A New Biochemical Approach to Detect Oxidative Stress in Infertile Women Undergoing Assisted Reproductive Technology Procedures

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ABSTRACT

Oxidative stress negatively affects folliculogenesis and embryo development. However, a reliable and biologically accurate indicator of oxidative stress does not yet exist. On these bases, the aim of this study was to assess -and compare- blood and follicular fluid (FF) redox status in 45 infertile subjects (and 45 age-matched controls) undergoing in vitro fertilization (IVF) and to establish its connection with the outcome of IVF.

Blood and FF were obtained at the time of egg retrieval and immediately analyzed. Firstly, ROS production in blood leukocytes and in granulosa cells was assessed. Oxidative stress markers in blood and in granulosa cells resulted significantly (p<0.001) increased in infertile patients compared to controls. Then, a redox index was obtained in plasma and in FF of patients and controls.

The main findings emerging from our study in infertile women are: i) blood oxidative stress reflects FF oxidative stress as demonstrated by the significant correlation between blood redox markers and FF redox markers; ii) a significant correlation between oxidative stress parameters and the considered IVF outcomes is present. We suggest the strict monitoring of the redox parameters for the improvement of assisted reproductive techniques success rate and infertility management.

Keywords: oxidative stress; in vitro fertilization (IVF); infertile women; follicular fluid (FF); granulosa cells
INTRODUCTION

Oxidative stress is a condition caused by an imbalance between Reactive Oxygen or Nitrogen Species (ROS/RNS) production and/or a decrease in antioxidant defense systems. Firstly, this process is responsible for an adaptive response consisting in the induction of an antioxidant response and, following antioxidant depletion, in cellular injury and dysfunction [1-3].

Redox imbalance in serum and in follicular fluid (FF) has been suggested to be responsible for anomalous oocyte development, due to DNA and cell membrane damage, which would then result in reduced egg quality but also in altered fertilization, embryo quality, implantation, and embryonic development [4,5].

Several studies have reported signs of oxidative stress in serum and in FF of infertile women [6-8] but data on the simultaneous presence of oxidative stress markers in blood (particularly plasma and leukocytes), FF and granulosa cells are lacking. Such information would be useful for a better understanding of the ethiopathogenic mechanisms involved in infertility due to the still debated role of oxidative stress in infertile patients.

Interestingly, FF composition reflects all the metabolic and hormonal processes occurring in the microenvironment of the maturing oocyte and can represent a predictor of outcome parameters such as fertilization, embryo cleavage and pregnancy rates in IVF [9].

At present, in literature, contrasting results related to the detrimental or beneficial effects of ROS during in vitro fertilization (IVF) procedures are present. Some authors suggested that oxidative stress alters the oocyte and embryo quality and thus the fertilization rate [10] while other authors [11] reported that signs of lipid peroxidation in FF do not reflect the reproductive potential of oocytes. Furthermore, it has been shown that higher ROS levels and signs of lipid peroxidation [12,13] are present in women who became pregnant by IVF but also that higher total antioxidant capacity (TAC) is associated to increased fertilization potential in women undergoing IVF [7].

However, a general consensus on the role of oxidative stress in the processes regulating fertilization and embryonic development and the relationship between redox balance in blood and FF and oocyte, embryo quality and finally IVF outcome does not yet exist.

In this study we estimated both in infertile and control patients: 1- in blood samples: leukocyte ROS production and plasma oxidative stress markers; 2- in FF samples: granulosa cell ROS production and oxidative stress markers. Indeed, our primary aim was the identification of a new laboratory redox index useful for clinicians to gain in depth information to finally increase assisted reproductive techniques success rate.

RESULTS

Redox status in blood samples and in granulosa cells

In plasma of infertile patients signs of oxidative stress were present (Figure 1A) as indicated by the significantly decreased total antioxidant capacity (23.42±3.69 vs 17.86±4.02, p<0.0001) and the significantly increased TBARS level (an index of lipid peroxidation, 0.34±0.06 vs 0.97±0.32, p<0.0001). In infertile patients, redox alterations were also evident in FF (Figure 1B) where total antioxidant capacity was significantly reduced (21.35±2.72 vs 12.59±5.72, p<0.0001) and lipid peroxidation was significantly increased (0.25±0.03 vs 1.02±0.40, p<0.0001). The redox imbalance in infertile patients was also confirmed by the significantly increased levels of ROS production in leukocytes (lymphocyte, monocyte and granulocyte) and granulosa cells (Figure 1C, 1D) compared to controls.
FIGURE 1. Oxidative stress parameters in plasma and Follicular Fluid in infertile patients and controls.
Total antioxidant capacity and lipid peroxidation in plasma (A) and Follicular Fluid (B), leukocyte and granulosa cells ROS production (C,D) in infertile patients (n.45) and controls (n. 45). * indicates that differences are statistically significant at the p<0.05 level.

Redox Index calculated as ORAC/TBARS ratio as a mirror of oxidative stress
To achieve a reliable estimation of redox status in infertile patients and controls, we calculated, both in plasma and in FF, a Redox Index (RI) obtained as ORAC/TBARS ratio. In infertile patients plasma RI resulted about 3.4 folds (p<0.0001) and FF RI 6.0 folds (p<0.0001) lower than in controls (Figure 2).
FIGURE 2. Plasma and Follicular Fluid Redox Index in infertile patients and controls
Plasma Redox Index and Follicular Fluid Redox Index in infertile patients (n.45) and controls (n. 45).
* indicates that differences are statistically significant at the p<0.05 level.

Correlation between investigated parameters
As shown in Figure 3, plasma RI significantly correlates with lymphocyte ROS (r=0.557, p<0.0001), monocyte ROS (r=0.616, p<0.0001), granulocyte ROS (r=0.624, p<0.0001), FF TBARS levels (r=0.676, p<0.0001), granulosa cell ROS (r=0.340, p<0.05), but not with FF antioxidant capacity (r=0.076, p=0.6201).

FIGURE 3. Plasma Redox Index correlation Analyses
Relationship among the investigated redox parameters and plasma Redox Index. Statistically significance was considered at the p<0.05 level.

Figure 4 shows that FF RI significantly correlates with lymphocyte ROS (r=0.472, p<0.005), monocyte ROS (r=0.544, p<0.0001), granulocyte ROS (r=0.534, p<0.001), plasma antioxidant capacity (r=0.436, p<0.005), plasma TBARS (r=0.396, p<0.01), granulosa cell ROS (r=0.460, p<0.005).

**FIGURE 4. Follicular Fluid Redox Index correlation Analyses**

Relationship among the investigated redox parameters and Follicular Fluid Redox Index. All correlation analyses were statistically significant at the p<0.05 level.

Systemic oxidative stress parameters are related to ART outcome

In order to verify, in infertile patients, the possible role of redox alterations in the main outcomes of ART procedures, we performed suitable correlation analyses among the plasma/FF oxidative stress-related parameters and fertilization rate (%) /retrieved oocyte Metaphase II (%) indexes.

As shown in Table 1, % Metaphase II oocytes significantly correlated with plasma TBARS (r=0.431, p<0.01) and ORAC (r=0.307, p<0.05), lymphocyte ROS (r=0.540, p<0.001), monocyte ROS (r=0.452, p<0.01), granulocyte ROS (r=0.519, p<0.001), granulosa cell ROS (r=0.605, p<0.0001), FF TBARS (r=0.315, p<0.05) and both with plasma RI (r=0.338, p<0.05) and FF RI (r=0.339, p<0.05) but not with FF antioxidant capacity (r=0.230, p=0.1291).

Fertilization rate (%) significantly correlated with plasma TBARS (r=0.648, p<0.0001), ORAC (r=0.483, p<0.001), monocyte ROS (r=0.298, p<0.05), FF TBARS (r=0.388, p<0.01), with plasma RI (r=0.521, p<0.001) and FF RI (r=0.304, p<0.05) but not with lymphocyte ROS (r=0.223, p=0.1405), granulocyte ROS (r=0.237, p=0.1168), granulosa cell ROS (r=0.176, p=0.2474) FF antioxidant capacity (r=0.229, p=0.1305).
TAB 1. CORRELATION ANALYSES BETWEEN THE INVESTIGATED REDOX PARAMETERS AND IVF OUTCOME.

<table>
<thead>
<tr>
<th></th>
<th>Metaphase II (%)</th>
<th>Fertilization Rate (%)</th>
</tr>
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<tbody>
<tr>
<td>Plasma TBARS</td>
<td>P&lt;0.0031 *</td>
<td>P&lt;0.0001 *</td>
</tr>
<tr>
<td>Plasma ORAC</td>
<td>P&lt;0.0404 *</td>
<td>P=0.0008 *</td>
</tr>
<tr>
<td>Plasma Redox Index (RI)</td>
<td>P&lt;0.0233 *</td>
<td>P&lt;0.0002 *</td>
</tr>
<tr>
<td>Lymphocyte ROS</td>
<td>P&lt;0.0001 *</td>
<td>P=0.1405</td>
</tr>
<tr>
<td>Monocyte ROS</td>
<td>P&lt;0.0018 *</td>
<td>P=0.0472 *</td>
</tr>
<tr>
<td>Granulocyte ROS</td>
<td>P&lt;0.0003 *</td>
<td>P=0.1168</td>
</tr>
<tr>
<td>FF TBARS</td>
<td>P&lt;0.0349 *</td>
<td>P&lt;0.0085 *</td>
</tr>
<tr>
<td>FF ORAC</td>
<td>P=0.1291</td>
<td>P=0.1305</td>
</tr>
<tr>
<td>FF Redox Index (RI)</td>
<td>P&lt;0.0229 *</td>
<td>P=0.0424 *</td>
</tr>
<tr>
<td>Granulosa cell ROS</td>
<td>P&lt;0.0001 *</td>
<td>P=0.2474</td>
</tr>
</tbody>
</table>

* Pearson rank correlation analysis was performed in unfertile patients. A value of p<0.05 was considered statistically significant.

DISCUSSION

The primary objective of this study was to compare blood and FF redox status in infertile subjects undergoing IVF and to establish its connection with the outcome of IVF. As a secondary objective, in order to achieve a good estimation of global redox status, besides to the common assayed systemic oxidative stress markers (TBARS and ORAC), we analyzed ROS production in blood lymphocytes, monocytes and granulocytes and in granulosa cells. A new parameter, Redox Index (RI, calculated as ORAC/TBARS ratio) was obtained in plasma and in FF of patients and controls as well. Our results indicate that in infertile patients a marked redox imbalance both in plasma and in FF is evident. Furthermore, the extent of oxidative stress shows significant correlation with the IVF outcome (Metaphase II (%), Fertilization rate (%)). Our data clearly demonstrate, in the considered population of infertile women undergoing IVF, that blood redox status reflects FF redox status thus giving valuable information on ovarian physiology and oocyte environment/follicular microenvironment. Interestingly, both plasma and FF RI show significant correlations with the considered indicators of IVF outcome. Although a certain amount of ROS is required under physiological conditions, an altered balance between pro-oxidant and antioxidant molecules may have deleterious effects on folliculogenesis and adequate embryo development [19]. Indeed, ROS deriving from several sources (i.e. the electron leakage of the inner mitochondrial membrane during oxidative phosphorylation) can damage cellular macromolecules during follicle growth. Consequently, antioxidants represent important defense mechanisms which allow cells to function within an oxidative environment, including the transient rise in ROS activity that accompanies ovulation [20]. Oxidative stress, which displays fundamental role in follicle development and oocyte maturation, has been studied by several strategies. In our study, markers of oxidative stress both in blood and in FF and ROS production in leukocyte subpopulations from infertile patients resulted significantly increased compared to controls. Indeed, as we previously reported [21], peripheral leukocytes represent a reliable model for studying the pathophysiology of oxidative stress-mediated homeostasis variations, which can be responsible for cell dysfunction and cell injury. Leukocytes reflect the condition of the whole organism and represent a valuable model to study systemic oxidative stress-related disorders [22]. Indeed, one of
the main result of our study is the significant correlation among leukocyte ROS production both with plasma RI, FF RI and granulosa cell ROS.

In reproductive functions the follicular microenvironment plays a critical role in oocyte maturation by providing an adequate environment for oocyte growth. FF provides several information about the biochemical status of follicle because it contains several metabolites useful for oocyte development. Changes in FF composition might influence oocyte quality, affecting fertilization, early embryonic development and subsequent pregnancy [23]. In particular FF contains molecules whose cysteine residues may be involved in sensing and buffering the local redox conditions which represent a critical issue in reproduction. In fact, in FF, several important proteins regulating follicle growth and oocyte quality, exhibit cysteine residues at specific points, whose oxidation would result in functional loss. Therefore, preservation of controlled redox conditions in the FF is essential for the fine-tuned oocyte maturation process [13]. In contrast, its disturbance enhances the susceptibility to the establishment of reproductive disorders that would require the intervention of reproductive medicine technology. In FF are also present ROS and antioxidants produced by granulosa cells, endothelial cells and leukocytes [24]. Although ROS are essential in some female reproductive functions, including the ovulatory response [25], when in excess they might have a negative impact, especially on estradiol levels, which are an important predictor of ovarian response [26], damaging steroidogenesis and consequently oocyte maturation and ovulation [8, 27]. Our results indicate that signs of oxidative stress (increased lipid peroxidation markers and reduced antioxidant capacity) are present in FF of infertile patients in line with other reports showing FF oxidative stress and its correlation with poor-oocyte/embryo quality and low fertilization rate. [28]. Interestingly, FF Redox Index was not only significantly correlated with plasma TBARS, ORAC and plasma RI but also with leukocyte and granulosa cell ROS production. High 8OHdG levels in the granulosa cells of infertile women undergoing IVF have been negatively correlated with fertilization rate and embryo quality [29]. Supporting these data, other authors [30] demonstrated that increased 8OHdG concentrations in the FF of women undergoing IVF are associated with oocyte degeneration, suggesting that oxidative stress in the follicular compartment has deleterious effects on the oocyte.

Our data are in agreement with a report showing lower vitamin E concentrations in the FF of women with infertility related to endometriosis [31] but are in contrast with another study reporting both increased 8OHdG and an higher vitamin E concentration in the FF of women with endometriosis. These data probably indicate an up-regulation of the follicular antioxidant system in an attempt to neutralize, but not successfully, reactive species and to prevent oxidative damage to oocytes. [32] Another important result emerging from our study is the increased ROS production in granulosa cells from infertile patients. Granulosa cells ensure successful maturation and developmental competency of oocytes by providing nutrients and maturation-enabling factors. Moreover, they protect oocytes from oxidative stress injury through their own antioxidant system during maturation of oocytes [33,34]. Granulosa cells are particularly sensitive to ROS which play a key role in their apoptosis-induction [4,5,35,36], in particular endogenous H2O2 which is an important signaling molecule, but at the same time, when present at high levels may cause cell dysfunction and cell death. Accordingly, it has been shown that GSH depletion sensitizes granulosa cells to toxicant-induced apoptosis [37] but the mechanism underlying cell toxicity induced by ROS is less known. Among our main results we found that granulosa cell ROS production was significantly correlated with leukocyte ROS production and with FF RI.

The redox index (RI) that we established in our study populations both in plasma and in FF samples was obtained from the ratio between antioxidants and oxidative stress markers (ORAC/TBARS). In our opinion, together with cellular (leukocytes and granulosa cells) ROS production estimation, it can give valuable information about global oxidative stress occurrence.

In fact, the statistical analyses performed in our study groups indicate a strict relationship between plasma RI and FF RI and their significant correlation with the estimated blood/FF redox parameters, highlighting that systemic oxidative stress reflects FF oxidative stress.
In this regard, other authors [38], reported, in the peritoneal fluid of women with infertility due to endometriosis, lower antioxidant capacity compared to controls and indicating that serum compartment reflects the redox status of the peritoneal microenvironment. Increased plasma and FF oxidative stress markers and decreased levels of antioxidants have been already related to poor in vitro fertilization (IVF) outcome [39-41]. Indeed, several studies reported the relationship between follicular development, poor oocyte quality and fertilization, embryonic development, and decreased female fertility and oxidative stress, both at systemic and local level. [42-45]. However, due to the different strategies (analysis of various biological samples) and methodologies (analysis of systemic levels of antioxidants vs gene expression) used in the different studies about redox state in the ovary, results are often difficult to compare and occasionally contradictory.

This study is not without limitations. First, the study population is small. However, strict eligibility criteria are necessary to increase the internal validity, eliminating other factors that are potentially related to oxidative stress and compromised oocyte quality. Second, whether the analysis of a single follicle is representative of the set of follicles that responded to ovarian stimulation cannot be explained. However, because a longer time of anesthesia and repeated ovarian punctures might promote follicular and/or systemic oxidative stress, we chose, in order to increase the internal validity of the present study, to aspirate only the entire content of the first follicle of the first ovary punctured. Third, although our findings suggest plasma RI and leukocyte ROS production as potential predictors of positive IVF outcomes, larger studies are needed for validation.

We would underline that peripheral blood collection is easier, more practical, less invasive and less susceptible to sampling inadequacy than FF collection. In conclusion, studies performed so far indicate that oxidative stress negatively affects reproductive potential and is associated with poor gamete quality and abnormal early embryo development however, a clinically reliable, biologically accurate indicator of oxidative stress condition does not yet exist and is still difficult to identify.

We think that the use of the parameters here suggested could help in improving assisted reproductive techniques success rate and infertility management.
MATERIALS AND METHODS

Patients
The study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Review Board of the Careggi University-Hospital (reference n. 10709 approved on 27/04/2017). The study included a total of 45 infertile women (age 35.0±3.3 years) with BMI 22.7±4.0 kg/m² and duration of infertility >2 years (3.2 ± 1.9 years) undergoing IVF were recruited from April 2017 to September 2017. Out of these, 19 women with endometriosis (Stages III and IV) were included as the study group. The control group included 45 women (infertility due to tubal factor or male factor). Basic characteristics of the study population are summarized in Table 2.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PATIENTS</th>
<th>CONTROLS</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>35.0±3.3</td>
<td>34.5±4.5</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>22.8±4.1</td>
<td>21.0±1.5</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>3.2±2.0</td>
<td>1.8±0.8</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>8.3±3.8</td>
<td>6.0±2.2</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>5.9±3.1</td>
<td>4.4±2.1</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>57.1±24.8</td>
<td>48.5±3.1</td>
</tr>
<tr>
<td>AMH (ng/ml)</td>
<td>2.4±2.0</td>
<td>4.2±3.6</td>
</tr>
<tr>
<td>Smoking habits (%)</td>
<td>26.7</td>
<td>28.0</td>
</tr>
<tr>
<td>GnRH Antagonist (%)</td>
<td>100</td>
<td>100</td>
</tr>
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</table>

TAB 2. Clinical characteristics of 45 infertile patients and 45 age-matched controls.

From each patient peripheral blood samples were obtained at the time of egg retrieval, and FF from the mature follicles of each ovary was centrifuged and aliquots were frozen at -80°C until analysis. After centrifugation (1500xg for 15 minutes at 4°C), aliquots of plasma were used for experiments or stored at -80°C for further analyses. Granulosa cells were isolated from all aspirated FF using gradient centrifugation at oocyte retrieval and immediately analyzed. Patients were enrolled according to the following inclusion criteria:
(i) Absence of any metabolic or endocrine system-associated diseases (such as hyperprolactinaemia and thyroid dysfunction) and no history of cancer;
(ii) Serological markers for HBV, HCV, HIV all negative;
Patients gave their written informed consent and did not receive any monetary compensation for participating in the study. Basal follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) levels were tested on cycle day 3 of a spontaneous menstrual cycle prior to ovarian stimulation.
All recruited patients received the antagonist protocol for ovarian stimulation. The ovarian stimulation began with 125–225 IU of recombinant FSH (Gonal-F®; Merck Serono) from day 2 of the menstrual cycle, and the GnRH antagonist (Cetrotide; Merck Serono,) was introduced according to a multiple-dose protocol (0.25 mg/day) when a leading follicle of 14 mm and/ or estradiol concentrations of 400 pg/ml were reached. Triggering was performed when at least three follicles >17 mm were present with 0.2 mg of triptorelin SC (Decapeptyl, Ipsen Pharma) or Recombinant human chorionic gonadotrophin (HCG) (Ovitrelle®, Merck Serono) and oocyte retrieval was performed under sedation at the 36th hour following GnRHa.

FF collection and processing
Once separated oocytes were placed into culture media, while FF was collected in flasks [11]. Considerable caution was taken to pool FF uncontaminated with flush medium or blood.
Blood contamination was evaluated by visual inspection and samples that looked cloudy or bloodstained were discarded [14]. Only uncontaminated FF minimally stained with blood was used for analysis. Samples without oocytes or contaminated were discarded.

**ROS assessment by FACS analysis**

After collection, 100 μl EDTA-anticoagulated blood samples or FF samples was resuspended in 2 ml of BD FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA, USA), gently mixed, and incubated at RT in the dark for 10 min, as previously reported [15]. Next, cells were centrifuged, the supernatant discarded and cells washed twice in PBS. To determine the level of intracellular ROS generation, cells were incubated with H2DCF-DA (2.5 μM) (Invitrogen, CA, USA) in RPMI without serum and phenol red for 15 min at 37 °C. After labelling, cells were washed and resuspended in PBS and analysed immediately using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA). The sample flow rate was adjusted to about 1000 cells/s. For a single analysis, the fluorescence properties of 20000 cells were collected. The respective gates were defined using the distinctive forward-scatter and side-scatter properties of the individual cell populations. Moreover, the viability of the cells was controlled by flow cytometry with propidium iodide staining and was found to exceed 95%. Data was analysed using BD FACSDiva software (Becton-Dickinson, San Jose, CA, USA) [16].

**TBARS (Thiobarbituric Acid Reactive Substances) estimation**

Plasma and FF TBARS levels were measured using a TBARS assay kit (OXI-TEK, ENZO, USA) in accordance with previous report by our group [17]. Briefly, the adduct generated by reacting malondialdehyde with Thiobarbituric acid after 1h at 95 ºC was measured spectrofluorimetrically, with excitation at 530 nm and emission at 550 nm. TBARS were expressed in terms of malondialdehyde equivalent (nmol/ml).

**Total antioxidant capacity (TAC) assay**

The ORAC method (Oxygen Radical Absorbance Capacity), based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), was performed as previously described on plasma and FF samples [18]. Briefly, a fluorescein solution (6 nM) prepared daily from a 4 μM stock in 75 mM sodium phosphate buffer (pH 7.4), was used. Trolox (250 µM final concentration) was used as a standard. 70 µl of sample with 100 µl of fluorescein were pre-incubated for 30 min at 37 °C in each well, before rapidly adding AAPH solution (19mM final concentration). Fluorescence was measured with excitation at 485 nm and emission at 537 nm in a Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher Scientific Inc. MA, USA). Results were expressed as Trolox Equivalents (µM) and then normalized for protein concentration.

**ORAC/TBARS ratio as an index of oxidative stress**

ORAC/TBARS ratio was calculated in plasma and in FF of infertile and healthy subjects in order to obtain a measure of oxidative stress.

**Measurements of outcomes**

The rate of metaphase II oocytes retrieved and the fertilization rate were measured as outcomes of IVF procedure. In the intracytoplasmic sperm injection (ICSI) procedure, in order to determine the rate of metaphase II oocytes, oocytes retrieved at 35 h post-hCG administration were exposed briefly to 80 IU/ml hyaluronidase (Origio) followed by denudation of surrounding cumulus cells by stepwise mechanical stripping. Oocytes were then examined under an inverted microscope at a magnification of ×200 to assess the maturity stage (germinal vesicle, metaphase I, or metaphase II) and only those that have extruded the first polar body are selected for ICSI. In in-vitro fertilization and Embryo Transfer (IVF-ET), the oocytes are surrounded by cumulus and corona cells at the time of
insemination so that their maturity cannot easily be evaluated; the maturity stage assessment is performed 16–20 hours after insemination, once corona radiata has been removed using denuding pipettes. Fertilization rate (FR) is the percentage of correctly fertilized oocytes (zygotes with two pronuclei) per number of inseminated oocytes; fertilization was evaluated under an inverted microscope 16–20 hours after ICSI or IVF.

Statistical Analysis
All experiments were performed in triplicate. For descriptive aspects, owing to the symmetry of the distribution, data are summarized as mean±SD. The statistical significance of the differences observed in the various groups considered in this study was assessed using the ANOVA Bonferroni test. p<0.05 was accepted as statistically significant. Correlation analysis was performed using the Pearson’s test. All statistical operations data were processed using the Graph Pad Prism 5 software.

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AUTHOR CONTRIBUTIONS
Matteo Becatti, Claudia Fiorillo and Maria Elisabetta Coccia conceived and designed the experiments; Matteo Becatti, Rossella Fucci, Amanda Mannucci, Victoria Barygina performed the experiments; Matteo Becatti, Claudia Fiorillo, Marco Mugnaini and Niccolò Taddei analyzed the results; Luciana Criscuoli, Claudia Giachini, Francesco Bertocci, Rita Picone, Paolo Evangelisti, Francesca Rizzello, Cinzia Cozzi enrolled patients and collected biological samples; Matteo Becatti and Claudia Fiorillo drafted the paper.
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