Abstract. Background/Aim: Reactive oxygen species (ROS) are involved in critical biological processes in human reproduction. The aim of this study was to evaluate the association of embryo quality following in vitro fertilization (IVF), with ROS levels in the serum and follicular fluid (FF). Materials and Methods: Eighty-five participants underwent ovarian stimulation and IVF; ROS levels were measured in blood samples on the day of oocyte retrieval and in the FF from follicular aspirates using enzyme-linked immunosorbent assay. These values were associated with the quality of embryos generated. Results: Univariable zero-inflated Poisson model revealed that ROS levels at both oocyte retrieval and in FF were not associated with the number of grade I, II, III and IV embryos (p>0.05). Age, body mass index, stimulation protocol and smoking status were not associated with the number of embryos of any grade (p>0.05). Conclusion: Neither ROS levels in serum nor in FF are associated with the quality of embryos produced following IVF.

The parameters under which in vitro fertilization (IVF) is conducted are strictly controlled, although many factors believed to interfere with gamete quality, fertilization and embryo progression are currently under thorough investigation in an attempt to increase the potential for a positive outcome. Follicular fluid (FF) on this basis has recently emerged as an essential target towards investigation of subfertility factors and IVF failure, since it is directly involved in the development and maturation of the oocyte, providing a nourishing intrafollicular microenvironment.

Reactive oxygen species (ROS) are believed to play an important role in the function of the male and female reproductive system, as well as in embryonic development, since these are involved in critical biological processes in human reproduction (1, 2). ROS exert direct and indirect effects on the dynamics of the oocyte in terms of maturation and fertilization potential, either spontaneously or through assisted reproduction technologies (3, 4). There are reports favoring a direct link between ROS levels and various types of sub-fertility, although this association has not yet been established, possibly due to the selected methodological approaches and outcome evaluation of the respective studies (3, 5-13).

It appears that an optimum level of ROS is necessary to support adequate oocyte development, whilst the notable imbalance in the ovarian environment following controlled ovarian hyperstimulation results in excessively high ROS levels and consequently in oxidative stress. The adverse relationship between ROS levels in the FF and oocyte maturation and fertilization has been reported (14). Increased levels of ROS were found to be linked with failure to achieve pregnancy (15), albeit the standardization of ROS cut-off levels has been established at $10^7$ cps/400 μl FF, beyond which viable embryo formation was not favorable in women with tubal factor subfertility (7).

The rationale of our previous work (5, 16) was based on these inconsistencies, attempting to contribute substantial information towards resolution of these contradictory views. With the emergence of newer data, as with the recent report of Elizur et al. reporting a link between lower concentrations of ROS in the FF and high quality embryos (17), our aim was to provide a quantitative comparison of ROS levels in serum and FF on the day of oocyte retrieval (OR) to determine the association with high-quality
embryos in subfertile women undergoing IVF, using the two most common applied protocols for pituitary desensitization.

**Patients and Methods**

*Patient population and study design.* This was a single-center, prospective, cohort study performed at the Assisted Reproduction Unit of the Third Department of Obstetrics and Gynecology of the Athens University School of Medicine, Attikon Hospital, from September 2011 to December 2013. The study was approved by the Scientific Board (protocol no. 187/24-06-08) and Bioethics Committee of the hospital (approval no. 6/07-07-08). Informed consent was obtained from all patients included in this study. The primary analysis and the respective results have been published elsewhere (16). For the present study, a re-analysis was performed of the ROS values obtained during our initial measurements.

*Sample collection/analysis and embryological scoring.* In brief, the participants underwent controlled ovarian hyperstimulation for the IVF procedures at our clinic, with either gonadotropin-releasing hormone (GnRH) agonist or GnRH antagonist. FF was collected from the first follicular aspirate \( \Phi \geq 1.8 \text{ cm} \) and centrifuged at \( 112 \times g \) for 10 min, then frozen at –60°C, until assayed. An oocyte was retrieved either in the first complete aspirate or during follicular flushing (at a maximum of three attempts); the final aspirate was discarded following its examination for the presence of an oocyte, thus excluded as a sample from ROS measurements. Blood samples were collected 5 min before the OR. ROS values were measured by using a commercially available monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines (Raybiotech, Norcross, GA, USA).

The study population was divided into four study groups, according to embryo quality, as scored at day 3 following fertilization and before placement in the uterus. Grade I corresponds to very good, II to good, III to medium and IV to poor embryo quality. A grade I embryo is defined as having seven or more blastomeres morphological assessment at day 3, equally sized, with <20% fragmentation and no multinucleation, whereas a grade IV embryo is defined as a poor quality embryo of the minimum degree allowed for transfer to the uterus, having four or more blastomeres on day 3 with no cleavage arrest and ≤20% fragmentation (18).

**Results**

Ninety-five women were initially enrolled to participate in the study but sufficient data were only available for 85, with a mean age±SD of the women included in the analysis of 35.7±4.9 years. Demographics, clinical and IVF cycle characteristics of the study population were analytically presented in our previous report (16).
The number of women (and the corresponding percentage) per number of embryos obtained (0 to 8) stratified by each embryo quality category is presented in Table I and Figure 1: 44 women had 1 or 2 grade I embryos, 35 had 1 or 2 grade II, 36 had 1 or 2 grade III and only 11 had 1 or 2 grade IV embryos.

Assessing age, BMI, stimulation protocol and smoking status for potential confounding effect on the number of embryos of all grades, univariable Poisson models were used. None of the four factors were associated with the number of grade I ($p=0.407$, $p=0.414$, $p=0.774$ and $p=0.224$, respectively), II ($p=0.340$, $p=0.289$, $p=0.796$ and $p=0.897$, respectively), III ($p=0.540$, $p=0.401$, $p=0.996$ and $p=0.408$, respectively) or IV embryos ($p=0.445$, $p=0.452$, $p=0.461$ and $p=0.659$, respectively) (data not shown).

Regarding ROS at OR and in FF, models were fitted using as response variable the number of grade I to IV embryos and as explanatory variables ROS levels (at OR and in FF), reporting the beta coefficient with corresponding 95% confidence interval and $p$-values (Table II).

The models revealed that ROS levels at OR were not associated with the number of grade I, II, III and IV embryos ($p=0.061$, $p=0.652$, $p=0.787$ and $p=0.445$, respectively). Similarly, ROS in FF were not correlated with the number of grade I, II, III and IV embryos ($p=0.227$, $p=0.921$, $p=0.345$ and $p=0.788$, respectively).

**Discussion**

Data from 85 participants were analyzed in this prospective cohort study, with determination of ROS levels in both the serum and FF of sub-fertile women undergoing IVF with ovarian stimulation, and direct comparison with the quality grade of the obtained embryos. Our main objective was to demonstrate the potential occurrence of an association between day 3 embryo quality and ROS levels. Our analysis determined that no association exists between ROS levels measured at the day of the OR either in serum or in FF and the quality of embryos, irrespective of grade. Adjustments for age, BMI, smoking and IVF protocol did not affect the outcomes of this study, thus revealing an insignificant effect of these characteristics when comparing ROS values with the resulting embryo grades.

The relationship between ROS levels and IVF outcome has been investigated during recent years, with important findings, although with contradictory views and no solid evidence in any specific direction, either due to the existent variation in the methodology (5) or to the wide fluctuation of ROS levels in oocytes (9). In our recent study, we found that ROS levels in the serum and the FF were not associated with pregnancy outcomes and this was irrespective of the patient’s age, BMI or IVF protocol used (16). In light of newly published data, reporting a negative correlation...
between follicular ROS levels and quality of the oocyte, we proceeded to a second analysis of our results by retrieving and re-analyzing data from the initial cohort with statistical significance, using zero-inflated Poisson models. In order to shed more light on the pathophysiology of a putative such relationship, and more importantly on the nature of ROS involvement in IVF outcomes, we also compared serum ROS at the day of OR, values that have been reported to be the highest during controlled ovarian hyperstimulation, with the quality of embryos obtained.

Our findings are in accordance with previous reports, where neither ROS, nor antioxidant enzyme activities and concentration within FF have been associated with embryo quality in an IVF cycle, by including number of blastomeres and degree of fragmentation in the process of embryo classification according to quality (3, 9, 19).

In contrast, many studies have demonstrated a negative correlation of elevated ROS levels with embryo quality (7, 12, 20); others have determined a positive correlation (8), whilst others report that lower levels predict decreased fertilization potential thus reduced oocyte competence, presumably leading to poor embryo quality in the occurrence of fertilization (21, 22). Recently, Elizur et al. assessed ROS, specifically H_2O_2 levels in sibling follicles, with observation that the highest H_2O_2 values resulted in embryos of poor quality and the lowest ROS values corresponded to empty follicles; the authors linked the presence of increased ROS levels with follicular aging, incompetence to produce good embryos, and atresia (17).

A study by Prieto et al. underlined the importance of the imbalance between pro-oxidants and antioxidants (23), which exerts a special impact on folliculogenesis and embryonic development, negatively affecting oocyte and embryo quality, in women with endometriosis. Similarly, Das et al. (6) and Jana et al. (7) concluded that the effect of ROS on embryo formation is favorable up to a certain threshold, which appears to be around 100 cps, with detrimental effects of ROS becoming significant beyond this range, with a reduction in the oocyte fertilization potential and a subsequent generation of embryos with poorer quality. The general estimate so far is that a certain threshold of oxidative stress, with a physiological quantity of ROS, may be required for the maturation of a competent oocyte with the dynamics to generate a good-quality embryo. This theory was proposed in its initial form 15 years ago (3), together with evidence on the presence of ROS in the FF of women undergoing IVF, showing a direct relationship between elevated ROS levels and clinical pregnancy rates (p < 0.03), but not with the number of oocytes recovered or fertilized (p = 0.2). We believe that the ROS threshold in the FF varies among sub-fertile women as a consequence of various confounding factors, such as sub-fertility cause, indicating an individual intrafollicular status, and not the hormonal potential of the entire ovary. Interestingly, adjustment for confounding factors did not change the results obtained. This was rather peculiar as all the parameters examined exert a negative effect on IVF outcome parameters.

The visible limitations of our study are mainly attributed to its nature as a prospective cohort study: the lack of power calculation, blinding and proper randomization are linked with possible confounders and selection bias. Moreover, residual confounding, such as antral follicle count and response to stimulation, cannot be excluded and this might potentially have an effect on the strength of the association between baseline ROS levels and the a priori defined outcomes. Clinical limitations may also introduce a degree of effect on the reported outcomes of the study. In an attempt to preserve consistency throughout the measurements, ROS levels were determined by examining the first follicular aspirate and although there is an increased incidence of oocyte retrieval in the first aspirate, this may not be feasible until subsequent flushings. In an ideal but impractical methodological design, we should attempt to include all aspirates from each follicle individually through multiple vaginal punctures, a procedure that would introduce delay and elevated risk in clinical practice (24). In addition, follicular selection was performed according to follicular size, assuming that the representative size reflecting oocyte maturity and fecundation capacity is >18 mm (25), thus disregarding smaller follicles in the process.

Future studies should include greater sample sizes deriving from women undergoing IVF for different causes in order to highlight the critical role of oxidative stress markers and their optimum levels in the female reproductive system.

Conflicts of Interest

The Authors declare no conflict of interest. We also state that we have full control of all primary data and we agree to allow the Journal to review the data if requested.

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