 30% lower than secondary infertility, which was 70%.

Inclusion criteria were age 22–38 years and BMI 18–29 kg/m^2. The participants with the following criteria were excluded: history of any type of ovarian surgery, women with endometriosis, women under contraceptive treatment or supplement, and smoking.

All subjects were hyperstimulated by gonadotropin releasing hormone antagonist (GnRH-a) protocol. Decapeptyl (0.1 mg) was administered beginning on day 21 of the previous menstrual cycle for ovarian stimulation. After 1 to 2 days, ovarian stimulation protocol in IVF/ICSI cycles in patients of various age ranges commenced with 100 IU-450 IU of GnRH-a daily. The dosage of GnRH-a was adjusted according to ovarian response. Oocyte retrieval was performed 34–36 hours after hCG trigger. Embryo transfers were performed 3 to 5 days later using ultrasound guidance. Uncontaminated FF samples with flush medium or blood were collected and centrifuged at 600xg for 10min. The apparent FF supernatant was distributed into sterile tubes from Eppendroff and processed until used at -20 ° C.

Biochemical Analysis
Determination of Iron Concentration
Iron was measured on an automatic platform (Cobas C311, Germany). A colorimetric method was followed that measures the absorbance at 552nm of the colored complex of Fe^{+2}- ferrozine, which is produced from Fe^{+3} reduction by ascorbic acid [13].

Determination of Ferritin Concentration
Ferritin was measured on chemiluminescence immunoassay in an automatic platform (Cobas C311, Germany). The measuring cell in which the micro particles are captured on the electrode surface is aspired to the reacting mixture. A voltage was applied on the measuring cell, leading to chemiluminescent emission which was recorded by a photomultiplier [14].

Determination of Transferrin Concentration
Transferrin was measured on immunoturbidimetric assay by automatic platform (Cobas C311, Germany). Anti-transferrin antibodies react with the antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. Addition of poly ethylene glycol (PEG) allows the reaction to progress rapidly to the end point and increases sensitivity [15].

Determination of Ceruloplasmin Ferroxidase Activity
Ferroxidase ceruloplasmin (Cp) activity was measured according to Erel method (1998). The Cp. ferroxidase activity in FF was estimated by the end point measurement method. This system measures the changes of ferrous ion concentration in the reaction medium, which contains ferrous ions present in the substrate solution [16].

Enzyme activity (U/L) = $(C_1-C_2)/ t \times (V_t/V_s) = (C_1-C_2) \times 38.166$

C1: the substrate concentration at the beginning of the enzymatic reaction (60µm/L); C2: the substrate concentration at the end of the enzymatic reaction; Vt: total volume (1374 µl); Vs: sample volume (9 µl); T: the incubation time (4 min).

Protein Assay
The protein concentration in samples was calculated from the standard curve of bovine serum albumin (BSA, 7g / dL) using Biuret reagent [17].

Statistical management of the data
Analysis of data was performed utilizing the statistical package of SPSS-22 (Statistical Packages for Social Science –Version 22). Data were exhibited in simple measures, mean and standard deviation (±SD), with normal distribution. The significance of differences of various means (quantitative data from different groups and control group) were tested utilizing analysis of variance (ANOVA), whilst independent students' t-test was used for differences between two means. When the p value was less than 0.05, this was statistically considered to reflect a significant difference. While high significant of differences was considered whenever the p value was less than 0.01 [18].

Results
The IVF Outcomes
The present study showed a significantly higher number (P<0.05) in aspirated oocytes, meta-phase two (MII) oocyte, fertilized oocytes, and two-pronuclear zygote (2PN), of the PCOS group compared with the UI group and low AMH level group. Also, a significantly higher number (P<0.05) was observed in the transferred embryos in the PCOS group when compared with the UI group. In the low AMH level group, the G1 embryo showed significantly lower level (P<0.05) when compared with the PCOS group.

The numbers of aspirated oocytes, MII oocytes, and fertilized oocytes were significantly lower (P<0.05) in the low AMH level group than in the control. Non-significant (P>0.05) differences were found in the mean values oocyte maturation rate and cleavage rate between the four studied groups (Table- 1).

Table 1- The characteristics of IVF/ICSI outcomes in the unexplained infertility, PCOS, Low AMH, and control groups.

IVF/ICSI outcomes	Control group (n=25)	UI group (n=27)	PCOS group (n=21)	Low AMH level group (n=26)
Aspirated oocytes	9.25±4.75	6.26±3.18[a]	11.76±4.19[b,d]	3.95±2.43[a]
M II oocyte	9.00±4.99	6.15±3.27	11.19±4.45[b,d]	4.33±2.37[a]
Fertilized oocytes	9.04±3.84	6.19±2.23 [a]	11.76±4.19[b,d]	4.38±2.35[a]
Embryo at (2PN)	6.79±2.69	5.69±3.27	9.38±4.66[b,d]	3.66±2.08
Transferred embryo	3.8±1.5	4.88±1.3	4.7±1.9[b,d]	3.33±1.1
G1 embryo	3.6±1.9	3.87±2.6	4.0±2.12[d]	2.24±1.0
Oocyte maturation rate %	0.93±0.14	0.97±0.24	0.86±0.18	0.98±0.05
Cleavage rate %	0.65±0.28	0.8±0.26	0.62±0.25	0.81±0.23
Fertilization rate %	0.75±0.29	0.95±0.31[a]	0.8±0.23	0.87±0.19

ANOVA, the post-hoc test for multiple contrast was accompanied by [a] P<0.05 compared to control group and [b]P<0.05 compared with UI group. Regression analysis was done by the ANOVA test.; [c]P<0.05 compared with AMH group.

Iron Status and Ceruloplasmin Ferroxidase Activity
Iron showed significant higher level in the PCOS group when compared with the control group and UI group (Table-2). The mean ferritin level of FF in the PCOS group was slightly higher (P<0.05) than the control group. In contrast to the UI group, the mean amount of transferrin (TF) in FF was higher (P < 0.05) in the PCOS group. The average Cp ferroxidase activity in PCOS group was lower than that in the other groups, but with non- significant differences. In contrast with the controls and UI groups, PCOS group showed unique behavior of ceruloplasmin ferroxidase specific activity, demonstrating slightly lower levels (P<0.05).

Table 2- The mean (±SD) levels of iron status parameters and Cp. Ferroxidase activity in the unexplained infertility, PCOS, Low AMH, and control groups.

Parameters	Control group (n=25)	UI group (n=27)	PCOS group (n=21)	AMH group (n=26)
Iron (µg/dl)	48±14	43±20	63±28[a,b]	43±21
Ferritin (µg/dl)	38±11	39±16	48±16[a]	38±22
TF (mg/dl)	197±34	190±55	225±34[b]	180±47
Cp. ferroxidase activity (U/L)	524±50	482±100	474±131	485±66
Cp. Ferroxidase specific activity (U/g)	12.9±2.7	12.3±5.2	9.5±2.9[a,b]	10.7±2.3

[a]P<0.05 compared with control group; [b]P<0.05 compared with UI group.

Effects of Iron Status and Ceruloplasmin Ferroxidase Activity on Chemical Pregnancy

Each of the four groups was subdivided into pregnant and non-pregnant groups. The value of iron, as seen in Table- 3, was greater in the non-pregnant group in all classes, but the difference was significant only in PCOS. The average ferritin level was higher, but with non- significance, except for that of the low AMH level group. TF was dramatically low in pregnant as compared with non-pregnant PCOS patients. Although, in the UI and low AMH level groups, it was lower in the pregnant than non-pregnant group, TF was higher in the pregnant than non-pregnant control group. Mean Cp. Ferroxidase activity in pregnant women was higher in the control and UI groups than in non-pregnant women of the same groups. While, in PCOS and low AMH groups, this activity was lower in pregnant women but with non-significant difference.

Table 3- The results of FF Iron status and pregnancy outcomes in unexplained infertility, PCOS, Low AMH, and control groups.

Parameters		Control group (n=25)	UI group (n=27)	PCOS group (n=21)	AMH group (n=26)
Iron (µg/dl)	Pregnant	49.05±14.43	49.87±23.2	76±4.0*	52.11±21.64
	Non-Pregnant	45.67±13.03	42.66±24.29	63.66±24.8	40.38±23.28
Ferritin (µg/dl)	Pregnant	39.45±13.01	48.75±16.38	55.06±31.88	57.83±27.31*
	Non-Pregnant	38.09±9.83	35.5±14.75	47.2±16.5	27.34±8.83*
Transferrin (mg/dl)	Pregnant	197.59±28.72	183.87±57.51	190.33±18.03	162.55±39.03
	Non-Pregnant	196.33±40.19	193.66±54.14	225.15±42.95*	198.66±61.11
Cp. Ferroxidase activity (U/L)	Pregnant	526.08±60.77	521.37±38.71	400.33±151.84	477±30.43
	Non-Pregnant	523.5±42.46	516.88±36.2	473.61±115.79	505.77±37.46

Analysis performed by independent samples t-test; statistically significant when *$P<0.05$; no asterisk: $P \geq 0.05$ non-significant.

Discussion

In this study, all participants were subjected to the same ovarian stimulation protocol. Only PCOS group showed significant differences related to IVF outcomes, as well as iron, ferritin, transferrin, and specific activity of ferroxidase in this group. A study by Sanchez *et al*. (2014) observed that iron level in FF did not seem to affect embryo quality [19].

In the low AMH level group, AMH level was associated with low oocyte retrieval and poor embryo quality. AMH level is correlated with the number of oocytes retrieved after stimulation, and is the best biomarker for predicting poor and excessive ovarian response [20].

The number of aspirated oocytes and the number of fertilized oocytes were significantly lower in the UI group than in the control group, whereas fertilization rate was significantly higher , which disagrees with study of Wardah *et al*., (2018) that found non-significant differences in fertilization rates between the UI and control groups [21].

As hypothesized by Sanchez *et al*. (2014) in their study on follicular fluid from patients with endometrioma undergoing IVF, ferritin binds and oxidizes ferrous to ferric iron to be stored inside the ferritin cavity. Thus, depending on availability of iron, ferritin levels are modulated. Their study suggested that iron overload disturbs iron metabolism in the luteinized granulosa cells in proximal follicles, as demonstrated by high levels of ferritin and transferrin receptor protein 1 (TfR1) mRNA expression in these cells [19].

Iron metabolism is controlled by a series of transcriptionally regulated factors that coordinate the cellular defense against iron stress and inflammation by modulating the expression of the heavy (H)

subunits of ferritin and TfR1. Another role of ferritin is the control of the pro-oxidant activity of the metal and as an acute phase protein in response to inflammation, stress, and injury. Thus, its increase in the PCOS group may be either due to sequester iron overload in the FF or as a response to oxidative stress or inflammation [22]. In a previous work, total peroxide levels and oxidative stress index in the FF from women undergoing IVF showed higher values in the PCOS groups in comparison to control [23].

Some other evidence demonstrated that granulosa cells are capable to synthesize transferrin, which could be translocated to the oocyte. However, some data suggested that ovarian transferrin and transferrin receptors might not participate in local iron metabolism [24]. A previous study showed that transferrin level in FF was significantly increased in PCOS patients compared to the control group [25], which agrees with our study.

The Cp. Ferroxidase activity in the FF showed non-significant differences among the four studied groups, but showed a lower level in the PCOS group compared with the other groups. The mean value of Cp. Ferroxidase specific activity was lower, but non-significantly, in the PCOS group when compared with the control and UI groups. An earlier study indicated that the stress caused by oxidation leads to increase the levels of non-transferrin bound iron (NTBI) [26], which is taken up by the liver [27].The cellular increase of NTBI may be due to lower ferroxidase activity of CP. However, this activity is very important to convert Fe^{+3} to Fe^{+2} in order to bind to TF [28].

The mean level of ferritin in the FF of all groups was higher in pregnant than non-pregnant women, with a significant increase in the low AMH group. If the higher number of oocytes results in a higher number of available good quality embryos, it would be reasonable to expect a higher rate of positive pregnancy tests after *in vitro* fertilization treatment [29]. It was found that oxidative stress (reduced antioxidant capacity) is present in the FF of infertile patients, and it was correlated with poor-oocyte/embryo quality and low fertilization rates [30].

The mean level of TF in the FF of UI, PCOS, and low AMH groups was lower in pregnant than the non-pregnant women, except in the control group which showed higher levels in pregnant women. Also, there was a significant decrease of TF in pregnant women with PCOS. The total iron-binding capacity of serum (TIBC) has been observed to rise during the course of pregnancy. This rise in TIBC, coupled with a lowered percentage saturation of transferrin and a fall in hemoglobin concentration, has given rise to the concept of the "physiological anemia of pregnancy" [31].

The mean Cp. Ferroxidase activity for the control and UI groups was higher in pregnant than non-pregnant women, while in PCOS and low AMH level groups, it was lower in pregnant women. However, all these differences were non-significant. Pregnancy is linked with significant physiological changes, which increases the requirement of iron. Iron is required for additional erythrocyte production during pregnancy [32].

Conclusions

Since the follicular fluid is the surrounding environment of the ovum and its content may be useful as well as harmful, and since iron had an important role in providing oxygen to the oocyte, its increase in the follicular fluid leads to a reduction in the chances of pregnancy, as observed in the PCOS group, and thus increases the percentage of failure of the IVF process.

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