Serum iron status in association with pregnancy outcomes in infertile women undergoing IVF/ICSI

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Abstract. Iron status can affect the outcome of in vitro fertilization (IVF) in infertile women who undergoes this process. The aim of this study is to evaluate iron status, ceruloplasmin ferroxidase activity and their association with outcome of pregnancy prior to initiation of IVF/ICSI procedure. The participants were fertile women with male cause infertility (control; n=25), women with polycystic ovary syndrome (PCOS; n=21), women with low anti-Müllerian hormone level (AMH; n=26), and women with unexplained infertility (UI; n=27). Blood samples were obtained on the day of oocyte aspiration. Serum iron, ferritin, transferrin level, and ceruloplasmin ferroxidase activity were measured; the transferrin saturation, Total Iron Binding Capacity (TIBC), and Unsaturated Iron Binding Capacity (UIBC) were calculated. In the low AMH group, Ferritin showed a significantly lower level compared to the control and UI groups. In the PCOS group, ferritin, transferrin, TIBC, and ceruloplasmin ferroxidase activity were measured; the transferrin saturation, Total Iron Binding Capacity (TIBC), and Unsaturated Iron Binding Capacity (UIBC) were calculated. In the low AMH group, Ferritin showed a significantly lower level compared to the control and UI groups. Cp. ferroxidase activity in the PCOS group showed a lower level but of no significance compared to the other groups. In this study, it can be concluded that higher levels of iron, ferritin, and lower transferrin in pregnant PCOS women lead to increase chances of pregnancy following an IVF protocol.

Keywords: Iron, Ferritin, Transferrin, Ceruloplasmin ferroxidase activity, Infertility

1. Introduction

Infertility is the inability to achieve a successful pregnancy after one year or more of frequent unprotected sexual activity; it may also defined as the inability of pregnancy to conceive a live baby to birth [1]. Infertility has been estimated to affect about 186 million people around the world while male infertility leads to over half of all global infertility cases [2]. Ovulation disorders, including polycystic ovary syndrome (PCOS), hypothalamic dysfunction, ovarian insufficiency, tubal infertility, endometriosis and uterine and cervical causes are common etiology for female infertility [3]. In order to achieve the maximum targeted intervention and effective recovery, a comprehensive infertility diagnostic assessment is essential. The last resort approach for treating couples with infertility problems is by the in-vitro fertilization (IVF). The number of oocytes directly collected represents ovarian reactions and considers as most important clinical indicators for oocyte production, besides its ability to fully mature and successfully viable [4]. Several human studies have reported the presence of reactive oxygen species (ROS) in the female reproductive tract and their role in its physiological functions such as oocyte maturation, ovarian steroidogenesis and corpus maturation [5,6]. Also, ROS may be produced during embryo metabolism and its surroundings [7].

Iron is an element of crucial importance for a living cell and is found in a range of oxidation states; ferrous (Fe²⁺) and ferric (Fe³⁺). A study shows that women who do not receive enough iron can be affected by ovulation and likely poor ovulatory health, which can inhibit pregnancy at a rate 60% higher than women with adequate iron in their blood [8]. Previous hypothesis suggests that excess iron contributes to reduce in the development of the hormones LH and FSH, indicating impaired oocyte, low ovarian reserve, infertility,
and genetic diseases. Thus leads to increased iron levels in the blood, subfertility in females, as well as in patients with hemochromatosis [9].

Iron is an essential redox-active metal that has vital importance for the organism. It has pro-oxidant effects related to oxidative stress and inflammation. Iron is essential as a cofactor for the activity of several enzymes that catalyze redox reactions. Ceruloplasmin (Cp) is an example of such enzymes, it is a glycosylated multi-Copper (Cu), with iron oxidase activity (ferroxidase) which is synthesized mostly in the hepatic and carries 95% of the total Cu in the serum, and is also the predominant protein container in mammalian serum. Cp has the highest affinity for Fe²⁺ among other substrates [10]. Cp is a protein that responds to acute inflammatory phases, while an excess of Cu leads to an inflammatory response and an oxidative effect. This means that Cu has antioxidant and pro-oxidant impact [11,12]. In addition to Cp; ferritin and transferrin are important proteins for iron homeostasis. Ferritin is an intracellular iron storage protein that is a vital marker for iron resources [13,14]. On the other hand, transferrin, which is a glycoprotein in blood-plasma, plays a crucial function in transporting iron. It serves as the primary reservoir for ferric body. It passes iron into various tissues including liver, spleen, and bone marrow through the blood. It is a key biochemical marker of iron status in the body [15]. High transferrin levels are responsible for the formation of non-transferrin bound iron, a toxic form of iron that tends to induce oxidative stress [16]. A previous study found a higher level of ferritin in different types of infertility such as PCOS associated with obesity in addition to oligomenorrhea and less blood loss [17]. Another study linked infertility with anemia [18]. The aim of this study is to evaluate iron status and Cp ferroxidase activity in different types of infertility, and to explore their correlation with pregnancy outcomes as well as the success of pregnancy prior to the initiation of IVF/ICSI procedure.

2. Experimental section

2.1 Subjects

This study was approved by the Medical Ethics Committee at the University of Baghdad and a prospective case monitoring analysis at the IVF center in Al-Qema Hospital in Baghdad, Iraq. The study was carried out between November 2019 and January 2020. Oral consent was taken from the participants. The study involved 99 women who were prepared to undergo IVF / ICSI, which were classified into four groups; a control group (n=27) for women with male factor infertility (mean age = 30.44±6.2 years), a UI group (n=25) for women with unexplained cause of infertility (mean age = 30.2±7.3 years), a PCOS group (n=21) for infertile women with PCOS (mean age 27.5±5.1 years), and a low level of AMH group (n=26) for infertile women (mean age 31.23±6.4 years).

Women with PCOS were diagnosed by a gynecologist as described by European Society of Human Reproduction and Embryology (ESHRE) / American Society for Reproduction Medicine (ASRM) criteria in Rotterdam [19]. Metformin is used by infertile women with PCOS to control blood sugar. Patients considered as unexplained (UI) based on ASRM recommendations [20]. These tests included spermiogram, ovulation, hysterosalpingogram and ovarian reserve and laparoscopy tests. If the tests were normal, then patients will be admitted as UI.

The gonadotropin releasing hormone antagonist (GnRH-a) regimen was used in ovarian hyperstimulation in all subjects. This was carried out by administering Decapeptyl (0.1 mg) starting on day 21 of the previous menstrual cycle. After one to two days, a daily dose of 100 IU-450 IU of GnRH-a, was commenced according to their ovarian responses. A period of 34 to 36 hours after stimulation by the Human chorionic gonadotropin (hCG) is conducted. Using the ultrasound instructions, embryo transfers were performed 3 to 5 days later. On the day of oocyte aspiration, venous blood (5ml) was drawn from all subjects and allowed to coagulate in a gel tube, then subsequently centrifuged (3000rpm for 10 min). The separated serum was kept at -20 °C in sterile eppendorf tubes.
2.2 Methods

2.2.1 Determination of hormonal profile

The hormonal profile was measured to confirm the diagnosis of infertility cause. These hormones were leuitinizing hormone (LH), follicular stimulating hormone (FSH), anti-Müllerian hormone (AMH), thyroid stimulating hormone (TSH), prolactin, and estrogen (E2). Immunoassay for the \textit{in vitro} quantitative determination of LH, FSH, TSH, AMH and Prolactin in human serum was made using the electrochemiluminescence immunoassay “ECLI A” which was done using Elecsys and cobas e411immunoassay analyzers.

2.2.2 Determination of Iron concentration

Iron was measured on an automatic platform (Cobas C311, Germany). The iron reacts with acid at pH<2.0 which then reduced by ascorbate to ferrous iron. The Divalent iron ions form an iron-colored complex which can be detected at 552 nm [21].

2.2.3 Determination of Ferritin concentration

Ferritin was measured using an automatic platform electrochemiluminescence immunoassay (Cobas C311, Germany). The reaction involves four steps: incubation of the sample with a monoclonalin-ferritin, biotinylated and ruthenium-labeled antibody, bonding the sandwich (antibody-analyte complex) to biotin streptavidin-coated solid phase, then placing the reaction mixture inside the measuring unit, where the micro-particles are magnetically trapped on the electric surface. By applying a voltage to the electrode voltage, the photomultiplier measures the chemiluminescent emission [22].

2.2.4 Determination of transferrin concentration

Transferrin was measured using an automatic platform immunoturbidimetric assay (Cobas C311, Germany). The anti-transferrin antibodies react with antigen that is present in the sample to form the antigen / antibody complex. This is known as turbidimetric agglutination method [23].

2.2.5 Determination of Cp ferroxidase activity

The activity of Cp was measured according to Erel (1998) protocol. Serum Cp endpoint represents the changes in the ion concentration in the reaction medium containing ferrous ion in the substrate solution [24].

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\text{Enzyme activity (U/L)} = \frac{(C_1-C_2)}{t} \times \frac{(V_t/V_s)} = (C_1-C_2) \times 38.166
\]

Where;

\(C_1\) represents the substrate concentration (60μm/L) at the beginning of the enzyme reaction, \(C_2\) substrate concentration at the end of the enzyme reaction (calculated by the normal curve), \(V_t\) overall volume (i.e., 1374 μl); \(V_s\): volume of sample (i.e., 9 μl); \(T\): incubation time (i.e., 4 min).

2.2.6 Protein test

An updated Biuret system used the normal Bovian Serum Albumin (7g / dL) to calculate the protein concentration [25].
2.3 Statistical analysis

Data analysis was completed using the SPSS-22 Statistical Package for Social Science (Version 22). Data were exhibited normal distribution and expressed as mean and standard deviation (mean±SD). The significance of differences of various means (quantitative data from different groups and control group) was tested applying analysis of variance (ANOVA), whilst using independent students’ t-test for differences between two means. When the P value is less than 0.05; this statistically is considered a significance value. While it is considered a significant increase whenever the P value is less than 0.01 [26].

3. Results

3.1 Hormonal profile

The hormone analysis was carried out in a hormonal laboratory to confirm the diagnosis of different groups of etiologic infertile women. In the PCOS group, Luteinizing Hormone (LH) showed a significant increase (P<0.05) compared to control group, Follicle Stimulating Hormone (FSH) showed a significant decrease (P<0.05) compared to the UI and low AMH groups, whereas significant rise was observed in low AMH level group in comparison with control group. In PCOS group, LH/FSH ratio showed highly significant differences when compared with control, UI, and low AMH groups. Significant decrease level of serum progesterone (P<0.05) was observed in Low AMH level group compared to PCOS group. Mean serum AMH was significantly higher in the PCOS group (P<0.05) compared to control, UI and low AMH level groups, whereas mean AMH levels in the low AMH level group were slightly lower (P<0.05) than UI and control groups. There is no significant (P>0.05) differences among the four studied groups in mean serum Prolactin and Estradiol.

In UI group, the mean Thyroid Stimulating Hormones (TSH) level in serum was significantly (P<0.05) smaller than the control and PCOS groups.

Table 1. The hormonal profile of the four studied groups.

| Parameter          | Control group (n=25) | UI group (n=27) | PCOS group (n=21) | Low AMH level group (n=26) |
|--------------------|----------------------|----------------|-------------------|---------------------------|
| LH (mIU/ml)        | 5.00±1.8             | 6.63±2.04      | 8.03±4.23         | 6.06±1.24                 |
| FSH (mIU/ml)       | 6.33±1.4             | 7.53±1.68      | 5.86±1.59         | 8.22±2.42                 |
| LH/FSH ratio       | 0.82±0.32            | 0.9±0.26       | 1.41±0.68         | 0.76±0.19                 |
| Prolactin (ng/ml)  | 19.39±8.6            | 19.99±12.4     | 16.92±5.89        | 21.15±13.32               |
| Progesterone (ng/ml)| 0.39±0.29           | 0.36±0.19      | 0.57±0.54         | 0.28±0.13                 |
| AMH (ng/ml)        | 3.48±1.16            | 3.12±1.02      | 6.25±3.74         | 1.08±0.37                 |
| E₂ (pg/ml)         | 34.3±11.6            | 36±14.4        | 35.5±15.7         | 33.8±14.1                 |
| TSH (mIU/ml)       | 2.88±0.81            | 1.67±0.67      | 3.26±1.97         | 2.78±0.93                 |

The results were mean ± SD; the figures were evaluated by ANOVA followed by the Turkey’s multi-comparison Post Hoc test; *P<0.05 compared to control group; ‡P<0.05 compared to UI group; ³P<0.05 compared to PCOS.
group; CP<0.05; and dP<0.05 compared to low AMH level group. AMH: Anti- Müllerian Hormone, E2: Estrogen 2, FSH: Follicular Stimulating Hormone, LH: Luteinizing Hormone, TSH: Thyroid Stimulating Hormone.

3.2 Iron status and Cp Ferroxidase activity

As shown in (Table 2), Iron, Cp ferroxidase activity, and its specific activity were lower in PCOS group compared to control group with no significance. We observed significant lower (P < 0.05) value in Ferritin, Transferrin, Total Iron Binding Capacity (TIBC), and Unsaturated Iron Binding Capacity (UIBC) in PCOS and low AMH level groups compared to control and UI groups. Non sig in serum of PCOS group showed high level but non-significance (P>0.05) compared to control, UI, and low AMH level groups. Mean saturation of Transferrin, and hemoglobin levels in PCOS and low AMH level groups showed non-significance among the studied groups.

Table 2. The mean (±SD) level of iron status and Cp. Ferroxidase activity of the four studied groups.

| Parameters                  | Control group (n=25) | UI group (n=27) | PCOS group (n=21) | Low AMH level group (n=26) |
|-----------------------------|----------------------|-----------------|-------------------|---------------------------|
| Iron (µg/dl)                | 65±31                | 61±31           | 61±16             | 64±42                     |
| Ferritin (µg/dl)            | 32±7.3               | 36±25           | 22±9ª,b          | 20±10ª,b                  |
| TF (mg/dl)                  | 286±25               | 287±30          | 243±22ª,b        | 246±21ª,b                 |
| TIBC (mg/dl)                | 407± 46              | 406± 101        | 345± 30ª,b       | 351±48ª,b                 |
| UIBC (mg/dl)                | 342± 86              | 348± 32         | 283±33ª,b        | 287± 48ª,b                |
| Saturation TF %             | 16.6±9.4             | 16.5±10.2       | 17.8±5.4         | 17.3±12.1                 |
| Hb (g/dl)                   | 12.7±0.7             | 12.5±1.3        | 12.1±1.8         | 12.1±1.3                  |
| Cp ferroxidase activity (U/L)| 688±135             | 603±96          | 585±163          | 606±78                    |
| Cp Ferroxidase specific activity (U/g) | 9.9±2.5            | 8.8±3.5        | 8.2±2.3          | 8.4±1.4                   |

ªP<0.05 compared with control group; bP<0.05 compared with UI group; cP<0.05 compared with PCOS group; dP<0.05 compared with low AMH level group. Cp: Ceruloplasmin, Hb: Hemoglobin, TF: Transferrin, TIBC: Total Iron Binding Capacity, UIBC: Unsaturated Iron Binding Capacity.

3.3 Effect of Iron status and Cp. Ferroxidase activity on pregnancy

As shown in Table 3, each group was sub-divided to pregnant and non-pregnant group. Serum iron, and ferritin levels showed significant higher levels in pregnant when compared with non-pregnant groups in PCOS and UI groups. The mean level of serum transferrin showed significant (P<0.05) decrease in pregnant control, and UI groups in comparison to non-pregnant groups. The Cp. Ferroxidase activity and specific activity showed non-significant difference among pregnant and non-pregnant of all the four groups.
Table 3. Serum Iron statuses and pregnancy outcomes

| Parameters       | Pregnancy status | Control group (n=12/13) | UI group (n=8/19) | PCOS group (n=3/18) | Low AMH level group (n=9/17) |
|------------------|------------------|-------------------------|-------------------|---------------------|-------------------------------|
| Iron (µg/dl)     | Pregnant         | 65.29±27.67             | 71.62±10.6*       | 70±7.21*            | 68.44±32.94                  |
|                  | Non-Pregnant     | 60±32.34                | 58.11±33.52       | 59.65±9.57          | 70.44±59.2                   |
| Ferritin (µg/dl) | Pregnant         | 31.37±18.39             | 43±8.61*          | 26.33±4.07*         | 23.41±17.84                  |
|                  | Non-Pregnant     | 33.85±21.43             | 33.05±23.63       | 20.42±9.75          | 18.6±11.7                    |
| TF (mg/dl)       | Pregnant         | 268.03±34.42*           | 235.87±45.71*     | 233.66±27.02        | 242.55±40.82                 |
|                  | Non-Pregnant     | 304.41±55.07            | 306±70.57         | 245.5±24.35         | 249.38±42.44                 |
| Cp Ferroxidase   | Pregnant         | 731.6±151.41            | 658.25±100.47     | 542.6±216.33        | 611±61.09                    |
| activity (U/L)   | Non-Pregnant     | 659.08±122.69           | 550.33±129.4      | 583.16±165.11       | 605.66±54.68                 |

Analysis performed by independent samples t-test; statistically significant when *P<0.05; **P<0.01; no asterisk: P ≥0.05 non-significant. The (n) represent number of pregnant/ non-pregnant in each group.

4. Discussion

The hormonal profile for the studied groups showed that the enhanced release of the pulsatile GnRH in PCOS leads to elevated LH and lower FSH levels in most of the participants. High levels of LH tend to increase the development of androgen by follicular theca, while low FSH levels lead to ovulation [27]. It is likely that a higher proportion of women with PCOS will have a high LH / FSH ratio rather than a lower FSH production due to higher LH levels as a result of GnRH stimulation [28]. The mean level of AMH in PCOS was higher than in other groups; an increase in the number of small antral follicles could be due to the elevated AMH in the blood serum. However, in a comparison between normal ovaries, ovulatory and anovulatory PCOS; the production of AMH per granulosa cell was 75 times greater than that of normal ovaries per granule cell from an ovulating PCOS, and 20 times higher than that of the ovulatory PCOS [29]. This indicates that the increase in AMH is due to an intrinsic property in the granulosa cells of PCOS, which remains even after IVF stimulation [30]. Higher levels of AMH were also found in the follicular fluid [31], which means that increased AMH leads to increase FSH inhibition and can also lead to ovulation in women specifically with PCOS [32]. Since both progesterone and AMH are steroid-based grain cell drugs, the mean level of progesterone in women with PCOS was higher compared to women without PCOS on the day of activation, in the study involving IVF patients [33]. This study revealed significant increase in the level of TSH in women with PCOS compared to the UI group. This finding was in agreement with previous studies [34,35]; yet it differs with other studies [36,37]. Thyroid hormones have profound effects on reproduction and pregnancy. Thyrotropin releasing hormone (TRH) undergoes control of negative feedback to TSH through a short negative feedback loop; that is any increase in TSH will decrease the secretion of TRH.
which in turn will inhibit the secretion of prolactin and will also lead to the normalization of TSH levels. The relationships between TSH, prolactin and female infertility are multiple and complex. TRH is a powerful prolactin stimulator, and the association between hypothyroidism and hyperprolactinemia is well established. Hyperprolactinemia -for several reasons- can reduce pulsating secretion of gonadotropin, which release hormone secretion and interfere with ovulation [38]. Few studies have examined the effect of iron overload on fertility. The excess iron allows the reduction of LH and FSH production by the anterior pituitary gland. Free iron can generate ROS through Fenton reaction, causing oxidative stress, a condition that has a negative impact on oocyte development [9]. In the research by Luque-Ramírez et al. (2007) on PCOS demonstrated that decreased serum ferritin during metformin therapy resulted in a decrease in serum ferritin in these patients within three to six months [39]. However, our findings disagree with those of Sami et al., (2018) study in which a higher ferritin level measured in PCOS group compared to control group [40]. A study by Sathiyanarayanan et al., (2014) found higher ferritin level and lower iron levels in primary infertile women compared to the control group [8]. The Cp binds to copper; appears to be more important as a copper storage pool than as a transport protein; integrates iron and copper homeostasis [41]. It has the ability to oxidize extremely toxic ferrous iron to a relatively non-toxic ferric form and thus avoid oxidative damage to proteins, lipids and DNA. Cp is also an important antioxidant [42]. A study by Rawaa et al., (2017) [43] found higher level of serum Cp in infertile women compared to fertile women. One possible explanation for this rise is to promote the antioxidant function in the body [44].

Similar results were found by another study that found Cp activity is higher in infertile women compared to controls, and it found a correlation between Cu, Cp and oxidative stress markers, which may indicate their functions in endometriosis, and oxidative stress, and can be used as therapeutic markers for the foreseeable future [45]. Consequently, this inhibits the production of red blood cells when iron reserves are in an unsafe range. This means that the body's organs, including the ovaries and uterus, obtain less oxygen. Therefore, if reproductive organs are poorly supplied with oxygen, the content of their eggs is low and may not be viable [9]. The relationship between AMH and pregnancy depends on the quantity of oocytes and embryos obtained as well as the quality of embryos to be transferred. Although AMH may compromise pregnancy outcomes, low AMH levels do not affect embryonic developmental skills [46].

An increase in red cell mass and plasma volume was observed during pregnancy to meet the needs of the increased fetus and uterus. The plasma volume increases faster than the mass of red cell, which contributes to a decrease in the blood hemoglobin concentration. This decrease in hemoglobin levels reduces blood viscosity by increasing the total amount of red cells and thus increasing placental perfusion, in consequence enhancing the exchange of nutrients and gas between mother and fetus [47].

To explore the association of pregnancy outcome with the iron status; each group was divided into pregnant and non-pregnant groups. A study investigating the presence of iron transmitting proteins in ovarian cells is investigating the potential function of iron status in female reproduction [48]. Georgsen et al., (2020) found an inverse association between ferritin level and the ability to conceive suggesting that low ferritin level is associated with a more severe reproductive disturbance in women and recommended iron supplementation to achieve pregnancy [49]. In this study, transferrin level did not show significant difference between pregnant and non-pregnant groups of PCOS and AMH level groups. The function of this protein enables it to act as antioxidant since it binds excess iron [50]. Mean Cp serum, ferroxidase activity of pregnant for control, UI, and low AMH groups was non-significantly higher than non-pregnant with the exception of the PCOS group, where mean level of Cp. Ferroxidase activity was non-significantly lower in pregnant than non-pregnant . Serum Cp increases during pregnancy as its synthesis is stimulated in the liver under the influence of estrogen [51].
5. Conclusions

The increased iron levels in pregnant women may be due to the increased demand for oxygen, although this increase at certain levels may lead to increased levels of ROS. Ferritin, an iron storage protein, plays an important role in detoxifying ROS that may be resulted from elevated iron level. Iron status level may be useful for predicting pregnancy prior to the initiation of IVF treatment or failure for patients with insufficient level of iron due to various causes of infertility. Also, iron status could be used as potential therapeutic targets before IVF.

Declaration of Competing Interest

The authors declare no conflict of interest.

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