Evaluation of Oxidative and Nitrosative Stress Markers Related To Inflammation in The Cumulus Cells and Follicular Fluid of Women Undergoing Intracytoplasmic Sperm Injection: A Prospective Study

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Abstract

Background: Oxidative/nitrosative stress in the oocyte microenvironment could have an impact on intracytoplasmic sperm injection (ICSI) outcomes. The presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can stimulate the secretion of pro-inflammatory cytokines, leading to chronic inflammation and potentially affecting embryo as well as oocyte quality. This study aimed to examine the relationship of lipid peroxidation [measured by the malondialdehyde (MDA) assay] with protein carbonyl [measured by the 2,4 dinitrophenylhydrazine (DNPH) assay] levels in cumulus cells (CCs), as well as nitric oxide (NO), peroxynitrite (ONOO-), and C-reactive protein (CRP) levels in follicular fluid (FF). The potential relationship of these levels with ICSI outcome was also evaluated.

Materials and Methods: In this prospective study, 63 FF samples and their corresponding CCs were collected for ICSI procedures. Spectrophotometry was used to assess levels of DNPH, MDA, NO, and ONOO⁻. CRP levels were evaluated using an immunoturbidimetric assay.

Results: The patients under 37 years with normal ovarian reserve had significantly lower levels of MDA, DNPH, NO, ONOO⁻, and CRP compared to those over 37 years. Additionally, we observed higher levels of MDA, DNPH, NO, ONOO⁻, and CRP in the group with an oocyte maturity rate of less than 60%. No significant difference was observed between the DNPH levels and factors such as infertility duration, embryo quality, pregnancy, or the number of retrieved oocytes. A higher level of MDA, NO, ONOO⁻, and CRP was found to be significantly related to the lower number of retrieved oocytes, longer periods of infertility, poor embryo quality, and negative pregnancy outcomes.

Conclusion: Oxidative/nitrosative stress, linking to inflammation in the oocyte microenvironment, can be considered as a potentially useful biomarker for assessing the development and competence of oocytes and embryos and predicting ICSI outcomes.

Keywords: Cumulus Cells, Follicular Fluid, Maternal Age, Oxidative/Nitrosative Stress, Pregnancy

Introduction

During assisted reproductive technology (ART) procedures, assessment of oocyte quality is indeed crucial, as it directly affects the competence of embryonic development and rate of successful implantation. However, evaluation of oocyte and embryo quality mainly relies on morphological criteria, which have limitations in predicting successful pregnancy outcomes (1). Since quality of the oocyte and its microenvironment is important in early embryo development, several researchers are working to develop new non-invasive biomarkers by analyzing oocyte microenvironment components, such as follicular fluid (FF) and cumulus cells (CCs) to improve intracytoplasmic sperm injection (ICSI) outcomes (2, 3). Among the many reasons for ART failure, oxidative stress (OS) appears to be an important factor. OS refers to disruption of the balance between reactive oxygen/nitrogen species and the antioxidant system. In the female reproductive system, ROS plays physiological roles during oocyte maturation, embryo development, and pregnancy, while it may contribute in ART

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failure (4). Indeed, reactive nitrogen species (RNS), such as nitric oxide (NO), are involved in signaling molecules and control various aspects of reproductive physiology, including early embryonic development and implantation (5).

Recently, FF and CCs are considered as non-invasive biomarkers and they are used for prediction of *in vitro* fertilization (IVF) outcomes (3, 6). CCs are the specialized cells surrounding oocyte and they are connected to the oocyte cytoplasm. They form gap junctions, as channels that allow direct communication between the CCs and the oocyte (7). As a consequence of this close molecular dialogue, CCs play an important role in oocyte maturation and fertilization, as well as signaling and regulation of function. Moreover, high levels of ROS in the ovaries can negatively impact oocyte quality, leading to apoptosis in granulosa cells (GCs). This results in degeneration of the corpus luteum (6), deteriorating communications between oocytes and CCs, and disturbtion of preovulatory oocyte maturation (8). Oxidative damage occurs due to the spread of lipid peroxidation cascades, which can affect meiosis and ovulation. It can also contribute in the aging of ovaries (9). Indeed, FF components are either derived from plasma or secreted by GCs and include leukocytes and several mediators, such as growth factors, ROS, and antioxidants (3). We propose that FF biochemical characteristics are involved in oocyte quality and subsequently in fertilization, embryo development, and pregnancy. Furthermore, in our previous study, we showed that high level of OS could be one of the causes of ovarian aging, in FF (10). Several studies have shown that inflammation and oxidative stress are often associated with many diseases, while they can exacerbate each other. Indeed, ROS promotes pro-inflammatory cytokine secretion (11, 12). Additionally, several authors have reported that chronic inflammation could alter oocyte meiosis and reduce oocyte quality (13), it could also have an impact on ovarian aging (14).

In the field of ART, it is noted that approximately 85% of transferred embryos do not successfully implant, and only 20-25% of IVF attempts result in a live birth. As a result, various studies are being conducted to explore the potential of new biomarkers based on the analysis of oocyte microenvironment (2, 3, 6). The current study aimed to examine effect of the lipid peroxidation, as assessed by a malondialdehyde (MDA) assay, and protein carbonyl, as assessed by a dinitrophenylhydrazine (DNPH) assay, levels in CCs, as well as NO, peroxynitrite, and C-reactive protein (CRP) levels in FF, on the outcomes of ICSI. Additionally, the study aimed to assess status of the oxidative/nitrosative stress in patients of advanced age undergoing ICSI.

Material and Methods

Patients' characteristics

This prospective study included 63 women undergoing ICSI procedures, at the Fertilization Center IRIFIV in Casablanca, Morocco. All participants gave written permission for the utilization of FF and CC samples after being informed. The reasons for seeking consultation among the couples were female infertility in 28 cases, a combination of infertility factors in 12, and unexplained cause of infertility in 23 cases. Patients were disqualified from the study, if they have had any of the following conditions: i. Endocrine disorders or previous ovarian surgery that impacted the ovaries or the secretion of gonadotropins, ii. Undergoing hormone therapy, suffering from metabolic syndrome, having undergone pelvic surgery, having ovarian tumors, being morbidly obese, or having an autoimmune disease, and iii. Having polycystic ovary syndrome or endometriosis.

Ovarian stimulation procedure

All patients underwent stimulation through the use of follicle stimulating hormone (FSH, Orgalutran 0.25 IU and Gonal-F) according to the antagonist protocol. FSH (Gonal-F from Serono Laboratories, Saint Cloud, France) was administered daily through subcutaneous injections, with doses ranging from 150-225 IU/day or ¼ 300 IU/day, determined based on different factors, such as the patient ages, antral follicle count (AFC) in the early days of the cycle, and anti-müllerian hormone (AMH) concentration. The FSH dose was monitored and adjusted based on ultrasound results showing follicle growth (10, 15). On the 6th day of FSH administration, daily injections of the GnRH antagonist Ganirelix (Orgalutran VR, MSD Schering-Plough, France) were started. Injection of the human chorionic gonadotrophin (HCG, Gonadotrophins Chorioniques Ovitrelle VR, Merck Serono, Germany) was given when the triggering criteria were met, including presence of at least three follicles with 17 mm size (10).

Maturation rates of the oocytes were divided into two groups: "Group I" contained oocytes with a maturation rate of 60%, and "group II" consisted of FFs with a maturation rate of 60%. Level of AMH was evaluated for each patient on the third day of their menstrual cycle. AMH levels <1.1 ng/ml are thought to indicate a reduced ovarian reserve, whereas levels >1.1 ng/ml indicate a normal reserve. On the third-day of post-oocyte retrieval, quality of the embryos was classified into A-D subgroups based on morphological criteria, including number of the blastomeres, uniformity of the blastomeres, and fragmentation rate. An embryo was considered to be of the highest quality (A or B grade), if it have had 6-8 evenly sized blastomeres and a fragmentation rate of 25%.

Collection and preparation of the samples

Follicular fluid samples

FF samples was collected, and CCs were isolated for ICSI procedures. The FF was obtained from mature follicles, during the time of oocyte retrieval, and only clear

samples were utilized. These samples were purified using a Ficoll-based protocol (3 ml), as described by Ferrero et al. (16). The purified FF was then immediately stored at -20°C until it was assessed for nitric oxide, peroxynitrite, and CRP.

Cumulus cell samples

Following the oocyte pick-up procedure, the CCs were gently dislodged by aspirating them with a 100 micron pipette and then placed in a buffered culture medium from Gynemed company (Germany) with pH=7. The CC samples were transferred into a tube and disrupted with a lysis buffer with pH=7.5, consisting of 10 mM EDTA, 50 mM Tris, 1 mM PMSF, 1 mM glycerol, and 1 mM mercaptoethanol. The samples were stored at -20°C for the later analysis of lipid and protein oxidation.

Biochemical assay

The protein contents of CCs and FF were determined using the Bradford method (17) using bovine serum albumin (BSA, Sigma-Aldrich, Germany) as the standard. To evaluate lipid peroxidation, levels of MDA were measured, as a well-known end product of lipid peroxidation. This was performed using the thiobarbituric acid (TBA) assay, which is a commonly used method for determining MDA content (18). In this assay, high concentration of trichloroacetic acid (TCA) was added to release free MDA by treating it with TBA under acidic conditions and at high temperature approximately 100°C for 30 minutes. The reaction of two molecules of TBA with one MDA molecule generates a chromophore that absorbs light at 535 nm.

In short, 100 µl of the purified FF was mixed with 10% TCA and 0.67% TBA. The MDA concentration was expressed as micromole per microgram of protein, calculated using a molar extinction coefficient of 1.56×105 $M^{-1} cm^{-1}$.

Protein oxidation was measured by quantifying carbonyl groups through the 2,4-dinitrophenylhydrazine (DNPH) method (19). To perform the measurement, 50 µl of the sample solution was mixed with 10 mM DNPH in 0.5 M H_3PO_4, and after 10 minutes incubation, 400 µl of NaOH (6 M) was added. After a further 10 minutes of incubation at room temperature, absorbance was read at 450 nm. The results were expressed as µmol/µg protein of carbonyl groups and calculated using a molar extinction coefficient of 22,000 $M^{-1} cm^{-1}$.

Colorimetric assay of NO was carried out using the Griess reaction protocol as previously outlined by Arif et al. (20). This reaction involved mixing 0.02% naphthyl ethylenediamine dihydrochloride (NED), suspended in water, and 2% sulphanilamide (SA) in 5% phosphoric acid. In this procedure, nitrite is initially subjected to treat with the diazotizing agent SA in an acidic environment to generate a short-lived diazonium salt. The diazonium salt subsequently interacts with the coupling reagent NED to produce a robust azo compound, which exhibits a color ranging from pink to dark pink. The absorbance was measured at 540 nm, and quantity of nitrite was calculated based on µmoles/µg of protein using a reference $NaNO_2$ solution.

The procedure for measuring peroxynitrite ($ONOO^-$) levels involved the method described by Ben Anes and colleagues (21). The assay takes advantage of the ability of $ONOO^-$ to nitrate phenol, which leads to the formation of nitrophenol. To perform the assay, 100 µl of FF was placed in a glass test tube and combined with 5 mM phenol in a 50 mM sodium phosphate buffer. The mixture was then incubated for 2 hours at 37°C, followed by the addition of 100 µl of 0.1 N NaOH. Absorbance of the samples was then read at 412 nm, and $ONOO^-$ concentration was calculated by determining the yield of nitrophenol using a molar extinction coefficient of 4400 $M^{-1} cm^{-1}$.

The CRP level was assayed using a commercial kit (Cobas Tina-quant C - reactive protein IV, Switzerland) based on an immunoturbidimetric test on latex particles. Indeed, aggregation of the human CRP occurs when it is combined with latex particles coating with anti-CRP monoclonal antibodies. Particle clusters were measured by turbidimetry. CRP levels were expressed based on mg/l.

Statistical analysis

The results are reported as the mean ± the standard deviation (SD), and the differences between groups were analyzed through the Mann-Whitney U test, which was performed using the Statistical Package for the Social Sciences (SPSS, Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) software. A $P<0.05$ was considered to indicate a statistically significant difference.

Results

The study participants were 63 infertile women with ages ranging from 23 to 43 years, who received ICSI treatment. Assessment of MDA and DNPH levels were performed in CCs, while NO, $ONOO^-$ and CRP levels were assessed in FF. The outcomes of age, infertility length, oocyte maturity rate, embryo quality, number of oocytes, age combined with AMH, and pregnancies were separated into two groups: a lower group and a higher group, based on the statistical analysis.

As shown in Table 1, The levels of MDA and DNPH in CCs, as well as the levels of NO, peroxynitrite, and CRP in FF pools, were significantly lower in the patients younger than 37 years old compared to those who were 37 years old or older. Moreover, MDA, DNPH levels in CCs and NO, peroxynitrite, and CRP levels in FF pools were significantly lower in those with AMH >1.1 ng/ml than AMH <1.1 ng/ml. Statistical analysis did not show any significant difference between the older and younger women.

Table 1: Comparison of the oxidative and nitrosative stress markers, CRP levels, in human follicular fluid and cumulus cells with age and AMH combined to age

Subgroups/Biomarkers	Age (Y)		AMH (ng/ml)			
	<37 (n=34)	>37 (n=29)	<37		>37	
			< 1.1 (n=14)	> 1.1 (n=14)	<1.1 (n=15)	<1.1 (n=15)
MDA (CCs) µmol/µg of proteins	0.24 ± 0.18	0.34 ± 0.25	0.29 ± 0.17	0.18 ± 0.14	0.36 ± 0.32	0.36 ± 0.32
P values	0.04*		0.01*		0.03*	
DNPH (CCs) µmol/µg of proteins	0.007 ± 0.007	0.002 ± 0.001	0.0028 ± 0.0018	0.0018 ± 0.0012	0.0029 ± 0.0021	0.0029 ± 0.0021
P values	0.03*		0.02*		0.04*	
NO (FF) µmol/µg of proteins	0.15 ± 0.14	0.27 ± 0.27	0.25 ± 0.25	0.18 ± 0.14	0.35 ± 0.26	0.35 ± 0.26
P values	0.02*		0.04*		0.02*	
Peroxynitrite (FF) µmol/µg of proteins	0.006 ± 0.005	0.037 ± 0.028	0.005 ± 0.005	0.002 ± 0.001	0.01 ± 0.01	0.01 ± 0.01
P values	0.02*		0.05*		0.03*	
CRP (FF) mg/l	1.43 ± 1.39	2.83 ± 2.78	0.92 ± 0.60	1.82 ± 1.82	1.65 ± 1.65	3.18 ± 2.38
P values	0.007*		0.04*		0.01*	

Values are in mean ± standard deviation. Statistical significance was defined as P<0.05 (*; Significant), analyzed with the Mann-Whitney U-test. CRP; C-reactive protein, AMH; Anti-müllerian hormone, MDA; Malondialdehyde, CCs; Cumulus cells, DNPH; 2,4 dinitrophenylhydrazine, FF; Follicular fluid, and NO; Nitric oxide.

According to the data presented in Table 2, we noticed that the levels of MDA in CCs and the levels of NO, peroxynitrite, and CRP in FF were significantly higher in the patients who had been attempting conception for more than five years compared to those who had tried for five years or less. In addition, the patients with a low number of retrieved oocytes had elevated levels of MDA, NO, peroxynitrite, and CRP, in their CCs and FF pools. Furthermore, we noted in the group with a maturity rate of <60% a higher level of MDA, DNPH in CCs and NO, peroxynitrite, and CRP levels in FF compared to the group with a maturity rate of >60%. Statistical analysis did not show significant difference between the DNPH levels and the duration of infertility, embryo quality, pregnancy, or number of oocytes. On the other hand, we noticed significantly higher levels of MDA, NO, peroxynitrite, and CRP in the samples associated with oocytes that produced poor-quality embryos (grades C and D) compared to the samples associated with high-quality embryos (grades A and B). Finally, MDA levels in CCs and NO, peroxynitrite, and CRP levels in FF samples were significantly higher in women who had not been pregnant compared to women who had been pregnant (Table 2).

Table 2: Comparison of the oxidative and nitrosative stress markers, CRP level in human follicular fluid and cumulus cells with infertility length, number of oocytes and ICSI outcomes

Subgroups/ Biomarkers	Infertility length		Number of oocytes		Maturity rates		Embryos quality		Pregnancy	
	<5 (n=30)	>5 (n=33)	>6 (n=33)	<6 (n=30)	>60% (n=43)	<60% (n=20)	A-B (n=42)	C-D (n=21)	Positive (n=18)	Negative (n=45)
MDA (CCs) µmol/µg of proteins	0.22 ± 0.18	0.31 ± 0.23	0.23 ± 0.21	0.30 ± 0.18	0.24 ± 0.19	0.35 ± 0.24	0.21±0.16	0.33 ± 0.30	0.20 ± 0.18	0.30 ± 0.23
Pvalue	0.05*		0.03*		0.05*		0.05*		0.02*	
DNPH (CCs) µmol/µg of proteins	0.002 ± 0.001	0.002 ± 0.002	0.002 ± 0.001	0.002 ± 0.001	0.0017 ± 0.001	0.0026 ± 0.001	0.002±0.001	0.002 ± 0.001	0.002 ± 0.002	0.002 ± 0.002
P value	0.13		0.10		0.02*		0.35		0.11	
NO (FF) µmol/µg of proteins	0.16 ± 0.11	0.27 ± 0.27	0.16 ± 0.14	0.27 ± 0.26	0.13 ± 0.11	0.19 ± 0.12	0.18±0.18	0.31 ±0.31	0.16 ± 0.08	0.26 ± 0.26
P values	0.02*		0.04*		0.03*		0.05*		0.02*	
Peroxynitrite (FF) µmol/µg of proteins	0.004 ± 0.002	0.01 ± 0.01	0.003 ± 0.002	0.014 ± 0.014	0.003 ± 0.002	0.004 ± 0.004	0.004±0.003	0.006 ± 0.005	0.004 ± 0.002	0.009 ± 0.002
P values	0.03*		0.05*		0.05*		0.03*		0.04*	
CRP (FF) mg/l	1.42 ± 1.27	2.25 ± 2.25	1.47 ± 1.40	2.33 ± 2.04	1.60 ± 1.47	2.68 ± 2.60	1.60 ± 1.60	2.99 ± 2.99	1.21 ± 0.95	2.13 ± 2.13
P values	0.04*		0.03*		0.05*		0.03*		0.01*	

Values are in mean ± standard deviation. Statistical significance was defined as P<0.05 (*; Significant) analysed with the Mann-Whitney U-test. CRP; C-reactive protein, ICSI; Intracytoplasmic sperm injection, MDA; Malondialdehyde, CCs; Cumulus cells, DNPH; 2,4 dinitrophenylhydrazine, NO; Nitric oxide, and FF; Follicular fluid.

Discussion

Inflammation is a response to any disturbance of tissue integrity, triggered to restore tissue balance by activating various repair mechanisms. It is important to regulate these mechanisms properly to prevent an excessive and uncontrolled inflammatory response, which can lead to development of various female reproductive disorders (22). Moreover, the redox reactions involved in cellular oxidative stress play a crucial role in the pathogenesis of inflammation. Excess levels of free radicals and inflammatory markers in FF can have toxic effects on germ cells, oocytes, and their early development (13, 14).

It is widely recognized that under conditions of antioxidant scarcity, ROS or RNS can oxidize membrane phospholipids, proteins, or DNA. Oxidative/nitrosative stress can cause damage through multiple mechanisms. The breakdown of peptide bonds, cross-linking, and modifications to amino acid side chains can cause changes to protein function and antigenicity, which can trigger the immune system and amplify harm caused by the inflammatory response (23, 24). This immune response, which is linked to oxidative stress, is associated with various pathological conditions affecting female fertility (25).

In addition, macrophages act as the first line of defense against invading pathogens or foreign substances and they generate NO, which can be harmful to ovulation, menstruation, and apoptosis, particularly under certain inflammatory circumstances (26). Combination of NO and superoxide anion leads to the creation of the highly toxic oxidant peroxynitrite. This typically occurs only when the concentration of NO surpasses toxic levels, causing it to compete with superoxide dismutase in eliminating superoxide. Indeed, in clinical practice, CRP is extensively used as a reliable marker of inflammation. CRP level is related to the prediction of reproductive outcomes (27). Therefore, in the current study, we measured lipid peroxidation (accessed by MDA assay) and protein carbonyl (accessed by DNPH alkaline assay) levels in CCs, as well as NO, peroxynitrite, and CRP levels in FF of women undergoing ICSI and these levels were compared to ICSI outcomes.

In our study, we also noted that MDA, DNPH levels in CCs as well as NO, peroxynitrite, and CRP levels in FF pools were significantly lower in the patients younger than 37 years old compared to those who were 37 years or older. This finding was consistent with another research noted that advanced age was marked by elevated levels of inflammatory markers, such as CRP (19), and higher production of ROS, leading to oxidative damage with age (10). While the body requires oxidative stress and inflammation to function properly, they can also speed up aging process and development of age-related diseases (11, 14). Furthermore, in the both younger and older women, levels of MDA and DNPH in the CCs, as well as levels of NO, peroxynitrite, and CRP in the FF pools were significantly lower in those with AMH >1.1 ng/ml compared to those with AMH <1.1 ng/ml. As far as we know, AMH is generated solely by the GCs of preantral and small antral follicles and it is a reliable measurement method for ovarian reserve. This result can be explained by the excessive OS and inflammation level affecting production of glycoprotein hormones such as AMH (28). Therefore, inflammation appears to impact ovarian reserve negatively, but the precise mechanism behind its effect on follicles is not clear yet (29).

We also noted that MDA, NO, peroxynitrite, and CRP levels weresignificantly elevated in the patients attempted conception for over five years, compared to those who tried for five years or less. Indeed, a long duration of infertility can result in elevated psychological stress levels among infertile couples. Various studies suggested that a prolonged period of psychological stress could be a contributing source of oxidative stress, leading to inflammation in follicular cells (30).

In addition, CCs and FF pools from patients with a low number of retrieved oocytes displayed elevated levels of MDA, NO, peroxynitrite, and CRP. These results agree with the various reports showing that ROS levels could influence number of the oocytes retrieved (5, 8). Moreover, ovarian stimulation promoted an increase in the number of leukocytes and lymphocytes. It also repaired some immune alterations in infertile patients. Thus, ovarian stimulation might affect integrity of systemic inflammatory hematologic parameters (31). Therefore, the rate of CRP can predict number of the oocytes retrieved.

Furthermore, our data showed that MDA, and DNPH levels in CCs and intrafollicular levels of NO, ONOO$^-$, and CRP were associated with poor oocyte maturity <60%. This finding was consistent with another research demonstrated a negative correlation between levels of ROS and oocyte maturation (5, 8). In the same line, there are various studies suggested that ONOO- played role in the activation of gene expression in response to cellular damage and it had an impact on pathways involved in oocyte maturation (32). Indeed, high levels of NO caused disruption in meiosis development along with a delay in the restart or resumption of meiosis (5). We hypothesized that OS activated the NF-κB pathway, which has a significant impact on triggering cytokine production and inducing an inflammatory response. There is evidence indicating that during the process of maturation from a germinal vesicle to a fully developed oocyte,activity of NF-κB is tightly controlled and suppressed. In support, impaired oocyte maturation was associated with elevated levels of inflammatory markers in FF (13, 29, 31).

We noticed that level of MDA in CCs as well as levels of NO, peroxynitrite, and CRP in FF were significantly higher in the samples associated with oocytes producing low-quality embryos (grades C and D) compared to those associated with high-quality embryos (grades A and B). Previous findings demonstrated excessive OS damages of oocyte membrane phospholipids and impairment of

cell signaling pathways, resulting in poor mitochondria function and altering embryonic development (33). Along with this, higher concentrations of NO can inhibit embryo development (34). In turn, presence of ONOO⁻ can cause lipid peroxidation and cellular damage in the oocyte microenvironment, potentially lowering quality of the oocyte and embryo. It also hinder successful implantation (35). In addition, high level of OS could give rise to an inflammation process in poor embryo quality (36).

MDA, NO, ONOO⁻ and CRP levels were significantly higher in the non-pregnant group compared to the pregnant group. These findings are consistent with various reports suggesting that high ROS levels could predict pregnancy failure by IVF (37-39). Moreover, in the physiological condition, pregnancy may also induce micro-inflammation and synthesis of inflammatory markers (37). Furthermore, higher levels of CRP are associated with women's infertility (40).

Conclusion

The study indicated that markers of oxidative and nitrosative stress as well as CRP levels in the oocyte microenvironment may be useful to assess developmental competence of oocytes and embryos. Deeper investigations of mechanism underlying the oxidative/nitrosative stress and CRP level in the human oocyte microenvironment help promote the clinical application of these non-invasive biomarkers in the future.

Acknowledgments

Authors' Contributions

H.D., M.J., H.J., A.K., M.E., N.L., R.C.; Participated in the study design, Data collection and Evaluation, Drafting, and Statistical analysis. M.J., H.J., A.K., M.E.; Performed follicle collection and prepared oocytes for ICSI in this component of the study. H.D., R.C.; Contributed to the all experimental work, Data and statistical analysis, and Interpretation of data. N.L., R.C.; Were responsible for overall supervision. H.D.; Drafted the manuscript, which was revised by N.L., R.C. All authors read and approved the final manuscript.

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