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RESEARCH ARTICLE

Oxidative Stress Statue in Follicular Fluid of Infertile Women Treated with IVF/ICSI-ET

Rusul Hashem[1]*, Shatha Abdul Wadood[1], Qays A. Mahdi[2]

[1.] *Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq.*

[2.] *Kamal Al-Samarai IVF Hospital, Ministry of Health, Baghdad-Iraq.*

Abstract

Introduction Oxidative stress (OS) in follicular fluid (FF) may affect *in vitro* fertilization (IVF) outcomes in infertile women undergoing IVF/ICSI-ET. Aim of study to assess the effects of total peroxide (TPX) concentration, total antioxidant capacity (TAC), oxidative stress index (OSI), malondialdehyde concentration (MDA), glutathione-s-transferase (GST) activity, and superoxide dismutase activity (SOD) on IVF and pregnancy outcomes in PCOS, male cause infertility, and unexplained infertile (UI) patients undergoing IVF cycles. Subjects, material, and methods A prospective case control study was performed among (40) control group (male cause), (40) polycystic ovary syndrome (PCOS) group, and (45) unexplained infertility (UI) group, all of which undergoing IVF/ICSI-ET. FF samples were collected at the day of oocytes aspiration. TPX, TAC, MDA levels, GST, and SOD activities were measured in FF. oxidative stress index were calculated. Results When control, PCOS and UI groups were compared in term of IVF outcomes, there were no significant differences in M II oocyte, G1 embryo, maturity rate, cleavage rate, fertilization rate, and chemical pregnancy state among the three studied groups (P>0.05). In addition, comparison of OS parameters among the studied groups showed no significant differences in TAC, MDA, GST activity, GST specific activity, and SOD activity (P>0.05); however, significant differences in TPX, OSI percentage, and SOD specific activity (P<0.05) were found. Association analysis between OS parameters and IVF outcomes showed a significant positive correlation between GST activity (and GST specific activity) with G1 embryo, GST activity with pregnancy, as well as, SOD activity and maturity rate in control group (P<0.05). In addition, GST activity and GST specific activity showed significant positive correlation with both cleavage rate and fertilization rate, GST activity with pregnancy, as well as, TPX with cleavage rate and fertilization rate in PCOS group (P<0.05). OS parameters in UI group did not show any significant correlation with IVF outcomes (P>0.05). When control, PCOS and UI groups were subdivided according to pregnancy outcome, mean TPX and TAC concentrations were higher in pregnant group compared to non-pregnant group; while mean MDA and OSI were lower in pregnant group compared to non-pregnant group in the three studied groups. Antioxidant enzyme activities in pregnant group were higher compared to non-pregnant group in the three studied groups. Conclusion The results of this study demonstrated that the oxidant-antioxidant balance in the oocyte surroundings must be obtained to improve IVF and pregnancy outcomes in patients treated by IVF/ICSI-ET.

Keywords: *Oxidative stress, Follicular fluid, Polycystic ovary syndrome, Antioxidant, Intra-cytoplasmic sperm injection.*

Introduction

Oxidative stress is a state caused by imbalance between reactive oxygen species (ROS) generation, and decrease antioxidant systems which leads to oxidation of macromolecules (DNA, proteins, and lipids) [1]. However, OS plays important physiological role in regulation of a full range of the reproductive functions, such as oocyte maturation, ovarian steroidogenesis, corpus luteum formation, luteolysis, fetus development, and pregnancy state [2a].

OS also has an impact on steroid hormones production of granulosa cell, which is important indicator of ovarian response [3]. Studies on pathophysiology of couple's infertility have demonstrated that OS may be one of the causative components of female infertility [2a, 4].

IVF is the most widely recognized assisted reproduction technologies. This strategy regularly remains the main possibility of

having a baby for infertile couples. ROS in peritoneal fluid of endometriosis patients and in FF of patients experiencing IVF have been archived [5,6]. In previous reports, researchers concentrated on the microenvironment surrounding the oocyte.

They found that OS affects the reproductive potential [3, 7, 8]. Furthermore, OS is by all accounts an important explanation behind IVF failure [9]. All these studies highlighted that there is a complex connection amongst ROS and antioxidants in the ovaries. Antioxidant enzymes inside granulosa cells, cumulus cells, and FF each play a basic part in the protection of the oocyte. Glutathione system plays an important role in cell defense against ROS [2a].

Glutathione peroxidase, glutathione reductase and GST are antioxidant enzymes expressed in mammalian oviducts and play important role in the balance between intra and extracellular redox system [10]. The clinical pregnancy occurs due to the presence of higher levels of TAC; in addition, OS markers levels in the FF are inversely associated with ovarian stimulation efficiency in infertile patients [11]. SOD enzyme catalyzes dismutation of superoxide anion radicals to form hydrogen peroxide and molecular oxygen; hence, SOD plays an important role in the first line of antioxidant defense [12].

ROS production in the ovaries may cause plasma membrane damage by lipid peroxidation (LPO) of polyunsaturated fatty acids and give rise to cell injury [2b, 13]. Additionally, low levels of ROS in FF and hydrosalpingeal fluid could be an essential marker for predicting success in IVF outcomes and could be a potential marker for normal tubal secretory function [2c].

Conflict result found in another report, which believed that lower levels of oxygen available (hypoxia) in developing follicle could contain lower levels of MDA and TAC, but unfortunately, they would have a smaller choice of confirming a pregnancy [14a]. An earlier study had confirmed the excessive ROS production in granulosa cells during controlled ovarian hyperstimulation of PCOS women may adversely affect IVF success [15].

Furthermore, elevated ROS levels and MDA in FF are associated with poor quality of oocytes and embryos and low fertilization rate in IVF patients [9, 16]. However, previous reports demonstrated that ROS and antioxidant enzyme concentration and activities in FF have no impact on embryo quality in IVF patients [7, 17]. An old study has summarized that lower ROS levels predict decreased fertilization potential thus reduced oocyte competence, presumably leading to poor embryo quality in the occurrence of fertilization [18a].

In a previous report, this studied the importance of the imbalance between pro-oxidants and antioxidants that exerts a particular impact on folliculogenesis and embryonic development, negatively affecting oocyte and embryo quality, in women with endometriosis [19].

This controversy in the studies mentioned above, about oxidative stress statue, and their effects on IVF clinical outcome, is the main reason to perform our research study. Accordingly, this study aimed to investigate levels of biochemical parameters of OS such as TPX concentration, TAC concentration, MDA concentration, SOD activity, and GST activity in follicular fluid of women undergoing ovarian hyper stimulation, and IVF/ICSI-ET.

In addition, to explore the effect of the OS parameters on oocyte quality, maturation rate, cleavage rate, fertilization rate, embryo quality, and pregnancy in infertile patients with PCOS, UI, and, male factor who were undergoing IVF cycles.

Subject, Materials and Methods

Subjects

A prospective case control study conducted at Kamal Al-Samarai IVF Hospital in Baghdad-Iraq, from December 2017 to June 2018. The medical ethics committee in University of Baghdad approved this study protocol. All patients signed a written informed consent. The study included (125) infertile women undergoing IVF/ICSI were divided according to the etiology of infertility into: (40) women with female cause infertility group (PCOS) (mean age 31.02±0.93 years), (40) women with male cause infertility group (Control) (mean age 30.87±1.98 years), and (45) women with UI group (mean age 30.68±1.23).

The diagnosis of PCOS was according to the revised Rotterdam European Society of Human Reproduction and

Embryology/American Society for Reproductive Medicine Criteria [20]. Patients diagnosed with UI based on standard infertility tests, according to the guidelines of the Practice Committee of the American Society for Reproductive Medicine [21]. These tests included assessments of spermiogram, ovulation, hysterosalpingogram, and, if indicated, ovarian reserve tests and laparoscopy. If the results of all these tests were normal, patients were accepted as UI. Patients with adenomyosis, severe pelvic adhesions, thyroid disorders, hypoprolactemia, diabetes mellitus and cardiovascular diseases were excluded.

Controlled Ovarian Hyper Stimulation, Oocyte Collection, and FF Sampling

All subjects were hyper stimulated by gonadotropin releasing hormone (GnRH) antagonist protocol. Administration of 150-225 IU of recombinant FSH (Gonal-F®) injection from day two of menstrual cycle. GnRH antagonist (Cetrorelix) is given (0.25 mg) daily when the follicle reach (12-14mm), as detected by ultrasound. Cetrorelix and Gonal-F® are continued together until either two or three follicles reach 17-18 mm in the ovary.

Then, ovulation induction using recombinant human chorionic gonadotropin administration (rhCG 6500 IU, Ovitrelle®; Merck Serono, Italy) was done. Oocytes were picked up after 34 - 36 hours from hCG injection using needle aspiration with a transvaginal ultrasound transducer guidance. Uncontaminated FF samples were centrifuged at 3000xg for 10 min at room temperature. The clear supernatant of FF transferred to sterile tubes and stored at -20 ^{o}C until assayed.

Assessment of oocyte morphology and oocyte maturation

Nuclear maturation of oocytes was dictated by the identification of the first polar body. Oocytes morphology was assessed by metaphase II (MII) oocyte [22, 23].

Assessment of Fertilization, Cleavage, and Embryo Quality

After 18 hours of ICSI, fertilization results were evaluated (appearance of two pronuclei and two polar bodies). Then, cleavage has been done after 24 hours of fertilization. Embryologists graded embryo quality on the second day of insemination [24].

Embryo transfer (2–3 embryos) was done on day 2 or 3 of embryonic development. Additionally, injection of 1500 IU hCG and vaginal administration Duphstone (200mg of progesterone three times per day), daily until pregnancy test day (14 days post embryo transfer). The chemical pregnancy is confirmed by positive beta hCG pregnancy test.

IVF Outcomes

Oocyte Maturity rate = (Total No. of mature oocytes/Total No. of all oocytes) ×100

Cleavage rate = (Total No. of informed embryos/Total No. of fertilized Oocytes) ×100

Fertilization rate = (Total No. of zygotes (2 pro nucleus)/Total No. of mature oocytes MII) ×100

Analysis

Total Peroxide (TPX) Assay

Total peroxide concentrations in FF samples were measured using modified xylenol orange assay (FOX2) [25]. The assay is based on oxidation of ferrous to ferric ion by several kinds of peroxides within the samples to give ferric–xylenol orange complex. The absorbance of the solution was measured at 560 nm using spectrophotometer. TPX content of samples was measured by a standard solution of hydrogen peroxide.

Total Antioxidant Capacity (TAC)

Estimation of TAC depends on reaction of [Fe^{2+}-O-dianisidine] complex with hydrogen peroxide to produce hydroxyl radicals. Hydroxyl radical oxidize colorless O-dianisidine to dianisidyl radicals that is yellow-brown color, hence can be measured by spectrophotometer at 444 nm.

Antioxidants in the sample suppress color degree depending on their concentrations. Ascorbic acid (2.0 mM) was used as standard [26].

Oxidative Stress Index (OSI) Calculation

The percentage ratio of TPX to TAC was used to measure OSI [27].

Measurement of Malondialdehyde (MDA)

The reaction of MDA with thiobarbituric acid (TBA) gives a red compound, which can be measured by spectrophotometer at 535 nm [13].

Determination of Glutathione-S-transferase (GST) Activity

Activity of GST was measured, as previously described [28], according to the conjugation reaction of 1-chloro-2,4- dinitro-benzene (CDNB) with reduced glutathione (GSH). The reaction mixture contain phosphate buffer saline (1 M, pH 6.5), GSH (100 mM), CDNB (100 mM), and FF sample (100 µl). The increase in absorbance at 340 nm at 25°C was recorded. One unit of enzyme activity was defined as one µmol of GSH conjugated/min at 25°C.

$$\text{GST activity (U/ml)} = [(\Delta A_{340}/\text{time min})/0.0096] \times (Vt/Vs)$$

ΔA_{340}/min= (A_{340}/min) sample - (A_{340}/min) blank; Molar extinction coefficient of CDNB at (340nm) = 0.0096 μM^{-1}/cm; Vt= volume of test; Vs= volume of sample

$$\text{GST specific activity (U/mg)} = \frac{\text{GST activity } (\frac{U}{ml})}{\text{protein Conc.} (\frac{mg}{ml})}$$

Determination of Super Oxide Dismutase (SOD) Activity

The activity of SOD was estimated using indirect method (riboflavin/ nitro blue tetrazolium NBT method), as previously described [29]. The working mixture (15.125 ml) consisted of phosphate buffer (131.19mM, pH 7.8), L-Methionine (300 mg/10ml), NBT-2HCl (14.1 mg/10ml), Triton X-100 (100mg/10ml). To this mixture, (100µl) of FF sample and (10µl) of Riboflavin solution (4.4 mg/100ml) were added followed by illumination at 25 °C in aluminum foil lined box containing two fluorescent lamps (20-Watts) for 7 minutes. The absorbance measured immediately at wavelength 560 nm. One unit of SOD was defined as that amount of sample that causes a 50% decrease of the SOD inhibition NBT reduction in this assay. Therefore, the SOD activity in the sample can be expressed in riboflavin/NBT assay unit (U) using the following equation:

$$\text{SOD activity (U/ml)} = \frac{Sample\ inhibtion\ \% \times 2 \times 1000}{Max.inhibtion\ \% \times Vs\ (\mu l)}$$

Vs: volume of the sample
Maximum inhibition: was calculated from inhibition curve of each group.

Sample inhibition % = (AB_2-AB_1) _ (AS_2-AS_1) / (AB_2-AB_1) ×100.

Where:

AS1: absorbance of sample before illumination; AS2: absorbance of sample after illumination;

AB1: absorbance of blank before illumination;
AB2: absorbance of blank after illumination.

$$\text{SOD specific activity (U/mg)} = \frac{\text{SOD activity } (\frac{U}{ml})}{\text{protein Conc.} (\frac{mg}{ml})}$$

Protein assay

The protein concentration was determined by the modified biuret method, using bovine serum albumin (BSA 10 mg/ml) as the standard [30].

Statistical Analysis

Data analysis was done by utilizing SPSS for Windows, version 17(SPSS Inc. Chicago, IL, United States). Data were appeared as mean ± standard deviation. Statistical analysis performed by one-way ANOVA, Chi-square test, Person correlation, and Independent sample's T-test. A *p* value less than 0.05 was considered statistically significant.

Results

IVF outcomes

There were no significant differences in M II oocyte, G1 embryo, maturity rate, cleavage rate, fertilization rate, and chemical pregnancy state among the three studied groups (P>0.05) (Table 1).

Table 1: IVF outcomes of the studied groups

IVF/ICSI outcomes	Control (n=40)	PCOS (n=40)	UI (n=45)	*P* value ‡
M II oocyte	4.84±1.79	4.99±2.09	4.83±1.78	0.822
G1 embryo	2.08±1.35	2.01±1.51	2.09±1.30	0.792
Maturity rate	65.33±20.44	64.68±22.84	65.36±18.98	0.480
Cleavage rate	61.56±21.22	61.31±15.62	60.88±21.69	0.750
Fertilization rate	68.03±24.64	66.69±23.93	66.60±22.49	0.969
Chemical pregnancy = count (%)				0.405 †
Yes	7 (17.5%)	12 (30%)	12 (26.7%)	
No	33(82.5%)	28 (70%)	33 (73.3%)	
Total	40(100%)	40(100%)	45 (100%)	

Data are expressed as mean ± SD or numbers with percentages in parentheses. Statistical analyses were performed by ANOVA followed by Post Hoc test (Tukey's test) for multiple comparisons. ‡ ANOVA significance test (2-tailed). † Statistical analyses performed by the Chi-squared test for multiple comparisons.

Oxidative Stress Parameters

Data concerning all measurements of oxidative stress markers presented in Table 2. Markers of oxidative stress: mean TPX concentration and OSI were higher in PCOS group compared to control group and was statistically significant just in OSI (P<0.05). Also, mean TPX concentration and OSI in UI group were higher significantly compared to both PCOS and control groups (P<0.05). No significant differences were found in MDA and TAC concentrations among the three groups (P >0.05). Antioxidant enzymes in PCOS group showed lower activities compared with control group, and the only significant reduces was in SOD specific activity (P <0.05).

Table 2: Oxidant and antioxidants levels in FF of the studied groups

Parameters	Control (n=40)	PCOS (n=40)	UI (n=45)	*P* value ‡
FF. concentrations				
TPX (µM)	5.42±2.12	6.47±2.19	8.31±1.32 ¥, £	0.000
TAC (mM)	0.93±0.08	0.89±0.12	0.89±0.11	0.276
OSI %	0.58±0.24	0.72±0.27 ¥	0.94±0.16 ¥, £	0.000
MDA (µmol/L)	0.31±0.25	0.41±0.27	0.24±0.13	0.115
FF. enzymes activities				
GST activity (U/ml)	66.50±28.50	57.66±24.06	54.21±26.46	0.185
GST specific activity (U/mg)	1.19±0.58	0.98±0.52	0.98±0.48	0.177
SOD activity (U/ml)	13.03±2.62	12.11±4.28	11.94±2.29	0.376
SOD specific activity (U/mg)	0.23±0.09	0.18±0.06 ¥	0.18±0.04	0.004

Data were expressed as mean ± SD. Statistical analyses were performed by ANOVA followed by Post Hoc test (Turkey's test) for multiple comparisons.‡ ANOVA significance test (2-tailed). ¥P< 0.05 compared with control group. £P< 0.05 compared with PCOS group

Association between OS Parameters and IVF Outcomes in (C) Group

Association analyses between the studied parameters and IVF parameters (M II oocyte, G1 embryo, maturity rate, cleavage rate, fertilization rate, and pregnancy) showed that, GST activity was significantly and positively correlated with G1 embryo (r=0.479, P =0.021) and pregnancy (r=0.526, P=0.000); while GST specific activity was significantly and positively correlated with G1 embryo (r=0.437, P =0.048). Furthermore, SOD activity was significantly and positively correlated with maturity rate (r=0.419, P =0.047) (Figure 1). However, no significant associations were observed between the rest FF OS parameters (TPX, TAC, OSI, MDA, SOD specific activity) and IVF parameters (P>0.05).

Association between OS Parameters and IVF Outcomes in (PCOS) Group

Association analysis between parameters of OS and IVF parameters (M II oocyte, G1 embryo, maturity rate, cleavage rate, fertilization rate, and pregnancy) showed that FF.TPX concentration was significantly and positively correlated with cleavage rate (r=0.480, P=0.004) and fertilization rate (r=0.471, P=0.007). Furthermore, GST activity was significantly and positively correlated with cleavage rate (r=0.422, P=0.009), fertilization rate (r=0.415, P=0.016), and pregnancy (r=0.44, P=0.01); while, GST specific activity was significantly

and positively correlated with cleavage rate (r=0.437, *P*=0.006) and fertilization rate (r=0.438, *P*=0.011) as shown in (Figure 2).

However, no significant association observed between the other of OS parameters (TAC, OSI, MDA, SOD activity) and IVF parameters (*P* >0.05).

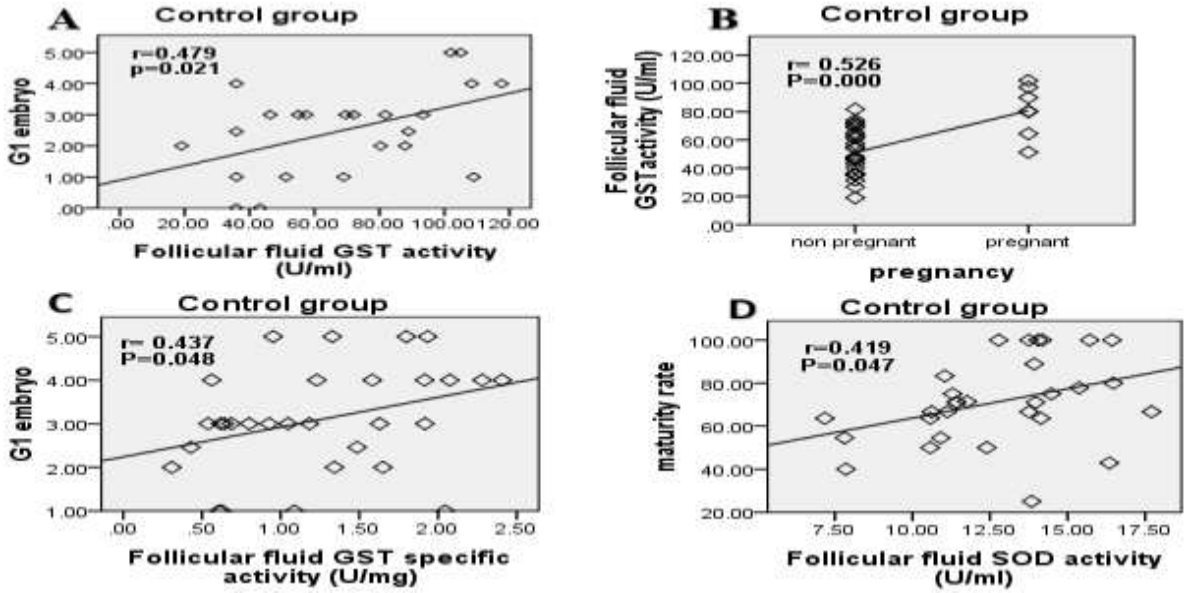

Figure 1: The association between antioxidant enzyme activities and IVF outcomes in control group. (A) FF.GST activity associated with G1 embryo. (B) FF.GST activity associated with pregnancy. (C) FF.GST specific activity associated with G1 embryo (D) FF.SOD activity associated with maturity rate

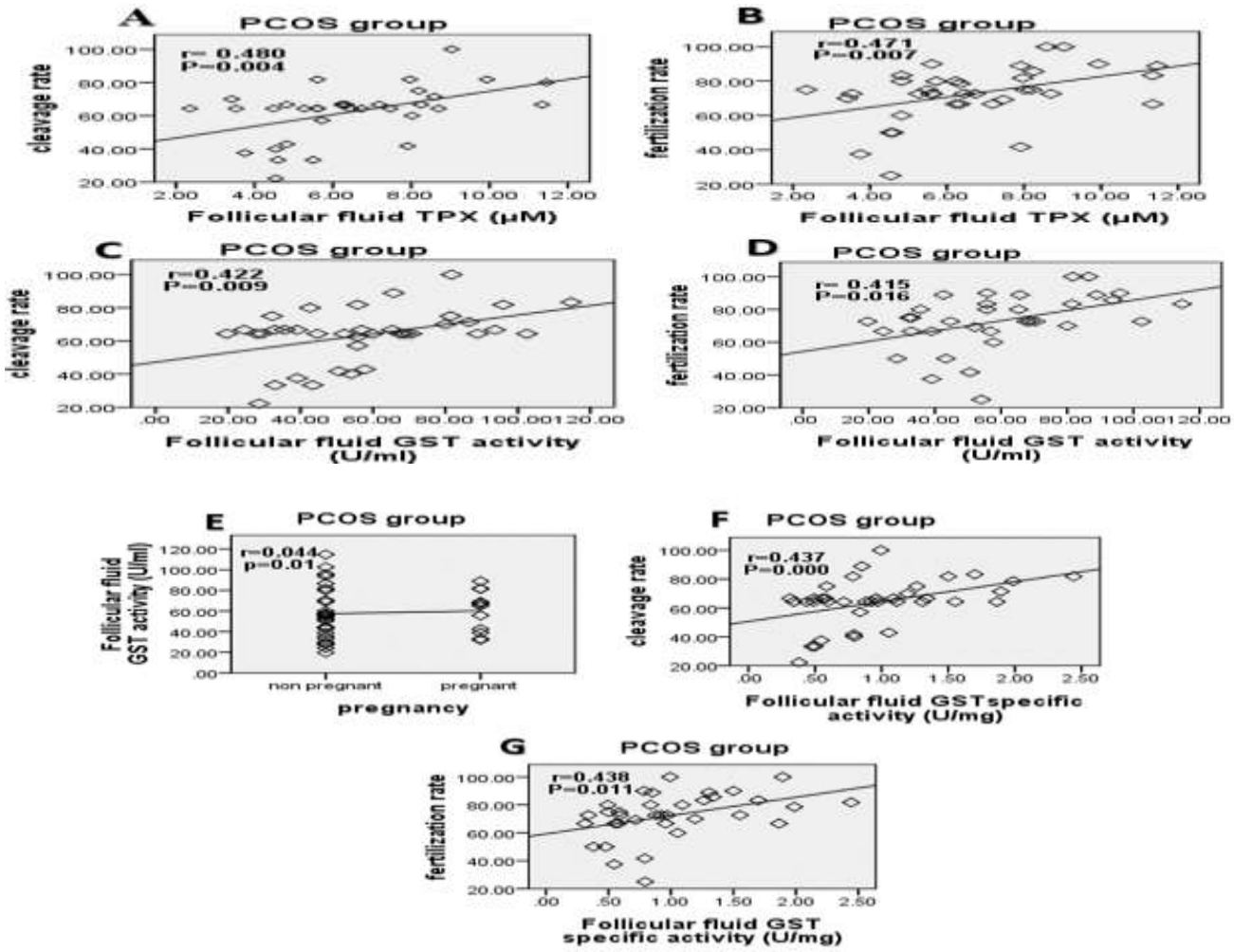

Figure 2: The association between antioxidant enzyme activities and IVF outcomes in PCOS group. (A) FF.TPX concentration associated with cleavage rate. (B) FF.TPX concentration associated with fertilization rate. (C) FF.GST activity associated with cleavage rate. (D) FF.GST activity associated with fertilization rate. (E) FF.GST activity associated with pregnancy. (F) FF.GST specific activity associated with cleavage rate. (G) FF.GST specific activity associated with fertilization rate

Oxidative Stress Parameters and Pregnancy Outcomes in (C) Group

As shown in Table3. Mean TPX and TAC concentrations were higher in pregnant group compared to non pregnant group and the difference was not statistically significant (P >0.05); while mean MDA concentrations were lower in pregnant group compared to non pregnant group and the difference was statistically significant (P =0.05). Antioxidant enzymes in pregnant group showed higher activities compared with non-pregnant group, and the only significant value was in GST activity (P <0.05).

Table 3: Oxidant and antioxidants levels in FF according to pregnancy outcome in (C) group

Parameters	non-pregnant group	pregnant group	*P* value
FF. concentrations			
TPX (µM)	5.54±1.53	5.87±1.66	0.66
TAC (mM)	0.87±0.03	0.94±0.11	0.13
OSI %	0.63±0.18	0.62±0.14	
MDA (µmol/L)	0.39±0.11	0.27±0.11	0.05
FF. enzymes activities			
GST activity (U/ml)	51.13±16.4	79.04±21.5	0.002
GST specific activity (U/mg)	1.06±0.41	1.40±0.49	0.08
SOD activity (U/ml)	12.06±2.6	12.65±2.04	0.59
SOD specific activity (U/mg)	0.186±0.05	0.22±0.02	0.06

Oxidative Stress Parameters and Pregnancy Outcomes in (PCOS) Group

As shown in Table 4. Mean TPX and TAC concentrations were higher in pregnant group compared to non-pregnant group; while mean MDA and OSI were lower in pregnant group compared to non pregnant group. Hence, the differences were not statistically significant (P >0.05). Antioxidant enzymes in pregnant group showed higher activities compared with non-pregnant group, and the only significant value was in GST activity (P <0.05).

Table 4: Oxidant and antioxidants levels in FF according to pregnancy outcome in (PCOS) group

Parameters	non-pregnant group	pregnant group	*P* value
FF. concentrations			
TPX (µM)	6.14±1.70	6.33±1.03	0.97
TAC (mM)	0.85±0.05	0.89±0.09	0.14
OSI %	0.72±0.17	0.70±0.13	0.84
MDA (µmol/L)	0.45±0.17	0.38±0.19	0.35
FF. enzymes activities			
GST activity (U/ml)	49.13±18.9	67.79±18.5	0.01
GST specific activity (U/mg)	0.95±0.41	0.98±0.30	0.84
SOD activity (U/ml)	10.48±4.44	12.25±3.92	0.27
SOD specific activity (U/mg)	0.176±0.03	0.18±0.04	0.62

Oxidative Stress Parameters and Pregnancy Outcomes in (UI) Group

As shown in Table 5. Mean TPX, TAC, GST activity, GST specific activity, SOD activity, and SOD specific activity were higher in pregnant group compared to non-pregnant group; while mean MDA and OSI were lower in pregnant group compared to non-pregnant group and the differences were not statistically significant (P >0.05).

Table 5: Oxidant and antioxidants levels in FF according to pregnancy outcome in (UI) group

Parameters	non-pregnant group	pregnant group	*P* value
FF. concentrations			
TPX (µM)	8.06±0.89	8.48±0.74	0.34
TAC (mM)	0.87±0.13	0.95±0.06	0.16
OSI %	0.93±0.11	0.89±0.11	0.55
MDA (µmol/L)	0.30±0.15	0.23±0.10	0.48
FF. enzymes activities			
GST activity (U/ml)	52.88±22.6	53.24±15.2	0.97
GST specific activity (U/mg)	0.95±0.32	0.99±0.23	0.80
SOD activity (U/ml)	11.46±2.55	12.73±1.67	0.30
SOD specific activity (U/mg)	0.18±0.03	0.20±0.04	0.20

Discussion

Follicular fluid TPX, TAC, and OSI

We compare the balance of the pro oxidant-antioxidant system in FF with the IVF outcomes. Markers reflecting OS statue were measured (TPX and TAC). In addition, OSI was calculated, which represent oxidative stress to total antioxidant status balance. However, OSI shows higher values if the fluid was under increased OS level. Oxidative metabolism is implicated in every stage of ovarian follicular growth and oocyte maturation [31].

As in many other systems, a physiological amount of ROS may be indicative of healthy developing oocytes, whereas excessively high levels may be indicative of OS [13]. The present study showed higher TPX concentration and OSI levels in FF of PCOS group compared to UI and control group while lower TAC concentration found in PCOS group compared to UI and control group. Our study is in agreement with previous reports, which studied OS parameters in FF of infertile females with PCOS compared to those with male factor infertility (control group) [32, 33].

It is worth mentioning that limited levels of OS is required for fetus development as well as, redox activity may be important for cell signaling process that is essential for fetus development, but extreme generation of ROS can be detrimental for fetus development. Physiological OS is important for human gestational sac remodeling process at trimester end, but pathophysiological mechanism of spontaneous miscarriage may be due to excessive OS levels [34].

As evident by our study, there was a good correlation between TPX with cleavage rate and fertilization rate in PCOS group. Therefore, these parameters reflect oocyte quality and initial embryo quality. Interestingly, this critical finding helped us to achieve the ROS threshold level beyond which it seems to be valuable, and is associated with the IVF outcomes. Unfortunately, the lack of a reference value in ordinary healthy women (unstimulated ovaries) makes it hard to decide whether the ROS levels observed in FF are in the pathological or physiological range. Despite there is a control group (male cause infertility group), but it may be possible that some normal women have an alternate response to ovarian hyperstimulation which leads to altered ROS levels. The present study showed that TPX levels were higher in a pregnant group as compared with a nonpregnant group for the control group, PCOS group, and UI group. Our results were supported by an old study, which reported that the pregnant women with male factor infertility and endometriosis had higher ROS level than non-pregnant women [35]. Conflict results were reported by a recent study, which they found that levels of total oxidant (TOS) in FF of pregnant group were lower compared to non pregnant group [36].

This discrepancy with results may be due to heterogeneity of their patient groups (unknown etiology, PCOS, male factor, tubal factor, endometriosis, and low ovarian reserve) Hence, that is why we subdivide our patients to three different etiologically infertility groups. This study revealed that TPX concentrations and OSI in FF of UI group where significantly higher than both PCOS group and control group. On the other hand, TAC and enzyme activities in FF of UI group showed lower levels compared to control group.

Hence, our data showed that TPX, TAC, OSI, MDA, and enzyme activities of UI group have no significant effect on IVF and pregnancy outcomes. Hence, the increased overall OSI in FF may not play an active role in IVF and pregnancy outcome in UI group. The results in this study revealed that, FF.TAC concentration were higher in pregnant group compared to non pregnant group in (control, PCOS, and UI) groups. Our results were in agreement with previous reports [14a, 36].

The possible explanation for higher production of intrafollicular ROS in pregnant group of the (control, PCOS, and UI) groups may be due to active metabolism during follicle growth that cause excessive ROS production, subsequently, may cause damage of oocytes. Therefore, to minimize the risk caused by higher ROS levels; higher TAC concentration and lower OSI are necessary to maintain intrafollicle pro-oxidant/antioxidant balance, and subsequent healthier reproductive system. Our explanations were in agreement with previous report [11]. The results in this study revealed that FF.TAC concentration have no significant effect on IVF and pregnancy outcomes.

This observation may have important a clinical implication is that the antioxidants in FF do not play an active role in improving IVF and pregnancy outcome. The important point is that intrafollicle TAC concentration did not appear to depend on the etiology of infertility, since it's concentration in pregnant group have been higher compared to non pregnant group in (control, PCOS, and UI) groups.

In contrast with the result of another study, which showed that the FF.TAC concentration depends on the etiology of infertility; hence the FF.TAC concentration was higher in PCOS with viable embryos compared to non viable embryos, while patients with FF.TAC concentration was higher in non PCOS with non viable embryos compared to viable embryos [18b].

MDA

The present study showed that MDA levels were higher in PCOS group as compared with control group. Our study was in agreement with another study, which showed that PCOS women had an elevated concentration of MDA compared to normal ovulatory controls treated with IVF/ICSI, as well as the study suggested that increased OS in PCOS is associated with inflammation that support insulin resistance which is closely linked to this syndrome [32].

The increased MDA levels in patients with PCOS treated with IVF/ICSI also documented by another study [37]. Our study showed, despite the OSI in FF of UI group were higher than control group and PCOS group, but, MDA concentration were lower than the other two groups. The scientific interpretation of this conflict observation that the antioxidant concentration in FF may be able to work as buffer to oppose the high toxic level of MDA and reduce its level in FF of UI group.

A previous study found that elevated levels of lipid peroxidation in FF might have negative effect on IVF clinical outcomes whereas; higher OS markers concentration in FF may reduce fertilization capacity [9]. However, the present study showed no significant association between fertilization rate and MDA concentration in PCOS group. In a study about MDA concentration in FF aspirates from dominant follicles of patients with UI, the studies found that FF. MDA concentration were higher in successful pregnancy group compared to unsuccessful pregnancy group [38]. Our study revealed lower MDA concentration in FF of pregnant group of (control, PCOS, and UI) groups. The reason is well known and it is due to lower OSI in FF of pregnant groups. Our results were in agreement with previous reports [39, 40].

Follicular Fluid Enzyme Activities

GST

In the present study, mean GST activity and specific activity in FF were lower in PCOS group compared to control group. Interestingly, since GST activity and specific activity were lower in FF and has significant direct (positive) effect on cleavage rate and fertilization rate in PCOS patient group. As a cause-effect relationship, we can hypothesize that lower GST activity may decrease cleavage rate and fertilization rate in PCOS infertile patients compared to control group.

A previous study demonstrated that the GST activity in FF was significantly lower in young women with low responders compared with oocyte donors and high responders, hence; may negatively influence ovarian response [41]. Another study studied the role of GST in follicular maturation; GST activity in both oocyte donors and patients were significantly lower in mature compared to immature oocyte. The study suggested that GST might have a role in follicle maturation by detoxifying xenobiotic process, hence contributing to oocyte normal development [42].

This study revealed that a significant positive association was found between FF.GST activity and specific activity with G1 embryo in control group, we can conclude that, higher FF GST activity may have a direct effect on embryo quality in control group undergoing IVF/ICSI-ET. Conflict result was found by a previous study, which studied GST activity in serum and FF in 31 women undergoing IVF.

They found that GST did not correlate significantly with embryo cell number [43]. Our study showed that GST activity in FF positively associated with pregnancy in control group and PCOS patients group, which may indicate a possible physiologic role in improving implantation.

The present study demonstrated that, GST activity in FF were significantly higher in pregnant group compared to non-pregnant group for both control group and PCOS group. Furthermore, GST activities in FF of UI group were slightly higher in pregnant group compared to non-pregnant group. In addition, GST specific activity in FF of (control, PCOS, and UI) groups showed higher (not statistically significant) levels in pregnant group compared to non-pregnant group. Wathlet *et al*.

Studied the expression of glutathione S transferase alpha 3 and glutathione S transferase alpha 4 in granulosa cells related to pregnancy and granulosa cells related to non-pregnancy. They found that the expression of the two studied genes were higher in pregnant group compared to non-pregnant group [44].

Its worth to mention that, our findings about GST activity in FF compared depending on pregnancy state in patients treated by IVF cycles have never been reported before. Interestingly, the significant positive correlation of FF.GST activity with pregnancy might be strengthen our finding about significant higher FF.GST activities in pregnant group compared to non pregnant group in both control group and PCOS group undergoing IVF/ICSI-ET.

SOD

In a case control study, which studied serum and FF SOD activity as well as Cu/Zn-SOD mRNA in PCOS patients, they found that FF SOD activity in PCOS cases lower than that in the control group [45]. SOD specific activity in FF from PCOS infertile women is significantly lower than that found in the control group [46a]. Similarly, our study showed that mean SOD specific activity in FF was significantly lower in PCOS group compared to control group.

Nevertheless, SOD specific activity did not correlate with IVF outcomes. An important point is a significant positive association between SOD activity and oocyte maturity rate in control group was found, which is supported by significantly higher SOD specific activity in control group compared to PCOS group; so we may presume that increased FF SOD activity may have a direct effect on oocyte maturation in control group undergoing IVF/ICSI-ET.

In addition, SOD activity and specific activity in FF of UI group showed lower levels compared to control group. Our study reported that SOD specific activity in the FF of the studied groups have no significant effect on pregnancy outcome. This is in a good agreement with a previous study [14b].

Furthermore, SOD activity and specific activity in FF of pregnant group were higher compared to non-pregnant group in (control, PCOS, and UI) groups. An earlier study had shown elevated SOD specific activity in FF of pregnant group compared to non pregnant group of patients with tubal factor infertility [40]. Conflicts results of a study by Sabatini *et al*. in which higher levels of SOD activity were present in FF from follicles whose oocytes did not fertilize compared with FF whose oocytes did fertilize. They conclude that SOD activity related inversely to fertilization of oocyte [46b].

We can conclude that a certain level of ROS in FF may be valuable and useful for oocyte quality and initial embryo quality in the PCOS group. In the UI group, the increased overall OSI in FF may not play an active role in IVF and pregnancy outcome. Moreover, although total antioxidants in FF may not play an active role in improving IVF and pregnancy outcome for the studied groups, antioxidant enzyme activities shown to play an active role in improving IVF and pregnancy outcome for the control group and PCOS group.

An interesting finding of our study that pregnancy outcome in patients undergoing IVF may be affected by the GST activity in the oocyte surroundings, regardless of the etiology of infertility. Finally, there was a similar trend observed in the studied markers and pregnancy outcome in the control group, PCOS group, and UI group.

Conflict of Interest

The Authors declare no conflict of interest.

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*Corresponding Author Email: al_zahra.al_baydhaa@yahoo.com

