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Scientific Research

Beneficial Effect of an Oral Antioxidant Supplementation (Fertimax2) on IVF-ICSI Outcomes: A Preliminary Clinical Study

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Abstract

Background: Considerable evidence points towards a significant role of oxidative stress (OS) in the pathogenesis of sperm dysfunction. OS as a result of an inappropriate balance between oxidants and antioxidants in the semen can cause DNA damage and lipid peroxidation leading to failure of conception, miscarriage or potentially even childhood cancer. The objective of this study was to investigate whether a male antioxidant therapy can improve semen parameters and the results of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Methods: A total of forty-eight infertile couples were considered. Male participants were administrated Fertimax2 antioxidant treatment for at least two months prior to their partner's IVF-ICSI cycle. Sperm parameters (volume, concentration, progressive motility) and the IVF-ICSI outcomes were compared before and after the antioxidant treatment. The primary outcome measures were oocyte fertilization, cleavage and good embryo quality rates; the secondary outcomes were biochemical pregnancies, clinical pregnancies and implantation rates. Results: The principal finding that emerged from this study was that antioxidant therapy resulted in significant improvements in fertilization (p = 00.2), cleavage (p = 0.004) and good-embryo quality (p = 0.002) rates accompanied by a marked increase in clinical pregnancy (18.7% versus 2.5%) and implantation (11.8% versus 1.02%) rates. No significant changes in routine sperm parameters were observed. Conclusion: The Fertimax2 antioxidant therapy appears to influence favorably chances of conception in couples undergoing assisted reproduction treatment (ART).

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Keywords

Sperm, Oxidative Stress, Antioxidants, Infertility Treatment, *IVF-ICS*

1. Introduction

Oxidative stress induced by reactive oxygen species (ROS) has been widely implicated in the physiopathology of male infertility [1] [2]. Indeed, low physiological levels of ROS play a crucial role in the acquisition of fertilizing ability and contribute to chromatin condensation, membrane remodeling and activation of intracellular signaling pathways, during epididymal maturation, capacitation and acrosome reaction [3]-[5], whereas excessive ROS production can be potentially toxic for spermatozoa sperm quality and function by two key mechanisms: the induction of lipid peroxidation and DNA damage [6] [7]. Detrimental modification of the sperm plasma membrane via the peroxidation of the fatty ac-id pattern disrupts its fluidity, which may impact negatively on the sperm concentration, motility and morphology [8] [9] and lead to impaired membrane fusion events such as the acrosome reaction and spermatozoon-oocyte fusion [10]. Furthermore, over-production of ROS may account for extensive sperm DNA structural damage at both nuclear and mitochondrial levels [11] [12]. The epidemiological evidence suggests that subsequent aberrant repair of such ROS-mediated DNA damage may result in impaired fertilization and embryo development [13] [14], low rates of implantation and the creation of mutations associated with the occurrence of miscarriage and a variety of pathologies in the offspring, including childhood cancer [15]-[17]. The origins of the OS could involve a number of endogenous factors such as infections [18], autoimmunity, varicocele [19], chronic disease [1] or exogenous factors including both environmental factors (high temperatures, electromagnetic radiation, pesticides and pollution) and lifestyle factors (advanced age, alcohol consumption, smoking, stress, obesity and poor diet) [1] [15] [20]. Prevention against sperm oxidative attack is generally provided by an array of enzymatic and non-enzymatic antioxidants of both spermatozoa and seminal plasma [21]. Under normal conditions, pro- and antioxidants are finely balanced in the sperm environment; however, a situation of OS can occur as a consequence of excessive generation of ROS and/or decrease in the antioxidant defense mechanisms in semen upsetting this balance [22].

In the attempts to identify potential therapeutic strategies in order to limit the deleterious effects of OS, beyond identification and amelioration of the underlying cause, interest in the use of oral antioxidant supplementation among subfertile and infertile men, is growing over the past few decades. The beneficial effect of numerous substances with recognized antioxidant properties (such as vitamins C and E, folate, zinc, selenium, carnitine, coenzyme Q10 and carotenoids) in patients with male factor infertility have been well documented in several reviews of clinical studies [23]-[26]. Some recent meta-analyses from randomized controlled trials concluded that complementary treatment with antioxidants may ameliorate male infertility by improving some sperm parameters and pregnancy outcomes [27]-[30]. Most of these studies investigated a wide variety of of antioxidants, as single agent or a 2 - 4 combined antioxidants (vitamin C + E, vitamin E + selenium, vitamin E + zinc, vitamin C + E + zinc, vitamin C + E + glutathione, L-carnithine + acetylcarnitine). Unfortunately, only four trials evaluated actually the effect of a combination therapy on sperm parameters and natural pregnancies [31], on vacuolization of the sperm head according to motile sperm organelle morphology examination (MSOME) criteria [32], on sperm DNA damage [33] and on pregnancy outcomes [34] respectively.

On theoretical grounds, an appropriate combination of antioxidants should be more effective than any single antioxidant since OS is a non-localized heterogeneous phenomenon. The Fertimax2 (DCMG™ Laboratory, France) is an antioxidant formulation whose components (**Table 1**) were identified as suitable to prevent or reduce sperm OS-related DNA damage [35]. In the purpose to identify novel combinations optimized for safety and efficacy, we therefore aimed in this study to evaluate whether Fertimax2 male supplementation increases the success rate of IVF-ICSI attempts.

2. Material and Methods

2.1. Patients and Study Design

A total of forty-eight participants for this study were recruited from couples referred to the reproductive medi-

Table 1. Substances of content for Fertimax2.

Composition	Daily dose/2 capsules
Vit C (Ascorbic Acid)	180 mg
Vit E (Tocopherol)	30 mg
Zinc	15 mg
Selenium	50 µg
L-Carnitine Tartrate	400 mg
Folic acid (Vitamin B9)	200 µg
Coenzyme Q10	40 mg

cine unit in the teaching Hospital in Sousse for a standard IVF (4 couples) or ICSI (44 couples) treatment with ejaculated spermatozoa and fresh embryo transfers. Female partners were 33.5 ± 5.1 years old with a normal FSH level (<10 IU/l) and without adverse affecting-implantation factors to prevent a female-factor bias. The average age of participants were 38.5 ± 5.5 years and exhibited no varicocele, genitourinary inflammation or infection and did not have any known genetic cause for an oligoasthenoteratozoospermia. The oral antioxidant treatment was administered to these men during 2 to 5 months at a daily dose of 2 capsules. The interval between the two sequential attempts did not usually exceed 9 months. Whatever, the second ICSI attempt was performed immediately after the antioxidant treatment, thus avoiding the risk of post-treatment impairment of sperm DNA integrity status.

2.2. Sperm Preparation

All semen samples were collected by masturbation after a period of three to five days of sexual abstinence and analyzed for basic sperm parameters, including sperm volume, count and motility according to World Health Organization recommendations (WHO 2010). A sperm-washing procedure was performed after centrifugation on a two-layer density (90% and 45%) gradient. Sperm concentration and motility were assessed after selection.

2.3. Ovarian Stimulation and Oocyte Retrieval

Based on age, prior evaluation of the ovarian function and previous IVF response, controlled ovarian stimulation was achieved using standard protocols including either long or short stimulation with agonist or antagonist. The protocols for inducing ovarian stimulation did not significantly differ between the two attempts. Ovulation was triggered by administration of 5000 to 10,000 IU of human chorionic gonadotrophin (hCG) when at least two follicles reached 18 mm in maximal diameter associated with a consistent rise in serum oestradiol concentration. Transvaginal oocyte retrieval was scheduled 36 hours after hCG injection and followed by standard IVF or ICSI fertilization procedures.

2.4. ART Procedure

ICSI was routinely performed on each metaphase II stage oocyte. For an IVF attempt, cumulus oocyte complexes (COCs) were inseminated with a concentration of 150,000 progressive motile sperm cells/ml. Injected oocytes or inseminated COCs were cultured using appropriate media overlaid with paraffin in a 37˚C and 6% CO_2 humidified air atmosphere.

On Day 1 (16 - 20 h after insemination/injection), fertilization was assessed by the presence of two pronuclei (2PN) and two polar bodies. On Day 2 and Day 3, each embryo was graded according to traditional morphological criteria, including size and shape of blastomeres and degree of fragmentation. Briefly, embryos were classified as good (morphology) if they had 1) equal-sized cells 2) 3 - 5 blastomeres at Day 2 and or 6 - 9 cells at Day 3 and 3) <20% cytoplasmic fragments of the embryonic surface. One to three best-scoring embryos were usually transferred to the patient's uterus 48 - 72 h post-oocyte collection, under ultrasound guidance. Of the 48 cycles, 4 and 9 proceeded to an embryo transfer (ET) at the blastocyst stage in the before and after-treatment attempt respectively. The luteal phase was supported with 400 mg/day of intravaginal progesterone, initiated on Day 1 after oocyte retrieval and continued until 8 weeks of gestation.

2.5. IVF-ICSI Outcomes

Primary endpoints were 1) fertilization rate (number of zygotes obtained per MII oocytes injected/inseminated), 2) cleavage rate (percentage of zygotes that underwent at least one cleavage or cell division) and 3) good quality embryo rate calculated from the number of cleaved embryos generated per IVF-ICSI cycle.

Biochemical, clinical pregnancy and implantation rates were other outcomes of interest. Chemical pregnancy was initially determined 14 days after ET by a positive quantitative serum β-hCG > 5 IU, followed by repeat serum pregnancy test in 2 to 4 days to ensure appropriately rising β-hCG levels. Clinical pregnancy was defined by ultrasound detection of an intrauterine gestational sac with a positive fetal heartbeat at 5 - 6 weeks following ET. Biochemical pregnancy (Number of positive β-hCG/Number of ET), clinical pregnancy (Number of heart beat/Number of ET) and implantation (Number of fetal sacs/Number of embryos transferred) rates were hence evaluated.

2.6. Statistical Analysis

Data of the study were analyzed using the commercial Statistical Package for the Social Studies (SPSS) version 18.0 software (Chicago, IL, USA). Quantitative variables are reported as counts or mean ± SD (standard deviation), while qualitative variables are reported as percentages. When the data were normally distributed, results were analyzed using the paired *t*-test. Differences were assessed with non-parametric Wilcoxon ranksum test for not normally distributed data. Noncontinuous variables were compared with Pearson's χ^2 test or χ^2 test with Yates' correction. A *p*-value ≤ 0.05 was considered statistically significant.

3. Results

The following variables were compared between the 48 IVF-ICSI cycles carried out before and after male antioxidant therapy.

3.1. Sperm Parameters

As shown in **Table 2**, there were no significant differences found in basic sperm characteristics (volume, count and progressive motility) after the treatment as compared with the before-treatment. It should be noted that assessment of sperm morphology was not performed as it was not enter in the routine practice within our ART unit (mean of baseline sperm abnormal morphology: 86.6% ± 10.2%).

3.2. IVF-ICSI Outcomes

When the main embryological outcome measures of the two sequential attempts were compared, fertilization rate, cleavage rate and the proportion of good-mophology embryos were significantly higher in IVF-ICSI cycles after therapy.

Following the transfer of similar mean number of embryos in the two cycles, a significant improvement in clinical pregnancy (<0.05) and implantation (<0.01) rates were detected between the before and after-antioxidant treatment. However, we noted an increase in the biochemical pregnancy rate (25% versus 10.2%) but without reaching significance. Of the 9 clinical pregnancies achieving by the partners after the oral antioxidant treatment, 5 (55.5%) were ongoing on the first-trimester (2 singleton, 2 twin and 1 triplet) and 4 were spontaneously lost. On the other hand, only 4 couples experienced a biochemical pregnancy resulted from the IVF-ICSI attempts that were performed before the antioxidant treatment, ending up to one live birth. For This couple, the subsequent cycle succeeded once again to carry out a viable pregnancy. These data are summarized in **Table 3**.

Table 2. Data for the semen parameters of patients before and after the therapy.

Variables assessed	Before therapy	After therapy	*p*-value
volume (ml)[a]	3.07 ± 1.5	3.1 ± 1.3	0.86 (NS)
Concentration ($\times 10^6$/ml)[b]	34.2 ± 33.7	36.8 ± 34.7	0.62 (NS)
Progressive motility (%)[b]	21.3 ± 12.1	22.08 ± 13.4	0.62 (NS)

Values are a mean ±SD. [a]Paired *t*-test. [b]Wilcoxon signed rank test. NS: not significant.

Table 3. Comparison of laboratory and clinical outcome measures in two sequential IVF-ICSI attempts performed before and after antioxidant therapy.

Variable	Before therapy	After therapy	*p*-value
No. Oocyte retrieved[a]	7.33 ± 4	6.52 ± 3.87	0.23 (NS)
Fertilization rate (%)[b]	53.5 ± 33.2	74.6 ± 20.7	0.002
Cleavage rate (%)[b]	79.8 ± 39.1	98.8 ± 5.4	0.004
Good embryo quality rate (%)[b]	48.7 ± 36.6	71.2 ± 28.2	0.002
No. embryo transfers	39	48	
Total No. of embryos transferred	98	110	
No. transferred embryos/cycle[a]	2.0 ± 1.1	2.0 ± 0.6	NS
Biochemical pregnancy rate/ET (%)[c]	10.2 (4/39)	25 (12/48)	NS
Clinical pregnancy rate/ET (%)[d]	2.5 (1/39)	18.7 (9/48)	<0.05
No. singleton	1	2	
No. twins	0	2	
No. triplets	0	1	
Implantation rate (%)[c]	1.02 (1/98)	11.8 (13/110)	<0.01

Values are a mean ±SD. [a]Paired *t*-test. [b]Wilcoxon signed rank test. [c]χ^2 test. [d]χ^2 test with Yates' correction. NS: not significant.

4. Discussion

As far as is known, the current study is the first to investigate the usefulness of Fertimax2 that provides a combination of many scavengers of ROS on IVF-ICSI outcomes. Even though this is an uncontrolled clinical study, the data obtained show clearly a significant improvement in laboratory and clinical endpoints following 3 months of antioxidant therapy.

It is well known that the completion of fertilization process and subsequent embryo development depends, in part, upon the sperm DNA integrity which may be affected by three major factors including DNA decondensation (tertiary structure), DNA fragmentation and formation of DNA addicts (primary and second structure) [36].

Firstly, an adequate sperm chromatin tertiary structure seems to be critical for correct epigenetic regulation and also for the very first cleavages early-embryogenesis [37]. Defective sperm chromatin packing could be either reduced compaction or supernormal compaction. Decreased compaction would increase the access to the DNA whereas a supernormal compaction of the sperm chromatin would jeopardize the timing of the rapid delivery of the sperm DNA in the ooplasm. Given the clear reliance on Zinc for optimal sperm chromatin stabilization [38], it seems that Zinc supplementation is reasonably required for men with sub-and infertility [39]. In our study, Fertimax2 appears to have a significant positive effect on fertilization rate (FR) probably mediated by improvement in sperm DNA protamination, thereby making spermatozoa less vulnerable to oxidative DNA damage [40]-[42]. For a group of 9 couples with complete fertilization failure in the first cycle, an embryo transfer was performed in all of female partners at the next attempt after therapy (data not shown). This result is consistent with the published literature. Junca *et al*. [43] observed an increase in DNA packing among patient treated with 15 mg of Zinc in association with vitamins B and quercetin (Condensyl®) as it was able to rescue hyaluronic acid (HA) binding capacity and sperm decondensation index (SDI). This treatment was followed by pregnancies after regular ICSI, artificial insemination or even spontaneous pregnancy. Similarly, Tunc *et al*. [33] recorded a small but statistically significant improvement in median levels of sperm DNA protamination with a 3 months antioxidant therapy (Menevit). This medication contains zinc (25 mg) and selenium (26 µg) another important cofactor in sperm DNA protamine packaging [44] that are believed to be most likely the active ingredients responsible for the observed improvement. However, a previous placebo-controlled randomized study [34] testing the Menevit when used in conjunction with IVF-ICSI was unable to detect any significant effect of antioxidant medication on FR and cleavage stage embryo quality despite an improvement in viable pregnancy. This is in contrast with our findings. It is possible that the lower values of selenium contained in the Menevit sup-

plement may be responsible for an increase in sperm protamination relatively insufficient to improve FR as compared with those contained in the Fertimax2 medication (50 µg). It has been postulated, in point of fact, that within and beyond optimum selenium concentrations that range between 50 and 70 µg/ml, the quality of the spermatozoon is altered [45].

The other parameters that have been also improved with oral antioxidant therapy are cleavage stage embryo quality rates. Unlike, Tremellen *et al*. [34] and Greco *et al*. [46] studies found no effect of oral antioxidant supplements with Menevit and a combined Vitamin C + Vitamin E respectively, on embryo development despite a significant reduction in sperm DNA damage and a boost in pregnancy rates. Keeping in mind evidences supported above, it would be expected that if sperm chromatin is unable to decondense after entering the ooplasma, fertilization or post-fertilization failure could occur due to defective sperm DNA namely DNA fragmentation. It was suggested that the outcome of sperm penetration into an oocyte depends on the amount and the type of DNA damage and the ability of the oocyte to repair this damage. In other words, there is a threshold level of sperm DNA fragmentation above which the oocyte repair capacity is overwhelmed and consequently the zygote or the early embryo will initiate apoptosis or developmental arrest [47]. Nevertheless, the influence of DNA fragmentation on fertilization process, embryo quality and development and subsequently on the reproductive outcome in assisted reproduction remain controversial. While a number of studies [48]-[50] have associated poor embryo quality with high DNA fragmentation in IVF-ICSI cycles, many other authors found that sperm DNA damage have no impact on embryological outcome, but possibly only on abortion rates [51]-[53]. This supports the hypothesis that the impact of damaged paternal DNA becomes manifest relatively late when the embryonic genome was transcribed giving the so-called "late paternal effect" by Tesarik *et al*. [54]. Accordingly, blastocyst culture is possibly a better marker of sperm DNA integrity than cleavage stage assessment. Among our cohort, nine participants underwent blastocyst culture since an increased number of good quality embryos were available, resulting in one biochemical pregnancy and one twin ongoing pregnancy.

Interestingly, Wirleitner *et al*. [32] observed that the percentage of Class I (with no vacuoles) spermatozoa according to MSOME criteria was significantly increased after an antioxidant supplementation (Fertilovit® M^{plus}) for atleast 2 months. Content of this supplement is quite comparable to Fertimax2 one (Vitamin C 100 mg, Vitamin E 100 mg, Folic acid 500 µg, Zinc 25 mg, Selenium 100 µg, N-acetyl-L-cysteine 50 mg, L-carnitine 300 mg, Citrulline 300 mg, Glutathione redox. 50 mg, Lycopene 4 mg, Coenzyme Q10). Considering the fact that selection of spermatozoa for ICSI using MSOME can improve results in ICSI cycles through an increase in the number of high morphology embryos formed [55], these results provide further evidence for the effectiveness of an appropriate combined antioxidative therapy on sperm quality.

Another important focus of this study was the question of whether this oral antioxidant supplement has the ability to translate these positive embryological changes into improved chances of pregnancy. The data obtained here illustrate clearly a marked improvement in the pregnancy and implantation rates at post-treatment ICSI cycle, which is most likely mediated by enhancement in sperm DNA integrity [26] [56]. This is concurrent with the observations reported by some studies [33] [34] [46]. Showell *et al*. [28] published the Cochrane review on the basis of meta-analysis of 34 trials that included 2876 couples evaluating the effect of oral supplementation with antioxidants for male partners of couples undergoing ART. The authors concluded that taking oral antioxidants had an associated statistically significant increase in live birth rate and pregnancy rate in subfertile couples.

Recently, Cavallini *et al*. [57] have demonstrated a reduced frequency of aneuploid sperm associated with more favorable ICSI outcomes, including the number of biochemical/clinical pregnancies and live births following a course of L-carnitine, acetyl-L-carnitine and cinnoxicam treatment. Here another point of greater concern is that some sperm DNA damage, if not repaired, may lead to a high risk of genetic disease transmission, compromising embryonic and subsequently post-natal development [58]. The possibility of the drug-induced degeneration of sperm cells with abnormal chromosome content may constitute an additional hypothesis that could explain our results as L-carnitine constitute one of the Fertimax2 ingredients.

In this study, we assumed that embryological and clinical IVF-ICSI data are more reliable outcome measures than sperm characteristics. Basic sperm parameters in terms of volume, count and progressive motility, morphology did not change significantly following antioxidant treatment. This in agreement with Tunc *et al*. study (Menevit) [33]. With Fertilovit® Mplus supplementation, Wirleitner *et al*. [32] is observed a drastic improvement in total and progressive sperm motility as well as in total sperm count in the group of oligoasthenoteratozoospermic (OAT) patients but not in the no-OAT group. Our results are well in line with these findings since it

can be seen that the average sperm parameters of our participants were only moderately impaired. Even as several reviews [26] [27] analyzing randomized trials showed an improvement in sperm quality (mainly motility, but also concentration and/or morphology), it is worth to note that these trials were small in size and heterogeneous concerning the studied population as well as type, dosage, and duration of antioxidant therapy.

Finally, we stress the point that our study was conducted on patients who had generally at least a first failed IVF-ICSI cycle and returned for another intervention cycle. This may have implications towards the validity of using such initial control data for pregnancy and implantation rates, as patients achieving pregnancy in a first cycle will probably not return. However, it will not affect therapy-induced changes in fertilization, cleavage and good embryo quality rates. Moreover, we did not estimate seminal oxidative stress or oxidative sperm DNA damage before and after therapy as a selection criterion for monitoring the response to antioxidant treatment, which is one of the study limitations. Further researches would be conducted in the context of this field.

5. Conclusions

Oxidative stress is one of the major causes of DNA damage that remains one of the most important concerns in ART, and in particular ICSI. There is now little doubt regarding the effectiveness of antioxidants on sperm quality and function which represent one promising approach that should be attempted after identification and amelioration of the underlying cause of OS [59]. This study adds to the growing body of evidence supporting that the use of antioxidant combinational therapy including several actors with different modes of action such as Fertimax2 is most likely to improve sperm DNA integrity, especially for those men undergoing IVF-ICSI treatment.

Although our data demonstrate the effectiveness of this antioxidant supplementation in the treatment of male infertility, these very preliminary observations need to be validated in a large sample and placebo-controlled study. Moreover, we emphasize the need for further studies to establish the optimal doses according to the recommended daily allowance [60] and duration of treatment or subpopulation of target patients who might benefit most from antioxidant therapy (isolated asthenozoospermia, oligoasthenoteratozoospermia, sperm DNA damage or all).

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Conflict of Interests

The author has declared that no conflict of interest exists.

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