



## Assessment of follicular fluid paraoxonase activity with pregnancy outcomes in women undergoing IVF/ICSI



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### Abstract

The search for a reliable marker to predict pregnancy success is not yet identified. This study aims to evaluate paraoxonase activities (PON1) in follicular fluid (FF) and their correlation with oocyte yield, fertilization, and clinical pregnancy (CP). **Methods:** The study included 99 women aged 22- 37 years old who were undergoing IVF/ICSI, were divided into fertile, which represents the control group consisting of 25 participants, and the infertile group, which was subdivided into 21 patients with PCOS, 26 patients with a low level of AMH, as well as 27 patients with unknown cause of infertility. The FF Basal PON1, salt stimulated PON1, and arylesterase activities were measured using the spectrophotometry method. **Results:** The PON1 basal activity of the PCOS group was higher ( $P < 0.05$ ) when compared with the control and UI groups. The PON1 arylesterase activity of the PCOS group was higher ( $P < 0.05$ ) when compared with control, UI, and low AMH groups. Each group showed a difference ( $P < 0.05$ ) in the pregnancy success in PCOS, low AMH, and UI groups but not in the control group. **Conclusion:** An increase in FF antioxidants seems to be a negative fertilization indicator, which may represent an adverse ovarian condition that triggered anti-oxidant behavior.

**Keywords:** Paraoxonase1 activity (PON1), clinical pregnancy (CP), follicular fluid (FF); infertility, in vitro fertilization (IVF).

### Introduction

Infertility is defined by the World Health Organization (WHO) as the failure in getting pregnant due to health problems in one of the couples or both, mostly, the female. According to WHO; the estimated prevalence of infertility is 1.9 percent among women aged 20-44 years [1].

Assistant reproductive technology is used to help infertile couples in achieving pregnancy, in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) is an important technique used as an effective procedure in female infertility treatment [2]. During a normal pregnancy, placental oxidative stress (OS) is present during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and protein kinases [3, 4]. Impaired ovarian function changes the reproductive health of women seriously and is an important factor in female infertility [5].

Follicular fluid contents of biomolecules, that are passing through blood –follicle barrier, reflect the nature of the microenvironment around the oocytes and it is a good indicator for assessing the IVF failure [6]. Paraoxonase 1 (PON1) (E.C. 3.1.8.1) is a liver-

synthesized and secreted calcium-dependent multi-functional enzyme that can be easily detected as systemic circulation molecules bound to HDL, and to a small degree, to other lipoproteins [7, 8]. PON1 have one of the broadest substrate specificities; it occupies three hydrolytic activities: paraoxonase activity, arylesterase activity, and lactonase activity [9].

PON1 is an antioxidant enzyme carried on HDL molecules that enable the enzyme to cross the blood-follicle barrier to act as a terminator for lipid peroxides produced from lipid peroxidation [10]. In earlier publications in our lab, we had studied total peroxide concentrations, as well as total antioxidant capacity, and OS index in serum and FF of patients undergoing IVF program to explore the possible relationships between local and systemic OS [11], and oxidative stress statue in follicular fluid from fertile and infertile patients that have PCOS, in addition to unexplained infertility (UI) [12].

In this study, we planned to assess FF PON1 activity and outcome parameters of pregnancy (rates of Cp, number of oocytes retrieved, and fertilization number). Also, explore the possibility of using the

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activity of this enzyme to monitor the clinical pregnancy outcome.

### **Materials and Methods**

#### **Sample selection**

This study is potential cohort research consisted of 99 women attending Al-Qima Hospital for Infertility and In Vitro Fertilization for IVF/ICSI program between October 2019, and June 2020. The studied groups were fertile women (control group; n= 25), and infertile patients were divided according to infertility cause to patients with PCOS (n=21), patients who have a low level of hormone AMH(26), and patients who were normal in their gynecology and clinical test in their blood but with no known cause of infertility (UI group; n=27)). The diagnosis of PCOS was done by a gynecologist depending on the Rotterdam criteria.

Inclusion criteria were: age 22–37 years, BMI 18–29(kg/m<sup>2</sup>), and no other gynecology diseases. The exclusion criteria included in this study were pregnancy, presence of cardiovascular, renal, liver, lung, diabetes mellitus diseases, and smoking.

All women signed an informed approval after explaining the aim of the study, and a protocol approval from our local ethically and scientifically institution was obtained.

#### **The protocol**

The protocol was done by the administration of gonadotrophin-release hormone-agonist (GnRH-a) that begins in menstruation cycle (MC) day 21, then all patients were treated with 3 ampoules of Recombinant human chorionic gonadotrophin (hCG) starting between two to five days of the next MC. This dose was attuned according to the outcome of the ultrasound examination when the size of the egg is appropriate. Then, inoculation of the hCG was made, and after 36 hours; the retrieval of the appropriately sized follicles was made [13].

Clear supernatant of FF, which was obtained by centrifugation (600×g; 10 min), was distributed in Eppendroff tubes then, stored at -20 °C until used. Serum was obtained from blood samples of all participants on the day of aspiration.

#### **Pregnancy outcomes**

The percentage ratio of oocyte maturity rate, cleavage rate, and fertilization rate was calculated [14, 15, 16]

#### **Biochemical analysis**

Various substrates (phenylacetate and paraoxon) were used to test the activities of PON1 (arylesterase and paraoxonase activities, respectively). These activities were measured using spectrophotometry method [17, 18].

Determination of FF total cholesterol, triglycerides, and HDL-c were done using kits from Spinreact (Spain). Immunoassay for the in vitro quantitative

determination of serum LH, FSH, TSH, AMH, and Prolactin was made using the electrochemiluminescence immune assay “ECLIA” which was done using Elecsys and Cobas e411 immunoassay analyzer.

#### **Statistical Analysis**

Data were displayed in simple measures; mean and standard deviation ( $\pm$ SD). The significance of differences of various means (quantitative data from different groups and control group) were tested utilizing analysis of variance (ANOVA), whilst using independent students' t-test for differences between two means. When the P-value was less than 0.05; this statistically was considered a significance value [19].

#### **Results and Discussion**

Table 1 shows the clinical characteristics in the serum of the four studied groups. In the present study, it is clear that mean age and infertility duration tend to be similar among the groups indicating that infertile women in our community seeking medical, and this is probably due to early marriage. It is well known that age-related decline of the biological capacity of a woman to reproduce and is primarily related to the poor developmental potential of women's gametes. Female ageing is the most significant determinant of IVF success [20]. The present study is comparable to other studies [21, 22].

The mean level of serum LH, LH/FSH ratio, Prolactin, AMH, and E2 showed non-significant ( $P > 0.05$ ) differences among the four studied groups.

However, the mean serum FSH of the PCOS group showed significant ( $P < 0.05$ ) lower levels compared to UI, and mean serum progesterone in the PCOS group showed significant ( $P < 0.05$ ) higher levels when compared with the low AMH group with non-significant differences with other groups. The mean serum TSH level of the UI group in comparison to control was significantly lower.

The current study findings of increased AMH levels (without significance) in the serum of PCOS patients compared with the four studied groups agreed with a previous study by Pellat *et al.*, (2007). It can be explained by the fact that PCOS patients have an increasing number of small antral follicles which are the major sites of producing AMH [23]. The study of Stracquadiano *et al.*, (2018) explained that this hormone has an inhibitory effect on the hormone FSH and thus prevents natural ovulation [24]; which explains what was found in this study where the mean of FSH level in PCOS women was lower.

The mean level of FFTG in the PCOS group was significantly higher when compared to control, UI, and low AMH groups, while, the mean level of FF TC and HDL-C showed non-significant differences ( $P > 0.05$ ) among the four studied groups.

Table 1. Clinical characteristics of the four studied groups.

Parameters	Control	UI	PCOS	AMH
Age (year)	30.2±7.9	30.44±6.2	27.71±5.1	31.23±6.4
Duration of infertility (year)	7.16±5.29	4.18±2.66	5.66±3.85	5.84±4.62
LH (m IU/ml)	5.00±1.76	6.84±2.23	8.03±4.23a	6.23±1.80
FSH (m IU/ml)	6.30±1.37	7.47±1.66	5.85±1.59b,d	8.40±2.40a
LH/FSH ratio	0.87±0.34	0.93±0.31	1.39±0.67a,b,d	0.74±0.18
Prolactin (ng/ml)	19.65±8.54	19.41±12.44	16.92±5.89	21.6±11.7
Progesterone(ng/ml)	0.37±0.28	0.35±0.19	0.53±0.55	0.26±0.13c
AMH (ng/ml)	3.45±1.44	3.11±1.00	6.25±3.74a,b,d	0.96±0.4a,b
E2 (dg/ml)	281.6±819.6	241.1±639	176.7±647.2	30.48±16.25
TSH (mIU/ml)	2.82±1.80	1.64±0.66a,c	3.23±1.97	2.61±1.17

Statistical analysis was performed by ANOVA followed by the Post Hoc test (Tukey's test) for multiple comparisons. The small letters represent a significant difference between groups: <sup>a</sup> with control; <sup>b</sup> with UI; <sup>c</sup> with PCOS; <sup>d</sup> with AMH

Table 2. Mean (±SD) of clinical characteristics in FF of the four studied groups.

Parameters	Control	UI	PCOS	AMH
Total protein g/dl	4.16±0.74	4.31±1.07	5.04±0.90 <sup>a, b</sup>	4.62±0.84
Total cholesterol(mg/dl)	38.56±8.8	40.08±16.07	37.21±9.1	33.16±10.9
Triglyceride (mg/dl)	19.21±17.7	22.64±12.6	37.53±26.4 <sup>a,b,d</sup>	11.79±3.9
HDL-C (mg/dl)	19.12±4.8	15.45±3.73 <sup>a</sup>	15.81±3.9	17.72±5.9

Statistical analysis was performed by ANOVA followed by the Post Hoc test (Tukey's test) for multiple comparisons. The small letters represent significant differences between groups: <sup>a</sup> with control; <sup>b</sup> with UI; <sup>c</sup> with PCOS; <sup>d</sup> with AMH

A cross-sectional study included fertile females, infertile females, and women with UI, which analyzed HDL particle lipids (cholesterol, phospholipids, and TG) in FF samples that displayed significant differences in HDL-C and TG between follicles variability among the groups [25].

The present study and other studies by Bacchetti *et al.*, and Kim *et al.* revealed that serum lipid profile concentrations were higher than that of FF from infertile women undergoing ovarian stimulation [26, 27].

The mean of the corresponding IVF outcomes; non-significant variations ( $P > 0.05$ ) were observed in the oocyte maturation rate and cleavage rate between the four studied groups (Table 3).

In pregnancy outcome parameters, statistical significance was found between PCOS women, control women, UI women, and AMH women in aspirated oocytes, MII oocytes, fertilized oocytes, 2PN, transferred embryo, G1 embryo, and fertilization rate.

PCOS patients had higher serum AMH levels than controls. The low AMH level group showed a lower number in the number of aspirated oocytes, MII oocytes, the number of fertilized oocytes, the number of embryos at 2 PN, the number of the transferred embryo, and embryo frequency (G1) when compared with control and PCOS women that have higher AMH level, which was in agreement with that of a previous study [28].

In this study, the number of aspirated oocytes and the number of fertilized oocytes were significantly lower in the UI group than in the control and PCOS groups. The number of MII oocytes, the number of embryos at 2 PN, and the number of the transferred embryo

were significantly lower in the UI group than in the PCOS group.

Fertilization rates were significantly higher in the unexplained infertility group than in the control group. A study by Alasmari *et al.*, (2018) found non-significant differences in fertilization rates between the unexplained infertility group and group with male factor infertility [29].

Liu *et al.*, (2020) studied thyroid autoimmunity and its association with pregnancy outcomes. They did not find significant alterations in the pregnancy rate and other IVF parameters [30].

Figure (1) represents a comparison between primary and secondary infertility. The rate of primary infertility is higher than that of secondary infertility in the entire samples of control women, PCOS women, and women with low AMH. These results are comparable to the results obtained from some studies dealing with the prevalence of primary and secondary infertility, in which the rate of primary infertility is more frequent than that of secondary infertility [31, 32].

As shown in Table 4, the mean level of FF PON1 basal activity of the PCOS group was higher significantly ( $P < 0.05$ ) when compared with that of control and UI groups. The mean level of FF PON1 (s-s) activity of the PCOS group was higher significantly ( $P < 0.05$ ) when compared with that of control, UI, and low AMH level groups. The mean level of FF arylesterase activity of the PCOS group showed a higher level significantly when compared with that of control, UI, and low AMH groups.

These results disagree with a previous study by Göktoğla *et al.*, (2017) they reported that PON1 and arylesterase activities in FF of women with PCOS

were significantly lower in comparison with control (male factor infertility) [33].

Browne *et al.*, (2008) have reported a positive correlation of elevated PON-1 rates with oocyte and embryo quality and discovered PON-1 arylesterase activity in human follicular fluid of patients undergoing IVF [34]. Besides, the potential advantage of evaluating PON-1 as a biomarker in the clinical diagnosis or treatment of oxidative stress was proposed [35]. Valkenburg *et al.*, (2008) found in their study increased OS in PCOS women, which agreed with our result where the antioxidant PON1 activity was higher in the PCOS group to rebalance the higher OS [28], which may affect characteristics of embryos that correlate adversely with OS [36].

Proteomic analysis of follicular fluid documented 31 up-regulated proteins and 18 down-regulated proteins with different functions, only 5 of which have a function in the genital system. Of these proteins is PON1 with its two enzyme activities (paraoxonase/arylesterase) which are related to oxidative stress [28].

The means of FF PON1 activities (basal and s-s) in the pregnant control group were higher than those of the non-pregnant group. The differences were statistically significant ( $P < 0.05$ ) only in FF basal PON1 activity. Moreover, the mean of FF arylesterase activity in the pregnant control group showed a lower level significantly ( $P < 0.05$ ) compared to that of the non-pregnant group (Table 5).

In the pregnant UI group, the mean of FF PON1 activities (basal, s-s, and arylesterase) were lower than those of the non-pregnant group, and the differences were statistically significant ( $P < 0.05$ ) only in FF arylesterase activity.

In the pregnant PCOS group, the mean of FF PON1 activities (basal and arylesterase) were lower than those of the non-pregnant group. The differences were statistically significant ( $P < 0.05$ ) only in FF basal PON1 activity. Moreover, the mean of FF PON1 activity (s-s) in the pregnant PCOS group was higher but non-significant ( $P > 0.05$ ) than those of the non-pregnant group.

In pregnant women of the low AMH level group, the mean of FF PON1 activities (basal and arylesterase) was higher than those of the non-pregnant group. The differences were statistically significant ( $P < 0.05$ ) only in FF basal PON1 activity. Moreover, the mean of FF PON1 activity (s-s) in pregnant of low AMH level group was lower but non-significant ( $P > 0.05$ ) than those of the non-pregnant group.

Additionally, non-significant ( $P > 0.05$ ) differences were found in the biochemical pregnancy rate between the four groups. However, each group showed a significant difference among pregnant and non-pregnant groups in PCOS, low AMH, and UI groups but not in the control group.

Conflict results from studies that have been taken into consideration the correlation between AMH level and IVF outcome such as the study by Terasaka *et al.*, (2017) who found that IVF outcome was lower when this hormone is very low. Their results confirmed the fact that despite the low AMH level; it is a good marker for the follicular pool while it may reduce pregnancy success [36].

In women with a very low ovarian pool, their age represents a very important factor in predicting pregnancy rate. It was found that the achievement of the IVF process is related to AMH level, when this hormone is in the normal range (the pool of follicles in the ovary is high) the rate of pregnancy is higher in comparison with women with lower level [37] or with women with higher AMH level such that in PCOS women [38].

In a fairly large study looking at fecundity rates in a normal population, the authors showed that women with low serum AMH levels achieved similar pregnancy rates compared to those with normal or high AMH levels. They concluded that neither low nor high AMH levels relative to normal AMH were associated with fecundity in unassisted conceptions in a cohort of fertile women with a history of one or two prior losses [39].

Our findings are confirmed by Attaran *et al.*, (2000) study, which found that pregnant women with male infertility had a higher level of FF ROS than women with no pregnancy which may be used as a marker of IVF outcomes [40]. Likewise, other experiments examined the level of total oxidants before and after both oocyte pick upstage and embryo transfer stage. They reported that in the clinically pregnant group, total oxidants were higher relative to the non-pregnant clinically-treated group of patients with IVF treatment. They described how the oxidant-antioxidant balance is essential to IVF performance rather than TAC alone [41]. Conflict findings reported by Ozturk *et al.*, (2018) showed that FF's total oxidants in pregnant women were lower than in non-pregnant IVF cases, but statistically significant differences were not observed. They believed that the total oxidants appear to be an inadequate indicator of the result of IVF clinical pregnancy [42]. A potential reason for the higher production of intrafollicular ROS in pregnant (control and low AMH) groups, that result during follicle development, is the active metabolism which may result in increased ROS production and oocyte destruction. There is a need for higher TAC concentrations in pregnant groups to improve the intrafollicular prooxidant/antioxidant balance to reduce the damage from elevated rates of ROS; this was consistent with previous studies [43, 11].

**Table 3: Characteristics of IVF/ICSI outcomes in the four studied groups.**

Parameters	Control	UI	PCOS	AMH
Aspirated oocytes	9.04±4.77	6.22±3.11 <sup>a,c</sup>	11.76±4.19	3.95±2.43 <sup>a,c</sup>
MII oocyte	8.72±5.08	6.07±3.23 <sup>c</sup>	11.19±4.45	3.99±2.44 <sup>a,c</sup>
Fertilized oocytes	9.04±4.84	6.19±3.23 <sup>a,c</sup>	11.76±4.19	4.38±2.35 <sup>a,c</sup>
Embryo at (2PN)	6.79±4.69	5.69±3.27 <sup>c</sup>	9.38±4.66	3.66±2.08 <sup>a,c</sup>
Transferred embryo	5.70±3.71	4.88±3.05 <sup>c</sup>	8±4.76	3.33±1.87 <sup>c</sup>
G1embryo	4.18±3.72	3.88±2.80	5.73±5.22	2.37±1.25 <sup>c</sup>
Oocyte maturation rate %	0.93±0.14	0.79±0.24	0.86±0.18	0.98±0.05
Cleavage rate%	0.65±0.28	0.80±0.26	0.62±0.25	0.81±0.23
Fertilization rate%	0.75±0.29	0.95±0.31 <sup>a</sup>	0.80±0.23	0.87±0.19

Statistical analysis was performed by ANOVA followed by Post Hoc test (Tukey's test) for multiple comparisons. The small letters represent a significant difference between groups: <sup>a</sup> with control; <sup>b</sup> with UI; <sup>c</sup> with PCOS; <sup>d</sup> with AMH

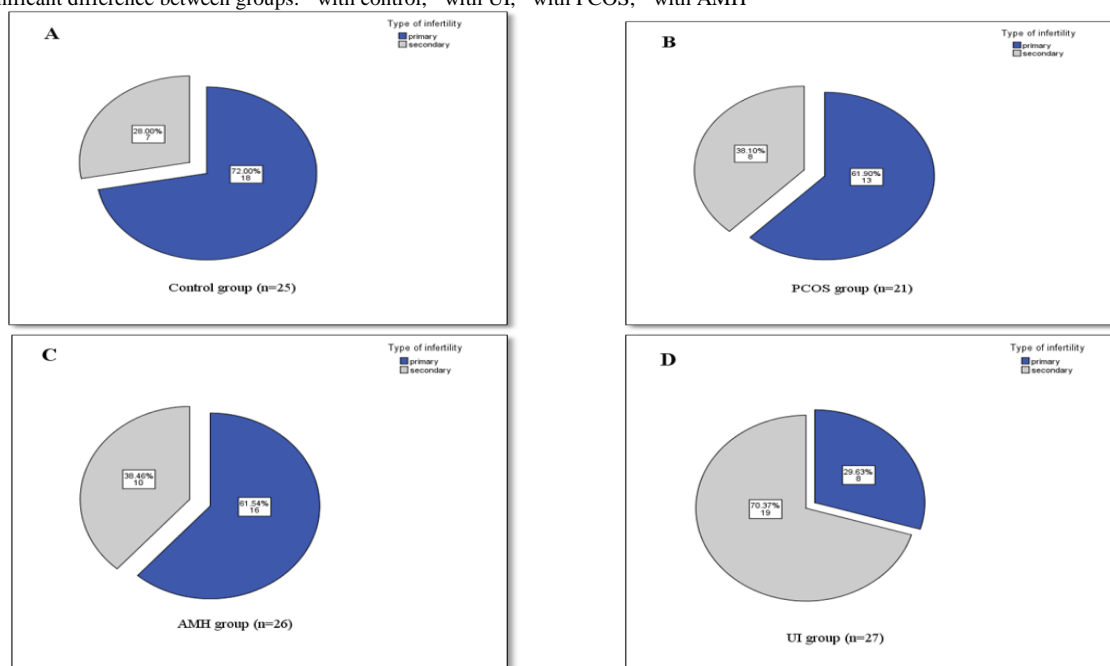


Fig. 1. Pie-chart showing the percentages and numbers of infertility types of: Control group, (B) PCOS group, (C) AMH group, (D) UI group

**Table 4. Mean (±SD) of FF Paraoxonase1 activities and specific activities in the four studied groups**

Parameters	Control group	UI group	PCOS group	AMH group
PON (Basal)U/L	39.22 ± 15.5	42.85 ± 18.2	56.38 ± 18.1 <sup>a, b</sup>	49.27 ± 13.3
PON sp. activity (Basal) U/g	0.95 ± 0.44	1.07 ± 0.68	1.16 ± 0.49	1.12 ± 0.34
PON (s-s)U/L	52.69 ± 16.2	61.16 ± 31.9	79.16 ± 23.4 <sup>a,b,d</sup>	60.97 ± 29.9
PON sp. activity (Salt) U/g	1.26 ± 0.43	1.47 ± 0.69	1.65 ± 0.7	1.36 ± 0.62
Arylesterase KU/L	29.70 ± 8.2	32.62 ± 8.7	38.53 ± 8.8 <sup>a,b,d</sup>	29.04 ± 7.7
Arylesterase sp. activity KU/g	0.71 ± 0.19	0.78 ± 0.19	0.77 ± 0.14	0.66 ± 0.23

**Table 5. Association of PON1 activities with pregnancy outcomes.**

Parameters		PON1 activities			
		Control	UI	PCOS	AMH
PON1 Basal	Pregnant	46.7±12.4*	39.3±18.8	41.4±3.5	56.2±8.7*
	Non pregnant	31.7±15.1	44.3±14.7	59±18.4**	42.7±13.2
PON1(s-s)	Pregnant	56.8±13.7	58.3±21.8	89.7±21	60.8±25.4
	Non-pregnant	48.5±18.1	62.4±30.6	77.2±23.8	63.2±38
Arylesterase	Pregnant	25.6±5.3*	26.3±7.3*	36.2±9	29.8±6.3
	Non-pregnant	33.7±8.7	34.4±7.7	38.9±9	28.4±9.7

Analysis performed by independent samples t-test; statistically significant \*P<0.05; \*\*P<0.01; no asterisk: P ≥0.05

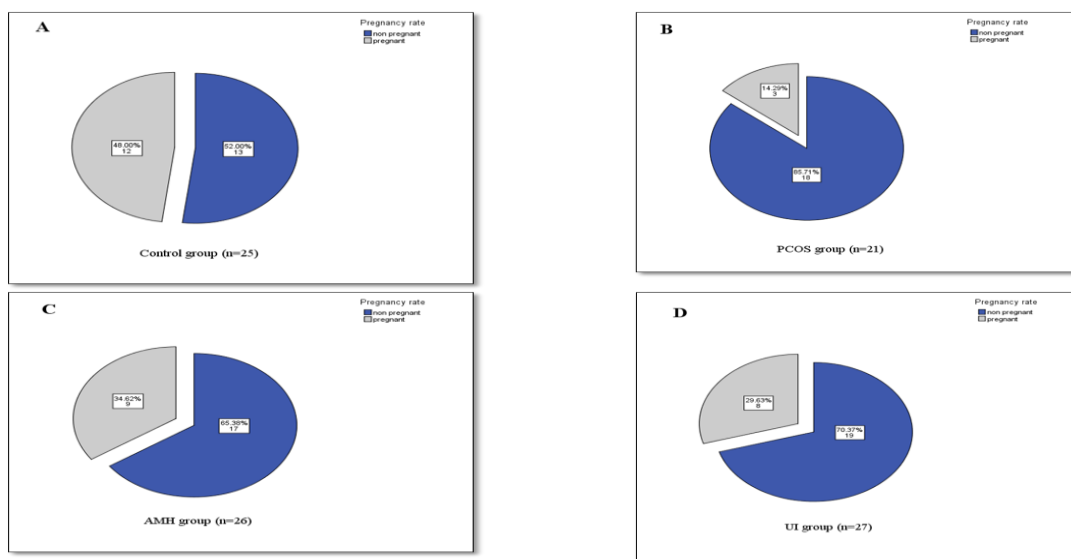


Fig. 2. Pie-chart showing the percentages and numbers of pregnancy rate in (A): Control group, (B): PCOS group, (C): AMH group, (D): UI group

### Conclusion

The essential point is that the high level of OS leads to pregnancy failure since PON1 activity in PCOS and UI groups is higher for non-pregnant women when compared to pregnant women. So, it is possible to measure the activity of this enzyme for predicting the failure or success of pregnancy.

Ovarian stimulation during IVF treatment was connected with higher oxidative stress and reduced PON1 activity in pregnant PCOS and UI groups. Importantly, an increase in FF antioxidant role seems to be a negative fertilization indicator, which may represent an adverse ovarian condition that triggered anti-oxidant behavior.

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## تقييم فعالية إنزيم Paraoxonase في السائل الجريبي مع نتائج الحمل لدى النساء اللاتي يخضعن للتلقيح الاصطناعي / الحقن المجهرى

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### الخلاصة

لم يتم تحديد البحث عن علامة موثوقة للتنبؤ بنجاح الحمل. تهدف هذه الدراسة إلى تقييم فعالية إنزيم (PON1) في السائل الجريبي (FF) وعلاقتها بإنتاجية البويضات والتخصيب والحمل السريري (CP). الطريقة: اشتملت الدراسة على 99 امرأة تتراوح أعمارهن بين 22 و 37 عامًا ممن خضعوا لعمليات التلقيح الصناعي / الحقن المجهرى، تم تقسيمهم إلى مجموعة خصبه تمثل مجموعة السيطرة المكونة من 25 مشاركًا، ومجموعة العقم التي تم تقسيمها إلى 21 مريضًا مصابًا بمتلازمة تكيس المبايض، و 26. المرضى الذين يعانون من انخفاض مستوى هرمون AMH، وكذلك 27 مريضًا مجهول السبب من العقم (UI). تم قياس فعالية FF Basal PON1 و PON1 المحفز بالملح وفعالية Arylesterase باستخدام طريقة القياس الطيفي. النتائج: كان فعالية PON1 basal لمجموعة PCOS أعلى ( $P > 0.05$ ) عند مقارنته بمجموعات السيطرة و UI. كان فعالية PON1 arylesterase لمجموعة PCOS أعلى ( $P > 0.05$ ) عند مقارنته بمجموعات السيطرة و UI ومجموعه AMH المنخفضة. أظهرت كل مجموعة فرقًا ( $P > 0.05$ ) في نجاح الحمل في مجموعات متلازمة تكيس المبايض، وانخفاض هرمون AMH، و UI ولكن ليس في مجموعة السيطرة. الخلاصة: يبدو أن الزيادة في مضادات الأكسدة FF مؤشر سلبي للإخصاب، والذي قد يمثل حالة ضارة للمبيض أدت إلى سلوك مضاد للأكسدة