Serum oxidizability and antioxidant status in patients undergoing in vitro fertilization

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Objective: To evaluate the serum oxidizability and antioxidant status in women undergoing an in vitro fertilization (IVF) cycle and to assess the possible relationship of the oxidizability indexes with the pregnancy rate.
Design: Prospective, longitudinal study.
Setting: Public university and public university hospital.
Patient(s): Systematically recruited cohort of 125 women undergoing either IVF or intracytoplasmic sperm injection (ICSI).
Intervention(s): Serum samples were collected before the beginning of the use of gonadotropins (basal) and the day of human chorionic gonadotropin (hCG) administration (final) during an IVF cycle.
Main Outcome Measure(s): The Cu^{2+}-induced serum oxidation in terms of the oxidation rate in the lag (V_{lag}) and propagation (V_{max}) phases and the time at which the oxidation rate is maximal (t_{max}), and measurements of serum total antioxidant activity (TAA), tocopherol, hydrophilic antioxidants, malondialdehyde, and nitric oxide.
Result(s): Albumin, urate, bilirubin, α-tocopherol and γ-tocopherol, TAA, and t_{max} statistically significantly decreased after the IVF cycle. Conception cycles were associated with a serum more prone to oxidation compared with nonconception cycles. In multivariate logistic regression analysis, the difference (final-basal) of the oxidation index V_{lag} (OR 1.394) and the body mass index (OR 0.785) were independent predictors of pregnancy.
Conclusion(s): Treatment with IVF induces the production of reactive oxygen species (ROS), which is reflected in a serum less protected against oxidation. The results also suggest a role for ROS in the occurrence of conception in IVF. (Fertil Steril® 2010;94:1279–86. ©2010 by American Society for Reproductive Medicine.)

Key Words: Antioxidant, in vitro fertilization, IVF-ICSI outcome, reactive oxygen species, serum oxidizability

Oxidative stress is related to a number of human pathophysiologic conditions, such as cardiovascular disease, cancer, diabetes, neurodegenerative diseases, and aging. A link between oxidative stress and male infertility has received substantial scientific support (1–8). Human spermatozoa are redox active cells capable of generating reactive oxygen species (ROS), which are important in sperm capacitation (9). However, excess ROS can lead to pathologic damage to spermatozoa and subsequently can disrupt their capacity for fertilization. The involvement of free radicals in female infertility is less well-known. It has been suggested that oxidative stress might play a role in endometriosis development and endometriosis-associated infertility (10, 11). Also, ROS can alter embryonic development and lead to early embryo death (12–14). In this respect, diabetes, a condition characterized by high free radical production, represents a model of oxidative stress related to embryo toxicity (15).

Oxidative stress in women during an in vitro fertilization (IVF) cycle has received little attention. In a previous work, we found that the IVF cycle was associated with changes in the physicochemical properties of low density lipoproteins, affecting the susceptibility of the particles to in vitro oxidation (16). These changes took place between two extreme stages of the cycle, when the ovarian suppression was attained (minimal serum estradiol levels) and when human chorionic gonadotropin (hCG) was administered (supraphysiologic estradiol levels). However, during a natural cycle no changes in the serum redox status could be ascertained (17). We wondered whether IVF treatment could induce oxidative stress and, if so, whether this condition would be connected with pregnancy. To address these questions, we analyzed the serum oxidizability and antioxidant status in women undergoing an IVF cycle. Because oxidative stress is known to be associated with dyslipidemia, we also determined the serum lipid profile to establish appropriate associations independent of confounding factors.

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MATERIALS AND METHODS

Materials

We obtained 2,2'-azobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1,3,3-tetraethoxypropane, dimethylsulfoxide (DMSO), α-tocopherol, γ-tocopherol, and δ-tocopherol from Sigma-Aldrich (Gillingham, Dorset, United Kingdom). All other reagents used were the highest purity available.

Patients

The population under study consisted of 125 women undergoing either IVF or ICSI at the Human Reproductive Unit of Cruces Hospital, Vizcaya, Spain. Criteria for participation in the study included [1] maximum age 40 years, [2] no vitamin supplementation, [3] no hormone therapy during the previous 12 months, [4] no cardiovascular medical history, [5] no hypertensive disorders, and [6] no metabolic disease. The women underwent a general biochemical study as well as the standard infertility work-up before IVF. The ovarian cycle management protocol was previously published elsewhere (18). Briefly, it consisted of down-regulation with the gonadotropin-releasing hormone analogue triptorelin acetate (Decapeptyl; Ipsen Pharma, Barcelona, Spain) administered subcutaneously from the 20th day of the pretreatment cycle. Ovarian suppression was defined as serum E_2 levels <40 pg/mL and follicular size <10 mm in diameter. Recombinant follicle-stimulating hormone (FSH, Gonal F; Merk Serono, Madrid, Spain) and human gonadotropins (Menopur; Ferring, Madrid, Spain) administration was started when ovarian suppression had been achieved. Recombinant hCG (250 μg of Ovitrelle; Merck Serono, Madrid, Spain) was administered subcutaneously when at least three follicles had reached 18.5 mm in mean diameter. Cycles with E_2 levels >4000 pg/mL were canceled (with the exception of two cycles with values up to 5000 pg/mL, where the patients agreed to assume the corresponding risks) or underwent coasting. Coasting cycles were not included in this study. Clinical pregnancy was defined as a gestational sac detected at vaginal ultrasound 4 weeks after embryo transfer.

Two blood samples were selected for analysis in [1] the midfollicular phase of a natural cycle (1 or 2 months before starting gonadotropin stimulation) (basal), and [2] the day when hCG was administered (E_{2max}).

The ethics committee of the hospital approved the human subject protocol, and the university research board approved the study protocol. The women gave informed consent for participation in the study.

Serum Samples Isolation

After the women had undergone an overnight fast, we collected blood samples by venipuncture into tubes without anticoagulant. Serum was recovered by centrifugation at 1300 $\times$ g for 30 minutes at 15°C and was stored at –80°C until the day of the experiment.

Serum Biochemical Analysis

An aliquot of each serum sample was analyzed for various biochemical parameters (total cholesterol, triglycerides, apolipoproteins A1 and B, bilirubin, albumin, and urate) using commercially available kits. Serum 17β-estradiol (E_2) was quantified by radioimmunoassay (Orion Diagnostica, Espoo, Finland) designated for direct quantitative in vitro measurement of unconjugated E_2. The 17β-estradiol area under the curve (AUC_{E2}) was calculated from the day with E_{2min} to the day of maximal stimulation (E_{2max}) by use of the trapezoid rule.

Cu^{2+}-Induced Oxidation of Serum

Unfractionated serum was oxidized with copper (Cu^{2+}), as described previously elsewhere (19, 20). Copper-induced oxidation was monitored at 37°C by continuous recording of absorbance at 245 nm (7-ketocholesterol and conjugated dienic hydroperoxide formation) and 268 nm (dienals) by the use of a Kontron spectrophotometer (Uvikon 943; Kontron AG, Eching, Munich) equipped with an automatic cell changer for 10 samples. Measurements were carried out in quartz cuvettes containing 20 μL of serum in 1 mL of a phosphate-buffered solution (pH = 7.4) with NaCl (146 mM), natrium citrate (720 μM), and $CuCl_2$ (100 μM).

The absorbance readings were made every 3 minutes over 5 hours. The increase in absorbance was plotted against time. From the curve, three phases—lag, propagation, and decomposition—were detected (Fig. 1). During the lag phase, the serum antioxidants are consumed and only a marginal oxidation of lipids takes place. When serum is depleted of its antioxidants, oxidizable lipids (mostly polyunsaturated fatty acids [PUFA] and cholesterol) are rapidly oxidized to lipid hydroperoxides and 7-ketocholesterol (propagation phase), which are then converted to a variety of other products, including reactive aldehydes (decomposition phase).

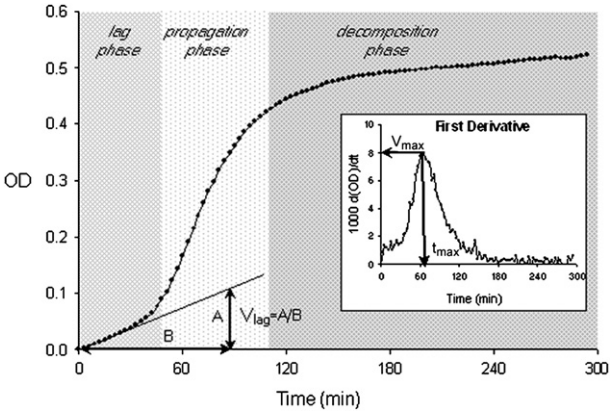
From the curves, the following oxidation parameters were calculated: [1] the rate of increase of absorbance in the lag phase preceding oxidation (V_{lag} in optical density [OD] units/minute), [2] the maximal accumulation rate of absorbing products (V_{max}), and [3] the time at which the oxidation rate is maximal (t_{max} in minutes). Both V_{max} and t_{max} were given by the peak of the first derivative, that is, the change of rate of oxidation as a function of time. All sample determinations were carried out in duplicate. The within-run and between-run coefficients of variation obtained for t_{max}, V_{lag}, and V_{max} were 1.2–2.2, 3.1–4.8, and 2.2–2.9 %, respectively.

Total Antioxidant Activity

The serum total antioxidant activity (TAA) was measured by the $ABTS^+$ decolorization method (21). One milliliter of the $ABTS^+$ solution (0.70 $\pm$ 0.01 OD at 734 nm) was added to 10 μL of serum (3/10 v/v diluted in H_2O) or Trolox standards (0-2 mM concentration), and the absorbance reading was taken 30 seconds after initial mixing every minute up to 12.5 minutes by Uvikon 943 spectrophotometer. Appropriate solvent blanks were run in each assay. All the determinations were carried out

FIGURE 1

Representative curve illustrating the kinetics of Cu^{2+}-induced serum oxidation. V_{lag}, rate of accumulation of absorbing products during the lag phase; V_{max}, maximal rate of accumulation of absorbing products; t_{max}, time at which the oxidation rate is maximal. The V_{max} and t_{max} are determined from the first derivative of the time course. OD, optical density.



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in duplicate. The TAA of the serum samples was calculated by comparing the area under the curve of the reaction of the serum with the area under the curve of the reaction of Trolox standard.

Malondialdehyde

Malondialdehyde (MDA) was measured by reverse phase high-performance liquid chromatography (HPLC). Serum was mixed with butylated hydroxytoluene (BHT) (100 mM in ethanol), 10% trichloroacetic acid (TCA), and 2 M acetic acid (5:1:5:5 v/v/v/v) in that order, then frozen in liquid N_2 for 15 minutes. After thawing, the precipitate was removed by centrifugation at 20,000 × *g* for 10 minutes, and 120 μL of the supernatant was mixed with 20 μL milliQ water and 100 μL of 0.71% thiobarbituric acid (TBA) in 0.1 M NaOH and heated to 96°C for 60 minutes. After cooling in an ice-water bath, the samples were filtered (0.45 μm) and injected into the HPLC system.

The MDA-$(TBA)_2$ adduct was separated using a C18 column (Waters Spherisorb ODS2, 5-μm particle size, 150 × 4.6 mm) at ambient temperature. The mobile phase consisted of 50 mM KH_2PO_4 (pH 6.8)/acetonitrile (83/17, v/v) and was filtered (PVDF, 0.45 μm) and degassed before use. The flow rate was 1 mL/minute, and the MDA-$(TBA)_2$ adduct was detected at 532 nm. The data were collected and integrated by the use of KromaSystem 2000 software (Bio-Tek/Kontron Instruments, Milano, Italy). The concentration of MDA was calculated from the peak area, based on a calibration curve prepared by using 1,1,3,3-tetra-ethoxypropane as MDA standard. Calibration curves were run daily.

Vitamin E

The serum α-tocopherol and γ-tocopherol content was determined by reverse phase HPLC after lipid extraction. We diluted 250 μL of serum in 625 μL of cold ethanol containing 0.05% BHT, 1% pyrogallol, and 2 nmol δ-tocopherol as internal standard, which was then vortexed for 30 seconds. Then 1 mL of heptane was added to the mixture and vortexed for 2 minutes. After centrifugation (600 × *g* for 5 minutes at 4°C), the upper heptane phase was collected, and the extraction was repeated with the ethanolic phase. The collected heptane phases were dried under vacuum in a Savant SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA), and the residue was dissolved in 20 μL of 2-propanol plus 80 μL of mobile phase, filtered (nylon, 0.45 μm), and injected into the HPLC system. The α-tocopherol and γ-tocopherol were separated using a C18 column (Water Spherisorb ODS2, 5-μm particle size, 150 × 4.6 mm; Barcelona, Spain) at ambient temperature. Isocratic separation was performed in methanol/ethanol/isopropanol (65:33.25:1.75) with 1 mL/minute flow and detection at 295 nm. Calibration curves were run daily.

Nitric Oxide

Nitric oxide was determined in 96-well plates by an adaptation of the method described by Miranda et al. (22), based on the simultaneous detection of nitrate and nitrite, as NO-derived metabolites (23). The method involves reduction of nitrate by vanadium(III) and detection with Griess reagent. Briefly, two volumes of ethanol per sample volume were added to the tubes, thoroughly mixed, and kept in ice for 10 minutes. After centrifugation (14,000 × *g* for 10 minutes at 4°C), to each well 100 μL of supernatant was added, followed by 100 μL of the Griess reagent freshly prepared (mix one volume of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in water with one volume of 2% sulfanilamide in 5% HCl), and 100 μL of 51 mM VCl_3 in 1 M HCl. Plates were vigorously shaken and incubated for 60 minutes at 37°C. Absorbance was registered at 540 nm. The interassay and intra-assay coefficients of variation were 8%, and 4%, respectively. The recovery was 97.8 ± 1.8%.

Statistical Analysis

Statistical analysis was performed with Statistics Package for Social Sciences for Windows, version 16.0 (SPSS, Inc., Chicago, IL). Results were expressed as mean ± standard deviation (SD). The comparisons of a variable between basal and final stages of a cycle were analyzed by the Student's *t*-test for paired data, and for nonpaired data when the comparisons were between groups (pregnancy YES or NO). The Wilcoxon rank-sum test was used for comparison when data for a particular variable were not normally distributed. All *P* values were two-sided, and $P<.05$ was considered statistically significant. Pearson's correlation was used to determine the relationship between covariates. Stepwise forward multivariate logistic regression analysis was used for the evaluation of age, body mass index, number of oocytes retrieved, infertility cause, Δt_{max}, ΔV_{lag}, and Δα-tocopherol as

TABLE 1

Characteristics of the study population.

Variable	Mean (range)
Age (y)	34.3 (23–40)
Body weight (kg)	63.8 (45–90)
Height (m)	1.63 (1.50–1.77)
Body mass index (kg/m^2)	24.3 (18.3–36.5)
Years of infertility	5.1 (2–15)
Infertility causes (%)	
Male	35.2
Female	12.8
Both male and female	47.2
Unknown	4.8
Ongoing pregnancy rate per transfer (%)	30

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predictors of clinical pregnancy in IVF. Stratified models were generated for infertility cause.

RESULTS

Age, anthropometric characteristics, and infertility causes of the study population are shown in Table 1. The causes of infertility were classified as [1] male subfertility, [2] female subfertility, [3] both male and female, and [4] unknown subfertility. Male factor infertility was defined as a subnormal sperm analysis according to the World Health Organization criteria (24), except for motility, which was studied according to our reference values (25), and also in cases with previous failures of six intrauterine insemination cycles or IVF fertilization failures, in the absence of female abnormalities.

The serum lipid profile and antioxidant status before (basal) and after (final) an IVF cycle are shown in Table 2. The serum levels of total cholesterol and apoB were reduced during the treatment. Triglycerides increased the day of the hCG administration. HDL-cholesterol (HDL-c) was not significantly altered. Several patients were found to have a high body mass index (BMI). Because BMI has been commonly associated with an altered lipid profile, it is an important risk factor in diseases such as insulin resistance and atherosclerosis. Bivariate regression analysis showed that BMI positively correlated with triglycerides ($r = 0.226$, $P<.01$) and negatively with high-density lipoprotein (HDL) cholesterol ($r = -0.326$, $P<.01$) (data not shown).

The serum oxidant/antioxidant status was tackled by different approaches: determination of serum oxidizability, and quantification of α-tocopherol and γ-tocopherol, hydrophilic antioxidants (albumin, urate, bilirubin), lipid oxidation products (malondialdehyde), and nitric oxide. The serum resistance to in vitro oxidation was measured as an alternative

TABLE 2

Serum lipid profile and oxidant/antioxidant status during an in vitro fertilization cycle.

	Basal	Final	*P* value[a]
Total cholesterol (mg/dL)	198 ± 31	178 ± 26	<.01
Triglycerides (mg/dL)	70 ± 25	77 ± 26	<.01
HDL-c (mg/dL)	63 ± 16	63 ± 14	NS
ApoB (mg/dL)	86 ± 18	76 ± 15	<.01
ApoA1 (mg/dL)	173 ± 32	175 ± 28	NS
V_{lag} (10^4 × OD/min)	8.6 ± 3.2	8.8 ± 3.3	NS
V_{max} (10^4 × OD/min)	25.0 ± 0.65	25.5 ± 6.3	NS
t_{max} (min)	82.8 ± 12.8	80.7 ± 12.4	<.01
TAA (mM)	2.46 ± 0.47	2.43 ± 0.47	<.05
α-tocopherol (μM)	29.3 ± 5.1	26.6 ± 4.7	<.01
γ-tocopherol (μM)	2.90 ± 0.77	2.78 ± 0.93	<.05
MDA (μM)	0.59 ± 0.23	0.60 ± 0.25	NS
NO (μM)	25.9 ± 10.8	26.9 ± 10.2	NS
Albumin (g/L)	45 ± 3	43 ± 3	<.01
Bilirubin (mg/dL)	0.50 ± 0.23	0.43 ± 0.15	<.01
Urate (mg/dL)	4.01 ± 0.85	3.89 ± 0.79	<.05

Note: Sera from women before (basal) and after (final) an IVF cycle were used to analyze the lipid profile and the indicated indices of the oxidant/antioxidant status. The oxidation kinetics was monitored at 268 nm. Values are mean ± standard deviation. HDL-c, high-density lipoprotein cholesterol; MDA, malondialdehyde; NS, not statistically significant; TAA, total antioxidant activity; t_{max}, time at which oxidation rate is maximal; V_{lag}, rate of increase of absorbance in the lag phase preceding oxidation; V_{max}, maximal accumulation rate of absorbing products.

[a] Statistical analysis by Student's *t*-test for paired data.

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TABLE 3

Age, body mass index, and reproductive parameters in conception and nonconception cycles.

	Pregnancy	No pregnancy	*P* value[a]
Age (y)	33.7 ± 2.9 (23–39)	34.6 ± 3.1 (26–40)	NS
Body mass index (kg/m^2)	23.2 ± 3.9 (19.3–35.1)	24.4 ± 3.9 (18.3–36.5)	NS
Maximal E_2 levels (pg/mL)	1918 ± 701 (764–3883)	1864 ± 936 (326–4756)	NS
AUC_{E2} (pg/mL)[b]	6057 ± 2699 (2728–14285)	5918 ± 3059 (1024–15137)	NS
Obtained oocytes	12.8 ± 5.8 (3–28)	9.6 ± 6.2 (0–29)	<.05
Transferred embryos	3.4 ± 0.8 (1–4)	2.1 ± 1.5 (0–4)	<.05

Note: The number of patients was 37 (pregnancy) and 88 (no pregnancy). Values are mean ± standard deviation (range). NS, not statistically significant.

[a] Statistical analysis by Student's *t*-test for unpaired data.

[b] AUC_{E2}, E_2 area under the curve, calculated from the ovarian suppression to the day of E_2max.

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assay to the low-density lipoprotein (LDL) oxidizability. The serum approach has the advantage of taking into account the complex interrelated effects of the various factors that are present in the plasma and influence lipid oxidation. Results showed that t_{max} decreased statistically significantly at the end of the cycle, reflecting a serum more prone to oxidation. Similar results were obtained when serum oxidation kinetics were registered at 245 nm. The oxidation indexes obtained from both approaches were statistically significantly correlated ($P<.001$) (data not shown). Likewise, the serum concentration of the antioxidant vitamin E (α-tocopherol and γ-tocopherol) was lower at the end of the cycle, as was the total antioxidant activity (TAA). Albumin, bilirubin, and urate also decreased after the IVF cycle. The BMI per se was not found to be associated with any of the oxidation markers.

Together these results suggest that the IVF therapy leading to the ovarian stimulation provokes an oxidative stress, which can be detected in the serum, basically by a reduced time to attain oxidation, decreased TAA, and depletion of hydrophilic antioxidants and vitamin E. The central question arises whether these changes have any relationship with pregnancy achievement. The next part of the study consisted of comparing the serum oxidizability and antioxidant potency variations during an IVF cycle between women who achieved pregnancy and those who did not succeed. Reproductive parameters in both groups are shown in Table 3. There were no differences in age, BMI, maximal E_2 levels, or the area under the curve of E_2 (AUC_{E2}). However, the number of obtained oocytes was higher in conception cycles.

With respect to the oxidative stress markers, no differences were found for either group at the beginning of the treatment (basal). In Table 4, only the indexes of serum oxidizability that statistically significantly differed after the cycle (basal versus final) are shown. As can be seen, the studied parameters followed a different profile of variation after the IVF treatment depending on whether pregnancy was achieved. Thus, in conception cycles significant reduction of t_{max} and α-tocopherol and increased V_{lag} were found, thus indicating a serum less protected against oxidation. In nonconception cycles, only α-tocopherol showed a statistically significant decrease at the final state compared with the initial one (see Table 4A). Despite these two patterns, mean changes were not statistically significantly different between conception and nonconception cycles when the whole population was studied, probably due to the high variability of response and the presence of some confounding factors. When the male factor as the sole cause of infertility was excluded, a similar pattern of variation was observed (see Table 4B), but in this case statistically significant differences between conception and nonconception cycles were found for t_{max} ($P<.05$) and V_{lag} ($P<.01$).

A multivariate logistic regression analysis was performed to identify predictive factors of pregnancy (Table 5). The following variables were tested: age, BMI, number of oocytes retrieved, infertility cause, variations (final minus basal) of t_{max}, V_{lag}, and α-tocopherol. The probability of achieving pregnancy after a cycle was directly associated with only the number of retrieved oocytes (OR 1.094; 95% CI, 1.024–1.169) and ΔV_{lag} (OR 1.146; 95% CI, 0.999–1.316) when the total study population was considered (model 1). In this model, the cause of infertility was introduced as a dichotomous variable (sole male infertility factor versus other factors). After stratification for this variable (model 2, infertility factors other than sole male factor), the probability of a conception cycle was directly associated with ΔV_{lag} (OR 1.394; 95% CI, 1.119–1.736) and inversely with the woman's BMI (OR 0.785; 95% CI, 0.649–0.950). In this population (when the confounding sole male factor of infertility was excluded), model 2 rendered an overall 77% of the cases correctly classified for pregnancy outcome. By contrast, when the male factor was the only cause of infertility, the only statistically significant predictor variable of pregnancy was the number of retrieved oocytes (model 3), stressing the existence of two populations with different behaviors.

DISCUSSION

In this work we describe, for the first time, serum oxidizability and antioxidant status in women undergoing an IVF cycle. We found that the serum is less protected from oxidation after the cycle, showing a lower resistance to in vitro oxidation,

TABLE 4

Variations of serum α-tocopherol and oxidation indexes after an in vitro fertilization, according to the achievement of pregnancy.

Whole population (n = 125)	Pregnancy (n = 37)			No pregnancy (n = 88)		
	Basal	Final	*P* value[a]	Basal	Final	*P* value[a]
t_{max} 245 (min)[b]	76.1 ± 11.8	70.5 ± 10.9	<.01	78.6 ± 18.6	76.2 ± 14.6	NS
t_{max} 268 (min)[b]	83.6 ± 12.6	77.4 ± 11.4	<.01	85.0 ± 19.0	82.7 ± 14.6	NS
V_{lag}268 (10^4 × OD/min)[c]	8.4 ± 3.6	9.7 ± 4.2	<.05	8.7 ± 3.1	8.9 ± 3.4	NS
α-tocopherol (μM)	28.8 ± 5.1	26.4 ± 5.4	<.01	29.5 ± 5.2	26.6 ± 4.8	<.01
Excluding male-infertility factor (n = 81)	**Pregnancy (n = 24)**			**No pregnancy (n = 57)**		
	Basal	**Final**	***P* value[a]**	**Basal**	**Final**	***P* value[a]**
t_{max} 245 (min)[b]	76.8 ± 12.7	70.3 ± 11.9	<.05	76.4 ± 14.7	76.1 ± 14.5	NS
t_{max} 268 (min)[b]	84.0 ± 14.5	76.6 ± 12.2	<.05	83.2 ± 15.5	82.5 ± 13.8	NS
V_{lag}268 (10^4 × OD/min)[c]	8.7 ± 3.9	10.6 ± 4.7	<.01	8.7 ± 3.3	8.7 ± 3.1	NS
α-tocopherol (μM)	28.8 ± 5.8	26.3 ± 5.9	<.05	29.6 ± 4.9	26.5 ± 4.9	<.01

Note: Sera from women before (basal) and after (final) an IVF cycle were used to analyze the indicated indices of serum oxidizability. The oxidation kinetics was monitored at 245 and 268 nm. Values are mean ± standard deviation. NS, not statistically significant; OD, optical density.

[a] Statistical analysis by Student's *t*-test for paired data.

[b] t_{max}, time at which the oxidation rate is maximal.

[c] V_{lag}, rate of increase of absorbance in the lag phase preceding oxidation.

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reduced TAA, and decreased levels of hydrophilic antioxidants and vitamin E. These results strongly suggest increased ROS production and the presence of oxidative stress. The TAA depends on several factors such as the serum composition and the antioxidant content; albumin, bilirubin, and urate are the major water soluble antioxidants that contribute to the TAA (26, 27). The data presented here were consistent, as we observed decreased levels of albumin, bilirubin, and urate together with lower TAA at the end of the cycle.

Moreover, a linear correlation ($P<.01$) between the serum concentrations of albumin, bilirubin, or urate and the TAA could be found (data not shown). The levels of vitamin E, one of the major antioxidants in the blood, also decreased with the treatment. Due to its hydrophobicity, the vitamin E contribution to the TAA may not be as important as that of water-soluble antioxidants; however, it is the principal molecule responsible for the protection of lipoproteins from oxidation in the circulation. The levels of α-tocopherol were also found to correlate with the TAA ($P<.01$).

The hydrophilic antioxidant vitamin C also contributes to the TAA. Ascorbate levels have been reported to change in response to gonadotropins during a spontaneous cycle. The rapid depletion of ascorbate induced by luteinizing hormone (LH) is the basis for a bioassay (28) that was much used for measurement of LH before the advent of the immunoassay. Changes in ascorbate secretion have been associated with the vitamin requirements in steroidogenesis. The demand for ascorbate is also important in relation to mature follicles. In a preliminary study, a strong correlation was observed between follicular fluid and serum concentrations of ascorbate at the time of oocyte recovery in women undergoing IVF procedures (29). An active uptake by the follicle against a concentration gradient seemed to occur, suggesting the sequestration of ascorbate to facilitate follicular development during the approach to ovulation and postovulatory steroidogenesis (30). Although vitamin C levels were not determined in our study, ascorbate fluctuations could take place during the IVF cycle in response to the gonadotropin treatment, thus contributing in part to a reduced TAA.

Serum oxidizability indicates the susceptibility of lipids to oxidative stress. The kinetics of oxidation, as observed by spectroscopic monitoring of absorbance at either 245 or 268 nm, can be expressed by the maximal rate of oxidation (V_{max}) and the time at which this rate is achieved (t_{max}). The V_{max} is related to the amount of oxidizable lipids, mostly PUFA and cholesterol, whereas t_{max} depends on the concentrations of antioxidants in the serum (19). Reduced in vitro susceptibility for the oxidation of serum, reflected by a decreased t_{max}, would probably be the result of a depletion of the antioxidants and a reduced TAA after the IVF cycle. The V_{max} did not change during the cycle, thus suggesting that the levels of PUFA or cholesterol in the blood remain unchanged.

According to Niki (31), in the lipid peroxidation process the length of the inhibition period (lag time or t_{max}) inversely correlates with the initiation oxidation rate (V_{lag}). In our study,

TABLE 5

Predictive variables of conception cycles in in vitro fertilization by multivariate logistic regression analysis.

Predictive variable	β	*P* value	Odds ratio	95% CI
Model 1[a]				
Oocytes retrieved	0.090	.008	1.094	1.024–1.169
ΔV_{lag} ($10^4 \times$ OD/min)	0.137	.052	1.146	0.999–1.316
Model 2[b]				
BMI (kg/m^2)	–0.242	.013	0.785	0.649–0.950
ΔV_{lag} ($10^4 \times$ OD/min)	0.332	.003	1.394	1.119–1.736
Model 3[c]				
Oocytes retrieved	0.105	.050	1.110	1.000–1.233

Note: β = regression coeffcient; CI = confidence interval; OD = optical density.

[a] Total study population (125 cases). Independent variables included in the logistic model were age, body mass index, number of oocytes retrieved, infertility cause (dichotomous variable), variations (final-basal) of t_{max}, V_{lag}, and α-tocopherol. The ΔV_{lag}, variation (final-basal) of the rate of accumulation of absorbing products during the lag phase. The Δt_{max}, variation (final-basal) of the time at which the oxidation rate is maximal.

[b] Infertility causes other than sole male factor (81 cases).

[c] Sole male infertility cause (44 cases). Independent variables included in the logistic models 2 and 3 were age, body mass index (BMI), number of oocytes retrieved, Δt_{max}, ΔV_{lag}, and $\Delta\alpha$-tocopherol. β, regression coefficient.

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these two parameters were highly correlated (r = –0.638, $P<10^{-6}$). At a fixed rate of radical chain initiation (as is the case of our experimental conditions), V_{lag} is inversely correlated with the potency of radical scavengers in the medium. The observed increase in V_{lag}, along with the reduction of hydrophilic antioxidants, α-tocopherol, and the TAA, reinforces the notion that serum antioxidant defenses become weaker after an IVF cycle. Moreover, in multivariate logistic regression, the variation (final minus basal) of V_{lag} during a cycle was an independent predictor of pregnancy. Women with one unit increase in V_{lag} were 1.39 times more likely to achieve pregnancy.

Another independent predictor of pregnancy was BMI, which was inversely associated with conception cycles (Table 5, model 2). Each one unit (kg/m^2) increase in BMI was associated with a 22% decreased probability of becoming pregnant. Both inadequate and excessive energy intakes have been associated with reduced fertility among women. Moreover, irregular menstrual cycles, ovulatory dysfunction, and later age at menarche have been associated with both low and high BMI (32, 33). The effects of body weight and weight changes on oxidative stress have been scantly investigated. Trevisan et al. (34) reported that BMI is modestly but positively correlated with oxidative stress in women; BMI and dyslipidemia have also been associated with oxidative stress in various pathologic states (35). In our study population, oxidative stress expressed in terms of reduced resistance to in vitro oxidation and decreased antioxidant potency was related to the IVF cycle independent of BMI or lipid profile. A similar lack of association between BMI and oxidative stress has been reported in relation to coronary lipid risk factors (36) or inflammatory markers (37).

Our results emphasize the importance of the contribution of the oxidation kinetic index V_{lag} per se to the pregnancy rate in women under assisted reproduction, independent of age, BMI, or lipid profile. The alterations of the serum antioxidant status associated with the IVF treatment could reflect local changes, particularly in reproductive organs and tissues in connection with the fertilization process. Several studies have related oxidative stress and infertility. Excessive levels of ROS may cause damage to DNA and other molecules inside the oocyte, which are critical for the oocyte maturing process. As a consequence, embryonic fragmentation often takes place during IVF therapy (38). However, despite their toxicity, free radicals also have a role in the physiology of reproduction. The ovulation process is associated with the formation of free radicals (39, 40). In particular, O_2^- generated by NADPH/NADH oxidoreductases is present in the preovulatory follicle, and the NO/NOS system in the ovary is necessary for follicle rupture during ovulation (40). In addition, free radicals mediate the correct implantation of the fertilized oocyte in the maternal uterus and in its subsequent development (41). In this respect, Wiener-Megnazi et al. (42) reported that certain oxidative stress biomarkers located in the follicular fluid were related to the IVF outcome. They found a good correlation between the biomarker and the maximal E_2 levels, number of mature oocytes, and number of cleaved embryos, and they proposed that a certain threshold of oxidative stress may be required for the conception to occur in IVF (42).

In conclusion, the IVF therapy leading to the ovarian stimulation produces a perturbation in the oxidant-antioxidant balance that causes the serum to be less protected against oxidation. The fact that the serum oxidizability index V_{lag}

is a statistically significant predictor of the occurrence of pregnancy suggests a role for ROS in IVF conceptions. To date, there is little information on the correlations between systemic and local levels of ROS in IVF therapy. The role of ROS in fertility requires further investigation, more specifically by approaches using human reproductive tissues.

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