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ARTICLE

Oxidative stress in follicular fluid of young women with low response compared with fertile oocyte donors

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Abstract The aim of this study was to determine the concentrations of oxidative stress markers, antioxidant enzymes and cytokines in the follicular fluid of young women with low response in ovarian stimulation cycles compared with high responders and fertile oocyte donors of the same age, to assess the impact of oxidative stress on ovarian reserve. The activity of follicular fluid antioxidant enzymes glutathione transferase, glutathione reductase and glutathione peroxidase was significantly lower in young women with reduced ovarian reserve compared with that in high responders and oocyte donors. Follicular fluid concentrations of oxidative stress marker malondialdehyde combined with 4-hydroxyalkenals and nitric oxide were higher in low responders than in high responders and oocyte donors. Significant differences between low responders and donors in concentrations of IL-2, IL-6, IL-8 and vascular endothelial growth factor were observed, with higher concentrations in low responders. However, IL-10 concentration was lower in low responders than in high responders and donors. No significant differences were found in follicular fluid concentrations of tumour necrosis factor alpha between the three groups. These results demonstrate that different concentrations of oxidative stress markers, oxidant enzymes and cytokines in low responders compared with high responders and oocyte donors may negatively impact ovarian response.

KEYWORDS: follicular fluid, low ovarian reserve, oxidative stress

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Introduction

IVF is one of the most common assisted reproduction techniques. This method is a widely accepted infertility treatment and often remains the only chance for infertile couples of having a baby. Unfortunately, the success of this technique, measured as an average pregnancy rate per cycle, is only 30–40% (Das et al., 2006; Gerris et al., 1999). Remarkably, reduced ovarian reserve (follicle number remaining in the ovarian pool), irrespective of age, is also associated with a decrease in pregnancy rate (Moolenaar et al., 2013).

Among the many reasons for the IVF failure, oxidative stress seems to be an important factor (Sikka, 2004). In the female reproductive system, oxidative stress and antioxidants perform physiological roles during folliculogenesis and oocyte maturation and may also be related to conditions that limit the success of assisted reproductive techniques (Pacella et al., 2012). Although there is also some evidence for the role of reactive oxygen species (ROS) in the pathophysiology of infertility and assisted fertility (Agarwal and Allamaneni, 2004; Gupta et al., 2009), the existing data are conflicting and the effect of oxidative stress on the outcome of IVF is not clear (Fujimoto et al., 2011; Jozwik et al., 1999).

Several studies have focused on the micro-environment surrounding the oocyte and on ROS and antioxidants found in the follicular fluid (Agarwal et al., 2012; Revelli et al., 2009). Follicular fluid creates the micro-environment for the developing oocyte and has a direct impact on oocyte quality, implantation and early embryo development. An imbalance in ROS production in ovarian follicular fluid may have an adverse effect on the above processes. Otherwise, oocyte growth and maturation appear to be affected by nutritional imbalances, hormonal disturbances and physical conditions of the micro-environment, such as oxidative stress (Borowiecka et al., 2012).

Sufficient antioxidant capacity of follicular fluid during oocyte retrieval has also been associated with characteristics of IVF success (Aydin et al., 2013a, 2013b; Younis et al., 2012); however, studies analysing the relationship between the level of oxidative stress in the follicular fluid and reduced ovarian reserve/response in women undergoing gonadotrophin stimulation are lacking. Blood collected from women undergoing ovarian stimulation for IVF or intrauterine insemination (IUI) allow a unique opportunity to investigate associations between different oxidative stress metabolites and various events in the reproductive process (Younis et al., 2014). Thus far, little is known about how alteration of the follicular environment as a result of oxidative stress leads to a reduced ovarian reserve.

The objective of this study was to determine the concentrations of oxidative stress markers, antioxidant enzymes and cytokines in the follicular fluid of young women with low response in ovarian stimulation cycles compared with fertile oocyte donors and high responder patients of the same age, to assess the oxidative stress impact on ovarian reserve (Núñez-Calonge et al., 2014).

To our knowledge, no study in the literature has examined the correlation between follicular fluid total oxidant and antioxidant concentrations and low response to gonadotrophin stimulation in young women undergoing assisted reproductive treatment.

Materials and methods

Study participants and ovarian stimulation protocols

This prospective clinical study was conducted at Clinica Tambre during the period of November 2013 to June 2015, was approved by the Ethical Review Board of the Hospital de la Princesa (Madrid, Spain) on 27 September 2012 (reference PI-648), and included 30 healthy fertile oocyte donors, eight women with high response (>10 oocytes retrieved) to ovarian stimulation and 22 women with low response (<5 oocytes retrieved) in at least one previous ovarian stimulation cycle.

Patients were recruited according to the following criteria: (i) absence of any apparent abnormality of the reproductive system, as revealed by their medical history, clinical examinations and common hormonal tests; (ii) absence of any metabolic or endocrine system-associated diseases, such as hyperprolactinaemia, thyroid dysfunction or polycystic ovary syndrome as defined by the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004); (iii) absence of any surgical history regarding the reproductive system; (iv) normal ovulatory cycle, with cycle lengths between 25 and 35 days; (v) age of women between 28 and 35 years; (vi) body mass index (BMI) between 19 and 26 kg/m^2; and (vii) absence of any smoking history in women. Patients gave their written informed consent and did not receive any monetary compensation for participating in the study (Aydin et al., 2013a, 2013b).

Women were included as donors after being thoroughly informed about oocyte donation and if they fulfilled the criteria to be admitted into the oocyte donation programme. In short, oocyte donors were 18–35 years old, with a complete medical history, which included the absence of current or past exposure to radiation or hazardous chemical substances, drug abuse and past reproductive history. All had a normal physical and gynaecological examination, BMI between 19 and 26 kg/m^2, no family history of hereditary or chromosomal diseases, normal karyotype and negative screening for sexually transmitted diseases (STD) (Garcia-Velasco et al., 2003).

Donors gave their written informed consent and did not receive any monetary compensation for participating in the study. Neither the donor nor the patients took any drugs, antioxidant-vitamin supplements, or any medication that might affect the results of the present study.

Patients (low and high responders) received the long gonadotrophin-releasing hormone agonist (GnRHa) protocol for ovarian stimulation. To achieve pituitary desensitization, hormone agonist triptorelin acetate (Decapeptyl, Ipsen Pharma, Barcelona, Spain) was initiated on day 21 of the previous cycle at a dose of 0.1 mg (Mercé et al., 2007). As soon as menstruation began, ovarian stimulation was initiated with 225–300 U/day rec-FSH (Gonal-F®; Merck Serono, Madrid, Spain). Recombinant human chorionic gonadotrophin (HCG) (Ovitrelle®, Merck Serono, Madrid,Spain) was applied when ≥2 follicles reached ≥17 mm; and oocyte retrieval was performed under sedation at the 36th hour following HCG. Donors received the antagonist protocol for ovarian stimulation. The ovarian stimulation began with 125–225 IU of recombinant FSH (Gonal-F®; Merck Serono, Madrid, Spain) from day 2 of the

menstrual cycle, and the GnRH antagonist (Cetrotide; Serono, Madrid, Spain) was introduced according to a multiple-dose protocol (0.25 mg/day) when a leading follicle of 14 mm and/or oestradiol 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, Barcelona, Spain) and oocyte retrieval was performed under sedation at the 36th hour following GnRHa. In all groups, the first control (ultrasonography and serum oestradiol) was performed after 5 days of stimulation, and the daily dose of FSH was adjusted individually according to the ovarian response.

Fertilization and embryo transfer

Intracytoplasmic sperm injection (ICSI) was used to fertilize the oocytes 4–6 h after oocyte retrieval. All ICSI cycles were performed according to routine procedures (Van Steirteghem et al., 1993) using selected spermatozoa from a swim-up sperm retrieval using polyvinylpyrrolidone medium for partial sperm immobilization (Origio, Denmark). Pronuclear scoring was done 16 to 18 h after sperm injection. The fertilization rate was calculated as the percentage of metaphase II oocytes with two pronuclei (Nuñez-Calonge et al., 2012). Embryo quality was assessed before every embryo transfer, and a maximum of two embryos were transferred to all patients 72 h after micro-injection.

Embryo transfer was performed 72 h after oocyte retrieval using the Wallace catheter (The Edwards-Wallace Embryo Replacement Catheter, Sims, Portex Ltd, Kent, UK). Pregnancy was defined as a spontaneous rise in a β CG concentration at least 10 days after transfer. Clinical pregnancy implied the presence of an intrauterine gestational sac and fetal heart beat on an ultrasound performed at 7 weeks' gestation (Nuñez-Calonge et al., 2012).

Follicular fluid collection and processing

Oocytes were separated and placed into culture media, whereas follicular fluid was collected in flasks (Jozwik et al., 1999). Considerable care was taken to pool follicular fluid uncontaminated with flush medium or blood. The presence or absence of blood contamination was graded by visual inspection, and samples that looked cloudy or bloodstained were discarded (Berker et al., 2009). Only uncontaminated follicular fluid minimally stained with blood were kept for further determinations. The samples without oocytes or contaminated samples were discarded (Kazemi et al., 2013).

At oocyte retrieval, and after removing the oocytes, follicular aspirates were centrifuged at 600*g* for 10 min and the supernatant stored at −70°C for a maximum of 2 weeks, until assessed for:

- Antioxidant enzymes: glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST).
- Oxidative stress markers: malondialdehyde (MDA) and nitric oxide metabolites (NOx) and cytokines as inflammatory markers: interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), tumour necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF).

Enzymatic antioxidant defences

GPx, GR and GST activities were measured in follicular fluid and analysed spectrophotometrically according to the manufacturer's instructions (Cayman Chemical; Ann Arbor, MI, USA) (Park et al., 2011). GST activity was measured spectrophotometrically by measuring formation of the conjugate of reduced glutathione (GSH) and 1-chloro-2.4-dinitrobenzene (CDNB) at 340 nm. Briefly, 10 μl of follicular fluid sample was transferred into a 190 μl incubation mixture consisting of 10 μl GSH, 180 μl CDNB in potassium phosphate buffer (pH = 6.5). The product formation was continuously measured for 5 min. GPx activity was measured spectrophotometrically, in which GPx activity was coupled to the oxidation of NADPH by GR. Each 770 μl reaction mixture consisted of 350 μl buffer (pH = 7.6), 350 μl NADH reagent containing β-nicotinamide-adenine dinucleotide phosphate, GSH, GR and 70 μl samples. The reaction was initiated by an addition of 350 μl of tert-Butyl hydroperoxide. The decrease in optical density at 340 nm due to the oxidation of NADPH was monitored for 3 min in this coupled assay at +25°C. The units of enzymatic activity were calculated using an extinction coefficient of 6220 mmol/cm for NADPH (Kireev et al., 2007a, 2007b).

GR assay kit measures activity of this enzyme by measuring the rate of NADPH oxidation. The reaction mixture contained 0.1 mol/l potassium phosphate buffer pH 7.4, 1 mmol/l glutathione disulfide (GSSG), and 0.16 mmol/l NADPH and appropriate amounts of follicular samples. The rate of NADPH oxidation was monitored at 25°C with a spectrophotometer, after the decrease of absorbance at 340 nm (extinction coefficient 340 nm = 6.22 mmol/l^{-1} cm^{-1}). The blank did not contain GSSG and the activity was calculated after subtraction of the blank value. Reaction rate proportionality was assessed by using different sample aliquots for the activity measurements. Enzyme activities were normalized according to follicular fluid protein content (Kireev et al., 2010).

Oxidative stress markers

Lipid peroxidation assay

The concentrations of MDA in combination with 4-hydroxyalkenals (4-HAE) were analysed using a commercial kit (Oxford Biomedical Research, MI, USA) according to the manufacturer's instructions.

In brief, an aliquot (70 μl) of the sample was added to a reaction mixture containing 227.5 μl of the chromogenic reagent N-methyl-2-phenylindole in acetonitrile, which reacts with MDA and 4-HAE. Afterwards, 25.5 μl of methanesulfonic acid were added (Kireev et al., 2013).

Samples were incubated at 45°C for 60 min, and then centrifuged at 15,000*g* for 10 min. The absorbance of the supernatant was measured by microplate reader (Dynex Technologies) at 586 nm.

NOx determination

NOx concentration was measured by the Griess reaction as NO_{2-} concentration after NO_3 reduction to NO_{2-}. Briefly, after incubation of the follicular fluid with *Escherichia coli* NO_3 reductase and NADPH+ (37°C, 30 min), 1 ml of Griess reagent (0.5% naphthylenediaminedihydrochloride, 5% sulphonilamide,

Table 1 Patients characteristics in the low responder (<5 oocytes), high responder and control groups.

Variable	*Low responder patients*	*High responder patients*	*Control (donors)*	*P-value*
Age (years)	30.6 ± 3.1	31.1 ± 2.4	29.7 ± 4.1	NS
BMI (kg/m^2)	22.7 ± 3.2	23.5 ± 2.1	22.6 ± 2.8	NS
AMH (ng/ml)	5.85 ± 1.3	6.34 ± 1.2	6.75 ± 2.3	NS
Basal FSH (IU/l)	5.2 ± 1.3	5.6 ± 2.8	4.9 ± 3.1	NS
Total FSH administered (IU)	1897 ± 256	2087 ± 243	1767 ± 346	NS
Oestradiol value of HCG/GnRH day (pg/ml)	1567 ± 198[a]	2897 ± 356	2763 ± 321	0.02
Total oocytes retrieved	3.2 ± 1.2[b]	12.3 ± 3.4	10.5 ± 4.8	0.001

Values are mean ± SD.
AMH = anti-Mullerian hormone; BMI = Body mass index; GnRH = gonadotrophin-releasing hormone; HCG = human chorionic gonadotrophin; NS = not significant.
The value of oestradiol[a] and number of retrieved oocytes[b] are significantly different among the low and high responder patients.

25% H_3PO_4) was added. The reaction was performed at 22°C for 20 min, and the absorbance at 546 nm was measured, using $NaNO_2$ solution as standard (Kireev et al., 2010).

Cytokines as inflammatory markers

IL-2, IL-6, IL-8, IL-10 and tumour necrosis factor alpha (TNF-α) were measured by an ultrasensitive enzyme-linked immunosorbent assay (Leti Pharma SL, Barcelona, Spain). Concentration of VEGF in follicular fluid was determined with a commercially available ELISA kit (human VEGF; Diaclone SAS, Besancon Cedex, France). Intra-assay and inter-assay coefficient of variation (CV) was 6.2% and 4.3% for VEGF, respectively (Oliveira et al., 2005). To avoid possible bias due to follicular fluid volume variability, the concentrations of protein were measured. Protein concentration was performed by BioRad Assay kit (Bio-Rad Lab) based on the Lowry method (Lowry et al., 1951).

Statistics

The concentrations of antioxidant enzymes, oxidative stress markers and cytokines were compared between three groups: low responder patients, high responder patients and donors.

For determinations with a standard deviation (SD) of the sample of 0.05, 0.07 statistical significance, a power of 89% and an error factor of 0.6, a sample size of at least 8 cases in each arm allows us detecting differences in average of 0.1.

The results are expressed as the mean ± SD and the difference was considered statistically significant at $P < 0.05$. Differences in the follicular antioxidant profile, oxidative stress markers and cytokines between the three groups of patients were assessed by one-way ANOVA using Tukey's post test for multiple comparisons. Statistics were obtained using Win STAT (R. Fitch Software).

Results

The clinical characteristics of the study population are represented in Table 1, by age group and response levels in terms of BMI, anti-Müllerian hormone (AMH), amount of basal FSH, amount of administered FSH, the oestradiol value on the day of HCG/GnRH injection and number of oocytes retrieved.

No statistical difference was found in the amount of total gonadotrophin used for ovarian stimulation for poor responders, compared with non-poor responders groups. Serum oestradiol concentrations on the day of HCG injection were lower in poor responders ($P = 0.02$) (Table 1). As the response levels were defined according to oocyte numbers, the number of retrieved oocytes was significantly different between the poor and non-poor responders ($P = 0.001$).

Outcomes of assisted reproduction cycles in low (<5 oocytes) responders, high responders and donors are represented in Table 2, in terms of mature oocyte (%), fertilization rate, cleavage rate, good quality embryos and clinical pregnancy rate per cycle. No statistical difference was found in the number of mature oocytes, fertilization and cleavage rates. However, the number of good quality embryos and clinical pregnancy rate was significantly higher in the donors group ($P < 0.01$ and 0.001, respectively).

Antioxidant enzymes

The three groups of women, donors and patients were compared in terms of the concentrations of antioxidant enzymes measured in follicular fluid: GST, GR and GPx activity. The results are shown in Figure 1. Significant variations between the three groups in concentrations of enzymes directly involved in oxidative stress were observed. In particular, follicular fluid antioxidant enzyme GST, GR and GPx activity were significantly lower in young women with reduced ovarian reserve compared with oocyte donors and patients with a high response. The mean GPx activity in the follicular fluid of donors was 8.532 ± 0.2141 nmol/min/mg and 6.797 ± 0.117 nmol/min/mg in low response patients and 8.0614 ± 0.1773 nmol/min/mg in high response patients ($P < 0.0001$ low response patients compared with control and high response groups) (Figure 1A) and the mean GR activity in the follicular fluid of donors was 1.534 ± 0.0532 nmol/min/mg, 0.882 ± 0.0142 nmol/min/mg in low response patients and 1.342 ± 0.041 nmol/min/mg in high response patients ($P < 0.0001$ low response patients compared with control and high response groups) (Figure 1B). The mean GST activity in the follicular

Table 2 Outcomes of assisted reproduction cycles in low responders (<5 oocytes), high responders and control groups.

Variable	*Low responder patients*	*High responder patients*	*Control (donors)*	*P-value*
Mature oocyte (%)	64/84 (76.2)	115/144 (79.9)	250/314 (79.6)	NS
Fertilization (%)	49/64 (76.5)	89/115 (77.6)	196/250 (78.4)	NS
Cleavage (%)	45/49 (91.8)	83/89 (93.3)	187/196 (95.4)	NS
Good quality embryo (%)	27/45 (60)	53/83 (63.9)	130/187 (69.5)[a]	<0.01
Clinical pregnancy rate per cycle (%)	7/22 (32)	4/8 (50)	20/30[b] (66)	<0.001

The values of good quality embryos[a] and clinical pregnancy rate[b] are significantly different among donors and patients (low responder and high responder patients).
NS = not significant.

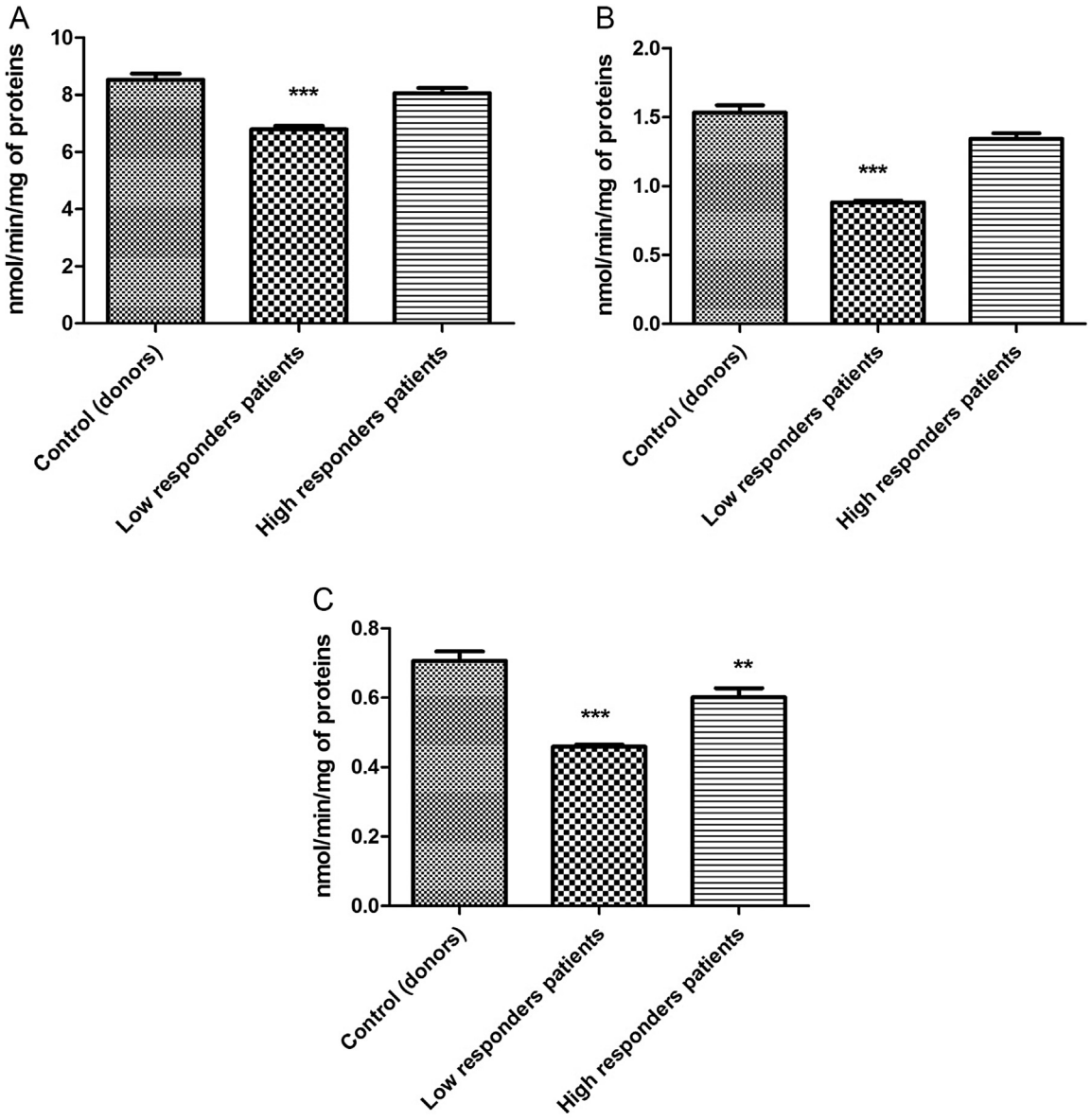

Figure 1 Activity of glutathione peroxidase (GPx) (A), glutathione reductase (GR) (B) and glutathione S-transferase (GST) (C) (nmol/min/mg of proteins) in follicular fluid from donors and patients (low and high response). Data represent mean ± SEM. GPx and GR: *** $P < 0.0001$ low response patients compared with control and high response groups. GST: *** $P < 0.0001$ low response patients compared with control; ** $P < 0.001$ high response patients compared with low response patients. One-way ANOVA analysis for GPx ($F = 22.79$, $P < 0.0001$), for GR ($F = 58.92$, $P < 0.0001$) and for GST ($F = 30.82$, $P < 0.0001$).

fluid of donors was 0.7062 ± 0.0278 nmol/min/mg, 0.459 ± 0.052 nmol/min/mg in low response patients and 0.601 ± 0.0102 nmol/min/mg in high response patients ($P < 0.0001$ low response patients compared with control; $P < 0.001$ high response patients compared with low response patients) (Figure 1C).

Oxidative stress markers

Data concerning all measurements of oxidative stress markers are presented in Figure 2. Follicular fluid concentration of MDA combined with 4-HAE was increased significantly in young women with low response as compared with oocyte donors. The mean MDA concentration in the follicular fluid of donors was 25.3 ± 1.4 μmol/mg, 38.437 ± 1.185 μmol/mg in low response patients and 27.081 ± 0.388 μmol/mg in high response patients ($P < 0.0001$ low response patients compared with control and high response groups) (Figure 2A).

NOx concentration was 0.365 ± 0.0259 nmol/mg in donors, 1.436 ± 0.095 nmol/mg in patients with low ovarian response and 0.592 ± 0.046 in patients with a high response to ovarian stimulation ($P < 0.0001$ low response patients compared with control; $P < 0.003$ high response patients compared with control; $P < 0.0008$ high response patients compared with low response patients) (Figure 2B).

Inflammatory markers

Concentrations of cytokines in follicular fluid as inflammatory markers were compared between donors and patients. Results are shown in Figures 3 and 4. Significant differences between the three groups in concentrations of IL-2, IL-6, Il-8 and IL-10, have been observed. Follicular fluid IL-2 concentration was significantly higher in young women with reduced ovarian reserve (0.0752 ± 0.0058 pg/mg of proteins) compared with oocyte donors (0.0311 ± 0.003 pg/mg of proteins) ($P < 0.0001$ low response patients compared with control and $P < 0.024$ high response patients compared with low response patients) (Figure 3A).

The concentration of IL-6 was also higher in low response patients (0.083 ± 0.0061 pg/mg of proteins) compared with donors (0.023 ± 0.002 pg/mg of proteins) ($P < 0.0001$ low response patients compared with control and $P < 0.0037$ high response patients compared with low response patients) (Figure 3B). Follicular fluid IL-8 concentration was significantly higher in young women with reduced ovarian reserve (2.768 ± 0.1816 pg/mg of proteins) compared with oocyte donors (1.857 ± 0.119 pg/mg of proteins) ($P < 0.0001$); Figure 3C). The concentration of IL-10 was low in the group of women with low response (0.1006 ± 0.004972 pg/mg of proteins) compared with donors (0.1602 ± 0.008437 pg/mg of proteins) ($P < 0.0001$ low response patients compared with control and high response groups) (Figure 3D).

Follicular fluid VEGF concentration was almost double in patients with low response (20.093 ± 0.804 pg/mg of proteins) compared with donors (12.604 ± 0.551 pg/mg of proteins) ($P < 0.001$ low response patients compared with control; $P < 0.009$ high response patients compared with control) (Figure 4).

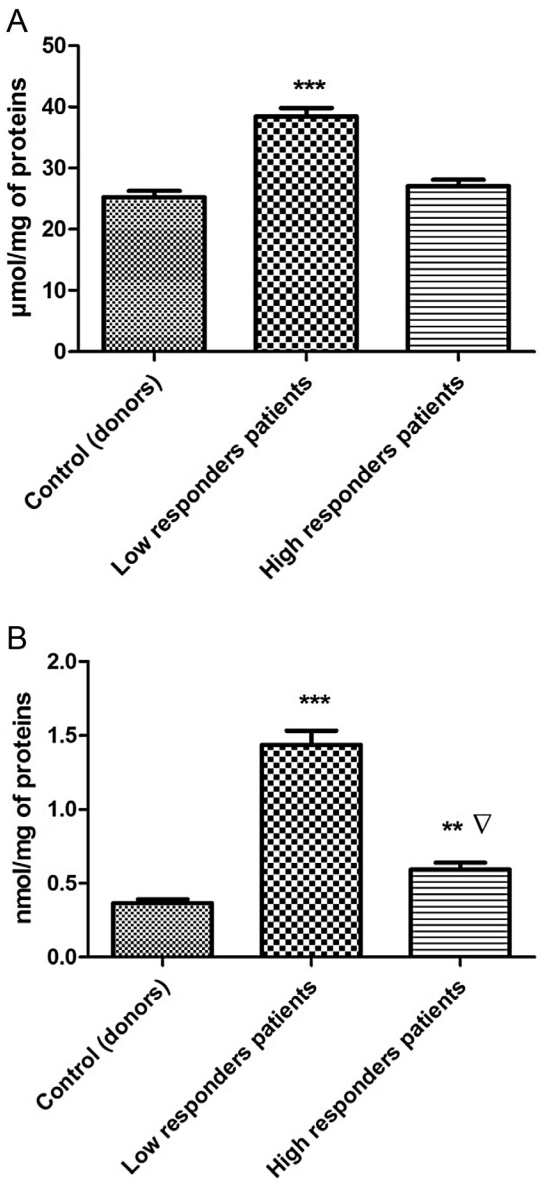

Figure 2 Malondialdehyde (MDA)+4-hydroxyalkenals content (μmol/mg of proteins) (A) and concentration of nitric oxide metabolites (NOx) (nmol/mg of proteins) (B) in follicular fluid from donors and patients (low and high response). Data represent mean ± SEM. MDA+4-hydroxyalkenals: ***$P < 0.0001$ low response patients compared with control and high response groups. NOx: *** $P < 0.0001$ low response patients compared with control; ** $P < 0.003$ high response patients compared with control; Δ-$P < 0.0008$ high response patients compared with low response patients. One-way ANOVA analysis for MDA+4-hydroxyalkenals ($F = 32.2$, $P < 0.0001$) and for NOx ($F = 76.84$, $P < 0.0001$).

No significant differences were found in follicular fluid concentrations of TNF-α between the three groups (data not shown).

Discussion

The biochemical composition of follicular fluid includes proteins, sugars, ROS, antioxidants and hormones (O'Gorman

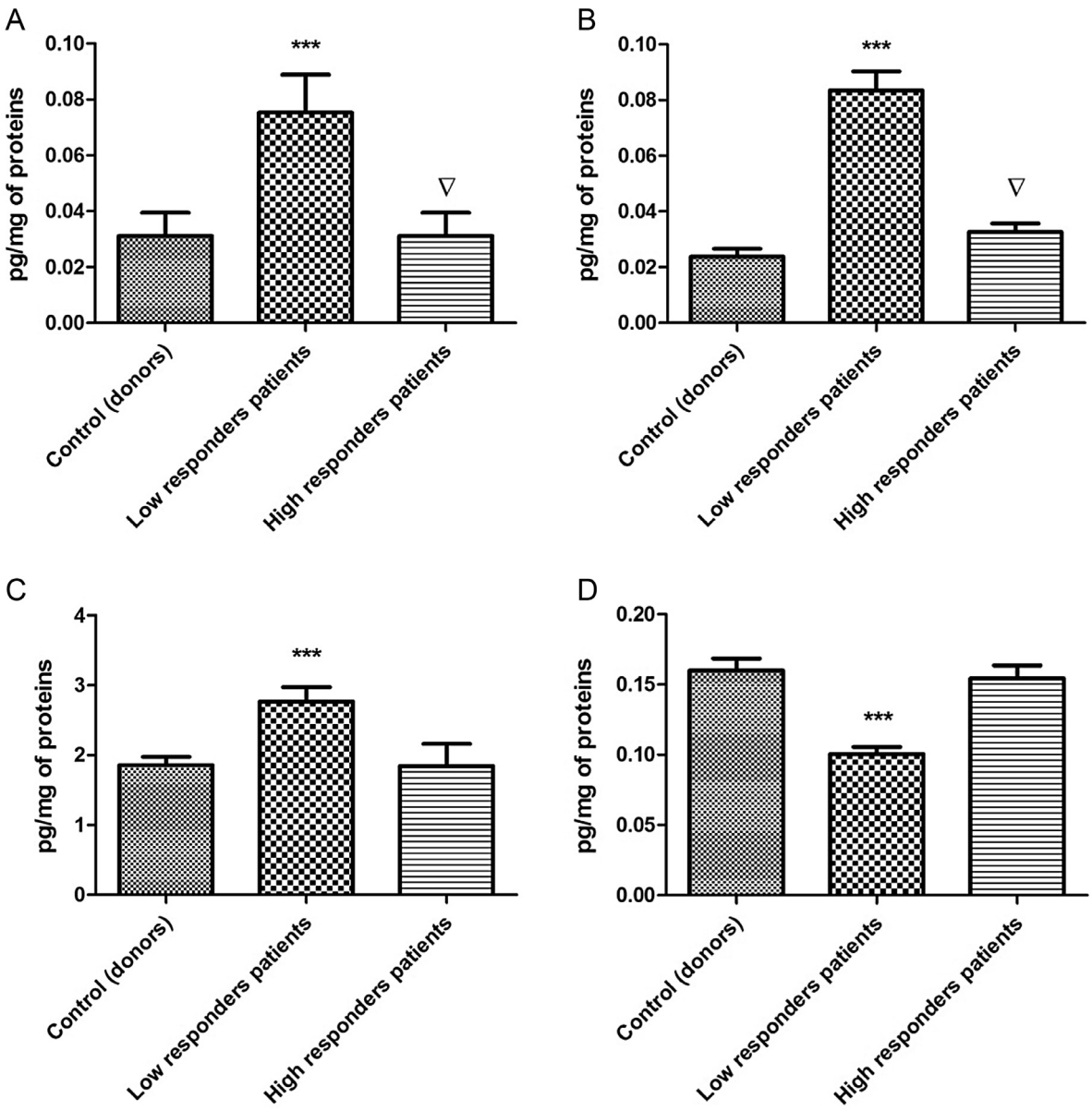

Figure 3 The concentrations of pro/anti-inflammatory cytokines IL-2 (A), IL-6 (B), IL-8 (C) and IL-10 (D) (pg/mg of proteins) in follicular fluid from donors and patients (low and high response). Data represent mean ± SEM. IL-2: *** $P < 0.0001$ low response patients compared with control; Δ- $P < 0.024$ high response patients compared with low response patients. IL-6: *** $P < 0.0001$ low response patients compared with control; Δ- $P < 0.0037$ high response patients compared with low response patients. IL-8: *** $P < 0.0001$ low response patients compared with control. IL-10: *** $P < 0.0001$ low response patients compared with control and high response groups. One-way ANOVA analysis for IL-2 ($F = 5.98$, $P = 0.015$), IL-6 ($F = 41.86$, $P < 0.0001$), IL-8 ($F = 8.76$, $P = 0.0004$), IL-10 ($F = 17.88$, $P < 0.0003$).

et al., 2013). These mediators may have a direct effect on the maturation ability and the quality of oocytes (Agarwal et al., 2012). Moreover, the oxidant–antioxidant state of follicular fluid and its effects on oocyte and IVF outcomes has been of great interest in recent years (Oyawoye et al., 2003; Revelli et al., 2009).

The aim of this study was to investigate the relationship between oxidative stress and inflammatory markers in follicular fluids and low response to ovarian stimulation cycles in a group of young women compared with fertile oocyte donors (controls) and high responders of the same age. The concentrations of MDA combined with 4-hydroxyalkenals were higher in low responders compared with high responders and donors, whereas high responders had similar concentrations to those of donors. Both increased markers (MDA combined with 4-hydroxyalkenals) indicated that the follicular fluid of low response patients had a higher degree of oxidative stress than that of the donors and high response patients.

Oral et al. (2006), investigated the impact of oxidative stress on pregnancy success by monitoring MDA concentrations in follicular fluid, and pregnancy rates were found to

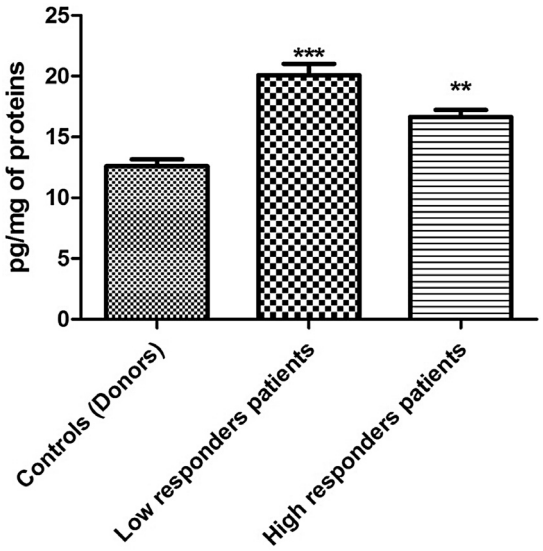

Figure 4 The concentration of vascular endothelial growth factor (VEGF) in follicular fluid from donors and patients (low and high response). Data represent mean ± SEM. *** $P < 0.001$ low response patients compared with control; ** $P < 0.009$ high response patients compared with control. One-way ANOVA analysis for VEGF ($F = 28.69$, $P < 0.0001$).

be lower with higher MDA concentrations, similarly to the study of Yalçınkaya et al. (2013).

Borowiecka et al. (2012) also demonstrated that elevated concentrations of lipid and protein peroxidation in follicular fluid may have a negative impact on IVF outcomes. These findings support the idea that the increased concentration of oxidative stress markers in follicular fluid may play an important role in reducing fertilization capacity (Borowiecka et al., 2012). However, the data from this study show no significant modification of fertilization rate between the three groups of patients on the basis of the ovarian response to ovarian stimulation.

It has been demonstrated that ovarian stimulation also induces ROS production and perturbation in the oxidant–antioxidant balance, leading to oxidative stress (Agarwal et al., 2012). At the same time, the GnRH antagonist protocol has been associated with increased oxidation stress compared with the agonist protocol (Celik et al., 2012), but the authors concluded that the relationship between GnRH analogues and oxidative stress and its implication in follicular growth needed to be addressed in further studies.

Palini et al. (2014), showed that in women undergoing a long agonist protocol of ovarian stimulation, there were reduced plasma antioxidant concentrations after gonadotrophin administration but not after pituitary suppression. Taking into account possible relationships between the oxidant profile and ovarian stimulation protocol, and to avoid bias, this study was carried out with the same pattern of stimulation for both groups of patients: low responders and high responders (long protocol with GnRHa), compared with the short protocol with antagonist in donors. When comparing oxidative stress levels between patients with high and low response (treated both with agonists), significant differences were detected from the same magnitude as those found between low response patients and oocyte donors. However no significant differences were found between levels of oxidative stress in high response patients treated with agonists and donors treated with antagonists We can hypothesize that the pattern of stimulation is not playing a role in the observed differences in both pro-oxidant and antioxidant substances in the follicular fluid.

The results of this study also show that concentrations of the other markers of oxidative stress, such as NO, were significantly higher in the group of women with low response compared with the control group. In addition, the group of high responders demonstrated low concentrations of NOx metabolites compared with low responders.

Different studies have demonstrated that NO is expressed by human granulosa cells and therefore present in significant amounts in human follicular fluid, where it plays several physiological roles (Agarwal et al., 2012). It has also been detected in the follicular fluid after gonadotrophin stimulation (Vignini et al., 2008).

Multiple enzyme systems and soluble factors maintain the redox state of cells (Kireev et al., 2007a, 2007b) and the glutathione system plays a fundamental role in cellular defence against ROS (Agarwal et al., 2012). Antioxidant enzymes such a GPx, GR and GST are constantly expressed in mammalian oviducts and have important roles for keeping intra and extracellular redox balance (El Mouatassim et al., 2000). Velthut et al. (2013) demonstrated that the achievement of clinical pregnancies is favoured by the presence of an elevated systemic total antioxidant status and that concentrations of follicular oxidative stress markers are inversely correlated with ovarian stimulation efficiency in infertile patients.

Among individuals undergoing IVF, the mean GSH-Px activity in fluids of follicles yielding oocytes that were subsequently fertilized, was higher when compared with that of other follicles in which the oocytes failed to fertilize (Babuska et al., 2012). In this study, the mean serum GSH and GSH-Px activity were significantly higher in the donor groups than in the low response groups, allowing a better shape of the follicles and thus a higher probability of maturation and fertilization (Babuska et al., 2012).

So, low ovarian response is associated with significantly lower levels of GPx, GR and GST activity in the follicular fluid of women undergoing IVF treatment, compared with oocyte donors with normal response to ovarian stimulation (Babuska et al., 2012). These data, which correlate the retrieved oocyte number with higher GPx, GR and GST concentrations, support the idea that growth of a higher number of mature follicles is associated with the availability of enough antioxidant enzymes (Agarwal et al., 2012; Babuska et al., 2012). In this study, oocyte donors, who can be considered as healthy control subjects, presented the best follicular antioxidant profile in comparison with those with low response or even patients with high response, confirming the presence of oxidative stress and reduced antioxidant capacity in follicular fluid from women with reproductive failure.

According to this study's results, low responders had statistically significantly higher follicular fluid concentrations of IL-2, IL-6, IL-8 and VEGF compared with those of both high responders and donors. Nevertheless, the concentration of anti-inflammatory cytokine (IL-10) was lower in the group of

women with low ovarian response than in both high responders and donors.

Taghavi et al. (2014) reported that the concentrations of IL-6, IL-8 and macrophage migration inhibitory factor (MIF) were significantly increased in women with poor ovarian response compared with normal women (Taghavi et al., 2014). Younis et al. (2012), showed that ovarian stimulation caused a significant increase in serum IL-6 activity in women undergoing IVF or IUI. The high concentrations of IL-6 were positively associated with pregnancy, indicating a possible physiological role in improving implantation (Taghavi et al., 2014). Other authors have demonstrated that IL-6 is able to diminish aromatase activity within follicles, which results in a decreased intrafollicular oestradiol concentration, fertility and fertilizing capacity (Pellicer et al., 1998). It has been also proposed that IL-2 and IL-6 may partly act via inhibition of FSH-induced progesterone release (Field et al., 2014); indeed, higher concentrations of IL-6 are found in the follicular fluid of patients with ovarian hyperstimulation syndrome (OHSS), where progesterone concentrations are characteristically low (Borman et al., 2004; Engmann and Benadiva, 2010).

Studies by Schafer et al. have demonstrated that an increase in oxidative stress leads to an increase in VEGF gene expression. Moreover, eliminating ROS in turn leads to a decrease in VEGF concentrations, proving the specificity of oxidative stress reactions (Schafer et al., 2003).

In most of the studies carried out to investigate the possible role played by ROS and antioxidants in assisted reproductive treatments, the number of oocytes retrieved and/or fertilized and/or number of embryos developed were considered as endpoints (Borowiecka et al., 2012; Lee et al., 2004; Younis et al., 2012). This study has shown that the follicular fluid concentrations of antioxidant enzymes, oxidative stress markers and cytokines are associated with higher oxidative stress levels in young women with low ovarian response, indicating an "enhanced ageing" of the follicles.

This is the first study analysing the relationship between oxidative stress and low response in young patients undergoing ovarian stimulation cycles compared with fertile oocyte donors and high response patients. The results show that the low ovarian response in young women is associated with the presence in the follicular fluid of higher concentrations of oxidative stress and inflammation markers. Knowledge of these perturbations could lead to the potential development of antioxidant therapies for these poorer prognosis women undergoing IVF treatment. Currently, the trend is to use antioxidant supplementation in infertile patients with the aim of strengthening the antioxidant defence mechanism.

In conclusion, the primary results of this study demonstrate that antioxidant, inflammatory status and follicular oxidative stress markers are related with ovarian response in young women undergoing infertility treatment with ovarian stimulation-IVF. Although the small numbers of women in the groups with different numbers of oocytes retrieved is a weakness of this investigation, it is shown that reduced follicular total antioxidant status is a characteristic of low response patients. The findings suggest a role in the pathogenesis of poor ovarian response (POR) in women undergoing infertility treatment with ovarian stimulation-IVF.

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