Hypolipidemic and Antioxidant Capacity of Spirulina and Exercise

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Abstract

Introduction: In order to reduce cardiovascular diseases risk factors, a healthy diet must include dietary antioxidants from different sources (e.g. *Spirulina maxima*) and regular practice of exercise should be promoted.

There is a little evidence from animal studies that *Spirulina maxima* and exercise, decreases cardiovascular diseases risks factors. However, very few studies have proven the independent or synergistic effect of *Spirulina maxima* plus exercise in humans. Therefore, this study pretends to address this independent and synergistic effect in overweight and obese subjects participating in a systematic physical exercise program at moderate intensity on general fitness, plasma lipid profile and antioxidant capacity in overweight and obese subjects.

Methods and analysis: Through a randomized, double-blind, placebo-controlled, and a counterbalanced crossover study design, eighty healthy overweight, and obese subjects will be evaluated during a twelve-week isoenergetic diet, accompanied by 4.5 g a day of *Spirulina maxima* intake and/or a physical systematic exercise program at moderate intensity. Body composition, oxygen uptake, heart rate, capillary blood lactate, plasma concentrations of triacylglycerols, total, low and high-density lipoprotein cholesterol, antioxidant status, lipid oxidation, protein carbonyls, superoxide dismutase, catalase, glutathione, glutathione peroxidase, glutathione reductase, and paraoxonase will be assessed.

Ethics and dissemination: This study and all the procedures have been approved by the Universidad Autonoma de Ciudad Juarez Bioethics Committee. Moreover,

findings will be disseminated through peer-reviewed journals, national and international conferences.

Trial registration number: ClinicalTrials.gov: NCT02837666.

Keywords: Spirulina, dyslipidemias, oxidative stress, exercise, body fat, antioxidant.

Strengths and limitations of this study

- Working with obese people, one of the main risk factors for cardiovascular diseases.
- This study can be extrapolated to different populations.
- Double-blind randomized controlled trial.
- There are not possible limitations to perform all the procedures described in the present paper.

Background

Cardiovascular diseases (CVD) are the leading causes of death worldwide. Each year, 17.5 million people die because of these diseases [1]. Dyslipidemias are a predisposing factor for CVD and are characterized by high concentrations of triacylglycerols (TAG), total cholesterol (TC), low-density lipoproteins cholesterol (LDL-c), and low concentrations of high-density lipoproteins cholesterol (HDL-c) [2]. Besides dyslipidemias, oxidative stress (OxS) is also observed to rise in obesity [3, 4], and it is also a predisposing factor for CVD [5-8].

As a way to reduce OxS, dyslipidemias and the CVD incidence, it has been proposed the intake of antioxidants that come from fruit and vegetable-rich diet or nutritional supplements, mainly by unprocessed foods [9, 10]. In this sense, cyanobacterium *Spirulina* is an important source of antioxidants, currently associated with cardiovascular protection properties [9, 11].

For centuries, *Spirulina maxima* (*S. maxima*) has been cultivated and used as a nutritional supplement due to its content of amino acids and essential fatty acids, vitamin C, vitamin E, tocopherols, and phycocyanins [12]. Recently, *S. maxima* studies have focused in verifying the biological activity of its components, including hypolipidemic and antioxidant effects [13-15]. However, most studies have been conducted in animal models, with only a few studies focused on the biological effect in humans.

Furthermore, it is known that the practice of systematic physical exercise ameliorates CVD risks [10, 16]; and it has been observed that also plays a role in OxS, especially physical exercise at high intensity (PEHI) [17]. PEHI considerably increases general metabolism, oxygen uptake (VO_2), and mitochondrial activity; thus increasing the reactive oxygen species (ROS) production[18]. Instead, physical exercise of moderated intensity (PEMI) has the best protective effect exerted against CVD, mainly due to physiological adaptations, including the expression of antioxidant enzymes [19], which stop the formation and propagation of ROS, and improving redox status of the organism [20, 21].

Algae and cyanobacteria have shown promising pharmacological properties providing health benefits and physical improvements [22]. These effects are

attributed to their specific profile in bioactive compounds such as uncommon carotenoids, phenolic compounds, tocopherols besides their well-known content in vitamins and good quality proteins associated to their specific chemical structure and interaction with biological membranes [23].

In this sense, there is evidence that the cyanobacteria *Spirulina*, in addition to exercise, lower CVD risks. This was mainly observed in animal models [24-26]. However, no studies in humans under *Spirulina* and exercise experimental designs proving these benefits have been reported yet. Therefore, the ISESE study will analyses the independent and synergistic effect of the intake of *S. maxima* with a PEMI on general fitness, plasma lipid profile, and redox status in overweight and obese subjects.

Methods/Design

Hypothesis

Spirulina maxima intake and a dosed physical activity program will improve lipids profile, general fitness and antioxidant status in overweight and obese subjects.

Objectives

The ISESE project's main objective is to demonstrate that ingestion of *S. maxima* and a dosed physical activity program will decrease, both independently and synergistically, some CVD risks in overweight and obese subjects.

Outcome measures

92 The primary outcome measure will be changes in the plasma lipid profile after a
93 six-week of treatment (plasma TAG, TC, HDL-c, and LDL-c by using standardized
94 enzymatic methods).

95 The secondary outcome measure will be changes in general fitness (maximal
96 oxygen uptake (VO_2 max), heart rate (HR), in onset blood lactate accumulation,
97 and body fat mass); as well as changes in redox status (malondialdehyde (MDA),
98 protein carbonyls, paraoxonase (PON1), superoxide dismutase (SOD), catalase
99 glutathione (GSH), glutathione reductase (GR), and glutathione peroxidase (GPx).

100 **Participants' eligibility**

101 The inclusion criteria will be the age between 18 and 45 years old of both sexes:
102 40 overweight (Body mass index (BMI): 25-29.9 kg/m^2) and 40 obese (BMI: >30
103 kg/m^2). The subjects´ exclusion criteria will be: drinking more than 100 mL of
104 alcohol a week, taking drugs and/or food or vitamin supplements, having a chronic
105 disease, or having a physical or electrocardiographic injury that prevents them from
106 engaging in any regular physical exercise. The elimination criteria will be that the
107 program subjects´ attendance will be lower than 80% and/or 20% change in their
108 body weight during study.

109 **Call to participation, ethics and safety procedures**

110 University students of Ciudad Juarez, Mexico with overweight and obesity will be
111 personally invited to participate. Participants will be informed of the purpose of the
112 study, and of the physical, clinical and biochemical procedures, benefits, physical
113 intensity tests, and risks, all before beginning the clinical trial. To guarantee the

good physical and mental health of the participants at the beginning of all the studies, a medical clinical laboratory study examination and an electrocardiography at rest tests will be performed. Noting that investigators are trained in cardiopulmonary reanimation and that the laboratory has the necessary communication tools and channels to perform emergency procedures. This study and all the procedures have been registered appropriately in ClinicalTrials.gov database (NCT02837666) and approved by the Bioethics Committee of the Universidad Autonoma de Ciudad Juarez (Supplementary files). That participants' acceptance formalized by means of written informed consent and their anonymity and confidentiality will be strictly enforced. This study will be conducted in compliance with the principles of the Declaration of Helsinki.

Baseline measurements

A day before beginning the treatment, subjects will visit the laboratory for baseline measurements. Body mass will be measured with subjects lightly dressed and barefoot in an electronic balance, and standing height with a stadiometer. Body fat percentage will be measured by plethysmography method (BodPod, USA), electric bioimpedance (in body 160, US), and by dual-energy X-ray absorptiometry (DXA) (Prodigy v6.8; GE Lunar, Milwaukee, WI) according to manual guidelines. Anthropometric parameters will be taken by the standardized method of the International Society for the Advancement of Kinanthropometry (ISAK), and according with ISAK Anthropometry Accreditation protocols [27]. Researchers are standardized in handling equipment and procedures.

Study design

The study consists of *S. maxima* treatment during 14 weeks in a randomized, double-blind, placebo-controlled, and counterbalanced crossover design, aimed to eliminate inter-individual bias as well as boosting the reliability of final results; moreover, this design is commonly used in clinical trials in order to have better results and conclusions [28]. Eligible participants (n= 80) will be divided into two groups, one with systematic practice of physical exercise (GEx) and other one without it (GNEx). The participants of each group will be randomized in equal proportions between *S. maxima* and placebo treatment, receiving either 4.5 g daily, in a non-transparent capsule during 12 weeks (6 weeks for the first treatment - 2 weeks of washout period - 6 weeks for the second treatment) (Figure 1). Before any reported allergic reaction occurs, the treatment will be discontinued.

To maintain the overall quality and legitimacy of the clinical trial, code interruptions should occur only in exceptional circumstances when knowledge of the actual treatment is essential for further management of the participant. Investigators are encouraged to discuss with a Medical Advisor if they believe that unblinding is necessary. Unblinding should not necessarily be a reason for study discontinuation.

A student outside the research team will feed data into the computer in separate datasheets, so that the researchers can analyze the data without having access to information about the allocation treatments, storing all the participants' files in numerical order in a safe and accessible place. The participants´ files will be stored for a period of five years after the end of the study.

159 The sample size was determined by using the statistical program G*Power [29],
160 selecting a sample of 73 subjects with $\alpha = 0.05$ and $p = 0.85$. This sample will be
161 fixed to 80 subjects in order to anticipate the possible desertions from the study.

162 **Maximum intensity tests**

163 Each subject will participate in four stress tests performed at maximum intensity
164 (MIT) during the study; during MIT record, consumed O_2 and produced CO_2 will be
165 taken by a gas analyzer (Cortex MetaLyzer® 3B, Germany), HR with a Polar H7
166 sensor (Polar Electro, Lake Success, NY), and lactate in capillary blood samples
167 will be assayed in a YSI 1500 Sport Lactate Analyzer (YSI Life sciences, OH,
168 USA). The MIT protocol consists of using a cycle ergometer (Monark ergomedic
169 828 E; Monark exercise AB, 105 Vansbro, Sweden) initiating with a workload of 50-
170 75 W with increments of 15-30 W every 3 min for until 15 min or when the subject
171 could no longer pedal more than 40 revolutions/min, finishing the test when
172 reaching 90-100 % of the maximum HR reserve; MIT will not be valid for less than
173 9 min (Figure 2). At the end of each increment load (3 min), capillary blood
174 samples will be taken to determine lactate, and the physical perceived effort will be
175 registered by using the Borg scale. Measurements of HR, glucose and blood
176 pressure will serve to take care of the subject's health during each MIT.

177 Before the first MIT, subjects will decide to be in the GEx or GNEx, and then they
178 will be divided randomly for the treatment, which consists in a daily dosage of 4.5 g
179 of Spirulina or placebo in capsules which will be recommended the subjects to
180 consume them before each meal every day during six weeks. The day after the
181 first supplementation period, subjects will come back to perform the second MIT

with identical conditions to the first one. The third MIT will be performed after a washout period of two weeks to remove the effects of treatment and avoid any possible delayed effect of *S. maxima* in the organism. The last MIT will be performed after the second treatment with identical conditions to the previous three times. General fitness parameters will be measured the same day of all the MITs to have accurate results.

Randomization

All subjects who consent to participation and who meet the inclusion criteria will be randomly assigned by an investigator who will not have any other interference in the clinical study; he will create a database with all the treatments through the duration of the study. Therefore, randomization will be carried out without any influence of the principal investigators. Participants will be randomly assigned to *S maxima* or placebo treatment with a 1:1 allocation according to a computer-generated random schedule stratified by site and the baseline score of the Action Arm Research Test using permuted blocks of random sizes with an online, central randomization service (TENALEA). Block sizes will not be disclosed, to ensure concealment.

Adherence assessments

Multiple methods will be used to assess treatment adherence including pill counting; reasons for non-compliance, and use of the capsules. Participants will return each week to receive new capsules. All treatment data will be recorded on the appropriate case report form.

Dietary analysis

All participants will be subjected to a nutritional survey to define the daily calories required to establish a custom diet. Dietary intake will be monitored through retrospective methods including the 24 h recall (which investigates intake over a specific day), and the food frequency questionnaire (a summary of usual intake of different categories of foods). These accurate and validated techniques allow quantifying the types and quantities of foods and beverages consumed during the period of interest in the past. Dietary intake evaluations will be performed at each MIT of the study in order to record the possible variability in food consumption patterns.

Physical exercise protocol

General health assessment and physical activity questionnaires (PAR-Q and YOU) will be conducted; this will be done to ensure that there are no physical impediments to exercise. GEx participants are going to exercise five days a week with the following protocol: between 5 and 10 min of warm-up exercise, between 20-30 min muscular endurance exercise and 20-30 minutes of aerobic exercise (cardiovascular exercise): walking, jogging, running and/or cycling. Three days a week aerobic intensities will be between 60% and 80% and two days between 70% and 90% of the maximum HR reserve, and five final minutes of stretching.

Sample collection and biochemical analysis

Blood samples (8 mL) will be collected before and after each MIT from the antecubital vein into ethylenediaminetetraacetic acid (EDTA) tubes after 10-12 h of

fasting, plasma from blood samples will be obtained by refrigerated centrifugation (4°C) at 5 000 g for 10 minutes. Plasma glucose TC, HDL-c, LDL-c, and TAG concentrations will be analyzed by using standard enzymatic procedures (Jas Diagnostics, Inc. Mexico) with a spectrophotometer (Genesys 10 UV; Thermo Electron Corporation, USA). Plasma will be used to measure MDA and GSH content and activity of GPx and GR according to Jimenez-Osorio *et al.* [30], total antioxidant status (TAS) by 2,2-diphenyl-1-picrylhydrazyl (DPPH) technique as previously described by Koren *et al.* [31], oxygen radical absorbance capacity (ORAC) following Prior *et al*. [32] methodology. The erythrocytes will be washed with cold 0.9% saline solution twice and are going to be used for SOD and catalase activity assays as previously described by Jimenez-Osorio *et al.* [30], and plasma PON1 content will be measured by following Perez-Herrera *et al.* [33] methods.

Statistical analysis

To evaluate data distribution, normality and homoscedasticity tests on each of response variables analyzed will be conducted. In order to analyze differences between variables before and after the study and between the groups, an ANOVA test will be performed. To analyze possible associations between variables, a bivariate correlation analysis will be used. To analyze independence between variables, a multiple regression analysis will be performed. The software to use is GraphPad Prism 6.

Discussion

Cardiovascular diseases are the leading cause of mortality worldwide, with OxS, overweight, obesity, and dyslipidemias being the main risk factors. Many drugs aimed at reducing these risk factors have often collateral side effects when used, and many people often use complementary medicine instead. For this and other reasons, the pharmacological industries have focused on developing new treatment options[34]; *S. maxima* research has proven benefits to reduce CVD risk factors, isolated or in combination with systematic practice of physical exercise, mainly in animal models [9, 35, 36].

This study is summarized and described in experimental design, procedures, compliance with ethical principles, and statistical analyses. Since the double-blind design, compressively described the methodology and a higher number of participants than other studies, it is expected to get a better support for the hypothesis that *S. maxima* will have hypolipidemic and antioxidant effect against intense exercise and obesity. It is noteworthy that, not every overweight or obese patient has issues with lipid metabolism, nowadays the association between the augmentation of body fat and lipid disturbances is well known [37]; for that reason, the primary focus of this protocol is prevention and it justifies why we do not consider dyslipidemia as an inclusion criterion; even though, they could present this condition.

Finally, it is known that systematic practice of exercise and a balanced diet not only favors reduction of weight in obese people, but it generally decreases cardiometabolic risks [2], and thus it probably generates an increase in antioxidant capacity [38]; however, no studies on the metabolic oxidation decreased by

administration of *Spirulina* in people with obesity were identified. In conclusion, well-designed trials are needed to clarify the value of *S. maxima* supplementation in clinical practice [39]and its complementary effect with or without exercise against dyslipidemias and OxS in overweight and obesity, a fact practically unknown at this time[40]. The later establishes the importance of this study.

Competing interests

The authors declare that they have no competing interests.

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Contributors

All authors contributed to the development of the study protocol and to the critical revision of the paper and approved the final version. RPHT, ARJ, and JALD will be involved in patients' recruitment. MAHL and AWM will analyze the data, MAHL and MAJO will do the lipid profile analyses, RUR, ARJ and MAHL will do the body composition measurements of all patients, LARC and JPC will do all the redox status analysis.

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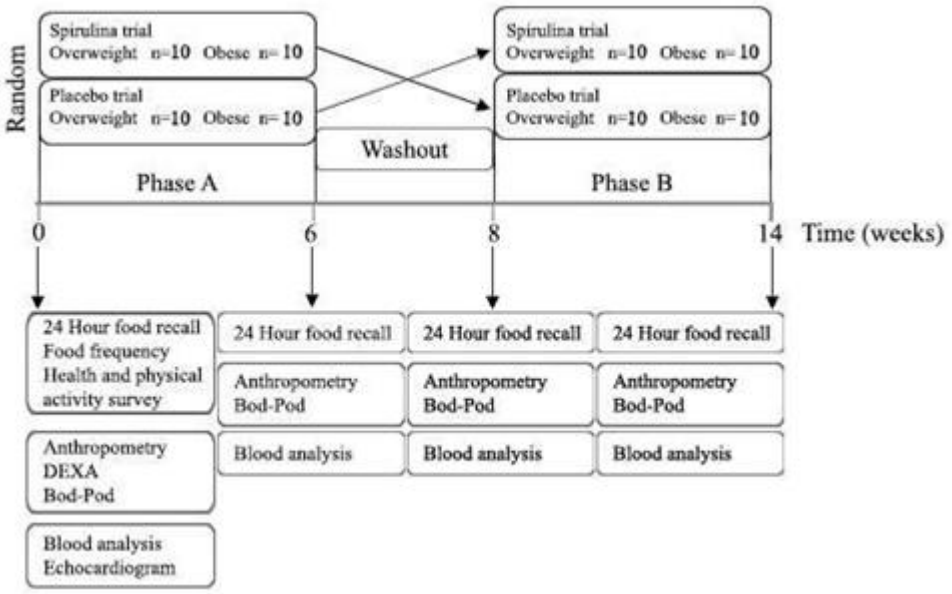

Figure 1. Experimental design for independent and synergistic effect of *Spirulina maxima* and exercise. Same color means the same group of participants.

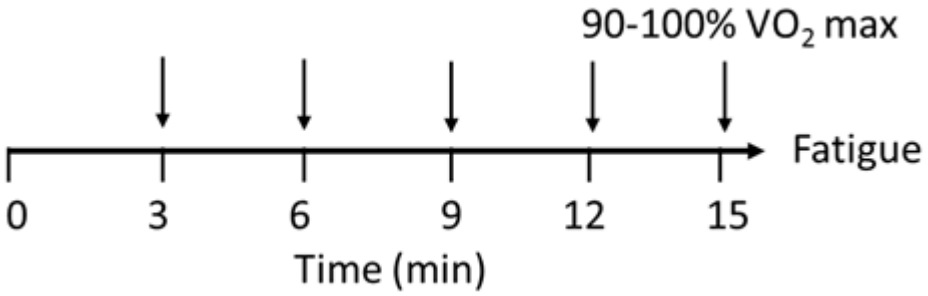

Figure 2. Design of maximum intensity tests. ↓ indicates the interval at which the resistance will be increased; in addition, physical perceived effort, heart rate, and capillary blood samples will be measured.