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Review Article

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Effect of Dietary Intake of Supplement Fertylor15 Containing Astaxanthin and Other Compounds on Human Sperm: In Vivo and In Vitro Study

Guido Ambrosini[1], Alessandra Andrisani[1], Gabriella Donà[2], Chiara Sabbadin[3], Elena Tibaldi[2], Anna Maria Brunati[2], Claudia Radu[3], Eugenio Ragazzi[4], Decio Armanini[3] and Luciana Bordin[2*]

[1]*Department of Women's and Chilren's Health, University of Padua, Padua, Italy*

[2]*Department of Molecular Medicine-Biological Chemistry, University of Padua, Padua Italy*

[3]*Department of Medicine-Endocrinology, University of Padua, Padua, Italy*

[4]*Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Padua, Italy*

[*]Corresponding author
Luciana Bordin, Department of Molecular Medicine-Biological Chemistry, University of Padua, Padua Italy

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Abstract

Chronic stress and endocrine disorders that result from environmental pollution, reactive oxygen species (ROS), and genetic abnormalities, are responsible of the idiopathic infertility. The aim of this study was to investigate the effect of a daily intake of supplement containing astaxanthin (Asta), known to ameliorate sperm viability in in vitro conditions, and other elements, on semen quality. Sperm from 34 volunteers were analyzed before (T_0) and after (T_1) 30 days of intake of supplement containing Asta, zinc, selenium, arginine, vitamins C and E, Coenzyme Q10 and folic acid. Semen parameters (sperm concentration, motility, morphology) and biochemical properties (rafts shifting, tyrosine phosphorylation (Tyr-P) and acrosome reaction (AR)) were evaluated. In additional 18 volunteers, Asta, all other components or both were analyzed also in in vitro conditions. Results show an improvement of sperm quality with a decrease of membrane-bound IgG. The percentages of cells achieving capacitation and AR were increased (28±8 to 52±10%, $p<0.0001$, and 24±5 to 46±7%, $p<0.0001$, respectively). The in vitro study assessed that Asta and other compounds had a synergistic positive effect on all the biochemical parameters, with no sperm DNA fragmentation. In conclusion, this dietary intake would be helpful in the treatment of no-complicated idiopathic infertility.

Keywords: Human Sperm Capacitation; Agglutination; Acrosome Reaction; Astaxanthin

Introduction

Male infertility affects almost the 15% of all reproductive age men and decreased semen quality is responsible for ~25% of cases of infertility with about 3.3-4.7 million only in the USA [1, 2]. Not yet established the etiology, it has been proposed that the suboptimal semen quality can be ascribed to physiological, infective, environmental and genetic factors, including oxidative stress (OS) [2-6]. Sperm acquire fertilizing ability only following an extreme maturation process, called capacitation, which involves a series of transformations including the cholesterol depletion of the sperm outer membrane with lipid reorganization, and the generation of a strictly controlled amount of ROS leading to the protein tyrosine phosphorylation (Tyr-P) process in the head region and the AR [7, 8].

Fecundating ability is seriously affected by exogenous and endogenous reactive oxygen species (ROS), which can induce DNA fragmentation, protein degradation and lipid peroxidation-related membrane denaturation [8, 9]. In addition to OS, reduced spermatogenesis, which lessens the number of sperm produced in the ejaculate, low motility and membrane weakness, in absence of any other pathophysiological disorders, co-operate to lower semen quality [10].

All these factors led to focus on the potential benefits of nutraceutical/vitamin supplements to prevent the OS in semen and improve sperm parameters [3, 10-12]. Among others, Asta (3, 3'-dihydroxy-beta, beta-carotene-4, 4'-Dione), which belongs to the xanthophyll subclass of carotenoids, stands out for its powerful antioxidant behavior and has been shown to be very helpful in different diseases. The two terminal rings joined by a polyene chain with conjugated double bonds gives the strong antioxidant properties in a wide range of applications, such as in food, cosmetic, nutraceutical, and pharmaceutical industries. Together with its antioxidant activity, which is 65 times more powerful than vitamin C and 54 times stronger than β-carotene, Asta has been also shown to promote human sperm capacitation (7) and prevents potential virus protein binding to sperm membrane [8, 13-15].

Other compounds with potential effectiveness in ameliorating both semen and sperm parameters included zinc (Zn), which was found statistically significant for strengthening cell membrane and reducing the DNA breaks, arginine and folic acid for helping spermatogenesis, CoQ10 and selenium for improving motility and vitamins C and E for their antioxidant synergic action, with vitamin C locating the cytoplasm and vitamin E inserting in the lipid bilayer [12, 16-20].

In almost all the previous studies on food supplements, the main parameters evaluated in the ejaculated were limited to sample volume, motility, morphology, concentration and, in some cases, sperm chromatin integrity or DNA damage [11]. The aim of this study was to evaluate the possibility of improving semen and sperm parameters by a simple intake of supplement Fertylor15 (Leonardo Medica Srl), containing astaxanthin (Asta), previously shown to ameliorate sperm viability *in vitro* conditions, and other elements able to enhance semen quality. We have assessed the semen quality evaluating biochemical parameters involved in sperm viability and functioning. We also compared the results with those obtained *in vitro*, to determine the involvement of Asta and of other components.

Experimental Section

Materials

Pure sperm wash buffer (PSW) was obtained from Nidacon International AB (Göteborg, Sweden). The supplement, Fertylor15, containing 15 mg Asta, 60 mg and 30 mg of vitamin C and E, respectively, 0.2 mg folic acid, 7.5 mg zinc, 0.06 mg selenium, 100 mg arginine and 10 mg Coenzyme Q10, for the treatment of recruited patients and the substances for the ex-vivo experiments were supplied by Leonardo Medica S.r.l. (Vinci- Florence, Italy). All other reagents were purchased from Sigma-Aldrich (Milan, Italy).

Study Population

Fifty-eight male donors (Table 1 Supplementary) from couples, who had failed to conceive after at least 1 year of regular unprotected intercourse, were enrolled at the Centre of Assisted Reproduction - U.O.C. Obstetrics and Gynecology Clinic – Padua. The female partners of the patients resulted normal after gynecologic evaluations. Inclusion criteria: Age from 25 to 50 years; Seminal volume >1.5 ml; Total sperm number >39 mil; Concentration >15 mil/ml; Total motility >30%; Vitality >58%; Normal morphology >4%; pH ≥7.2; Leucocytes <1.0/ml.

Exclusion criteria: Subjects who presented genitourinary or endocrinological pathologies, cancer, infectious, autoimmune diseases or anti-sperm antibodies were excluded from the study. Forty patients (age range: 29–50 years, average age: 38.8±5.6 years) accepted to participate to the study and to take supplement, but only 34 completed the treatment; the remaining 18 patients were enrolled for the in vitro study. At the end of the treatment (day 31), all volunteers were questioned about any side effects found during the treatment, but all the participants declared none.

All recruited patients, gave their informed written consent and provided detailed lifestyle histories. The Ethics Committee for Research and Clinical Trials of our University approved the study (Protocol number: 3924/AO16).

In vivo study

Study design

After 3-5 days of sexual abstinence, semen samples were collected by masturbation in a sterile container and assessed for sperm parameters (T0). Recruited patients (Figure 1) were asked to take a dose of Supplement once/a day for 30 days. At the end of supplementation (T1), 34 patients returned, and their semen was subjected to the same analysis carried out at T0. Patients were instructed to maintain the same time lapse of sexual abstinence as for T0 (Table 1 Supplementary). Six patients (number 12, 18, 23, 24, 31 and 34) dropped out.

Seminal Parameters

Semen samples were processed for analysis after liquefaction for 30 min at 37°C for sperm count, motility, morphology, volume, and pH, according to the World Health Organization criteria [21].

Computer assisted sperm analysis (CASA)

Sperm motility, hyper-activation VAP (average path velocity), VSL (straight-line velocity), ALH (amplitude of lateral head displacement) and LIN (linearity, VSL/VCL) were analyzed using a computer-assisted sperm analyzer (CASA). A minimum of 100 cells and 5 fields were analyzed for each aliquot.

Sample Preparation

After semen analysis, seminal plasma and sperm cells were separated by centrifugation at room temperature (750×g for 10 min). Sperm were washed with PSW, resuspended in the same buffer at a concentration of 80×10^6 sperm cells /mL and incubated for up to 180 min in capacitating conditions at 37°C. At the end of incubation, the samples were analyzed for the evaluation of agglutination and presence of immunoglobulin bound to the sperm cells, membrane rafts localization, not viable cells and acrosome reaction.

In vitro study

For the in vitro study, assuming that the plasma bioavailability reported for each substance could mirror the concentration also in seminal fluid, we prepared the following treatments indicating with S the concentration expected after supplement intake, 2S or following, multiple of the S concentration. Asta, supposed to have a bioavailability around to 1 μM after 1 month intake, was used from 0.5 μM to 5 μM; Vit C, supposed about 60 μM after supplement intake was used from 30 μM to 300 μM; Vit E, from 7μM to 70 μM (5S); folic acid, from 46 to 460nM (5S); Zn, from 12.5 μM to 125 μM; Se, from 175nM to 1.75μM; Arg from 12.5 μM to 125 μM and CoQ10, from 0.5 μM to 5 μM [22-29].

After routinely spermiogram, semen from volunteers, who did not agree to take supplements (n=18), was divided in aliquots and incubated for 3 hours at 30°C, in absence of effectors (Control) or presence of Asta alone, Other components (except Asta), or Asta and Other components (Whole Supplement) (S = 0.3 μM Asta, 60 μM Vitamin C, 14 μM vitamin E, 90 nM folic acid, 23 nM zinc, 350 nM selenium, 23 nM arginine and 0.5 μM Coenzyme Q10). After incubation, aliquots were separately examined for CTB, NVC and ARC as described below.

Evaluation of Sperm Agglutination State

Sperm samples from recruited subjects were pelleted by centrifugation at room temperature (750×g for 10 min), washed and resuspended in PSW and analyzed in order to test sperm

agglutination state (i.e. small groups of cells with head-to-head contact) by contrast phase microscopy. Samples were classified as "positive", if the agglutination was ≥30% of the cells counted or "negative if it was absent or less. For each sample, at least 200 cells were considered.

Evaluation of IgG Bound to the Sperm Membrane

Sperm (20×10^6) from each sample, were resuspended in 200 µL of PBS (containing 1 mM sodium orthovanadate and protease inhibitor cocktail), sonicated three times (30 seconds followed by a 10 seconds rest period each), and then treated with 2 mM (final concentration) of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate hydrate (CHAPS) at 0 °C for 10 min. Aliquots (3×10^6 cells) were analyzed by Western blotting on 10%SDS-PAGE and immuno-revealed with anti-human IgG, or anti-tubulin antibody as loading control [30]. Bands corresponding to the total IgG were densitometrically estimated. The percentage of decrease in IgG bound was calculated as Δ IgG % = (1- IgG T_1 /IgG T_0) %, considering the amount of IgG before supplement intake (T_0) as 100% for each patient.

Evaluation of Membrane Rafts (CTB)

GM1 membrane raft marker was visualized by staining with the CTB-FITC [31, 32]. Briefly suspensions of cells (15×10^6 cells) from each sample were mixed with an equal volume of CTB (50 µg/ml) fixed in 2% paraformaldehyde, mounted on poly-L-lysine slides and viewed using the Leica DMI6000CS fluorescence microscope (Leica Microsystems, Wetzlar, Germany); DIC: differential interference contrast; Oil immersion lens: 100x/1.4; Camera: DFC365FX; Leica Application Suite (LAS-AF) 3.1.1. Software (Leica Microsystems). For each sample, at least 200 cells were analyzed and sperm showing fluorescence over the head were counted.

AR Evaluation

Acrosome status was monitored with FITC-PNA in conjunction with DNA-specific fluorochrome PI as a viability test according to [7, 30]. Briefly, aliquots (15×10^6 cells) of each sample were incubated the presence of 10 µM Ca2+ ionophore A23187 or DMSO [7, 30], washed, resuspended in PBS, marked for 10 min at room temperature with 12 µM PI, washed with PBS, fixed with 2% (w/v) paraformaldehyde and layed on poly-l-lysine-treated slides. After permeabilization with 0.2% (v/v) Triton X-100 for 15 min at 4°C slides were stained with FITC-PNA /mL for 15 min at 37°C in the dark, washed and mounted. At least 200 cells were evaluated for each sample. Only sperm cells showing evenly distributed fluorescence over the acrosomal region were considered acrosome intact.

Alkaline Comet assay

Alkaline Comet assay protocol was performed according to with slight modifications. Aliquots of 6 volunteers' samples, including cells treated with H_2O_2 (0, 0.03, 0.15 and 0.30%) to induce cells ssDNA break, as positive controls, were diluted to a concentration of 10×106 spermatozoa/ml and 20 µL was mixed with 50 µL of 0.7% low melting point agarose [33].

The mixture was placed on two different pre-treated slides with (1.2% normal melting point agarose gel), covered with coverslips and allowed to gel on at 4°C for 15 min. The slides were treated for 1 hour in lysing solution (2.5 M NaCl, 100 mM Na2-ethylenediaminetetraacetic acid, 10 mM Tris, pH 10, with 10% dimethyl sulfoxide and 1% Triton X-100 added fresh) and then placed in an electrophoresis tank with electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13.0) kept in the dark and cold for 30 min. Electrophoresis was performed at 25 V and 300 mA for 25 min. The slides were incubated in the neutralizing solution (0.4 M Tris–HCl, pH 7.5) for 5min, and finally dehydrated in cold 100% methanol for 3 min. All Comet assay samples were stained with DAPI and evaluated using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany), counting at least 200 spermatozoa per sample. Sperm cells were classified in fragmented and non-fragmented sperm, and values were presented as percentage of sperm with DNA fragmentation (SDF). Three different blinded laboratory technicians independently performed all microscope analysis.

Statistical Analysis

The sample size was estimated using G * Power 3.1.9.2 considering an effect size (Cohen's d) of 0.5, an alpha error probability of 0.05 and a power of 0.80, and a possible subject dropout of 15% [34]. Results are expressed as means ± SD. Comparisons of parameters between times T_0 and T_1 were obtained with Student's t-test for paired data; comparison involving the percentage of decrease in IgG bound was obtained using Mann–Whitney U test. Statistical significance was set at $p<0.05$ (two-tailed). All statistical analyses were performed with JMP® 13 software (SAS Institute, Cary, NC, USA).

Results

Sperm Agglutination

Semen from patients was analyzed before and after one month of supplement intake as described in the flow chart (Figure 1).

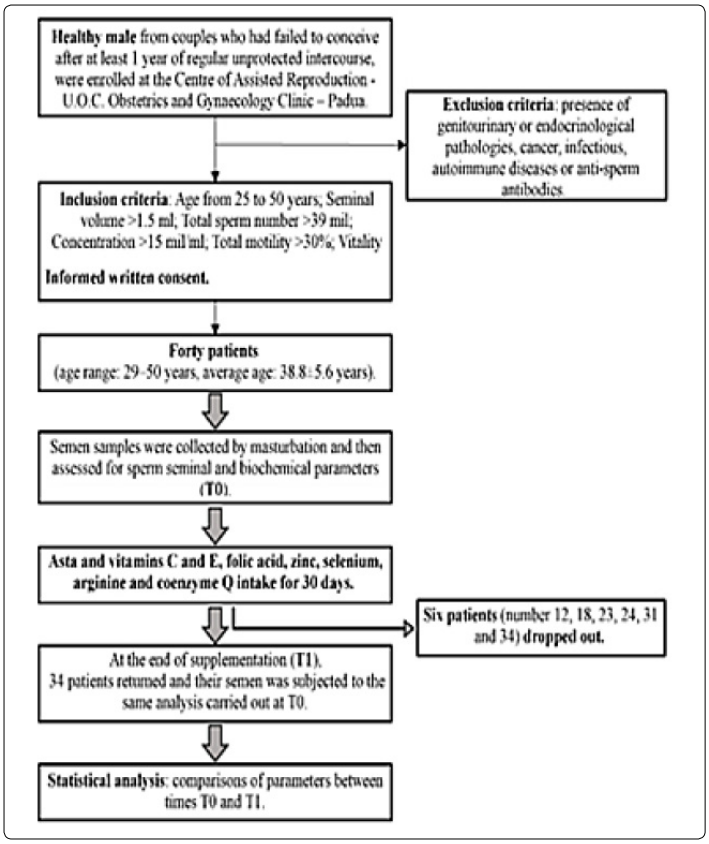

Figure 1: Flow diagram of the controlled intervention clinical study.

At T_0, i.e. before starting supplement intake, 19 samples (56%) showed agglutination state, leading to small groups of cells (2, 3 or 4 for each group) with head-to-head contact (Fig. 2, panel A). After treatment (T_1), a net reduction in the samples presenting head-to head contacts was evident, above all among volunteers who had agglutinations at T_0 (positive) (Fig. 2A, compare panel T_1 and T_0). Only 4 samples (12%) showed no benefit at the end of the treatment (Fig. 2, panel B) carrying persistent cell-to-cell adhesion also if in reduced groups of two or three cells.

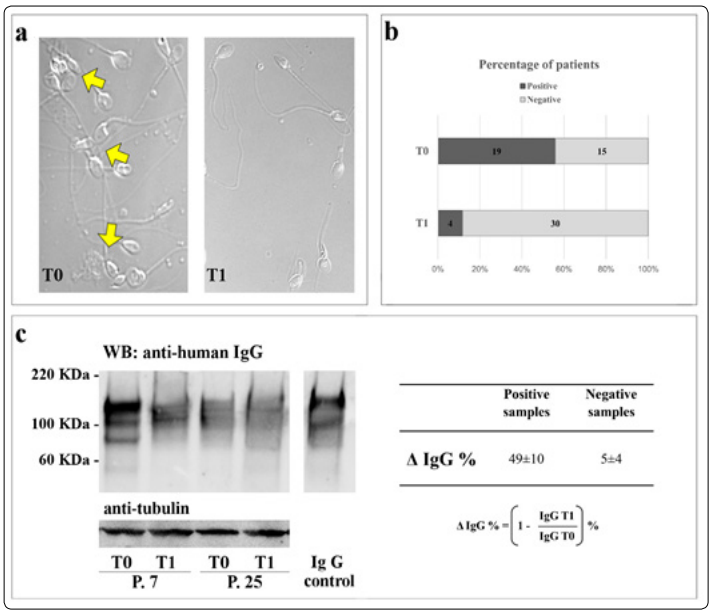

Figure 2: Sperm samples from recruited subjects, pelleted, washed and resuspended in PSW as described in Methods were analyzed in order to test sperm agglutination state (i.e. small groups of cells with head-to-head contact) and presence of immunoglobulins bound to the plasma membrane. (**a**) Contrast phase microscopy images of sperm from patients before (T_0) and after Fertylor15 intake (T_1). (**b**) Samples from all the patients were analyzed and classified as "positive", if the agglutination was ≥30% of the cells counted or "negative if it was absent or less. Percentage of positive or negative patients at T_0 and T_1 were reported. (c) Western blot analysis of total lysates of sperm from patients at T_0 and T_1, obtained as described in Methods. Aliquots from each samples corresponding to 3×106 cells were loaded and analyzed by SDS-PAGE (10%), transferred to nitrocellulose and immuno-revealed with anti-human immunoglobulins G (IgG) antibody and then with anti-tubulin antibody as loading control. The image is representative of a positive and a negative sample (patient 7 and 25, respectively). Bands corresponding to the total IgG were densitometrically estimated and statistically analyzed. The percentage of the decrease of bound IgG was calculated as **Δ IgG % = (1- IgG T_1 /IgG T_0) %**, considering the amount of IgG before Fertylor intake (T_0) as 100% for each patient. The average decrease (Δ IgG %) in positive and negative samples was reported as means±SD. Δ IgG % was significantly different between the positive and negative groups (p<0.0001, Mann-Whitney U-test).

Samples containing agglutinated sperm were classified into the group of positive and showed a high molecular weight band ranging from 200 to 80 kDa, indicating the presence of a great amount of sperm-bound IgG (Fig. 2, panel C). At T_1, samples from positive volunteers showed less evident IgG band. The reduction of the amount of IgG bound to sperm membrane surface, ΔIgG %, was significantly different between the positive and negative groups, (p<0.0001, Mann-Whitney U-test), being much higher in the positives.

Spermiograms Parameters

Semen parameters comparison before and after supplement intake showed no significant difference when considered as average means (Table 1).

Table 1: Seminal parameters (sperm concentrations, progressive motility, normal morphology) and sperm motility values (VAP, VSL, LIN and ALH) of recruited patients, before (T_0) and after 30 days of supplement intake (T_1). Values are means±SD.

	T_0	T_1
Concentration (106 sperm/ml)	49±28	49±30
Progressive motility (%)	33±17	36±16
Normal morphology (%)	13±7	12±5
VAP(μm/s)	62±7	63±10
VSL (μm/s)	55±9	54±9
LIN (%)	67±6	68±5
ALH (μm)	2.9±0.5	3.0±0.4

Comparison T_1 vs T_0 with Student's t-test for paired data indicated not significant difference for all the parameters.

However, considering the differences among all samples (Fig. 3), when each volunteer was singularly analyzed, only cell concentration appeared to be not modified by supplement treatment, being alterations suitable with the common sample variability (panels A and D). On the opposite, after treatment, 61% of patients (n=21) showed an improvement of the progressive motility, with an increase of more than 80-100% in 7 patients (panel B).Similarly, 18 patients (53%) showed an improvement of normal sperm morphology according to the WHO guidelines [21] (panel C and D).

Figure 3: Seminal parameters of recruited patients, before (T_0) and after 30 days of Fertylor15 intake (T_1): (a) sperm concentrations, (b) progressive motility and (c) normal morphology. (d) Percentage of patients with increase or decrease of seminal parameters

Capacitation and Viability

To evaluate the effect of supplement intake on sperm viability, cells, isolated from semen were analyzed for the CTB-raft shifting after capacitation [11].

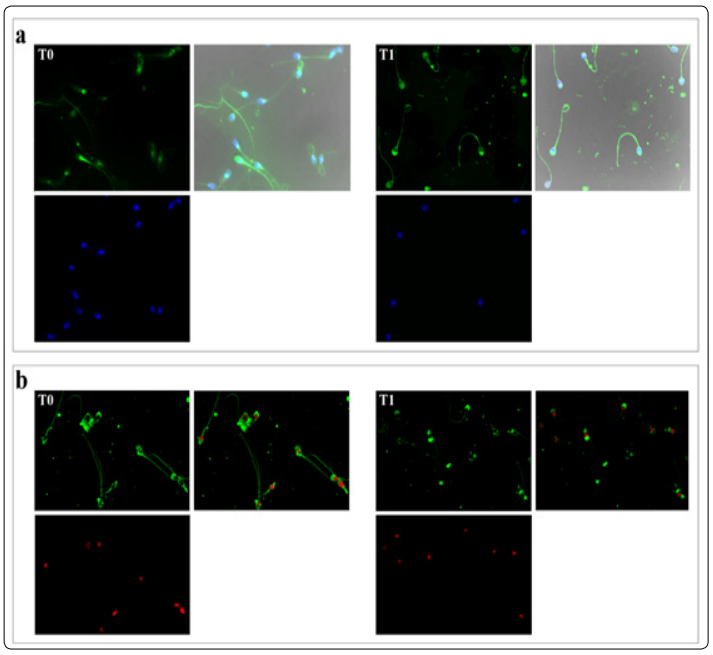

Figure 4: Capacitation (panels a) and Acrosome reaction (panels b) of sperm samples from volunteers before (T0) and after (T1) one month of Fertylor intake.

Semen from recruited subjects were centrifuged, washed, and incubated for 180 min at 37°C in capacitating conditions. Panels (**a**): sperm were analyzed by immunofluorescence cytochemistry with cholera toxin subunit B-FITC labelling (CTB) for evaluation of membrane rafts localization. After staining, sperm were treated with Hoechst to visualize the nucleus. (**b**) In order to induce AR, aliquots of each sample were incubated for 30 min at 37°C, in the presence of Ca2+ ionophore A23187. After incubation sperm were treated with propidium iodide, to assess not viable cells (NVC) and then stained with FITC-PNA for evaluation of acrosome reacted cells (ARC).

Each panel shows the phase contrast image, the specific staining and the merge image, respectively. The image is representative of study population. At T_0 after capacitating incubation, sperm could not carry out the complete membrane rearrangement needed for the correct capacitation (Fig. 4, panels A, T_0). CTB labelling was spotted mainly in the flagellum and mid piece and, only partially, in the lower part of the head (panel A, T_0). At T_1, a larger number of sperm showed a clear membrane rearrangement as indicated by the CTB labelling present all over the cell, but, principally, in the upper part of the head (panel A, T_1). Nucleus are well defined in both T_0 and T_1 samples as showed by the Hoechst-blue-staining and by the following PI determination, showing nuclear membrane integrity (Fig. 4 and table 2).

Parallel increase of the percentage of cells reaching the AR, represented by cell showing the loss of the acrosome with a perfect nuclear envelopment integrity, is reported (Fig. 4 panel B and table 2). When compared, at T_1 the percentage of ARC almost doubled the one present at T_0 in all samples (Table 2).

Table 2: Biochemical parameters of sperm from patients before (T_0) and after Fertylor15 intake (T_1).

	T_0	T_1
CTB head (%)	28±8	52±10 ***
ARC (%)	24±5	46±7 ***
NVC (%)	8±2	7±1 ***

p<0.0001 comparison T1 vs T0. Student's t-test for paired data.

Semen samples from recruited subjects were centrifuged, washed, incubated for 180 min at 37°C in capacitating conditions and then analyzed with cholera toxin subunit B-FITC labelling (CTB) for evaluation of membrane rafts, by immunofluorescence cytochemistry. Number of cells showing CTB labelling in the head, expressed as % of total number of cells, were detected and reported. In order to induce AR, aliquots of each sample were incubated for 30 min at 37°C, in the presence of Ca2+ ionophore A23187. After incubation sperm were treated with propidium iodide, to assess not viable cells (NVC) and then stained with FITC-PNA for evaluationof acrosome reacted cells (ARC) by immunofluorescence cytochemistry? Percentage of cells undergoing Ca2+ Ionophore-induced AR and NVC were also detected and reported

In vitro experiments

To determine the effect of Asta and of other substances, both were added separately or in combination to sperm from additional 12 volunteers in in vitro incubation (see Methods).

Table 3: Effect of Asta, other components, or whole supplement. On membrane rafts (CTB head), not viable cells (NVC) and acrosome reacted cells (ARC) by immunofluorescence cytochemistry, as described in methods.

	control	Asta	other components	whole supplement
CTB head (%)	35±5	49±7 ***	35±4	61±5 *** † † †
ARC (%)	32±4	44±6 ***	33±4	54±5 *** † † †
NVC (%)	9±1	7±1 **	8±1	7±2 *

Values are expressed as % of total number of cells means±SD

*** p<0.0001, ** p<0.01, * p<0.05, comparison Control vs other treatments, Student's t-test for paired data.

† † † p<0.0001, comparison Asta vs Whole supplement, Student's t-test for paired data.

Raft shifting (CTB), ARC and NVC were compared among samples (Table 3). Both for CTB labelling and ARC percentages, Asta effect compared to that of other compounds was much higher (CTB head counting for 49±7 *vs* 35±4, and ARC 44±6 vs 33±4, p<0.0001). However, when added altogether, whole supplement induced a larger percentage of capacitated sperm (CTB 61±5 and ARC 54±5, p<0.0001.

We also evaluated the dose response effect of Asta, other elements, or whole suppl, on CTB and membrane integrity. As showed in Fig. 5, none of the concentrations used (i.e. from half the expected concentration in plasma after supplement intake, S, or its multiple) affected the percentage of cells capacitated (CTB and AR) (Fig. 5A, left and right panel, respectively) which remained practically identical to that obtained with S. In addition, no nuclear degeneration, represented by DNA breaks (Comet analysis, lower right panel) was detected in each condition.

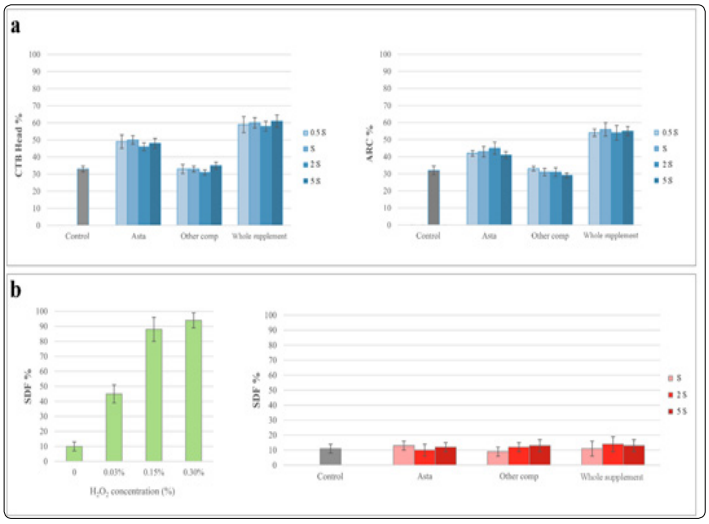

Figure 5: Semen samples from volunteers (n=6) were divided in aliquots and incubated in absence (control) or presence of increasing concentrations (0,5S, S, 2S, 5S,) of Asta, Other comp, or whole supplement, as described in methods. a) Samples were evaluated for membrane rafts (CTB head) (left panel) and ARC (right panel) by immunofluorescence cytochemistry b) Sperm with DNA fragmentation (SDF) was evaluated by alkaline Comet assay performed in cells incubated with increasing concentrations of H2O2 (positive control showing sperm with DNA fragments, left histogram) and in absence (Control) or presence of increasing concentration of asta, other comp, or whole supplement (right histogram). Values are expressed as % of total number of cells means±SD

Discussion

In the last decade, increasing attention has been devoted to the improvement of seminal parameters, according to the continuous decline of the semen quality, with particular attention to the potential correlation with dietary habits. Several studies, from cross-sectional, case-control, retrospective to prospective observational ones, have assessed the associations between diet and semen quality and/or fertilizing ability, but the results remain controversial [11]. Many studies have supported the possibility that diets, rich in some nutrients such as omega-3 fatty acids, some antioxidants (vitamin E, vitamin C, β-carotene,), other vitamins (vitamin D and folate) could improve semen quality [11]. Since observational studies may prove associations but not causation, an effective explanation about cause/effect cannot yet be identified.

In this study, we evaluated the effect of the supplement Fertylor15 intake by comparing sperm physiology and functioning. Consistent with previous in vitro findings about the beneficial effect of Asta [8, 9], our results showed that the studied supplements increase the percentage of cells reaching capacitation and AR, as well as the percentage of cells with progressive motility.

The in vitro experiments performed in the presence of Asta and/or other compounds led to some considerations. i) No detrimental effect was detected either at concentration assumed to be present in plasma from supplement intake, or even at higher. In fact, no nucleus degeneration leading to DNA fragmentation, or cell impairment were present in all the sample analyzed. Being Vit C, E, and CoQ10 anti-oxidants, a potential side effect would be an exaggerate decrease of the ROS endogenous content, which would seriously impair cell survival [7]. In our study, however, this harmful effect on cell viability and functioning was not observed. ii) Asta effect was improved when combined with other elements, whereas incubation with other elements, in the absence of Asta, did not induce any significant change in sperm capacitation or AR. It is arguable that the insertion of Asta in the bilayer, with the nonpolar middle segment inserted in the lipid portion of membrane bilayer, can be facilitated by the lipophilic portion of vit E and CoQ10. In addition, the ionone rings present at both terminal ends of Asta and representing the polar portions, can cooperate with other polar elements, such as Zn, Vit C, S. These substances can regulate both Asta positioning inside the bilayer and the consequent contacts with both proteins and lipids, constituents of the membrane. Steric/redox interactions blocking lipid rafts in the non-capacitated state would be released more easily, thus letting a larger number of cells reach the suitable conformation to undergo the AR. iii) The percentage of capacitated cells did not display a dose-dependent response to any of the treatment assayed (Asta, Other Comp, Whole Suppl), but was stable at the same level for all the concentration tested (from 0.5 to 5S) tested, as if no further improvement could be achievable in those conditions.

The intrinsic limitations of this study concern both the reduced number of samples and limited population, and the duration of treatment. In fact, study population involved only patients with satisfying semen parameters, in absence of other patho/physiological complication. Further studies are required to investigate if similar positive effects would come from same supplementation in asthenozoospermic, teratozoospermic or oligospermic patients. In addition, considering that spermatogenesis is a process requiring about 72 days, it would be interesting to evaluate volunteers after a longer period of intake, about 3 months or more, to evidence if improvement in sperm counts, morphology and motility can be achieved too.

Despite these limitations, this study would be helpful in the treatment of no-complicated idiopathic infertility in couples looking for pregnancy, because of safety of the treatment even at much higher concentrations, of the components.

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