research article

Effect of the oral intake of astaxanthin on semen parameters in patients with oligo-astheno-teratozoospermia: a randomized double-blind placebo-controlled trial

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Background. Higher concentrations of seminal reactive oxygen species may be related to male infertility. Astaxanthin with high antioxidant activity can have an impact on the prevention and treatment of various health conditions, including cancer. However, efficacy studies on astaxanthin in patients with oligospermia with/without astheno- or teratozoospermia (O±A±T) have not yet been reported. Our aim was to evaluate the effect of the oral intake of astaxanthin on semen parameters.

Patients and methods. In a randomized double-blind trial, 80 men with O±A±T were allocated to intervention with 16 mg astaxanthin orally daily or placebo. At baseline and after three months basic semen parameters, sperm deoxyribonucleic acid (DNA) fragmentation and mitochondrial membrane potential (MMP) of spermatozoa and serum follicle-stimulating hormone (FSH) value were measured.

Results. Analysis of the results of 72 patients completing the study (37 in the study group, 35 in the placebo group) did not show any statistically significant change, in the astaxanthin group no improvements in the total number of spermatozoa, concentration of spermatozoa, total motility of spermatozoa, morphology of spermatozoa, DNA fragmentation and mitochondrial membrane potential of spermatozoa or serum FSH were determined. In the placebo group, statistically significant changes in the total number and concentration of spermatozoa were determined.

Conclusions. The oral intake of astaxanthin did not affect any semen parameters in patients with O±A±T.

Key words: antioxidant; male infertility; oligo-astheno-teratozoospermia; semen quality; DNA fragmentation; cancer

Introduction

Currently, almost every seventh couple is infertile. The male factor as the single or additional reason for infertility is presented in nearly 50% of infertile couples.[1] World Health Organization (WHO) defines oligo-astheno-teratozoospermia (OAT) as the concentration and the proportions of motile and morphologically normal spermatozoa below the reference values.[2]

One of the many pathophysiological factors of male infertility are higher concentrations of seminal reactive oxygen species (ROS).[3] The uncontrolled production of these molecules may be harmful to cells and different biomolecules such as carbohydrates, amino acids, proteins, lipids, and deoxyribonucleic acid (DNA), which can be damaged. ROS can have a negative influence on sperm function and quality[4-7] due to the reduced motility of spermatozoa[8], DNA damage[9-11] and the

impaired integrity of the cellular membrane.[7,12,13] It was revealed that infertile men with low semen concentration, poor semen motility, and a high proportion of morphologically abnormal spermatozoa were associated with an increased risk of testicular cancer.[14,15] Several antioxidants are present within the ejaculate as protection against excessive ROS-induced lipid peroxidation.[16] It has been confirmed that antioxidant capacity in semen is decreased in infertile men and men with testicular cancer with a high ROS proportion, compared to men with a normal proportion of ROS.[17–19]

Sperm function tests have arisen as a supplement to standard semen analysis.[20] The DNA integrity of spermatozoa has a critical impact in determining sperm competence[21,22] which is related to ROS levels[9,10] and can be measured by sperm DNA fragmentation (SDF) assays.[21,22] A recent meta-analysis of 28 studies demonstrated that SDF is more accurate in determining the function of spermatozoa compared to conventional semen parameters.[23] A study of apoptotic DNA fragmentation in human spermatozoa found a significant increase in apoptotic SDF in the semen of testicular cancer patients post-orchiectomy and in OAT patients compared to a control group of healthy men.[24] In addition to DNA integrity, mitochondria have an important impact on cell function[25], and the sperm mitochondrial membrane potential (MMP) contributes some benefit to male fertility potential and sperm quality.[26] Combined SDF and MMP may act as better predictors of natural conception than standard semen parameters.[27]

Regarding the role of seminal antioxidant capacity in male infertility, several trials on the impact of antioxidants on semen characteristics have been performed. The review of randomized and placebo-controlled trials affirmed the favorable impact of some types of antioxidants on different semen characteristics.[28] Several studies have shown favorable effects, especially on the motility of spermatozoa.[29-34] In some trials, antioxidant combinations (coenzyme Q10, vitamin C, vitamin E, selenium, zinc, L-carnitine, etc.) were assessed.[35–40] The most recent Cochrane review shows that for couples attending fertility clinics, pregnancy rates and live births may be improved with antioxidant supplementation in subfertile males.[41]

As one of many antioxidants, ketocarotenoid astaxanthin has the potential to prevent and treat various diseases, including diabetes, liver and renal diseases, cancer, chronic inflammatory diseases, cardiovascular diseases, eye and skin diseases, metabolic syndrome, gastrointestinal diseases, and neurodegenerative diseases.[42] Astaxanthin accumulates in the microalga *Haematococcus pluvialis*, and compared to vitamin E, this compound has up to 100 times higher antioxidant activity.[43] Astaxanthin is a food supplement and widely available over-the-counter. Until now, there were two "in vitro" studies that first showed the positive effect of astaxanthin on sperm capacitation in 24 healthy, fertile men[44]; subsequently, the positive effect on sperm capacitation in 27 men from couples who did not succeed to conceive after at least twelve months of regular unprotected intercourse was confirmed.[45] On the other hand, there has been only one clinical trial investigating the influence of astaxanthin on male fertility published until now. The trial included a low number of patients with a history of male infertility – 11 patients in the study group and 19 patients in the placebo group; however, these patients were enrolled without regard for semen characteristics.[46] Favorable changes in inhibin B concentration, sperm linear velocity and ROS levels, and pregnancy rate were determined. However, the efficacy of astaxanthin oral intake on semen parameters in OAT patients treated for infertility has not yet been reported.

The aim of this prospective randomized double-blind placebo-controlled clinical trial was to assess the effect of three-month intake of astaxanthin in infertile men with oligospermia with/without astheno- or teratozoospermia (O±A±T) on basic semen parameters, DNA fragmentation and mitochondrial membrane potential of spermatozoa, and serum level of follicle-stimulating hormone (FSH).

Patients and methods

The prospective randomized double-blind placebo-controlled trial was performed from November 2014 to January 2019 at the outpatient infertility clinic, Department of Human Reproduction, Division of Obstetrics and Gynecology of the University Medical Centre Ljubljana, Slovenia. The study was approved by the Slovenian National Medical Ethics Committee (consent number 145/02/14) and was registered at ClinicalTrials.gov, NCT02310087. All participants were enrolled after they provided written informed consent to participate in the trial.

Participants

A total of 80 infertile men with O±A±T were enrolled after they signed a written informed consent

to participate in this trial. They were considered O±A±T after at least two previous semen analysis (seminogram) and andrological examination in the frame of their infertility treatment after their partner being unable to conceive for at least 12 months of unprotected sexual intercourse or after a failed assisted conception procedure. Semen quality was defined as O±A±T according to the WHO 2010 guidelines: oligospermia (O) − sperm concentration < 15 million/ml; asthenozoospermia (A) − progressive motility of spermatozoa < 32%; teratozoospermia (T) − < 4% spermatozoa with normal morphology.[20] The exclusion criteria were smoking more than 20 cigarettes per day, genetic causes of infertility, endocrinopathies, genital tract infections, undescended testis, systemic diseases, history of testicular cancer and treatment with other drugs and food supplements, such as antioxidants, during the last three months before enrolling in this study.

Intervention and methods

Eighty eligible infertile men with O±A±T were allocated at random to daily intake of 16 mg of astaxanthin capsules (Astasan) or placebo capsules, both produced by the same manufacturer (Sensilab). Astaxanthin capsules contained astaxanthin, vitamin E as a stabilizer, safflower oil, gelatin, water and glycerine. Placebo capsules were identical both in appearance and taste, containing gelatine, water and glycerine only. A computerized randomization table was used for the purpose of randomization. A random allocation sequence was generated and participants were enrolled and assigned to interventions by a third party, thus ensuring that both the enrolled participants and researchers were blinded. At baseline and after three months of treatment, all participants answered a questionnaire about their age, height, weight, smoking status and the history of current occupational exposure to high temperatures, ultraviolet or radiology radiation, chemicals, or a predominant sedentary job in order to exclude the potential effect of these factors on semen quality. The semen samples were obtained by masturbation after 2–5 days of sexual abstinence. Semen quality was assessed according to the WHO guidelines.[20] The total number, concentration, motility and morphology were determined by international standard laboratory microscopic analyses.[47]

Sperm DNA fragmentation in our study was evaluated using a TUNEL assay – measuring DNA fragmentation in spermatozoa using terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labelling.[48] The calculated threshold value for the TUNEL assay to distinguish between normal and abnormal semen samples in men was 20%.[49] MMP was measured by means of 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) staining as an indicator of mitochondrial membrane integrity and the mitochondrial potential capacity to generate ATP by oxidative phosphorylation.[50] Propidium iodide (PI) was used as a supravital fluorescent dye and stained spermatozoa were analyzed by flow cytometry. MMP was considered to be normal in our laboratory setting when the proportion of spermatozoa with normal MMP (attributed to cells with high fluorescence signals) was higher than 60%.[27] The serum level of FSH was measured by a solid-phase, two-site chemiluminescent immunometric assay. The normal FSH values for males in our laboratory setting were 0.7−11.1 IU/L.

Sample size calculation

To estimate the sample size for the study, we anticipated an increase in sperm concentration in the astaxanthin group, as reported in the only clinical study until now.[46] With the assumption of a 34% increase as reported, with alpha 0.05 and power 80%, 32 cases were needed in each group. To account for drop-outs, we aimed to recruit 40 cases in each group.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Science (SPSS), version 25, IBM Corp, Armonk, NY, USA. An independent Student's t-test was used to analyze normally distributed data, and the Mann-Whitney test was used to analyze data that were not distributed normally. A paired t-test or the Wilcoxon test were applied to analyze the differences in the semen variables within groups. The chi-square test was applied to compare the exposure to occupational factors between groups. The results of continuous variables were expressed as the mean ± SD. P-values < 0.05 were considered significant.

Results

A group of 256 men was assessed for eligibility; half (126 men, 49.2%) declined to participate, and 50 did not meet the inclusion criteria; 80 men were eligible and willing to participate and these indi-
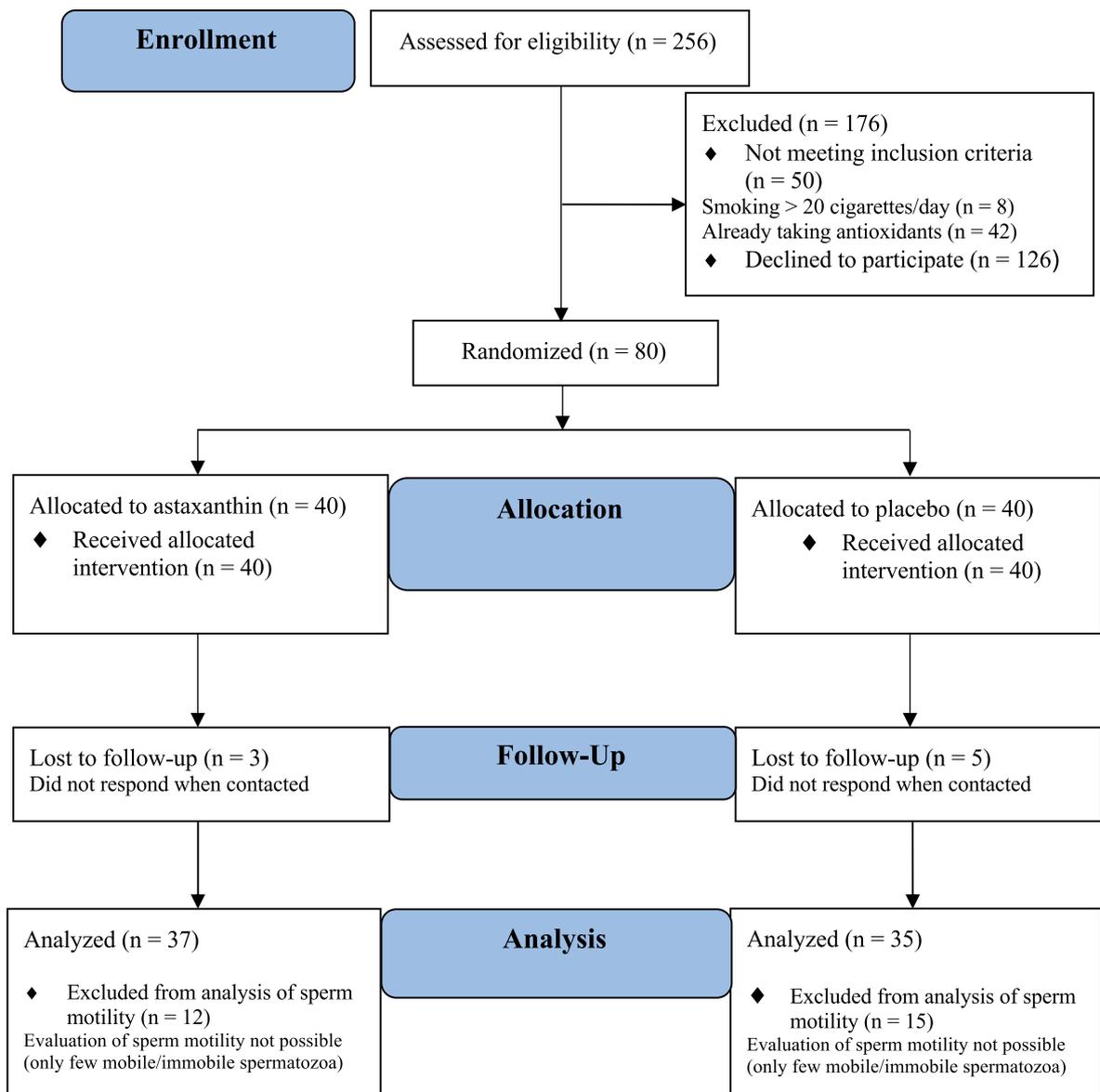
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┌─────────────────────┐   ┌─────────────────────────────────────┐
│    Enrollment       │   │  Assessed for eligibility (n = 256) │
└─────────────────────┘   └─────────────────────────────────────┘
                                           │
                                           │      ┌──────────────────────────────────────┐
                                           │      │ Excluded (n = 176)                   │
                                           │      │  ♦  Not meeting inclusion criteria   │
                                           ├─────►│        (n = 50)                      │
                                           │      │ Smoking > 20 cigarettes/day (n = 8)  │
                                           │      │ Already taking antioxidants (n = 42) │
                                           │      │  ♦  Declined to participate (n = 126)│
                                           │      └──────────────────────────────────────┘
                                           ▼
                              ┌──────────────────────────┐
                              │  Randomized (n = 80)     │
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Figure structure:

Allocated to astaxanthin (n = 40)
♦ Received allocated intervention (n = 40)

Allocation

Allocated to placebo (n = 40)
♦ Received allocated intervention (n = 40)

Lost to follow-up (n = 3)
Did not respond when contacted

Follow-Up

Lost to follow-up (n = 5)
Did not respond when contacted

Analyzed (n = 37)
♦ Excluded from analysis of sperm motility (n = 12)
Evaluation of sperm motility not possible (only few mobile/immobile spermatozoa)

Analysis

Analyzed (n = 35)
♦ Excluded from analysis of sperm motility (n = 15)
Evaluation of sperm motility not possible (only few mobile/immobile spermatozoa)

FIGURE 1. CONSORT flow chart of study presenting the inclusion criteria, randomization and follow-up of participants.

viduals were randomized. Eight patients in both groups (10%) dropped out for personal reasons during the treatment, and thus, 72 patients completed the trial. No adverse effects during the treatment were reported. Finally, we analyzed the effect of treatment in 37 patients in the astaxanthin and 35 patients in the placebo group (Figure 1).

Five patients were not included in the statistical analyses on changes in sperm total number and concentration and 27 patients were not included in the statistical analyses on the motility of spermatozoa as in these patients only a few mobile or immobile spermatozoa in the semen sample were present. At baseline, all patients in the astaxanthin group were in accordance with WHO criteria for OAT. In the placebo group, all patients were in accordance with the WHO criteria for oligo-teratozoospermia and two-thirds for asthenozoospermia.

The epidemiological and semen characteristics of the included patients are summarized in Table 1. There were no statistically significant differences between all parameters of both groups of patients upon enrollment. In addition, exposure of patients to chemicals, ultraviolet rays, X-ray radiation, high temperature, and a sedentary job according to their history (questionnaire) have not been related to the semen parameters in the studied population of men.

The pre- and post-treatment semen parameters and hormonal levels of both groups of patients are displayed in Table 2. The three-month treatment of patients with astaxanthin did not affect the semen parameters or serum FSH levels in any way. Interestingly, the significant change after the three-month period was the increased total number (16.0 ± 21.1 *vs.* 38.4 ± 69.2 million/ejaculate, p = 0.002) and concentration (5.7 ± 5.9 *vs.* 10.2 ± 15.7 million/ml, p = 0.024) of spermatozoa in the placebo group, but not in the astaxanthin group (24.6 ± 28.6 *vs.* 31.7 ± 33.4 million/ejaculate, p = 0.186, and 7.0 ± 5.6 *vs.* 9.2 ± 7.9 million/ml, p = 0.100).

To address the increase in the concentration of spermatozoa in the placebo group, we separately analyzed the seasonal inclusion of patients into the trial to review the potential seasonal changes of semen quality. Nineteen patients in the astaxanthin group and 18 patients in the placebo group were enrolled in the study during the winter/spring period, and 18 patients in the astaxanthin group and 17 patients in the placebo group were enrolled during the summer/fall seasons. There was no significant difference in the seasonal inclusion of patients in the trial (p = 0.995).

Discussion

The present study is the first study of astaxanthin efficacy on semen parameters in oligo-asthenoteratozoospermic patients who were treated for infertility. Our results did not show any significant improvements in the basic semen parameters or at the cellular level (SDF, MMP) or serum FSH values after oral intake of astaxanthin. In previously published studies on the effects of antioxidants in semen quality[28,32,38,39], the semen quality was defined according to the WHO 1999 guidelines. On the other hand, our patients were included based on their semen quality in line with the WHO 2010 guidelines, which have lower threshold values. Therefore, our patients were enrolled in the study with considerably poorer semen quality compared to patients in previous studies, which followed older WHO guidelines. Considering cancer patients, both chemotherapy and radiation have measurable effects on the sperm number, motility, morphology, and DNA integrity.[51] Although treatment usually represents the greatest threat to the fertility of male survivors, the underlying malignancy may additionally affect semen production and quality. In cases of testicular cancer, the rates of oligospermia and azoospermia at presentation are

TABLE 1. Comparison of baseline characteristics of patients in astaxanthin and placebo groups

Characteristic	Astaxanthin (n = 37)	Placebo (n = 35)	p-value
Age (years)	35.0 ± 5.2	36.4 ± 5.5	0.276
BMI (kg/m²)	25.9 ± 3.8	27.1 ± 4.6	0.162
Smoking (no. of cigarettes smoked / day)	4.8 ± 6.8	3.6 ± 6.8	0.264
Sperm concentration (million /ml)*	6.9 ± 5.7	5.4 ± 5.8	0.297
Total sperm number (million/ejaculate)*	23.9 ± 28.4	15.6 ± 21.0	0.164
Total sperm motility (%)**	29.9 ± 14.8	38.2 ± 14.8	0.063
Progressive sperm motility (%)**	25.1 ± 14.5	33.3 ± 14.6	0.063
Sperm morphology (%)	1.8 ± 2.1	1.9 ± 2.0	0.663
Sperm head defect (%)	95.1 ± 16.2	97.5 ± 3.3	0.936
Sperm neck + midpiece defect (%)	2.9 ± 16.4	0.2 ± 0.8	0.981
Sperm tail defect (%)	0.2 ± 0.6	0.5 ± 1.0	0.424
Leukocytes with peroxidase (million/ml)	0.3 ± 0.3	0.5 ± 0.8	0.976
Mitochondrial membrane potential (%)***	27.5 ± 18.5	26.9 ± 15.3	0.888
DNA fragmentation (%)	50.5 ± 23.9	45.8 ± 21.8	0.424
FSH (IU/l)	9.7 ± 7.8	9.2 ± 5.8	0.919
Chemicals at work (% exposed)	5.4	14.3	0.204
Ultraviolet rays at work (% exposed)	2.7	0	0.327
X-ray at work (% exposed)	0	11.4	0.051
High temperature at work (% exposed)	13.5	14.3	0.925
Sedentary job (% exposed)	45.9	40.0	0.611

Results are presented as average (± SD) or percentage (%). Differences at baseline assessed with independent Student t-test, Mann-Whitney U-test or Chi-square test, as appropriate.

* = the analysis for total sperm number and sperm concentration was made for 34/37 patients from the astaxanthin group and 35/35 patients from the placebo group, since the excluded patients only had a few mobile or immobile spermatozoa in the semen sample;

** = the analysis for total and progressive sperm motility was made for 25/37 patients from the astaxanthin group and 20/35 patients from placebo group, since the excluded patients only had a few mobile or immobile spermatozoa in the semen sample;

*** = the proportion of spermatozoa with normal MMP;

BMI = body mass index; DNA = deoxyribonucleic acid; FSH = follicle-stimulating hormone

50% and 10%, respectively.[52–55] Similarly, Hodgkin Lymphoma is associated with oligospermia and azoospermia before the treatment, and depending on the treatment regimen, also after.[56–58] According to poor starting values of basic sperm parameters in our patients, in addition to their low MMP and very high DNA fragmentation, it can be concluded that our patients, in general, had poorer semen quality as subjects in the previous studies due to the lower threshold of the new WHO guidelines.

TABLE 2. Comparison of basic semen parameters, sperm MMP, DNA fragmentation, serum FSH values in astaxanthin and placebo groups after three-month treatment period

Parameter	Astaxanthin (n = 37)			Placebo (n = 35)		
	Baseline	After 3 months	p-value	Baseline	After 3 months	p-value
Sperm concentration (million /ml)*	7.0 ± 5.6	9.2 ± 7.9	0.100	5.7 ± 5.9	10.2 ± 15.7	0.024
Total sperm number (million/ejaculate)*	24.6 ± 28.6	31.7 ± 33.4	0.186	16.0 ± 21.1	38.4 ± 69.2	0.002
Total sperm motility (%)**	32.3 ± 14.0	37.9 ± 14.7	0.172	38.0 ± 15.0	43.1 ± 12.8	0.164
Progressive sperm motility (%)**	27.3 ± 14.0	33.0 ± 14.7	0.171	33.1 ± 14.7	38.1 ± 12.8	0.176
Sperm morphology (%)	1.8 ± 2.1	1.6 ± 1.4	0.471	1.9 ± 2.0	2.0 ± 1.7	0.913
Sperm head defect (%)	95.1 ± 16.2	97.8 ± 2.2	0.785	97.5 ± 3.3	97.2 ± 2.7	0.836
Sperm neck + midpiece defect (%)	2.9 ± 16.4	0.2 ± 0.7	0.916	0.2 ± 0.8	0.3 ± 0.8	0.472
Sperm tail defect (%)	0.2 ± 0.6	0.4 ± 0.9	0.253	0.5 ± 1.0	0.5 ± 0.9	0.872
Leukocytes with peroxidase (million/ml)	0.3 ± 0.3	0.5 ± 0.7	0.155	0.5 ± 0.8	0.6 ± 0.9	0.365
Mitochondrial membrane potential (%)***	27.5 ± 18.5	26.3 ± 18.5	0.375	26.9 ± 15.3	27.1 ± 15.9	0.925
DNA fragmentation (%)	50.5 ± 23.9	51.2 ± 17.9	0.958	45.8 ± 21.8	49.8 ± 16.9	0.116
FSH IU/l)	9.7 ± 7.8	10.0 ± 8.3	0.200	9.2 ± 5.8	9.7 ± 6.5	0.345

Results presented as average (±SD) or percentage (%). Differences within groups between baseline and after 3 months assessed with paired t-test and Wilcoxon test, as appropriate.

* = the analysis for total sperm number and sperm concentration was made for 33/37 patients from the astaxanthin group and 34/35 patients from the placebo group, since the excluded patients only had a few mobile or immobile spermatozoa in the semen sample;

** = the analysis for total and progressive sperm motility was made for 22/37 patients from the astaxanthin group and 16/35 patients from the placebo group, since the excluded patients only had a few mobile or immobile spermatozoa in the semen sample;

***: the proportion of spermatozoa with normal sperm mitochondrial membrane potential (MMP);

DNA = deoxyribonucleic acid; FSH = follicle-stimulating hormone

Under these conditions, our study revealed that antioxidant astaxanthin could not improve semen quality in cases of very poor semen quality, which could also be of great importance for cancer patients.

Our results are in contrast to a previous study by Comhaire, which determined favorable changes in the inhibin B concentration, sperm linear velocity and ROS levels, and pregnancy rate after astaxanthin treatment in the same dosage of astaxanthin as in our study. However, the study enrolled a low number of infertile males (11 in the study group and 19 in the placebo group) and was not based on semen analysis but rather on a more than twelve months' infertility history of female partners having no verifiable cause of infertility.[46] Interestingly, another recent study, similar to our study, showed that different antioxidants did not affect the semen quality in a larger group of infertile patients.[59] Similar results of no effect of antioxidants were found in some other studies.[38,60–63] These data show that antioxidants are interesting, but the clinical data should be comprehended with prudence.

The improved total number and concentrations of spermatozoa after three months of treatment with a placebo could reflect the beneficial psychological effect of placebo on patients. In the literature, the strength of expectations is connected to the degree of the placebo effect.[64,65] The appearance of a medication, past experience, verbal information, and relationship between a patient and physician may influence the patient's expectations.[66] However, previously published reviews on the effects of oral antioxidants on semen quality did not show any positive effect of the placebo in the placebo-controlled studies.[28,67]

On average, the FSH value in the studied population was relatively high thus indicating the imbalance of the pituitary-gonadal endocrine axis and hypogonadism with a serious damage of spermatogenesis. In infertile men, a higher concentration of FSH is considered to be a reliable indicator of germinal epithelial damage, and was shown to be associated with azoospermia and severe oligozoospermia.[68] This indicates that the studied population of men in our study was facing severe male infertility (severe OAT).

The advantage of our prospective, double-blind study includes the good randomization of patients for astaxanthin or placebo treatment. At baseline, both groups were comparable by their characteristics. We included patients based on their previous seminograms, and they were all clinically examined by an experienced andrologist. Additionally, strict exclusion criteria were used. Besides, this is the first clinical study of the efficacy of astaxanthin oral intake on semen parameters in severe oligo-astheno-teratozoospermic patients treated for infertility. The number of patients included is higher compared to similar placebo-controlled studies of other antioxidants.[32,39,69,70] Astaxanthin group was homogenous because all patients accorded with WHO 2010 criteria for O+A+T. In the placebo group, two-thirds of patients also met the criteria for O+A+T; the average progressive motility of spermatozoa was just above the reference value (33.1 ± 14.7%), but the average total motility of spermatozoa was below the reference value. Therefore, the placebo group was also considered to be homogenous. Nevertheless, our study had some limitations, since one-third of patients had only a few mobile or immobile spermatozoa during the semen analysis; therefore, the measurements and statistical analysis for the total number, concentration and motility of spermatozoa in these patients were not possible. In addition, a relatively high proportion of patients declined to participate in this study.

As our study was performed with humans who gave consent to participate, it is our ethical obligation to also report the results with no evidence of an effect.[71,72] Also, our study delivers an important fact that the clinical study on the astaxanthin effect in oligo-astheno-teratozoospermia was carried out. Unreported or unpublished negative findings introduce a bias in meta-analysis.[73] Last but not least, the results could be an important part of future scientifically fair and flawless meta-analyses and reviews of the effects of antioxidants in infertile men.

Conclusions

In conclusion, the oral intake of 16 mg astaxanthin daily for three months did not significantly improve the semen parameters in patients with oligo-astheno-teratozoospermia compared to placebo. Our findings are important for the treatment and counselling on possible improvements in semen quality and fertilizing potential in infertile, and also post-testicular cancer patients. As a wide range of different antioxidant supplements is available, including astaxanthin, these findings are also significant concerning the efficacy of supplements in the treatment of male infertility.

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