Non-enzymatic antioxidant defense and polymorphic changes in male infertility

running title: **Non-enzymatic antioxidant defense and male infertility**

Supplementary Materials 1.

Material and methods

Seminological studies

Semen abnormalities (quantitative and qualitative) applied to oligozoospermia, asthenozoospermia, azoospermia, teratozoospermia, necrozoospermia, combined oligozoospermia–asthenozoospermia–teratozoospermia OAT II, OAT III, cryptozoospermia, polyzoospermia, cryptoteratozoospermia, leukospermia or combined disorders. The control group comprised 87 men with normozoospermia (no abnormalities in a seminal examination; [29]). All participants stated their conscious and voluntary agreement to participate in the study, received information on the objectives, and completed a questionnaire. The collection of biological material and the semiological tests were conducted by a qualified workers' clinic (immediately after the collection of the semen sample). The authors of this paper also analyzed semen samples. Evaluations applied to morphological parameters such as volume, time of liquefaction, sperm density (number per milliliter of ejaculate), motility (with specification of types of movement), presence of agglutination, presence of leukocytes, and sperm morphology.

On this basis, normozoospermia or specific seminological abnormalities were diagnosed, which allowed us to place patients in either the infertile or control group. In addition to semen (>1.5 mL), whole blood samples (about 15 mL) and serum samples (1.5 mL) were also collected. The serum was separated from the blood by centrifugation at 3500 cycles per minute for 10 min. Serum and semen were stored in Eppendorf-type tubes. Full blood and serum samples were conserved at −80°C (New Brunswick Scientific-86 Co. Inc. 175 Ultra Low-Temperature Freezer, Freshwater Blvd., Enfield, Connecticut, Edison, NJ, USA), while semen was kept in liquid nitrogen (−196°C).

The study participants were men who voluntarily attended a private clinic for infertility treatment to obtain a semiological evaluation. Men who fulfilled the norms of the WHO (2010) [29] regarding semen parameters were qualified to join the control group (normozoospermia), while those with diagnosed impairments in any of the parameters (about norms of WHO 2010 [29]) were qualified to join the infertile group. We did not apply any specific recommendations regarding the age of participants. We also did not introduce any specified parameters that might influence exclusion from the study. The average age of infertile men was 31.29 years, while in the control group, it was 29.02 years ($p=0.05$). Surveys that each participant voluntarily filled out provided information about their health and potential environmental or genetic burdens (in the survey, there were questions about age, residence, kind of work, exposure to stress factors – physical or chemical, lifestyle, diet, innate or acquired diseases, diseases in family members, and medication use. Participants received complete information about the nature of the study project and voluntarily agreed to participate.

Supplementary Materials 2.

Material and methods

Methods used for semiological analysis

Seminological analyses were based on macro- and microscopic analysis of ejaculate to verify parameters such as semen volume, time of liquefaction, sperm density (count per milliliter of ejaculate), motility (regarding types of movement), presence of agglutination, presence of leukocytes, and percentage of pathological forms. After comparing the WHO reference values (2010) [29], subjects were assigned to either the infertile or control group. The criteria of normozoospermia (control group) in our study were defined based on the following reference values: semen volume (≥1.5 mL), time of liquefaction (<60 min.), sperm density (≥15 million·mL^{-1}), sperm motility–types of movement A, B, C, D (progressive motility ≥32%), agglutination (lack of agglutinates and sperm aggregates), presence of leukocytes (<1 million·mL^{-1}), and morphology (≥4% of correct forms). Deviation from these norms constituted a basis for classification in the infertile group, in which we singled out the following diagnoses: asthenozoospermia (n=15; 19.74%), azoospermia (n=10; 13.16%), cryptozoospermia (n=3; 3.95%), necrozoospermia (n=1; 1.32%), oligoasthenoteratozoospermia OAT (n=14; 18.42%), oligozoospermia (n=7; 9.21%), polyzoospermia (n=4; 5.26%), and combined disorders (n=22; 28.95%).

Supplementary Materials 3.

Material and methods

Standard semen evaluation

Macroscopic evaluation of semen

Volume (mL): measurement was carried out in a measuring cylinder with a conical base. Reaction (pH): The measurement was made with a Spezial–Indicator paper indicator (pH range 7.2–9.7; Macherey–Nagel GmgH & Co., Düren, Nordrhein–Westfalen, Germany) by putting a drop of semen on it. After 30 s, the color obtained at the point of contact was compared with the colors in the template provided by the manufacturer. Measurements were made within one hour of ejaculation.

Microscopic evaluation of semen

A Makler sperm counting chamber (Sefi Medical Instruments, Haifa, Israel) inserted into a light microscope (Carl Zeiss Jena, Jena, Germany) with Ph2 phase contrast was used for microscopic semen evaluation. A small drop of semen, well mixed by pipetting, was placed in the center of a Makler chamber, and the chamber was covered with a coverslip. The preparations were then viewed under a magnification of 200× at room temperature.

The microscopic evaluations focused on the following parameters:

1. Concentration and motility of sperm in semen

The principles of using a hemocytometer were used in the assessments performed in Makler's chamber. During observations, the entire surface of the preparation was first viewed, and then sperm within 10 squares arranged along the diagonal mesh were counted (in the case of oligozoospermia, concentrations were in the hundreds of thousands per mL, while lower sperm counts were observed in all areas of the chamber-100 fields). The type of movement was then assessed, distinguishing among fast (type A) and slow (type B), forward (type A) and slow (type B) sperm, non-progressive (type C) sperm, and non-moving (type D) sperm.

2. Morphological characteristics

Sperm were observed under an immersion microscope with a magnification of 1000 times (100× objective) after prior staining with a semen smear using the Schorr method:

Reagents: Haemotoxylin Papanicolaou No. 1; ammonium alcohol (95 mL 75% ethanol + 5 mL 25% (250 mL·L^{-1}) ammonium hydroxide); and Schorr's solution.

Preparation of smear: about 20 µL of the sample was applied (50–100×10^{6} sperms·mL^{-1}) to a glass slide, smeared, and allowed to dry.

Dyeing Procedure:

the slide was rinsed with running water (12–15 dips);

the smear was immersed in Haemotoxylin Papanicolaou No. 1 for 1–2 min;

the slide was rinsed with running water (12–15 dips);

the smear was immersed in ammonium alcohol 5 times for 5 min;

the slide was rinsed with running water (12–15 dips);

the smear was immersed in 50% ethanol (500 mL·L^{-1}) for 5 min;

the smear was immersed in Schorr's dye for 3–5 min;

the smear was immersed in 50% ethanol (500 mL·L^{-1}) for 5 min;

the smear was immersed in 75% ethanol (750 mL·L^{-1}) for 5 min;

the smear was immersed in 95% ethanol (950 mL·L^{-1}) for 5 min;

the smear was immersed in absolute ethanol 2 times for 5 min;

the smear was immersed in xylene (dimethylbenzene) twice for 5 min.

Taking into account the criteria included in the Kruger classification [31], after staining with the Schorr method, sperm were considered normal if they exhibited:

1. An oval head with a regular and clean contour (front view) or resembling a flattened pear (in profile). Sperm were divided by staining into two parts – the front, which was less saturated and covered the nuclear zone (acrosome), and the rear, which had a more compact atomic mass. The length of the head was 4–5 µm, the width was 2–3 µm, and the length-to-width ratio was 1.5 to 2.

2. The intermediate part of the withers comprised a simple thickening in the extension of its long axis and may have been surrounded by a small cytoplasmic residue, 4–5 µm in length.

3. The central part of the twine was thin, tapering regularly and measuring 8–9 times the length of the head. In our analyses of spermiograms, the ejaculate was treated as usual (normozoospermic) when the volume of sperm ranged from 2 to 6 mL, the number of sperm ranged from 20 to 250 million mL^{-1}, the percentage of sperm in the progressive movement (type A + B) was not less than 50%, and the rate of normal sperm was at least 30% (abnormal maximum 70%).

Supplementary Materials 4.

Material and methods

Chemical element analysis

Quantitative analyses of chemical elements were conducted with mass spectrometry with inductively coupled plasma (ICP–MS; Agilent Technologies 7500CE ICP-MS apparatus). Before examination, sample mineralization was conducted. Digestion was conducted using the microwave method (Discovery SP–D, CEM Corporation, Matthews, North Carolina,

USA). Samples were analyzed using helium collision mode using a quadrupole mass spectrometry system (Agilent Technologies, Inc., Headquarters, Santa Clara, CA, USA). Before digestion, samples were vortexed to achieve a homogeneous state, and then they were pipetted to prevent settling before extraction. Blind reagents were prepared by adding deionized water instead of samples. Samples were placed in a microwave cooker and digested according to the serum digesting protocol. At the end of digestion, samples were preserved in a refrigerator at a temperature of 4°C until further testing. Mineralization was applied to all chemical elements, so no separate mineralization was performed. The effectiveness of the applied method and good recoveries of mercury were experimentally confirmed. Mineralizing biological samples in a microwave oven in hermetically closed Teflon-coated containers prevents the volatile loss of mercury. Calibration curves were received using the weight method. Certified reference materials were used as quality controls of serum samples (Seronorm Trace Elements Serum L–1 and L–2). For each sample series, certified reference material (NCS ZC73016 chicken) from China National Analysis Center for Iron and Steel was used to fulfill quality control requirements.

The preparatory stage of the ICP-MS method comprised blood mineralization. Quantitative analyses of chemical elements were conducted with mass spectrometry with inductively coupled plasma (ICP–MS). This method is particular and makes it possible to determine chemical element concentrations with relatively small amounts of material. Before examination, sample mineralization was conducted. Full blood was transferred to calibrated tubes made of borosilicate glass (25 mL). After mineralization, redistilled water (up to 8 mL) was added to the solution, which was then transferred to polyethylene containers. Samples were prepared in clean hood class 100 to prevent contamination with atmospheric particles.

Digestion

Digestion was conducted using the microwave method (Discovery SP–D, CEM Corporation, Matthews, North Carolina, USA). Subsequently, samples were tested for vestigial minerals with an Agilent 7500CE ICP–MS (quadrupole mass spectrometry system using helium collision mode, Agilent Technologies, Inc., Headquarters, Santa Clara, CA, USA). Before digestion, all samples were thoroughly vortexed to achieve a homogeneous matrix. Samples were immediately pipetted to prevent settling before extraction. Then, 250 µL samples were dosed into glass vessels designed for microwave digestion and washed with acid. Blind reagents were prepared by adding deionized water instead of samples. Nest, 300 µL concentrated nitric acid (HNO_3) (Ultrex purity, Fisher, Thermo Fisher Scientific, Waltham, MA, USA), 200 µL concentrated hydrochloric acid (HCl) (Ultrex, Fisher, Thermo Fisher Scientific, Waltham, MA, USA) and 100 µL of unstable 30% hydrogen peroxide solution (H_2O_2) (Ultrex, Fisher, Thermo Fisher Scientific, Waltham, MA, USA) were added to each vial with serum samples. Deionized water was added to obtain a final volume of 2.0 mL. Samples were closed tightly, placed in a microwave cooker, and digested according to serum digesting protocol. At the end of digestion, all samples were removed and left to cool to room temperature. In a clean hood, samples were transferred to polypropylene tubes (acid washed; capacity 15 mL) enriched with multi-elemental internal patterns to obtain final concentrations of 10 $ng \cdot mL^{-1}$ of indium, scandium, and praseodymium and diluted to the final volume with deionized (DI) H_2O. Samples were preserved in a monitor refrigerator at a nominal temperature of 4°C until further testing. The mineralization method was applied to all chemical elements; no separate mineralization was performed.

The effectiveness of the applied method and good recoveries of mercury were experimentally confirmed. Microwave digestion with concentrated nitric acid and hydrogen peroxide was used to break down the analyzed material. To 2 mL of 30% hydrogen peroxide was added 8 mL of 69–70% nitric acid (Baker Instra Analyzed, J.T. Baker Chemical Company, Avantor, Phillipsburg, NJ, USA). Subsequently, samples were exposed to microwaves for 5 min at 190°C (time of escalation: 5 min.), and finally, 5 min at 210°C (time of escalation: 5 min.) to

ensure the complete dissolution of organic matter. Digested solutions were transferred to single calibrated tubes and mixed with deionized water (0.05 $\mu S \cdot cm^{-1}$) to achieve a final volume of 50 mL.

Quantitative Analyses

Quantitative analyses of trace elements were based on the ICP–MS technique (inductively coupled plasma mass spectrometry). The Agilent Technologies 7500CE ICP-MS apparatus is equipped with a micro–miss nebulizer; a double-pass spray chamber cooled with the Peltier method, and a peristaltic pump. Argon 5.0 (99.999% cleanness) was used as the carrier gas. The apparatus is also equipped with a torch with a reduction system (shield torch) that eliminates interference. The vacuum system is made up of a rotary pump and a turbo-molecular pump. Quadrupol is a mass separator with hyperbolical sticks. The detector operates in two modes, i.e., digital and analog, making it possible to work in nine volume grades. All analyses were performed in the presence of 45Sc, 89Y, and 159Tb as internal patterns to minimize the effect of the matrices and ensure long-term stability. This procedure was also conducted for blind samples to detect pollution. Simultaneously, for each sample series, certified reference material (NCS ZC73016 chicken) from the China National Analysis Center for Iron and Steel was used to meet the relevant quality control requirements. For this material, recoveries were obtained in the 90–110%, and uncertainty of measurements was established at <10%. An ICP–MS Agilent 7500 CE was used to measure the concentrations of chemical elements. It was equipped with a nebulizer (micro-mist) and a mist chamber, which was thermoelectrically cooled via the Peltier effect (double-pass).

It also featured a reaction chamber ORS (octopole reaction system) to eliminate polyatomic interference and interference from doubly charged ions. In the reaction chamber, we used hydrogen 6.0 and helium 6.0 (99.9999% cleanness) as reaction gases to eliminate interference. Highly clean argon 5.0 (99,999% cleanness) was used as the transferring gas (pressure 200 bar). Samples were passed along at a speed of 0.01–0.5 rpm. Additionally, each analysis was performed with ^{45}Sc, ^{89}Y, and ^{159}Tb as internal patterns to maintain the stability of the apparatus and minimize matrix effects.

Mineralization

The mineralization of biological samples in a microwave oven in hermetically closed Teflon-coated containers prevents the volatile loss of mercury Hg. After mineralization, containers are cooled to room temperature, and then the solution is transferred to a regular vessel. Problems with mercury may arise from the fact that in the presence of nitric acid, a reduction of mercury may occur. Additionally, the adsorption of mercury on hydrophobic surfaces of Teflon-coated vessels used for mineralization may create a problem or cause a memory effect, which is typical for mercury. In this respect, 20 tubes were checked by adding mercury to assess the influence of mineralization in the oven on recovery efficiency. For the sake of comparison, we analyzed samples with additional patterns that were not mineralized; other elements were not analyzed, and the amount of acid and final sample volume remained the same. Therefore, it is obvious that mineralization does not result in a loss of analytes; recoveries were excellent, i.e., in samples conserved with EDTA, they were close to 100%, while in the case of samples with lithium heparin, they were around 90%. Mineralization occurred in Teflon-coated containers, which, after mineralization, were cooled to prevent losses. The low pH of the solution after mineralization and quantitative transfer to the final volume effectively prevented Hg loss. The problem of the influence of pH on Hg loss was examined by Rosain and Wai (1973) [32]. They found that the process of mercury mineralization does not result in the loss of this element.

The first calibration pattern on the curve was 50 $ng \cdot L^{-1}$. The recovery of mercury from samples fortified in this way was almost 100% in the case of tubes with EDTA, whilst in the case of heparin, it was over 90%. Recoveries are lower for samples analyzed without mineralization, as nebulizer and plasma processes may interfere with non-mineralized EDTA and

heparin. Patterns without matrices were also mineralized to obtain final concentrations after transfer to 25 mL of around 10 $ng \cdot L^{-1}$, 25 $ng \cdot L^{-1}$, and 50 $ng \cdot L^{-1}$. Again, the obtained recoveries were satisfactory. During each measurement, the same conditions were applied. An internal pattern was applied to monitor the conditions. The apparatus receiving the signal from the internal pattern calculated the current results while eliminating the influence of environmental factors (changes in flow caused by the decrease of opening diameter in the cone due to the sedimentation of calcium).

Calibration and reference materials

The calibration curves were produced using the weight method. A mixture of nitric acid (V) and water served as the blind sample and was exposed under the mineralization conditions along with the other samples. Multi-elemental patterns were used with the appropriate conditions for ICP examinations. Because patterns were multi-elemental, preparing separate curves (with different ranges) for each chemical element was impossible. Certified reference materials were used as quality controls (QC) in ICP–MS analyses of serum samples. We applied reference materials Seronorm Trace Elements Serum L–1 and Serum L–2, which were marked at the beginning and the end of each group consisting of 20 samples. Simultaneously, for each sample series, certified reference material (NCS ZC73016 chicken) from the China National Analysis Center for Iron and Steel was used to fulfill quality control requirements. For this material, recoveries obtained were 90–110%, and measurement uncertainty was established at 10%.

Supplementary Materials 5.

Material and methods

Lipid peroxidation intensity

An examination of the MDA (lipid peroxidation marker) level in serum was conducted with a commercially available set manufactured by Cayman Chemicals Co. Ltd. (TBARS Assay Kit, Item No. 10009055, Cayman Chemical Company, Ann Arbor, MI, USA). The procedure of MDA (lipid peroxidation marker) examination was based on the measurement of the concentration of substances reacting with thiobarbituric acid (TBARS), which is a reflection of lipid peroxidation intensity. The presence of more unsaturated lipids causes an increment in TBARS value. Adduct MDA–TBA is produced in the reaction of MDA with TBA at high temperatures (90–100°C) and in conditions of acidic pH. The procedure was performed colorimetrically.

Supplementary Materials 6.

Material and methods

Molecular Analysis

The applied DNA isolation kit (Epicentre an Illumina Co.; Cat. No MCD85201, Madison, WI, USA) consisted of Red Cell Lysis Solution, Tissue & Cell Lysis Solution (as well as T&C Lysis Solution 2×), MPC Protein Precipitation Reagent, RNase A, Proteinase K, and TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). After isolation, DNA was evaluated regarding its usefulness for further analysis. DNA isolation kit (Epicentre an Illumina Co.; Cat. No MCD85201, Madison, WI, USA) consisted of Red Cell Lysis Solution, Tissue & Cell Lysis Solution (as well as T&C Lysis Solution 2×), MPC Protein Precipitation Reagent, RNase A, Proteinase K, and TE buffer. After isolation, DNA was evaluated. Quantities

(ng·μL^{-1}) and pureness (A260/A280) were measured with NanoDrop 2000 (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA). In this respect, 2 μL of DNA in TE buffer was adequate for effective measurements (the measurement apparatus was first calibrated with 2 μL of pure TE buffer–blank). DNA concentration in the 50–150 ng·μL^{-1} and pureness in the range of 1.6–1.9. were considered adequate (values were established based on optimization and the available literature [33,34]). Horizontal electrophoresis was used (horizontally placed gel covered with 1×TBE buffer). The advantages of this method are the lack of risk of leakage of electrolytes and the effortless transfer of heat [35]. In our study, we used 2% agarose gel. The PCR reaction mix was prepared sterilely (UVP UV3 HEPA PCR Workstation chamber). The order of reagents was established during optimization, guaranteeing optimal activity and minimizing the use of unspecific products. Microtubes containing 1 μL of previously isolated DNA were transferred 19 μL of reaction mix with sterile pipettes and single tips (final reaction volume: 20 μL). These were placed in a thermal cycler, where a programmed protocol was performed. Applied starters, PCR reaction conditions (pre-denaturation, denaturation, annealing, elongation, and additional elongation), as well as the conditions of digestion with restrictive enzymes for respective polymorphism, were established based on the available literature [36, 37].

Supplementary Materials 7.

Material and methods

Polymorphism of Gene *IL–4*v.*C589T* (rs2243250)

In the analysis of the polymorphism of the *IL–4*v.*C589T* (rs2243250) gene starter, forward 5'–TAAACTTGGGAGAACATGGT–3' and reverse 5'–TGGGGAAAGATAGAGTAATA–3' were used (Sigma-Aldrich, St. Louis, MO, USA). Before application, they were diluted with TE buffer according to the Sigma Technical Datasheet (forward: 1281 μL of TE; reverse: 1163 μL of TE). PCR reaction products were sectioned through electrophoresis with an apparatus designed for horizontal electrophoresis (MS Major Science MP–300V, GenBiotech, Roseti, C1427 BWB, Buenos Aires, Argentina) at 110 V for about 60 min on 2% agarose gel. In the final step, each sample was digested with a restrictive enzyme (*AvaII*) to obtain restriction fragments with a specific length (PCR–RFLP). Based on the image of the restriction fragments, it was possible to distinguish particular genotypes (heterozygote, wild-type homozygote, and mutated homozygote). The conditions of electrophoresis after digestion were about 90 min. On 2% agarose gel at a voltage of 110 V.