A double-blind randomized placebo-controlled clinical study to verify the efficacy of TetraSOD® in the improvement of semen quality in patients with idiopathic infertility.

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CONFIDENTIAL

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1.6 Ethical Committee

All the materials have been reviewed by CEIC Hospital Clínic de Barcelona (Barcelona)

2 Introduction and justification

2.1 Background information

Infertility is an increasing global public health problem present in about 15-20% of the population of reproductive age, affecting as many as 186 million people worldwide [1]. The main cause of this increase is the age of women, but it must be highlighted that in 20% of the cases are due to an exclusive male factor, and in 30-40% of the cases a combined cause is found (male and female factor). Therefore, a male factor is present in approximately 50% of the infertile couples, a large number of them of unknown or idiopathic origin [2].

Spermatogenesis is a complex biological process that requires a highly regulated genetic and hormonal program in a singular environment created by the interaction with different cell types to orchestrate a successful differentiation process. This process occurs periodically every 72 days during a man's fertile life [3]. It has been demonstrated that semen quality is deteriorating over the time, perhaps as a result of exposition to several environmental factors related with lifestyle: drug use (such us tobacco, alcohol, marijuana, cocaine, opioids and anabolic agents), diet and overweight, disorders of the sleep-wake cycle and working conditions (continuous exposition to heat sources or toxic substances) that could impact both directly and indirectly on the complex process of spermatogenesis [4].

Currently, the impact of oxidative stress, a cellular state product of an imbalance between the generation of highly unstable molecules known as reactive oxygen species (ROS) and the antioxidant cellular capacity in male fertility is being deeply investigated [5]. High levels of oxidative stress in semen have been associated with both lower sperm concentration, sperm motility and acrosome integrity and higher sperm DNA damage and mitochondrial activity [6-10]. Moreover, it has been demonstrated the negative correlation of high levels of ROS in semen with the sperm fertilization ability and embryo quality after IVF cycles [9,11,12]. Sperm are particularly susceptible to the oxidative effects of ROS, due to the large amounts of unsaturated fatty acids in the cell membrane (lipid peroxidation). Lipid oxidation process leads to loss of membrane integrity, increased its permeability, the inactivation of cellular enzymes, structural DNA damage and cellular apoptosis.

Recent clinical trials have demonstrated the high prevalence of sperm DNA damage by up to 80% of men diagnosed with idiopathic male infertility [5-7]. This DNA damage produced during spermatogenesis or sperm maturation process could be the result of an increase of reactive oxygen species (ROS) in male reproductive tract, which are related to different known factors (chronic systemic disease, use of some drugs, radiation or pesticides, febrile processes, old age and environmental factors related to lifestyle: smoking, obesity, alcohol) and other unknown so far.

Recently, Agarwal A. et al [13], have demonstrated the usefulness of measuring oxidation-reduction potential (ORP) in semen and seminal fluid by a novel analyzer, "Mioxsys", patented by Aytu BioScience, as a diagnostic test of male fertility capacity. This allows assessing the degree of oxidative stress in the sample (assessing the balance between oxidizing and antioxidant substances), in a simple, cheap and real-time manner, which has been shown to be closely related to seminal quality; the higher ORP, the worse sperm motility.[11] Moreover, it has been

shown that the mean ORP value does not change when it is measured at different times after the collection of the sample (between 0-120 min) [13] .

2.2 Justification

Antioxidant supplementation has gained relevance within routine practices in patients with reproductive problems [5]. Different studies have shown the beneficial effect of antioxidant consumption against oxidative damage caused by environmental and pathological components, improving sperm characteristics associated with the seminal analysis. There are scientific evidences about the improvement of male fertility and higher rates of live newborn after antioxidant treatment in subfertile men [14].

TetraSOD® is a unique commercial product comprised of 100% lyophilized biomass of the marine microalgae *Tetraselmis chuii* strain CCFM03, which is currently marketed for food and nutraceutical applications around the world by the company Fitoplancton Marino, S.L. This microalgae product is characterized by a high content in the antioxidant enzyme superoxide dismutase (SOD), as it is produced using own (patent pending) technology developed by the company. Results of *in vitro* studies with human cell lines suggest that TetraSOD® stimulates the cellular protective mechanisms against oxidative stress, including up-regulation of genes encoding SOD, glutathione peroxidase (GPx) and catalase (CAT), as well as the nuclear factor erythroid 2-related factor 2 (NRF2) and the heme oxygenase-1 (HMOX1) inducible phase II detoxifying enzyme.

A pilot study has been previously performed in which 40 subjects were recruited and divided into two groups of 20 subjects each. In summary, the results obtained in this pilot study with the highest dose of TetraSOD® (250 mg/day) were promising, since a high statistically significant response was observed in three of the four studied parameters after three months of treatment.

Taking into account these results, a new extended double-blind randomized and placebo-controlled clinical study will be performed to confirm the positive effects of dietary supplementation with 250 mg of TetraSOD® during 3 months (corresponding to the time of a complete spermatogenic cycle) in sperm quality.

The main objective of our study is to demonstrate the usefulness and safety of TetraSOD® treatment in the improvement of sperm quality including sperm DNA fragmentation. As we mention previously, oxidative stress is one of the main causes of sperm DNA damage. Nowadays, a diagnostic test for the study of the sperm DNA fragmentation exists, but it requires a specific sperm sample processing and the results are obtained in delay-time. In our study we are going to also assess the correlation between sperm DNA fragmentation and sORP (static oxidation reduction potential) degree by the use of "Mioxsys". Therefore, the availability of a simple and cheap instantaneous response time diagnostic test seems promising for the reproductive clinics. It allows us to advance in the diagnosis of male fertility potential and in the identification of those patients susceptible to antioxidant treatment [15] in order to improve their reproductive results, increasing the chances of pregnancy both spontaneously or after assisted reproductive treatment [9,12].

3 Objectives of the study

3.1 Main objective

- To evaluate the efficacy of TetraSOD® in improving the sperm quality in patients with asthenozoospermia (low sperm motility), oligozoospermia (low sperm concentration) or oligoastenozoospermia (low sperm concentration and motility) after 3 months of treatment.

3.2 Secondary objectives

- To evaluate the improvement in the sperm oxidative stress in infertile patients after 3 months of treatment with TetraSOD®.
- To evaluate the efficacy of TetraSOD® in improving sperm DNA integrity in infertile patients.
- To establish a prognostic correlation value between sORP levels, sperm DNA fragmentation, and seminal parameters.
- To evaluate the safety of TetraSOD®.

3.3 Main variables

- Seminal parameters as described in the WHO laboratory manual for the Examination and processing of human semen, Fifth Edition (manual of the WHO 2010) [16] including vitality and leukospermia.

3.4 Secondary variables

- Sociodemographic variables.
- Evaluation of the degree of sperm DNA fragmentation in semen samples using the COMET assay.
- Evaluation of sORP by Mioxsys.
- Sperm ROS intracellular and sperm DNA oxidation.
- Adverse events attributable to the treatment or study recommendations.

4 Study Design

Monocentric, prospective, randomized, double-blind and placebo-controled clinical study to evaluate clinical parameters, seminal quality, oxidative stress and DNA fragmentation, in astheno/oligozoospermic patients attending to the Andrology unit of the "Hospital Clínic de Barcelona".

They will be randomized into two groups: experimental group (TSOD food supplement) and control group (Placebo). The experimental group will receive a daily dose of 250 mg of TetraSOD® during three months and the control group will receive a daily dose of placebo during the same period of time. After three months of treatment, both groups will stop using TetraSOD® or placebo and a wash-out period of 3 months will start.

The intervention group will be assigned to each patient according to the assigned code in order of inclusion to the study and that will serve for the randomization of the patients. The code lists will be previously prepared through an informatic program. The ratio of experimental and control group will be 1:1. The randomization numbers will be distributed by blocks. The study is designed to make the assignment double-blind; therefore, the randomization sequence will be hidden and only visible by the study monitor.

The Placebo and TetraSOD® capsules will be prepared in such a way that they are indistinguishable from each other. The participants in the study, as well as the technicians in contact with the patients, in charge of preparing questionnaires, distributing capsules, taking samples, etc., will not be able to know which capsules correspond to each experimental group.

5 Selection of participants

Adult asthenozoospermic, oligozoospermic or oligoastenozoospermic males who come to the Uroandrology unit of "Hospital Clínic de Barcelona" of Barcelona.

5.1 Inclusion criteria

- Age: 18 to 45
- Male patients with idiopathic infertility classified with asthenozoospermia, oligozoospermia or oligoastenozoospermia after seminal assessment.
- Not achieving pregnancy after at least one year of intercourse with the same partner without protective measures

5.2 Exclusion criteria

- Azoospermia (absence of spermatozoa) or severe oligozoospermia (< 5 x 10^6 spermatozoa/ml of ejaculate)
- Testicular torsion or prostatitis
- Urinary retention and infections
- Drug consumption
- Hormone treatments
- Recent surgical interventions
- Diabetes
- Kidney or liver disease
- Leukocytosis
- Antioxidant supplement consumption in the last 3 months
- BMI>30Kg/m^2
- Endocrinopathies, hypo and hyperthyroidism
- Chromosomal anomalies (XX, XYY, XXY)
- Treatments with anticoagulants
- Radiotherapy/Chemotherapy
- Participation in another clinical study prior to inclusion in this study that could affect the objectives of the current study

6 Treatment and study calendar

The investigator will recruit the patients as they come to consultation and meet the inclusion and exclusion criteria. The assignment of the study treatment will be carried out randomly.

The expected duration for the recruitment of patients will be 1 year. After the patient recruitment, they will attend to 3 visits (initial visit, 3 months and 6 months visits).

6.1 Baseline Visit (V0)

In this visit the patients will sign the informed consent form (if they agree to participate in the study) after reading the patients information sheet and receiving all the information about the study by investigators. The investigator will ensure that patients meet all the inclusion/exclusion criteria in order to be finally included in the study. The investigator will give to the patients the product needed until the next visit.

In order to characterize the population of patients, the following demographic parameters will be collected in this visit:

- Age
- Studies and degrees
- Weight, height and BMI
- Professions
- Infertility period
- Years of marriage
- Physical activity
- Smoking history
- Blood analytics, to determine hormones (testosterone, Prolactin, FSH, LH, inhibin)

Semen samples will be collected in order to do the following analysis:

- Seminal analysis as described in the manual of the WHO 2010 (15)
- Sperm DNA fragmentation
- SORP
- Sperm ROS intracellular and sperm DNA oxidation

Semen samples will be obtained as described in the manual of the WHO 2010 section 2.2 (15) but after a minimum of 3 days and a maximum of 5 days of sexual abstinence. The sample will be taken by masturbation and ejaculated into a clean, wide-mouthed container that has been confirmed to be non-toxic for spermatozoa, administered by the hospital. The total ejaculated sample will be delivered between 30-45 minutes maximum after the collection. Prior to collection, a thorough hand wash will be required. The investigator will be responsible for the samples, both in the custody and conservation. After the sample extraction a code will be assigned which will be the only identification of the sample, thus ensuring confidentiality of the data in accordance with the provisions of the LOPD. Once the sample has been used for research purposes, it will be destroyed; so, it won't be preserved.

The results of the hormones will be taken from the habitual blood analysis that take place in routine clinical practice.

- Seminal analysis will be performed as described in the manual of the WHO 2010[16].
- Sperm DNA fragmentation will be performed using COMET assay.
- SOPR will be measured by the Mioxsys technique.
- Sperm ROS intracellular and sperm DNA oxidation will be measured by flow cytometry evaluating the sperm intracellular ROS (superoxide anion, hydroxyl radical, hydrogen peroxide) and 8-hydroxydeoxyguanosine (8OHdG) concentrations, respectively.

6.2 3 months Visit (V3months)

During this visit semen samples will be collected as was done at V0. The following analyses will be performed:

- Semen analysis as described in the manual of the WHO 2010 (15)
- Sperm DNA fragmentation
- SORP
- Sperm ROS intracellular and sperm DNA oxidation
- Blood analytics, to determine hormones (testosterone, Prolactin, FSH, LH, inhibin)

The analyses will be performed as described in V0.

Adverse events attributable to the treatment will be recorded.

Patients will be asked to stop the treatment.

6.3 6 months Visit (V6months)

During this visit semen samples will be collected as was done at V0. The following analysis will be performed:

- Semen analysis as described in the manual of the WHO 2010 (15)
- Sperm DNA fragmentation: if an improvement of this parameter is not detected in V3, it will not be evaluated at this visit.
- SORP
- Sperm ROS intracellular and sperm DNA oxidation: if an improvement of this parameter is not detected in V3, it will not be evaluated at this visit.
- Blood analytics, to determine hormones (testosterone, Prolactin, FSH, LH, inhibin)

The analyses will be performed as described in V0.

Adverse events attributable to the treatment will be recorded.

Table 1: study schedule

	V0	V3 months	V6 months
Inclusion/exclusion criteria	x		
Sign Informed consent	x		
Delivery of product	x		
Sociodemographic data	x		
Semen samples collection	x	x	x
Seminal analysis	x	x	x
Sperm DNA fragmentation	x	x	x*
Static Oxidation Reduction Potential	x	x	x
Sperm ROS intracellular and sperm DNA oxidation	x	x	x*
Hormones	x	x	x
Collection of excess product and empty blisters		x	
Adverse events**		x	x

* If an improvement of this parameter is not obtained in V3, it will not be evaluated at this visit.
** Adverse events will be collected from the first intake of the product and during the 6 months of the study.

7 Statistical analysis

7.1 Sample size

The sample size has been calculated using the on-line tool GRANMO v7.12 (https://www.imim.es/ofertadeserveis/software-public/granmo). Data obtained in the previous pilot trial regarding total sperm count per ejaculate were used as a reference. With the aim to determine differences between the mean of the two experimental groups, and accepting an alpha risk of 0.01 and a beta risk of 0.2 in a two sided test, a total of 46 individuals per group are necessary to recognize as statistically significant a difference greater than or equal to 20 x 10^6 spermatozoa/ml of ejaculate. The common standard deviation is assumed to be 25 (the maximum value in the previous pilot trial), with an anticipated drop-out rate of 20%. In that way, a total of 100 subjects, 50 per experimental group, will be recruited for the present clinical trial.

7.2 Statistical analysis

A descriptive statistic of all the variables collected will be made.

The categorical variables will be presented in the form of lists of frequencies and proportions. For the quantitative variables, central tendency (mean and median) and dispersion (standard deviation, maximum and minimum values) will also be presented.

Initial demographic and anthropometric characteristics of patients in TetraSOD® and placebo groups will be compared with independent t-test for continuous variables and chi square test for categorical ones. The non-parametric Mann-Whitney test will be applied to evaluate the effects of the dietary supplementation with TetraSOD® group vs. placebo group at the same time of study (time 0, 90 d or 180 d). Within each experimental group (TetraSOD® and placebo), the non-parametric Friedman test for matched-pairs will be applied in order to determine statistically significant differences of data recorded at the three sampling times (0, 90 d and 180 d). Significance will be accepted for $p<0.05$. All analyses will be conducted with Prism 6 software.

8 Ethical and legal considerations

This study will be performed in accordance with Declaration of Helsinki in its latest revised version (Fortaleza, 2013)

This study will be developed in accordance with what is established in this protocol and with the Law 14/2007 of 3rd July on Biomedical Research.

Informed consent will be requested from patients prior to their inclusion in the study.

9 Data processing and archive. Confidentiality of data.

The data used in this study will be collected with the intention of use them for the completion of the study.

The processing, communication and transfer of personal data of all participants will be in accordance with EU Regulation 2016/679 of the European Parliament compliance and of the Council of 27th April 2016 on the protection of natural persons with regard to the processing of personal data and the free movement of data, being mandatory from May 25th 2018, and to Organic Law 3/2018, of December 5th, on the Protection of Personal Data and guarantee of digital rights. The legal basis justifying the processing of the data is the consent signed by the patient, in accordance with Article 9 of EU Regulation 2016/679.

The data collected for these studies will be identified only by a code, so no information will be included to identify the participants. Only the study investigators and his collaborators with the right of access to the source data (medical history) will be able to relate the data collected in the study to the patient's medical history.

The identity of the participants will not be available to any other person except for a medical emergency or legal requirement.

They may have access to the identified personal information, health authorities, the Research Ethics Committee and staff authorized by the study promoter, when it would be necessary to check data and procedures of the study, but always maintaining confidentiality in accordance with current legislation.

Only the encrypted data will be transferred to third parties and other countries, which in no case will contain information that can identify the participant directly (such as first and last name, initials, address, social security number, etc.). In the assumption that this assignment occurred, it would be for the same purpose as the study described and guaranteeing confidentiality.

If a transfer of coded data is made outside the EU, whether in entities related to the hospital where the patient participates, to service providers or to researchers collaborating with us, the data of the participants will be protected by safeguards such as contracts or other mechanisms established by data protection authorities.

As promoters of the project we are committed to carry out the processing of the data in accordance with EU Regulation 2016/679 and, therefore, to keep a record of the processing activities and to do a risk assessment of the treatments, to know what measures we will have to implement and how to do so.

In addition to the rights already contemplated by the previous legislation (access, modification, opposition and cancellation of data, deletion in the new Regulation) participants can now also limit the processing of data collected for the project that are incorrect, request a copy or transfer to a third party (portability). To exercise these rights, they should contact the principal investigator of the study or the Data Protection Officer of "Hospital Clínico" of Barcelona through protecciodades@clinic.cat. They also have the right to contact the Data Protection Agency if they are not satisfied.

Data cannot be deleted even if a patient leaves the study, to ensure the validity of the investigation and to comply with legal duties and drug authorization requirements.

The Investigator and the Promoter are obliged to retain the data collected for the study at least up to 15 years after its completion. Subsequently, personal information will only be retained by the center for health care and by the promoter for other scientific research purposes if the patient has consented to it, and if permitted by applicable law and ethical requirements.

10 Management of biological samples.

Semen samples will be collected at the time of inclusion in all the visits in the study.

- Objective: Seminal analysis, sORP, sperm DNA fragmentation, sperm ROS intracellular and sperm DNA oxidation analysis necessary for the study.
- Samples will be analyzed in the site of the study.
- Principal investigator will be responsible for the samples, both in the custody and conservation of them.
- Once samples are used for research purposes, they will be pre-processed and stored for a possible analysis in the future. Therefore, the consent of the project will be included in the informed consent.

11 Financing

This study will be funded by the study promoter: FITOPLACTON MARINO, S.L.

12 Publication policy

Promoter will publish the results of the Study in scientific publications and will assume the responsibility of preparing the final or partial reports, as well as communicating them to whoever corresponds. For this purpose, the principal investigator will deliver to the promoter

the clinical data obtained during the study and stipulated in the protocol for the preparation of the final report with the signature of the principal investigator.
The lack of authorization for the publication of the Results will not prevent the promoter, the principal investigator or the center from using said Results in their professional activities.

When the results are presented in meetings or published in scientific journals, the right of authors or inventors to be recorded as such will be respected in any case. Likewise, mention will be made of the center, as the place where the study has been carried out, and of the promoter who has financed the study.

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