

Controlled, prospective, observational study on the efficiency and tolerability of a combination of potential Nrf2-inducing antioxidants and micronutrients as pre-treatment for ICSI in dyspermic patients with previous failure

R. RAGO¹, M. GALLO¹, A. DAL LAGO¹, E. LICATA¹, G. PACIOTTI¹, M. AMODEI¹, C. MENEGHINI¹, C. FABIANI¹, G. DANI¹, C. LIBERANOME¹, D. ANTONACI¹, R. CORNO¹, D. MIRIELLO¹, G. GIUFFRIDA², B. GIAMMUSSO³

¹Physiopathology of Reproduction and Andrology Unit, Sandro Pertini Hospital, Rome, Italy

²Assisted Reproduction Center, Catania, Italy

³Andrology Unit, Morgagni Hospital, Catania, Italy

Abstract. – **OBJECTIVE:** To evaluate the efficiency of pre-treatment in dyspermic males in IVF couples with a combination of micronutrients, for the purpose of improving the fertilization rate, the implantation rate and the outcome of the pregnancy.

PATIENTS AND METHODS: This controlled prospective clinical study was performed in two medically assisted reproduction centers. 59 males with mild oligo-astheno-teratospermia (OAT) were admitted to the study. All of them had a history of previous *in vitro* fertilization (IVF) attempts with female partners aged < 40 diagnosed having tubal or idiopathic infertility. The subjects upon enrolment underwent a semen test and afterward were treated with alpha lipoic acid and glutathione (Fertiplus SOD®, Idi-Pharma, Catania, Italy) for 4 weeks (short-term). The primary endpoints that were evaluated are the following: fertilization rate (mean fertilization), implantation rate and pregnancy rate.

RESULTS: At the end of this study all the males (mean age 39.5 ± 5.1) reported in not having any side effects during the administration of Fertiplus. Their female partners (mean age 34.9 ± 4.5) underwent IVF using the ICSI technique. The number of oocytes retrieved and inseminated was not statistically different in comparison to previous attempts, but with the same number of oocytes treated, the fertilization rate per couple demonstrated statistically significant increase ($p < 0.001$). We did not observe a percentage increase in evolutionary embryos, but we noticed an improvement in embryo quality per individual couple ($p < 0.001$), associated with a net increase in the implantation rate per couple ($p < 0.001$) in

terms of clinical pregnancy. The estimated miscarriage risk after treatment was five times lower ($p < 0.001$).

CONCLUSIONS: Short-term treatment with micronutrients in dyspermic subjects can improve the reproductive outcome of the IVF procedure.

Key Words:

Superoxide dismutase (SOD), Reactive oxygen species (ROS), Antioxidants, Micronutrients, Infertility, IVF.

Introduction

In the mature spermatozoa, unlike other cells, the high concentration of unsaturated lipids is associated with a relative insufficiency of reactive oxygen species elimination mechanisms^{1,2}. This lack is, therefore, balanced with the strength of the antioxidant system present in the seminal plasma. Many studies³⁻⁵ have shown that seminal plasma contains significant amounts of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) dependent activity, associated with significant concentrations of antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E) and GSH. This antioxidant system works in an integrated way and if the process of hydrogen peroxide elimination doesn't work in the presence of transition metals, then toxic hydroxyl radicals are produced. Therefore, the whole system must work in synergy and an alteration of

one of the components can potentially lead to a harmful accumulation of reactive oxygen species (ROS). Physiologically, homeostasis between free radicals and antioxidant substances is guaranteed by very complex systems; the most efficient seems to be the system mediated by the Nrf2 (nuclear factor [erythroid-derived 2]-like 2 transcription factor) pathway, which regulates a wide variety of antioxidant cytoprotective enzymes through a promotion sequence known as ARE (antioxidant response element)⁶. Being able to activate the endogenous antioxidant system would offer undoubted advantages, since the production of antioxidant substances would be proportionate to the oxidative stress levels and lead to a correct balance of free radicals and antioxidant substances. Recent *in vitro* and *in vivo* trials support the theory that Nrf2 activation strategies could effectively combat oxidative stress. What is more, Nrf2 deficiency has been associated with poor sperm quality, so much so that Nrf2 could be considered a marker of male factor infertility or subfertility^{7,8}. Diterpene carnosol and polyphenol hydroxytyrosol are two of the most efficient molecules able to activate the ARE-induced Nrf2 system⁹⁻¹¹. All these indications clarify the concept that if oxidative stress is not compensated during spermatogenesis and/or epididymal maturation, then we will have an irreversibly impair motility and acrosome reaction, leading to a state of reduced fertility. The harmful impact of radicals and toxic products on spermatogenesis has been studied in depth, particularly as regards the negative effects of ROS on sperm structures¹²⁻¹⁵. Their excessive production and/or reduction in the spermatozoa damage the sperm DNA, reduce sperm motility and alter the membrane¹⁶⁻¹⁸. Certain andrological disorders, such as varicoceles and genital inflammation, are associated with an increased risk of sperm lipid peroxidation^{19,20}. Observational studies^{21,22} have found a lower rate of aneuploid spermatozoa in men who consumed more foods with antioxidant properties. From these observations, numerous clinical findings have suggested that antioxidant supplementation can help to preserve the balance between ROS production and its clearance, and improves sperm quality. It is, therefore, important to evaluate the impact of oxidative damage on the sperm used in IVF procedures^{23,24}. Identifying and treating the cause of increased ROS production should be the first step for all male patients that present subfertility/infertility, since supplementation with antioxidant nutrients could improve seminal plasma scavengers capacity.

However, there are numerous disputes in literature regarding the efficacy of antioxidant therapy in infertile men²⁵⁻²⁷. The various outcomes obtained from different researches could be due to variables such as biochemical differences between the various antioxidants used and the non-standard compositions and doses of the compounds used. We, therefore, conducted a prospective multicenter observational study designed to assess the efficacy of a combination of enzymatic and non-enzymatic antioxidants (microencapsulated SOD, Extramel[®]), alpha lipoic acid and glutathione (low dose), zinc, B vitamins, and a complex of micronutrients (ORISOD) of plant origin containing substances such as hydroxytyrosol and carnosol able to activate the endogenous intracellular antioxidant system (Nrf). These substances are contained in a product called Fertiplus SOD (Idi-Pharma, Catania, Italy), which was administered to all male in the 30 days before the IVF procedure, with the aim of assessing the effects of the therapy in terms of fertilization, implantation and clinical pregnancy.

Patients and Methods

Study Group Selection

The trial adhered to the Helsinki Declaration and the protocol was approved by the Institutional Review Boards. All patients signed a written informed consent before entering the study. 59 couples preparing for ICSI procedures were enrolled in accordance with the established inclusion criteria: male partners aged between 30 and 50 years, suffering from infertility characterized by alteration of at least one of the seminal parameters (such as concentration/ml, progressive motility and morphology), who had already undergone at least one of ICSI cycle in the 6 months preceding the study. Couples in which the male partner presented endocrine diseases of the hypothalamus-pituitary-testicular axis, varicoceles, accessory gland infections (MAGI) or chromosomal disorders were excluded from the study. Female partners were aged < 40 and had a diagnosis of tubal or idiopathic infertility. During the enrolment phase (before therapy - Pre), all the males underwent the following examination: clinical history, internal medicine/andrology check-up, semen analysis²⁸, testicular Doppler ultrasound, hormone screening (FSH, LH, total testosterone, PRL) and microbiological screening for *Chlamydia* and *Mycoplasma*. All patients with a sperm

concentration $<1 \times 10^6/\text{ml}$ underwent a genetic screening of Y chromosome microdeletions. During the second medical examination (post therapy-Post), after having assessed the relative test results and obtained informed consent, a therapy with Fertipus SOD (50 mg) 1 tablet/day for 30 days was prescribed before undergoing IVF treatment with ICSI.

Ovarian Stimulation Protocol

All the female partners completed the following ovarian folliculogenesis stimulation protocol: r-hFSH 225 IU s.c. from the second day of the menstrual cycle combined with a GnRh antagonist from the 6th day of their menstrual cycle. When the follicular diameter reached 18-20 mm, hCG 10.000 IU s.c. was administered. Transvaginal oocyte retrieval (TVOR) was performed 36 h after hCG administration. The luteal phase was supported by intravaginal progesterone 600 mg/day²⁹. Oocytes in metaphase II were used for ICSI. Embryo transfer was performed on the 3rd day after oocytes retrieval. The blood test for β -subunit of human chorionic gonadotropin (β hCG) assay was scheduled 12 days after embryo transfer. In this case, the β hCG was monitored until the gestational chamber was visible on the ultrasound.

Statistical Analysis

Critical value for α in the study was taken 0.05. Measures of the identified variables were collected before and after the treatment for each couple. Since we found an abnormal distribution of these variables (Shapiro-Wilk test $p < 0.001$) we then used the Wilcoxon signed-rank test to perform the sta-

tistical analysis. The clinical pregnancy rate (number of clinical pregnancy vs. number of transfers) was evaluated on the whole pool of patients and the equality of proportions was tested with Pearson χ^2 statistic test. The risk of abortion was evaluated by Z-test on risk ratio. The correlation with age was evaluated by Pearson's product-moment correlation and linear model fitting. The total number of test performed was 9 and the cases of statistical relevant difference always showed a $p < 5 \text{ E-}4$. Therefore, no post hoc correction was evaluated.

Results

All 59 patients completed the study without side effects due to the therapy. The statistical analysis of the pre- and post-treatment seminal parameters did not reveal any statistically significant differences. The mean FSH, LH, total testosterone and PRL concentrations were within normal limits and comparable in the two groups, with no statistically significant differences. The statistical analysis did not reveal significant differences between the two groups as regards oocyte retrieval during metaphase II in phase Pre and Post (5.8 ± 2.3 vs. 6.0 ± 2.7). The mean Pre and Post oocyte fertilization percentages were respectively 52.8% vs. 86.3%. In both groups, the fertilization rate was not normally distributed. Despite this, a statistically significant difference emerged in the Pre vs. Post fertilization percentage. In fact the fertilization rate per couple presented a statistically significant difference of 33.6% (median of 40.0%) Wilcoxon signed rank test $p = 1.4 \text{ E-}8$ (Figure 1). No statistically significant differences were observed

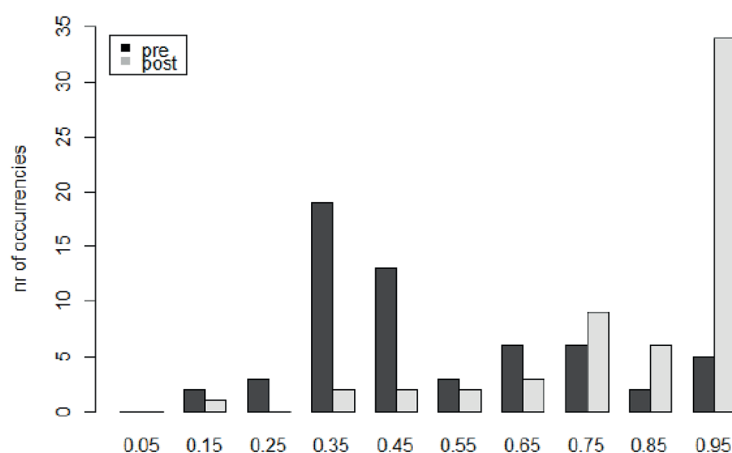


Figure 1. Fertilization rate.

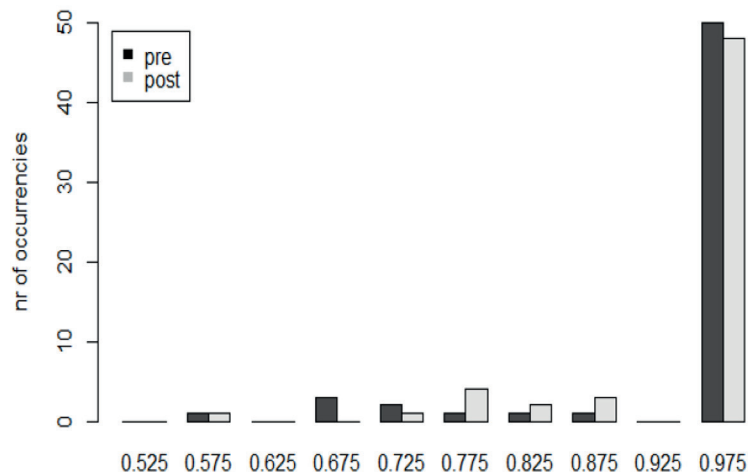


Figure 2. Ratio of embryo development.

between the two groups as regards the number of embryos obtained (95.94% vs. 96.21%) (Figure 2), but a statistically significant increase of good-quality embryos (Type A) per couple of 19.8% (median of 27.5% Wilcoxon test $p=2.6 \text{ E-}04$) was observed together with a statistically significant reduction in type-B embryos of 14.7% (median of 28.3% Wilcoxon test $p=7.7 \text{ E-}04$) (Figure 3). From the treated male group (Post) we obtained 26 pregnancies out of 58 transfers (44.8%), while in the Pre-group we obtained 5 pregnancies out of 56 transfers performed (8.9%). Therefore, a statistically significant increase in clinical pregnancies of 35.9% was observed ($p=4.2 \text{ E-}05$). There were no multiple pregnancies. From the paired-type test, which evaluates the specific effect of antioxidant treatment, the implantation rate

in 54 couples, conducted during both the pre- and post-treatment phase, was 4.6% in pre-treatment and 20.3% in post-treatment. Our findings, therefore, reveal a statistically significant increase in the implantation rate equivalent to 15.8% (median 33.3%, $p=3.8 \text{ E-}04$). All the pregnancies (5) achieved in the pre-treatment group (Pre) miscarriage, while in the post-treatment couples (Post) just 5 of the 26 pregnancies failed to develop (19%). This shows a reduction in the relative risk ($p=2 \text{ E-}05$). Moreover, to assess whether the age factor could influence the effectiveness of treatment regarding the reproductive outcome, we performed correlations between the age of the male and female partners and the fertilization rate, embryo formation rate, embryo quality, pregnancy and miscarriage rate. We found no statistically significant corre-

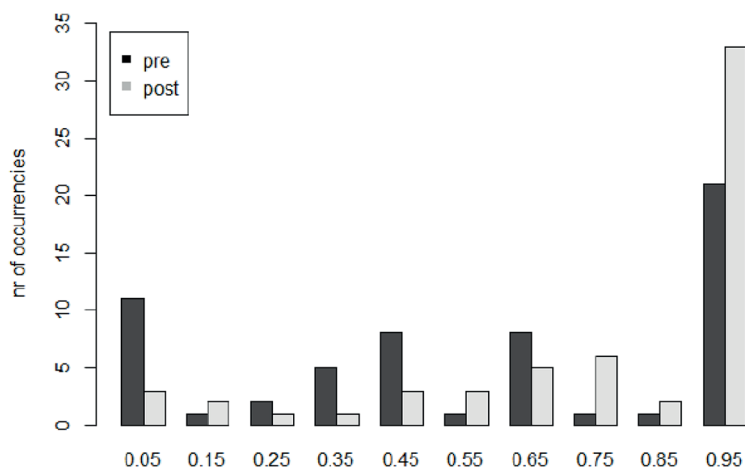


Figure 3. Ratio of highest quality embryo (type A) development.

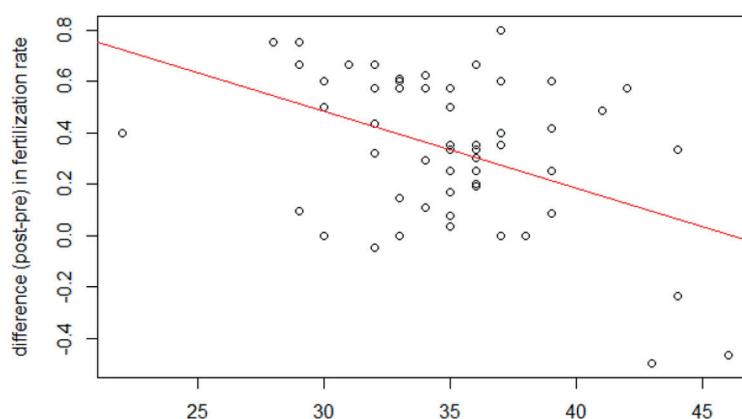


Figure 4. Relationship between fertilization rate and maternal age.

lation between the male age and the fertilization rate ($p=0.40$). Instead, we found a statistically significant correlation between the age of the female partner and the fertilization rate ($p=4 \text{ E-}4$), which decreased by 3% for each additional year (Figure 4). There was no statistically significant link between the age of partners and the embryo formation rate, embryo quality and the number of miscarriages. As regards clinical pregnancies, we did not find any statistically significant correlation with male age ($p=0.16$), but only with female age ($p=7.6 \text{ E-}3$), with a decrease of 2% per year.

Discussion

Poor eating habits, incorrect lifestyles and unfavorable environmental conditions increasingly appear to be the main cause of the decline in male fertility. Over recent years, reproduction specialists, particularly andrologists, gynecologists and physiopathologists, have focused on the role of reactive oxygen species (ROS) in an attempt to understand the pathogenetic causes of idiopathic infertility. ROS are oxidant agents and unstable, incomplete molecules with an unpaired electron, available and ready to react with other molecules. Free radicals are produced by leukocytes, which often represent the first line of defense against infections. When high levels of leukocytes are reached in the seminal fluid, the spermatozoa may undergo structural and genomic alterations. Other factors that lead to free radical formation include unbalanced diets, cigarette smoking, alcohol, ionizing radiation such as excessive exposure to the sun's rays, pollutant gasses and toxic materials or substances, including some drugs. Phy-

siologically, the oxygen used in our body, causes the production and release of pro-oxidant free radicals which are neutralized by the intervention of antioxidant substances present not only at an intracellular level, like in the spermatozoa (scavenger enzymes) but also at an extracellular level, like in the seminal plasma (superoxide dismutase, citric acid, ascorbic acid, tocopherol, uric acid, glutathione, taurine, hypotaurine, pyruvate and albumin). When the oxidative balance is upset, this can lead to an oxidative stress condition that leads to a free-radical disease in the sperm. This can cause a reduction in the fluidity and deconstruction of the cell membrane (lipid oxidation), a quantitative and qualitative reduction in sperm motility, and DNA fragmentation, making it difficult for the sperm to penetrate and fertilize the oocytes^{30,31}. Many antioxidant substances have been proposed over recent years as possible effective therapeutic aids in infertile males with idiopathic dyspermia caused by oxidative stress. Literature includes numerous scientific evidence of the effects of antioxidant therapy on sperm quality and on reproductive outcome of infertile couples, but only a few of these studies have produced consistent results. The results of these studies are often contradictory and refer to small groups of patients whose inclusion criteria are often different. As already mentioned, small quantities of ROS are needed for capacitation and acrosome reaction. Paradoxically, a significant reduction in their concentration, after the incongruous administration of antioxidant micronutrients, can even have a negative effect on fertility. Likewise, improved seminal parameters do not necessarily entail increased fertility, and so individuals with normal sperm count, motility, vitality and mor-

phology can still be sub-fertile. Because of this, the primary outcome of every study must be the pregnancy rate. Very few works described in the literature have evaluated this³²⁻⁴². Abel et al³⁵ conducted a randomized prospective study to evaluate the effect of clomiphene citrate or vitamin C treatment in 179 infertile men. The authors did not achieve a statistically significant difference in the pregnancy rate in the 2 treatment groups. The same results were observed by Hargreave et al³⁶ in a randomized prospective study to evaluate the effect of treatment with mesterolone or vitamin C in 368 infertile men. Paradiso Galatioto et al³⁷ evaluated the effects of antioxidant therapy on sperm quality and the spontaneous pregnancy rate in 20 oligozoospermic subjects, demonstrating a statistically significant increase in the sperm count, but with no significant modifications as regards motility, morphology and spontaneous pregnancy rate. The authors, therefore, concluded that antioxidant therapy based on a combination of NAC and micronutrient supplementation could help to improve the number of sperm in oligozoospermic males, although this was not associated with a statistically significant increase in spontaneous pregnancies after 12 months. Meanwhile, other authors have demonstrated that antioxidant therapy, particularly if administered to men with a low selenium concentration in their seminal plasma, improves seminal characteristics and leads to an increased pregnancy rate percentage in partners^{34,41,43}. Also Dinkova et al⁴⁴ showed as a treatment with myo-inositol significantly improved sperm motility, increasing the likelihood of achieving a spontaneous pregnancy. The clinical heterogeneity of the results published in literature, the different drugs used and the different doses administered, together with a lack of case studies and different inclusion criteria contribute to a lack of substantial evidence⁴³. In consideration of the above, our study aimed to evaluate whether short-term treatment with antioxidants could affect the reproductive capacity of infertile couples. That is to say whether the components of Fertipus SOD, which act on the level of the cellular and mitochondrial membranes, improving sperm quality and reducing apoptotic events, could act in synergy in the etiopathogenesis of dyspermia with positive effects on the reproductive outcome. The evaluation of our data does not highlight a statistically significant difference in the seminal parameters and hormonal assays of pre- and post-antioxidant therapy patients. Instead, there is a significant difference between the fertilization

rate of Pre vs. Post patients ($p=7 \text{ E-}03$), with an increase in the fertilization rate per couple of 33.5% ($p<1 \text{ E-}05$). As regards the percentage of type-A embryos, we observed a statistically significant increase per couple of 19.8% ($p=2.6 \text{ E-}04$), confirming the findings already described in literature. The 26 pregnancies obtained from 58 transfers in post-treatment patients demonstrate a statistically significant increase in both the clinical pregnancy rate (35.9%) and in the implantation rate (15.8%), with a five times reduction in the relative risk of miscarriage after treatment ($p=2 \text{ E-}05$).

Conclusions

We can state that antioxidant therapy produces an improvement in terms of pregnancy rate. The various components of the drug work in synergy to improve not only the formation of the embryo, but also implantation and clinical pregnancy. Moreover, to fully understand the action of the drug we performed some correlations between the age of the subjects and the reproductive outcome, precisely to define whether a particular age group is more likely to benefit from the drug's action. The comparative analysis between male age, fertilization rate ($p=0.40$) and clinical pregnancy ($p=0.16$) did not reveal any major statistical significance ($p=0.40$), while we found a statistically significant correlation between the age of the female partner, fertilization rate ($p=4 \text{ E-}4$) (which fell by 3% for every additional year) and clinical pregnancy ($p=7.6 \text{ E-}3$). What is more, we found no statistically significant correlation between the age of partners and embryo formation rate, embryo quality and cases of miscarriage. As regards the risk of miscarriage, when comparing the ages of the 21 post-treatment couples who achieved a successful pregnancy with the 5 who were unsuccessful, it was impossible to find a link between age and the adverse event. Therefore, the results obtained from our study clearly indicate that the use of antioxidants and, particularly, micronutrients able to activate superoxide dismutase, represent a valid method for improving seminal parameters and, subsequently, for an improved IVF outcome. Superoxide dismutase (SOD), like other antioxidant molecules, has demonstrated measurable and significant sperm DNA fragmentation/decondensation reduction activity both *in vivo* and *in vitro*. In fact, by catalyzing the conversion of the superoxide into hydrogen peroxide, SOD protects the sperm cell from ROS, particularly

preventing lipid peroxidation of the plasma membranes. Although we only studied a limited number of cases, our findings suggest that antioxidant therapy can be indicated for preparing the male partner of the couple undergoing assisted reproduction, and is particularly effective in cases of patients with dyspermia who are, therefore, ideal candidates for ICSI. The study results suggest that a combination of enzymatic antioxidants (micro-encapsulated SOD, Extramel[®], alpha lipoic acid and low-dose glutathione), zinc, B vitamins, with a complex of micronutrients (ORISOD) of plant origin containing hydroxytyrosol and carnosol can restore redox homeostasis, partly by activating the endogenous endocellular antioxidant system (Nrf).

Conflict of interest

The authors declare no conflicts of interest.

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