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Effect of metabolic and antioxidant supplementation on sperm parameters in oligo-astheno-teratozoospermia, with and without varicocele: A double-blind placebo-controlled study

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Summary

Sperm require high energy to perform their specialised function, and it is vital that essential nutrients are available when they develop, capacitate and acquire motility. However, spermatozoa are vulnerable to lack of energy and excess amounts of reactive oxygen species, which can impair function, subsequently leading to immobilisation, acrosomal reaction impairment, DNA fragmentation and possibly cell death. This monocentric, randomised, double-blind, placebo-controlled trial investigated the effect of 6 months of supplementation with L-carnitine, acetyl-L-carnitine and other micronutrients on sperm quality in 104 subjects with oligo- and/or astheno- and/or teratozoospermia with or without varicocele. In 94 patients who completed the study, sperm concentration was higher in supplemented patients compared to placebo (statistically significant change $p = .0186$). Total sperm count positively changed in supplemented group with a significant difference with placebo group ($p = .0117$). Both progressive and total motility were higher in supplemented patients; the difference with placebo is significant ($p = .0088$ and $p = .0120$ respectively). A small difference (not statistically significant) was also observed in the semen volume in favour of the experimental group. All of these parameters are, in general, more evident in those suffering varicocele. In conclusion, supplementation with metabolic and antioxidant compounds could be efficacious when included in strategies to improve infertility.

KEYWORDS

antioxidant, oligo-astheno-teratozoospermia, sperm, spermiogram, varicocele

1 | INTRODUCTION

Infertility is the inability of a sexually active, noncontracepting couple to achieve spontaneous pregnancy within one year. Worldwide, the incidence of infertility is about 15%, of which, in general, 50% can be attributed to a male-associated factor. This can be reported with or without abnormal semen parameters (WHO, 2000). Male fertility can be affected by many factors ranging from congenital, malignancies, endocrine, immunologic, infectious as well as lifestyle factors. On the other hand, in 30%–40% of cases, no obvious male infertility-associated factor is found (idiopathic male infertility; Nieschlag, Behre, & Nieschlag, 2010).

Varicocele is defined as an abnormal dilatation of scrotal veins, and data report a general prevalence of 15% in the healthy male population, whereas it is 40% in infertile men (Nagler, Luntz, & Martinis, 1997). Although the pathophysiologic mechanisms are not yet

completely known, varicocele has adverse effects on spermatogenesis and, to date, is considered as the most common among the known causes of male infertility (Practice Committee of American Society for Reproductive Medicine and the Society for Male Reproduction and Urology, 2014).

Spermatozoa have very high energy requirements as sperm maturation as well as sperm function such as capacitation and motility is all highly energy-dependent (Talwar & Hayatnagarkar, 2015). Many factors that negatively affect semen quality act through decreasing energy availability by mitochondrial dysfunction (Amaral, Lourenço, Marques, & Ramalho-Santos, 2013). Spermatozoa are also vulnerable to reactive oxygen species (ROS) because plasma membranes and cytoplasm are rich in polyunsaturated fatty acids (Agarwal et al., 2014). In particular, ROS accumulation leads to membrane damage, membrane instability and functional alterations causing cell death (Agarwal et al., 2014). Latest evidence demonstrates an association between high ROS levels and increased mitochondrial DNA (mtDNA) copy number with decreased mtDNA integrity (Bonanno et al., 2016). Oxidative stress (OS) occurs when there is an imbalance between oxidants and antioxidants (Agarwal, Hamada, & Esteves, 2012). However, for normal sperm cell function including chromatin compaction in maturing spermatozoa during epididymal transit, a delicate redox balance of reduction and oxidation is required (Wright, Milne, & Leeson, 2014). In general, an oxidative milieu may lead to cellular degeneration by apoptosis or necrosis, and a reducing milieu could favour cell survival (Durackova, 2014). Thus, a therapeutic strategy would need to use supplements to increase sperm energy metabolism, minimise free radical damage to sperm and improve the cellular processes connected with the formation and maturation of sperm.

L-Carnitine and acetyl-L-carnitine play an important role in spermatozoa energy metabolism (Agarwal & Said, 2004; Zhou, Liu, & Zhai, 2007). Many clinical studies have shown that their oral administration to asthenozoospermic subjects increases the percentage of mobile spermatozoa, progressive rapid motility, average speed and linearity sperm index (Balercia et al., 2005; Lenzi et al., 2004). Selenium is an essential component of several major metabolic pathways: antioxidant defence systems, thyroid hormone metabolism and immune function (Brown & Arthur, 2001). Coenzyme Q10 is concentrated in the mitochondria of the midpiece of sperm, and its levels show a significant correlation with sperm count and with sperm motility. Furthermore, the CoQ10 may be deficient in varicocele leading to higher sensitivity to oxidative damage (Balercia et al., 2004). Fructose, citric acid, vitamin C, vitamin B12 and zinc are related to increased damage to the sperm genetic material, synthesis of coenzymes, metabolism and energy production (Chia, Ong, Chua, Ho, & Tay, 2000; Dawson, Harris, Teter, & Powell, 1992; Moslemi & Tavanbakhsh, 2011).

Thus, the objective of this trial was to evaluate, utilising a randomised, double-blind, placebo-controlled design, the effect of supplementation with selected naturally occurring compounds formulation on sperm quality. The effect was evaluated in subjects with oligo- and/or astheno- and or teratozoospermia, as well as with or without varicocele.

2 | MATERIALS AND METHODS

Between December 2014 and June 2015, 104 infertile patients with oligo- and/or astheno- and/or teratozoospermia with an average age of 32.5 years (range 18–48) were enrolled in this single-centre, randomised, double-blind, placebo-controlled trial to determine the effect of antioxidant supplementation on semen quality. All participants were enrolled from our andrology clinic at the Department of Gynecological-Obstetric Sciences and Urological Sciences, "Sapienza" Rome University. The block randomisation method was used to randomise subjects into groups that result in equal sample sizes to ensure a balance across groups over time. At study start, 52 patients with varicocele grade I-III (confirmed with Doppler ultrasound) and 52 patients without varicocele were divided into two groups consisting of the supplementation arm and the placebo arm. Ten patients dropped out from the study leaving 45 patients with varicocele and 49 without varicocele.

The supplementation formulation (Proxeed Plus from Sigma-Tau HealthScience, Utrecht, the Netherlands) consisted of 1,000 mg of L-carnitine, 725 mg of fumarate, 500 mg of acetyl-L-carnitine, 1,000 mg of fructose, 50 mg of citric acid, 50 μg of selenium, 20 mg of coenzyme Q10, 90 mg of vitamin C, 10 mg of zinc, 200 μg of folic acid and 1.5 μg of vitamin B12. Placebo, provided from the same company, was made with excipients (sucrose, silica (anti-caking), lemon flavour, acesulfame K (E950) sweetener) of the supplementation without the active compounds.

Subjects received supplement or placebo (two sachets daily for 6 months) according to the randomisation schedule (nQuery Advisor nTerim 2.0 (2012) program) and were instructed of the method of use. One evaluation of a spermiogram was carried out at the beginning of treatment (V1) to examine semen parameters in each patient. At the end of the 6-month treatment (V2), a consecutive spermiogram was collected. Together with semen analysis, before and after treatment, we collected demographic data (age, weight, height), physical examination, blood pressure, medical history and intake of previous/concomitant therapies.

Semen samples were collected after 3–5 days of sexual abstinence, and variables taken into consideration were ejaculate volume, total sperm count, progressive motility, total motility and sperm morphology. Classification of the spermiograms was made according to the WHO guidelines (2010; 5th edition guidelines).

In our trial, we have included men with oligo-, astheno- and/or teratozoospermia, with or without varicocele, men aged between 18 and 50 years, men from couples with history of difficulty conceiving for more than 12 months. The varicocele patients were not surgically treated before and during the treatment. Patients without varicocele were suffering from idiopathic male infertility, and these men presented with no previous history of diseases affecting fertility. Every patient underwent a complete check-up to exclude any other cause of infertility (history, examination, complete ultrasound and Doppler, hormones and genetic tests) with no difference between varicocele and nonvaricocele patients. Fertile female partners were required with regular menstrual cycles, age <40 and couples not looking for fertility-related procedures such as artificial insemination (AI), in

vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) for next 90 days.

Subjects with known hypersensitivity to any of the treatment compound, history of undescended testes or cancer, endocrine disorders, history of post-pubertal mumps, genitourinary surgery, obstructive azoospermia or obstructive pathology of the urogenital system, autoimmune disease, cystic fibrosis, history of taking any therapy affecting fertility within last 3 months, excessive consumption of alcohol or regular use of illicit or "recreational" drugs, positive serology for HIV, subjects following any special diet, any condition which in the opinion of the investigator might put the subject at risk by participation in this study and subjects involved in any other clinical trials were excluded from the trial. Endpoints of the study were sperm concentration, semen volume, total sperm count, total motility, progressive motility and percentage of normal sperm morphology.

The Ethical Committee of the Department of Gynecological-Obstetric Sciences and Urological Sciences, "Sapienza" Rome University, approved the study protocol (Institute Ethical Approval Number PXP-001A). The study was 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. Every patient signed an informed consent to participate in the study.

2.1 | Sample size

Planning to carry out the analysis of covariance in a factorial design with two groups (Proxeed or Placebo, with and without varicocele), defined $f = \sigma m/\sigma = 0.25$ and the correlation coefficient (R^2) between the baseline and final equal to 0.50, chosen $\alpha = .05$ (significance) and $\beta = .20$ (power of 80%), it was necessary to enrol at least 88 patients equally distributed in 22 units for each subgroup. However, in anticipation of having about 15% of dropout, 104 patients (52 per arm) were enrolled.

2.2 | Statistical analysis

All continuous variables have been reported as mean, median, standard deviations, minimum and maximum values. Discrete and nominal variables have been reported as frequency and percentage in contingency tables. The basal homogeneity of groups has been tested, on the continuous variables, by the analysis of variance (ANOVA) with two levels (drug and varicocele). The Shapiro–Wilk test was adopted for checking the normal distribution of the data. In the present analysis, no discrete variables were considered for testing the homogeneity of groups.

All the study endpoints considered in the present analysis were evaluated, on the complete sample, by the analysis of covariance for a model with two classification levels. The independent variable was the value detected at the baseline visit, while the dependent variable was the value detected at the end of treatment. The Wilcoxon rank-sum test was adopted for comparing the two groups at baseline, while the Wilcoxon signed rank test was used in the comparisons before/after by group. Having defined as "Responder," for each parameter, a patient who improved in response at final visit, a responder analysis was also carried out. The Chi-squared test was adopted for detecting possible differences between the two treatment groups. All the above analyses (apart from the ANCOVA) were repeated for the comparison of the two groups separately by the presence of varicocele. Considering the low power, due to the small size, the responses of the tests presented separately for the presence of the varicocele have to be evaluated accordingly. SAS® Vers. 9.4 was used for performing all the analyses.

3 | RESULTS

In total, 94 (of 104) patients completed the study. Table 1 summarises demographic and baseline characteristics of the population by treatment group. The results of the homogeneity tests show that the two groups were well-balanced. The descriptive analyses show that at baseline, all sperm parameters in patients suffering from varicocele were lower when compared to the nonvaricocele group.

Adverse events (Table 2) that did not lead to stopping the therapy occurred only in the treatment group. All events were not serious: four patients had nausea and three vertigo or headache.

The results of the inferential analyses of the semen parameters are presented in Table 3. As for the ANCOVA, the *p*-values refer to the intention-to-treat population (ITT). The last observation carried forward (LOCF) method was used for replacing the missing data. The analyses are also presented for varicocele patients (Table 4) and nonvaricocele patients (Table 5). The Wilcoxon rank-sum test was adopted for calculating the *p*-values. In analysing the comparisons before/after in the placebo group, a significant difference in some parameters was observed. Therefore, the results of all the tests were also included in both the tables and the text. The responder analysis for all the parameters was carried out for all the groups and included in Table 6.

3.1 | Sperm concentration

The overall results for the sperm concentration in all subjects are summarised in Table 3. In the placebo group, were $41.4 \pm 17.9 \times 10^6$/ml at baseline and $43.7 \pm 13.6 \times 10^6$/ml at final visit. In the supplemented group, sperm concentration was $40.8 \pm 18.2 \times 10^6$/ml at baseline and $51.4 \pm 13.9 \times 10^6$/ml at final visit. While for the placebo group no change was observed ($p = .5244$), the increase in sperm concentration for the treatment group was significant ($p = .0026$). Before the treatment (at baseline), the sperm concentration in the placebo group did not differ from that in the treatment group ($p = .8453$). At the end of the trial, the difference between both groups was significant ($p = .0186$) in favour of the supplemented group.

TABLE 1 Baseline characteristics

Parameter	Statistics	Placebo	Supplemented	Total
Age (years)	*N*	52	52	104
	Missing	0	0	0
	Mean	32.5	32.5	32.5 (p = .9792)
	Std. Deviation	6.7	6.7	6.7
	Median	33.0	32.0	32.3
	Range	19.0–48.6	18.8–48.4	18.8–48.6
Height (cm)	*N*	52	52	104
	Missing	0	0	0
	Mean	178.6	177.2	177.9 (p = .2487)
	Std. Deviation	6.0	6.0	6.0
	Median	180.0	177.0	178.0
	Range	168.0–190.0	163.0–192.0	163.0–192.0
Weight (kg)	*N*	52	52	104
	Missing	0	0	0
	Mean	76.4	75.1	75.8 (p = .4242)
	Std. Deviation	8.7	7.7	8.2
	Median	75.0	75.0	75.0
	Range	62.0–94.0	62.0–93.0	62.0–94.0
HR (b/min)	*N*	52	52	104
	Missing	0	0	0
	Mean	70.8	70.4	70.6 (p = .6295)
	Std. Deviation	4.5	3.5	4.0
	Median	70.0	70.0	70.0
	Range	60–80	60–78	60–80
SBP (mmHg)	*N*	52	52	104
	Missing	0	0	0
	Mean	119.4	117.2	118.3 (p = .0961)
	Std. Deviation	7.3	6.3	6.9
	Median	120.0	120.0	120.0
	Range	100–130	110–130	100–130
DBP (mmHg)	*N*	52	52	104
	Missing	0	0	0
	Mean	72.4	73.2	72.8 (p = .4707)
	Std. Deviation	5.8	5.5	5.7
	Median	70.0	70.0	70.0
	Range	60–90	60–85	60–90

Results from ANOVA with two levels (drug and varicocele).

In varicocele patients (Table 4), mean sperm concentrations in the placebo group were 38.7 ± 18.1 × 10^6/ml at baseline and 39.9 ± 17.2 × 10^6/ml at the final visit (p = .7572). In the supplemented group, sperm concentration significantly (p = .0403) increased from 38.5 ± 19.0 × 10^6/ml at baseline to 50.2 ± 17.9 × 10^6/ml at the final visit. Before the treatment, the placebo and treatment groups showed no difference (p = .9708). The comparison of the changes from baseline between the two groups showed a nonsignificant difference (p = .1391) in favour of the supplemented group.

The results of sperm concentration at baseline in nonvaricocele patients (Table 5) in the placebo group were 44.1 ± 17.5 × 10^6/ml and 47.2 ± 7.8 × 10^6/ml at final visit (p = .5318). In the supplemented group were 43.2 ± 17.3 × 10^6/ml at baseline and 52.5 ± 9.4 × 10^6/ml at final visit (p = .0354). Before the treatment, the placebo and treatment groups showed no difference (p = .8048). The comparison of the changes from baseline between the two groups showed no difference (p = .2460) in favour of the supplemented group.

TABLE 2 Listing of adverse events

Treatment group	Id. no.	Age	Description (PT term)	Seriousness	Relationship	Action taken
Supplemented	36	32	Nausea	Not serious	Probable	None
			Gastro-oesophageal reflux disease	Not serious	Probable	None
	67	27	Nausea	Not serious	Possible	None
			Vertigo	Not serious	Possible	None
	68	21	Headache	Not serious	Possible	None
			Nausea	Not serious	Possible	None
	85	28	Headache	Not serious	Possible	None
			Nausea	Not serious	Possible	None

The responder analysis (Table 6) showed that 73.3% of supplemented patients versus 51.0% of the patients in the placebo group increased from baseline (p = .0262).

3.2 | Semen volume

The overall results for the semen volume are summarised in Table 3. In the placebo group were 3.0 ± 1.0 ml at baseline and 2.9 ± 1.0 ml at final visit. In the supplemented group were 3.1 ± 1.2 ml at baseline and 3.2 ± 0.9 ml at final visit. No difference was observed in both the placebo group (p = .6787) and the supplemented group (p = .6271). There was also no difference for the comparison between the two groups at baseline visit (p = .7499) and final visit (p = .1313).

In varicocele patients/Table 4), mean semen volume at baseline in the placebo group was 2.7 ± 0.7 and 2.4 ± 1.1 ml at final visit. In the supplemented group were 2.9 ± 1.2 ml at baseline and 3.2 ± 1.2 ml at final visit. No difference before/after was observed in both the placebo group (p = .2250) and the supplemented group (p = .3632). Comparing the two groups at baseline (p = .8761) and at the end of the study showed no difference (p = .1273).

As for nonvaricocele patients (Table 5), the semen volume in the placebo group was 3.2 ± 1.1 and 3.3 ± 0.8 ml at final visit. In the supplemented group was 3.4 ± 1.2 ml at baseline and 3.3 ± 0.6 ml at the final visit. Furthermore, for nonvaricocele patients, no difference before/after was observed in both the placebo group (p = .7711) and the supplemented group (p = .8753). The data at baseline (p = .6144) and at the end of the study (p = .5026) also did not differ.

The responder analysis (Table 6) did not show difference between the two groups; 48.9% of supplemented patients versus 46.9% in the placebo group were considered as responders at final visit (p = .8500).

3.3 | Total sperm count

The overall results for the total sperm count in all subjects are summarised in Table 3. In the placebo group were 113.1 ± 37.4 × 10^6 at baseline and 127.8 ± 61.4 × 10^6 at final visit. In the supplemented group, sperm count was 114.2 ± 37.8 × 10^6 at baseline and 163.5 ± 64.3 × 10^6 at the final visit. While for the placebo group no change was observed (p = .2030), the increase in the supplemented group was highly significant (p < .0001). While at baseline no difference (p = .8658) was observed between the two groups, the two groups differed at the end of the study in favour of the supplemented group as was confirmed by the inferential analysis with p = .0117.

In the varicocele group (Table 4), total sperm count in the placebo was 100.5 ± 41.9 × 10^6 at baseline and 102.4 ± 77.2 × 10^6 at the final visit (p = .5749). In the supplemented group had a sperm concentration of 96.3 ± 36.1 × 10^6 at baseline and of 158.8 ± 90.1 × 10^6 at final visit (p = .0009). While at baseline the two groups were balanced (p = .8764), a statistical difference in favour of the supplemented group was observed at final visit (p = .0066).

The results of total sperm count for the group without varicocele (Table 5) were 125.6 ± 27.7 × 10^6 at baseline and 152.1 ± 23.9 × 10^6 at final visit (p = .0022). In the supplemented group, results were 132.0 ± 30.9 × 10^6 at baseline and 167.6 ± 28.5 × 10^6 at final visit (p = .0005). No significant difference between the two groups was observed at baseline (p = .4259) and final visit (p = .2460).

The responder analysis (Table 6) showed that 82.2% of supplemented patients versus 55.1% in the placebo group increased from baseline (p = .0048).

3.4 | Progressive motility

The overall results for the progressive motility are summarised in Table 3. In the placebo group were 23.0 ± 7.8% at baseline and 24.5 ± 7.2% at final visit (p = .1567). In the supplemented group were 23.4 ± 6.1% at baseline and 28.6 ± 8.2% at final visit (p = .0012). While no difference was observed at baseline (p = .6701), the difference between the two groups at study end was significant with p = .0088 in favour of the supplemented group.

Analysing varicocele patients (Table 4), we had 21.8 ± 6.2% at baseline and 23.6 ± 6.8% at final visit in the placebo group (p = .1570) and 23.1 ± 5.2% at baseline and 27.4 ± 7.2% at final visit in the treated group (p = .0149). No difference between the two groups was observed at baseline (p = .3096) and at study end (p = .1686).

As for the group without varicocele (Table 5), the results were 24.2 ± 9.1% at baseline and 25.4 ± 7.7% at final visit in the placebo arm (p = .4866) and 23.7 ± 7.0% at baseline and 29.6 ± 9.0% at final

visit in the treated arm (p = .0311). Also for this comparison, the test was not statistically significant for both the baseline (p = .8907) and final visit (p = .2040).

The responder analysis (Table 6) showed that 73.3% of supplemented patients versus 51.0% in the placebo group increased from baseline (p = .0262).

3.5 | Total motility

The overall results for total motility are summarised in Table 3. In the placebo group, the means were 32.6 ± 9.2% at baseline and 34.6 ± 7.1% at final visit (p = .1483). In the supplemented group were 31.7 ± 8.2% at baseline and 39.0 ± 8.0% at final visit with a high statistically significance

TABLE 3 Sperm parameters. Absolute values of baseline and final

Parameter	Groups	Statistics	Baseline	Final	p-Values before/after**
Sperm concentration (10^6 ml)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	41.4	43.7	.5244
		Std. Deviation	17.9	13.6	
		Median	38.3	44.0	
		Range	11.0; 79.0	16.0; 79.0	
	Supplemented	*N*	52	45	
		Missing	0	7	
		Mean	40.8	51.4	.0026
		Std. Deviation	18.2	13.9	
		Median	39.0	49.0	
		Range	12.3; 77.0	28.0; 86.0	
		p-Values by visit*	.8453	.0186	
Volume of ejaculate (ml)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	3.0	2.9	.6787
		Std. Deviation	1.0	1.0	
		Median	2.8	3.0	
		Range	1.3; 6.3	1.1; 5.1	
	Supplemented	*N*	52	45	
		Missing	0	7	
		Mean	3.1	3.2	.6271
		Std. Deviation	1.2	0.9	
		Median	2.8	3.2	
		Range	1.4; 6.0	1.1; 5.5	
		p-Values by visit*	.7499	.1313	
Total sperm count (10^6)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	113.1	127.8	.2030
		Std. Deviation	37.4	61.4	
		Median	107.6	136.7	
		Range	30.0; 197.6	24.0; 270.0	
	Supplemented	*N*	52	45	
		Missing	0	7	
		Mean	114.2	163.5	<.0001
		Std. Deviation	37.8	64.3	
		Median	112.1	158.4	
		Range	43.2; 205.8	48.4; 369.6	
		p-Values by visit*	.8658	.0117	

(Continues)

TABLE 3 (Continued)

Parameter	Groups	Statistics	Baseline	Final	*p*-Values before/after**
Progressive motility (%)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	23.0	24.5	.1567
		Std. Deviation	7.8	7.2	
		Median	22.3	23.0	
		Range	5.9; 43.2	8.1; 44.0	
	Supplemented	*N*	52	45	
		Missing	0	7	
		Mean	23.4	28.6	.0012
		Std. Deviation	6.1	8.2	
		Median	23.2	27.0	
		Range	12.0; 40.0	15.0; 57.9	
		p-Values by visit*	.6701	.0088	
Total motility (%)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	32.6	34.6	.1483
		Std. Deviation	9.2	7.1	
		Median	32.0	35.0	
		Range	8.0; 55.0	12.0; 49.2	
	Supplemented	*N*	52	45	
		Missing	0	7	
		Mean	31.7	39.0	<.0001
		Std. Deviation	8.2	8.0	
		Median	31.3	37.5	
		Range	18.9; 48.0	29.0; 65.3	
		p-Values by visit*	.5239	.0120	
Sperm morphology—typical (%)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	21.1	15.7	.0146
		Std. Deviation	16.2	9.4	
		Median	15.0	15.0	
		Range	3.0; 59.0	3.0; 52.0	
	Supplemented	*N*	52	44	
		Missing	0	8	
		Mean	23.5	17.7	.0055
		Std. Deviation	14.6	15.2	
		Median	20.0	13.5	
		Range	5.0; 64.0	3.0; 77.0	
		p-Values by visit*	.2062	.3791	

(Continues)

($p < .0001$). While the two groups were balanced at baseline ($p = .5239$), the difference between the two groups at study end was statistically significant ($p = .0120$) in favour of the supplemented group.

In the varicocele group (Table 4), the results in the placebo group were 31.3 ± 8.2% at baseline and 34.5 ± 6.9% at final visit ($p = .1214$). In the treated group were 31.5 ± 8.1% at baseline and 37.5 ± 7.1%

TABLE 3 (Continued)

Parameter	Groups	Statistics	Baseline	Final	*p*-Values before/after**
Sperm morphology—atypical (%)	Placebo	*N*	52	49	
		Missing	0	3	
		Mean	78.9	84.1	.0105
		Std. Deviation	15.4	9.3	
		Median	82.5	85.0	
		Range	41.0; 97.0	48.0; 97.0	
	Supplemented	*N*	52	45	
		Missing	0	7	
		Mean	80.2	82.5	.1310
		Std. Deviation	16.6	15.2	
		Median	85.0	86.0	
		Range	22.0; 96.0	23.0; 100.0	
		p-Values by visit*	.5379	.5081	

*The *p*-values for the Baseline visit are derived from the comparison between the two groups with the Wilcoxon rank-sum test. The *p*-values for the final visit are derived from the ANCOVA on the ITT population.
**The *p*-values before/after by treatment group are derived from the Wilcoxon signed rank test.

at final visit ($p = .0065$). No difference was observed at baseline ($p = .7836$) and at study end ($p = .3164$).

As for the group without varicocele (Table 5), the results were 33.9 ± 10.2% at baseline and 34.7 ± 7.5% at final visit in the placebo group ($p = .5604$) and 31.8 ± 8.4% at baseline and 40.2 ± 8.7% at final visit in the treated group ($p = .0028$). The two groups were balanced at baseline ($p = .5396$). A statistical difference in favour of the supplemented group was reported in those without varicocele ($p = .0257$).

The responder analysis (Table 6) showed that 68.9% of supplemented patients versus 53.1% in the placebo group increased from baseline ($p = .1166$).

3.6 | Normal sperm morphology

Analysing typical morphology (Table 3), the results were 21.1 ± 16.2% at baseline and 15.7 ± 9.4% at final visit in the placebo group ($p = .0146$) and 23.5 ± 14.6% at baseline and 17.7 ± 15.2% at final visit in the supplemented group ($p = .0055$). No significant difference between the two groups was observed at baseline ($p = .2062$) and at study end ($p = .3791$).

Looking at atypical morphology (Table 3), the results were 78.9 ± 15.4% at baseline and 84.1 ± 9.3% at final visit in the placebo group ($p = .0105$) and 80.2 ± 16.6% at baseline and 82.5 ± 15.2% at final visit in the supplemented group ($p = .1310$). No significant difference between the two groups was observed at baseline ($p = .5379$) and at study end ($p = .5081$).

3.7 | Pregnancy rate

Twelve pregnancies occurred during follow-up time: 10 in the supplementation group (nine nonvaricocele and one varicocele) and two in the placebo group (one nonvaricocele and one varicocele). One spontaneous abortion was reported in the placebo arm.

4 | DISCUSSION

Male infertility is a medical condition and a relevant social problem that has a strong impact on well-being. From various studies, it has emerged that seminal oxidative stress and sperm DNA damage must be taken into account as a critical factor in the aetiology of semen alterations and infertility (Saalu, 2010; Vessey et al., 2016).

When levels of free radicals, and in particular ROS, are increased and antioxidant levels are decreased, OS occurs (Agarwal, Roychoudhury, Bjugstad, & Chou, 2016). OS has a negative effect on sperm quality parameters and was shown to impact the genetic content carried by these specialised cells. Human mitochondrial DNA gene alterations were associated with many pathological conditions, and this damage per se is also linked as a cause of poor sperm quality. Thus, targeting OS is a strategy to increase fertility and spermatozoa number and quality (Agarwal et al., 2016).

Varicocele is associated with an increase in ROS production and seminal OS leading to sperm dysfunction. Mitochondria are a key source of ROS production especially when they are damaged or dysfunctional due to lack of proper substrates and cofactors as well as if there are any gene mutations affecting the respiratory chain. These DNA variants probably underlie mitochondrial dysfunction leading to impaired ATP synthesis and ultimately interfere with sperm motility and fertility status (Heidari et al., 2016).

Nonenzymatic antioxidants including vitamins (mainly vitamins A, B, C, E), glutathione as well as metabolic coenzymes such as pantothenic acid, coenzyme Q10, carnitines (L-carnitine and

acetyl-L-carnitine) and micronutrients (zinc, selenium, copper) are often deficient causing a general diminution in the antioxidant status as well as mitochondrial dysfunction (Jeulin & Lewin, 1996; Virmani, Ali, Pinto, Zerelli, & Binienda, 2016). Nutrients such as zinc, folic acid, vitamin B12, L-carnitine and acetyl-L-carnitine are also associated with sperm formation and maturation (Adams et al., 1998; Ebisch, Thomas, Peters, Braat, & Steegers-Theunissen, 2007; Jeulin & Lewin, 1996; Watanabe et al., 2003).

Studies demonstrate that using these substances has a beneficial effect on fertility, in particular sperm quality and are therefore

TABLE 4 Sperm parameters. Patients with varicocele. Absolute values of baseline and final visits

Parameter	Groups	Statistics	Baseline	Final	*p*-Values before/after**
Sperm concentration (10^6 ml)	Placebo	*N*	26	24	
		Missing	0	2	
		Mean	38.7	39.9	.7572
		Std. Deviation	18.1	17.2	
		Median	32.0	34.8	
		Range	17.5; 76.0	16.0; 79.0	
	Supplemented	*N*	26	21	
		Missing	0	5	
		Mean	38.5	50.2	.0403
		Std. Deviation	19.0	17.9	
		Median	32.5	45.0	
		Range	12.3; 76.0	28.0; 86.0	
		p-Values by visit*	.9708	.1391	
Volume of ejaculate (ml)	Placebo	*N*	26	24	
		Missing	0	2	
		Mean	2.7	2.4	.2250
		Std. Deviation	0.7	1.1	
		Median	2.7	2.2	
		Range	1.3; 4.1	1.1; 5.0	
	Supplemented	*N*	26	21	
		Missing	0	5	
		Mean	2.9	3.2	.3632
		Std. Deviation	1.2	1.2	
		Median	2.6	3.2	
		Range	1.4; 5.6	1.1; 5.5	
		p-Values by visit*	.8761	.1273	
Total sperm count (10^6)	Placebo	*N*	26	24	
		Missing	0	2	
		Mean	100.5	102.4	.5749
		Std. Deviation	41.9	77.2	
		Median	94.9	77.7	
		Range	30.0; 197.6	24.0; 270.0	
	Supplemented	*N*	26	21	
		Missing	0	5	
		Mean	96.3	158.8	.0009
		Std. Deviation	36.1	90.1	
		Median	96.1	126.0	
		Range	43.2; 190.6	48.4; 369.6	
		p-Values by visit*	.8764	.0066	

(Continues)

TABLE 4 (Continued)

Parameter	Groups	Statistics	Baseline	Final	*p*-Values before/after**
Progressive motility (%)	Placebo	*N*	26	24	
		Missing	0	2	
		Mean	21.8	23.6	.1570
		Std. Deviation	6.2	6.8	
		Median	22.3	22.0	
		Range	10.0; 40.0	15.0; 44.0	
	Supplemented	*N*	26	21	
		Missing	0	5	
		Mean	23.1	27.4	.0149
		Std. Deviation	5.2	7.2	
		Median	23.4	27.0	
		Range	13.0; 33.3	15.0; 41.7	
		p-Values by visit*	.3096	.1686	
Total motility (%)	Placebo	*N*	26	24	
		Missing	0	2	
		Mean	31.3	34.5	.1214
		Std. Deviation	8.2	6.9	
		Median	31.5	35.0	
		Range	8.0; 45.0	18.0; 49.0	
	Supplemented	*N*	26	21	
		Missing	0	5	
		Mean	31.5	37.5	.0065
		Std. Deviation	8.1	7.1	
		Median	30.4	36.0	
		Range	21.0; 46.7	29.0; 55.0	
		p-Values by visit*	.7836	.3164	

*The *p*-values for the Baseline visit are derived from the comparison between the actual baseline values of the two groups with the Wilcoxon rank-sum test. The *p*-values for the final visit are derived from the comparison on the differences before/after between the two groups with the Wilcoxon rank-sum test.
**The *p*-values before/after by treatment group are derived from the Wilcoxon signed rank test.

recommended as potentially effective therapy for the treatment of male infertility (Walczak-Jedrzejowska, Wolski, & Slowikowska-Hilczer, 2013).

L-Carnitine together with acetyl-L-carnitine is a safe treatment commonly used because of their capacity in improving sperm quality and pregnancy rate in males suffering from astheno-teratozoospermia (Wang et al., 2010). Selenium is essential for testis development, spermatogenesis and final sperm quality. It acts via a positive antioxidant action through glutathione peroxidase enzymes (Moslemi & Tavanbakhsh, 2011). Both vitamin E and zinc play a role in antioxidant balance regulation and are able to improve sperm concentration, percentage of progressively motile sperm and consequently pregnancy rate (Ajina, Sallem, Haouas, & Mehdi, 2016; Chen et al., 2012). Coenzyme Q10 levels show a significant correlation with sperm count and with sperm motility (Festa et al., 2014; Mancini et al., 1994). Administration of coenzyme Q10 to men with idiopathic asthenozoospermia results in an increase in sperm motility (Balercia et al., 2004).

The real association between varicocele and fertility status is still not completely clarified, but a recent meta-analysis showed a significant improvement in semen parameters in patients undergoing varicocelectomy (Agarwal et al., 2007). Furthermore, after surgical treatment, a reversal in the sperm DNA damage was also evidenced (Zini & Dohle, 2011). Gual-Frau et al. (2015) confirmed a beneficial effect of antioxidant compounds in patients suffering from grade I varicocele. In their study, patients showed an average relative reduction of 22.1% in sperm DNA fragmentation ($p = .02$) with 31.3% fewer highly degraded sperm cells ($p = .07$), and the total number of sperm cells was also significantly increased after 3 months of treatment. Another recent trial, conducted on patients with high-grade varicocele and randomised to receive surgical treatment or L-carnitine supplementation, reported good results in all sperm parameters: motility changed from 21.7% to 35.4% (vs. 33.9%–47.5% in L-carnitine group), normal sperm morphology changed from 46.3% to 60% (vs. 56.6%–69.7% in L-carnitine group) and volume

of semen changed from 3.5 to 4.2 ml (vs. 2.9–4.3 ml in L-carnitine group). The authors concluded that supplementary treatment was as effective as varicocelectomy in improving semen parameters and can be used as an alternative to surgery (Sofimajidpour, Ghaderi, & Ganji, 2016).

Lastly, in a Cochrane analysis, men taking oral dietary supplementation for infertility were able to obtain better live birth rates in couples undergoing assisted reproductive techniques (Showell et al., 2014).

Comparing effectiveness of varicocelectomy and medical therapy to treat these cases of infertility is difficult, and trials are limited

TABLE 5 Sperm parameters. Patients without varicocele. Absolute values of baseline and final visits

Parameter	Groups	Statistics	Baseline	Final	*p*-Values before/after**
Sperm concentration (10^6 ml)	Placebo	*N*	26	25	
		Missing	0	1	
		Mean	44.1	47.2	.5318
		Std. Deviation	17.5	7.8	
		Median	42.3	48.5	
		Range	11.0; 79.0	30.0; 65.0	
	Supplemented	*N*	26	24	
		Missing	0	2	
		Mean	43.2	52.5	.0354
		Std. Deviation	17.3	9.4	
		Median	42.4	50.5	
		Range	19.0; 77.0	37.7; 78.0	
		p-Values by visit*	.8048	.2460	
Volume of ejaculate(ml)	Placebo	*N*	26	25	
		Missing	0	1	
		Mean	3.2	3.3	.7711
		Std. Deviation	1.1	0.8	
		Median	3.0	3.1	
		Range	1.5; 6.3	2.0; 5.1	
	Supplemented	*N*	26	24	
		Missing	0	2	
		Mean	3.4	3.3	.8753
		Std. Deviation	1.2	0.6	
		Median	3.3	3.2	
		Range	1.9; 6.0	2.0; 4.5	
		p-Values by visit*	.6144	.5026	
Total sperm count (10^6)	Placebo	*N*	26	25	
		Missing	0	1	
		Mean	125.6	152.1	.0022
		Std. Deviation	27.7	23.9	
		Median	118.0	154.0	
		Range	69.3; 178.6	107.5; 200.9	
	Supplemented	*N*	26	24	
		Missing	0	2	
		Mean	132.0	167.6	.0005
		Std. Deviation	30.9	28.5	
		Median	132.2	163.8	
		Range	68.4; 205.8	107.5; 220.8	
		p-Values by visit*	.4259	.2460	

(Continues)

TABLE 5 (Continued)

Parameter	Groups	Statistics	Baseline	Final	*p*-Values before/after**
Progressive motility (%)	Placebo	*N*	26	25	
		Missing	0	1	
		Mean	24.2	25.4	.4866
		Std. Deviation	9.1	7.7	
		Median	23.9	25.0	
		Range	5.9; 43.2	8.1; 40.0	
	Supplemented	*N*	26	24	
		Missing	0	2	
		Mean	23.7	29.6	.0311
		Std. Deviation	7.0	9.0	
		Median	23.2	27.5	
		Range	12.0; 40.0	15.0; 57.9	
		p-Values by visit*	.8907	.2040	
Total motility (%)	Placebo	*N*	26	25	
		Missing	0	1	
		Mean	33.9	34.7	.5604
		Std. Deviation	10.2	7.5	
		Median	32.0	35.0	
		Range	15.5; 55.0	12.0; 49.2	
	Supplemented	*N*	26	24	
		Missing	0	2	
		Mean	31.8	40.2	.0028
		Std. Deviation	8.4	8.7	
		Median	31.4	37.8	
		Range	18.9; 48.0	29.0; 65.3	
		p-Values by visit*	.5396	.0257	

*The *p*-values for the Baseline visit are derived from the comparison between the actual baseline values of the two groups with the Wilcoxon rank-sum test. The *p*-values for the final visit are derived from the comparison on the differences before/after between the two groups with the Wilcoxon rank-sum test.
**The *p*-values before/after by treatment group are derived from the Wilcoxon signed rank test.

by small case studies and nonrandomisation. There is only one report with a direct comparison between L-carnitine and varicocelectomy in patients with grade II/III varicocele. The authors describe a statistically significant improvement in sperm count, motility and morphology after treatment, and results are not different between different treatment methods. The main limitations of the study are the inclusion criteria, small sample size and that is not a randomised double-blind placebo-controlled (DBPC) study (Sofimajidpour et al., 2016).

Our trial evaluated the utilisation of a combination of metabolic substances, antioxidants and micronutrients to improve sperm parameters. For a better understanding of the action of the supplementation, we applied a DBPC system and very specific inclusion and exclusion criteria. Furthermore, in consideration of the still not clear effect of varicocele on male fertility, we divided our cohort into infertile varicocele patients and into idiopathic infertile nonvaricocele patients. At the end of the experimental treatment, we observed a marked increase in sperm count and concentration together with increases in motility, progressive motility and morphology. All differences between treatment and placebo groups were statistically significant in both varicocele and nonvaricocele patients. A small difference (not statistically significant) was also observed in the semen volume in favour of experimental group. Differences, in general, are more evident in those suffering from varicocele, and this can be probably explained with the major OS and ROS-mediated damage that is usually associated with this condition. At the moment, it is not possible to conclude whether the medical treatment is inferior or superior to varicocelectomy in those with varicocele. Affirmation whether or not oral supplementation can replace surgery has yet to be properly established. Nevertheless, it is important to take into consideration that the role of oral supplements in clinical practice in the two groups is completely different, and one could possibly rather speak about an association between surgery and oral supplementation be more appropriate.

TABLE 6 Responder analysis

Parameter	Placebo		Supplemented		*p*-Values
	Non responders	Responders	Non responders	Responders	
Sperm concentration (10^6 ml)	24 (49.0%)	25 (51.0%)	12 (26.7%)	33 (73.3%)	.0262
W varicocele	12 (50.0%)	12 (50.0%)	6 (28.6%)	15 (71.4%)	.1432
W/O varicocele	12 (48.0%)	13 (52.0%)	6 (25.0%)	18 (75.0%)	.0950
Volume of ejaculate (ml)	26 (53.1%)	23 (46.9%)	23 (51.1%)	22 (48.9%)	.8500
W varicocele	16 (66.7%)	8 (33.3%)	9 (42.9%)	12 (57.1%)	.1088
W/O varicocele	10 (40.0%)	15 (60.0%)	14 (58.3%)	10 (41.7%)	.1994
Total sperm count (10^6)	22 (44.9%)	27 (55.1%)	8 (17.8%)	37 (82.2%)	.0048
W varicocele	15 (62.5%)	9 (37.5%)	4 (19.0%)	17 (81.0%)	.0032
W/O varicocele	7 (28.0%)	18 (72.0%)	4 (16.7%)	20 (83.3%)	.3419
Progressive motility (%)	24 (49.0%)	25 (51.0%)	12 (26.7%)	33 (73.3%)	.0262
W varicocele	12 (50.0%)	12 (50.0%)	5 (23.8%)	16 (76.2%)	.0706
W/O varicocele	12 (48.0%)	13 (52.0%)	7 (29.2%)	17 (70.8%)	.1762
Total motility (%)	23 (46.9%)	26 (53.1%)	14 (31.1%)	31 (68.9%)	.1166
W varicocele	11 (45.8%)	13 (54.2%)	7 (33.3%)	14 (66.7%)	.3932
W/O varicocele	12 (48.0%)	13 (52.0%)	7 (29.2%)	17 (70.8%)	.1762

Although pregnancy rate was not an endpoint of the study, it is interesting to notice that of 12 pregnancies occurred during the follow-up time, 10 were reported in supplementation group.

The safety of the formulation was assured by its composition, and tolerability was confirmed by the almost total absence of adverse effects during all the treatment. We did not compare the effect of this treatment with surgical treatment of varicocele, and we did not evaluate DNA fragmentation and level of ROS. Furthermore, latest evidences report that evaluating OS can be a diagnostic tool in predicting the best responders to supplementation (Vessey et al., 2016). Oxidative stress is a cause of male infertility with significant negative effect on semen parameters, and varicocele is an additional cause of poor sperm quality. The use of carnitines and other functional substances can form part of an efficacious strategy to handle male infertility in both the nonvaricocele and the varicocele subjects. Indeed, we plan future studies to examine role of energy metabolism, OS and ROS in particular to gain a better understanding of underlying mechanisms and thereby help us to determine the best strategies for male infertility treatment.

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