Antioxidative enzyme-related gene expression levels in cumulus cells during in vitro fertilization: A comparison between normal and poor ovarian responders

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Abstract

Background: Preeminent interfering factor caused in vitro fertilization (IVF) failure has been broadly emphasized on poor ovarian response (POR) to controlled ovarian hyperstimulation (COH). The specific etiology and precise infertility treatment in POR patients, until now, has not been established and leads to detrimental effects on physical or psychological patient life qualities. To date, one biological stress phenomena, oxidative stress, is broadly implicated in varieties of female reproductive pathological conditions including poor oocyte quantity and quality. Hence, the present study is principally aimed to investigate oxidative stress scavenging system; enzymatic antioxidant-related gene expression level (*SOD1*, *SOD2* and *GPx4*), in cumulus cells retrieved from poor ovarian responders (POR) and normal ovarian responders (NOR).

Methods: A cross-sectional analytic study; fifty-six infertile women, aged between 25-49 years-old who underwent IVF procedure, were enrolled into this study with 28 participants in POR group (1-4 retrieved oocytes) and 28 participants in NOR group (5-15 oocytes). The cumulus cells from first punctured follicle were processed for mRNA gene expression levels of enzymatic antioxidant-related genes (Superoxide dismutase: *SOD1*, *SOD2* and Glutathione peroxidase: *GPx4*) by quantitative real-time PCR (qPCR) and their association to other reproductive biological factors.

Results: The relative mRNA expression of *SOD1*, *SOD2* and *GPx4* neither differed between poor and normal responders nor correlated with other relevant reproductive biological cohorts. However, the relative *SOD2* mRNA expression level in patients who received human menopausal gonadotropin (hMG) was notably higher than the recombinant follicle stimulating hormone (rFSH) group (P = 0.017).

Conclusions: Our major findings indicated that the enzymatic antioxidant-related gene mRNA expression in the cumulus cells do not associate with the ovarian response and other clinical outcomes (i.e. fertilization and pregnancy rates) after IVF procedure. However, the enzymatic antioxidant-related genes positively relate with type of gonadotropin administration.

Introduction

According to stress from the social environment and modern lifestyles, the incidence of couples who encounter infertility has dramatically increased. Assisted reproductive technology (ART) including in vitro fertilization (IVF) has been introduced and is widely accepted as standard treatment procedure for couples with tubal factor such as bilateral tubal obstruction, severe male factor, unexplained infertility or couples who failed the initial infertility treatment. However, overall success rates for infertility treatment by IVF are described to be within a range between 30% - 40% [1]. Currently, one of the major obstacles causing IVF failure has been emphasized on poor ovarian response (POR) to controlled ovarian hyperstimulation (COH). Obviously, low numbers of collectable oocytes, impairment of embryo development and IVF outcomes are a consequence of POR [2]. Furthermore, clinical pregnancy rates in patients with POR is eventually lower than normal ovarian response (NOR) patients (defined as 10-15

retrieved oocytes) [2]. Although multiple POR etiologies are proposed such as ovarian aging, premature ovarian insufficiency, ovarian tumor or other ovarian pathologies including endometrioma [3], some infertile women who present with normal baseline characteristics and receive standard doses of gonadotropin still encounter a problem of poor ovarian response without any identifiable causes. The specific etiology together with precise infertility treatment in POR patients, until now, has not been established which leads to recurrent IVF failure and has detrimental effects on physical or psychological patient life qualities [4].

Recently, one biological stress phenomena, oxidative stress, was broadly implicated in varieties of pathological conditions. Oxidative stress refers to an imbalance between pro-oxidant and antioxidant (enzymatic and non-enzymatic antioxidants) homeostasis that leads to an excessive amount of reactive oxygen species (ROS), reactive nitrogen species (RNS) or other free radicals [5.] The excessive accumulation of ROS or other free radicals could interfere with normal cells, organ structures and functions through the following mechanisms; opening of ion channel resulting in unstable mitochondrial membrane potential, lipid peroxidation, protein modifications and DNA oxidation [6]. In terms of female reproductive dysfunction, a number of previous studies demonstrated that polycystic ovary syndrome (PCOS), endometriosis and preeclampsia was negatively affected by oxidative stress [7] while oxidative stress related to POR and poor IVF outcomes are imperceptibly progressed. A previous study in 1995 initially examined the enzymatic antioxidant activity in follicular fluid which documented lower Glutathione peroxidase (GPx) activity in the follicular fluid of non-fertilized oocytes compared to fertilized group [8]. Another research study in 2006 compared different gene expressions in cumulus cells retrieved during IVF procedures between old and young women. The findings presented a gradual decreased level of messenger RNA (mRNA) coding for enzymatic antioxidants; Superoxide dismutase (*SOD1, SOD2*) and catalase in older women [9]. Notable scientific support was again published in 2009 when a low level of cumulus cell-SOD was significantly correlated with IVF failure [10]. To emphasize the role of oxidative stress markers in IVF, follicular fluid-enzymatic antioxidant profile was recently comprehensive studied [11]. The research data indicated that the activities of GPx, Glutathione reductase (GR) and Glutathione S-transferase (GST) from patients with reduced ovarian response was lower than age-matched healthy oocyte donors [11]. Furthermore, collective data in 2018 described that follicular fluid-total glutathione (GSH) activity markedly decreased in patients with low fertilization rate after ICSI (intracytoplasmic sperm injection) [12].

From the above-mentioned data, the oxidative stress phenomenon with decreased enzymatic antioxidant capacity may result in senescence of ovary in patients with poor ovarian response resulting in poor IVF outcomes. However, studies on enzymatic antioxidant-related gene expression levels; *SOD1* and *SOD2* and *GPx4*, which are considered potent lipid hydroperoxide scavengers in cumulus cells, and how they are related to ovarian response status have only been partially investigated. Thus, this study principally aimed to I) evaluate enzymatic antioxidant-related gene expression levels (*SOD1*, *SOD2* and *GPx* (isoform 4; *GPx4*) in cumulus cells which are under similar milieu as it presents in the oocytes between poor ovarian responders (POR) and normal ovarian responders (NOR) to COH and II) determine any correlation

between enzymatic antioxidant-related genes to other reproductive biological factors including IVF outcomes and clinical pregnancy rate.

Methods

Ethical approval

This cross-sectional study was conducted at the Infertility Clinic, Reproductive Medicine Division, Department of Obstetrics and Gynecology, King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand from February 1, 2017 to April 30, 2018. The study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand on January 27, 2017 (IRB No.688/59). Written informed consent were obtained from all participants. This research has been registered at clinicaltrials.gov (NCT03155438).

Participants

Fifty-six infertile women, aged between 25-49 years old who underwent IVF were enrolled into this study with 28 participants in normal ovarian response group (NOR) and 28 participants in poor ovarian response group (POR). Patients were categorized to the normal and the poor ovarian response groups according to the number of retrieved oocytes at ovum pick-up (OPU) day.

Poor ovarian response in the present study was defined as 1-4 retrieved oocytes. The control group or normal ovarian response group, age-matched with POR group, consisted of women with 5-15 retrieved oocyte. Exclusion criteria were women with active ovarian pathologies such as ovarian endometrioma or basal follicle stimulating hormone (FSH) level more than 20 IU/L or women with ovarian hyperstimulation syndrome. None of the participants were taking any antioxidant and vitamin supplements other than folic acid.

Sample size calculation

Sample size calculation was analyzed according to the previous study by Donabela et al [13] with mean of *SOD1* relative gene expression in healthy women control group; 2.06 $2^{-\Delta \Delta CT}$ (SD = 0.53). To compare two different means, the sample size calculation was performed with the following expectation; *SOD1* gene expression level in POR was lower than NOR by 20%, resulting in a number of 25 women for each study group. With an addition of 10% probability for data loss, a total of 56 women were finally included in the present study, 28 patients with NOR and 28 women with POR.

Ovarian stimulation protocol

As the patients were categorized into normal responders and poor responders according to their results on the OPU day, ovarian stimulation protocol for each patient was managed and adjusted to patient baseline characteristics under judgement of attending physicians. Both patient groups received flexible gonadotropin-releasing hormone (GnRH) antagonist protocol. Ovarian stimulation initiated on day 3 of

menstruation cycle with 200-375 U/day of recombinant follicle stimulating hormone (rFSH; Follitropin alpha; Gonal-f®, Merck Serono SA, Switzerland), Follitropin beta (Puregon®, MSD, France) or human menopausal gonadotropin (hMG; Menopur®, Ferring Pharmaceuticals, St Prex, Switzerland). Dosages were adjusted according to the ovarian response. The GnRH antagonist; Ganerelix (Orgalutran®, N.V. Organon, The Netherlands), was given daily at a dosage of 250 mcg starting when leading follicles reached 14 mm. Finally, 250 mcg of recombinant human chorionic gonadotropin (hCG) (Ovidrel®, Serono, Rockland, MA, USA) was administered when the presence of at least three leading follicles (or most of leading follicles in the poor ovarian response group) reached more than 17 mm in diameter. The OPU was performed at 36-38 hours after hCG administration.

Oocyte and cumulus cell retrieval

Oocyte retrieval was carried out using 16-gauge double lumen and ovum aspiration needle (Cook Medical, Queensland, Australia) under transvaginal ultrasound guidance. Nutrient Mixture (Ham) F-10 (1X) (Gibco®, Life Technologies, New York, USA), containing 25 mM HEPEs (Sigma, St. Louis, MO, USA) and L-Glutamine (Sigma), was used as flushing media. Only oocyte with cumulus cells (cumulus cell oocyte complex; COCs) from first punctured follicle exceeding 18-20 mm in diameter of each patient was collected for further mRNA gene expression analysis.

Cumulus cell retrieval protocol is described briefly as the following: the collected COCs were stored in fertilization media (LifeGlobal, Guilford, CT, USA) containing 10% human serum albumin (LifeGlobal) and incubated at 37°C in 5% CO_2 for 2 to 3 hours. The COCs were then exposed to 70 IU hyaluronidase (type VIII from bovine testes, Sigma). The cumulus cells were mechanically separated from oocyte under stereo-microscopy (2X magnification). The oocyte from the first punctured follicle was further cultured separately from other sibling oocytes. To avoid vaginal cell and white blood cell contamination, cumulus cells were washed with phosphate-buffered saline solution and assessed under light microscope (10X magnification). Each sample was stored in 15 mL-tube and further processed for the mRNA gene expression study.

Evaluation of antioxidative enzyme-related genes (*SOD1*, *SOD2* and *GPx4*) mRNA expression

The cumulus cell total RNA extraction from each individual COCs was performed using ReliaPrep™ RNA Miniprep System (Promega, Madison, WI, USA). RNA concentration was then measured with NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The first-strand complementary DNA (cDNA) was generated from 50 ng total RNA by using Reverse Transcription System (Promega). The cDNA concentration was again measured with Nanodrop spectrophotometer. Gene-specific primers for the subject sequences, housekeeping gene (*GAPDH*) and target genes (*SOD1*, *SOD2* and *GPx4*) were predesigned by integrated DNA technologies (PrimeTime Mini qPCR Assay, Integrated DNA Technologies, Singapore) (Table1). Conventional PCR was carried out according to the manufacturer's instruction (Multiplex PCR kit, Qiagen, Valencia, CA, USA) as following: Taq activation (95°C, 15 min) and 38 cycles of denaturation (94 °C, 30 sec), annealing for 30 sec at a different

temperature for each primer (*GAPDH*, 52 °C; *SOD1*, 52 °C; *SOD2*, 56 °C; *GPx4*, 52 °C) and then extension at 72°C for 20 sec. The final extension was performed at 72 °C for 1 min. Visualization of the PCR product was evaluated using UV light (Gel Documentation System, Bio-Rad, Hercules, CA, USA). Only samples with confirmation of *GAPDH* expression were proceeded to quantitative real-time polymerase chain reaction (qPCR) with total volume of 20 µl (consisted of cDNA 50 ng, 10 µl qPCR master mix (Luna Universal Probe qPCR master mix, New England Biolabs, Ipswich, MA, USA) and 500 nM of qPCR Primer and probes (PrimeTime Mini qPCR Assay) (Table 1). All reactions were performed in duplicate and processed with qPCR (StepOnePlus Instrument, Applied Biosystem, Foster City, CA) according to following conditions: 95 °C for 60 sec, followed by 45 cycles at 95 °C for 15 sec and 56 °C for 15 sec. Two replications were calculated for each gene expression. Relative quantification of mRNA expression was calculated using $2^{-\Delta\Delta CT}$ method [14].

Baseline characteristics, in vitro fertilization (IVF), embryo transfer and clinical pregnancy outcomes

Patients were determined to undergo fresh or frozen-thawed embryo transfer on the basis of their clinical indication. Clinical pregnancy was defined as the presence of intrauterine gestational sac 4 weeks after embryo transfer. Clinical data such as baseline characteristics, infertility history, basal hormonal profiles, ultrasonography result, IVF and embryo profiles were obtained from medical records.

Statistical analysis

All data analyses were performed using SPSS statistic software package version 22 (SPSS Inc, Chicago, IL, USA). The nonparametric Mann-Whitney test was used for data analysis to compare variables between normal and poor responders when data was absent of normal distribution. Independent t-test for continuous variables and Chi-square test for categorical variables were performed when data distributed normally. The level of significance was set at $P < 0.05$.

Results

Baseline characteristics, in vitro fertilization (IVF), embryo transfer and clinical pregnancy outcomes

A total of 56 infertile women who met the inclusion criteria were enrolled into the present study. Only cumulus cells from women with confirmation of *GAPDH* expression were included in the antioxidative enzyme-related genes expression level analysis; 28 participants in the poor ovarian response group and 28 participants in the normal ovarian response group. The mean age of total participants was 38.6 years old. There were no significant differences between POR (39.3 ± 3.7 years old) and NOR group (37.9 ± 2.9 years old) ($P = 0.15$) (Table 2). The other baseline characteristics including body mass index, duration of infertility, number of previous medically assisted reproduction (MAR) attempts were also not significantly different (Table 2). Regarding the baseline of hormonal profiles, the data demonstrated that the level of FSH was marginally higher in POR than those in NOR group ($P = 0.05$) (Table 3).

As shown in Table 4, the selected types of gonadotropin administration were significantly different between the two groups; physicians preferred to prescribe rFSH in NOR (78.6%) than in POR group (35.7%), whereas hMG were administered more in POR than in NOR group, 64.3% and 21.4%, respectively. However, the total dose of gonadotropin and the duration of ovarian stimulation were comparable between both groups. As expected, estradiol level on the day of hCG trigger and the numbers of retrieved oocytes in the normal responder group were significantly higher than the poor responder group ($P < 0.01$).

According to the IVF outcomes, the oocyte maturation rate did not differ between the two groups ($P = 0.72$). Additionally, there were no significant differences of fertilization rate after ICSI between the two groups ($P = 0.34$). More than 90.0% of participants in POR group underwent fresh embryo transfer, compared with 75.0% in NOR group. Similar to IVF outcomes, clinical pregnancy rates per aspiration were comparable (21.4%) between poor responders and normal responders (Table 4).

Relative mRNA expression level of antioxidative enzyme-related genes

The present findings revealed that the relative mRNA expression level of all enzymatic antioxidant-related genes obtained from cumulus cells did not significantly differ between POR and NOR groups (Table 5). When participants were categorized according to the type of gonadotropin administration, the relative *SOD2* mRNA expression level in patients who received hMG was notably higher than in those who obtained rFSH ($P = 0.017$) (Table 6). However, no significant differences were found in the other antioxidative enzyme-related gene mRNA expression between the two gonadotropin administration cohorts (Table 6).

The evaluation of cumulus cell mRNA gene expression level from each oocyte per patient revealed that the relative mRNA expression levels were not significantly different between the fertilization competence groups, i.e., normal feralization and non-fertilization. (Table 7). Similarly, none of the antioxidative enzyme-related genes expression levels were significantly different between pregnant and non-pregnant groups (Table 8).

Discussion

Reactive oxygen species (ROS) induced oxidative stress has been well documented to be balanced by antioxidant scavenging systems. The impairment of antioxidant activity could negatively affect female reproductive functions in various ways including alteration of ovarian steroidogenesis, oocyte maturation, ovulation or embryo development. The aim of this study was to determine the mRNA expression levels of enzymatic antioxidant-related genes (*SOD1*, *SOD2* and *GPx4*) in cumulus cells and their differences between patients with poor and normal ovarian response to the controlled ovarian hyperstimulation in IVF procedure. However, this study notably revealed that the relative mRNA expression levels of all antioxidant coding genes in the cumulus cells were not different between the two participant groups. Considering the IVF outcomes represented here by the fertilization status and the clinical pregnancy rates, there were no differences in the level of enzymatic antioxidant-related gene mRNA expression.

Interestingly, the significantly higher level of *SOD2* mRNA expression was observed in patients who received hMG for ovarian stimulation.

SOD1 and *SOD2* gene encoding enzymatic antioxidant, present in cytoplasm (Cu/ZnSOD) and mitochondria (MnSOD), mainly functions as a superoxide anion ($O_2^{\cdot-}$) scavenger [15]. *SOD1* and *SOD2* were previously evidenced their existence in human cumulus and granulosa cells [16]. In addition, they have been considered as one of the crucial factors that minimizes oocyte damage related to the oxidative stress environment during follicular growth, oocyte maturation and ovulation [16]. A recent 2019 study revealed that the antioxidant enzymes mRNA expression level in cumulus cells was lower in POR patients than young oocyte donors [17]. However, our findings indicated that the levels of *SOD* isoform 1 and 2 (*SOD1* and *SOD2*) mRNA expression were not different between the cumulus cells retrieved from POR and NOR participants.

Regarding another antioxidative enzyme, the family of selenium containing-glutathione-dependent peroxidase (GPx) is composed of four major isoforms; GPx1-GPx4 [18]. GPx4, a monomeric enzyme containing a single selenocysteine moiety, performs the potent function as a lipid peroxidation inhibitor [18]. The remarkable research studies on GPx4 activity have been mainly focused on male reproductive physiology such as subfertility in mice caused by mutation of GPx4 active site selenocysteine (GPx4_U46S), whereas the research data in female is confined [19]. Recently, relevant supporting data on the role of GPx family in the female reproductive physiology; ovarian microenvironment/ follicular fluid, was reported [11]. There is some evidence to suggest that GPx activity markedly decreased in poor IVF responders, whereas the lipid peroxidation level increased [11]. In contrast, the GPx4 mRNA expression level did not significantly differ between the two participant groups included in the current study. While more investigations are needed, this so far modest group of collective findings appears to suggest that cumulus cell-antioxidative enzymes (SOD1, SOD2 and GPx4 isoforms) may not be exerting crucial roles and functions in POR patients. The contradictory results could be principally deliberated by the age difference between the participants from our group and Nuñez-Calonge's research group. The average age of patients in our study was 38 years old (POR = 39.3 ± 3.7 years old and NOR = 37.9 ± 2.9 years old), while the average age of POR patients was 30.6 ± 3.1 years old and those of healthy oocyte donors was 29.7 ± 4.1 years old in the study of Nuñez-Calonge et al. [11]. Previous studies have suggested that *SOD* gene expression and activity significantly changed as age-dependence [9,20]. Similar to *SOD*, *GPx* activity gradually declined by cell aging [21]. In addition to the direct effect on the lipid peroxides scavenging system, GPx4 was shown to be involved in mitochondrial membrane potential (ΔΨm) regulation under the oxidative stress condition [22]. There is also some evidence demonstrating that the frequency of cumulus-granulosa mitochondrial membrane rupture was higher in advanced-age than young participant groups [23]. This phenomenon might reflect exquisite changes in the oxidative phosphorylation capacity, i.e. GPx4 activity specifically in the advanced-age patients [21]. Constantly, the global gene expression analysis of aged oocytes, represented by cumulus cell-oocyte complexes (COCs), in animal research models notably addressed the reduction of mitochondrial and cytosolic antioxidant gene-mRNA expression including peroxiredoxin 3 (Prdx3), thioredoxin 2 (Txn2) and superoxide dismutase 1 (Sod1)

[24,25]. With relevant supporting scientific data, it might be possible to explain our contrasting findings from other research studies which found that enzymatic antioxidant-related genes (*SOD1*, *SOD2* and *GPx4*) in cumulus cells retrieved from advanced-age patients may not play prominent roles in determining poor or normal ovarian response to COH. The present findings could only support the existence of *SOD* and *GPx* isoforms in human cumulus cells, while their roles in age-related ovarian response are still to be further investigated.

According to the IVF outcomes, none of the genes in our study were correlated with fertilization or pregnancy rates. Although various evidences indicated the correlation between oxidative stress and IVF outcomes [26], the gradual decrease of antioxidant activity in advanced-age related cohorts, both POR and NOR participants, as presented in our study should be considered. Indisputably, oocyte quantity and quality have tremendously declined in advanced-age patients [27]. However, fertilization competence has been also influenced by various factors such as sperm quality, microenvironment during the IVF procedure, while clinical pregnancy depends on diverse factors such as embryo quality including ploidy status, uterine receptivity as well as maternal co-morbidities. Thus, it is not surprising that the indifferent IVF outcomes between POR and NOR groups are demonstrated in our study.

Regarding patient groups categorized by the type of gonadotropin administration, the mRNA expression level of *SOD2* in patients who received hMG was notably higher than rFSH group. The hMG, composed of FSH and luteinizing hormone (LH), is generally known as hormonally active medication for infertility treatment [28]. Due to the crucial role on antioxidant system, LH-like exogenous hormone could stimulate *SOD1* activity in human corpus luteum [29]. Besides, the recent study in other mammalian species, bovine luteal cell cultures revealed that mRNA and protein expression levels of *SOD1* and *SOD* 2 were also mediated by LH supplementation via cAMP-dependent pathway [30]. Theoretically, one key of ovarian function regulation through pituitary gonadotropins is the action of LH via LH receptor (LHR) [31]. In female reproductive physiology, the LHR expressed in various cell types including cumulus cells; both compacted and expanded cumulus cells [32]. Relative to our results, the major finding on the markedly high SOD2 mRNA expression in patients receiving hMG might contribute to the dominant effect of LH. However, the specific mechanism of hMG related to SOD2 mRNA expression in cumulus cells may be of interest to elucidate the underlying pathway.

Conclusions

Our findings indicated that the enzymatic antioxidant-related gene mRNA expression (*SOD1*, *SOD2* and *GPx4*) in cumulus cells did not associate with ovarian response and IVF outcomes. However, one of these genes' mRNA expression was significantly related to type of gonadotropin administration. Future research should be conducted to elucidate the different gonadotropin preparations related with oxidative stress status to ultimately achieve optimal ovarian stimulation protocol, especially in poor ovarian response patients.

Abbreviations

ART: assisted reproductive technology; COCs: cumulus cell oocyte complex; COH: controlled ovarian hyperstimulation; GPx: glutathione peroxidase; GSH: total glutathione; GR: glutathione reductase; GST: glutathione S-transferase; hCG: human chorionic gonadotropin; hMG: human menopausal gonadotropin; IVF: in vitro fertilization; LH: luteinizing hormone; LHR: LH receptor; mRNA: messenger RNA; NOR: normal ovarian response; OPU: ovum pick-up; PCOS: polycystic ovary syndrome; POR: poor ovarian response (POR); qPCR: real-time PCR; rFSH: recombinant follicle stimulating hormone; ROS: reactive oxygen species, RNS: reactive nitrogen species; SOD: superoxide dismutase

Declarations

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand on January 27, 2017 (IRB No.688/59). Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

References

1. The European IVF-monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, et al. Assisted reproductive technology in Europe, 2013: results generated from European registers by ESHRE. Hum Reprod. 2017;32(10):1957-73.

2. Polyzos NP, Sunkara SK. Sub-optimal responders following controlled ovarian stimulation: an overlooked group? Hum Reprod. 2015;30(9):2005-8.

3. Oudendijk JF, Yarde F, Eijkemans MJC, Broekmans FJM, Broer SL. The poor responder in IVF: is the prognosis always poor? A systematic review. Hum Reprod Update. 2012;18(1):1-11.

4. Domar AD, Rooney K, Hacker MR, Sakkas D, Dodge LE. Burden of care is the primary reason why insured women terminate in vitro fertilization treatment. Fertil Steril. 2018;109:1121-6.

5. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3:28. doi:10.1186/1477-7827-3-28.

6. Burton GJ. Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol. 2011;25:287-99.

7. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol.2012;10:49. doi: 10.1186/1477-7827-10-49.

8. Paszkowski T, Traub AI, Robinson SY, McMaster D. Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta. 1995;236:173-80.

9. Tatone C, Carbone MC, Falone S, Aimola P, Giardinelli A, Caserta D, et al. Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells. Mol Hum Reprod. 2006;12(11):655-660.

10. Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. Mol Hum Reprod. 2009;15(7):411-9.

11. Nuñez-Calonge R, Cortés S, Gutierrez-Gonzalez LM, Kireev R, Vara E, Ortega L, et al. Oxidative stress in follicular fluid of young women with low response compared with fertile oocyte donors. Reprod Biomed Online. 2016;32:446-56.

12. Nishihara T, Matsumoto K, Hosoi Y, Morimoto Y. Evaluation of antioxidant status and oxidative stress markers in follicular fluid for human in vitro fertilization outcome. Reprod Med Biol. 2018;17:481-6.

13. Donabela FC, Meola J, Padovan CC, de Paz CC, Navarro PA. Higher SOD1 gene expression in cumulus cells from infertile women with moderate and severe endometriosis. Reprod Sci. 2015;22(11):1452-60.

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402-8.

15. von Mengden L, Klamt F, Smitz J. Redox biology of human cumulus cells: basic concepts, impact on oocyte quality and potential clinical use. Antioxid Redox Signal. 2020;32(8):522-35.

16. Kala M, Shaikh MV, Nivsarkar M. Equilibrium between anti-oxidants and reactive oxygen species: a requisite for oocyte development and maturation. Reprod Med Biol. 2017;16:28-35.

17. Nuñez-Calonge R, Rancan L, Cortés S, Vara E, Andrés C, Caballero P, et al. Apoptotic markers and antioxidant enzymes have altered expression in cumulus and granulosa cells of young women with poor response to ovarian stimulation. J Reprod Biol Endocrinol. 2019;3(1):1-6.

18. Ufer C, Wang CC. The roles of glutathione peroxidases during embryo development. Front Mol Neurosci. 2011;4:12. doi: 10.3389/fnmol.2011.00012.

19. Ingold I, Aichler M, Yefremova E, Roveri A, Buday K, Doll S, et al. Expression of a catalytically inactive mutant form of glutathione peroxidase 4 (Gpx4) confers a dominant-negative effect in male fertility. J Biol Chem. 2015;290(23):14668-78.

20. Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R, et al. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Mol Hum Reprod. 2003;9(11):639-43.

21. Eichenlaub-Ritter U, Wieczorek M, Luke S, Seidel T. Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. Mitochondrion. 2011;11:783-96.

22. Kulinsky VI, Kolesnichenko LS. Mitochondrial glutathione. Biochemistry (Mosc). 2007;72(7):698-701.

23. de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M, Looman CW, et al. Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. Biol Reprod. 2004;70:41975-84.

24. Chang TS, Cho CS, Park S, Yu S, Kang SW, Rhee SG. Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. J Biol Chem. 2004;279(40):41975-84.

25. Lim J, Luderer U. Oxidative damage increases and antioxidant gene expression decreases with aging in the mouse ovary. Biol Reprod. 2011;84:775-82.

26. Ávila J, González-Fernández R, Rotoli D, Hernández J, Palumbo A. Oxidative stress in granulosa-lutein cells from in vitro fertilization patients. Reprod Sci. 2016;23(12):1656-61.

27. Grisendi V, Mastellari E, La Marca A. Ovarian reserve markers to identify poor responders in the context of Poseidon Classification. Front Endocrinol (Lausanne). 2019;10:281. doi: 10.3389/fendo.2019.00281.

28. Afnan M. Identifying real differences in live birth rates between HMG and rFSH in IVF. Reprod Biomed Online. 2009;18 Suppl 2:25-30.

29. Sugino N, Takiguchi S, Kashida S, Karube A, Nakamura Y, Kato H. Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. Mol Hum Reprod.

2000;6(1):19-25.

30. Kawaguchi S, Sakumoto R, Okuda K. Induction of the expressions of antioxidant enzymes by luteinizing hormone in the bovine corpus luteum. J Reprod Dev. 2013;59(3):219-24.

31. Choi J, Smitz J. Luteinizing hormone and human chorionic gonadotropin: origins of difference. Mol Cell Endocrinol. 2014;383:203-13.

32. Jeppesen JV, Kristensen SG, Nielsen ME, Humaidan P, Dal Canto M, Fadini R, et al. LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. J Clin Endocrinol Metab. 2012;97:E1524-31. doi: 10.1210/jc.2012-1427.

Tables

Table 1 Primer sequence for relative quantification of mRNA expression in cumulus cells

Gene	Forward Primer	Reverse Primer	PCR product (bp)
SOD1	TTAATGCTTCCCCACACCTT	CCTCGGAACCAGGACCT	126
SOD2	TGCTCCCACACATCAATCC	GGTTGGCTTGGTTTCAATAAGG	100
GPx4	TGCCCGTCGATGTCCTT	CCTTTGCCGCCTACTGAAG	140

* Reference gene: GAPDH TaqMan® (Gene expression assay ID hs02758991_g1; Applied Biosystems, Foster City, CA)

Table 2 Comparison of baseline characteristics between poor and normal ovarian response groups

Characteristics	Poor response group (*n* = 28)	Normal response group (*n* = 28)	*P*-value
Age (years)	39.3 ± 3.7	37.9 ± 2.9	0.15
Infertility			0.33
- Primary	24 (85.7%)	20 (71.4%)	
- Secondary	4 (14.3%)	8 (28.6%)	
BMI[a] (kg/m^2)	20.9 ± 2.8	22.0 ± 2.4	0.16
Partner age (years)	39.9 ± 5.8	38.3 ± 5.5	0.29
Duration of infertility	4.4 ± 3.8	5.4 ± 3.5	0.28
Cause of infertility			
- Unexplained	14 (50%)	10 (35.7%)	0.56
- Tubal factor	7 (25%)	9 (32.1%)	
- Male factor	7 (25%)	9 (32.1%)	
Previous MAR[b] treatment			
- IUI[c] (cycles)	2.1 ± 1.9	2.9 ± 1.8	0.10
- IVF (cycles)	0.6 ± 0.8	0.8 ± 1.3	0.42

Data are presented as mean ± SD or n (%).

[a] BMI = Body mass index

[b] MAR = Medical assisted reproduction

[c] IUI = Intrauterine insemination

Table 3 Antral follicle counts and baseline hormonal profiles between poor and normal ovarian response groups

Variable	Poor response group (n = 28)	Normal response group (n = 28)	P-value
Antral follicles count	6.1 ± 1.8	7.4 ± 1.9	0.08
FSH (IU/L)	9.1 ± 1.5	8.2 ± 1.6	0.05
LH (IU/L)	5.4 ± 1.7	4.5 ± 1.5	0.07
Estradiol (ng/ml)	32.2 ± 22.2	26.4 ± 14.5	0.24

Data are presented as mean ± SD.

Table 4 Characteristics of IVF cycles and outcomes between poor and normal ovarian response groups

Variable	Poor response group (n = 28)	Normal response group (n = 28)	P-value
Gonadotropin drug			
- rFSH	10 (35.7%)	22 (78.6%)	0.001
- hMG	18 (64.3%)	6 (21.4%)	0.001
Total gonadotropin dose (unit)	2240.0 ± 445.0	2322.2 ± 451.5	0.50
Duration of ovarian stimulation (day)	8.7 ± 0.9	9.2 ± 1.1	0.12
Estradiol level on the day of hCG trigger	981.2 ± 481.0	1653.4 ± 631.0	<0.01
Number of retrieved oocytes	3 (2.2,4)	7.5 (6,9.8)	<0.01
Oocyte maturation rate (%)	82.7 ± 18.7	80.9 ± 17.4	0.72
Fertilization rate (%)	82.7 ± 23.4	76.7 ± 24.0	0.34
Embryo transfer			
- Fresh transfer	26 (92.9%)	21 (75.0%)	
- Frozen-thaw transfer	2 (7.1%)	6 (21.4%)	
- No available embryo	0	1 (3.6%)	
Day of embryo transfer			
- Day 3	19 (67.9%)	6 (21.4%)	
- Day 4	3 (10.7%)	3 (10.7%)	
- Day 5	5 (17.9%)	15 (57.1%)	
- No of transferred embryo	1 (3.6%)	3 (10.7%)	
Clinical pregnancy rate per aspiration (%)	6 (21.4%)	6 (21.4%)	

Data are presented as mean ± SD or n (%).

Table 5 Comparison of relative mRNA expression levels in cumulus cells between poor and normal ovarian response groups

Gene	Poor response group (n = 28)	Normal response group (n = 28)	P-value
SOD1	0.2567 (0 - 3.1483)	0 (0 - 0. 3455)	0.59
SOD2	5.0062 (2.1971 - 17.4628)	6.8266 (0-64.8226)	0.87
GPx4	0.0009 (0-0.9950)	0 (0-0.4229)	0.25

Data are presented as median (interquartile range).

Table 6 Relative mRNA expression levels in cumulus cells between patients who received rFSH and hMG

Gene	rFSH (n = 32)	hMG (n = 24)	P-value
SOD1	0.039204 (0-2.7332)	0.2567 (0 - 4.3843)	0.28
SOD2	2.5535 (0 - 20.2133)	13.8478 (0 - 44.5888)	0.017
GPx4	0 (0 - 0.0540)	0.0297 (0 - 1.1919)	0.088

Data are presented as median (interquartile range).

Table 7 Comparison of Relative mRNA expression levels in cumulus cells between normal fertilization and non-fertilization groups

Gene	Normal fertilization (n = 44)	Non-fertilization (n = 12)	P-value
SOD1	0.2289 (0 - 3.8337)	0.1766 (0 - 2.4073)	0.87
SOD2	5.9838 (0.0137- 34.7013)	3.4363 (0.8239 - 24.8194)	1.00
GPx4	0 (0 - 0.8031)	0 (0 - 1.2554)	0.93

Data are presented as median (interquartile range).

Table 8 Comparison of relative mRNA expression levels in cumulus cells between pregnant groups and non-pregnant groups

Gene	Pregnant (n = 12)	Non-pregnant (n = 44)	P-value
SOD1	0 (0 - 2.463)	0.2556 (0 - 3.8337)	0.28
SOD2	3.9913(0.5536 - 32.1446)	5.9838 (0.1417 - 32.0436)	0.87
GPx4	0.1606 (0 - 0.7898)	0 (0 - 0.7704)	0.52

Data are presented as median (interquartile range).