Original Communication

Dietary Supplementation of Antioxidants Improves Semen Quality of IVF Patients in Terms of Motility, Sperm Count, and Nuclear Vacuolization

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Abstract: *Background:* This study aimed to investigate the influence of an oral antioxidative supplementation on sperm quality of in vitro fertilization (IVF) patients, as analyzed by sperm motility according to the WHO criteria and motile sperm organelle morphology examination (MSOME). Methods: Semen samples were collected from 147 patients before undergoing an IVF/intracytoplasmic morphologically-selected sperm injection (IMSI) cycle and 2 – 12 months after an antioxidative supplementation. Semen analysis was evaluated according to WHO and MSOME criteria. Spermatozoa were grouped according to the size of nuclear vacuoles within the sperm's heads. Patients were divided into oligoasthenoteratozoospermic (OAT) and non-OAT men. Between first and second semen analysis, patients were supplemented orally with an antioxidative preparation. *Results:* After the antioxidative therapy we observed a significant reduction in the percentage of immotile sperm cells in the patients. Additionally, the percentage of class I spermatozoa according to MSOME criteria was significantly higher after antioxidative supplementation. In OAT patients the percentage of class I sperm was found to be increased, although not significantly. However, we observed a drastic improvement in sperm motility as well as in total sperm count in this group. *Conclusion:* The results demonstrated a considerable improvement in semen quality, notably in OAT patients. Considering the putative relationship between semen quality on the one hand and reactive oxygen species on the other, the observed changes in the sperm parameters indicate that a decline in semen quality, and even subtle morphological changes, might be associated with oxidative stress. Our findings suggest that an antioxidative and micronutrient supplementation has a remarkable benefit for IVF patients having restricted sperm parameters, in particular.

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Key words: IVF, sperm quality, IMSI, MSOME, nuclear vacuoles, oligoasthenoteratozoospermia, oxidative stress, antioxidative supplementation

Introduction

Assisted reproductive technology (ART) has helped overcome human infertility for more than three decades. Nevertheless, bypassing the natural barriers of reproduction still raises questions about the danger of possible negative effects in terms of implantation failure, abortion, or congenital malformations of the offspring. By comparing ART cycles and natural births, different studies observed an increased risk for children conceived after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). Thereby the occurrence of chromosomal aneuploidies [1], imprinting disorders [2], or other defects [3, 4] were elevated. Even though it is not unequivocally clear whether these anomalies are related to the ART procedures, it is reasonable that sperm quality can affect pregnancy and birth in terms of fertilization, implantation, and abortion rates as well as health of the offspring [5]. Therefore selecting spermatozoa for ICSI is a delicate and important step, which is primarily done according to morphological criteria.

In fact, several publications indicate that morphology is a crucial parameter for sperm quality. Poor sperm morphology was associated with acrosome anomalies, abnormalities in chromosome number, alterations in chromatin packaging, or increased DNA damage [6–8]. Moreover, spermatozoa from infertile men were shown to have higher incidence of DNA damage such as DNA fragmentation as compared to sperm from fertile men [9]. These observations are of particular importance for ART outcome. Sperm DNA damage was found to impair reproductive outcomes [9–11].

A detailed examination of subtle sperm morphology by MSOME was first introduced by Bartoov *et al.* 10 years ago [10]. It allows the examination of the sperm's fine morphology *in vivo* at very high magnification (6000–12,500x), thus providing the possibility of detailed sperm analysis, in particular assessment of the sperm head. MSOME enables observation of so-called nuclear vacuoles, which cannot be detected by lower magnifications. MSOME was subsequently applied to complement ICSI, and subsequently intracytoplasmic morphologically-selected sperm injection (IMSI) was successfully established in ART. Using the MSOME technique, spermatozoa can be categorized according to the presence and size of nuclear vacuoles within the sperm head. It has been clearly demonstrated that the size and number of these vacuoles in human sperm impede the outcome of ART considerably [12, 13]. In accordance with these findings, a large number of studies that used the stringent MSOME criteria in ART cycles displayed a significant improvement in implantation and pregnancy rates and a statistically significant reduction in miscarriage rates [10, 14–18].

Within the last decade it has become more and more accepted that environmental as well as certain individual factors can have an impact on human sperm quality. Thereby, life style factors such as smoking, physical training, the state of nutrition, and other personal and environmental factors were postulated to influence sperm quality according to WHO criteria [19–21]. Additionally, it was recently demonstrated that the subtle sperm morphology defined by MSOME criteria declines during aging [22]. These findings indicate that several sperm parameters such as morphology or motility are not inalterable in perpetuity but can be influenced in a positive or negative manner by a multitude of intrinsic and extrinsic factors.

Beside genetic reasons, oxidative stress (OS) has been considered as a major contributory factor to reduced male fertility [23]. Oxidative stress occurs when the formation or presence of oxidants such as reactive oxygen species (ROS) overshoots the pool of antioxidants, molecules that are able to scavenge these reactive species. Although the production of ROS as by-products of our life is a physiological process and low amounts of ROS are crucial to mediate signals between cells and also act as intracellular signal transduction factors, e. g., in sperm-oocyte interaction [24], an imbalance might lead to damage of the sperm's membrane, DNA, or enzymes. It has been shown in animal model systems and confirmed in human male infertility patients that inadequate ROS quenching or rise in ROS production in the seminal plasma caused by inflammation, aging, malnutrition, genetic reasons, or excessive pollution might impair sperm's motility and morphology, and in consequence its function [24].

The objective of this study was to evaluate the effect of antioxidative supplementation on sperm parameters in IVF patients. Not only semen quality in

terms of sperm concentration and motility according to WHO was taken into account, but also vacuolization of the sperm head, as detected by MSOME. We evaluated the semen samples of 147 men undergoing ART before and after a 2 – 12 month treatment period with an oral antioxidant supplement.

Material and methods

In the period from January 2008 to July 2011 a total of 147 patients from our IVF clinic in Bregenz (Austria) were included in this study. The semen samples of patients undergoing IMSI cycles were analyzed with respect to their morphology by MSOME and motility and sperm count by WHO criteria (WHO Laboratory Manual for the Examination and Processing of Human Semen, Fifth Edition, 2010).

For the study, patients were recruited according to following criteria: They consented to an antioxidative supplementation, they had no or were not planning to have a testicular sperm extraction (TESE), they exhibited no indication of azoospermia and did not have any known genetic reasons for an oligoasthenoteratozoospermia (OAT) syndrome (such as chromosomal aberrations, e. g., Klinefelter syndrome or other genetic defects). Moreover, they had had no chemotherapy or exposure to other noxious agents in the past and did not have any other medication with a known influence on sperm quality. After the first semen analysis all patients were treated orally with an antioxidant supplement (Fertilovit® M^{plus}) for at least 2 months, twice daily. Content of the supplement is given in Table I. The different components of Fertilovit® M^{plus} were found to be suitable substances for protecting sperm from oxidative stress, or to have other supportive effects. The semen samples were examined during IMSI again after a 2 – 12 month period of antioxidative therapy (mean duration, 3.5 months). No side effects of the antioxidative intake were noted.

The patients' ages ranged from 28 to 61 years (mean 39.3, 25th and 75th percentile between 35 and 43 years). Sperm concentration and percentage of motility were assessed according to the WHO criteria. According to this assessment patients were grouped into OAT and non-OAT patients. A sperm-washing procedure was performed after centrifugation on a three-layer gradient of pure sperm, as previously described [5]. Briefly, post-ejaculated liquefied semen was gently placed onto the gradient and centrifuged at 375 x g for 20 minutes at room temperature. The sperm cell pellet was suspended in human tubular fluid (HTF) medium supplemented with human serum albumin (HSA, LifeGlobal, Ontario, Canada) and centrifuged for 10 minutes. After this washing step, the samples were kept at room temperature. For MSOME analysis, 1 μL of the sperm suspension was transferred in glass bottom dish (WPI, Berlin, Germany) in a micro-droplet with polyvinyl pyrrolidone (PVP). Analysis of MSOME criteria was performed under 6000x magnification on a Nomarski interferential Leica AM 6000 inverted microscope (Leica, Germany). MSOME classification was determined in three categories according to the shape [25] and the presence of nuclear vacuoles as modified from Vanderzwalmen *et al.* [5]. Spermatozoa were classified as grade I when showing normal shape, size, and no vacuoles or only small vacuole(s) (< 4 % of the sperm's head). Grade II were spermatozoa with normal shape and size, but with one or more vacuoles (> 4 % of the sperm's head). Finally, grade III included spermatozoa with abnormal shape and/or size with or without vacuoles.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 17.0 for Windows (SPSS Inc., USA). For analysis of the statistical significance of differences between-groups [sample volume, total motile sperm count (TMSC) and MSOME criteria], the non-parametric Wilcoxon test was applied.

Table I: Substances of content for Fertilovit® M^{plus}

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

For all patients a daily intake of 2 capsules was recommended.

Results

The study population comprised a total of 147 IVF patients. Patients were grouped into OAT (sperm concentration < 15 Mio/mL and progressive motility < 32 %) and non-OAT (sperm concentration ≥ 15 Mio/mL and/or progressive motility ≥ 32 %). According to these criteria 39 men were classified as OAT and 108 as non-OAT (see Table II). From these 108 non-OAT patients, 57 were found to have normozoospermia and 51 had either restrictions in sperm motility or concentration (data not shown). The mean age at the onset of the therapy for OAT patients was 40.1 years and for non-OAT men 39.0 years. By comparing the semen parameters of the first semen analysis, OAT patients revealed lower sperm motility (total motility for OAT patients 20.9 % vs. 54.0 %) and sperm concentration (2.5 Mio/mL vs. 20.9 Mio/mL), as expected. Additionally, we observed smaller ejaculation volume (2.5 vs. 3.0 mL) and, interestingly, a lower percentage of the MSOME class I spermatozoa (2.6 % vs. 5.9 %) in the OAT group.

The second semen analysis was performed in a period of 2 to 12 months after an antioxidative supplementation (mean: 3.2 months for OAT, 3.5 for non-OAT patients). We found significantly more progressive motile sperm in the OAT group in the second semen analysis (0.4 % vs. 2.4 %, $p < 0.05$), Table II. The total sperm number of the OAT group was significantly increased (2.5 Mio ± 5.0 vs. 6.7 Mio ± 9.8, $p < .0.05$), but not in the non-OAT group.

Additionally, a highly significant increase in non-linear motility was observed (8.0 % vs. 28.6 %), $p < 0.001$. In concordance to this observation the percentage of immotile sperm was dramatically decreased (79.1 % vs. 49.5 %, $p < 0.001$). In the non-OAT group we observed no significant increase of progressive motility (4.2 % vs. 5.0 %) or in total motility, (54.0 vs. 56.7 %).

Regarding the subtle sperm morphology, we observed a tendency toward a higher number of MSOME class I spermatozoa in OAT patients (2.6 % vs. 5.0 %), and a significant rise of MSOME class I spermatozoa in the total patient number (5.0 vs. 6.6 %); $p < 0.05$. There were also more class I spermatozoa in the non-OAT patients semen in the second spermiogram (7.2 % vs. 5.9 %), although the rise was not significant.

Discussion

Spermatozoa are particularly sensitive to oxidative stress. First, the sperm's cell membrane contains a multitude of unsaturated fatty acids, which are prone to ROS attack. Second, the sperm's genetic material is highly condensed, which might protect the DNA from ROS, but on the other hand, due to condensation, the sperm DNA is transcriptionally inactive and therefore unable to scavenge elevated ROS concentrations via transcription of ROS-inactivating enzymes.

An imbalance between ROS and antioxidative substances has been hypothesized as a cause for several clinical pictures of infertility. In fact, there are several associations of ineffective ROS scavenging and human disease [26]. In addition, poor sperm quality caused by several intrinsic or extrinsic factors, such as inflammation, irradiation, or malnutrition can be explained by an increase of ROS levels [27]. Advanced male age is also associated with increased ROS levels [28] and might therefore offer an explanation for the decline of sperm quality in advanced age. A number of publications showed a beneficial effect of the intake of antioxidants such as ascorbic acid, tocopherol, glutathione, N-acetyl-L-cysteine, lycopene, and coenzyme Q.

Akmal and colleagues observed a significant improvement in sperm motility in oligozoospermic men after 2 months of oral supplementation with ascorbic acid [29]. Moslemi and Tavanbakhsh reported an amelioration of semen parameters in asthenozoospermia patients with vitamin E and selenium [30]. A placebo-controlled, double-blind randomized study revealed a positive effect of coenzyme Q on sperm motility and number in patients affected by idiopathic asthenozoospermia [31]. In a preliminary report including 30 infertile men, Gupta and Kumar demonstrated an improvement in sperm concentration and motility with the carotene lycopene, which has antioxidative and anti-inflammatory properties [32]. Folate was shown to reduce the probability of aneuploidy and disomy in sperm [33]. Zinc has antioxidative properties and is a cofactor of the ROS-scavenging enzyme, glutathione peroxidase. Decreased zinc concentrations in seminal plasma were found in infertile men [34]. N-acetylcysteine (NAC) was reported to prevent oxidative stress in animal model systems and humans, thus preserving male fertility [35, 36]. Moreover, NAC is a precursor of the ROS-scavenging tripeptide, glutathione. Some studies show a positive effect of nitric oxide (NO)-donating amino acids like citrulline or arginine, attributed to improved blood circulation and alleged reduction of oxidative stress [36]. Although several studies implicate that the redox balance and the intake of antioxidative drugs have an impact on semen quality according to WHO criteria in terms of cell number or motility [38–41], there is still an ongoing discussion

Table II: Routine semen assessment before and after antioxidative supplementation.

		All patients			Non-OAT patients			OAT patients		
		Before treatment	After anti-oxidative intake	p-value	Before treatment	After anti-oxidative intake	p-value	Before treatment	After anti-oxidative intake	p-value
Number of patients		147			108			39		
Mean age at onset of therapy (years)		39.3			39			40.1		
Sample volume (ml)		2.9±1.5	2.4±1.4	**	3.0±1.3	2.4±1.2	***	2.5±1.8	2.5±1.7	n.s.
Sperm count (Mio/ml)		16.0±19.7	19.1±22.7	n.s.	20.9±20.7	23.6±24.3	n.s.	2.5±5.0	6.7±9.8	*
Total sperm count (TSC)		43.8±54.6	38.4±42.8	n.s	58.4±56.9	47.9±44.4	n.s.	3.3±3.5	12.2 ±17.5	*
Sperm motility	Total motile sperm count (TMSC)	45.2±24.4	55.1±22.2	***	54.0±18.7	56.7±19.6	n.s.	20.9±21.6	50.5±27.8	***
	Grade a	3.1±5.9	4.3±6.6	n.s.	4.2 ±6.5	5.0±6.8	n.s.	0.4±1.4	2.4±5.3	*
	Grade b	27.7±19.1	30.5±20.4	n.s.	34.8±16.4	31.2±18.5	n.s.	8.0±10.4	28.6±24.9	***
	Grade c	14.4±14.6	20.3±18.8	**	15.0±13.0	20.6±16.5	**	12.5±18.1	19.6±24.1	n.s.
	Grade d	54.8±24.4	44.9±22.2	***	46.0±18.7	43.3±19.6	n.s.	79.1±21.6	49.5±27.8	***
MSOME criteria	Grade I	5.0±5.5	6.6±6.7	*	5.9 ±5.7	7.2±6.4	n.s.	2.6±4.0	5.0±7.2	n.s.
	Grade II	39.5±15.7	42.8±14.7	n.s.	44.1±12.9	44.7±13.7	n.s.	26.8±16.0	37.4±15.8	**
	Grade III	55.5±18.4	50.6±18.3	*	50.0±15.2	48.0±16.9	n.s.	70.6± 18.1	57.7±20.1	**

Oligoasthenoteratozoospermia was defined according to WHO criteria. MSOME classification was done with modifications according to Vanderzwalmen *et al.*, 2008. OAT= oligoasthenoteratozoospermia; n.s. = not significant; * = $p<0.05$; **= $p<0.01$; *** = $p<0.001$.

about these effects. Our results are well in line with these findings. We found a substantial improvement in sperm quality after a dietary supplementation in terms of motility, number, and moreover the sperm's morphology.

In the context of the importance of antioxidants to maintain redox balance and prevent oxidative stress, nutrition has to be mentioned as a crucial aspect. In a recent questionnaire in our center, the included 1499 male IVF patients reported their mean fruit and vegetable intake to be 1.3 portions/day [42]. This is far below the recommended intake as given in the Joint FAO/WHO Expert Consultation on diet recommending a minimum of 3–5 servings/day. Men attending a fertility clinic should be reminded of the importance of healthy nutrition habits, might be advised to reconsider their foodstuffs, alcohol, and tobacco consumption; men who feel they will not be able to change their dietary habits might be advised to take a suitable antioxidative supplement.

Another important focus of this study was the question of whether an oral antioxidative treatment also has an effect on the appearance of nuclear vacuoles in the sperm head. We observed that the percentage of sperm with no vacuoles (Class I) was significantly increased after the antioxidant supplementation. Although it is not unequivocally clear whether nuclear vacuoles reflect an increase of DNA damage [6,7], failure in chromatin condensation [8], or other subcellular events, it is quite obvious that nuclear vacuoles do not present a physiological, but a pathological state. Given that vacuoles might reflect defective DNA-packaging, epigenetic alternations, or DNA fragmentation on a morphological level and considering the fact that nuclear vacuoles affect IVF outcome, our results provide further evidence for the effectiveness of an oral antioxidative therapy on sperm quality. As long as the origin of the nuclear vacuolization is not fully understood, the pathways of influencing the vacuolization rate are only hypothetical. Different pathways can be discussed such as improvement of sperm condensation by better protamination, influenced by improved blood circulation and nutritive support, reduced DNA fragmentation by reduced oxidative stress or decreased inflammation inside the urogenital tract, or improved intracellular processes by improved mitochondrial activity. Further studies are needed to address this topic.

Our data show an improvement of sperm parameters after antioxidant supplementation. In addition to a higher percentage of motile sperm and an increased sperm count, a positive effect on sperm morphology, in terms of vacuole formation, was observed. To our knowledge, this is the first time that a reduction of the number of sperm with vacuoles due to antioxidant supplementation has been reported. Given that sperm quality is one major determinant for the success of any fertility treatment, and that sperm morphology has been found to reflect sperm health, it can be concluded that these findings should influence counseling of male patients prior to fertility treatment.

Footnotes

ART: assisted reproductive technology
FAO: Food and Agriculture Organization
HSA: Human serum albumin
HTF: Human tubular fluid
ICSI: intracytoplasmic sperm injection
IMSI: intracytoplasmic morphologically selected sperm injection
IVF: in vitro fertilization
MSOME: motile sperm organelle morphology examination
NAC: N-acetylcysteine
OAT: oligoasthenoteratozoospermia
OS: Oxidative stress
PVP: Polyvinyl pyrrolidone
ROS: reactive oxygen species
SPSS: Statistical Package for Social Sciences
TESE: testicular sperm extraction

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