Impacts of zinc enriched spirulina as an adjunct to conventional treatment of patients with Chronic Obstructive Pulmonary Disease: Study protocol for a North African randomized controlled trial (SPIRICOPD)

Effets de la spiruline enrichie en zinc comme adjuvant au traitement conventionnel des patients atteints de broncho-pneumopathie chronique obstructive: Protocole de recherche pour un essai clinique contrôlé randomisé Nord-Africain (SPIRICOPD)

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Abstract

Introduction: Chronic Obstructive Pulmonary Disease (COPD) is a respiratory condition strongly related to oxidative stress. Recent studies have explored the potential benefits of nutritional supplementation in managing several conditions.
Aim: To investigate the effects of zinc-enriched Spirulina Supplementation (ZSS) on patients with COPD.
Methods: This study is a monocentric randomized controlled clinical trial. COPD-eligible adult male patients under 65 years of age, receiving regular medical treatment, will be included. They will continue their conventional medical treatment following the GOLD 2023 guidelines for COPD. Ninety subjects will be randomly assigned to either the experimental group (G1) or the control group (G2) in a 1:1 ratio. G1 will receive ZSS (1 g/day) for 2 months, while G2 will continue only basic medical treatment. The study will assess oxidant-antioxidant balance, lung function through pulmonary function tests, functional capacity via the 6-minute walking test, lipid profile, dyspnea, and health-related quality of life. All outcomes will be measured at baseline and two months later after supplementation completion. After the 8-week treatment, patients will be followed up for an additional 4 weeks. Ethics approval was obtained from the faculty of medicine of Sousse ethics committee.
Expected results: It is expected that ZSS can positively impact the oxidant/antioxidant balance in patients with COPD after two months of supplementation. The authors anticipate that ZSS may improve various physiological parameters, such as lung function, exercise capacity, endothelial function, and metabolic profile, leading to an overall improvement in the quality of life of patients with COPD.

Key words: Chronic obstructive pulmonary disease, Spirulina, Oxidative Stress, pulmonary function tests, endothelial function.

Résumé

Contexte: La broncho-pneumopathie chronique obstructive (BPCO) est une affection respiratoire fortement liée au stress oxydatif. Des études récentes ont exploré les avantages de la supplémentation nutritionnelle dans la prise en charge de plusieurs pathologies. Notre étude vise à examiner les effets d'une supplémentation en spiruline enrichie en zinc (SSZ) chez les patients atteints de BPCO.
Méthodes: Il s'agit d'un essai clinique contrôlé randomisé monocentrique incluant des hommes âgés de moins de 65 ans, suivis pour BPCO et bénéficiant d'un traitement médical conventionnel selon les recommandations GOLD2023. Quatre-vingt-dix sujets seront répartis au hasard entre le groupe expérimental (G1) et le groupe contrôle (G2) dans un ratio 1:1. Le G1 recevra la SSZ (1 g/jour) pendant 2 mois, tandis que le G2 poursuivra uniquement le traitement médical de base. L'étude évaluera la balance oxydant-antioxydant, la fonction pulmonaire, la capacité à l'exercice, le profil lipidique, la dyspnée et la qualité de vie. Tous les paramètres seront évalués au départ, immédiatement après la fin de la supplémentation, puis un mois plus tard. L'approbation éthique a été obtenue auprès du comité d'éthique de la faculté de médecine de Sousse.
Résultats attendus: On s'attend à ce que la SSZ puisse avoir un impact positif sur l'équilibre oxydant/antioxydant chez les patients atteints de BPCO. Les auteurs prévoient que la SSZ peut améliorer divers paramètres physiologiques, tels que la fonction pulmonaire, la capacité d'exercice, la fonction endothéliale et le profil métabolique, conduisant à une amélioration globale de la qualité de vie des patients atteints de BPCO.

Mots clés: Broncho-pneumopathie chronique obstructive, spiruline, stress oxydatif, explorations fonctionnelles respiratoires, fonction endothéliale.

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Introduction

Background and rationale

Chronic Obstructive pulmonary disease (COPD) is a respiratory condition characterized by progressive and irreversible airflow limitation (1). According to the World Health Organization (WHO), COPD is as a major public health challenge, causing 3.23 million deaths globally in 2019 and ranking as the third leading cause of death worldwide (2).

The disease is primarily associated with significant exposure to harmful particles or gases, with smoking being the main risk factor (3). Research indicates that prolonged exposure to cigarette smoke depletes antioxidants, leads to systemic lipid peroxidation, and triggers inappropriate activation of inflammatory responses (4).

Oxidative stress is a key factor in the development of diseases like COPD, resulting from an imbalance in the body's oxidation-reduction system, leading to increased free radicals and tissue damage (5). Antioxidants, often found naturally in herbs, help prevent or reduce the harmful effects of reactive oxygen species (ROS) (6). As clinicians look for safer and more cost-effective treatments, antioxidant therapy is emerging as a promising preventive approach for COPD. Although human clinical studies are limited, spirulina's notable antioxidant activity suggests it could be an effective treatment option (7). This filamentous cyanobacterium (blue-green algae), widely used in diets, is extensively cultivated for its biological benefits and dietary supplements (7, 8). It provides a high content of proteins and vitamins, such as vitamin B12, minerals, and carotenoids (9). Its key active component is phycocyanin, a distinctive blue pigment (10). Asghari et al. have suggested that spirulina could serve as a potential source of antioxidants, as it contains several components with antioxidant properties, such as phenolic compounds, tocopherols, β-carotenes, and phycocyanin (11, 12). Studies have shown that spirulina supplementation can decrease lipid peroxidation and increase the levels of reduced glutathione (GSH), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GPX) in the kidneys of rats (13).

Mani et al. demonstrated that combined treatment with bronchodilators and anti-inflammatory drugs resulted in improved pulmonary function. This improvement was evidenced by a notable increase in Forced Expiratory Volume in 1st second (FEV1) and the ratio of the FEV1 to the Forced Vital Capacity (FVC) over a 4-month period. Moreover, a marked reduction in FVC%, FEV1%, and FEV1/FVC% was demonstrated when spirulina supplementation was discontinued while basic treatment was continued in the subsequent two months (14).

M Ismail et al. demonstrated evidence of the imbalance in oxidant-antioxidant levels in patients with COPD compared to healthy subjects (controls) (7). They assessed the impact of spirulina on oxidative stress and antioxidant status of patients with COPD. The antioxidant status and lipid profile improved after 2 months of treatment with spirulina. Patients showed a significant reduction in serum levels of malondialdehyde (MDA), lipid hydroperoxide, and cholesterol, while the levels of GSH and vitamin C as well as SOD and Glutathione S-Transferase (GST) activity increased significantly (7). Lu et al. also pointed out the power of spirulina as an MDA-reducing factor (15). This involves preventing the generation of ROS and extinguishing them with antioxidants (16). Although the consumption of spirulina has been shown to enhance the body's antioxidant system, the evidence regarding its effects on this system is conflicting (17).

A systematic review of nine randomized clinical trials published between 2006 and 2019 on the antioxidant capacity of spirulina found a significant increase in Total Antioxidant Capacity (TAC) by spirulina supplementation (1-8g/d) in a period of 1 to 16 weeks (17).

Both COPD and smoking are established risk factors for cardiovascular disease, potentially involving endothelial dysfunction (18). COPD also is a leading risk factor for atherosclerosis (19). The heightened risk of cardiovascular complications in COPD may be attributed to various pathophysiological mechanisms, including endothelial dysfunction, chronic inflammation, and the systemic effects of smoking (20, 21). Given these risks, spirulina is actually used as an adjuvant therapy for metabolic and inflammatory disorders (17), providing excellent anti-inflammatory, lipid-lowering, immunomodulatory, weight-loss, and antioxidant activities (22, 23). In a prospective controlled clinical trial, the effects of spirulina supplementation on oxidative stress indicators and endothelial function were evaluated in 16 patients with systemic arterial hypertension. The study revealed that the administration of spirulina led to a reduction in markers of endothelial activation and damage (24). Research on spirulina's vascular benefits highlights its role in promoting Nitric Oxide (NO) production by endothelial cells and its association with cyclooxygenase release, which aids in blood vessel dilation (22). Recent findings suggest that spirulina maxima supplementation enhances endothelial function by decreasing blood pressure, arterial stiffness index (IS), and weight in overweight patients with hypertension (25).

Our study is exploratory, as there are no existing reports on the effects of spirulina supplementation on endothelial damage in patients with COPD. Further randomized clinical trials are required to verify spirulina's efficacy in treating COPD and to confirm its health benefits. Notably, to the best of our knowledge, this is the first study to assess the impact of zinc-enriched spirulina in African patients with COPD.

The objectives of the following survey which will be conducted in a population of Tunisian patients with COPD are:

Main objective

To evaluate the impact of zinc-enriched spirulina supplementation on oxidative stress in Tunisian patients with COPD.

Secondary objectives

The secondary objectives are to assess the effects of zinc-enriched spirulina supplementation on lung function, lipid and metabolic profiles, endothelial function, incapacity,

and social disadvantage in Tunisian patients with COPD.

Methods

Our research will rely on validated objective tools to enhance the accuracy and usefulness of the results.

Study design

This is a monocentric randomized controlled clinical trial, which will be carried out in Tunisia (laboratory of physiology and functional explorations, Sousse). The trial has been designed according to the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT). The study flow chart is presented in Figure 1.

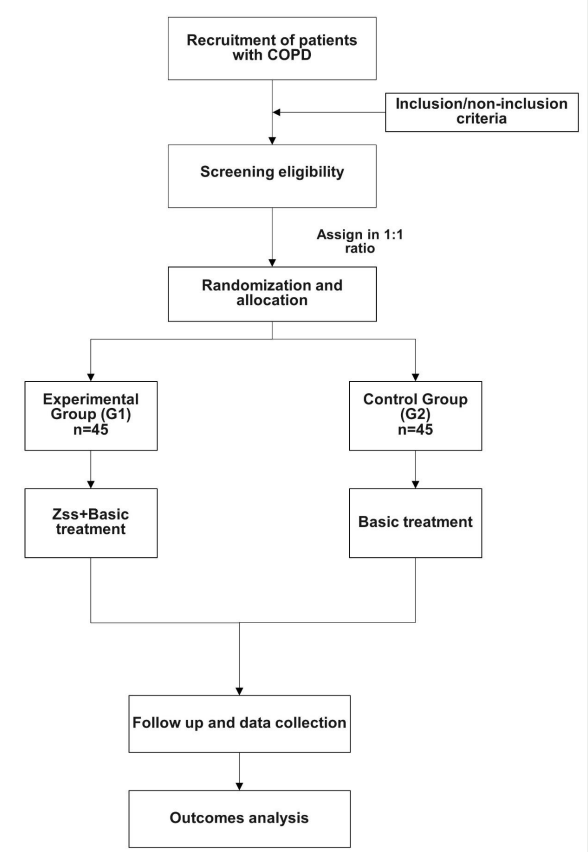

Figure 1. The flow chart of the study design
ZSS: Zinc-enriched Spirulina Supplementation; **COPD**: Chronic Obstructive Pulmonary Disease

Study population

It will consist of a sample of adult male patients under 65 years old treated for COPD GOLD 1, 2, or 3 for a minimum of 3 months, recruited from referrals to the laboratory of physiology and functional explorations of Farhat Hached Hospital, Sousse, Tunisia for PFT.

Before participant recruitment, comprehensive information about the clinical study will be provided to potential participants. This information will include the study's objectives, the distinctions between the experimental and the control groups, the study schedule, and potential risks and benefits. An informative document is prepared for this purpose. All patients will be asked to review and sign a written informed consent form prior to the commencement of any research procedures, indicating their voluntary participation and understanding of the study protocol.

The diagnosis of COPD will be confirmed through PFT in accordance with GOLD criteria which require a post-bronchodilator FEV1/FVC ratio of less than 0.7. Additionally, COPD obstruction severity will be classified as mild, moderate, or severe according to GOLD guidelines (26) respectively: postbronchodilator FEV1/FVC <70% and postbronchodilator FEV1 ≥80% predicted value; postbronchodilator FEV1/FVC <70% and postbronchodilator FEV1 ≥50% predicted value and postbronchodilator FEV1 <80% predicted value, postbronchodilator FEV1/FVC <70% and postbronchodilator FEV1 ≥30% predicted value and postbronchodilator FEV1 <50% predicted value. Two groups will be constituted, and patients will be randomly allocated to the experimental (G1) or control group (G2) at a 1:1 ratio.

- the experimental group (group G1): will receive regular basic treatment associated with the ZSS for two months.
- the control group (group G2): will receive only the regular basic treatment. This group will be formed by subjects with COPD of the same severity stages as G1 and will serve as a control for G1.

The non-inclusion criteria will be:

- absolute or relative contra indications to the 6-Minute Walking Test (6MWT) (signs of unstable angina or myocardial infarction within the previous month, resting heart rate ≥120 bpm, systolic blood pressure (SBP) ≥180 mmHg, diastolic blood pressure (DBP) ≥100 mmHg) (27)
- patients with limited physical activity who cannot perform 6MWT (A known neuromuscular, orthopedic, rheumatologic, or vascular disorders affecting the ability to exercise)
- imperfect performance of the required maneuvers
- contra indications to plethysmography
- patient who has been in further clinical trials in the previous year
- degradation of clinical condition leading to hospitalization within 3 months prior to the study
- chronic respiratory disease other than COPD as asthma
- history of chronic diseases (cardiac, gastrointestinal, renal, otorhinolaryngologic, neurological)
- recent episode of acute upper respiratory tract infection within the three weeks preceding the evaluation
- acute exacerbation of COPD in the last 4 weeks
- clinical manifestations of allergic disorders (skin allergy, urticaria, atopic dermatitis)

Sample size

To estimate the total sample size, we employed the following formula (27):

$$N = \frac{(r+1)\ (Z\alpha_{/2} + Z_{1\text{-}\beta})^2\ \ \delta^2)}{r\ d^2}$$

"N" is equal to $n_1 + n_2$ ("n_1" and "n_2" are the sample sizes for the two groups of experimental and control

groups);"$Z_{\alpha/2}$": normal deviate at a level of significance (3.29 for 0.1% level of significance); "$Z_{1-\beta}$": normal deviate at 1-β% power with β% of type II error (= 2.33 at 99% statistical power); "r" (equal to n_1/n_2): ratio of the sample size required for the two groups (r = 1 gives the sample size distribution as 1:1 for the experimental and control groups);"δ" and "d" are the pooled standard-deviation (SD) and the difference of the SOD means of the two groups determined at day0 and day30. Given the pioneer character of this study, these two values were obtained from a previous study aiming to compare oxidative stress, antioxidant status, and lipid profile between COPD patients and controls (healthy individuals) (7). In the abovenamed study, 30 COPD and 20 healthy controls were included. The SOD means±SD of the COPD group at day0 and day30, were 67.25±6.43 and 74.76±5.64 U/ml, respectively.

The total sample size was 42 COPD (21 in each group). The assumption of 20% of nonattendance during the third/fourth visits gives a revised sample of 52 COPD [= 42/ (1-0.20)].

To better elucidate the effect of ZSS in patients with COPD, according to severity status, we will include a total of 45 patients in the experimental group and a total of 45 patients in the control group. For each group, we will include 15 COPD GOLD I patients, 15 COPD GOLD II patients, and 15 COPD GOLD III patients. Therefore, a total of 90 eligible patients will be enrolled according to the inclusion and non-inclusion criteria and randomly assigned to G1 and G2 at a 1:1 ratio. Randomization will be performed in permuted blocks using the random sequence application with 10 blocks of 9.

Patient recruitment for the study is currently in progress.

Data collection processes and experimental procedures

Medical questionnaire

The questions will be asked in the local Arabic language by the investigating physician.

Clinical data will be acquired by completing a medical questionnaire based on the American Thoracic Society (ATS) (28). The age of participants will be determined in full years. Height (cm) and weight (kg) will be measured using a height gauge, with participants maintaining straight back postures without any heavy clothing or shoes. Body-mass-index (BMI) will be calculated (BMI=weight/height2). According to BMI, three groups of patients will be identified (0. Underweight [BMI < 18.5 kg/m²], 1. Normal weight, or overweight, [BMI range 18.5-29.9 kg/m²], 2. Obesity [BMI≥30 kg/m²]). Education, occupation, smoking status, and comorbidities will also be noted.

The following medical histories will be checked: diabetes, hypertension, cardiovascular diseases, signs and history of respiratory disorders (dyspnea, chronic bronchitis, tuberculosis), and thyroid disorders. Current treatment and compliance will be recorded. The disease course and control level will be reported.

Dyspnea

It will be assessed by the Modified Medical Research Council (mMRC) dyspnea scale, the most commonly used validated subjective scale to assess shortness of breath in patients with respiratory disease (29). The mMRC scale ranges from 0 to 4, with 0 indicating breathlessness only during vigorous exercise. The scale is graded and progressive for symptoms 1 to 3. Four indicates that the subject is too breathless to leave the house or is breathless when getting dressed.

Dyspnea will also be assessed using the Visual Analog Scale (VAS) (30)

COPD Assessment Test (CAT)

The CAT is one of the preferred questionnaires used for the assessment of health status in COPD (31). It is practical, easy to use, and can be filled in within a short time. It was developed and validated in 2009 (32). It covers a variety of symptoms as cough and phlegm, and it is used for monitoring long-term follow-up of COPD. It consists of eight elements, each presented as a semantic six-point differential scale. Higher scores are associated with poorer health status.

The Hospital Anxiety and Depression Scale (HADS)

HADS is a short and widely used tool for measuring psychological morbidity (33). It is comprised of 14 items and includes two subscales - anxiety and depression. Participants rate each item on a four-point scale, with the maximum score being 21 for anxiety and 21 for depression. If a participant scores 11 or higher on either subscale, it is considered a significant case of psychological morbidity. Scores between 8-10 are classified as borderline, and scores between 0-7 are considered normal.

Pittsburgh Sleep Quality Index (PSQI)

The PSQI questionnaire is designed to evaluate the quality and disturbances of sleep over a period of one month. It includes 19 self-assessment questions that are organized into 7 categories: sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disorders, medication usage for sleep, and daytime sleepiness. Each category is rated on a scale of 0 to 3, and the sum of the scores for all 7 categories generates a score ranging from 0 to 21. A cutoff point of PSQI≤5 is used to indicate good sleep quality, while a score of PSQI>5 indicates poor sleep quality (34).

GIRERD questionnaire

It is a simple and reliable self-administered questionnaire to estimate the compliance level, i.e., whether the treatment is regularly taken and according to the prescription. The questionnaire is composed of 6 questions, the answers to which are either "yes" or "no". Each "yes" response is assigned a value of 1 point, while each "no" response is assigned a value of 0 points. The sum of the points for each question gives a score ranging from 0 to 6 (35).

Biological data

Blood samples will be collected from all patients in G1 and G2 twice within a period of two months: prior to the start of ZSS, and on the 60th day of the ZSS intervention.

The samples will be sent immediately to the hematology and biochemistry laboratories.
Two groups of patients will be defined according to the hemoglobin content (0. Non-anemic [hemoglobin level greater than or equal to 13 g/100 ml], 1. anemic [hemoglobin level less than 13 g/100 ml]).
The following biological data will be interpreted according to the usual laboratory values:
- oxidative stress enzymes including SOD and GPX; and TAC
- C-Reactive Protein (CRP)
- Interleukin-6 (IL-6)
- Glycated hemoglobin (HbA1C)
- Lipid status i.e., total triglycerides (TG), total cholesterol (TC), High-Density Lipoprotein Cholesterol (HDL-C) and Low-Density Lipoprotein Cholesterol (LDL-C)
- Uric acid
- Homocysteine

Plethysmography measurement
Plethysmography will be carried out according to the recent guidelines using a daily calibrated bodybox (plethysmograph) (Medisoft, Dinant, Belgium) (36, 37).
The following parameters will be measured or calculated before and after Bronchodilator Responsiveness Testing (BDR) according to the international guidelines (37): FVC (L), FEV1 (L), slow vital capacity (SVC, L), peak expiratory flow (PEF, L/S), Forced Mid-Expiratory Flow (FEF25-75%, L/S), FEV1/FVC ratio (absolute value), Total Lung Capacity (TLC, L), Functional Residual Capacity(FRC, L) and Residual Volume (RV, L). The results will be expressed as a percentage of international norms. The international plethysmographic reference values of the global lung function initiative (GLI) will be used (GLI 2012 for spirometric data and GLI 2021 for static lung volumes (38-40). Therefore, patients will be requested to discontinue the use of bronchodilator medications, with a recommended duration of six hours for short-acting bronchodilators and 12 to 48 hours for long-acting bronchodilators.
Plethysmography is non-invasive and, therefore would not raise any questions about patient safety. According to official guidelines, spirometry is the fundamental test for making a confident diagnosis of obstructive airway disorders such as COPD and the most effective way for determining the severity of lung diseases and following up. Spirometry is a physiological measure of breath. This test aims to evaluate inspiratory and expiratory flow rates, as well as lung volume subcomponents. Through spirometry outcomes, pulmonary function abnormalities could be classified. This clinical approach is based on the fact that the test measures/calculates two basic components – air flow and lung volume.
Plethysmography tests enable the measurement of both mobilizable and non-mobilizable lung volumes. The principle of the test is as follows: the patient is closed in a sealed box and breathes normally through the pneumotachograph. An occlusion at his mouth is then performed. The patient must continue to breathe against this occlusion. The increase in the rib cage volume causes an increase in the pressure inside the sealed box. The slope of the variation of mouth pressure and box pressure during the occlusion is proportional to the FRC of the patient. After the occlusion, the patient performs a vital capacity maneuver so that TLC and RV can be calculated.

Bronchodilator Responsiveness Testing
We will perform a BDR in all our study patients according to recent international recommendations (37). The plethysmography will be reperformed 15 minutes after inhalation of an aerosol of short-acting bronchodilator medication (Salbutamol), administered through an inhaler device. The patient will inhale four 100µg puffs every 30 seconds (total dose 400 µg). A change of >10% of the predicted value in FEV1 or FVC is in favor of a significant BDR (41).

Double CO and NO diffusion with Helium (TLCO/NO)
The simultaneous measurement of lung transfer factor for carbon monoxide (TLCO) and nitric oxide (TLNO) has emerged as a potent technique (double diffusion (TLCO/NO)) for investigating the alveolar-capillary gas exchange (39). The execution and interpretation will be conducted according to recent international recommendations (39, 42).
The TLNO system is a single-breath TLCO system that has been enhanced by the inclusion of nitric oxide in the inspiratory gas mixture, and the use of an NO analyzer. Only the inspiratory bag is supported, and the mixture is prepared from two different bottles (CO-He and NO). Standard gas analyzers were adopted for both NO and CO/He measurements. This technique allows evaluation of the subcomponents of transfer factor (TL) which are specifically associated with the alveolar or pulmonary capillary structures.
Double diffusion allows measuring simultaneously the transfer factors for NO and CO. Further, two components: the alveolar-capillary membrane conductance (Dm) and the pulmonary capillary blood volume (Vc) could be determined using the Roughton-Forster equation. The inclusion of new parameters such as transfer coefficient for nitric oxide (KNO), Dm, and Vc has proven to be valuable for identifying and managing various cardio-respiratory diseases at an early stage. The principle of the test is as follows: First, the patients are often instructed to undergo a period of quiet tidal breathing before performing a full expiration to residual volume followed by a full vital capacity volume of a gas mixture contained in an inspiratory bag with typically 45-50 ppm of NO, 13-14% He, 0.280% CO, 21% O2 and balance of N2. Following a breath-holding period of approximately 4-6 s, the subject is instructed to exhale continuously and completely. A washout volume is initially discarded, followed by the collection, in an expiratory bag, of a sample volume corresponding to a fraction of the alveolar gas. The concentrations of He, CO, and NO are then measured using an analyzer.
To develop predictive equations for TLNO, TLCO, DmCO, and Vc, researchers combined data from various studies that employed breath-hold times ranging from 4 to 10 seconds, with an average of around 6 seconds.
The TLCO/NO measurement option is supported by

Medisoft BodyBox systems (Medisoft, Dinant, Belgium) accessible in our laboratory.

Six-minute walking test

The 6MWT will be performed outdoors (between 8 and 12 o'clock) according to international guidelines (43). The course will be indicated at every meter, and two markers will be used to indicate the start and end of the circuit. Patients will be asked to walk as quickly as possible within 6min along flat ground of 40 m long at a steady pace, in order to cover the largest distance in 6 minutes. However, we will explain that they can stop if they feel it is necessary and then restart. No encouragement will be given. The patient will be informed of the remaining time every minute. Data will be measured at rest and at the end of the test (eg; dyspnea (VAS), heart rate (beats/minutes), oxyhemoglobin saturation (SpO2, %); systolic and diastolic blood pressure (mmHg)) and the 6-Minute Walking Distance (6MWD m, %)). The number of stops will be noted. The test instructions given to the patients will be those recommended by the international guidelines (43). Heart rate will be expressed as an absolute value (bpm) and as a percentage of the predicted maximal heart rate. The ΔSpO2 (SpO2End- SpO2Rest) will be calculated. The predicted 6MWD and the lower limit of normal (LLN) will be calculated according to local norms (44).

Endothelial Function

The quantification of cutaneous perfusion changes in real-time through Laser Doppler flowmetry (LDF) is a non-invasive approach to microvascular function evaluation. This assessment can prove valuable in evaluating the onset and progression of various cardiometabolic disorders (45).

LDF relies on the detection of the doppler shift of laser light that is backscattered by moving red blood cells in the microvasculature, using light-beating spectroscopy. The frequency of the backscattered light is changed in proportion to the velocity of the red blood cells (46).

To prevent any potential interference with the recording, participants will be instructed not to wear clothing or accessories that may disrupt the process. Additionally, they will be required to avoid any significant physical effort before the test and assume a supine position during the recording. In order to minimize any microvascular blood flow artifacts induced by the heart and respiratory rates, patients will be asked to refrain from speaking. The recording will take place in a temperature-controlled (20-26°C), air-conditioned room to ensure optimal conditions after at least 4 to 6h fasting and no caffeine intake for at least 4 to 6h (45-48). Measurements will be performed on the nondominant arm of each patient. The evaluation will be conducted through LDF during post-occlusive reactive hyperemia (PORH). The PORH test enables the measurement of the increase in skin blood flow resulting from arterial occlusion. Typically conducted on the forearm, the procedure involves temporarily obstructing the brachial or cubital arteries using a pressure cuff, at a pressure 50 mmHg above the SBP, and measuring the flow on the ventral surface of the forearm (49). Commonly used indices for characterizing microvascular response include peak flow (PF) after occlusion release, time to peak flow (tPF), half recovery flow (HRF), time to half recovery flow (tHRF), duration of hyperemia, post-ischemic increase in flow compared to baseline, and area under the curve (50). In this study, a Laser Doppler Perfusion Monitor of the type PERIFLUX SYSTEM 5000 of the company PERIMED will be used. The procedure will begin with placing a pneumatic cuff around the upper arm, followed by positioning an LDF probe on the ventral face of the forearm, at least 6 cm away from the cuff, over a lesion-free measuring point without any major vessels such as cubital or radial arteries. The measurement process will involve three stages: first, baseline microvascular flow will be recorded for three minutes, followed by inducing transient ischemia by inflating the cuff to at least 10 mmHg above systolic blood pressure for three minutes. Finally, the cuff will be rapidly deflated, and PORH (stage 3) will be recorded for three minutes (45).

Used definitions

Plethysmographic definitions and classifications

Subjects whose results fall outside the 5th and 95th percentile limits (-1.645 and +1.645 z-score) of the healthy population will be identified as having unusually low or high results, respectively. The severity of lung function impairment will be assessed using z-score values as follow: z-scores >−1.645 are normal, z-scores between −1.65 and −2.5 are mild, z-scores between −2.51 and −4 are moderate, and z-scores <−4.1 are severe (41).

To diagnose COPD using spirometry, the GOLD guidelines recommend conducting a forced vital capacity maneuver that reveals a post-bronchodilator FEV1/FVC ratio of less than 0.7. Additionally, the severity of airflow obstruction in COPD can be determined by measuring FEV1. It is then classified into four stages or grades: GOLD 1, 2, 3, and 4, respectively GOLD I: FEV1 ≥80% predicted. GOLD II: FEV1 50–79% predicted. GOLD III: FEV1 30–49% predicted. GOLD IV: FEV1 <30% predicted (51, 52).

Definitions and classifications for the 6MWT

When an individual performs a 6MWT, the covered distance (6MWD) is compared to the lower limit of the normal (LLN) value (27, 44). If the individual's 6MWD is below the LLN, it is generally regarded as an abnormal finding and could be indicative of walking intolerance or impaired functional capacity.

Three levels of walking intolerance can be determined based on an individual's 6MWD. The levels are defined as very severe walking intolerance (when 6MWD is less than 150m), severe walking intolerance (when 6MWD is between 150m and 350m), and moderate walking intolerance (when 6MWD is between 350m and the lower limit of normal (LIN)) (53).

A drop in arterial oxygen saturation of five points or more is considered clinically significant and indicative of desaturation in the 6MWT (54). A dyspnea score exceeding five on a VAS is considered pathological (27). The cessation of walking during the test is regarded as a sign of poor prognosis (27). If the heart rate at the end of the test is below 60% of the individual's maximum

predicted value, this can indicate the presence of chronotropic insufficiency (44).

Definitions and classifications for the TLCO/NO

To distinguish between normal biological or technical variability of the measurement and a clinically significant change in diffusing capacity, it is essential to document the intra- and inter-session variability of TLCO and TLNO measurements. For healthy individuals and those with pulmonary pathophysiology, an average value obtained from two TLNO and TLCO trials conducted within a 4–10-minute interval, with differences within 17 and 3 mL/min/mmHg, respectively, is considered acceptable. The inter-session reproducibility of TLCO and TLNO in healthy individuals and those with pulmonary pathophysiology is around 5 and 20 mL/min/mmHg, respectively, a week-to-week or month-to-month (42).

TLNO, TLCO, KNO, KCO, Dm, and Vc will be reported in absolute values and as predicted values from regression equations, with the corresponding LLN, upper limit of normal (ULN), and z-scores of North African reference values (55, 56) or international ones established by Zavorsky et al. in 2017 (42).

Definitions and classifications for the endothelial function

This test is used to measure the increase in skin blood flow above the basal level after arterial occlusion. The laser doppler profile is interpreted independently of the other profiles. Indeed, no threshold or normal value is determined or published. Variations in the endothelial response to PORH are, therefore, measured and interpreted with reference to baseline (47). Maruhashi et al. suggested a cut-off point of less than 7.1% for diagnosing Flow-Mediated Dilation (FMD) endothelial dysfunction (57).

Conduct of the study

The baseline data for lung function, oxidative stress, antioxidant status, incapacity data, endothelial function parameters, lipid profile, and other biological tests mentioned above will be established experimentally. This ensures that any changes observed in these parameters are solely attributable to the intervention of ZSS. Subjects will be instructed to maintain their usual diet and refrain from taking any dietary supplements.

Every patient (from G1 and G2) will complete the evaluation process in four steps: 1) visit 1 [day 0]: clinical evaluation followed by endothelial function, blood sampling, PFT, 6MWT, questionnaires responses and familiarization of ZSS; 2)visit 2[first month]: reevaluation of PFT, 6MWT and all questionnaires; 3)Visit 3 [second month]: reevaluation of all parameters; 4)visit 4 [third month]: clinical evaluation followed by endothelial function, PFT, 6MWT and all questionnaires responses.

All evaluations will be performed at the same time of day, to avoid the different physiological responses caused by the circadian cycle's influence. All patients will maintain their basic usual medication. Only patients in the experimental group will be supplemented with zinc-enriched spirulina. The duration of the supplementation will be two months at a dose of 1 gram per day, i.e. two tablets per morning.

After orientation and familiarization with the procedures, the patients will start the intervention protocol.

The study will take over three months for each patient.

The design of the SPIRICOPD study is summarized in table 1.

Table 1. Summary table of participants' follow-up

	Study period				
	Enrollment	**Allocation**	**Intervention/ZSS**		**Close**
Timepoint (weeks)	-1	0	4	8	12
Visit		1	2	3	4
Enrollment					
Eligibility Screen	×				
Informed consent		×			
Allocation		×			
Interventions					
ZSS			→		
Basic treatment	→	→	→	→	→
Assessments					
PFT		×	×	×	×
Endothelial function		×	×	×	×
6MNWT		×	×	×	×
Oxidative/antioxidative status		×		×	
Lipid profile		×		×	
CRP/ IL6		×		×	
HBA1C		×		×	
CBC		×		×	
CAT		×	×	×	×
Dyspnea Score (mMRC)		×	×	×	×
HADS		×	×	×	×
PSQI		×	×	×	×
Safety Assessments					
Adverse event record GIRERD Questionnaire		→	→	→	→
Close					×

ZSS: Zinc-enriched Spirulina Supplementation, **PFT**: Pulmonary Function Tests, **6MWT**: 6-minute walking test, **CRP**: C-Reactive Protein, **IL-6**: Interleukin-6, **HBA1C**: Glycated Hemoglobin, **CBC**: Cell Blood Count, **CAT**: COPD Assessment Test, **mMRC**: modified Medical Research Council Dyspnea, **HADS**: Hospital Anxiety and Depression Scale, **PSQI**: Pittsburgh Sleep Quality Index

Termination and withdrawal criteria

All participants will be informed of their right to withdraw from the study. The reasons for the withdrawal will be recorded in their case report file (CRF). The criteria for stopping treatment and pulling patients out of the study are:

- Participant experiencing adverse events.
- Participant discovering serious illness and needs to be treated during the study.
- Participant suffering from a severe exacerbation.
- Participant showing non-compliance

Compliance

In this trial, compliance will be quantified by observance. The researchers will make every reasonable effort to follow the patients during the study by telephone once a week.

GIRERD questionnaire for compliance assessment will be used during the follow-up.

Safety assessment and adverse events

During the intervention process, we will collect information on any adverse events that occur during the study and investigate if they are related to the intervention. If deemed necessary, we will provide additional medical care for the participants. All adverse events will be documented in the CRF and reported to the Ethics Committee within 24 hours. If any severe adverse event occurs, ZSS will be discontinued, and the patient will receive appropriate treatment. The event will be reported to the Ethics Committee as well.

Statistical analysis

The statistical analysis will be performed with SPSS version 25 software. A descriptive analysis of the sample will be performed. The methods will be adapted to the types of variables and their distributions.
The sociodemographic, clinical, and functional characteristics of the included subjects will be described by the usual parameters. The significance level will be set at $p<0.05$.
We will assess the normality of quantitative variables using the Kolmogorov-Smirnov test. Normally distributed data will be reported as mean ± standard deviation, while skewed data will be presented as median and interquartile range. Qualitative variables will be expressed in absolute frequencies (n) and relative frequencies (percentages). The Mann-Whitney U test will be used to compare two medians. The Spearman (or Pearson) correlation coefficient will be used to study the association between two quantitative variables.
We will employ an intention-to-treat analysis, allocating participants regardless of their completion of the intervention. Any missing outcome data will be addressed using a mixed-model approach. Subsequently, a per-protocol analysis will be conducted, focusing solely on participants who completed all outcome assessments and adhered to the intervention protocol.
Paired-sample t-test or signed rank sum test will be used to compare the difference between the two groups or within one group, for pre-ZSS and post-ZSS measures. For categorical variables, the McNemar test will be employed to detect differences for pre-ZSS and post-ZSS measures.
Analysis of covariance will be employed to assess differences while accounting for potential confounding factors.
To evaluate the effect size, Cohen's d will be calculated.

Data privacy

The persons having direct access to the collected data (investigating physician, methodological referent) will take all the necessary precautions to ensure the confidentiality of the information relating to the study (identity of the participant, contact details, medical data, results of performed tests).
The patient will have the right to access personal information concerning his own investigation.
Any dissemination or communication of the results will consist solely of anonymized statistical data.

Quality control

The study protocol has been reviewed and revised several times. Data quality control will be performed. In case of missing or aberrant data, it will be necessary to make the appropriate corrections. The verification must be carried out only by a member of the medical team responsible for the survey.

Data management

All data will be collected by a trained and qualified investigator and recorded in CRF. The original record will not be changed once CRF is completed. A basic data entry with proofreading will be performed by the investigating physician on SPSS software, version 25. The source data will be checked by a physician involved in the research. The data will be validated according to the data management plan defined jointly by the investigator and the scientific directors. All data will be kept for the entire study period and then archived.

Ethical and legal considerations

The study will be conducted in compliance with the 'Ethical principles for medical research involving Human subjects' of the Helsinki Declaration (available from: https://www.wma.net/wp-content/uploads/2016/11/ethics_manual_arabic.pdf, accessed June 20th, 2022). Approval for the study was obtained from the faculty of medicine of Sousse ethics committee (approval number 146/2022). Written informed consent will be obtained from all subjects.

Evaluation criteria

The effects of ZSS on the antioxidant status of COPD patients will be considered as the primary endpoint. The secondary outcomes will include the effects of ZSS on symptom scores (assessed by the mMRC); social disadvantage assessed by the CAT questionnaire and HADS; exercise tolerance measured by 6MWT; pulmonary function assessed by FEV1, the ratio FEV1/FVC, FEV1 as a percentage of predicted value (FEV1%), static pulmonary volumes (TLC, FRC, RV) and characteristics of alveolocapillary diffusion assessed by the TLCO/NO test; endothelial function; inflammatory status including CRP and IL6; lipid profile according to TC, TG, LDL-C, and HDL-C; HbA1C levels; PSQI and GIRERD questionnaire for compliance assessment during the follow-up.

Discussion

Expected results

We anticipate that ZSS will not only delay the decline of lung function but also improve pulmonary function

and enhance the quality of life in patients with COPD. Furthermore, we hypothesize that ZSS will lead to a favorable balance in oxidant/antioxidant levels, and improve exercise capacity, endothelial function, and metabolic profile in these patients.

Our hypothesis also suggests that ZSS will result in improved vascular function after 2 months of supplementation. This hypothesis prompted us to investigate the potential positive effects of ZSS on additional endothelium-related COPD risk factors in our selected patient group.

Strengths and limitations of the study

To the best of the authors' knowledge, this is the first study from a low-income country reporting the impact of Zinc-enriched spirulina supplementation on the deficiency, incapacity, and social disadvantage data in patients with COPD. This is a pilot study in developing countries. It will provide preliminary data on the effects of zinc-enriched spirulina supplementation in patients with COPD, which can be used to design more in-depth studies in the future.

The present study has some limitations:

- the non-inclusion of female patients.
- the short duration of the supplementation of only two months, which will only allow us to evaluate the short-term effects of spirulina.
- the absence of a placebo in the control group may introduce a potential source of bias when assessing the impacts of ZSS.

Dissemination

Once terminated, we will submit the study results to a peer-reviewed scientific journal for publication.

Trial status

The study is open in the recruiting phase.

The funding sponsor has no role in the research activity. All authors are independent of the funder.

Abbreviation list

BDR: Bronchodilator Responsiveness Testing; **BMI**: Body-mass-index; **CAT**: COPD Assessment Test; **CBC**: Cell Blood Count; **COPD**: Obstructive Pulmonary Disease; **CRF**: Case Report File; **CRP**: C-Reactive Protein; **Dm**: Alveolar Capillary Membrane Conductance; **FEF25-75**: Forced Mid-Expiratory Flow; **FEV1**: Forced Expiratory Volume in 1 second; **FMD**: Flow-Mediated Dilation; **FRC**: Functional Residual Capacity; **FVC**: Forced Vital Capacity; **DBP**: diastolic blood pressure; **GOLD**: Global Initiative for Chronic Obstructive Lung Disease; **GPX**: Glutathione Peroxidase; **GSH**: reduced glutathione; **GST**: Glutathione S-Transferase; **HADS**: Hospital Anxiety and Depression Scale; **HbA1C**: Glycated Hemoglobin; **HDL-C**: High-Density Lipoprotein Cholesterol; **HRF**: half recovery flow; **IL6**: Interleukin-6; **IS**: Arterial Stiffness Index; **KNO**: Transfer Coefficient for Nitric Oxide; **LDF**: Laser Doppler flowmetry; **LDL-C**: Low-Density Lipoprotein cholesterol; **LLN**: Lower Limit of Normal; **MDA**: Malondialdehyde; **mMRC**: modified Medical Research Council Dyspnea; **NO**: Nitric Oxide; **PF**: Peak Flow; **PEF**: Peak Expiratory Flow; **PFT**: Pulmonary Function Tests; **PORH**: Post-Occlusive Reactive Hyperemia; **PSQI**: Pittsburgh Sleep Quality Index; **ROS**: Reactive Oxygen Species; **RV**: Residual Volume; **SBP**: systolic blood pressure; **SVC**: Slow Vital Capacity; **SOD**: Superoxide Dismutase; **TAC**: Total Antioxidant Capacity; **TC**: Total Cholesterol; **TG**:Triglycerides; **tHRF**: time to half recovery flow; **TL**: Transfer Factor; **TLC**: Total Lung Capacity; **TLCO**: Lung Transfer Factor for Carbon Monoxide; **TLNO**: Lung Transfer Factor for Nitric Oxide; **tPF**: Time to Peak Flow; **ULN**: Upper Limit of Normal; **VAS**: Visual Analog Scale; **Vc**: Pulmonary Capillary Blood Volume; **WHO**: World Health Organization; **ZSS**: Zinc-enriched Spirulina Supplementation; **6MWD**: 6-minute walking distance, **6MWT**: 6-minute walking test

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