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Estradiol and Progesterone Levels are Related to Redox Status in the Follicular Fluid During In Vitro Fertilization

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Studies have reported a possible association between the levels of oxidative stress biomarkers in follicular fluid (FF) and infertility treatment outcomes. FF analysis can provide important information about oocyte quality. This study aimed to evaluate the possible correlation between oxidative stress biomarker and intrafollicular hormone levels and clinical and laboratory parameters in women during controlled ovarian stimulation. These women were undergoing in vitro fertilization with intracytoplasmic sperm injection (ICSI).The FF samples were acquired from September 2012 to February 2014 from women undergoing private fertility treatment in Rio de Janeiro, Brazil. A total of 196 women who were undergoing ICSI and had different infertility diagnoses were recruited. The FF from each patient (average patient age of 36.3 ± 4.3 years) was collected following puncture of just one follicle with the largest diameter. After ruling out blood contamination by spectrophotometry, 163 patient samples were utilized in the study. In the FF, the progesterone levels were negatively correlated with (a) hydrogen peroxide scavenging capacity (HPSC) ($r = -0.294$, $P < 0.0001$), (b) total number of follicles ($r = -0.246$, $P < 0.001$) and (c) total number of oocytes punctured ($r = -0.268$, $P = 0.0001$). The concentration of serum estradiol exhibited a positive correlation with intrafollicular HPSC ($r = 0.165$, $P = 0.037$). Our data indicate that the FF levels of estradiol and progesterone are related to the FF redox status, which is closely associated with the number of oocytes obtained during ICSI procedures.

Key Words: oxidative stress, infertility, ICSI, catalase activity, assisted reproduction, fertility

Abbreviations: ART, assisted reproductive technology;BMI, body mass index; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; E2, estradiol;FF, follicular fluid;FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; HPSC, hydrogen peroxide scavenging capacity;ICSI, intracytoplasmic sperm injection;IVF, in vitro fertilization;LH, luteinizing hormone; MDA, malondialdehyde;P4, progesterone; ROS, reactive oxygen species;SOD, superoxide dismutase;TBA, thiobarbituric acid;TBARS, thiobarbituric acid reactive substances.

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Considering the biomedical advances in the area of assisted reproductive technology (ART), the search for biomarkers to evaluate oocyte quality and pregnancy outcome has intensified over the years. Techniques assessing embryo quality through genetic sequencing are often efficient [1]; however, they are invasive and do not prevent the production of surplus embryos, which has raised legal and ethical issues in several countries [2, 3]. Analysis of a biomarker of oocyte quality before its insemination would be a noninvasive procedure that would be easier and faster than sequencing, with a lower cost. Moreover, gaining the knowledge about the oocyte environment conducive to the improvement of oocyte quality is also intended.

The follicular fluid (FF) is in close contact with granulosa cells and the oocyte, and it contains several molecules derived from them [4]. Thus, it mediates communication within the follicle and orchestrates oocyte quality, ultimately having a direct impact on early embryo development. The FF harbors different hormones produced by the cells of the follicular microenvironment [5-8], as well as other molecules related to the redox homeostasis [9-12]. Although biomarkers of oxidative stress, hormones, and other molecules have already been evaluated in the FF [12, 13], there is no consensus in the literature about their influence on the oocyte physiology [9, 10, 14, 15]. This lack of consensus can be related to the criteria used for the admission of FF samples within these studies; the methodological differences in the measurement of analytes, patient age, body mass index (BMI), infertility or other disease diagnosis; human ability with ART procedure; or the psychophysiological characteristics of each woman (or couple). Many of these aspects could influence the FF content and reflect on oocyte and embryo quality.

Oxidative stress is defined as an imbalance between oxidants and antioxidants in which oxidants prevail, leading to a disruption of redox signaling and/or molecular damage. The structural changes in biomolecules caused by reactive oxygen species (ROS) can alter cellular function and physiological processes, playing an important role in a range of common diseases and degenerative conditions [13]. Several reproduction steps can be affected by ROS, including ovarian steroidogenesis, oocyte maturation, blastocyst formation, implantation, luteolysis, and luteal maintenance during pregnancy [16].

The ROS and lipid peroxidation levels were increased, and the total antioxidant capacity was decreased in the FF of oocytes lacking a meiotic spindle compared with oocytes bearing a preserved meiotic spindle [17]. Furthermore, infertile women with endometriosis exhibited greater granulosa cell ROS production than control women [18]. Thus, there was a positive correlation between granulosa cell ROS production and cellular damage, as evaluated by FF thiobarbituric acid reactive substances (TBARS) [18]. The FF from oocytes that produced embryos with severe fragmentation was enriched with significantly higher nitrogen oxides (NOx) levels than from embryos with very minor fragmentation [19]. FF glutathione was positively associated with greater high-quality embryos in a study with FF obtained from a single-dominant follicle [20].

Thus, we investigated possible correlations between oxidative stress biomarkers and the hormonal levels in the FF of women undergoing the intracytoplasmic sperm injection (ICSI) procedure (with different clinical conditions), adopting a reproducible, affordable, and easy methodology to exclude contaminated samples with blood elements, to find possible markers that would allow the selection of the female gamete most suitable for insemination. Analysis of these correlations may be of clinical relevance and may help the understanding of different associations between diverse clinical aspects of infertility and the variation in hormonal levels in reproductive cycles.

Materials and Methods

Ethics statement and participants

Women undergoing ICSI treatment at Fertipraxis Clinic (Rio de Janeiro, RJ, Brazil), a human reproduction clinic, between September 2012 and February 2014 were enrolled in

the study. This center for human reproduction is certified by the Brazilian Health Regulatory Agency (ANVISA) and the Latin American Network of Assisted Reproduction (REDLARA). The study was conducted with the approval of the Research Ethics Committee of the Maternity Hospital of Federal University of Rio de Janeiro and was registered with the Brazilian platform of research under the following numbers: CAAE: 02213812.4.0000.5275 and the number of purpose: 91.930; this study was approved on September 6, 2012. All participants provided written informed consent, and the procedures were in accordance with the Declaration of Helsinki.

A total of 196 women undergoing ICSI with different infertility diagnoses were recruited to participate in the study, regardless of ethnicity, age, cause of infertility, or diagnosis of their male partner's infertility. It is important to note that ICSI is the most utilized ART in Brazil regardless of the male infertility factor; thus, ICSI was the ART technique chosen for the study.

Clinical data were obtained from medical records and included patient age, BMI, height, size of the first follicle punctured, number of follicles, oocytes retrieved, embryo quality, and pregnancy and other relevant data (Fig. 1 and Table 3). The serum hormone levels were obtained from medical records at different times as described in Fig. 1A.

Assisted reproduction procedures and FF sampling

Controlled ovarian stimulation. Controlled ovarian stimulation was performed according the clinical protocol as previously described and according to the specific clinical requirements of the patients [21]. Briefly, on the second to third day of menstruation, ovarian stimulation was initiated with synthetic follicle-stimulating hormone (FSH) alone (Gonal-F, Merck-Serono, Italy; or Bravelle, Ferring Pharmaceutical, Germany) or FSH and luteinizing hormone (LH) (Pergoveris, Merck-Serono, Italy; or Menopur, Ferring Pharmaceutical, Germany) treatments. FSH dosages varied from 150 to 300 IU/day, and LH dosages ranged from 75 to 300 IU/day. The gonadotropin-releasing hormone antagonist cetrorelix acetate

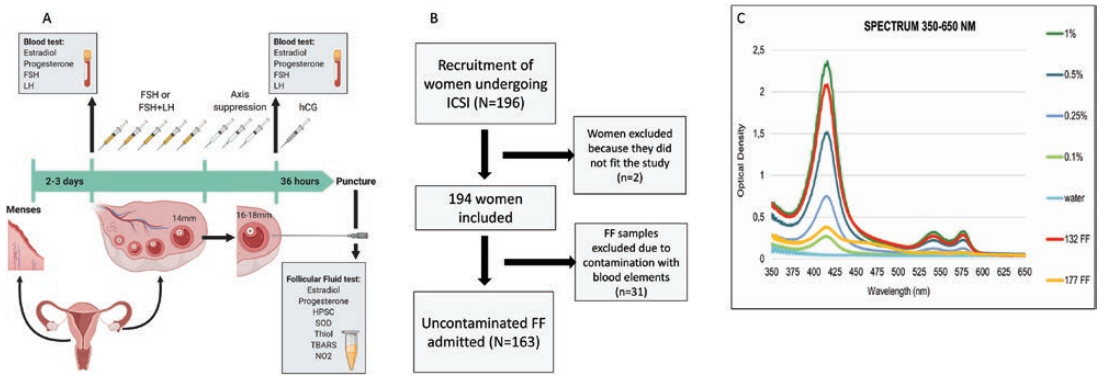

Figure 1. (A.) Schematic of the ovarian stimulation protocol. Two to 3 days after the start of menses, the ovarian stimulation protocol (FSH or FSH + LH) started based on the clinician criteria. Pituitary suppression began when the first follicle reached 14 mm in diameter. Recombinant hCG was given when at least one follicle reached 18 mm. Hormone serum levels were obtained before the ovarian stimulation and before hCG administration. Levels of estradiol, progesterone, HPSC, SOD, reduced thiol (Thiol), TBARS, and NO_2 were evaluated in the follicular fluid (FF). (B.) Flowchart demonstrating the recruitment of patients undergoing IVF/ICSI and FF sample admission or exclusion. (C.) Spectrum for blood contamination evaluation in FF samples. The spectrum 350-650 nm for blood contamination evaluation in FF samples shows different blood concentrations diluted in deionized water (1%, 0.5%, 0.25%, and 0.1%) from a blood sample. Note: 12.1 g/dL hemoglobin = 100% blood elements. Only deionized water (water); 1 FF sample without blood contamination, yellow line (177 FF); 1 FF sample with blood contamination, red line (74 FF). Abbreviations: HPSC, hydrogen peroxide scavenging capacity; IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

(Cetrotide 0.25 mg, Merck-Serono, Italy) was administered to induce hypophysis suppression whenever the first follicle was ≥14 mm.

When at least one follicle had reached 18 mm or at least 2 follicles had reached 16 mm (assessed by ultrasound), human chorionic gonadotropin (hCG) (Ovidrel 250 µg, Merck-Serono, Italy) was administered to mimic the LH peak. Thirty-five hours post-Ovidrel administration, the oocytes were retrieved, and FF was obtained during the follicular aspiration procedure.

Follicular aspiration. Briefly, follicular aspiration was undertaken using a transvaginal ultrasound probe (Medison SonoAce X8) and a 17G single lumen oocyte aspiration needle (Wallace) for ≥5 follicles with a diameter of at least 16 mm or a 17G double lumen oocyte aspiration needle (Swemed, Vitrolife) for <5 follicles with a 16 mm diameter connected to a vacuum system used for follicular aspiration; after oocyte retrieval by the embryologist, the FF was placed in a cryopreservation tube (Nunc, Thermo Scientific) and stored in a liquid nitrogen container. Approximately 4 to 6 hours after oocyte retrieval, the ICSI procedure was performed. The FF from each patient was collected following puncture of just one follicle with the largest diameter (>17 mm) to reduce blood contamination via rupture of small blood vessels during follicular aspiration (Fig. 1). The FF sample for each patient was centrifuged at 12000*g* for 15 minutes at 4°C, and the FF supernatant was sorted into aliquots for different assays to avoid possible losses associated with freezing and thawing cycles [22] and were stored at −80°C until the assay.

The exclusion criteria were as follows: smoking, positive for hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), or thyroid dysfunction.

Blood contamination evaluation

Few studies have used a clear methodology for the exclusion of blood-contaminated FF samples that can alter the biochemical profile of the specimen [23, 24]; most studies use visual inspection or do not mention the criterion used [10, 13, 14, 18-20, 25-40]. Here, the presence or absence of blood contamination was graded by visual inspection and spectrophotometric analysis. Although visual analysis can determine which FF samples need to be discarded due to a high degree of blood contamination [41], this methodology is subjective. Spectrophotometric analysis was performed to detect the presence of hemoglobin in FF samples (Fig. 1C). However, there is no consensus or definition on the acceptable limit of contamination with blood elements in the FF sample with the use of this technique [8, 42]. Thus, a dilution curve with human blood samples (collected into EDTA tubes from one female healthy volunteer and measured using an automated colorimetric assay) with an initial concentration of 12.1 g/dL was performed. The oxygenated hemoglobin showed the typical maximal absorption at 415 nm and the double peak at 540 and 575 nm [43], so the absorbances of the blood and FF samples were measured in the range of 350 to 650 nm using a spectrophotometer (Spectramax Paradigm, Molecular Devices) and analyzed by Softmax Pro 6.2.1 software (Molecular Devices, Inc.). The spectrum was analyzed, and the absorbance values at 415 nm were used to determine the degree of contamination. All FF samples without culture medium contamination and with less than 0.035 g/dL of hemoglobin (less than 0.3% of blood elements), which showed a single absorbance peak of approximately 450 nm "uncontaminated fluid" (n = 163), were included for further analysis [42]. All experiments were performed in duplicate.

Determination of the hormone levels in FF

The FF 17β-estradiol levels were determined using an ImmuChem Coated Tube 17β-Estradiol iodine 125 (^{125}I) RIA Kit (MPBio, cat# 07-238105, using the antibody MP Bio 07-238110,

RRID:AB_2847903) [44], whereas the FF progesterone levels were determined using ImmuChem Double Antibody Progesterone ^{125}I RIA Kit (MPBio, cat#, 07-170102, using the antibody MP Bio 07-270110, RRID:AB_2847904) [45], according to the manufacturer's instructions. For the measurement of ^{125}I, an automatic gamma counter (PerkinElmer Wizard 1470) was used. The FF samples were diluted 1:2000 v/v for 17β-estradiol and 1:3000 v/v for progesterone assays, both diluted in steroid-free gelatin phosphate buffer (0.1 M PBS pH 7.0, containing 5% BSA, 1% gelatin, 0.1% sodium azide). The results were expressed as pg/mL or ng/mL, respectively. Sensitivity of the 17β-estradiol assay was 10 pg/mL and intra-assay variation was 5.6%. Sensitivity of the progesterone assay was 0.35 ng/mL and intra-assay variation was 4.9%. All samples were measured in the same assay. All experiments were performed in duplicate.

Determination of lipid peroxidation in FF

Malondialdehyde (MDA) [46] levels (a product of fatty acid peroxidation) were measured in FF by the thiobarbituric acid (TBA) assay [47]. Briefly, the mixture containing TBA reagent was prepared (10 µL, sodium dodecyl sulfate [SDS] 8.1%; 74 µL, trichloroacetic acid [TCA] 10%; 74 µL, TBA 0.8%, and 32 µL, distilled water) and mixed with 10 µL of each thawed FF sample. Then, the mixture was incubated in a water bath at 95°C for 1 hour and cooled on ice. TCA is used for protein precipitation, and TBA reacts with MDA to give a red compound, which can be read at 532 nm. Butanol was used to extract the red compound prior to spectrophotometer measurements (Asys UVM340, Bedfordshire, UK). The results were expressed as MDA ng/µL. Sensitivity was 9 µmol/mL. All samples were measured in the same assay. All experiments were performed in duplicate.

Determination of superoxide dismutase in FF

The superoxide dismutase (SOD) activity was assayed using a colorimetric assay kit (SOD Assay kit, Cayman Chemical Company, catalog number 706002) following the manufacturer's instructions. The absorbance was measured at 450 nm in a spectrophotometer (Asys UVM340, UK). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The results were expressed as SOD activity units per mL (U/mL). The range of the assay was from 0.005 to 0.050 U SOD/mL. All samples were measured in the same assay. Intra-assay coefficient of variation was 3.2%. All experiments were performed in duplicate.

Determination of hydrogen peroxide scavenging capacity (HPSC) in FF

The HPSC was evaluated in FF samples following adaptation of Aebi's method [48, 49]. Briefly, the FF samples were diluted (1:75) in Tris-HCL 0.1 mol/L pH 7.5 buffer. Then, 250 µL of 40 µM H_2O_2 solution was added to 250 µL of diluted sample or the catalase standard curve standards, and all samples were incubated at 37°C for 30 minutes. Then, 50 µL of samples and standards were placed on 96-well microplates in duplicate and 100 µL of a solution containing 0.4 U/mL horseradish peroxidase (HRP), Tris-HCL 0.1 mol/L pH 7.5 buffer, and Ampliflu Red (Sigma-Aldrich). In this reaction, HRP uses Ampliflu Red as an electron donor during the reduction of H_2O_2 to water, and fluorescent resorufin is formed. Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively, and it was measured with a spectrofluorometer (Victor X42030 Multilabel Reader, PerkinElmer) at 37°C. The results were expressed as arbitrary units of intensity (A.U.). The samples were measured in 3 different assays. All experiments were performed in duplicate.

Determination of nitrite in FF

The concentration of nitrite (NO_2) in FF was measured through the detection of nitric oxide (NO) in the gas phase by the chemiluminescence of this reaction with ozone, producing

energy in the form of light, which is proportional to the level of NO in the samples, using a Nitric Oxide Analyzer (NOA, Sievers 280i, General Electric). First, the calibration curve was constructed using a $NaNO_2$ solution (0.125, 0.25, 0.5, 1, and 2.5 μM) as the standard solution. The FF samples (100 μL) underwent deproteinization with 100% cold ethanol (200 μL), mixed and centrifuged at 1000*g* for 10 minutes, and the supernatant (20 μL) was injected into a glass purge vessel, as previously described [50]. The FF NO_2 concentration was determined by interpolation of the NO_2 standard curve. The data were expressed in μM. Sensitivity of the assay was 1 picomole. All samples were measured in the same assay. All experiments were performed in duplicate.

Determination of the reduced thiol levels in FF

The reduced thiol group levels in FF were measured using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, DTNB). The Ellman method for assaying thiols is based on the reactions of thiols with DTNB [51]. For each FF sample, 1 blank tube and 2 sample tubes were prepared for reading. The blank tube contained FF sample (20 μL), Tris-EDTA buffer solution pH 8.0 (130 μL), and methanol (830 μL); the sample tubes were prepared with the same reagents as the blank tube plus 10 mM DTNB (10 μL). Then, the samples were mixed, incubated at 25°C for 15 minutes, and centrifuged at 3000*g* for 15 minutes. The supernatant was used, and the absorbance was measured at 412 nm by a spectrophotometer (PerkinElmer LambdaBio+, UK). The results were expressed as μmol DTNB reduced/mL. All samples were measured in the same assay. All experiments were performed in duplicate.

Determination of the total protein in FF

The total protein concentration of the FF samples was determined by a colorimetric method using a commercial kit (Pierce BCA Protein Assay Kit, Thermo Scientific) according to the manufacturer's instructions and was measured using a spectrophotometer (Asys UVM340, Bedfordshire, UK).

Statistical analysis

Data are presented as medians, means ± standard deviations. Normal distribution was verified in all data measured with the D´Agostino-Pearson test. Spearman correlation analysis was used for correlations. The Kruskal-Wallis test was used to analyze the differences between the groups. Student's *t* test was used for normally distributed continuous variables and the Mann-Whitney U test for nonnormally distributed continuous variables. For qualitative characteristics, the chi-square test was used to determine a significant relationship between 2 nominal variables. To stratify populations with different redox status, each redox marker concentration was subjected to cluster analysis as used before [52]. Clustering using the K-Means algorithm was divided into 3 clusters: high, medium, and low, using MATLAB 7.5.0 software (MathWorks). Differences were considered to be significant if $P < 0.05$. Statistical analysis was performed using GraphPad Prism v.6 software (GraphPad Software, Inc) and SPSS v.24 software (IBM Corporation).

Results

A total of 196 FF samples of women undergoing the ICSI procedure were collected and submitted to spectrophotometry analysis for their blood levels, and this analysis resulted in 163 blood-free FF samples enrolled for further analysis (Fig. 1B). The mean patient age was 36.25 ± 4.32 years (22-48), the mean height was 1.63 ± 0.06 meters (1.46-1.82), and the mean BMI was 23.31 ± 3.82 kg/m^2 (15.60-38.06). More than 70% of the patients were 31 to 40 years of age and had a normal BMI (18.5-24.9 kg/m^2). The 3 main causes of infertility diagnosis were unexplained infertility (30.06%; n = 49), male factor (25.77%; n = 42), and

tubal factor (13.5%; n = 22). The distribution of women (n = 163) by age, BMI, and infertility diagnosis are illustrated in Fig. 2.

The FF estradiol level was positively correlated with the total FSH dose (r = 0.165; P = 0.019; n = 158, Fig. 3A), total LH dose (r = 0.173; P = 0.018; n = 149, Fig. 3B), and serum estradiol concentration on the day of hCG administration (r = 0.482; P < 0.0001; n = 117, Fig. 3C) and negatively correlated with the serum progesterone dose on the day of hCG administration (r = − 0.183; P = 0.023; n = 119, Fig. 3D). However, the FF progesterone level did not exhibit a correlation with the same parameters as observed for the FF estradiol level (Fig. 4A). The FF progesterone level was negatively correlated with the number of follicles punctured (r = −0.246; P = 0.001; n = 157, Fig. 5A), the number of oocytes retrieved (r = −0.268; P < 0.0001; n = 157, Fig. 5B), FF HPSC (r = −0.294; P < 0.0001; n = 157, Fig. 5C) and FF SOD (r = −0.237; P = 0.002; n = 152, Fig. 5D) and was positively associated with reduced thiol levels in FF (r = 0.209; P = 0.004; n = 157, Fig. 5E). No correlation was observed between the FF progesterone and NO_2 concentrations (Fig. 5F).

K-means clustering was used to detect possible differences in follicle and oocyte numbers related to oxidative stress biomarkers. The levels of progesterone and oxidative stress biomarkers were divided into 3 groups (low, medium, and high), and the numbers of follicles punctured and oocytes retrieved were plotted (Figs. 6, 7). The number of punctured follicles was higher in the high FF HPSC group than in the low group (Fig. 6A). No significant differences were observed related to SOD, reduced thiols, TBARS, and NO_2 (Fig. 6B to E). Interestingly, the number of follicles punctured was higher in the low FF progesterone concentration group than in the medium group (Fig. 6F).

The number of retrieved oocytes was higher in the high FF HPSC group than in the low and medium groups (Fig. 7A). No significant differences were observed related to SOD, reduced thiols, TBARS, or NO_2 (Fig. 7B to E). The number of oocytes was higher in the low FF progesterone concentration group than in the other groups (medium and high FF progesterone) (Fig. 7F).

As we did not follow the exact oocyte from the studied FF to the embryo transfer process, it was not our aim to evaluate pregnancy rates. However, as shown in Table 3, the women were divided into 2 groups according to whether they were pregnant (those who became pregnant after embryo fresh transfer) or nonpregnant (those who did not become pregnant after embryo fresh transfer). In our study, 127 women had the embryo transferred in the same cycle of the follicular puncture (41 women became pregnant and 86 women did not become pregnant. Pregnant women were associated with significantly higher FF estradiol levels than nonpregnant women (P < 0.05). Embryo transfer occurred on days 3 or 5. Biochemical pregnancy was ascertained though positive β-hCG

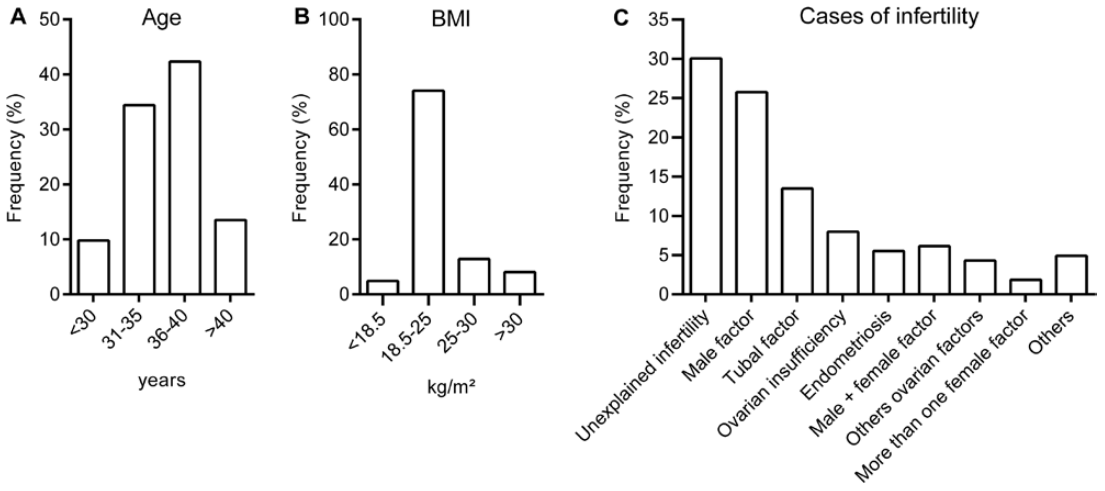

Figure 2. Main demographic parameters of the women enrolled in the study. (A.) Age (years); (B.) body mass index (BMI, kg/m^2); and (C.) infertility diagnosis.

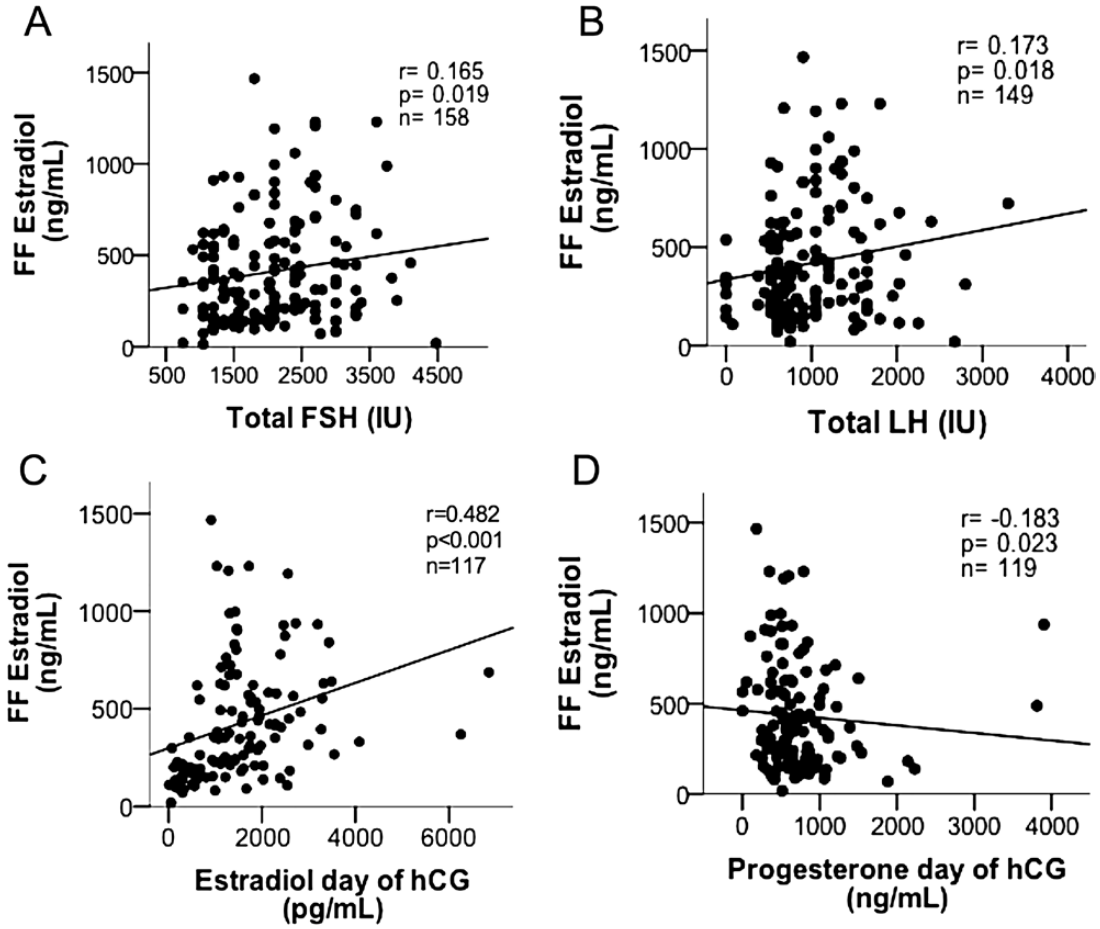

Figure 3. Correlations between the estradiol level in FF with (A.) total follicle-stimulating hormone (FSH) used during the ovulation induction cycle; (B.) total luteinizing hormone (LH) used during the ovulation induction cycle; (C.) serum estradiol on the day of hCG administration; and (D.) serum progesterone on the day of hCG administration. Abbreviations: FF, follicular fluid; hCG, human chorionic gonadotropin; n, number of samples; p, *P* value; r, Spearman's rho.

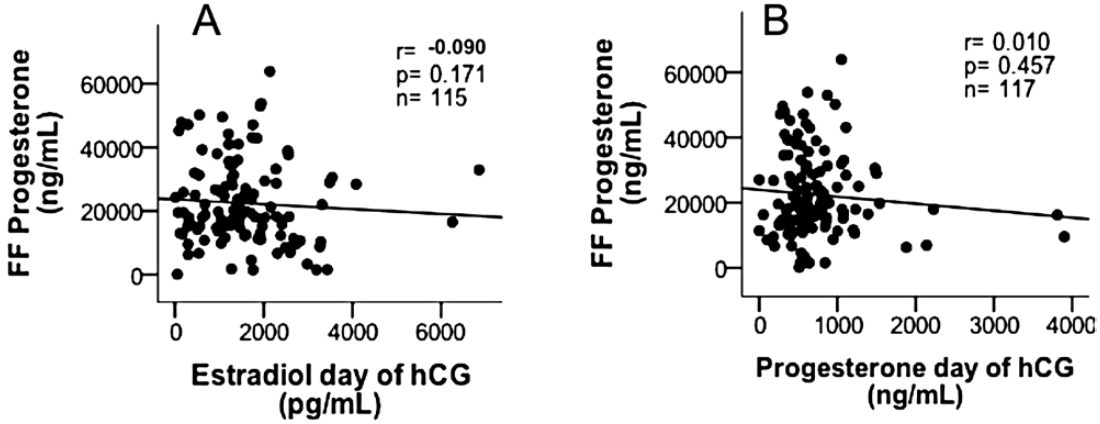

Figure 4. Correlations between the progesterone level in FF with (A.) serum estradiol on the day of hCG administration and (B.) serum progesterone on the day of hCG administration. Abbreviations: FF, follicular fluid; hCG, human chorionic gonadotropin; n, number of samples; p, *P* value; r, Spearman's rho.

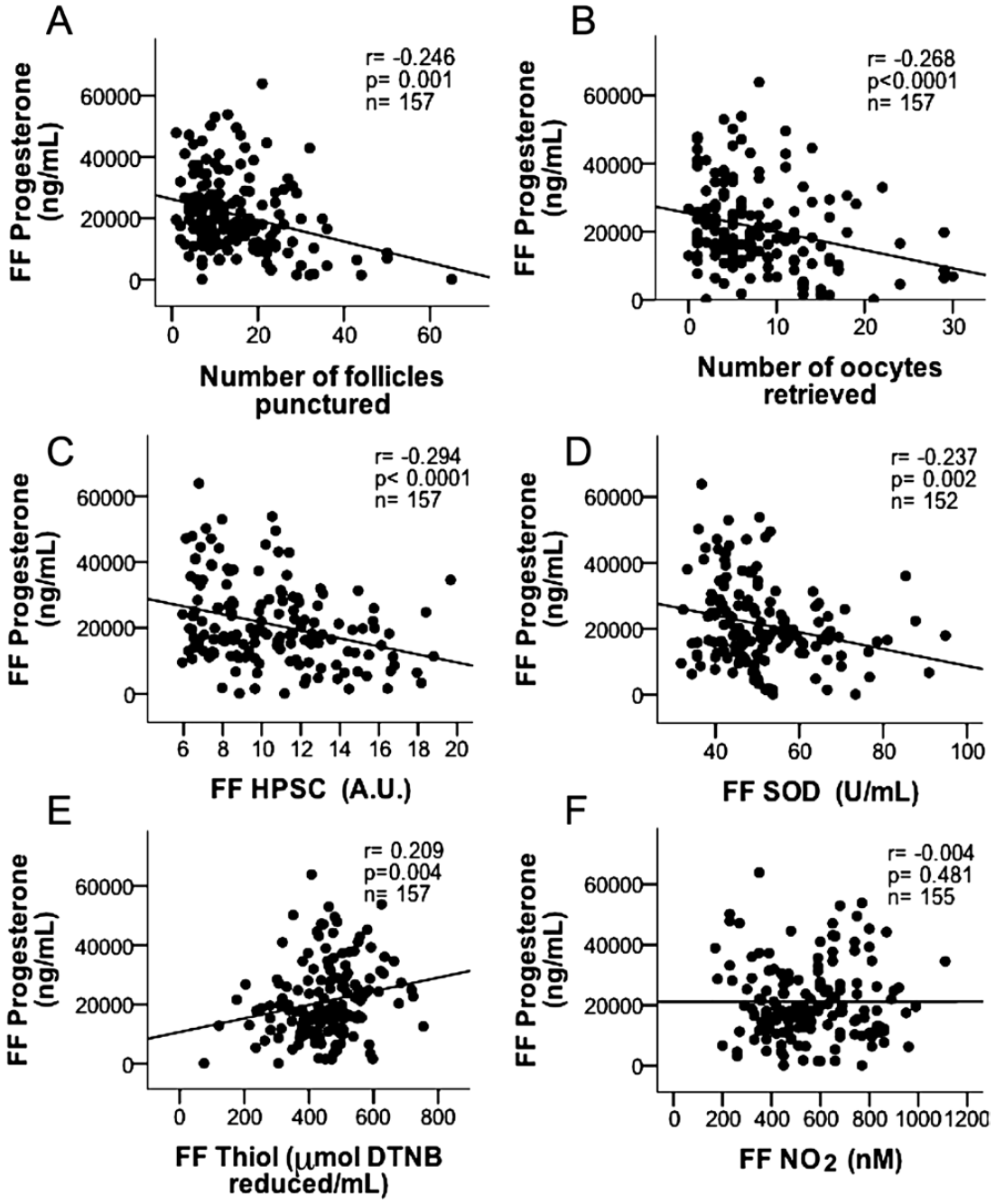

Figure 5. Correlations between the progesterone level in FF with (A.) number of follicles punctured; (B.) number of oocytes retrieved; (C.) hydrogen peroxide scavenging capacity (FF HPSC); (D.) superoxide dismutase (FF SOD); (E.) reduced thiol (FF thiol); and (F.) FF NO_2. Abbreviations: FF, follicular fluid; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; n, number of samples; p, P value; r, Spearman's rho.

15 days after embryo transfer, and clinical pregnancy was confirmed by ultrasound at 6 to 7 weeks of gestation.

Data concerning all correlations of oxidative stress markers, antioxidant proteins, and hormone levels in FF versus clinical data (i.e., age, BMI, serum hormones on day of oocyte retrieval, numbers of follicles and oocytes retrieved) are presented in Tables 1, 2.

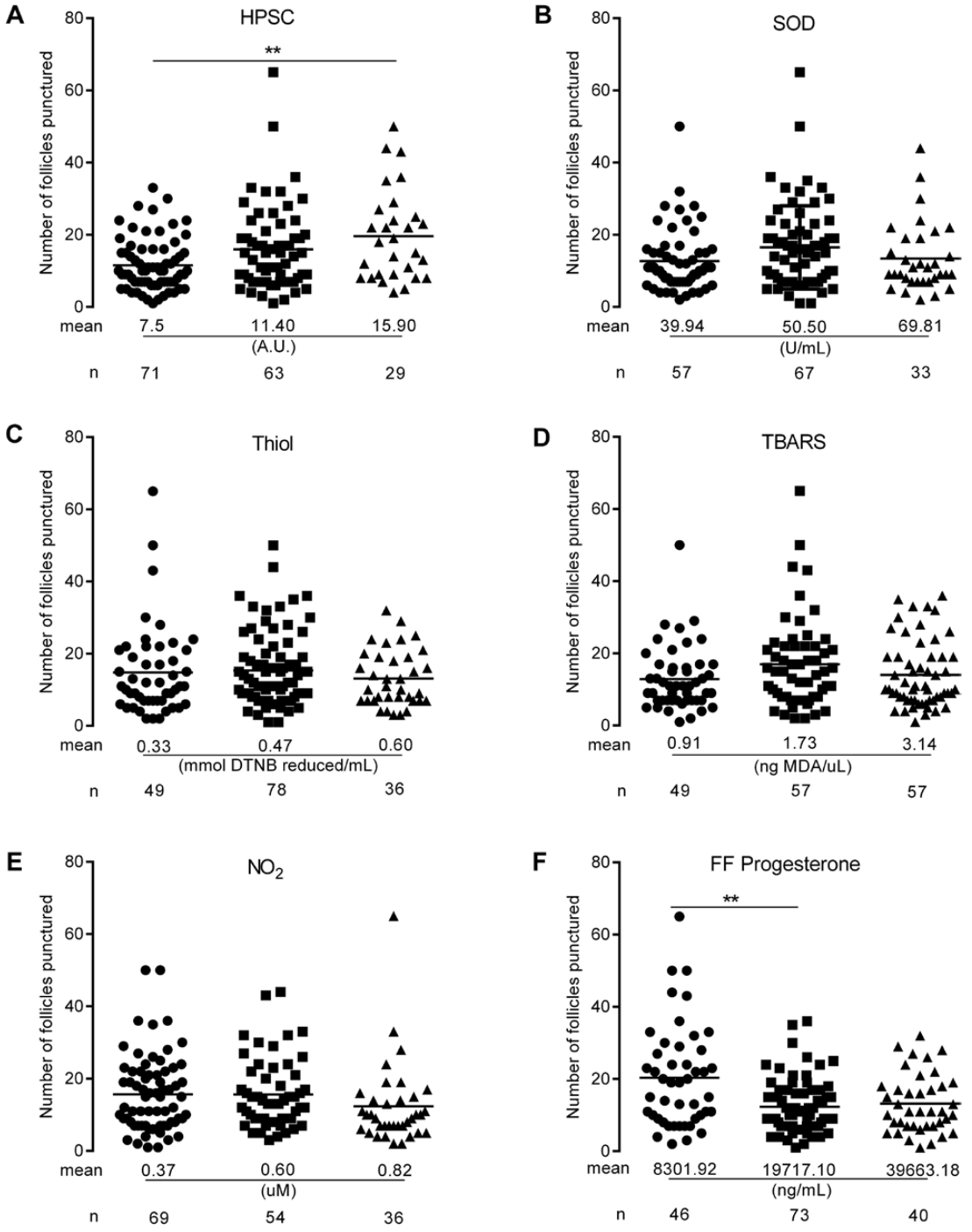

Figure 6. Number of total follicles punctured after ovarian stimulation. (A.) Hydrogen peroxide scavenging capacity (HPSC); (B.) superoxide dismutase (SOD); (C.) thiol; (D.) thiobarbituric acid reactive substances (TBARS); (E.) NO_2; and (F.) Follicular fluid (FF) progesterone. Oxidative stress biomarkers and intrafollicular steroid hormones were stratified into 3 groups: low (●), medium (■), and high (▲). ** $P < 0.01$. TBARS results are expressed as ng malondialdehyde (MDA)/μL.

Discussion

Instead of collecting the FF of different follicles, we aimed to focus on the analysis of the largest follicle and its correlation of oxidative stress biomarkers with hormonal parameters.

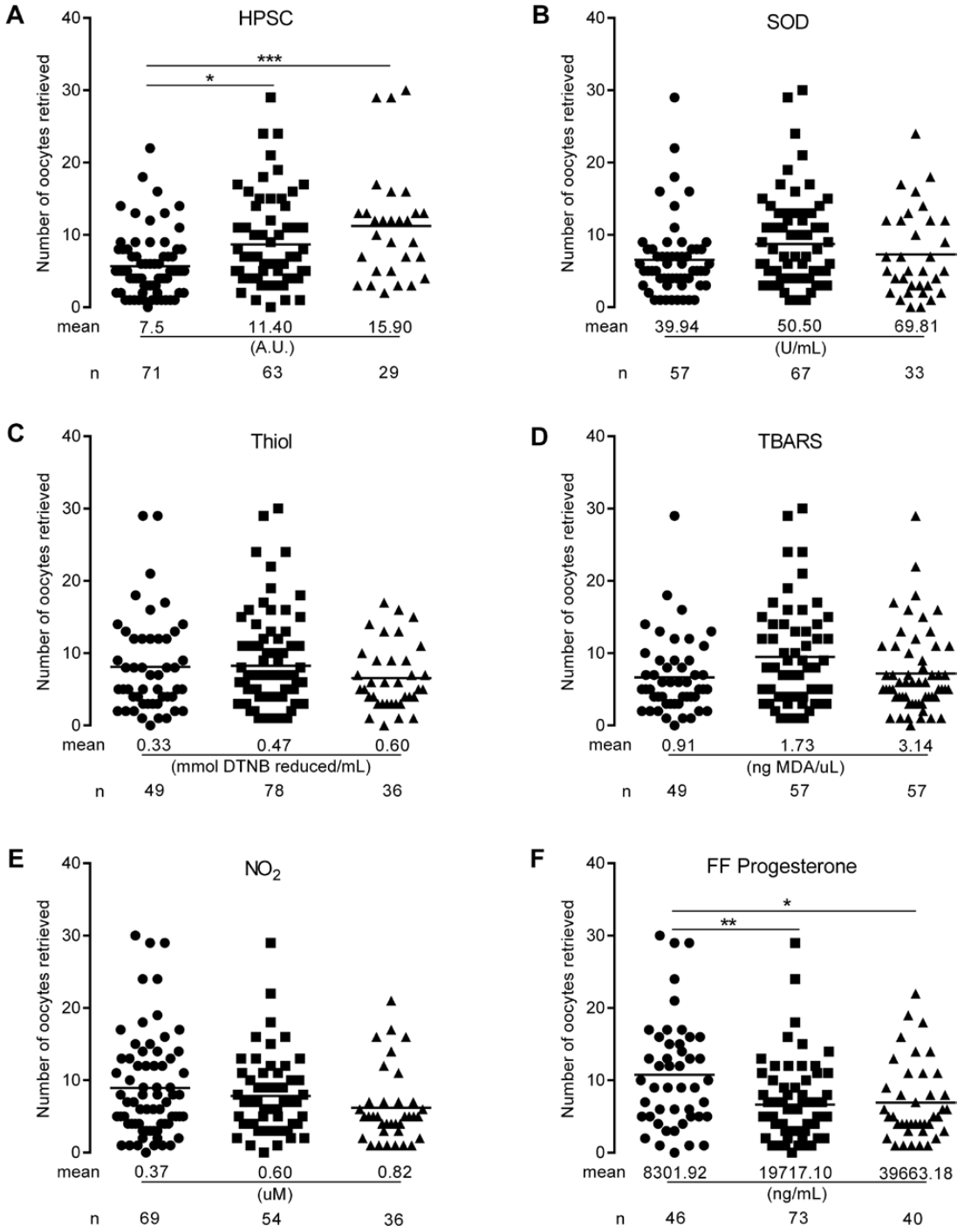

Figure 7. Number of total oocytes retrieved after ovarian stimulation. (A.) Hydrogen peroxide scavenging capacity (HPSC); (B.) superoxide dismutase (SOD); (C.) thiol; (D.) thiobarbituric acid reactive substances (TBARS); (E.) NO_2; and (F.) Follicular fluid (FF) progesterone. Oxidative stress biomarkers and intrafollicular steroid hormones were stratified into 3 groups: low (●), medium (■) and high (▲). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. TBARS results are expressed as ng malondialdehyde (MDA)/μL.

This choice was based on the fact that the larger follicle would have the greater probability to achieve dominance and would be ovulated (normal reproduction process) or be chosen to follow the fertilization procedure. In addition, normally, the largest follicle has an increased capacity to produce hormones and compared with the physiology of smaller follicles, the

Table 1. Correlations Detected Between Hormone Levels and Clinical Data

Spearman's correlation		Age	BMI	Total FSH	Total LH	FSH day of hCG	LH day of hCG	E2 day of hCG	P4 day of hCG	Follicles punctured	Oocytes retrieved
Age		1.000	0.067	**0.194**	**0.229**	**0.253**	0.126	**-0.285**	-0.063	**-0.360**	**-0.280**
BMI			1.000	0.058	0.108	**-0.340**	0.071	-0.054	-0.069	-0.046	0.096
Total FSH				1.000	**0.725**	**0.531**	-0.034	0.041	0.021	**-0.145**	-0.118
Total LH					1.000	**0.394**	-0.059	0.045	-0.126	**-0.212**	**-0.190**
Serum FSH	On the day					1.000	0.112	-0.012	**0.158**	**-0.256**	-0.146
Serum LH	of hCG						1.000	-0.141	0.019	**-0.288**	**-0.239**
Serum E2								1.000	**0.218**	**0.652**	**0.609**
Serum P4									1.000	**0.327**	**0.349**
Follicles punctured										1.000	**0.894**
Oocytes retrieved											1.000

Bold values indicate statistically significant relationships. N = 114-163.
Abbreviations: BMI, body mass index; E2, estradiol; FF, follicular fluid; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; P4, progesterone.

Table 2. Correlations Detected Between Oxidative Stress Biomarkers in Follicular Fluid and Hormone Levels

Spearman's correlation	E2 day of hCG	P4 day of hCG	Follicles punctured	Oocytes retrieved	FF HPSC	FF SOD	FF Thiol	FF TBARS	FF NO2	FF E2	FF P4
Serum E2 on the day of hCG	1.000	**0.218**	**0.652**	**0.609**	**0.165**	-0.034	0.079	0.030	-0.103	**0.482**	-0.090
Serum P4 on the day of hCG		1.000	**0.327**	**0.349**	-0.077	**-0.172**	0.070	-0.114	**-0.173**	**-0.183**	0.010
Follicles punctured			1.000	**0.894**	**0.220**	0.096	-0.015	-0.010	**-0.153**	0.009	**-0.246**
Oocytes retrieved				1.000	**0.268**	0.099	-0.050	-0.019	**-0.151**	-0.025	**-0.268**
FF HPSC					1.000	**0.347**	0.128	0.066	-0.123	-0.027	**-0.294**
FF SOD						1.000	-0.105	-0.050	-0.127	0.002	**-0.237**
FF Thiol							1.000	0.019	0.024	0.066	**0.209**
FF TBARS								1.000	0.053	0.005	0.050
FF NO_2									1.000	0.029	-0.004
FF E2										1.000	0.024
FF P4											1.000

Bold values indicate statistically significant relationships. N = 114-163.
Abbreviations: E2, estradiol; FF, follicular fluid; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; HPSC, hydrogen peroxide scavenging capacity; LH, luteinizing hormone; P4, progesterone; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

Table 3. Characteristics of Women Who Achieved Pregnancy (n = 41) Compared With Nonpregnant Women (n = 86)

	Pregnant		Nonpregnant		
	Mean ± SD or %	n	Mean ± SD or %	n	*P* value
Age (years)	35.7 ± 2.9	41	36.4 ± 4.8	86	0.33
BMI (kg/m^2)	23.9 ± 4.3	39	23.2 ± 3.9	82	0.31
Infertility					0.56#
Primary (%)	65.8	27	70.9	61	-
Secondary (%)	34.2	14	29.1	25	-
Causes of infertility					0.22#
Unexplained (%)	34.1	14	30.2	26	-
Male factor (%)	29.3	12	24.4	21	-
Tubal factor (%)	17.1	7	14.0	12	-
Ovarian insuficiency (%)	-	0	8.1	7	-
Endometriosis (%)	4.9	2	5.8	5	-
Male+female factor (%)	9.7	4	2.3	2	-
Other ovarian factors (%)	-	0	4.7	4	-
More than one female factor (%)	-	0	3.5	3	-
Others(%)	4.9	2	7.0	6	-
Doses of gonadotropin (FSH+LH)	3105 ± 1354	41	3042 ± 1268	85	0.89
FSH (IU)	2130 ± 803	41	2083 ± 811	85	0.86
LH (IU)	975 ± 642	41	960 ± 598	85	0.98
Days of stimulation	10.1 ± 1.8	41	9.7 ± 1.69	83	0.42
Serum estradiol on day of hCG (pg/mL)	1772 ± 1129	35	1387 ± 839	61	0.09
Serum progesterone on day of hCG (ng/mL)	633 ± 231	37	766 ± 661	61	0.76
Total number of follicles	15.3 ± 8.2	41	13.4 ± 8.1	86	0.18
Total number of oocytes	8 ± 4.7	41	7.3 ± 5.0	86	0.35
Embryo quality (day 3)					0.19#
Grades 1 and 2	68.75%	11	64.28%	18	
Grades 3 and 4	25%	4	10.72%	3	
UFADEB	6.25%	1	25%	7	
Number of embryos transferred	2.1 ± 0.5	41	2.0 ± 0.7	82	0.53
Fertilization rate	78.4 ± 19.7	41	80.5 ± 20.6	85	0.51
FF HPSC (A.U.)	10.7 ± 3.6	41	10.5 ± 3.2	86	0.88
FF SOD (U/mL)	48.4 ± 10.9	39	51.1 ± 11.9	84	0.18
FF Thiol (mmol DTNB reduced/mL)	0.48 ± 0.09	41	0.46 ± 0.12	86	0.35
FF TBARS (ng MDA/μL)	1.80 ± 0.99	39	1.95 ± 0.88	85	0.28
FF NO_2 (μM)	0.54 ± 0.19	40	0.57 ± 0.17	83	0.37##
FF estradiol (pg/mL)	$50 \times 10^4 \pm 32 \times 10^4$	41	$37 \times 10^4 \pm 24 \times 10^4$	82	**0.03***
FF progesterone (ng/mL)	$2.4 \times 10^4 \pm 1.2 \times 10^4$	40	$2.1 \times 10^4 \pm 1.2 \times 10^4$	83	0.10

Mann-Whitney U test was used in analyses unless otherwise indicated.
Grade 1 = equally sized blastomeres, no fragmentation; grade 2 = equally or unequally sized blastomeres with <20% fragmentation; grade 3 = equally or unequally sized blastomeres with 20%-50% fragmentation; and grade 4 = equally or unequally sized blastomeres with >50% fragmentation.
Abbreviations: BMI, body mass index; FF, follicular fluid; HPSC, hydrogen peroxide scavenging capacity; MDA, malondialdehyde; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; UFADEB, unfertilized, anomalous, or degenerate embryo.
#Chi-square test (#)
##Unpaired t-test (##)
*Significant difference ($P < 0.05$)

physiology of the largest follicle would best correspond to the environment of the ovulated oocyte [40]. The choice of the first follicle is preferable to the pool of different follicles since we were able to individualize the microenvironment of each follicle.

In our study, we were able to show that high levels of FF HPSC were positively associated with the highest total number of follicles (Fig. 6A) and oocytes punctured (Fig. 7A),

which suggests a possible protective effect of estradiol and its relationship with a successful outcome. Additionally, the serum estradiol concentration was positively correlated with intrafollicular HPSC (Tables 1 and 2). It has been shown that estradiol is able to protect granulosa cells from oxidative stress-induced apoptosis [53, 54]. As HPSC is directly related to the levels of small antioxidants molecules in FF, our results suggest that estradiol stimulates follicular cells to release these molecules to the extracellular milieu. Since ovaries are the main site of estrogen synthesis in premenopausal women [55], it is plausible that serum estradiol levels positively correlate with the levels of this hormone in FF. As expected, we found a positive correlation between the serum estradiol concentration on the day of hCG administration and the intrafollicular estradiol level (Fig. 3C), suggesting that an increase in serum estradiol is accompanied by intrafollicular production of the same hormone, as observed by others [56, 57].

We also found a higher concentration of estradiol in the FF of women who became pregnant following ART than in the FF of women for whom the ART treatments were not successful (Table 3). In agreement with our observations, women who became pregnant on in vitro fertilization (IVF) cycles presented with higher levels of estradiol in the FF than those who did not become pregnant [58-61]. However, in a recent study in which the authors evaluated the steroid levels by LC-MS/MS, they did not find any correlation between the FF estradiol levels and pregnancy [40]. We did not observe differences between pregnant and nonpregnant women in relation to serum estradiol concentrations on the day of hCG administration (Table 3), which is in agreement with most studies [10, 62-65], except one [66].

We also found a positive correlation between the FF estradiol levels and the total FSH dose or total LH dose used during an ovulation induction cycle (Fig. 3A and 3B). LH and FSH are both important for estradiol production since FSH induces the enzymatic machinery necessary for estradiol synthesis, and estradiol is not produced until LH stimulates the thecal cells to secrete androgen, which can be converted into estradiol by granulosa cells aromatase [67].

Sawada and Carlson (1966) demonstrated that superoxide ($O_2^{\bullet -}$) is involved in progesterone release once insertion of SOD into the corpus luteum of rats treated with physiological doses of LH inhibited progesterone secretion [68]. Moreover, plasma progesterone levels were significantly decreased in SOD1-deficient female mice, but plasma estradiol levels remained unaltered. It is important to highlight that SOD1 deficiency increases $O_2^{\bullet -}$ availability, which was also observed in the ovary [69]. Interestingly, in rat corpus luteum cells treated with low doses of LH, the insertion of SOD or catalase dose-dependently inhibited progesterone secretion. However, at high doses of LH, the electroporation of SOD or catalase produced the opposite response, with an increase in progesterone secretion [68]. Thus, progesterone secretion is likely to depend on the level of $O_2^{\bullet -}$ produced since a moderate elevation of $O_2^{\bullet -}$ level is associated with an increase in its secretion, whereas higher levels of $O_2^{\bullet -}$ appear to decrease or inhibit progesterone [68, 70]. Thus, the negative correlation between serum or FF progesterone levels and SOD activity found in the present study reinforce the possible involvement of superoxide on progesterone secretion since lower SOD activity is related to higher levels of superoxide.

Reduced thiol groups are present in many biological molecules, including glutathione. In its reduced form, glutathione is the main nonprotein thiol present in animal cells and protects them by removing reactive metabolites by their conjugation with reactive forms, such as H_2O_2 [71]. HPSCs reflect the activity of small molecules that have the capacity to detoxify H_2O_2. We found a negative correlation between HPSC and progesterone in FF and a positive correlation between the progesterone and thiol levels in FF (Fig. 5C and 5E). The correlation between progesterone and reduced thiol groups suggests that progesterone production is decreased by ROS, since higher levels of reduced thiol groups reflects a lower ROS availability. Moreover, it was demonstrated that high concentrations of H_2O_2 inhibit progesterone production in the culture of luteinized granulosa cells High concentrations of H_2O_2 inhibit progesterone production in the culture of luteinized granulosa cells [72, 73]. We believe that the levels of reduced thiol groups present in FF, together with the SOD and

HPSC levels, create a protective effect against reactive metabolites and are involved in the progesterone production/inhibition process since it can detoxify H_2O_2.

When analyzing the number of follicles and oocytes punctured, we observed a negative association with the FF progesterone levels (Figs 5A, 5B, 6F, and 7F). Interestingly, the serum progesterone levels on the day of hCG were positively correlated with the total number of follicles and oocytes punctured (Tables 1 and 2).

As with all studies, ours has limitations. First, we did not assess nutrition, physical activity, or psychological status at admission; second, we did not discriminate the analyses for infertility diagnosis or age, due to the low number of cases. One of the strengths of our study was the use of FF samples with very low levels of blood contamination. Certainly, the use of equipment with increased sensitivity in the detection of blood molecules would allow more accurate results [74]. However, such methods use expensive equipment and require well-trained professionals; therefore, these methods are not fast or inexpensive. It is important to say that a clear and reproducible method needs to be used to discriminate blood-contaminated FF samples to allow for a better comparison of information between different studies. In this context, our study described a rapid, inexpensive, and easily reproducible method to exclude contaminated samples. Regardless of the method used, the most important objective is to define the maximum acceptable amount of blood elements and/or blood present in the sample for a better comparison between different studies.

In conclusion, the intrafollicular production of estradiol and progesterone are linked to the redox status of the follicular microenvironment, which reflects the number of oocytes obtained during the ICSI procedure. In fact, there must be adequate levels of hormones, oxidant molecules and antioxidants, and other factors within the follicle that result in a good quality oocyte. In a dynamic environment with hormonal variations throughout the day, understanding the role of each substance will help us to propose new strategies to achieve success in the treatment of infertile couples. Thus, additional studies are required to better understand the follicular microenvironment, contributing to improvement in the ICSI procedure and decreased costs for patients with infertility.

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Data Availability: Restrictions apply to the availability of data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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