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Antioxidants reduce oxidative stress in follicular fluid of aged women undergoing IVF

Alice Luddi¹, Angela Capaldo¹, Riccardo Focarelli², Martina Gori¹, Giuseppe Morgante¹, Paola Piomboni^{1*} 
and Vincenzo De Leo¹

Abstract

Background: The status characterized by the imbalance between pro-oxidants and antioxidants molecules, defined as oxidative stress, has been suggested to be involved in the pathogenesis of subfertility in females. This study aims to evaluate the impact of a complete micronutrients supplementation on oxidative stress levels in follicular microenvironment as well as on in vitro fertilization (IVF) outcome.

Methods: This preliminary study was conducted between January 2014 and July 2015 at the Siena University Hospital Infertility Clinic. Serum and follicular fluid were collected from infertile women aged > 39 years who underwent two in vitro fertilization cycles: in the first cycle they were treated with GnRH-antagonist protocol and gonadotropins for controlled ovarian hyperstimulation, whereas in the second cycle ovarian stimulation protocol was associated to micronutrients supplementation, starting three months earlier. Protein oxidation levels and total antioxidant capacity in serum and in follicular fluid were evaluated in IVF cycles with or without micronutrients supplementation. Differences in IVF outcome parameters were statistically evaluated.

Results: Two-dimensional electrophoresis analyses demonstrated that when patients assumed micronutrients before IVF cycles, follicular fluid and serum proteins were protected from oxidative damage. Comparable results were obtained when total antioxidant capacity was measured. Moreover, the mean number of good quality oocytes retrieved when patients received micronutrients supplementation was significantly increased.

Conclusion: The additional treatment with micronutrients, starting three months before IVF cycles, protects the follicular microenvironment from oxidative stress, thus increasing the number of good quality oocytes recovered at the pick up.

Keywords: Micronutrients, In vitro fertilization, Follicular fluid, Oxidative stress, Oocyte quality

Abbreviations: FF, Follicular fluid; GnRH, Gonadotropin-releasing hormone; hCG, Human chorionic gonadotropin; ICSI, Intra-cytoplasmic sperm injection; IVF, In vitro fertilization; OS, Oxidative stress; ROS, Reactive oxygen species; VDR/RXR- α , Vitamin D receptor and retinoid X receptor-alpha heterodimer

Background

Recent studies on the pathophysiology of couple's infertility have shown that oxidative stress (OS) may be one of the causative factors of female infertility [1–3]. The OS is currently defined as an increase in the level of reactive oxygen species (ROS) due to the decreased

antioxidant capacity of the cells to scavenge and remove these free radicals [4]. The oxidizing species are products of normal metabolic activity that, at physiological concentration, play pivotal roles both at cellular and systemic level. Numerous protective mechanisms that prevent the formation of reactive species, or their removal before they can damage cell components, have been identified [5]. Indeed, the impairment of this physiological balance between ROS production and elimination, may result in the alteration of cellular structures and

* Correspondence: paola.piomboni@unisi.it

¹Department Molecular and Developmental Medicine, University of Siena, Policlinico Le Scotte Viale Bracci, Siena 53100, Italy
Full list of author information is available at the end of the article

macromolecules ultimately leading to cell death [6]. The main targets of ROS are lipids, nucleic acids, carbohydrates and proteins [7]. Oxidative reactions may completely alter physical/chemical properties of proteins by proteolysis, structural modifications, aberrant combinations, oxidation of the side chains of amino acids [8]. In fact, the oxidation of the -R groups of amino acids, cause the variation of the isoelectric point, whereas the formation of intramolecular bonds and/or protein cleavage into peptide fragments induces alteration of the molecular weight [9–12]. Moreover, proteins having in their backbone cysteine and methionine, sulfur-containing amino acids, are more susceptible to the attack of ROS, since thiols may undergo reversible oxidization [11, 13].

It appears that oxidative stress plays an essential physiological role in the modulation of a full spectrum of reproductive functions, such as oocyte maturation, ovarian steroidogenesis, formation of the corpus luteum and luteolysis, fertilization, embryo development and pregnancy [2, 4, 14]. The impact of OS on the reproductive potential has been investigated by several studies focused on the microenvironment surrounding the oocyte [15–20]; all these study clearly highlighted that there is a complex relationship between ROS and antioxidants in the ovary. In particular, oxidative damage has been implicated as a causal factor in the oocytes quality. The failure of antioxidant defense mechanisms to neutralize OS, allows free radicals to alter the cellular redox state, with decreased total antioxidant capacity in follicles accompanied by poor oocyte fertilization [1, 16, 21, 22].

Numerous protective substance that counteract the formation of reactive species, have been hitherto identified [23, 24] and numerous commercial preparations containing antioxidant micronutrients have been developed. Among the others, the particular micronutrients supplement, we assessed in this study, represents a suitable preparation for its broad composition, including vitamins, folates and minerals. Vitamin E and A have been reported as potent non-enzymatic antioxidants that play a significant role in protecting the integrity of the cell membrane and in promoting reproductive function. In humans it has been shown that vitamin E reacts with free radicals transforming them in hydroperoxides, substance safely metabolized by the cell; in addition, vitamin E interacts and neutralizes three important ROS, e.g. superoxide anion, hydrogen peroxide and the hydroxyl radical [23]. Finally, vitamin E is also able to interrupt the process of lipid peroxidation acting as a chain-breaking antioxidant.

It has been demonstrated that Vitamin A and carotenoids are able to protect the cells from superoxide radicals and, most importantly, a decrease in their serum concentration has been associated with anovulation [25].

Also folate has been reported as effective antioxidants, so that women receiving folic acid supplementation had a better quality oocytes and a higher degree of mature oocytes compared with women who did not receive folic acid supplementation [26].

Based on these observations, this study aimed to evaluate the effectiveness of a complete micronutrients supplementation on the level of oxidative stress, both in serum and in follicular fluid (FF), of infertile women aged >39 years who undergo assisted reproductive technologies.

Methods

Patients

The study was approved by Ethic Committees of Siena University. Signed informed consent was obtained from all patients who participated in the study, performed at the Center for Couple Sterility, Siena University Hospital, between January 2014 and July 2015. A total of 18 patients aged >39 years undergoing IVF/ICSI treatment were included in the study. All patients have been unable to conceive naturally for at least 1 year before entering the study. The reasons for the couples' infertility were male factor infertility ($n = 8$), polycystic ovary syndrome ($n = 6$), tubal occlusion ($n = 3$) and endometriosis ($n = 1$). The physical and demographic characteristics of all recruited women are shown in Table 1. All patients underwent two IVF cycles within a period of 9–12 months: in the first cycle they were treated with gonadotropins for ovulation induction, while in the second cycle gonadotropin treatment was associated to micronutrients supplementation (Elevit, Bayer; 1 cp/day), starting three months earlier.

Ovulation induction

Ovarian stimulation was performed administering recombinant gonadotropins (Pergoveris, Merck-Serono, Rome, Italy) at a dose of 150–300 IU per day from the 1st or 2nd day of spontaneous or induced menstruation. The dose of gonadotropins was adjusted according to ovarian response, as detected by ultrasound examination.

Table 1 Characteristics of patients who underwent the two IVF cycles, without (untreated) and with (treated) micronutrients supplementation

| Variable | Untreated | Treated | <i>P</i> value |
|--------------------------------------|-------------|-------------|----------------|
| No. of patients | | 18 | — |
| Age (yrs) | | 40.3 ± 1.2 | — |
| Duration of infertility (months) | | 26.1 ± 8.2 | — |
| Body mass index (kg/m ²) | | 22.5 ± 3.0 | — |
| Duration of stimulation (days) | 11.5 ± 0.9 | 12.1 ± 1.4 | NS |
| E2 peak level | 2,315 ± 490 | 2,270 ± 515 | NS |

Note: Values are mean ± SD. NS not significant

As soon as the dominant follicle reached 14 mm in diameter, a gonadotropin-releasing hormone (GnRH) antagonist was administered daily, until the day of ovulation triggering which was obtained by HCG injection, when at least three follicles of size >18 mm were present in the ovaries. The oocyte pick-up was performed 34–36 h after the human chorionic gonadotropin (hCG) injection. Follicular fluid was aspirated and after collecting the oocytes the fluid was centrifuged for 10 min at 1500 rpm. Only follicular fluid samples with no macroscopic evidence of blood were selected. For each patient, at the day of oocyte pickup, blood serum samples for comparative analysis was collected.

Oocyte morphology

Oocyte morphology was evaluated according to previously published criteria [27]. Dimorphisms were subdivided into intracytoplasmic and extracytoplasmic. In the first case we evaluated the presence of incorporations, refractile bodies, vacuoles, aggregation of the smooth endoplasmic reticulum and dense granulation. The assessment of extracytoplasmic dimorphism was based on the first polar body morphology, perivitelline space size and granularity, zona pellucida defects and shape anomalies. We considered high quality oocytes those cells without any of the previously described alterations. The evaluation was carried out by two different embryologists, blinded to the study protocol.

The FRAP assay

The total antioxidant capacity (TAC) was measured as ferric-reducing ability of follicular fluid and serum (FRAP), according to Benzie and Strain [28], by spectrophotometric quantification. Briefly, working FRAP reagent was prepared by combining in the ratio of 10:1:1 a 300 mM acetate buffer pH3.6, 10 mM 2,4,6-tri-(2-pyridyl)-1,3,5-triazine, in 40 mM HCl and 20 mM FeCl₃. A 50 µl aliquot of follicular fluid or serum was added to 1 ml of FRAP reagent and 10 min later the absorbance measurement was taken at 593 nm. As standard reference, 50 µl of the standard (FeSO₄ 1 mM) was added to 1 ml of FRAP reagent. All measurements were taken at room temperature with samples protected from direct sunlight.

Follicular fluid and blood serum proteins analysis

Free -SH groups labeling with MPB

This assay was carried out according to a modified protocol as described by de Lamirande and Gagnon [29]. A solution of 3-N-maleimidopropionyl biocytin (MPB; Sigma Aldrich, St Louis, MO, USA) 1 mM in Tris-HCl 15 mM pH 6.8 was added to equivalent amount of FF proteins, in a final volume of 100 µl. Protein concentration

was determined by Bicinchoninic Acid Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instruction. The mixture was then heated at 95 °C for 5 min and immediately processed for 2D electrophoresis.

Two-dimensional electrophoresis

Two-dimensional (2D) electrophoresis was carried out as described: 30 µg of total protein were resuspended in rehydration solution and mixed with 0.2 % immobilized pH gradient buffer (IPG, GE Healthcare, Uppsala, Sweden) with a 3–11 nonlinear pH range. Samples were loaded onto Immobiline DryStrips with immobilized nonlinear pH gradient, ranging from pH 3 to 11 (GE Healthcare). Isoelectric focusing was performed as described by Görg et al. [30] and Bjellqvist et al. [31]. Isoelectric focused strips were equilibrated for 15 min with 50 mM Tris-HCl pH 6.8 containing 30 % glycerol, 6 M urea, and 2 % sodium dodecyl sulfate (SDS) and for an additional 5 min in the same solution containing 2.5 % iodoacetamide (IAA) and 0.1 % bromophenol blue, and then placed on a 6–16 % polyacrylamide linear gradient SDS gel according to Laemmli et al. [32].

Western blotting

For western blotting analysis, proteins were electroblotted from polyacrylamide gels to nitrocellulose according to Towbin et al. [33]. Membranes were then incubated with streptavidin conjugated to peroxidase in TBS containing 0.2 % Tween 20, with 1 % non-fat dry milk. Reactivity was detected using an Immuno-Star HRP Chemiluminescent kit (Bio-Rad Microsciences) and revealed with an XRS instrument ChemiDoc (Bio-Rad Microsciences, Hemel Hempstead, UK). Images were then processed using the Quantity One® 4.5.7 and PDQuest™ 7.4.0 softwares (Bio-Rad Microsciences) for spots identification and quantification as pixel/mm².

Statistical analysis

Statistical analysis was performed with the software Graph pad Prism 4 (GraphPad Software, San Diego, CA, USA). The standardized values of skewness and kurtosis will be used to verify the normal distribution of data. Parametric or non-parametric tests were used when appropriate. Statistical significance will be set at a value of $P < 0.05$.

Results

Demographic features and clinical data of patients are presented in Table 1. Total FSH-LH units, number of days of stimulation, peak E2 levels at hCG administration were not significantly different between the two IVF cycles (without or with micronutrients supplementation) in the enrolled patients. In order to detect the oxidative stress levels, we measured the total antioxidant capacity

and ROS induced-damage on proteins both in follicular fluid and in serum. By means of the FRAP assay we measured total antioxidant capacity showing a significant increase in TAC both in follicular fluid and in serum of women treated with micronutrient supplementation (Fig. 1). In order to confirm these data, we analyzed all samples by two dimensional electrophoresis to monitor the presence of free-SH residues in serum and follicular fluid proteins. In Fig. 2, a representative 2D-electrophoresis of serum and follicular fluid proteins is showed. By this procedure, numerous spots distributed in a pH range of about 4–7 with an apparent molecular weight ranging between 15 and 75 kDa (Fig. 2a and c) were evident in patients not receiving micronutrient supplementation (untreated) during the first IVF cycle. When the same analysis was performed in patients receiving a micronutrient supplementation in the second IVF cycle, starting from three months before controlled ovarian stimulation, we observed an increased number of proteins with free thiol groups (Fig. 2b and d). Therefore, a significant recovery of a physiological level in the overall oxidative stress status in these biological fluids after treatment is demonstrated. Indeed, both in blood serum and follicular fluid of treated women, 2D electrophoresis detected numerous spots distributed between pH 4 and 7, with a wide molecular weight ranging from about 60 to 200 kDa (Fig. 2b and d). The quantitative analysis of main spots enabled us to highlight a statistically significant increase in spot intensity in both serum and follicular fluid recovered from women treated with micronutrient supplementation ($p < 0.05$) in comparison to untreated (Fig. 2). As regard to IVF outcome parameters, we observed that the mean number of retrieved

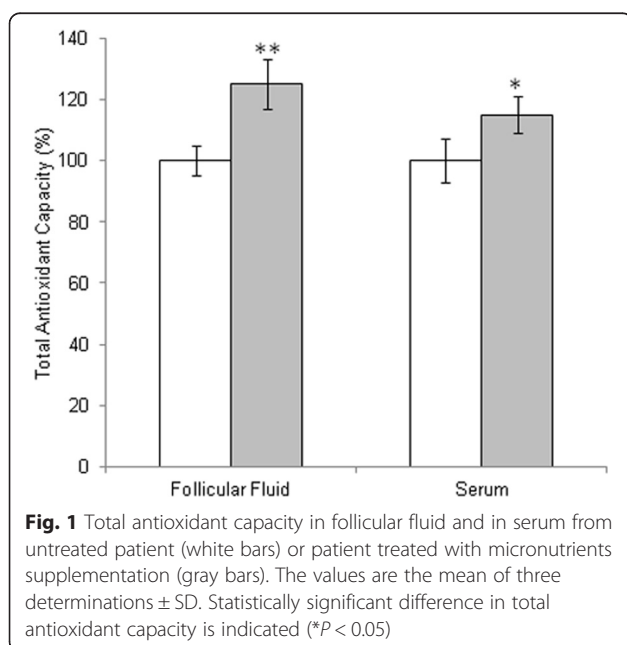
oocytes did not differ between the two cycles, whereas the mean number of oocytes not suitable for injection was significantly reduced (1.20 ± 0.77 vs. 1.88 ± 1.01 ; $P = 0.01$) in the micronutrients supplemented cycles. Fertilization and cleavage rates as well as the mean number of top-quality transferred embryos did not differ significantly (Table 2). Nevertheless, in the treated group, we registered a total of 3 ongoing pregnancies (pregnancy rate = 17.7 %), which represent an encouraging results in aged women undergoing to IVF.

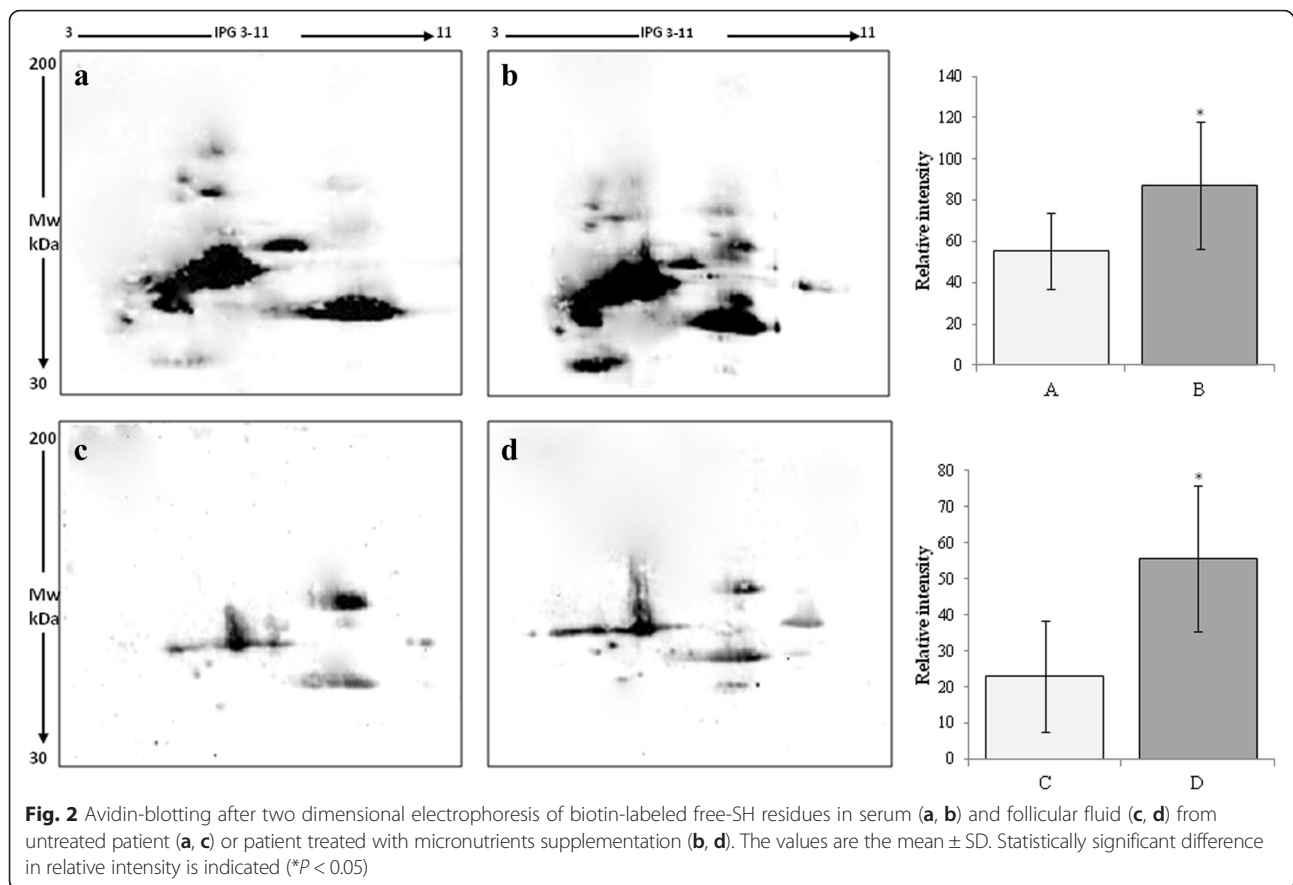
Discussion

Data from this study suggest that patients assuming micronutrients before IVF cycles have a high level of free-SH groups in follicular fluid and serum proteins, indicating a protective activity from oxidative damages by this supplementation.

The oxidative stress and the ROS level in biological fluid may be indirectly measured by a proteomic approach, that allow the accurate evaluation of oxidative stress induced-damage on proteins [34, 35]. The main target of free radicals are proteins that undergo to oxidation of the side chains of amino acids [9, 12] where cysteine and methionine are key components in the mechanism involved in redox balance, since thiols group (-SH) may be oxidized in an almost irreversible manner [13, 36, 37]. Indeed, an inverse correlation between levels of free-SH group in proteins from biological fluids and oxidative stress may be established. By this way, we showed in both serum and follicular fluid a dramatic increase in the level of protein free -SH groups in women treated with a micronutrients supplementation, suggesting a significant decrease of the oxidative stress in the follicular microenvironment. In this study, we confirmed the efficacy of micronutrient supplementation also by means of FRAP, a sensitive and reproducible approach commonly used to monitor total antioxidant capacity in biological fluid. This strong correlation is very important since confirm our proteomic strategy as an excellent approach to study ROS levels in biological sample. It should be outlined that the direct evaluation of the oxidative stress levels in biological fluids is very difficult because the cellular elements that produce them are absent, and because the radical species are generated and dissolve continuously. The demonstrated close relation between FRAP results and protein free -SH groups in biological samples will allow the researchers to specifically evaluate the effect of ROS by indirectly measuring the protein damage levels.

The supplementation seems to be effective not only in diminishing oxidative stress, but also in increasing oocyte quality; in fact, a significant decrease in the mean number of oocytes not suitable for injection could be observed. Our data confirmed numerous publications





reporting the antioxidant capability of several micronutrients that, accordingly, are regarded as potent antioxidants. It has been reported that folates effectively scavenge oxidizing free radicals by inhibiting lipid peroxidation [34], thus playing a key role in oocyte quality and maturation [35]. In this regard, poor folate status seems to be detrimental also due to its involvement in cell

Table 2 Oocyte maturity and embryo score in the same patients who didn't received (Untreated) or received (Treated) micronutrients supplementation form three months

| Characteristic | Untreated | Treated | <i>P</i> value |
|--------------------------------------|-----------------|-----------------|----------------|
| No. of retrieved oocytes | 6.76 \pm 4.12 | 6.37 \pm 3.31 | NS |
| No. of MII oocytes | 5.14 \pm 3.49 | 4.07 \pm 3.04 | NS |
| No. of not suitable oocytes (GV-DEG) | 1.88 \pm 1.01 | 1.2 \pm 0.77 | 0.01 |
| Fertilization rate | 0.69 \pm 0.15 | 0.71 \pm 0.28 | NS |
| Cleavage rate | 0.84 \pm 0.04 | 0.85 \pm 0.07 | NS |
| No. of embryos transferred | 2.01 \pm 0.55 | 1.96 \pm 0.75 | NS |
| Embryo score grade 1 (%) | 0.96 \pm 0.5 | 0.91 \pm 0.45 | NS |
| Embryo score grade 2 (%) | 0.87 \pm 0.75 | 0.84 \pm 0.71 | NS |
| Embryo score grade 3 (%) | 0.29 \pm 0.64 | 0.30 \pm 0.5 | NS |

Note: Values are mean \pm SD. The embryos were scored according to the criteria established by Ebner et al. [27]. *DEG* degenerated oocytes, *MI* metaphase II, *NS* not significant; *GV* germinal vesicle

division (e.g. of oogonia and/or granulosa cells), inflammatory cytokine production and defective methylation reactions [38]. The latter, in turn, are the main regulators of DNA synthesis, a pivotal process in oocytes development. It has been reported that several enzymes involved in DNA synthesis are vitamin- or zinc-dependent [39]. Zinc might also affect female fertility due to its antioxidant properties: in fact, zinc can counteract the oxidation by binding sulphhydryl groups in proteins and by occupying binding sites for iron and copper in lipids, proteins and DNA [40, 41]. In this regard, the supplementation of a chelated form of zinc may contribute to the reduction of oxidative stress, while retaining safety since assumed at dose far below the tolerable upper level of 40 mg/day. Also the potential toxicity of cations, such as iron and calcium, is hindered by their low dose. Moreover, it is remarkable that women with normal menstrual cycles could have deficiency of such compounds. An influence of these mineral on oocyte development and fertilization process has been highlighted. It has been reported that the consumption of iron supplements and non-heme iron from foods may decrease the risk of ovulatory infertility [41], and that oxidative stress in unfertilized mouse oocytes induces abnormal calcium oscillation [42].

Also vitamins seems to play a key role in oocyte maturation, since their deficiencies have been linked to a diminished fertility in animals [43]. In a recent study, we have demonstrated that vitamin receptor VDR/RXR- α (vitamin D receptor and retinoid X receptor- α heterodimer) are involved in follicular fluid homeostasis, suggesting the existence of a close functional correlation among follicular protein and micronutrients [44]. VDR has been hypothesized to control several human genes, thus vitamin D aberrant endogenous production or dietary up-take may severely affect the follicular physiology [42]. Vitamin D supplementation has been recently demonstrated to increase the serum soluble form of Receptor for Advanced Glycation End-products (sRAGE) [45]. sRAGE was also detected in HFF and its incremented concentration has been associated to positive IVF outcomes [46, 47]. These soluble receptors exert a “scavenger” activity of advanced glycation end-products (AGEs), potent pro-inflammatory molecules that cause ROS generation. The AGE/sRAGE interaction, by preventing the binding of AGE to cellular receptor (cRAGE), counterbalances the well known detrimental effects of AGE [48]. In summary, by inducing the expression of sRAGE through VDR/RXR- α , vitamin D may modulate AGE/RAGE-system, thus diminishing follicular fluid oxidative stress.

Vitamin E has been reported as a scavenger of lipid peroxyl radicals and suppresses the generation of lipid hydroperoxides from cell membrane phospholipids, by acting synergistically with other antioxidant nutrients including vitamin C, carotene, melatonin and selenium [23]. The latter is reported to act both as an antioxidant and anti-inflammatory mediator. The antioxidant activity is achieved by the reduction of lipid and hydrogen peroxides, thus lowering free radicals and ROS spreading. Moreover, selenium supplementation plays a pivotal role in regulating thyroid function by improving the conversion of plasma thyroxine (T4) into the active hormone, T3 [49].

Our data suggest that the appropriate administration of these micronutrients may therefore have significant effects on redox status of follicular microenvironment and, ultimately, on IVF outcome.

The evidence that oocyte maturation, ovulation, luteolysis and follicle atresia are affected by ROS unbalance along with the demonstration that the oxidative stress might be influenced by micronutrients intake, supports our results reporting the effectiveness of this micronutrients supplementation also in term of oocyte quality. To our knowledge, only limited information is available on the impact of dietary micronutrients supplementation on IVF outcome.

Conclusion

In conclusion, the biochemical evaluations carried out in the present preliminary study points out that a micronutrients

supplementation may decrease oxidative stress both in serum and in follicular fluid proteins and it is positively associated with oocytes quality. These information may be helpful for clinicians in the management of women undergoing IVF treatment.

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Availability of data and materials

All data are available by contacting Dr. Alice Luddi at luddi@unisi.it.

Authors' contributions

LA, study design, data collection and analysis, manuscript writing and revision; CA, proteomic analysis, oocyte/embryo scoring; FR, data collection and manuscript revision; GM, data collection; PP, study design, oocyte/embryo scoring, data analysis, manuscript revision and expert knowledge; MG, oocyte aspirations, expert knowledge; DLV, oocyte aspirations, study design and coordination, data analysis, manuscript revision and expert knowledge. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by Ethic Committees of Siena University (CEAVSE). Signed informed consent was obtained from all patients who participated in the study, performed at the Center for Couple Sterility, Siena University Hospital, between January 2014 and July 2015.

Author details

¹Department Molecular and Developmental Medicine, University of Siena, Policlinico Le Scotte Viale Bracci, Siena 53100, Italy. ²Life Sciences Department, University of Siena, Siena 53100, Italy.

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