The Impact of Antioxidant Food Supplementation on Seminal Antioxidant Capacity, Sperm DNA Fragmentation and Sperm Chromatin Quality in Subfertile Men with Oligoastenoteratozoospermia Randomized Clinical Trial

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MATERIALS AND METHODS (STUDY PROTOCOL)

1. Patient Selection

Forty-eight subfertile men diagnosed with idiopathic OAT in the Urology Clinics of Ondokuz Mayis Univerity (OMU) Medical Faculty Hospital between March and November 2021 was included in this study. Ethics committee approval (2021/108) was obtained from Clinical Research Ethics Committee at OMU. All participants could not achieve pregnancy despite their regular sexual intercourse for 12 months or longer, and their female partners had normal fertility examinations. All patients were evaluated with OAT after two semen analyses in the last six months. Physical examination data, hormonal, and genetic evaluations of all patients were normal and were accepted as idiopathic infertile. Exclusion criteria of the patients were undergoing to sterilization procedures such as vasectomy, medical treatment or drug affecting the reproductive function or metabolism, taking multivitamins or herbal products, use of anticoagulants, sperm concentration <5 million/mL, untreated hypothyroidism or uncontrolled diabetes mellitus and medical conditions such as cancer, heart disease or cirrhosis.

2. Antioxidant Food Supplement

By computer-assisted simple randomization (www.randomizer.org) patients who agreed to participate in the study were divided into Group 1 (n=24) and Group 2 (n=24). A food supplement containing 2000 mg carnitine, 2000 mg fructose, 932 mg acetyl L-carnitine, 225 mg vitamin C, 115 mg citric acid, 50 mg coenzyme Q10, 14 mg zinc, 115 µg selenium, 3750

µg vitamin B12, 500 µg folic acid (Alfasigma Health Science, Trento, Italy) was given daily as an antioxidant to the patients in Group 1. We did not suggest the use of any supplements to Group 2. All subjects were recommended to do moderate physical activity for at least 45 minutes (at least 150 minutes-600 METs per week) 3-4 days a week for three months.

3. Hormone Analyses

Hormone analyses (FSH, LH, prolactin, estradiol, and total testosterone) were performed using the ELISA method by drawing peripheral venous blood samples from the patients between 7 am – 11 am after at least 12 hours of fasting.

4. Body Mass Index (BMI) and Physical Activity Assessment

BMI of the participants was calculated using the formula kg/m2. All patients participating in the study were recommended to get moderate physical activity at least 45 minutes 3-4 times a week for three months. Physical activity assessments were made using the International Physical Activity Questionnaire (IPAQ) Short form, in which total scores included the sum of walking, moderate-intensity activity, and duration (minutes) and frequency (days) of vigorous activity (Meh et al. 2021).

5. Semen Collection and Preparation

Semen samples were collected from all patients at the 0th and 3rd month of examinations after 2-7 days of sexual abstinence. After incubation for 20-30 minutes at 37ºC, liquefied samples were analyzed according to the World Health Organization (WHO) 2010 guidelines (World Health 2010). Semen samples were then treated to evaluate the total seminal antioxidant capacity (TAC), sperm DNA fragmentation (SDF) and sperm DNA chromatin condensation. For the analysis of SDF, sperm concentrations of samples were adjusted to 2.5×106 /mL (Sharma et al. 2021). Semen samples were centrifuged at 2000 rpm for 8 minutes to separate seminal plasma from the sperm pellet. Then, seminal plasma was centrifuged at

8000 rpm for 10 minutes at 4°C and was stored at -70ºC until TAC measurements. A portion of the semen pellet was fixed for SDF analysis. The remaining pallet was used to determine sperm chromatin condensation with aniline blue staining.

6. Measurement of Seminal Total Antioxidant Capacity

TAC was measured by the colorimetric assay using the antioxidant Assay Kit (Cayman Chemical, Michigan, USA) according to the manufacturer's recommendations (Mahfouz et al. 2009). All seminal plasma samples were diluted from 1:10 to 1:20 prior to analysis. Briefly, Trolox standards were diluted sequentially with assay buffer, and then 10 µl of each was added to the 96-well plate. Afterward, 10 µl of each sample was added to the sample wells. All samples and standards were placed in duplicate. Chromogen 10 µl metmyoglobin and 150 µl ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) were added to both samples and standards. To all wells 40 µl of 441 µM hydrogen peroxide was added and then the plate was incubated for 5 minutes on a shaker at room temperature. The absorbances of the standards and samples were measured at 750 nm using a microplate spectrophotometer (Multiscan GO, Thermo Scientific, Finland) after incubation.

Calculations of each standard and sample were made to evaluate the assay. A standard Trolox curve was plotted with the mean absorbance of the standards. The TACs of the samples were calculated according to the formula using the linear regression of that standard curve and the average of the absorbance of samples: Antioxidant (mM) = [(Sample average absorbance) - (y-intercept)/ Slope] x Dilution

7. Assessment of Sperm Chromatin Condensation by Aniline Blue Staining

After the semen sample of each patient was immediately separated from the seminal plasma, some of the sperm pellet was washed twice with 1x phosphate-buffered saline (PBS) (Gibco, NY, USA). The spermatozoa were then spread on clean slides and the smears were air

dried for approximately 15 minutes. Dried smears were fixed with 3% glutaraldehyde at room temperature for 30 minutes. Afterward, all slides were immersed in a 5% aniline blue solution (Sigma, Steinheim, Germany) in 4% glacial acetic acid (Sigma, Steinheim, Germany) for 15 minutes (Hekim et al. 2021). After staining, 200 sperm were counted at least on each slide at 1000x magnification at a light microscope. Pale blue spermatozoa that received less or no staining were considered protamine-rich, and spermatozoa partially or completely stained dark blue were evaluated as histone-rich.

8. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) for Direct Detection of SDF

SDF was analysed with TUNEL using the commercial In situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to instructions of the manufacturer (Hekim et al. 2021). After removing the seminal plasma, the pellet was washed with 1xPBS. For the positive control of the analysis, 30% hydrogen peroxide solution was placed on the fresh semen pellet of one of the samples and incubated for 15 minutes at 37°C. Afterward, all samples were fixed with 4% paraformaldehyde (PFA) (Merck KGaA, Darmstadt, Germany) for 20 minutes at room temperature. Then, PFA was removed, and the samples were washed twice with 1xPBS by centrifugation at 2000 rpm for 8 minutes. Sperm samples were added onto phosphate-buffered (PB) sucrose on polylysine-coated slides. They were kept in a humid and dark environment at 4°C overnight. The next day, slides were washed twice with 1xPBS for 5 minutes and kept in freshly prepared 0.1% sodium citrate and 0.1% Triton X-100 permeabilization solution on ice. For TUNEL reaction, label solution was mixed with enzyme solution, and 50 µl of the mix was dropped onto the washed slides at the end of permeabilization. Negative controls were obtained by adding only label solution. Slides were incubated for 60 minutes at 37°C in a humid and dark environment. Afterward, the slides were washed three times with 1xPBS and a mounting medium with DAPI (Fluoroshield, Sigma, Missouri, USA) was added and covered with a

coverslip. Samples were immediately examined and photographed using an excitation wavelength of 461 nm and 519 nm (green) detection under a fluorescent microscope (BX51, Olympus Life and Material Sciences). Photographs were analysed with the Image J program (LOCI, University of Wisconsin) and at least 500 cells were evaluated from each sample. While blue fluorescence stained all nuclei, sperm nuclei showing green fluorescence were considered to have fragmented DNA. SDF was calculated as the number of sperm nuclei stained green as a percentage of the total sperm nuclei identified as blue in the same area.

9. Statistical Analysis

The conformity of the dependent and independent continuous variables used in the study to the normal distribution was analysed with the Kolmogorov Smirnov test. The comparison of the arithmetic mean of independent groups (with and without antioxidants), Student's t-test was used for variables with normal distribution, and Mann Whitney U test was performed for variables that did not fit a normal distribution. Comparisons of arithmetic means in dependent groups (pre-treatment and post-treatment) were analysed with Paired t-test for normally distributed variables and Wilcoxon test for variables found to be non-normally distributed. Statistical data analysis was performed using IBM SPSS Statistics Version 22.0 (Armonk, NY, USA). $P<0.05$ was considered statistically significant