Progress in Nutrition 2024; Vol. 26, N. 2: e2024018 DOI: 10.23751/pn.v26i2.15817

Original article

Evaluation of the efficacy and absorption of a nutraceutical product: Pilot study

Laura Tomaino[1], Francesca Gori[2], Cinzia Dellanoce[3], Alessandra Piontini[4], Maria Rosaria Ingenito[4], Vittoria Di Donna[5], Filomena Napolitano[6], Vincenzo Soresi[7], Andrea Fratter[8,9], Luisella Vigna[4]

[1]Emergency Medicine Residency Program, Marche Polytechnic University, Ancona, Italy; [2]Department of Anesthesia, Critical Care and Emergency, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy; [3]CNR Istituto di Fisiologia Clinica - ASST Grande Ospedale Metropolitano Niguarda, Milano, Italy; [4]Center of Obesity and Work, Occupational Health Unit; Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milano, Italy; [5]Occupational Medicine Residency Program, Università degli Studi di Milano, Italy; [6]Laboratory of Clinical Chemistry and Microbiology, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy; [7]Head physician emeritus on Pneumology, Ospedale Niguarda, Milano, Italy; [8]Pharmaceutical and Pharmacological Sciences, Università di Padova; [9]Italian Society of Nutraceutical Formulators (SIFNut), Vice-President

Abstract. *Background and aim*: Oxidative stress is a process involved in aging and numerous human pathologies. The aim of this study was to evaluate the safety and efficacy of MITOFAST® in increasing the levels of markers linked to oxidative stress in plasma and red blood cells of healthy subjects. *Methods*: Prospective pilot study conducted on 22 healthy adult subjects. All participants received two sachets/day for 30 days of a commercial supplement containing a blend of antioxidant micronutrients (Coenzyme Q10, resveratrol, vitamin C, folic acid, and N-acetylcysteine). At enrolment (T0) and after 4 weeks (T1), blood samples were taken to evaluate homocysteine, gluthatione, coenzyme Q10, cysteinylglycine, cysteine, vitamin C, and vitamin E. Furthermore, any side effects reported by the subjects were evaluated. P-value <0.05 was considered statistically significant. *Results:* Vitamin E, coenzyme Q10, and total plasma gluthatione levels significantly increased, whereas vitamin C remained constant after treatment. Homocysteine concentration decreased due to folic acid content (p= 0.003). *Conclusions*: The overall increase in the levels of antioxidant molecules measured, together with the reduction of cysteinylglycine and homocysteine, indicate an antioxidant activity of MITOFAST®.

Key words: antioxidant, aging, oxidative stress, skin aging, coenzyme Q10

Introduction

Oxidative stress is a term used to indicate an imbalance between the production of oxidant species and endogenous antioxidant defenses which can cause damage to biological systems. Oxidative stress is involved in a wide range of human diseases, such as atherosclerosis and cardiovascular diseases, cancer, diabetes, and Alzheimer's disease, to name few. However, the extent to which oxidative stress affects diseases onset is quite variable.

Reactive oxygen species (ROS), the mainly produced oxidant species, are physiologically produced through endogenous or exogenous pathways. The first pathway is represented by the activity of electron transport chain enzymes, as well as the action of xanthine dehydrogenase, nitric oxide synthase, and NADPH oxidase (1-4). The exogenous pathways include the

introduction of determined foods (5), alcohol metabolism (6,7), smoking (8), and ultraviolet irradiation (UV) (9-12).

Small fluctuations in ROS concentration are involved in cell signalling and the control of various physiologic functions, such as the erythropoietin production, and regulation of the vascular wall relaxation (13). However, an excess of ROS production leads to damage of proteins, lipids, lipoproteins, and DNA (14). The human body has two main mechanisms to counteract the excessive ROS production: the presence of endogenous antioxidant enzymes (i.e. superoxide dismutase, catalase, glutathione peroxidase) and through the antioxidant molecules introduced with the diet (mainly vitamin E (VitE), vitamin C (VitC), and polyphenols). Improving antioxidant levels can reduce the risk of oxidative stress-induced diseases.

When dietary intake of such antioxidant molecules is not sufficient, due to an unbalanced nutrition pattern or to impaired malabsorption, it is possible to use commercial preparations that help supplement micronutrients deficiency. Several researches, both in experimental and clinical setting, demonstrated that antioxidants in natural products, vegetables, fruits, soy products, tea or in powered concentrate juice/blends prevent the oxidation reactions associated with human diseases, such as cardiovascular disease or cancer (15).

MITOFAST® (Mitochon, Bologna, Italy) is a new commercial product composed of a mixture of antioxidants that would have the potential to provide antioxidant molecules and rebalance the oxidative state. Specifically, it contains coenzyme Q10 (CoQ10), VitC, resveratrol, folic acid, and N-acetylcysteine (NAC). The antioxidant properties of these molecules are well known (16-20). In general, they exert their protective activity through different mechanisms, such as acting as ROS scavengers, limiting oxidative damage to cell structures and macromolecules (21-23), and/or activating/silencing genes that encode antioxidant enzymes or, on the contrary, which promote oxidative stress (24-27).

Given that oxidative stress is associated with a wide range of pathologies, preventing or decreasing oxidative injury could be an important tool to reduce the risk of oxidative stress-dependent human diseases. This could occur by stimulating the immune system or endogenous antioxidant defenses, reducing oxidative damage to DNA or protecting the endothelial barrier. To the best of our knowledge, there are no studies investigating the effects of supplementation via MITOFAST®.

The aim of this pilot study was to evaluate the absorption and the effectiveness of the antioxidants agents contained in MITOFAST®, a commercial oral supplement, which could help increase the levels of endogenous antioxidant defenses, prevent skin aging, and increase the sense of energy in healthy adults.

Materials and methods

Study design

This was an uncontrolled, single-center, open label pilot clinical study. Participants were recruited during nutritional education programme organized by the Occupational Medicine Department, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico - in Milan on a voluntary basis from May to July 2021. Participants received no monetary incentive, and all provided written informed consent. The study was performed following approval by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan (Reg. no. 1370). All procedures involving human participants were performed in accordance with the ethical standards of the Ethics Committee of the Fondazione Ca' Granda of Milan and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Before recruitment, each subject underwent a medical evaluation and, if the inclusion criteria were satisfactory, the participant was enrolled in the study. Inclusion criteria: (age range 18-65 years, BMI 22,5-35, healthy, without ongoing home medical treatment). The following exclusion criteria were applied: administration of omega-3 fatty acids, acetylsalicylic acid, nonsteroidal anti-inflammatory drugs, or corticosteroids in the month prior to study entry; pregnancy or breastfeeding; blood disorders (coagulation disorders, or therapy with coumadin anticoagulants); history of cancer; type 1 or type 2 diabetes mellitus; and immune (rheumatoid arthritis, lupus, etc.) or inflammatory

Table 1. Formulation of the dietary supplement MITOFAST®.

	Content of a sachet	RNV %
Coenzyme Q10, mg	100	-
Resveratrol, mg	40	-
N-acetylcysteine, mg	150	-
N-acetylglucosamine, mg	150	-
Vitamin C, mg	20	25%
Folic acid, µg	400	200%

Abbreviations: RNV: Reference nutritional values.

(ulcerative colitis, Crohn's disease, etc.) diseases likely to modify biological markers of inflammation; pancreatic insufficiency; and kidney failure. Failure to comply with the guidelines and non-attendance at follow-up visits led to exclusion.

The study protocol consisted in the supplementation of MITOFAST® sachet twice a day at morning and afternoon on empty stomach, for a treatment period of 4 consecutive weeks. MITOFAST® was provided for free by Mitochon (Bologna, Italy), for the entire duration of the study. Each sachet of MITOFAST® contained CoQ10, resveratrol, NAC, N-acetylglucosamine, VitC, and folic acid, in the exact amounts showed in Table 1.

Anthropometrics and blood samples before supplementation (baseline, T0) and after 4 week of treatment (T1) were recorded by health personnel trained for this study, during a clinical examination including also a dietary interview. The collected blood biochemical parameters included: homocysteine (Hcy), VitC, glutathione (GSH, total, reduced and oxidized form), CoQ10, VitE, cysteine (CYS), and cysteinyl glycine (CYSGLY). CYS, CYSGLY, and GSH were quantified in both plasma and red blood cells.

The eating habits of participants were collected by a food frequency questionnaire (FFQ), namely the "Grana Padano Observatory" (GPO) (28,29), which was administered during the dietary interview both at T0 and T1, the. Subjects were asked to not modify their nutritional habits during the study. During the supplementation, physicians were available to evaluate possible adverse effects.

Daily levels of macro- and micro-nutrients intake were extrapolated from results to a self-administered patient questionnaire concerning the dietary habits and number of weekly servings of the different food types. The nutritional data thus obtained were processed using GPO software, freely provided online on the website http://www.osservatorio.granapadano.it, as described elsewhere (28,29).

At T1, a satisfaction questionnaire to find the possible positive effects derived from the supplementation (i.e. tiredness reduction, improvement of the skin condition, improvement of muscle or tendon aches) were filled by study participants. As well, compliance to the supplementation program was also assessed.

Method for the determination of homocysteine and glutathione in red blood cells

Three mL of whole blood was collected by venipuncture into EDTA tubes and plasma was separated from the cellular portion by centrifugation at 4000 g for 5 min at 4 °C to prevent thiol oxidation. Plasma and red blood cells were transferred to test tubes and stored at -80 °C until analysis. Plasma for total Hcy and red blood cells for total and reduced GSH were measured according to methods validated in the laboratory of the ASST Ospedale Metropolitano Niguarda (30). Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) and 4-fluoro-7- sulfamoylbenzofurazan (ABD-F) were used as reducing and derivatizing agents, respectively. After protein precipitation with trichloroacetic acid (TCA), the sample was centrifuged at 14.000 g for 10 min at 4 °C; clear supernatant (100 µL) was incubated 90 min at room temperature with ABD-F before chromatographic analysis. Thiol separation was performed by isocratic high-performance liquid chromatography (HPLC) analysis on a 5 µm Discovery C18 analytical column (250×4.6 mm i.d, Supelco, Sigma-Aldrich) at room temperature, eluated with a solution of 0.1 M acetate buffer, pH 4.0 / methanol, 81:19 (v/v) at a flow rate of 1 mL/min. Fluorescence intensities were measured with an excitation at 390 nm and emission at 510 nm, using a Jasco fluorescence spectrophotometer. The concentration of GSH oxidized (GSH EOX) forms was calculated as the difference between total and reduced forms. GSH forms encompass the dimers, the mixed disulphide forms and the protein mixed disulphide forms (31).

Method for the determination of vitamin c and coenzyme Q10

Three mL of whole blood was collected by venipuncture into tubes and serum was separated from the cellular portion by centrifugation at 4000 g for 10 min at 4 °C. A portion of serum were transferred to test tubes and diluted 1:2 (v/v) with internal standard VitC and stored at -80 °C until analysis and 1 mL was frozen until the CoQ10 analysis. An HPLC isocratic system equipped UV detector (Shimadzu) was used for both analyses. Commercial kits were used for VitC end CoQ10 assay (Chromsystems Instruments & Chemicals GmbH, Munich).

Method for the determination of vitamin E

Three mL of whole blood was collected by venipuncture into tubes and plasma was separated from the cellular portion by centrifugation at 2000 g for 5 min. Plasma was then stored at -80 °C away from the light until the moment of analysis. An HPLC isocratic system equipped UV detector (Shimadzu) was used for analysis. Commercial kit was used for VitE detection (Chromsystems Instruments & Chemicals GmbH, Munich).

Statistical analysis

All continuous variables are presented as mean ± standard deviation (SD) or as median [interquartiles range], as appropriate. The analysis was performed at the start of the supplementation (T0) and after 4 weeks (T1). Comparisons of parameters between T0 and T1 were performed with paried-sample t-test. P-value < 0.05 was assumed as significant. Data were analyzed with SIGMAPLOT 11.0 software.

Results

Characteristics of the subjects at baseline

Twenty-six participants were enrolled in this study, but only 22 (16 women and 6 men) complied with the prescribed supplement for the full 4 week-period. Four subjects quit consuming the product due to the bitter taste which caused stomach uneasiness.

Overall, the study sample aged 50 ± 15 years and presented a mean Body Mass Index (BMI) of 29.80 kg/m^2. Differences in educational level were observed: university degree (n= 13, 55% of study participants), high school diploma (n= 10, 36%), and secondary school (n= 2, 9%). Rate of compliance was high, without any return of the MITOFAST® sachet left at the end of the treatment.

Effects of the product on antioxidant status

Table 2 shows the blood samples analysis at T0 and T1. Some participants presented micronutrient deficit at T0, particularly CoQ10 (50%) and total plasma GSH (86%). At T1, a significant increase in plasma levels of many of the tested micronutrients was recorded. In particular, the levels of CoQ10 ($p<0.001$) and total GSH ($p<0.001$) increased, both micronutrients in which MITOFAST® is rich directly, in the case of CoQ10, or indirectly by providing precursors, as in the case of GSH. Eleven participants had levels of CoQ10 lower than 4.5 μg/dL at T0 while, after 4 weeks of treatment, 14 even reached supraphysiological levels. The reason is that MITOFAST® contains 100 mg of CoQ10, so each participant assumed 200 mg of CoQ10 every day, which is a higher dose than the average amount introduced through the diet.

Similarly, plasma VitE levels also significantly increased after supplementation compared to T0, ranging from 1855 ± 586 μg/dL at T0 to 2058 ± 559 μg/dL at T1 ($p< 0.001$).

However, the levels of VitC remained the same after supplementation ($p= 0.415$). In fact, VitC at the start of the supplementation was 1024 ± 470 μg/dL, without significantly change after 4 weeks (1074 ± 418 μg/dL). Only 3 subjects had low levels of VitC at T0.

As regard CYS, a significant reduction in its concentrations in red blood cells was recorded ($p= 0.015$), due to a decrease in the presence of CYS in its oxidized form ($p = 0.006$). The same trend was obtained for CYSGLY levels.

Table 2. Blood sample analysis.

	Reference values	T0	T1	p-value
CoQ10, µg/dL	4.5 - 11	5.52 ± 2.22	15.66 ± 6.12	**0.001**
VitE, µg/dL	500 - 2000	1855 ± 586	2058 ± 559	**0.001**
GSH PT, µmol/L	9.2 - 13.4	8.50 ± 2.69	13.04 ± 3.53	**0.001**
GSH ET, µmol/L	1275 - 2700	2124 ± 461	1901 ± 416	0.077
GSH ER, µmol/L	600 - 1700	481 ± 186	250 ± 143	**0.001**
GSH OX, µmol/L	400 - 1300	1511 [1328; 2034]	1687 [1334; 2026]	0.846
VitC, µg/dL	460 - 1490	1024 ± 470	1074 ± 418	0.415
CYS PT, µmol/L	181 - 267	219.62 ± 7.12	217.70 ± 8.29	0.089
CYS ET, µmol/L	11.9 - 48.3	26.98 ± 6.40	23.13 ± 5.64	**0.015**
CYS ER, µmol/L	0.6 - 7.6	2.40 [1.70; 2.90]	2.20 [1.70; 310]	0.766
CYS EOX, µmol/L	9 - 27	24.59 ± 6.83	19.93 ± 7.01	**0.006**
CYSGLY PT, µmol/L	26.6 - 43.4	34.72 ± 1.40	34.82 ± 2.08	0.847
CYSGLY ET, µmol/L	1.2 - 9.2	3.70 [3.10; 4.0]	4.00 [3.40; 4.50]	**0.020**
CYSGLY ER, µmol/L	0.2 - 1.5	1.62 ± 0.080	1.35 ± 0.77	0.099
CYSGLY EOX, µmol/L	0.6 - 7.2	2.70 [2.30; 3.30]	2.10 [1.60; 2.80]	**0.010**
Hcy PT, µmol/L	4 - 15	10.85 ± 2.32	9.45 ± 1.88	**0.003**

Data are presented as mean ± standard deviation (SD), median [interquartiles range], or number (percentage) as appropriate. Satistical significance was set for a p-value ≤0.05. Abbreviations: CoQ10: coenzyme Q10; VitE: vitamin E; GSH ET: plasma total gluthatione; GSH ET: total gluthatione in red blood cells; GSH ER: reduced gluthatione in red blood cells; GSH EOX: oxized glutatione in red blood cells; CYS PT: plasma total cystein; CYS ET: total cystein in red blood cells; CYS ER: reduced cystein in red blood cells; CYS EOX: oxized cystein in red blood cells; CYSGLY PT: plasma total cysteinylglycine; CYSGLY ET: total cysteinylglycine in red blood cells; CYSGLY ER: reduced cysteinylglycine in red blood cells; CYSGLY EOX: oxized cysteinylglycine in red blood cells; Hcy PT: plasma homocysteine

Plasma Hcy significantly decreased after 4 weeks of supplementation, ranging from 10.85 ± 2.32 µmol/L at T0 to 9.45 ± 1.88 µmol/L at T1 (p = 0.003), because MITOFAST® contains 800 µg of folic acid. Only one subject presented Hcy levels higher than 15 µmol/L at T0 but, after 4 weeks, returned in physiological range.

Effects of product on dietary intake

Overall, the findings of the FFQ analysis showed an average energy consumption of 1718 ± 449 kcal/day. Macronutrient daily consumption amounted to 65 ± 16 of fats (of which 22 ± 9 g saturated fats, and 9 ± 3 g poly-unsaturated), 201 ± 54 g carbohydrates (of which 84 ± 35 g sugars), 26 ± 11 g fiber, 69 ± 16 g proteins, and 14 ± 23 g alcohol (7 ± 10 g/day and 35 ± 37 g/day for females and males, respectively). Table 3 shows micronutrients intake, assessed by GPO questionnaire.

Table 3. Average estimated micronutrients intake as assessed by the FFQ OGP.

Micronutrient	Calculated Intake	Reference value of the northern Italian population
Calcium, mg	987 ± 280	> 1000 mg
Iron, mg	12 ± 4	≥ 18 mg in females ≥ 10 mg in males
Zinc, mg	10 ± 2	≥ 8 mg in females ≥ 11 mg in males
Vitamin A, µg	1390 ± 647	≥ 700 µg in females ≥ 600 µg in males
Vitamin D, µg	2 ± 1	≥ 15 µg
Vitamin E, mg	12 ± 4	≥ 12 mg in females ≥ 13mg in males
Folates, µg	384 ± 141	≥ 400 µg
Vitamin B12, µg	4 ± 1	≥ 2.4 µg
Vitamin C, mg	167 ± 77	≥85 mg in females ≥ 105 mg in males

Data are presented as mean ± standard deviation (SD).

Satisfaction assessments

Overall, among study participants a high satisfaction rate was observed ah the end study. They reported an improvement in energy levels during the day (63%), a reduction in muscles and articular aches (32%), and an improvement in skin condition (36%).

Of note, only 4 subjects abandoned the study due to the bitter taste which caused stomach uneasiness.

Discussion

In this pilot clinical study, the potential beneficial effect of MITOFAST® in modifying the concentrations of some oxidative stress markers in the plasma and red blood cells of healthy adult subjects was assessed. MITOFAST® contains a combination of active components that show antioxidant activity. At the end of the study, in the population analyzed, a significant increase in VitE, GSH, CoQ10, and a reduction in Hcy was found, with possible positive effects on health.

The results showed that, overall, this product integrates the quantity of antioxidants without particular side effects. These preliminary data demonstrate that supplementation provided micro- and macronutrients in line with northern Italian Mediterranean dietary pattern (32) and that it increased the levels of endogenous antioxidant molecules in agreement with previous evidence on the effects of supplementation of individual compounds. In particular, this study demonstrated that MITOFAST® allows effective integration of CoQ10, ensuring real intestinal absorption.

The findings of this study show a reduction of Hcy between T0 and T1. Hcy is known to be an independent risk factor for atherosclerotic diseases (33). High levels of Hcy are associated with inflammation and apoptosis of the endothelial cells (34) because it is incorporated into proteins, irreversibly modifying their structure and function, thus making them biologically inactive (35). An alternative hypothesis is based on the association between Hcy and hydrogen sulfide (H_2S). H_2S has a protective role against the vascular damage-induced by Hcy, reducing oxidative stress through the increase of the levels of superoxide dismutase, catalase, GSH, and endothelial nitric oxide synthase (36). Hyperhomocysteinemia leads to a deficiency of H_2S probably because this gas is produced by the same enzymes that are involved in the metabolism of Hcy.

High levels of Hcy are also associated with higher blood pressure, especially in males (37). Maintaining optimal levels of blood Hcy is therefore essential for the cardiovascular health (38).

In this study, the levels of plasma Hcy significantly decreased after 4 weeks of supplementation, due to the folic acid content in MITOFAST®. In fact, the increased intake of folic acid provides the methyl group for the conversion of Hcy into methionine (39) with a consequent decrease in Hcy levels. Folic Acid, vitamin B6, and vitamin B12, together, play a role in Hcy metabolism. Folic acid, once introduced into the body, is metabolized into methyl-tetrahydrofolate which reacts with vitamin B12, giving it the methyl group. Vitamin B12 becomes methyl-cobalamin which donates the methyl group to Hcy converting it into methionine. Through this process folic acid can reduce the concentration of Hcy.

A recent meta-analysis that analyzed 884 randomized controlled trials on the supplementation of 27 different types of micronutrients, showed that there are moderate to high quality evidence that folic acid, as well as CoQ10, reduces cardiovascular disease risk factors. Specifically, folic acid supplementation decreased stroke risk, whereas CoQ10 decreased all-cause mortality events. Folic acid also seems to have an antioxidant effect, increasing both serum GSH and total antioxidant capacity (TAC) and, simultaneously, reducing malondialdehyde (MDA) (18).

In regard to CoQ10, as mentioned above, this study demonstrated that MITOFAST® is an excellent source of CoQ10, capable to ensure a real absorption of the micronutrient. The CoQ10 plasma response to the oral ingestion of various CoQ10 commercial formulations has been examined in the literature with debated results (40-42).

CoQ10 is a fat-soluble molecule, mainly synthesized in the tissue body with high metabolic activity (such as heart, kidneys, liver, and muscle) and only a small amount is introduced with the diet (e.g., salmon and tuna, organ meats in particular hearth and liver, whole grains) (43). CoQ10 has a central role in the electron transport chain during aerobic cellular respiration (44),

and acts as antioxidant molecule, preventing the lipid peroxidation of cellular membranes (45). For its important properties, it is used as a supplement in the treatment of many chronic diseases (46). In this study, a significant three-fold increase in the plasma levels of CoQ10 after 4 weeks of supplementation was recorded. At T0, 50% of the subjects had insufficient levels of CoQ10. After 4 weeks, 4 subjects (18%) reached normal levels while 14 participants (63%) presented a supraphysiological concentration of CoQ10. Moreover, at the end of the treatment, 11 subjects (50%) reported feeling more energic during the day together with a reduced sense of fatigue. Fifty-five percent of those who reported and energy improvement, started from suboptimal CoQ10 values at baseline. The higher blood concentration of CoQ10 achieved after supplementation can lead to a consequent increase in mitochondrial activity. CoQ10 could facilitate the flow of electrons from complex I or complex II, and thus create a higher electrochemical gradient for the synthesis of adenosine triphosphate.

The increased consumption of foods containing high concentrations of CoQ10 (e.g. meat, fish, nuts, and some vegetable oils) or the supplementation of this molecule could be useful to sustain optimal physiological function, not only in subjects who are deficient, but also for those who have sub-/optimal serum levels (47,48). Supplementation with CoQ10 is safe: most side effects reported in human studies are limited to gastrointestinal symptoms, such as nausea, stomachache, and diarrhoea. Moreover, in our study, participants consumed a total of 200 mg of CoQ10 per day, being the tolerated doses up to 1200 mg per day for a prolonged period or 3000 mg for a short period (19).

Furthermore, some evidence supports the beneficial effects of CoQ10 supplementation on oxidative status (49), determining a significant increase of TAC levels and reduction in MDA levels (50). However, the effects on superoxide dismutase and catalase are controversial (49). CoQ10 also exerts its antioxidant function by regenerating VitE from its reduced form, the tocopheroxyl radical (51). For this reason, this study analyzed plasma levels of VitE before and after supplementation, expecting an increase in its blood concentration. In fact, plasma VitE levels increased significantly after 4 weeks supplementation. This improvement can also be explained by VitC contained in the MITOFAST® supplement. In fact, VitC can also convert oxidized VitE back to its reduced form.

VitC performs numerous functions in the human body, mainly thanks to its reducing power. It is an important endogenous plasma antioxidant, which can act both as a direct scavenger of free radicals and as a regenerator of VitE in its reduced form. It has an essential role in collagen synthesis and in maintaining the integrity of connective tissue, as well as vasodilator effect because it prevents the oxidation of tetrahydrobiopterin and thus regulates the production of nitric oxide (52). Finally, VitC is involved in the metabolism of various hormones, as well as in the conversion of iron (Fe) from the oxidized (Fe^{3+}), introduced with the diet, to the reduced (Fe^{2+}) form, thus making it soluble and more bioavailable.

The findings of this study did not show an increase in VitC levels between T0 and T1, but VitC could have been used to convert VitE back to its reduced form or as a direct antioxidant for ROS neutralization.

MITOFAST® also contains NAC. Once absorbed, NAC is divided into acetate and CYS, the limiting amino acid in the synthesis of GSH, a molecule formed by 3 amino acids (CYS, glutamate, and glycine). NAC supplementation is therefore useful for providing precursors for endogenous GSH synthesis. There is even the hypothesis that NAC has an anti-inflammatory power, but currently randomized clinical trials have provided various and conflicting results regarding this aspect (53). According to a recent systematic review and meta-analysis of last years, NAC would reduce the serum level of C-reactive protein and interleukin-6, but would have no effect on other inflammatory markers (53).

We measured the levels of GSH in plasma and in red blood cells, where GSH is present in high concentrations to protect the erythrocytes from oxidative stress. Only total GSH in plasma significantly increased, while that in red blood cells slightly decreased after supplementation. Reduced and oxidized GSH in the red blood cells was also measured. The levels of GSH EOX remained similar, but the levels of reduced GSH (GSH ER) decreased at T1.

GSH is one of the most intracellular antioxidants in the body, with the function of counteracting the

effects of ROS inside the cells that damage lipids, proteins, and DNA. It can act directly as an antioxidant thanks to its sulfhydryl group (54) or as a cofactor of the GSH peroxidase enzyme, active against peroxides. The active (reduced) form of GSH can be regenerated through the action of the GSH reductase enzyme, with consumption of NADPH or through the contribution of VitC.

Direct oral consumption of GSH is not effective because it is a tripeptide, a target for gastrointestinal peptidases and proteases. The intestinal enzyme γ-glutamyl transpeptidase degrades GSH, reducing its bioavailability. Moreover, GSH lacks specific transporters in enterocytes, contributing to its poor bioavailability.

In the human body, GSH is present in high concentrations within hepatocytes and erythrocytes. In the liver, it is involved in detoxification from xenobiotics (55), whereas in the erythrocytes it protects the cells themselves from ROS produced by the autoxidation of hemoglobin, since its binding with oxygen produces superoxide anion (56).

At baseline, only six subjects presented physiological levels of GSH ER, while the rest of the participants had low levels of the tripeptide. On the other hand, 68% of them had supraphysiological levels of GSH EOX at T0. After 4 weeks of MITOFAST® supplementation, the levels of GSH ER significantly decreased overall. The low levels of GSH ER coupled with the high levels of GSH EOX could be indicative of an error during the storage of the test tubes that caused the oxidation of the GSH. Anyhow, we can see how the increase of the concentration of total plasma GSH is an indication that the NAC contained in MITOFAST® was used for its biosynthesis. The increase of the total GSH means that there was an increase of the utilization of CYS, the limiting amino acid in the synthesis of the tripeptide.

Therefore, we decided to measure CYS levels in erythrocytes before and after supplementation, expecting a decrease in concentrations of the amino acid caused by its utilization in the synthesis of GSH. A significative decrease in CYS levels in the red blood cells was observed, confirming our hypothesis. If there had not been the storage problems mentioned above, we would probably have found an increase in GSH ER in red blood cells, also demonstrated by the increase in CYSGLY levels due to the higher quantity of metabolized GSH. CYSGLY, together with glutamate, is the product of GSH degradation.

According to the satisfaction questionnaire, 23% of the subjects reported an improvement in their skin quality. A possible explanation for this improvement could be the presence of the N-acetylglucosamine, a molecule used for the synthesis of hyaluronic acid which have an important role in the maintenance of the hydration of the connective tissue and of the skin. Up to date, there are not studies that supports that an oral supplementation of N-acetylglucosamine helps with the endogenous biosynthesis of hyaluronic acid in the body and, in this study, we did not consider any parameter indicative of this process. Supplementation with N-acetylglucosamine needs to be further investigated, in order to define a direct correlation with the increase of the hyaluronic acid synthesis and therefore the beneficial effects for the skin and for the joint's health. The improvement of skin condition could also be associated with the antioxidant function of the VitE, VitC, and CoQ10 which protect the skin from the ROS formed due to UV irradiation. VitC also has an important role in the collagen biosynthesis, so it may have directly contributed to the improved skin.

Gastrointestinal problems such as stomach pain and bloating caused by supplementation were the main side effect that led to the interruption of the supplementation and withdrawal from the stu. Therefore, we can conclude that the product could be improved in the terms of its digestion and absorption. This is not a case-control study and thus one of the limiting factors of this study is the lack of a control group. Others limiting factors are the small number of subjects and the lack of parameters measuring the possible positive effect of resveratrol on health: for example, the measurement of the C-reactive protein in the blood before and after supplementation could indicate a possible improvement in levels of systemic inflammation. Further studies with a higher sample size are needed to confirm the findings of this pilot study.

In conclusion, the aging process is accompanied by oxidative stress, with worsening of physiological functions. A diet, even if balanced, hardly guarantees a sufficiently high quantity of nutraceuticals that can

have a beneficial role on the subject's health. Supplementation with nutraceutical products helps to achieve an effective dose of these molecules, allowing them to perform their beneficial role. In this pilot study, MITOFAST® was found to be a useful supplement for increasing the presence of endogenous antioxidant molecules, feeling less fatigue, and maintaining adeguate elasticity of the skin and joints.

Conflict of Interest: The authors have no conflict of interest to declare.

Funding Statement: The authors report no founding. At the time of writing there is no ongoing agreement with the company producing MITOFAST®.

References

1. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44. doi: 10.1113/jphysiol.2003.049478.
2. Li X, Fang P, Mai J, Choi ET, Wang H, Yang XF. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. J Hematol Oncol. 2013;6:19. doi: 10.1186/1756-8722-6-19.
3. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med. 2000; 29(3-4):222-30. doi: 10.1016/s0891-5849(00)00317-8.
4. Hrycay EG, Bandiera SM. Involvement of Cytochrome P450 in Reactive Oxygen Species Formation and Cancer. Adv Pharmacol. 2015;74:35-84. doi: 10.1016/bs.apha.2015.03.003.
5. de Roos B, Duthie GG. Role of dietary pro-oxidants in the maintenance of health and resilience to oxidative stress. Mol Nutr Food Res. 2015 Jul;59(7):1229-48. doi: 10.1002/mnfr.201400568.
6. Das SK, Vasudevan DM. Alcohol-induced oxidative stress. Life Sci. 2007;81(3):177-87. doi: 10.1016/j.lfs.2007.05.005.
7. Bailey SM, Pietsch EC, Cunningham CC. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. Free Radic Biol Med. 1999;27(7-8): 891-900. doi: 10.1016/s0891-5849(99)00138-0.
8. van der Vaart H, Postma DS, Timens W, ten Hacken NH. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. Thorax. 2004;59(8):713-21. doi: 10.1136/thx.2003.012468.
9. Prasad A, Pospíšil P. Ultraweak photon emission induced by visible light and ultraviolet A radiation via photoactivated skin chromophores: in vivo charge coupled device imaging. J Biomed Opt. 2012;17(8):085004. doi: 10.1117/1.JBO.17.8.085004.
10. Rinnerthaler M, Bischof J, Streubel MK, Trost A, Richter K. Oxidative stress in aging human skin. Biomolecules. 2015;5(2):545-89. doi: 10.3390/biom5020545.
11. Berneburg M, Gattermann N, Stege H, et al. Chronically ultraviolet-exposed human skin shows a higher mutation frequency of mitochondrial DNA as compared to unexposed skin and the hematopoietic system. Photochem Photobiol. 1997;66(2):271-5. doi:10.1111/j.1751-1097.1997.tb08654.x.
12. Bickers DR, Athar M. Oxidative stress in the pathogenesis of skin disease. J Invest Dermatol. 2006;126(12):2565-75. doi: 10.1038/sj.jid.5700340.
13. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47-95. doi: 10.1152/physrev.00018.2001.
14. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci. 2008;4(2):89-96.
15. Bhardwaj RL, Pandey S. Juice blends--a way of utilization of under-utilized fruits, vegetables, and spices: a review. Crit Rev Food Sci Nutr. 2011;51(6):563-70. doi: 10.1080/10408391003710654.
16. Milisav I, Ribarič S, Poljsak B. Antioxidant Vitamins and Ageing. Subcell Biochem. 2018; 90: 1-23. doi: 10.1007/978-981-13-2835-0_1.
17. Koushki M, Lakzaei M, Khodabandehloo H, Hosseini H, Meshkani R, Panahi G. Therapeutic effect of resveratrol supplementation on oxidative stress: a systematic review and meta-analysis of randomised controlled trials. Postgrad Med J. 2020; 96(1134): 197-205. doi: 10.1136/postgradmedj-2019-136415.
18. Asbaghi O, Ghanavati M, Ashtary-Larky D, et al. Effects of Folic Acid Supplementation on Oxidative Stress Markers: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Antioxidants (Basel). 2021; 10(6): 871. doi: 10.3390/antiox10060871.
19. Aaseth J, Alexander J, Alehagen U. Coenzyme Q10 supplementation - In ageing and disease. Mech Ageing Dev. 2021 Jul;197:111521. doi: 10.1016/j.mad.2021.111521.
20. Raghu G, Berk M, Campochiaro PA, et al. The Multifaceted Therapeutic Role of N-Acetylcysteine (NAC) in Disorders Characterized by Oxidative Stress. Curr Neuropharmacol. 2021; 19(8): 1202-1224. doi: 10.2174/1570159X19666201230144109.
21. Prior RL, Cao G. Antioxidant phytochemicals in fruits and vegetables: dietary and health implications. Hortic Sci. 2000;35:588-592.
22. Paganaga G, Miller N, Rice-Evans CA. The polypohenolic content of fruit and vegetables and their antioxidant activities: what does a serving constitute? Free Radic Res. 1999;30:153-162.
23. Halvorsen BL, Holte K, Myhrstad MC, et al. A systematic screening of total antioxidants in dietary plants. J Nutr. 2002;132:461-471.
24. Wild AC, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression: insights into transcriptional

control of antioxidant defenses. Free Radic Res. 2000; 32:281-301.
25. Hayes JD, McLellan LI. Glutathione and glutathione dependent enzymes represent a coordinately regulated defense against oxidative stress. Free Radic Res. 1999;31:273-300.
26. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J. 1996;10:709-720.
27. Talalay P. Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors. 2000;12:5-11.
28. Festi D, Colecchia A, Pini S, et al. Development and application of a simple and powerful tool for nutrition and lifestyle education for the Italian general population by general practitioners and family paediatricians. MJNM. 2009;2:139-144.
29. Vigna L, Cassinelli L, Tirelli AS, et al. 25(OH)D Levels in Relation to Gender, Overweight, Insulin Resistance, and Inflammation in a Cross-Sectional Cohort of Northern Italian Workers: Evidence in Support of Preventive Health Care Programs. J Am Coll Nutr. 2017; 36(4): 253-260. doi: 10.1080/07315724.2016.1264280.
30. Dellanoce C, Cozzi L, Zuddas S, Pratali L, Accinni R. Determination of different forms of aminothiols in red blood cells without washing erythrocytes. Biomed Chromatogr. 2014; 28(3): 327-31. doi: 10.1002/bmc.3056.
31. Svardal AM, Mansoor MA, Ueland PM. Determination of reduced, oxidized, and protein-bound glutathione in human plasma with precolumn derivatization with monobromobimane and liquid chromatography. Anal Biochem. 1990; 184(2): 338-46. doi: 10.1016/0003-2697(90)90691-2.
32. Serra-Majem L, Tomaino L, Dernini S, et al. Updating the Mediterranean Diet Pyramid towards Sustainability: Focus on Environmental Concerns. Int J Environ Res Public Health. 2020;17(23):8758. doi: 10.3390/ijerph17238758.
33. Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. Nutr J. 2015;14:6. doi: 10.1186/1475-2891-14-6.
34. Wu X, Zhang L, Miao Y, et al. Homocysteine causes vascular endothelial dysfunction by disrupting endoplasmic reticulum redox homeostasis. Redox Biol. 2019;20:46-59. doi: 10.1016/j.redox.2018.09.021.
35. Balint B, Jepchumba VK, Guéant JL, Guéant-Rodriguez RM. Mechanisms of homocysteine-induced damage to the endothelial, medial and adventitial layers of the arterial wall. Biochimie. 2020;173:100-106. doi: 10.1016/j.biochi.2020.02.012.
36. Yang Q, He GW. Imbalance of Homocysteine and H2S: Significance, Mechanisms, and Therapeutic Promise in Vascular Injury. Oxid Med Cell Longev. 2019;2019:7629673. doi: 10.1155/2019/7629673.
37. Lim U, Cassano PA. Homocysteine and blood pressure in the Third National Health and Nutrition Examination Survey, 1988-1994. Am J Epidemiol. 2002;156(12):1105-13. doi: 10.1093/aje/kwf157.
38. Gori F, Tirelli AS, Piontini A, et al. Metabolic Syndrome, Homocysteine and Uric Acid in Patients with Obesity; Experience from Obesity and Work Centre. Mediterranean Journal of Nutrition and Metabolism. 2021: 277-288.
39. Zaric BL, Obradovic M, Bajic V, Haidara MA, Jovanovic M, Isenovic ER. Homocysteine and Hyperhomocysteinaemia. Curr Med Chem. 2019;26(16):2948-2961. doi: 10.2174/0929867325666180313105949.
40. Bhagavan HN, Chopra RK. Coenzyme Q10: absorption, tissue uptake, metabolism and pharmacokinetics. Free Radic Res. 2006;40(5):445-53. doi: 10.1080/10715760600617843.
41. Bhagavan HN, Chopra RK. Plasma coenzyme Q10 response to oral ingestion of coenzyme Q10 formulations. Mitochondrion. 2007;7 Suppl:S78-88. doi: 10.1016/j.mito.2007.03.003.
42. Judy WV. The Single-dose Absorption and Steady-state Bioavailability of Different Coenzyme Q10 Formulations. Integr Med (Encinitas). 2022;21(1):28-34.
43. Saini R. Coenzyme Q10: The essential nutrient. J Pharm Bioallied Sci. 2011;3(3):466-7. doi: 10.4103/0975-7406.84471.
44. Aberg F, Appelkvist EL, Dallner G, Ernster L. Distribution and redox state of ubiquinones in rat and human tissues. Arch Biochem Biophys 1992;295:230–234.
45. Crane FL. Biochemical functions of coenzyme Q10. J Am Coll Nutr. 2001;20(6):591-8. doi: 10.1080/07315724.2001.10719063.
46. Gutierrez-Mariscal FM, Arenas-de Larriva AP, Limia-Perez L, Romero-Cabrera JL, Yubero-Serrano EM, López-Miranda J. Coenzyme Q10 Supplementation for the Reduction of Oxidative Stress: Clinical Implications in the Treatment of Chronic Diseases. Int J Mol Sci. 2020;21(21):7870. doi: 10.3390/ijms21217870 .
47. Arenas-Jal M, Suñé-Negre JM, García-Montoya E. Coenzyme Q10 supplementation: Efficacy, safety, and formulation challenges. Compr Rev Food Sci Food Saf. 2020;19(2):574-594. doi: 10.1111/1541-4337.12539 .
48. Langsjoen PH, Langsjoen AM. Overview of the use of CoQ10 in cardiovascular disease. Biofactors. 1999;9(2-4): 273-84. doi: 10.1002/biof.5520090224.
49. Akbari A, Mobini GR, Agah S, et al. Coenzyme Q10 supplementation and oxidative stress parameters: a systematic review and meta-analysis of clinical trials. Eur J Clin Pharmacol. 2020; 76(11): 1483-1499. doi: 10.1007/s00228-020-02919-8.
50. Sangsefidi ZS, Yaghoubi F, Hajiahmadi S, Hosseinzadeh M. The effect of coenzyme Q10 supplementation on oxidative stress: A systematic review and meta-analysis of randomized controlled clinical trials. Food Sci Nutr. 2020; 8(4): 1766-1776. doi: 10.1002/fsn3.1492.
51. Ouchi A, Nagaoka S, Mukai K. Tunneling effect in regeneration reaction of vitamin E by ubiquinol. J Phys Chem B. 2010;114(19):6601-7. doi: 10.1021/jp910856m.
52. Huang A, Vita JA, Venema RC, Keaney JF Jr. Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. J Biol Chem. 2000 Jun 9;275(23):17399-406. doi: 10.1074/jbc.M002248200.

53. Askari M, Faryabi R, Mozaffari H, Darooghegi Mofrad M. The effects of N-Acetylcysteine on serum level of inflammatory biomarkers in adults. Findings from a systematic review and meta-analysis of randomized clinical trials. Cytokine. 2020; 135: 155239. doi: 10.1016/j.cyto.2020.155239.
54. Rushworth GF, Megson IL. Existing and potential therapeutic uses for N- acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. Pharmacol Ther. 2014;141(2):150-9. doi: 10.1016/j.pharmthera.2013.09.006.
55. Raftos JE, Whillier S, Kuchel PW. Glutathione synthesis and turnover in the human erythrocyte: alignment of a model based on detailed enzyme kinetics with experimental data. J Biol Chem. 2010;285(31):23557-67. doi: 10.1074/jbc.M109.067017.
56. Rifkind JM, Mohanty JG, Nagababu E. The pathophysiology of extracellular hemoglobin associated with enhanced oxidative reactions. Front Physiol. 2015;5:500. doi: 10.3389/fphys.2014.00500.

Correspondence
Received: 11 March 2024
Accepted: 2 May 2024
Luisella Vigna, MD
Responsabile Centro Obesità e Lavoro, Centro accreditato Società Italiana Obesità – SIO Collaborating Center for Obesity Management of the European Association for the Study of Obesity - EASO COM
Responsabile Servizio di Promozione della Salute dei Lavoratori SC Medicina del Lavoro - Clinica del Lavoro L Devoto Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy tel 39 02 55032603-2592
E-mail: luisella.vigna@policlinico.mi.it