



Antioxidant Supplementation of Subfertile Men Improves Top-Blastocyst Rate in Couples Undergoing IVF/IMSI

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Abstract

In recent years, oxidative stress (OS) has been identified as important factor in male infertility. Thus, the intake of antioxidants to improve semen quality (in subfertile men) has been widely discussed. Improvements of semen quality after supplementation have been reported. However, this issue is still underevaluated. Critics complain a lack of data regarding firstly the use of antioxidants due to the heterogeneity between patient groups, nutritional supplements and treatment effect and secondly rare data in regard to the impact of supplementation on assisted reproduction technique (ART) outcome. In this study the effect of an antioxidant supplementation (AOS) on semen quality and therapy outcome of 92 couples undergoing fertility treatment was assessed. Semen analysis was performed during a first treatment cycle and data regarding treatment outcome were recorded. These were compared to a second treatment cycle of the same couples, prior to which the male partners had received AOS (Fertilovit[®] Mplus for 3 - 6 months). Semen samples were assessed according to WHO and MSOME (motile sperm organelle morphology examination) criteria. Parameters chosen for evaluation of treatment outcome were fertilization-, (top-)blastocyst rate, pregnancy- and clinical pregnancy rate. After AOS, we found a slight improvement of semen quality according to WHO – and a significant improvement according to MSOME criteria ($p < 0.01$). Even though – naturally – the age of the female partner had increased, we observed a rise of blastocyst rate after AOS. Top-blastocyst rate even improved significantly ($p < 0.05$). In addition to this, pregnancy- and clinical pregnancy rate showed a marked improvement. In summary, the use of a concomitant AOS might be discussed not only for men with impaired semen quality, but also for men with normozoospermia undergoing ART as it may contribute to improved semen parameters and an increased success of the treatment.

Keywords ART; Sperm; MSOME; Blastocyst quality; Antioxidant; Nutritional supplementation

Abbreviations

AOS: Antioxidant Supplementation; ART: Assisted Reproductive Techniques; BR: Blastocyst Rate; cPR: Clinical Pregnancy Rate; FR: Fertilization Rate; ICSI: Intracytoplasmic Sperm Injection; IMSI: Intracytoplasmic Morphologically Selected Sperm Injection; IVF: In Vitro Fertilization; MSOME: Motile Sperm Organelle Morphology Examination; OAF: Oocyte Activation Factor; OS: Oxidative Stress; PR: Pregnancy Rate; ROS: Reactive Oxygen Species; tBR: top-Blastocyst Rate; TUNEL: TdT-mediated dUTP-biotin Nick End Labelling; WHO: World Health Organization

Introduction

It is estimated that in the industrial nations an average of one in every 10 couples have problems with reproduction and stay childless without an appropriate fertility treatment. According to the World Health Organization (WHO) the estimated total proportion of male-factor-related infertility comes to approximately 46%.

Sperm quality is generally considered to be a proxy measure of male fertility. The initial semen analysis to evaluate the number of sperm, motility and morphology is mostly performed according to the current WHO criteria [1]. In addition to this, a multitude of other tests are meanwhile available to analyze semen quality in more detail e.g. TUNEL-assay, Comet assay), acritin orange, hyaluron-binding assay and, most importantly, motile sperm organelle morphology examination (MSOME) [2]. MSOME allows the investigation of subtle sperm morphology in vivo. It enables in particular the observation of nuclear vacuoles, which cannot be detected by lower magnifications. The origin of these vacuoles and their impact on fertility is still somewhat debated. However, the majority of studies substantiate that vacuoles represent pathologic conditions [3-5]. In accordance with this, the combination of MSOME and ICSI, also designated as intracytoplasmic morphologically selected sperm injection (IMSI) results in a significant improvement in implantation- and pregnancy rates and a statistically significant reduction in miscarriage rates [4-10].

Reasons for male subfertility are numerous and next to purely medical reasons such as infections, genetic or chromosomal disorders, use of drugs as during chemotherapy, radiation and environmental pollution are held responsible. In addition to this a man's age and his lifestyle have been found to have an impact on sperm quality as well [11-16]. Interestingly, according to various publications the presence of seminal oxidative stress (OS) plays a key role in male infertility. Normally, the controlled generation of reactive oxygen species (ROS) is associated with normal physiological functions. However, uncontrolled and excessive ROS might be an important factor in the pathophysiology of infertility. In fact, infertile men were shown to have increased levels of ROS in seminal plasma and spermatozoa, reduced antioxidative capacity, and increased number of mitochondrial DNA mutations or nuclear DNA fragmentations [17-19]. This raises the question whether semen quality can be positively influenced by an oral AOS. According to a large number of studies, AOS is mostly regarded as a helpful tool to quench ROS and thereby improve sperm quality in humans or in laboratory animals [20-23], but not without controversy. This might be due to the - still - little clinical data, the effectiveness of different antioxidants, the administered doses, the analyzed semen parameter(s), types of infertility and, probably, due to the heterogeneity of the studied

subpopulations. Additionally, critics have particularly emphasized the fact that only few of the clinical studies presented to date have evaluated the impact on outcome of fertility treatment [24]. For example, to date there is only limited data on the impact of antioxidant treatment on sperm head vacuolization. Yet nuclear vacuolization has been reported to have in turn a negative correlation to blastocyst- and top blastocyst rates [25,26,8]. Currently - to our knowledge - there is no data available assessing directly the association between antioxidant intake, vacuolization rate and the outcome of fertility treatment.

In this study we present data on the treatment outcome of IVF patients undergoing IVF/IMSI. Semen samples were collected during a first treatment cycle without AOS (control). Second sample was taken during the second cycle after the men had taken an AOS for a continuous period. Semen samples were evaluated according to WHO and MSOME criteria. Additionally, fertilization rate (FR), blastocyst rate (BR), top-blastocyst rate (tBR) as well as pregnancy- (PR) and clinical pregnancy- (cPR) rates were recorded and the results of both cycles were compared.

Material and Methods

In the period from January 2008 to July 2011 a total of 92 patients from our IVF clinic in Bregenz (Austria) were included in this study. Couples were recruited according to the following criteria: Male patients consented to have a dietary supplementation. Indications of azoospermia and patients with known genetic reasons for impaired spermatogenesis (such as chromosomal aberrations or other genetic defects) were excluded. Additionally, exposition to irradiation or chemotherapy in the past were further exclusion criteria. Only females at the age of ≤ 43 years at the start of the 1st cycle were included. Male size and weight and smoking were asked by questionnaire. Female BMI was assessed at the beginning of the first cycle.

Before starting the second cycle male patients were treated orally with a dietary supplement (Fertilovit® Mplus) for at least 3 months (one capsule twice daily, substances of content are given in Table 1).

Content	Daily dose/ 2 capsules
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 red.	50 mg
Lycopene	4 mg
Coenzyme Q10	15 mg

Table 1: Supplement facts of Fertilovit® Mplus. Dosages were given in micro- or milligram respectively.

The different components of the preparation were described as beneficial for protecting sperms from oxidative damage or having other supporting effects, respectively. The semen samples were examined by MSOME again after a 2-12 months course of antioxidative therapy. No undesired side effects of the supplementary intake were noted.

Analysis of semen samples included several sperm parameters such as ejaculation volume, sperm concentration and sperm motility according to WHO criteria [1]. Additionally, semen quality was assessed according to MSOME criteria (modified from Vanderzwalmen et al., 2008) [8]. Selection of spermatozoa was performed at 6000x magnification under a Nomarski interferential Leica AM 6000 inverted microscope (Leica, Germany). Grade I sperm was defined by normal shape and size, no vacuoles or only small vacuole(s) $<4\%$ of the sperm's head, grade II: normal shape and size but large vacuoles $>4\%$ of the sperm's head, grade III: large vacuoles $>4\%$ of the sperm's head and, additionally, abnormal shape and/or size of spermatozoa. The GnRH long protocol was applied for all cycles with daily injections of triptorelin (Decapeptyl®, Ferring Arzneimittel, Vienna, Austria) 0.1 mg/day, beginning in the mid-luteal phase of the preceding cycle for down-regulation of the pituitary gland. HMG (Merional®, IBSA, Lugano, Switzerland) 2-4x 75 IU/day was used for follicle stimulation.

To evaluate impact on treatment outcome, data on the following parameters were collected: Number of two-pronuclear stage (2PN), number of blastocysts, top-blastocyst rate (tBR), fertilization- (FR), pregnancy- (PR) and finally clinical pregnancy rate (cPR) were determined. Blastocysts with a degree of expansion of 2, 3, 4 and 5 and with A-grading for inner cell mass and trophectoderm, or a combination of A- and B-grading, were classified as top-blastocysts. (Top-) blastocyst rate was calculated by the number of (top-) blastocysts divided by the number of 2PN stages. Pregnancy rate (PR) was determined by urinary β -hCG level 14 days after transfer, clinical pregnancy rate (cPR) was defined as observation of fetal heartbeat(s) by ultrasound 6-8 weeks after ET.

Data was expressed as mean \pm standard deviation for parametric variables and analyzed by Student's t-test and chi-square test to evaluate the significance of data.

Results

The mean male age at the onset of the first IVF cycle was 39.2 years. Male patient cohort revealed moderate overweight (BMI: 26.0 at the first and BMI 26.1 at the second cycle). Twenty patients stated occasional or heavy smoking (19 patients at the 2nd cycle). According to the WHO criteria 32 patients were found to have normozoospermia, 29 men were classified as OAT, while 31 were found to have either asthenozoospermia or oligozoospermia. Lapse of time between the IVF attempts was 1.4 years (mean \pm 1.5)

By comparing the semen parameters before and after supplementation a highly significant increase in class I sperm could be observed after supplementation (6.0 \pm 5.8 versus 3.8 \pm 4.9, $p < 0.01$, see Table 2). The patients revealed a highly significant increase in class I sperm according to MSOME criteria (6.0 \pm 5.8 versus 3.8 \pm 4.9 before supplementation, $p < 0.01$, see table 2). However, no significant improvement of semen parameters in regard to sperm count and motility could be observed.

In a further step we evaluated and compared IVF outcome of both cycles (Table 3). Female ageing is normally associated with deterioration in oocyte number and quality, and subsequent in embryo quality. The mean female age at the onset of the first therapy was 36.8 years. Mean BMI was 23.0. Although the average age of the female patients had increased until the second cycle (38.1 years, $p=0.03$) and number of oocytes retrieved were marginally lower, we observed an increase in the blastocyst rate in the second IVF cycle after the men had been supplemented orally with the antioxidative preparation. Although this increase was not significant, the number of top-blastocysts augmented significantly ($p<0.05$). We also found a rise in PR and cPR, however this rise was not significant, due to the fact that the total number of patients included in this study was low.

	First cycle without Supplementation	Second cycle with Supplementation	p-value
Male characteristics			
Male age (years)	39.2 +/- 8.5	40.6 +/-8.5	n.s
Male BMI (kg/m ²)	26.0+/-3.0	26.1+/-3.1	n.s
Semen assessment			
Sample volume (ml)	2.9 +/- 1.5	2.3 +/- 1.4	< 0.01
Total sperm count (TSC)	44.3 +/- 49.5	49.4 +/- 41.5	n.s
Concentration (Mio/ml)	16.7 +/- 17.6	20.8 +/- 22.5	n.s
Sperm motility (%)			
Grade a	3.9 +/- 6.3	4.0 +/- 6.5	n.s
Grade b	30.6 +/- 18.7	29.0 +/- 19.6	n.s
Grade c	14.9 +/- 14.7	21.4 +/- 18.1	< 0.05
Grade d	50.6 +/- 24.2	45.6 +/- 22.1	n.s
Progressive Motility (%)	34.5 +/- 21.6	32.6 +/- 21.3	n.s
MSOME criteria (%)			
Class I	3.8 +/- 4.9	6.0 +/- 5.8	< 0.01
Class II	38.9 +/- 16.7	41.9 +/- 14.5	n.s
Class III	57.3 +/- 19.3	52.1 +/- 18.0	n.s

Table 2: Patients characteristics and results of semen analysis before and after supplementation.

Discussion

It is well known that spermatozoa are highly prone to OS. Enhanced OS hinders motility and development of normal sperm cells. It accelerates apoptosis, thus lowering sperm number, and in addition to this, ROS attack sperms' DNA integrity by base modification and strand breaks. Moreover, ROS can alter epigenetic modifications. This aspect is most important as modified genetic signatures can lead to diseases in the offspring [27].

Elevated levels of ROS are meanwhile even suggested to be a major cause of idiopathic male factor infertility, which is an increasingly common problem today. One promising approach is the reduction of OS in the male reproductive tract by application of oral antioxidants and a variety of studies have shown that this indeed can provide a means of ameliorating sperm quality and quantity as assessed by standard WHO criteria [22].

Female characteristics	First cycle without Supplementation	Second cycle with Supplementation	p-value
Female Age (years)	36.8 +/- 4.2	38.1 +/- 3.9	n.s
Female BMI (kg/m ²) (mean)	23.0+/-3.5	n.d.	
Stimulation dose (IU)	2451 +/- 745	2647 +/- 764	n.s.
Number of oocytes retrieved (total)	1127	1092	n.s.
Oocytes (mean)	12.4 +/- 5.9	12.1 +/- 5.7	
Number of 2PN (total)	672	659	
2PN (mean)	7.3 +/- 3.9	7.3 +/- 4.3	
FR (%)	59.6	60.4	n.s.
Number of blastocysts (total)	267	288	
Blastocysts (mean)	2.9 +/- 2.4	3.1 +/- 2.7	
Blastocyst Rate (%)	39.7	43.7	n.s.
Top-Blastocysts (mean)	0.4 +/- 1.1	0.6 +/- 1.0	
tBR (%) (Nb. of top-blastocysts)	5.5 (n= 37)	8.5 (n= 56)	< 0.05
Number of embryos transferred (mean)	1.9 +/- 0.4	1.9 +/- 0.3	n.s.
PR	34.8	44.5	n.s.
cPR	32.8	39.1	n.s.

Table 3: Patients characteristics and comparison of treatment outcome of the same couples. First cycle without supplementation (left) and in a second cycle after supplementation (right).

Non-enzymatic antioxidants include vitamins A, E, C, and B complex, glutathione and co-enzymes or co-factors of antioxidative enzymes such as coenzyme Q10, zinc or selenium. Various studies have documented a positive impact of such micronutritional and antioxidative supplementation for sperm quality. However, only a few studies established current medical evidence on the effect of oral antioxidants on sperm quality as evaluated according to MSOME criteria [25].

In addition, there has been a lot of criticism. Most studies investigated several sperm parameters, however, there is lack of studies evaluating the main outcome of any fertility-related study, namely pregnancy [22].

Therefore, after confirming the positive impact of an antioxidative preparation on semen parameters [25,26]. We examined the impact of

the same preparation on the treatment outcome of 92 couples undergoing IMSI. In the course of the study, we compared the therapy outcome of these couples who first underwent fertility treatment without any supplementation of the male partner with a second treatment cycle, during which adjuvant supplementation of the male partner was used. By analyzing the same cohort of patients we circumvent the problem of comparing populations with heterogeneity of medical history. Even though the age of the female partner therefore was higher during the second cycle, we were able to find a significant beneficial impact on the top-blastocyst rate as well as a marked improvement of PR and cPR. The improvement observed in spite of increased female age hints strongly at the male impact.

Historically, at first, it was thought that the success rates of ICSI are not even related to basic semen parameters [28-30]. Sperm were considered "mere vectors that carry the paternal genetic component to the oocyte" [31]. However, in several cases of recurrent negative IVF results in conventional IVF and ICSI attempts the influence of the paternal effect on early embryogenesis is suggested as a reason for IVF failure [32-34].

Today it is well known that various components of the spermatozoa actively participate in early embryonic development [35-37]. So-called early paternal effects are visible from day 1 to day 3 of development and include transfer of oocyte activation factor (OAF), which is critical for successful fertilization, centrosomes, which are crucial for cell division [38], as well as a population of RNAs with developmental importance [35,39].

Late paternal effects, however, are effective from day 3 onwards of embryonic development. Impaired development at this stage is usually linked to defects within the sperm nucleus. These include chromosomal aberrations, DNA fragmentation, as well as faulty epigenetic modifications of the DNA (such as DNA-methylation, histone tail modifications, telomere-shortening, targeted histone retention and protamine incorporation into the chromatin) [31].

The fact that we found no significant changes in FR but effects in top-blastocyst rate might therefore be seen as a hint to the fact that AOS is most effective in improving aspects of late paternal effects, that is, nuclear factors. This is consistent with previous findings that AOS can attenuate DNA fragmentation of spermatozoa [40]. Sperm DNA damage is thought to be induced by several mechanisms, including apoptosis during spermatogenesis, induction of DNA strand breaks during remodeling of sperm chromatin, DNA fragmentation induced by endogenous caspases and endonucleases, radiotherapy, chemotherapy, environmental toxicants and xenobiotics and DNA damage induced by oxidative stress [31].

Sites within the sperm genome particularly sensitive to OS include the telomeres as well as the peripheral nuclear compartment which contains histone-bound DNA (5-15%) [31]. Susceptibility to oxidative damage is particularly high in spermatozoa as compared to other cells because sperm lose the majority of cytosolic antioxidants at the time of spermiogenesis and at the same time have lower levels of DNA repair enzymes [31].

We therefore propose that AOS in subfertile male may help to quench ROS, thus contributing to protecting spermatozoa from OS. This is beneficial for success of fertility treatment by supporting DNA integrity and therefore late paternal effects on embryonic development.

This study might also hint at the fact that, given the correlation between ameliorating vacuolization and blastocyst formation rate through late paternal effects, that vacuolization, though still not fully understood, might develop in association with nuclear defects. For the clinician, the here presented data not only confirms previous studies [22,41] but directly emphasizes the correlation between sperm head vacuolization and success of fertility treatment and gives the opportunity to reduce the vacuolization grade and to improve the IVF outcome by nutritional supplementation. We therefore suggest, that the issue of sufficient AOS should be addressed when counseling and treating ART-patients, predominantly if morphology according to MSOME criteria is impaired. The same applies, if patients feel they will not be able to comply with recommendations regarding fruit and vegetable intake or if semen quality is strongly impaired with respect to WHO criteria, supplementation might be considered.

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