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Association of Follicular Fluid Antioxidants Activity with Aging and *In Vitro* Fertilization Outcome: A Cross-Sectional Study

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Abstract

Background: This research was aimed at assessing the relationship between the follicular fluid (FF) antioxidants activity, aging and *in vitro* fertilization (IVF) outcome.

Materials and Methods: The present cross-sectional study was carried out on 65 women undergoing IVF/intracytoplasmic sperm injection (IVF/ICSI) cycles due to unexplained infertility. Ovarian stimulation was performed using the long gonadotropin-releasing hormone (GnRH) agonist protocol. After ovum pickup, FF was collected and processed to measure the level of superoxide dismutase (SOD), catalase (CAT) activity, total antioxidant capacity (TAC) and glutathione (GSH). Day 3 after ICSI, fresh embryos were transferred and later, possible pregnancy was assessed. Patients participating in this study were divided into four groups on the basis of age and pregnancy outcome.

Results: SOD activity was not significantly different between the groups (P=0.218). GSH in the group whose participants were aged ≤35 years and were pregnant was higher than that in other groups. CAT activity in groups with younger participants was higher compared to the other groups. The mean TAC was higher in groups with pregnant participants compared to the non-pregnant women. Correlation analysis showed that: GSH level had a significant negative correlation with age (P<0.001, R -0.55) and a significant positive correlation with pregnancy (P=0.015, R=0.30). CAT level also had a significant negative correlation with age (P<0.001, R=-0.42) and the level of TAC had a significant positive correlation with pregnancy (P<0.001, R=0.59).

Conclusion: According to our results, the levels of TAC, GSH and CAT in younger and pregnant women were higher compared with those undergoing ICSI cycles. Given the correlation of FF antioxidant activity with age and pregnancy, it is necessary to carry out more research on these compounds and the maintenance of pregnancy.

Keywords: Aging, Catalase, Follicular Fluid Antioxidants, Glutathione, Infertility

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Introduction

The frequency of age-related infertility has increased in recent years (1). In age-related infertility, in addition to the ovarian reserve factor, there is another important factor that is specific to the microenvironment of oocyte development, namely follicular fluid (FF). The content of this fluid that surrounds the oocyte has a vital role in the quality and fertility of the oocyte and subsequent embryo development (2, 3). This microenvironment contains growth factors, granulosa cells, and steroids hormones, as well as factors that produce reactive oxygen species (ROS), such as leukocytes, cytokines, and macrophages (4). ROS imbalance in ovarian FF has a negative impact on the development of oocytes and embryos, as well as sequent pregnancy outcomes (5).

Antioxidants are known as potentially useful factors that can keep the equilibrium between ROS production and clearance. Antioxidant pathways occur in all species, allowing them to cope with oxidative conditions and assisting cells to repair ROS-caused damage. They also play important roles in eliminating toxic oxygen products. These mechanisms are classified as nonenzymatic or enzymatic (6). FF samples taken from women after controlled ovarian stimulation contain antioxidants such as superoxide dismutase (SOD), glutathione oxidase

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(GPX), catalase (CAT), peroxiredoxins (Prx), glutathione transferase (GST), and glutathione reductase (GR) (7-9). Therefore, evaluation of FF in different types of infertility, which requires the use of assisted reproductive technology (ART), may reveal many predictive factors for improvement of outcome *in vitro* fertilization (IVF) (8). Lower levels of ROS and higher total antioxidant capacity (TAC) have been identified as pregnancy predictors for IVF cycles (10).

Identifying the changes in FF antioxidant levels and other related components, as well as their clinical potentials provide helpful means, by which physicians can decide on treatment strategies to increase fertility in infertile couples.

In certain previous investigations, increased SOD activity, reduced CAT and GST, and a modest rise in both GSH-Px and GSSG reductase were shown in the FF of reproductive-aged women (11). Also, it was shown that decreased FF homocysteine concentrations can significantly enhance the oocyte maturation and embryo quality (12).

The question that is posed at this juncture is whether with advanced age, the status of antioxidants in the FF is also affected. Also, it is not clear which antioxidant is most affected by aging. Therefore, this research aimed at assessing the relationship between the FF antioxidants (such as SOD, GSH, CAT and TAC) activity, aging, and IVF outcome.

Materials and Methods

Subject selection and ovarian stimulation

This cross-sectional study was carried out on women undergoing IVF/ICSI cycles. The FF samples were obtained from women referring to the ACECR Infertility Research and Treatment Center, who were under infertility treatment cycles due to unexplained infertility. The research excluded all cases of severe male factor infertility and azoospermia.

Ethical approval was achieved from the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1396.843). Participants were recruited to partake in the research and were given a study description as well as an informed consent.

A total of 65 patients participated in this research, and were categorized on the basis of age and clinical pregnancy outcome (yes/no):

Group 1: females aged > 35 years and pregnant (n=15).

Group 2: females aged ≤ 35 years and pregnant (n=20).

Group 3: females aged > 35 years and non-pregnant (n=15).

Group 4: females aged ≤ 35 years and non-pregnant (n=15).

In this study, women who did not become pregnant without contraception after one year and whose follicle-stimulating hormone (FSH) levels were less than 10 were recruited. Data were obtained using a questionnaire and were stored with the patients' clinical records. Before the start of ovulation induction, all patients had a transvaginal ultrasound (TVU) scan to check their ovaries and other pelvic tissues.

Women who were using antioxidant vitamins, hormonal contraception, or other hormonal preparations were not allowed to participate. Thus, patients with details on the etiology of infertility with tubular factors, endometriosis, cardiovascular problems, diabetes, nutritional diseases, depression, cancer, hypertension, hyperthyroidism, uterine fibroids, endometriosis, ovarian cyst, polycystic ovary syndrome, smoking history, hydrosalpinx and severe ovarian hyperstimulation (OHSS) (characterized by an egg count above 20 and clinical signs such as increased abdominal fluid, shortness of breath, pericardial effusion, pleural effusion, electrolyte disturbances and oliguria), and 19>body mass index (BMI)>30, were excluded from this study.

Ovarian stimulation protocol

The lengthy gonadotropin-releasing hormone (GnRH) agonist treatment was used for ovarian stimulation. On the 21st day of the menstrual cycle, a subcutaneous injection of GnRH agonist (Dipherelin triptorelin; Ipsen Pharma, Paris, France) was used to downregulate the hormone. Gonal F (Merck Serono, Germany) was introduced on the second day of the cycle. Gonadotropin injections were continued until at least two follicles reached 17-18 mm in diameter, at which point ovitrelle (250 µg, Merck Serono, Germany) was administered to induce the last oocyte maturation phase.

Collection and processing of follicular fluid

Under TVU guidance, the follicles were collected 34-36 hours following the ovitrelle injection. The FF from the first aspirated follicle was collected separately in a sterile tube without adding more culture medium. The remaining follicles were placed in regular culture media in preparation for the routine IVF process. According to the conventional classifications, the first aspirated oocytes were classified as germinal vesicle (GV), metaphase I (MI) stage, or metaphase II (MII) stage (13). MII oocytes were analyzed morphologically, and oocytes were divided into three groups based on the number of abnormalities: grade I: no abnormalities, grade II: one abnormality, and grade III: at least two abnormalities (14). Oocytes were implanted in conventional culture medium, and their development was tracked after intracytoplasmic sperm injection (ICSI) procedure using spermatozoa. After the removal of the oocytes, the FF was centrifuged at 2700 rpm for 5 minutes to remove cellular components. The supernatant was stored at -80°C for assessment of SOD, CAT, TAC, and GSH activities. Then, the day 3 embryo was transferred after morphological examination according to

the standard classifications (15). Only FF with minimal contamination and no blood or culture medium was used. After fresh embryo transfer, chemical pregnancy detected by checking beta-hCG in the serum.

Measurement of superoxide dismutase activity

SOD activity was measured using a Randox test combination (16). Superoxide radicals were created by combining 2-(4-iodophenyl)3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) with xanthine and xanthine oxidase to give a red formazan dye. This reaction is inhibited by superoxide dismutase, which converts the superoxide radical to oxygen. SOD activity in cleaned erythrocyte hemolysates was measured at 505 nm using a CECIL 3000 SCANNING Spectrophotometer. The results were given in SOD U/ml.

Measurement of glutathione activity level

The GSH assay was carried out using the usual procedure (17). The standard curve was created with a 1 mM GSH solution and evaluated for GSH levels. In addition, 0.2 ml of FF or serum samples were mixed with 2.3 ml of potassium phosphate buffer (0.2 M, pH=7.6), followed by 0.5 ml of DTNB (0.001 M) in buffer. After 5 minutes, the absorbance of the reaction products was measured at 412 nm. Each sample's total protein concentration was determined, and GSH values were represented as nmol/mg protein.

Measurement of catalase activity

CAT activity was evaluated based on the method elucidated by Koroliuk et al. (18). In brief, this test is on the basis of the reduction rate of hydrogen peroxide per unit time due to the activity of CAT in the sample. Ammonium molybdate with hydrogen peroxide forms a yellowish complex that has a maximum light absorption at 510 nm. The enzyme CAT prevents this reaction by breaking down hydrogen peroxide. It has maximum light absorption. The enzyme CAT prevents this reaction by breaking down hydrogen peroxide. One U is the enzyme amount that breaks down one micromole of hydrogen peroxide in one minute. To measure the activity of Tris-HCl buffer CAT enzyme (0.05 mmol/l, PH=7.8), 15 mmol/l hydrogen peroxide was made and mixed with the sample and after 15 minutes 0.4 ammonium molybdate was added. Finally, the optical absorption of the samples and control against Buffer Tris-HCl at 510 nm were read by microplate spectrophotometer.

Measurement of catalase level

TAC in FF was determined in duplicate, first after defrosting and then again after 72 hours of storage at room temperature in the dark, using the ferric reducing/antioxidant power (FRAP) assay (14, 17, 18). TAC values obtained from this assay are proportional to those obtained from the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (19). In summary, 50 µl of FF was mixed with 1 ml of freshly made FRAP reagent, which contained 1 mM 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ), 2 mM $FeCl_3$, and 240 mM sodium acetate, at pH=3.6. Antioxidants in the sample cause the colorless Fe^{3+}–TPTZ complex to be reduced to an intense blue Fe^{2+}–TPTZ complex, which is detected using a spectrophotometer at 593 nm after incubation for 10 minutes at 25°C. A standard curve of 0-1 mM Fe^{2+}–TPTZ was prepared by diluting $FeSO_4.7H_2O$ (1 mM). TAC estimates are therefore given in mmol/l ferrous equivalents.

Statistical analysis

Statistical analyses were carried out with the help of the SPSS software version 23 (IBM Corp., Armonk, New York, USA). Since the data of the present study did not have a normal distribution (as attested by Kolmogorov-Smirnov test), non-parametric tests of Kruskal-Wallis and Spearman were used. Therefore, for pairwise comparison of the groups, Mann-Whitney U test was used. The data obtained from the present study were expressed as mean ± SD, and the significance level was set at $P<0.05$.

Results

In order to investigate the challenging relationship of antioxidant activity with age-related changes, fertilization rate, and pregnancy outcomes, we collected FF samples from women undergoing IVF/ICSI cycles due to unexplained infertility.

Comparison of physiological parameters in groups undergoing IVF/ICSI cycles

The mean age of the women in groups 1 to 4 were: 39 ± 2.29, 30.35 ± 2.73, 38.87 ± 2.10, and 31 ± 2.77, respectively.

The mean number of retrieved oocytes in the four mentioned groups was statistically significant (P=0.041). Groups 4 and 1 had the highest and lowest oocyte counts, respectively.

Statistical analysis showed that the mean number of MII oocytes in the four groups was not significantly different (P=0.260). Nonetheless, groups 1, 2 (1 and 2 were equal) and 4 had the lowest and highest MII oocyte counts, respectively.

Statistical analysis of the fertilization rate in each group showed a significant difference among the groups (P=0.042). The information of all four study groups is summarized in Table 1.

Comparison of oocyte and embryo morphological scores in groups undergoing IVF/ICSI cycles

There was no significant difference in oocyte morphological scores among the different groups (P=0.291). Embryo morphological scores also were not significantly different in the four study groups (P=0.188). The information of all study groups is summarized in Table 2.

Table 1: Comparison of physiological parameters in groups undergoing *in vitro* fertilization cycles

Groups/Variables	1[a] (n=15)	2[b] (n=20)	3[c] (n=15)	4[d] (n=15)	P value
Age (Y)	39 ± 2.29	30.35 ± 2.73	38.87 ± 2.10	31 ± 2.77	-
NO. Oocyte	8.07 ± 2.60	8.35 ± 2.23	9.13 ± 3.18	10.33 ± 2.09	0.041[abcd*] 0.532[ab] 0.270[ac] 0.017[ad*] 0.563[bc] 0.013[bd*] 0.103[cd]
	7 (5)	8 (3)	8 (3)	11 (4)	
NO. Oocyte MII	7 ± 2.07	7 ± 1.68	7.33 ± 3.65	8.27 ± 1.90	0.260
	6 (4)	7 (2)	7 (6)	8 (3)	
Proportion of MII oocytes (%)	88.03	85.02	78.12	81.46	0.656
Fertilization rate (%)	71.28	72.87	66.16	55.28	0.042[abcd*] 0.664[ab] 0.416[ac] 0.021[ad*] 0.299[bc] 0.007[bd*] 0.191[cd]

Data presented as n or mean ± SD or median (IQR). P<0.05 were considered significant. The values are compared by Kruskal-Wallis Test. *; Shows significant difference. [a]; Group 1: Females aged >35 years and pregnant, [b]; Group 2: Females aged ≤35 years and pregnant, [c]; Group 3: Females aged >35 years and non-pregnant, [d]; Group 4: Females aged ≤35 years and non-pregnant. MII rate; No. MII oocyte/all oocyte retrieved×100, Fertilization rate; No. oocyte with 2PN/No. MII oocyte injected ×100.

Table 2: Comparison of oocyte and embryo morphological score in groups undergoing *in vitro* fertilization cycles

Groups/Variables	Scoring	1 (n= 15)	2 (n=20)	3 (n=15)	4 (n=15)	P value
Oocyte morphological score	Grade I	3	7	7	3	0.291
	Grade II	11	9	4	5	
	Grade III	1	4	4	7	
Embryo morphological score	Good	9	10	5	4	0.188
	Fair	3	6	8	4	
	Poor	3	4	2	7	

Data presented as Number. The mean values are compared by Kruskal-Wallis Test. P<0.05 were considered significant. Group 1; Females aged >35 years and pregnant, Group 2; Females aged ≤35 years and pregnant, Group 3; Females aged >35 years and non-pregnant, and Group 4; Females aged ≤35 years and non-pregnant.

Levels of follicular fluid antioxidant activity in groups undergoing IVF/ICSI cycles

The levels of SOD activity in FF of all four groups were not significantly different (P=0.218).

However, the mean of GSH in FF was significantly different between the four groups (P<0.001).

Pairwise comparison of groups was performed to determine the differences in the level of GSH activity. The results showed that the level of activity of this enzyme in group 2 was significantly higher compared with group 3 (P<0.001). Also, the level of GSH activity in group 4 was significantly higher compared with groups 1 and 3 (P=0.016 and P<0.001, respectively).

Therefore, these results showed that the mean activity level of this enzyme is generally higher in younger women.

The mean of CAT activity in the four groups showed that there was a significant difference among them (P<0.001). Pairwise comparison of the groups was performed to determine the level of activity of CAT in each group. The results indicated that the mean of CAT activity in group 2 and group 4 was higher than that in the other groups. The activity level of this enzyme in group 3 was lower compared with all groups.

The four study groups were significantly different in terms of their mean TAC level (P<0.001). Pairwise comparison of the groups was performed to determine how different the level of TAC enzyme is among the study groups. The results showed that the mean TAC level in group 2 was significantly higher compared to groups 3 and 4 (P=0.004 and P<0.001, respectively). These results showed that the mean level of this enzyme was higher in pregnant women.

The results of FF antioxidant activity levels in the four study groups and their pairwise comparisons are presented in Table 3.

Table 3: Levels of FF antioxidant activity in groups undergoing *in vitro* fertilization cycles

Groups variables	1[a] (n=15)	2[b] (n=20)	3[c] (n=15)	4[d] (n=15)	P value
SOD activity (U/g)	0.23 ± 0.12	0.29 ± 0.26	0.22 ± 0.21	0.16 ± 0.11	0.218[abcd]
	0.17 (0.13)	0.25 (0.19)	0.17 (0.13)	0.11 (0.21)	
GSH (nmol/mg protein)	52.32 ± 12.54	66.29 ± 25.26	28.86 ± 3.45	64.71 ± 14.15	<0.001[abcd*] 0.064[ab] <0.001[ac*] 0.016[ad*] <0.001[bc*] 0.640[bd] <0.001[cd*]
	55.14 (13.14)	60.75 (24.75)	29.78 (5.07)	69.42 (19.50)	
CAT activity (mM/L)	1.44 ± 0.46	2.53 ± 1.55	0.96 ± 0.51	2.89 ± 1.55	<0.001[abcd*] 0.662[ab] 0.022[ac*] 0.003[ad*] 0.003[bc*] 0.473[bd] 0.001[cd*]
	1.44 (0.46)	2.52 (2.46)	0.79 (0.84)	3.10 (2.22)	
TAC activity (mMol/l)	0.69 ± 0.27	0.76 ± 1.30	0.58 ± 0.11	0.27 ± 0.11	<0.001[abcd*]
	0.70 (0.33)	0.75 (0.21)	0.64 (0.19)	0.29 (0.18)	0.250[ab] 0.198[ac] <0.001[ad*] 0.004[bc*] <0.001[bd*] <0.001[cd*]

Data presented as n or mean ± SD or median (IQR). The mean values are compared by Kruskal-Wallis Test. P<0.05 were considered significant. *; Shows Significant difference. [a]; Group 1: Females aged >35 years and pregnant, [b]; Group 2: Females aged ≤35 years and pregnant, [c]; Group 3: Females aged >35 years and non-pregnant, [d]; Group 4: Females aged ≤35 years and non-pregnant, FF; Follicular fluid, SOD; Superoxide dismutase, GSH; Glutathione, CAT; Catalase, and TAC; Total antioxidant capacity.

Correlation of age, pregnancy, and oocyte and embryo morphological score with the level of antioxidants activity Spearman correlation was used to investigate the relationship between age and pregnancy and the level of antioxidant activity. The results showed that GSH level has a significant inverse correlation with increasing age (P<0.001, r=-0.55), and a significant direct correlation with pregnancy (P=0.015, r=0.30). Therefore, it can be suggested that with increasing age, the level of GSH decreases, but this level increases during pregnancy. CAT level had a significant inverse correlation with increasing age (P<0.001, r=-0.42). Therefore, with increasing age, the level of this antioxidant decreases, and it is not correlated with positive pregnancy. The level of TAC, on the other hand, had no significant correlation with increasing age, but it had a significant direct correlation with positive pregnancy (P<0.001, r=0.59), as the level of this antioxidant increases with positive pregnancy. The data discussed in this section are summarized in Table 4.

Table 4: Correlations between age and pregnancy in relation to level of antioxidants activity

Variables	SOD (n=65)	GSH (n=65)	CAT (n=65)	TAC (n=65)
Age (Y)				
r	0.26	-0.55	-0.42	0.10
P value	0.835	<0.001*	<0.001*	0.417
Pregnancy				
r	0.19	0.30	0.08	0.59
P value	0.127	0.015*	0.498	<0.001*

Relationship between age, pregnancy and level of antioxidants were done by Spearman correlation. SOD; Superoxide dismutase, GSH; Glutathione, CAT; Catalase, TAC; Total antioxidant capacity, r; Correlation coefficient, *; Correlation is significant at the 0.01 level.

Spearman correlation was also used to investigate the correlation between oocyte morphological score and levels of antioxidants activity. The correlation between oocytes morphological score and levels of SOD, GSH, CAT, and TAC was not statistically significant (P=0.190, P=0.343. P=0.327 and P=0.190, respectively). Similarly, no statistically significant correlation was observed between embryo morphological score and the levels of SOD, GSH, CAT and TAC (P=0.315, P=0.852. P=0.853 and P=0.221, respectively).

Discussion

Considering the important effects of FF compounds on oocyte and embryo development, this research aimed to examine the impact of aging on the changes in FF antioxidant activity levels and their possible relationship with the outcome of IVF cycles. The main objective of the present study was to identify these changes and use them clinically to make decisions about treatment strategies in infertile couples.

According to our results, the proportion of MII oocytes (at ICSI) in the study groups was not significantly different. However, fertilization rates were significantly higher in pregnant women than in their non-pregnant counterparts.

The results of this study also show that the morphology scores of oocytes and embryos were not significantly different in all women undergoing IVF/ICSI cycles, while antioxidant levels and the outcomes of IVF cycles

were significantly different. These results may indicate the effects of antioxidants on non-morphological levels, including the molecular levels, which may affect the fertility potentials of oocytes and embryo implantation.

This investigation revealed that the SOD activity did not differ substantially in all of the studied infertile women. Nonetheless, mean SOD activity in younger and pregnant cases was higher than those undergoing IVF/ICSI cycles. Therefore, it could be argued that in the present study, changes in SOD activity were not age-related, as they were higher in groups with pregnancy outcomes. Human investigations have demonstrated that older women have lower levels of SOD and CAT in their FF compared with their younger counterparts, and that older women experience decreased fertilization rates and blastocyst development (20). A previous study showed that a statistically significant decrease was observed in the SOD activity of FF in polycystic ovary syndrome patients compared with the control group (21).

Our results also indicated that the mean level of GSH was higher in younger women. In addition, the mean SOD activity in younger and pregnant cases was more significantly different in comparison to other cases. Further, the level of GSH had a significant inverse association with age and a significant direct association with pregnancy. Indeed, both pregnancy and age contribute to changes in the GSH levels. The lower GSH content seen in endometriosis FF compared to the controls was also linked to low quality embryo (22). GSH plays a role in various biological activities, including cell proliferation, differentiation, and death (5). GSH, according to the literature, boosts gamete viability and fertilization (23). ROS are thought to be involved in the start of apoptosis, as ROS levels rise before any other signal associated with death in follicles. A statistically significant increase in atretic antral follicles was seen in rat ovaries after limiting GSH production with the inhibitor buthionine sulfoximine (BSO) (24). FSH is widely thought to prevent apoptosis in antral follicles, and surprisingly, FSH therapy enhances GSH production. The anti-apoptotic effect of FSH on granulosa cell death is significantly reduced by inhibiting GSH production with BSO in cultured follicles (25). A previous study on the effects of cyclophosphamide on the ovaries showed that this cancer drug causes follicular apoptosis as well as reduced GSH levels in the ovaries (24). Apoptosis in cultured preovulatory follicles is induced by oxidative stress, and the antioxidant GSH plays a role in regulating the anti-apoptotic impact of FSH on granulosa cells in preovulatory follicles (26). An investigation was conducted on blood samples and FF of the first-retrieved follicle from PCOS women, and the mean activity of GPx and GR, as well as GSH levels in the serum and FF were compared with the quality of the first follicle and resulting embryo. The mean GPx activity and GSH levels were considerably greater in the serum and FF of high-quality grade I embryos (27).

The present study showed that in younger women, regardless of pregnancy, the mean CAT activity was significantly higher compared to the older women. Moreover, a significant inverse association was detected between age and CAT activity. That is, as age increases, the activity of these antioxidants decreases significantly. In agreement with the present study, a decrease in CAT activity in FF was observed with advanced age in a previous study (20). In another study, select indicators such as CAT activity, TAC, and hydrogen peroxide (H_2O_2) were measured in FF samples derived from cow antral follicles. According to their results, although TAC rose dramatically, CAT activity and H_2O_2 dropped considerably as follicle size grew. Lower TAC and higher H_2O_2 levels in tiny follicles indicate an increase in ROS during the early stages of folliculogenesis. Because CAT levels are highest in the FF of small follicles in a low total TAC, CAT may serve as a major antioxidant defense in the early phases of folliculogenesis (28). In the present study, CAT activity in younger groups was significantly higher, which may be due to the fact that small-sized follicles (ovarian reservation) are more common in younger women. However, another study discovered that following FSH stimulation, CAT activity rose, and the degree of this rise was greater in large follicles than in medium or small follicles (29). According to some review studies, CAT plays a role in follicular formation, the estrous cycle, and steroidogenic events in the ovaries (5), and protects the DNA from oxidative damage (30). Furthermore, increased CAT activity was identified in obese and infertile women, revealing that the FF of obese women was associated with higher CAT activity, which indicates excessive oxidative stress (31). Results of the present study showed that with advanced age, the activity of this antioxidant decreases, and based on other studies CAT activity increases in pathologic cases. The distribution and oscillation of CAT during several ovarian cycles have been linked to gonadotropin regulation (32). As a result, CAT may be regarded as a protective factor neutralizing H_2O_2 and preserving ROS equilibrium. Measuring the activity of antioxidants may not be enough for a predictive marker alone. Therefore, future studies are recommended to assess the amount of balance between CAT activity and ROS associated with assisted reproductive technique (ART) outcomes.

Gonadotropin signaling modulates oocyte GSH levels throughout the preovulatory stage, according to both *in vivo* and *in vitro* investigations. FSH stimulation has been reported to increase ovarian GSH concentration *in vivo* (33). GSH, CAT, and SOD can also protect big antral follicles against apoptosis in rats (34). Reduced antioxidant systems have also been linked to age-related reproductive reduction (35, 36). Previous studies indicated that the ability of antioxidants to scavenge ROS is related to fertilization outcomes (5). According to the above explanations, it can be argued that high levels of CAT activity can create a level of confidence in the ROS-antioxidant balance. Increasing the ROS in different conditions leads to increased GSH and thus maintains the balance between ROS and antioxidants, consequently

reducing the destructive effects due to ROS. Given the association of age and gonadotropin with level of GSH and CAT activity, it is recommended to pay special attention to GSH and CAT in cases of age-related infertility.

The present study compared four different groups in terms of their mean TAC level, which was higher in the pregnant groups as opposed to the non-pregnant ones (regardless of the age). Also, pairwise comparisons and correlation analysis to investigate the changes in the level of TAC showed that pregnancy is more important than age as far as the changes in the level of this antioxidant are concerned. A previous study reported that TAC increased significantly as follicle size increased in estrous cycle (28). Another prospective cross-sectional study showed that FF TAC levels were higher in women with 'unexplained' (UE) or tubal factor (TF) infertility, while age did not affect FF TAC activity in general. Similarly, the results of the present study confirm this correlation between age and TAC activity. It has been demonstrated that low TAC is associated with fertilization incompetence, while high TAC is associated with embryo nonviability (optimum follicular TAC was ~0.68 mmol/l) (37). In the present study, TAC in younger and pregnant women was 0.76 mmol/l, which is higher compared with those undergoing IVF/ICSI cycles.

Since the antioxidant activity can be affected by different conditions, including increased FSH in menstrual cycle (33), age (10) and pregnancy, measuring the antioxidant activity, may not be enough for a predictive marker alone. Although the small sample size in this study is one of its limitations, based on data from the literature, it is suggested that the balance between antioxidants activity and the amount of ROS in FF and serum associated with the outcomes of IVF cycles, be addressed in future studies. Measurement of FF antioxidant activity/ROS ratio is necessary in women undergoing IVF cycles (related to aging and etiology of infertility). For this ratio, definition of a cutoff point could be predictive of the pregnancy outcome. Further studies on GSH and CAT activity are recommended to be performed with the aim of using these antioxidants for the prevention, diagnosis and treatment of infertility.

Conclusion

The present study represents the possible effects of FF antioxidants on fertility potentials of oocyte and embryo implantation at a molecular level. The level of the TAC was higher in pregnant women while the mean GSH and CAT levels were higher among younger women. The mean GSH and CAT levels decreased as age advanced. According to the results of this study, there is a correlation between GSH, age, and pregnancy, and it is necessary to carry out more research on FF antioxidants and their effects on maintenance of pregnancy.

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Authors' Contributions

M.A., R.N., M.H., P.D.; Participated in study design, Data collection, and Evaluation. R.N., M.A.; Collected the samples. M.A., M.H., E.Gh, S.Am., A.Z., S.Ad., M.M.; Analyzed antioxidants activity. P.D., M.A.; Carried out data analysis and Interpretation. P.D., R.N.; Wrote the first draft of the manuscript. All authors revised and approved the final version of the manuscript.

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