Journal of **R**eproduction & **I**nfertility
Volume 23, Issue no. 3
https://doi.org/10.18502/jri.v23i3.10010

Original Article

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Comparison of Follicular Fluid Paraoxonase 3 Level, Ovarian Hormones and Oocyte Quality between Fertile and Infertile Women

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Received: Aug. 25, 2021
Accepted: Jan. 30, 2022

Abstract

Background: The purpose of the current study was to evaluate the possible effect of follicular fluid paraoxonase 3 (PON 3) on oocyte quality and sex hormones.

Methods: This descriptive-analytical study was performed on totally 90 enrolled women including fifty infertile women presenting with polycystic ovaries and unilateral tubal factor and forty fertile women with male factor infertility referring to Umm-al-Banin Infertility Clinic in Dezful, Iran for in vitro fertilization during October 2018 to November 2019. Oocyte removal was carried out under transvaginal ultrasound guidance, and follicular fluid (FF) was removed and preserved to detect PON3, estrogen, and progesterone levels. In addition, oocyte number and quality were assessed and its association with PON3 activity in the FF was evaluated. One-way ANOVA and Fisher's least significant difference (LSD) were used for data analysis and $p \leq 0.05$ were considered statistically significant.

Results: A significant increase was observed in the total number of the oocytes and mature metaphase II oocytes with ≥20 *pg/ml* of PON3 concentration in the FF ($p \leq 0.05$). Moreover, a positive relationship was shown between the increased estradiol level in follicular fluid and PON3, so that the highest estradiol level was observed in the amount of 31-40 *pg/ml* of PON3 ($p \leq 0.05$).

Conclusion: According to the results, as the number of the mature oocytes increased, the amount of PON3 as well as estradiol levels in the FF increased. This research displays an increase in the level of PON3 with mature oocytes, thus supporting the indirect evidence for the function of PON3 in follicle development.

Keywords: Oocyte, Paraoxonase 3, Follicular fluid, Antioxidants, Assisted reproduction
To cite this article: Janati S, Behmanesh MA, Najafzadehvarzi H, Nezami B, Poormoosavi SM. Comparison of Follicular Fluid Paraoxonase 3 Level, Ovarian Hormones and Oocyte Quality between Fertile and Infertile Women. J Reprod Infertil. 2022;23(3):192-198. https://doi.org/10.18502/jri.v23i3.10010.

Introduction

Today, more attention is paid to the use of assisted reproductive techniques (1), and several applied researches have contributed to improvement of these methods (2). Nevertheless, failed pregnancy is observed in multiple infertile couples despite spending huge amounts of money and using various ART methods (3, 4). Various factors are involved in achieving a successful pregnancy with ART, which affect the process of follicle and embryo development (5).

Follicular fluid (FF) provides nourishment for oocyte development, and the increase or decrease in the composition of this fluid affects the morphology and quality of the oocyte (6). Oxidative

stress and the adverse effects of active oxygen in the body could decrease the quality of germ cells (7). Free radicals damage the cell membrane lipids and lead to their oxidation (8). Inside the cell, reactive oxygen species (ROS) are neutralized by antioxidants, and paraoxonases (PONs) are considered to be potent antioxidants in the serum and FF (9, 10). The PON gene family in humans has three members, including PONs1, PONs2, and PONs3. PONs3 is synthesized in the liver and carried in the bloodstream while bound to lipoproteins with specific weight to prevent their oxidation (9).

Recent reports have confirmed the activity of this enzyme in the FF, and its concentration has been shown to be three times higher in the FF compared to the serum (11). PON3 has potent antioxidant properties, and high levels of oxidative stress are reported to inhibit cell proliferation in the theca layer, thereby preventing hormone generation by the theca of the ovarian follicle. On the other hand, antioxidants avert the proliferation of theca cells; therefore, oxidative stress and antioxidant shortage play a key role in determining and changing the amount of the secreted hormone by the ovaries (12).

Natural FF antioxidants improve the influential factors in oocyte quality. Considering the remarkable antioxidant properties of PON3 and its high concentration in the FF, this enzyme also impacts oogenesis, oocyte quality, the amount of the hormones secreted by the ovarian follicle wall, and the fertility process. The purpose of the present study was to evaluate the possible effect of the PON3 activity and intrafollicular hormone levels of fertile and infertile women after ovarian stimulation. Moreover, the changes regarding the number and quality of oocytes were also compared in this study.

Methods

The study protocol was approved by the Ethics Committee of Dezful University of Medical Sciences, Dezful, Iran (IR.DUMS.REC.1396.27). A descriptive-analytical study was performed on FF of ninety fertile and infertile women who were candidates of in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) during October 2018 to November 2019 referring to Umm-al-Banin Infertility Clinic in Dezful, Iran. The participants included fifty infertile women presenting with polycystic ovaries and unilateral tubal factor and forty fertile women with male factor infertility who were candidates of IVF/ICSI. The subjects were enrolled in the study after providing informed consent, receiving primary examinations, and determining the cause of infertility and treatment protocol. To initiate the IVF/ICSI cycle, the patients required ovarian stimulation, which was carried out in accordance with the protocols.

The included participants with polycystic ovary syndrome and male factor infertility aged between 20 and 45 years and were scheduled for IVF/ICSI. Women with endocrine and metabolic diseases, follicle-stimulating hormone levle of >12, other causes of infertility, endometriosis (all stages), smoking, bilateral tubal factor, treated with different protocol of ovarian stimulation and empty follicle syndrome, history of recurrent miscarriage, and blood contamination of FF were all excluded from the study.

Ovarian stimulation: In this study, ovarian stimulation was carried out using the antagonist method. The patients completed the cycle of contraceptive LD use (30 *mg* of ethinyl estradiol (EE) and 0.3 *mg* of norgestrel or 0.15 *mg* of levonorgestrel). Stimulation occurred on the second day of menstruation by 150-300 daily doses of the recombinant gonadotropin of GONAL-f (Merck-Serono, Germany), which was determined depending on the age, weight, and the previous response of the subjects. Furthermore, follicular monitoring was performed by vaginal ultrasound on days 7-8, gonadotropin was re-adjusted, and the cycle continued based on the ovarian response and serum estradiol concentration.

At the next stage, cetrotide (Merck-Serono, Germany) was added to the treatment regimen subcutaneously (daily dose: 0.25 *mg*) when the dominant follicles reached the diameter of 14 *mm*, and the process continued until the day of the administration of the human chorionic gonadotropin (HCG). One vial of recombinant hCG (Ovidrel) was injected following the observance of at least three follicles with the diameter of ≥18 *mm* in ultrasound monitoring.

Oocyte retrieval and FF extraction: Oocyte retrieval was carried out 34-36 *hr* after hCG administration under transvaginal ultrasound guidance and intravenous anesthesia. In the next stage, the FF containing oocytes was assessed under an inverted microscope by an embryologist after the aspiration of follicles. Following that, the oocytes were removed and washed by a G-MOPS medium and incubated in a medium containing G-IVF with

a special mineral oil coating inside an incubator at the temperature of 37°*C* with 6% CO_2 and 5% O_2. The cumulus cells were isolated from the oocytes mechanically (*i.e.*, denuded) after the exposure of the oocytes to an HYASE medium containing 80 *IU/ml* of hyaluronidase for 30 *s*.

The denuded oocytes were assessed in terms of the nucleus and cytoplasm, and oocyte maturation was determined based on the size of the cumulus, size, and adhesion of granulosa cells, and the shape and color of the oocytes. Further assessments were carried out after the removal of the cumulus to prepare the oocytes for an ICSI procedure depending on the presence or absence of the polar body and germinal vesicles (GVs).

Mature metaphase-II oocytes extruded the first polar body, the cumulus cells were typically dilated and luteinized, and a solar radius pattern was detected in the corona radiata. On the other hand, the MI oocyte with intermediate maturity had no polar body, its cumulus cells were denser, and its germinal vesicle and nucleus had disappeared. At prophase I, the oocytes were clearly immature, and their corona radiata was dense. In addition, the cumulus cells were relatively fewer, and the prominent nucleoli and germinal vesicle were detected.

After oocyte retrieval by the embryologist, the isolated FF was centrifuged at 1,000 *rpm* for 20 *min* after oocyte removal in order to remove additional cells and materials. In the next stage, the supernatant was removed and placed in a cryopreservation tube (Nunc, Thermo Scientific, USA) and then preserved at the temperature of -198°*C* in a liquid nitrogen container. The activity of PON3 was determined. The samples were collected using an ELISA kit (MyBioSource, USA) and the laboratory kit (Azma Plast Co, Iran) was used to measure the levels of estrogen and progesterone. This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for PON has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PON present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for PON is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PON bound in the initial step. The color development is stopped and the intensity of the color is measured.

Outcome measures: The determined outcomes included the oocyte's number and quality. Therefore, after retrieval, oocytes were classified into three categories using Olympus inverted microscope. The categories were as follows:
metaphase II (MII, presence of first polar body), metaphase I (MI, absence of first polar body) and germinal vesicle oocytes), and degenerated oocytes. After the determination of paraoxonase levels, these levels were divided into four groups. The number of oocytes in each group was 184, 160, 300, and 242, respectively. The morphology of human pronuclear embryos is related to blastocyst development and implantation (13). Laboratory and biochemical analysis of estrogen and progesterone was done and paraoxonase 3 levels in FF were measured as described in the previous section. Clinical data were obtained from medical records which included patient age, BMI, cause, and duration of infertility.

Statistical analysis: Data analysis was performed in SPSS v 16 (IBM, USA) using one-way ANOVA to assess the variance of the groups and Fisher's least significant difference (LSD) procedure was conducted to evaluate the significant differences between the groups. In all the statistical analyses, $p \leq 0.05$ was considered significant.

Results

This descriptive-analytical study was performed on the FF of the ninety fertile and infertile women who were candidates of IVF/ICSI. The participants included a total of ninety women including fifty women presenting with polycystic ovaries and unilateral tubal factor and forty fertile women with male factor infertility who were candidates of IVF/ICSI.

According to the information in table 1, a significant decrease was observed in the number of the mature metaphase II oocytes following the assessment of the effect of the age on the number of oocytes in the individuals aged ≥35 years. Furthermore, a significant decrease was denoted in the total number of oocytes in the women aged ≥40 years. However, no significant changes were observed in any age groups in terms of the number of GVs and immature metaphase I oocytes.

Table 1 also shows the effect of the women's body mass index (BMI) on the number of oocytes. Accordingly, the total number of oocytes, mature

Table 1. Comparison of the average number of MII oocytes and other oocytes at different groups

Variables	Number of samples	Oocytes	MII	MI	GV, degenerated
Women Age					
25-30	25	12.42±4.3 [a]	4.42±1.9 [a]	4.48±1.6 [a]	3.43±1.1 [a]
31-35	35	12.63±3.1 [a]	4.27±1.4 [a]	4.15±1.8 [a]	3.82±1.2 [a]
36-40	20	10.24±2.4 [a]	2.44±0.5 [b]	4.25±1.2 [a]	3.72±0.4 [a]
41-45	10	6.7±2.4 [b]	1.74±0.4 [b]	3.4±0.3 [a]	2.7±0.5 [a]
Women BMI					
15-24.99	20	12.47±3.1 [a]	3.26±0.8 [a]	5.14±1.7 [a]	2.47±0.4 [a]
25-34.99	43	12.75±4.2 [a]	3.57±0.7 [a]	5.47±1.2 [a]	2.81±0. 5 [a]
35-44.99	27	6.82±1.9 [b]	1.87±0.4 [b]	2.78±0.8 [b]	2.42±0.3 [a]
Cause of infertility					
Male factor	40	10.48±2.3 [a]	4.53±0.74 [a]	4.4±0.7 [a]	1.97±0.5 [a]
Female factor	50	5.35±0.8 [b]	2.17±0.5 [b]	2.1±0.61[b]	1.59±0.3 [a]
Infertility duration (Year)					
≤10	45	10.42±1.4 [a]	5.28±0.34 [a]	2.35±0.3 [a]	1.82±0.9 [a]
10-20	30	5.23±0.4 [b]	2.15±0.5 [b]	2.37±0.7 [a]	1.43±0.7 [a]
≥20	15	5.14±1.44 [b]	1.46±0.46 [b]	2.44±0.5 [a]	1.36±0.22 [a]

a and b in each column indicate significant differences at p≤0.05

metaphase II oocytes (M II), and immature metaphase I (MI) oocytes significantly decreased in women with a BMI of >35 *kg/m*^{2}. On the other hand, BMI caused no significant change in the number of GV oocytes.

Table 1 shows the association between the cause of infertility and the number of oocytes. Accordingly, if female factors are the cause of infertility, the total number of oocytes, MII, and MI oocytes significantly decreases. However, none of the infertility factors caused a significant change in the number of immature GV oocytes. Regarding the association between the duration of infertility and the number of oocytes, the results presented in table 1 also show a significant decrease in the total number of oocytes and MII oocytes in the subjects who had been infertile for more than 10 years. On the other hand, no significant associations were observed between the number of MI oocytes and immature GV oocytes with the duration of infertility.

Table 2 shows the effect of the level of PON3 in the FF on the number of oocytes. Accordingly, the total number of oocytes and MII oocytes significantly decreased at lower levels of PON3 ≥20 *pg/ml* in the FF. However, no significant association was observed between the number of MI oocytes and GVs with the level of PON3 in the FF. According to the information in table 3, PON3 in the FF had significant effect on the level of estradiol. The amount of FF estradiol increased at higher PON3 levels, so a positive relationship was shown between the increased estradiol level in follicular fluid and PON3; accordingly, the highest estradiol level was observed in the amount of 31-40 *pg/ml* of PON3 (p≤0.05). On the other hand, no significant change was observed in the level of progesterone at any concentration of the PON3.

Table 2. Comparison of the average distribution of oocytes between different levels of paraoxonase 3 in FF

Paraoxonase (*pg/ml*)	Number of samples	Oocyte number	MII	MI	GV, degenerated
0-10	23	8.47±1.4 [b]	3.22±1.2 [b]	3.17±1.2 [a]	2.49±0.2 [a]
11-20	20	8.26±2.1 [b]	2.29±0.8 [b]	4.78±0.6 [a]	2.15±0.4 [a]
21-30	25	12.7±3.9 [a]	5.45±1.5 [a]	4.21±0.6 [a]	2.27±0.7 [a]
31-40	22	11.44 ±3.6 [a]	5.88±1.43 [a]	4.55±0.7 [a]	2.75±0.6 [a]

a and b in each column indicate significant differences at p≤0.05

Table 3. Comparison of the amount of paraoxonase 3 and the amount of estrogen and progesterone in follicular fluid

Paraoxonase 3 (*pg/ml*)	Number of samples	Estradiol (*pg/ml*)	Progesterone (*ng/dl*)
0-10	23	745.25±3.7 [c]	14.2±5.3 [a]
11-20	20	973.6±4.2 [c]	10.8±3.8 [a]
21-30	25	2459. 5±3.5 [b]	12.6±3.3 [a]
31-40	22	5670.3±4.9 [a]	14.5±4.5 [a]

a, b, and c in each column indicate significant differences at $p \leq 0.05$

Discussion

During the study period, totally ninety women were recruited including fifty women presenting with polycystic ovaries and unilateral tubal factor and forty fertile women with male factor infertility who were candidates of IVF/ICSI. A significant increase was observed in the total number of the oocytes and mature metaphase II oocytes with concentration of ≥20 *pg/ml* of PON3 in the FF. Moreover, as the number of the mature oocytes increased, the amount of PON3 as well as estradiol levels increased in the FF.

The paraoxonase (PON) gene group is combined of three types (PON1, PON2, and PON3) that share significant structural homology, are placed in tandem on chromosome 7 in humans, and have antioxidative effects (13). Paraoxonases are secreted from granulosa cells (10). The potent antioxidant properties of PON3 and its role in the proliferation of theca cells have been confirmed. On the other hand, high levels of oxidative stress could inhibit cell proliferation and prevent hormone production in the endogenous layer of the ovarian follicles, which indicates the key role of oxidative stress and antioxidant deficiency in determining and changing the amount of the hormones that are secreted by the ovaries (12).

According to Opuwari et al. 2016, paraoxonase could prevent the oxidation of low-density lipoprotein (LDL) and use lipid oxidation products as a substrate to reduce the intensity of oxidative stress in cells (14). Given the role of natural FF antioxidants in the improvement of the influential factors in oocyte quality, the remarkable antioxidant properties of PON3, and its high concentration in the FF, this enzyme seems to be essentially involved in oogenesis, oocyte quality, and the fertility process (15). Therefore, the evaluation of the follicular concentration of antioxidants (*e.g.*, PON3) could provide reliable data on the status of oocytes and ovaries, as well as the oxidative conditions affecting these parameters.

The study conducted by Primo-Parmo 1996 was the first to report the lower level of PON3 activity in the FF of infertile women in contrast to healthy participants (8). Also, it was indicated that fertilization ability of oocyte depends not only on the number of oocytes, but also on their quality (8). According to Meijide et al. 2017, high PON3 activity plays a key role in various stages of growth and maturity, as well as the quality of oocytes (16).

In a research performed by Bacchetti et al. 2019 on 100 infertile women and 55 fertile oocyte donors, PON3 levels were reported to be significantly higher in the FF. Moreover, PON3 activity in the FF obtained from large follicles was significantly higher in the fertile women compared to the infertile subjects (17). According to the results of the present study, the total number of the oocytes and MII oocytes significantly increased with ≥20 *pg/ml* of paraoxonase in the FF. However, no significant association was observed between MI oocytes and VGs. Rashidi et al. 2014 investigated the effects of high-density lipoprotein (HDL) in the FF on oocyte quality and fertility, considering PON1 as an HDL-related antioxidant. In the mentioned study, the subjects included 20 infertile overweight/obese women and 38 infertile women with normal BMI. The findings indicated the effect of PON1 activity on oocyte quality, as well as increased HDL oxidation and decreased PON1 activity (18). HDL is the only lipoprotein element in human follicular fluid and prepared cholesterol for de-novo steroidogenesis (13).

Closshey et al. 2007 performed research on 50 infertile couples with male or female factors during the ART treatment cycle to measure PON3, HDL, total antioxidant activity, and malondialdehyde, concluding that PON3 activity was directly

correlated with ART success and fertilization due to the antioxidant properties of the FF (19). Furthermore, Giordano et al. 2013 investigated the FF obtained from 14 infertile women undergoing IVF to determine the antioxidant effects of PON3. According to the obtained results, PON3 activity was higher in the FF compared to the serum. The further activity of PON3 in the FF also implied its insignificant association with fertilized oocytes (20).

According to the results of the current research and the aforementioned studies, since PON3 improves the function and maturity of oocytes by decreasing oxidative stress, the higher concentration of PON3 in the FF could increase MII oocytes and the total number of oocytes. Moreover, the effect of PON3 level in the FF on estradiol and progesterone level was assessed in this study. According to our findings, a positive relationship was shown between the increased estradiol level in follicular fluid and PON3, so that the highest estradiol level was observed in the amount of 30-40 *pg/ml* of PON3. Therefore, estradiol may have a positive impact on increasing PON. On the other hand, increased PON leads to higher estradiol levels through an antioxidant mechanism, thereby affecting the ovarian hormones. In another study, Pizarro et al. evaluated the role of PON family to determine the direct impact of estradiol on the increased translation of PON2 protein. The results of the mentioned study indicated the effect of paraoxonase on estradiol levels, which is consistent with our findings (21). It has been revealed that estradiol is able to defend granulosa cells from oxidative stress-induced apoptosis. Also, in one study, the observed concentration of estradiol was elevated in the FF of women who became pregnant after ART in comparison to FF of cases for whom the ART treatment cycles were unsuccessful (1).

It seems that the effect of catalytic activity of increased paraoxonase on estradiol levels causes the lack of secretion of this hormone in postmenopausal women leading to decreased level of paraoxonase in the serum or FF. A research in this regard was performed in 2001 to evaluate the level of antioxidants in menopausal women receiving hormone replacement therapies. According to the findings, the postmenopausal women had significantly lower paraoxonase protein levels in the serum. Moreover, the results of the mentioned study were indicative of decreased PON1 activity in the menopausal women diagnosed with type II diabetes (22). No research has reported an association between PON3 and progesterone, and our findings in this regard were not considered significant as no increasing or decreasing trend was observed. It is recommended to conduct more statistical and laboratory assessments in this regard.

One of the limitations of this study was the small sample size. A larger sample could help to provide more robust evidence of the effect of PON3 concentration in the FF on the number and maturity of oocytes. Also, this study failed to measure the concentration of PON3 in the serum and the FF at the same time. This could have helped to determine the effect of PON3 on sex hormone concentration in the FF and serum. Other limitations of our study were the lack of findings regarding the relationship between the fertility outcomes and the amount of PON3 in follicular fluid. One of the strengths of the study is that all steps of procedures from the selection of cases, analysis of indications, IVF process, ovum pick up, and embryo transfer were done by one gynecologist and an embryologist, which reduces the confounding factors caused by the operator.

Conclusion

According to the results, PON3 in the FF had direct effect on the increased total number of oocytes and number of MII oocytes with ≥20 *pg/ml* of PON3 concentration in the FF. Moreover, a positive relationship was shown between the increased estradiol level in follicular fluid and PON3, so that the highest estradiol level was observed in the amount of 31-40 *pg/ml* of PON3. This research displays an increase in the level of PON3 with mature oocytes, thus supporting indirect evidence for the function of PON3 in follicle development. However, no association was detected between PON3 and progesterone. Thus, additional studies are required to be performed in patients with polycystic ovary syndrome with different size and maturation phases to evaluate the amount of enzyme in FF and better understand the follicular microenvironment, contributing to improvement in the ICSI procedure.

Acknowledgement

The authors would like to acknowledge the financial support of Dezful University in this study. Authors have no conflict of interests to declare.

Conflict of Interest

The authors declare no conflict of interest.

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