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ORIGINAL ARTICLE

Male Infertility

Antioxidant treatment for oligoasthenoteratozoospermia and varicocele: a DBPC trial to evaluate the impact of age and body mass index

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Oxidative stress is one of the main mechanisms responsible for male infertility. Various conditions such as varicocele, obesity, advanced age, and lifestyle can lead to an increase in reactive oxygen species, causing an oxidative imbalance in the reproductive environment. Spermatozoa are sensitive to reactive oxygen species and require energy to carry out their main function of fertilizing the egg. Excessive reactive oxygen species can affect sperm metabolism, leading to immobility, impaired acrosome reaction, and cell death, thereby impairing reproductive success. This double-blind randomized study evaluated the effect of supplementation with L-carnitine, acetyl-L-carnitine, vitamins, and other nutrients on semen quality in 104 infertile patients with or without varicocele, while also investigating the impact of factors such as obesity and advanced age on treatment. Sperm concentration significantly increased in the supplemented group compared to the placebo group ($P = 0.0186$). Total sperm count also significantly increased in the supplemented group ($P = 0.0117$), as did sperm motility ($P = 0.0120$). The treatment had a positive effect on patients up to 35 years of age in terms of sperm concentration ($P = 0.0352$), while a body mass index (BMI) above 25 kg m^{-2} had a negative effect on sperm concentration ($P = 0.0110$). Results were not showing a net benefit in stratifying patients in accordance with their BMI since sperm quality increase was not affected by this parameter. In conclusion, antioxidant supplementation may be beneficial for infertile patients and has a more positive effect on younger patients with a normal weight.

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INTRODUCTION

The infertility affects around 17% of couples in the world and is defined as failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. Infertility can be caused by both female and male factors, as well as a combination of factors from both parties. Some cases can be classified as idiopathic, when the cause is unknown, adding another layer of complexity to the diagnosis.[1] Male infertility is responsible for 50% of cases. It can be attributed to factors such as varicocele, obesity, infections, diabetes, lifestyle choices such as smoking, and advanced paternal age.[2]

Varicocele is considered one of the main causes of male infertility and affects approximately 15% of the general population.[3] The precise mechanisms by which varicocele may impact male fertility remain incompletely understood. Nevertheless, there is a suggestion that varicocele could raise hydrostatic pressure in the pampiniform plexus and epididymis, consequently inducing alterations in the testicular microenvironment.[4] Additionally, varicocele can raise scrotal temperature, disrupting the temperature gradient due to impaired heat exchange. As a result, there is an increased production of free radicals that can potentially affect spermatogenesis and sperm maturation in the epididymis.[5] Obesity is also a significant factor contributing to infertility. Like varicocele, the exact mechanisms are not fully known. Nonetheless, studies have demonstrated that molecules associated with obesity and the inflammatory milieux have the potential to influence the male reproductive system, resulting in alterations to sperm functional parameters. This includes heightened levels of DNA fragmentation and oxidative stress, alongside a reduction in sperm motility.[6–8] Moreover, paternal age can pose a problem for couples

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trying to conceive, as hormonal levels, sexual function, and sperm production naturally decline with aging.[9]

Many factors that affect infertility are associated with oxidative stress.[10] Oxidative stress is caused by an imbalance between reactive oxygen species (ROS) and antioxidants. Typically, mitochondria generate a specific quantity of ROS through oxidative phosphorylation and the general metabolism of spermatozoa. These ROS play a vital role in fundamental fertilization processes, including capacitation, acrosome reaction, hyperactivation, binding of the spermatozoon to the zona pellucida, and cellular signaling.[10] However, when in excess, ROS becomes detrimental to the spermatozoon, as its membrane is rich in polyunsaturated fatty acids and susceptible to lipid peroxidation. Spermatozoa also lack essential cytoplasmic enzymes necessary for repairing oxidative stress-induced damage.[10] Lipid peroxidation can generate toxic compounds that can cause DNA fragmentation, consequently affecting the spermatozoa, resulting in reduced motility, impact on the acrosome reaction, as well as induction of apoptosis, thereby diminishing the chances of reproductive success.[11]

Several studies have been conducted to evaluate therapies to improve seminal quality and to decrease oxidative stress, such as treatment using antioxidants and metabolic compounds. Some commonly used antioxidants include vitamins C and E, selenium, zinc, coenzyme Q10 (CoQ10), and carnitines.[12,13]

Carnitines, fructose, vitamin C, vitamin B12, CoQ10, zinc, selenium, and folic acid play important roles in sperm health. Carnitines play a role in cellular energy metabolism and possess antioxidant properties. Fructose serves as a crucial carbohydrate, providing energy for spermatozoa. Vitamin C exhibits antioxidant properties, safeguarding sperm against oxidative stress. Additionally, vitamin B12 is indispensable for DNA production.[14,15] CoQ10 is related to cellular energy production and acts as an antioxidant in mitochondria. Zinc is important for testosterone production. Selenium protects sperm from oxidative damage, and it is necessary for testicle development and spermatogenesis. Folic acid plays a role in genetic material production.[12] All these compounds are associated with an improvement in semen parameters and reproductive success rates.[16–19] However, the results are conflicting due to the absence of high-quality randomized clinical trials.

This study is based on a double-blind placebo-controlled trial with the objective to evaluate the efficacy of antioxidant and metabolic compounds on sperm quality in participants experiencing oligo-, astheno-, and/or teratozoospermia, with or without varicocele. It also aims to evaluate if variables such as age and body mass index (BMI) have an impact on the treatment. Decision curve analysis (DCA) has been incorporated due to its crucial role in evaluating whether to include a compound in clinical practice and in determining if advantages can be discerned among different categories of patients.

PATIENTS AND METHODS

Patients and study design

In this study, the impact of antioxidant supplementation on semen quality was evaluated using a post hoc analysis of our database.[20,21] The trial included 104 infertile patients exhibiting oligo- and/or astheno- and/or teratozoospermia, with or without varicocele, in a monocentric randomized double-blind placebo-controlled design (**Figure 1**). All participants included were enrolled from the Andrology Clinic at the Department of Gynecological-Obstetric Sciences and Urological Sciences, Sapienza Rome University (Rome, Italy), and the data were collected between December 2014 and June 2015. The groups were divided between patients with grades I–III varicocele (52 patients) and

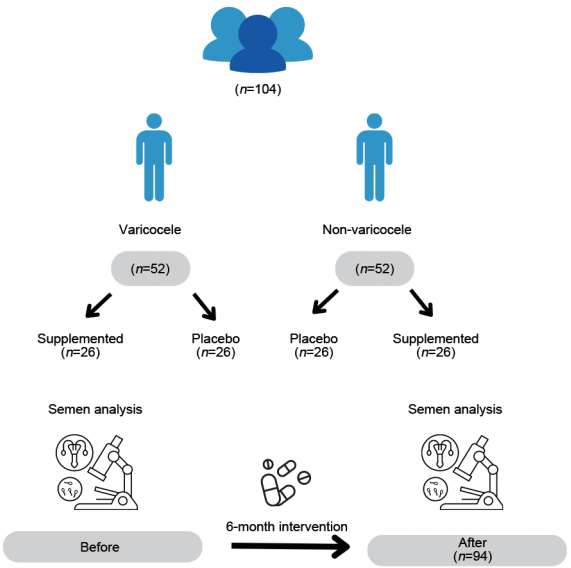

Figure 1: The study is designed as a double-blind controlled trial with 104 infertile patients, divided by the presence of varicocele. The volunteers received a supplementation with antioxidants and metabolites or placebo for a period of 6 months. Before the treatment, a semen analysis was conducted in each patient, and after 6 months another semen analysis was conducted. At the end of the study, 94 patients completed.

without varicocele (52 patients). Ten patients were excluded from the study leaving 45 patients with varicocele and 49 without varicocele. The study was approved by the Ethics Committee of the Department of Gynecological-Obstetric Sciences and Urological Sciences, Sapienza Rome University (Approval No. PXP-001A) and conducted in line with European Urology and Good Clinical Practice guidelines, with ethical principles laid down in the latest version of the Declaration of Helsinki. Informed consent was signed by every patient who participated in the study. The study was registered on clinicaltrial.gov (No. NCT04177667).

Inclusion and exclusion criteria

Patients were selected following a comprehensive check-up, which included a review of medical history, physical examination, ultrasound and Doppler assessment, hormone evaluation, and genetic tests. This extensive evaluation aimed to eliminate other potential causes of infertility for all participants, encompassing both male and female partners. The inclusion criteria comprised individuals aged between 18 years and 50 years, a diagnosis of oligo-, and/or astheno-, and/or teratozoospermia (with or without varicocele), and a history of infertility exceeding 12 months. Female partners needed to fulfill specific criteria, including having a regular menstrual cycle, being under the age of 40 years, and not currently undergoing any assisted reproductive technology (ART) procedures. The varicocele patients were not surgically treated before and during the treatment. Patients without varicocele were suffering from idiopathic male infertility, and had no other previous history of diseases affecting fertility. Exclusion criteria were: known hypersensitivity to any of the compound, history of undescended testes or cancer, endocrine disorders, history of postpubertal mumps, genitourinary surgery, obstructive azoospermia or obstructive pathology of the urogenital system, autoimmune disease, cystic fibrosis, history of taking any therapy affecting fertility, excessive consumption of alcohol or regular use of illicit or "recreational" drugs, positive serology for human immunodeficiency virus (HIV),

subjects following any special diet or taking antioxidants, any systemic condition which in the opinion of the investigator might put the subject at risk by participation in this study, or subjects involved in any other clinical trials. A block randomization method was employed to divide all patients into groups, ensuring the randomization of subjects and achieving equal sample sizes. This approach was implemented to maintain balance across the groups throughout the study duration. While pregnancy rate was not the primary endpoint of the study, we did report the number of pregnancies that occurred during the course of the study.

Intervention

Based on the randomization method, the volunteers received a supplementation formulation (Proxeed Plus from Alfasigma HealthScience, Utrecht, The Netherlands) or placebo daily for 6 months. The supplementation formulation consisted of 1000 mg L-carnitine, 725 mg fumarate, 500 mg acetyl-L-carnitine, 1000 mg fructose, 20 mg CoQ10, 90 mg vitamin C, 10 mg zinc, 200 μg folic acid, and 1.5 μg vitamin B12. The placebo was provided from the same company and was made with excipients (sucrose, silica anticaking, lemon flavor, and acesulfame K [E950] sweetener) of the supplementation without the active compounds. Before the initiation of the treatment, a semen analysis was performed to assess the semen parameters for each patient. Subsequently, 6 months after the completion of the treatment, another sample analysis was conducted. Additionally, we gathered demographic data (including age, weight, and height), conducted a physical examination, measured blood pressure, and documented medical history and the use of previous/concomitant therapies both before and after the treatment. The variables from the semen analysis were assessed as the primary outcome, with pregnancy rate considered a secondary outcome.

The semen samples were obtained by masturbation after 2–5 days of ejaculatory abstinence. After semen collection and liquefaction, ejaculate volume, total sperm count, total and progressive motility, as well as sperm morphology were evaluated based on the 5th (2010) edition of the World Health Organization (WHO) Guidelines.[1,2]

Statistical analyses

Planning to carry out the analysis of covariance in a factorial design with two groups (compound or placebo, with and without varicocele), defined f = σm/σ (σm is the sample size weighted) = 0.25 and the correlation coefficient (R^2) between the baseline and final equal to 0.50, chosen a = 0.05 and b = 0.20 (power of 80%). It was necessary to enroll at least 88 patients, equally distributed in 22 units for each subgroup. However, anticipating a dropout rate of about 15%, it was expected to enroll 104 patients (52 per arm).

This is a post hoc analysis with logistic regression models applied to evaluate four different outcomes (change at final visit from baseline of total number of spermatozoa, sperm concentration, sperm motility and sperm morphology) after 6 months of intervention on a group of patients aged between 18 years and 50 years. The two groups were balanced for the presence of varicocele. The outcome variables were categorized in binary variables when 1 was an increase of the difference at 6 months from baseline of at least 10% and 0 was not increase of the difference. The factors considered in this analysis were the following: group of treatment (active = 1, placebo = 0), BMI (>25 kg m^{-2} *vs* ≤25 kg m^{-2}), age (>35 years *vs* ≤35 years) and presence of varicocele (yes *vs* no). We considered eight subgroups, defined by two age classes of ≤35 years old and >35 years old; and two weight classes of BMI ≤25 kg m^{-2} and >25 kg m^{-2}. The BMI cut-off of 25 kg m^{-2} was suggested by clinicians, as it is typically regarded as the upper limit of the normal BMI range. The age cut-off of 35 years was selected because it is commonly seen as the age at which individuals in Western countries often begin seeking parenthood.

After identifying possible significant factor(s), an additional analysis was proposed in order to evaluate how the intervention was appropriate on the population at risk. For this evaluation, the decision curve analysis was performed. Statistical significance was defined when $P < 0.05$.

RESULTS

Among the 104 patients initially recruited, 94 successfully completed the study, representing a completion rate of 90.4%. The varicocele group consisted of 45 patients, while the nonvaricocele group included 49 patients. Ten individuals were excluded from the study due to insufficient adherence to the therapy, as evidenced by the return of several sachets of the compound/placebo, indicating noncompliance with the prescribed medication. The supplemented group experienced adverse effects, but none were serious. Four patients reported nausea, while three reported dizziness and headaches. The mean age of patients in both the placebo and treatment groups was 32.5 (range: 18–48) years.

For a complete overview of the supplementation effect on sperm, in the following sections, for each parameter, beside the results of the present post hoc analysis, some results of a previous analysis are presented.[20,21]

Sperm concentration

There was a statistically significant increase in sperm concentration observed in the overall patient group (comprising both varicocele and non-varicocele cases) undergoing antioxidant treatment compared to patients receiving the placebo ($P < 0.05$). In the placebo group, the sperm concentration (mean ± s.d.) started at $41.4 \times 10^6 \pm 17.9 \times 10^6$ ml^{-1} at the beginning of treatment and increased to $43.7 \times 10^6 \pm 13.6 \times 10^6$ ml^{-1} at the treatment's conclusion. In contrast, the active group began with a sperm concentration of $40.8 \times 10^6 \pm 18.2 \times 10^6$ ml^{-1}, which raised to $51.4 \times 10^6 \pm 13.9 \times 10^6$ ml^{-1} by the end of treatment. The proportion of patients experiencing an increase from the initial value was higher in the supplemented group (73.3%) compared to the placebo group (51.0%; $P < 0.05$).

Antioxidant treatment and age younger than 35 years yielded a positive result with an odds ratio of 2.23 (95% confidence interval [CI]: 1.03–4.82; $P < 0.05$) and odds ratio of 2.14 (95% CI: 1.05–4.33; $P < 0.05$), respectively. However, a BMI above 25 kg m^{-2} showed a negative result in the supplemented group with an odds ratio of 0.25 (95% CI: 0.09–0.73; $P < 0.05$). The varicocele had no effect on the sperm concentration values ($P > 0.05$). The data are described in **Table 1** and illustrated in **Figure 2**.

Total count

Total sperm count showed a statistically significant increase in the overall patient group (varicocele and non-varicocele) undergoing antioxidant treatment compared to patients who only received placebo ($P < 0.05$). At the initiation of treatment, the placebo group displayed a total sperm count (mean ± s.d.) of $113.1 \times 10^6 \pm 37.4 \times 10^6$, which rose to $127.8 \times 10^6 \pm 61.4 \times 10^6$ at the end of the treatment. In contrast, the supplemented group commenced with a total sperm count of $114.2 \times 10^6 \pm 37.8 \times 10^6$, experiencing an increase to $163.5 \times 10^6 \pm 64.3 \times 10^6$ after 6 months. The percentage of patients demonstrating an increase from the initial value was higher in the supplemented group (82.2%) compared to the placebo group (55.1%; $P < 0.05$).

As depicted in **Figure 2**, none of the evaluated factors (antioxidant treatment, BMI, age, and varicocele) influenced the final result of the total sperm count.

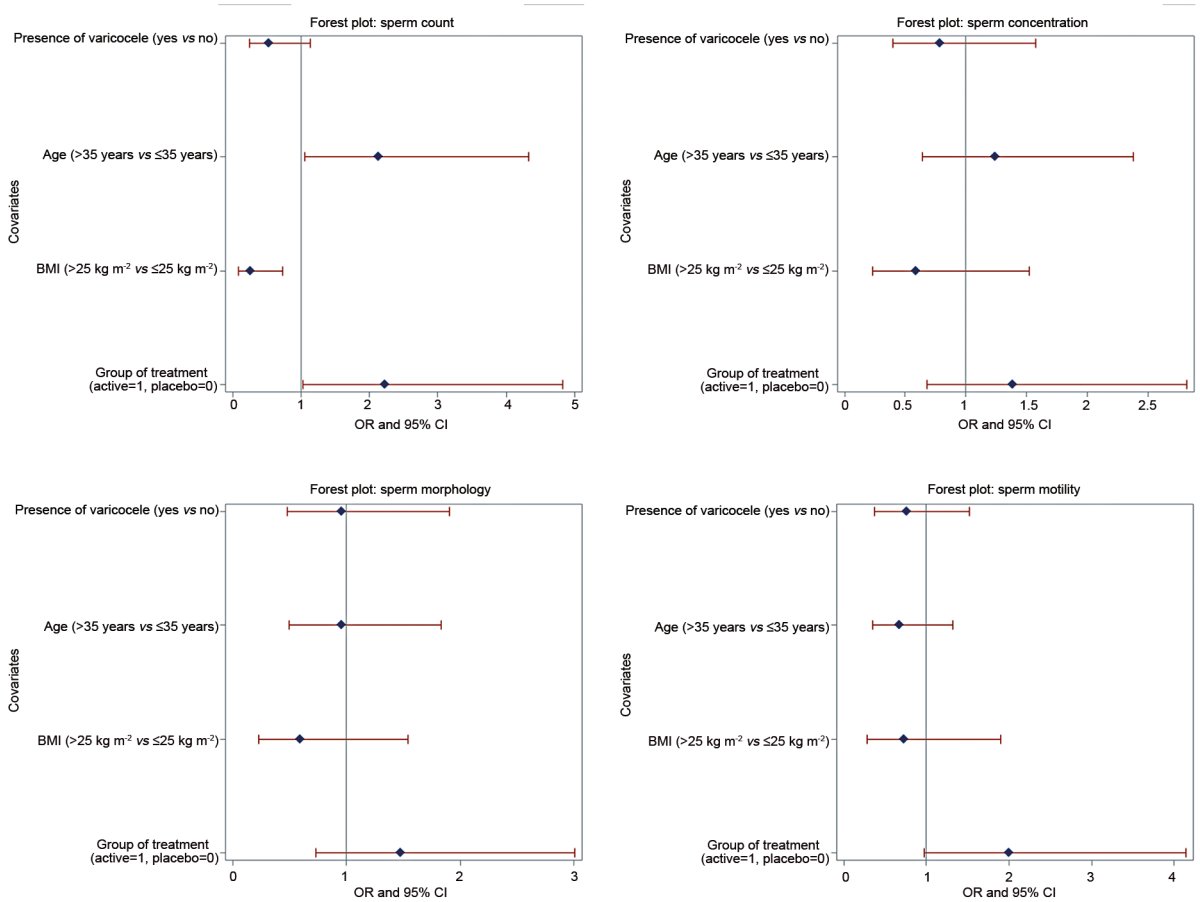

Figure 2: Forest plot summarizing the effect of antioxidant treatment in accordance with presence of varicocele, different age, different BMI and group of treatment. Each seminal parameter has been evaluated (sperm count, concentration, motility and morphology). CI: confidence interval; OR: odds ratio; BMI: body mass index.

Table 1: Effect of treatment and factor as body mass index, age, and presence of varicocele in each sperm parameters

Factor	*Sperm concentration*			*Total sperm count*			*Sperm morphology*			*Sperm total motility*		
	Point estimated	*95% CI*	*P*	*Point estimated*	*95% CI*	*P*	*Point estimated*	*95% CI*	*P*	*Point estimated*	*95% CI*	*P*
Group of treatment (active=1, placebo=0)	2.23	1.03–4.82	0.04	1.39	0.68–2.82	0.37	1.48	0.73–3.01	0.28	2.00	0.97–4.14	0.06
BMI (>25 kg m^{-2} *vs* ≤25 kg m^{-2})	0.25	0.09–0.73	0.01	0.59	0.23–1.52	0.27	0.60	0.23–1.54	0.29	0.73	0.28–1.90	0.52
Age (>35 years *vs* ≤35 years)	2.14	1.05–4.33	0.04	1.24	0.64–2.38	0.52	0.96	0.50–1.84	0.90	0.68	0.35–1.32	0.25
Presence of varicocele (yes *vs* no)	0.54	0.25–1.13	0.10	0.79	0.39–1.57	0.50	0.96	0.48–1.91	0.91	0.76	0.38–1.52	0.44

CI: confidence interval; BMI: body mass index

Total motility

The total sperm motility exhibited a significant increase in the supplemented patients in comparison to the placebo group ($P < 0.05$). Before treatment, the placebo group displayed a sperm motility (mean ± s.d.) of 32.6% ± 9.2%, which rose to 34.6% ± 7.1% at the end of the treatment. In contrast, the supplemented group started with 31.7% ± 8.2% and increased to 39.0% ± 8.0%.

The antioxidant treatment had a positive effect on sperm motility, although it did not reach statistical significance ($P > 0.05$), with an odds ratio of 2.00 (95% CI: 0.97–4.14; **Table 1**). BMI, age, and the presence or absence of varicocele had no effect on sperm motility values (**Figure 2**).

Sperm morphology

There was no significant difference in sperm morphology between the groups, both at the study's outset ($P > 0.05$) and its conclusion ($P > 0.05$). In the placebo group, the percentage of sperm with normal morphology (mean ± s.d.) was 21.1% ± 16.2% at baseline and decreased to 15.7% ± 9.4% after treatment ($P < 0.05$). Meanwhile, in the supplemented group, the percentage of sperm with normal morphology (mean ± s.d.) was 23.5% ± 14.6% before treatment and decreased to 17.7% ± 15.2% after treatment ($P < 0.05$).

Furthermore, antioxidant treatment, presence or absence of varicocele, BMI, and age had no effect on the outcome of sperm morphology (**Figure 2**).

Effect of BMI to the treatment

BMI was considered for the decision curve analysis. This curve was conducted only on the varicocele group considering the values of sperm concentration and motility. The curves are represented in **Figure 3**. The results were not showing a net benefit in stratifying

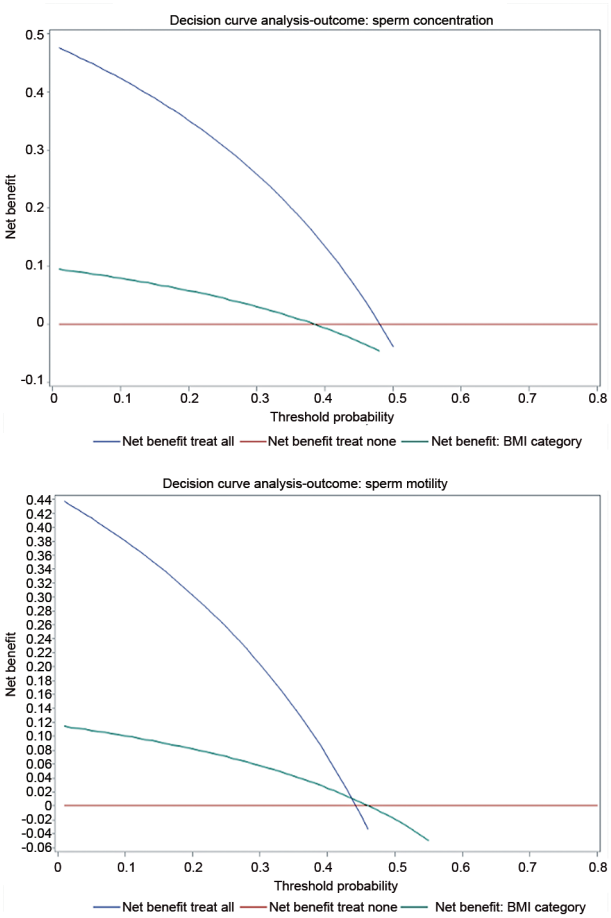

Figure 3: Representation of decision curve analysis, considering the BMI factor on the values of sperm concentration and motility in varicocele group. BMI: body mass index.

patients in accordance with their BMI since sperm quality increase was not affected by this parameter, therefore, the results suggested treat all the subjects regardless their BMI.

Pregnancy rate

A total of 12 pregnancies were documented post-treatment. Among these, ten pregnancies were observed in the supplementation group (comprising nine in the nonvaricocele group and one in the varicocele group), whereas two pregnancies were recorded in the placebo group (one in the non-varicocele group and one in the varicocele group). Notably, one spontaneous abortion was reported in the placebo group.

DISCUSSION

In recent years, there has been a significant increase in the incidence of male infertility.[1] At present, seminal analysis stands as the predominant diagnostic approach; however, its predictive efficacy is constrained. Consequently, novel diagnostic methods are being devised and implemented to assess sperm, taking into account its functionality and molecular aspects. Among these methods are tests for sperm DNA fragmentation, biochemical assays, and assessments for the presence of ROS. Within this framework, a novel concept in the realm of reproduction surfaced in 2019, as "male oxidative stress infertility (MOSI)".[22]

Studies suggest that high levels of ROS can negatively impact sperm quality by reducing sperm motility and sperm concentration, as well as causing damage to sperm DNA. These effects can hinder fertilization of the egg and increase the chances of spontaneous miscarriage.[10,22] Risk factors such as varicocele, obesity, and advanced age play an important role in the production of ROS. Additionally, many couples experience infertility without apparent cause, wherein they exhibit a normal semen analysis but present with infertility. Recent studies have delved into a potential connection between seminal oxidative stress and unexplained infertility. This is particularly pertinent because numerous patients with normal semen analysis results exhibit functional sperm dysfunction, characterized by increased levels of DNA fragmentation and diminished oxidative reduction potential.[3,6,9,10,22,23]

In order to improve and restore oxidative balance, physicians often recommend lifestyle changes, such as adopting a balanced diet rich in antioxidants, engaging in regular physical exercise, avoiding exposure to toxic substances, and reducing stress. Furthermore, supplementation with specific antioxidants, such as carnitines, vitamin C, vitamin E, zinc, and selenium, has been studied as a potential approach to enhance sperm quality and reduce oxidative stress.[12]

A meta-analysis indicated a general improvement in pregnancy rates when utilizing antioxidant supplementation, implying potential benefits for interventions directed at male fertility.[24] Additionally, a study by Yaris *et al*.[18] proposed that supplementation with selenium, CoQ10, L-carnitine, acetyl-L-carnitine, folic acid, and zinc could be advantageous in addressing male infertility.

Balercia *et al*.[25] conducted a placebo-controlled, double-blind and randomized trial evaluating the use of carnitine supplementation in 59 patients. They observed an increase in seminal parameters among those who received the treatment compared to those who received placebo. Additionally, there was an increase in pregnancy rates among patients treated with carnitine.[25]

Combining CoQ10 with other compounds has demonstrated efficacy in diminishing testicular oxidative stress, reducing sperm DNA fragmentation, and enhancing seminal parameters, particularly sperm motility. Additionally, CoQ10 treatment has shown an association with elevated pregnancy rates, both in natural conception and *in vitro* fertilization (IVF) settings.[16]

Another meta-analysis investigating vitamin C and E supplementation to improve success rates revealed that vitamin E is associated with an improvement in pregnancy rates. When combined with vitamin C, there was also an improvement in sperm motility, concentration, and morphology.[17]

However, there are conflicting findings in studies regarding the use of antioxidants to improve reproductive success. Some studies failed to show significant differences in improving seminal quality.[26–28] Therefore, further well-designed clinical studies are needed to properly evaluate the effect of antioxidants on male infertility.

We conducted a clinical trial employing a double-blind, placebo-controlled design to assess the potential benefits of antioxidant supplementation in the context of male infertility.[20,21] This study encompassed infertile patients, both with and without varicocele, and examined the influence of factors such as BMI and age on the outcomes of antioxidant therapy. Noteworthy improvements in seminal parameters, including sperm motility, sperm concentration, and total count, were observed in both groups. However, the enhancements were more pronounced in patients under the age of 35 years with a BMI below 25 kg m^{-2}. Conversely, patients with a BMI above 25 kg m^{-2} and those aged over 35 years exhibited a less favorable response, particularly in terms of sperm concentration. Therefore, we concluded that antioxidant supplementation is more effective in younger patients with a healthy body weight. On the other hand, decision curve analysis

results, which provide a comprehensive method for evaluating the clinical utility of using the compound, suggest treating all subjects regardless of their BMI. DCA, by offering insights into the real-world application of the compound, enables a direct comparison of the clinical impact of using the drug within specific patient groups, facilitating evidence-based decision-making regarding its applicability and efficacy within varied patient population. Finally, it is important to mention that, although not the primary focus of the study, 12 pregnancies occurred during the follow-up period, with 10 in the supplemented group and 2 in the placebo group.

However, our study has some limitations. We did not include tests for DNA fragmentation before and after treatment, nor did we assess oxidative stress through tests such as oxidation-reduction potential (ORP), which could have provided more comprehensive data on the effect of supplementation on sperm functionality. Therefore, more studies are needed to investigate these parameters for a more comprehensive understanding of the benefits of antioxidant supplementation in enhancing male infertility.

AUTHOR CONTRIBUTION

GMB, AC, AN, AR, GB, PF, and BFR take responsibility for the integrity of the data and accuracy of the data analysis while review concept and design of the review were developed by GMB, BFR, AV, AA, VM, UGF, and LC. All authors contributed to acquisition, analysis, and/or interpretation of data. Drafting of the manuscript was performed by GMB, BFR, UGF, PA, NdA, MF, FC, and AP. GMB, CB, GC, and LC are the study supervisor of the entire project. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors and Alfasigma HealthScience Company declared no competing interests.

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