ORIGINAL RESEARCH

Nutraceutical Support of Mitochondrial Function Associated With Reduction of Long-term Fatigue and Inflammation

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ABSTRACT

Objectives • To evaluate the effects of ATP 360, a nutraceutical energy formula, in people experiencing long-term fatigue affecting daily living. To explore the use of ex vivo mitochondrial stress testing to evaluate cellular energy improvements with nutraceutical support.

Study Design • An open-label study design was used with screening for long-term fatigue, scoring 50% or higher on the Piper Fatigue Scale. Eleven participants (8 women, 3 men) consumed the nutraceutical energy formula for 8 weeks, with a 1-week online evaluation and 4-week and 8-week follow-up visits. Eleven healthy people of similar age and BMI range donated blood for comparative evaluation of mitochondrial function in non-fatigued subjects.

Methods • Fatigue scoring was performed using the Piper Fatigue Scale. Other data included blood pressure readings and questionnaires for pain and wellness. Blood draws were performed. Serum was tested for cytokines using bead-based immunoassays. Leukocytes were tested for mitochondrial mass and mitochondrial membrane potential after 2-hour ex vivo inflammatory challenges with bacterial lipopolysaccharide using fluorescent probes, along with flow cytometry analysis.

Primary Outcome Measures • Change in fatigue and pain levels from baseline to 8 weeks.

Results • Reduction in long-term fatigue was rapid and highly significant after 1 week. Pain reduction reached statistical significance at 4 weeks. Wellness scores improved, especially mental functioning, sleep, increased emotional wellness, and increased energy/vitality. Diastolic blood pressure was reduced. Serum levels of TNF-α and interleukin 8 were reduced. At baseline, leukocyte mitochondrial responses to ex vivo inflammation were low compared to leukocytes from healthy non-fatigued people, showing a mild 21% increase after 4 weeks (not statistically significant), and returning to baseline at 8 weeks.

Conclusion • This proof-of-concept study showed that consumption of a proprietary nutraceutical energy formula resulted in rapid and sustained fatigue reduction associated with reduced pain and inflammatory cytokines and improved wellness. A mild increase in mitochondrial response to inflammation was seen at 4 weeks. A future study with longer duration should evaluate whether mitochondrial function may approach that of a healthy population.

Trial registration • This study was conducted in accordance with the ethical standards set forth in the Helsinki Declaration of 1975 (trial registration number NCT04261881) (*Altern Ther Health Med.* 2021;27(3):8-18).

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BACKGROUND

Fatigue is defined as persistent disrupted physical and/or mental tiredness that impacts quality of life and decreases the ability to perform activities of daily living. Fatigue is a common complaint presenting to practitioners. Up to 25% to 30% of visits to practitioners involve a complaint including fatigue.[1] When the general population is surveyed, the prevalence of fatigue appears to affect 30% to 50% of people. When the fatigue is persistent and severe, patients will be given the diagnosis of chronic fatigue syndrome which affects approximately 1% of the population by clinical evaluation and 3% by self-assessment.[2] Long-term fatigue can be an isolated symptom or a part of chronic disease. Children with significant long-term disease may also present with fatigue in up to 21% causing a negative impact on their lives.[3] While many patients suffer from fatigue, it is difficult to successfully treat. Infections and autoimmune diseases often involve fatigue as a symptom,[4] including in fibromyalgia,[4]

posttreatment Lyme disease syndrome,[5] and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). These illnesses have significant long-term fatigue as one of the primary symptoms, along with additional symptoms ranging from headaches, cognitive dysfunction, sleep disturbance, muscle and joint pain, to orthostatic intolerance.[6]

Pain is a common symptom of people in those who experience significant fatigue. The definition of chronic pain includes persistent pain in at least one area for greater than 3 months.[7] When US adults were surveyed for pain, approximately 20% were positive for chronic pain and 8% were positive for high-impact chronic pain (HICP) associated with significant disability.[8] Pain, much like the nonspecific symptom of fatigue, is difficult to treat. One of the reasons that fatigue and pain are difficult to treat is because practitioners tend to treat the symptoms without understanding the underlying pathology. Both fatigue and pain can arise from inflammation, where the perception of fatigue is regulated in the brain by pro-inflammatory cytokines, including interleukin 1α (IL-1α) and IL-1β.[9-11]

Mitochondria are the organelles in the cell responsible for cellular energy. From beta-oxidation to the citric acid cycle and finally to the electron transport chain, the mitochondria synthesize ATP.[12] Many environmental factors can negatively impact mitochondrial function, including infections, medicines, toxins, ischemia, aging, and poor nutrition.[13] Mitochondrial dysfunction has now been associated with over 40 major diseases and health problems, including type 2 diabetes, cancer, Alzheimer disease, and other neurodegenerative diseases.[14]

Upon studying the metabolic steps in the mitochondrial production of ATP, multiple key cofactors have been discovered. Research has been investigating several of these cofactors, such as Coenzyme Q_{10} (CoQ_{10}), carnitine, NADH, and several of the B vitamins, in their role in strengthening mitochondrial function. CoQ_{10} was found to increase the size, number, and volume of mitochondria during in vitro studies.[15] CoQ_{10} is a strong antioxidant and an electron carrier in the respiratory chain in the mitochondria. NAD/NADH is another cofactor critical for mitochondrial function.[14] A nutritional supplement containing NADH and CoQ_{10}, along with phospholipids, carnitine, and multiple other cofactors involved in mitochondrial function was found to significantly decrease fatigue in chronically fatigued patients in a clinical trial.[16]

Recently pyrroloquinoline quinone (PQQ) has been identified as another component involved in cellular energy metabolism.[17] Clinical trials of PQQ supplementation showed enhanced mitochondrial function along with a decrease in C-reactive protein (CRP) consistent with a decrease in inflammation and an increase in antioxidant function.[18] One of the unique functions of PQQ is its ability to increase mitochondrial biogenesis.[19]

A nutritional supplement, ATP 360, was designed to combine the multiple cofactors involved in supporting mitochondrial function and biogenesis. The addition of PQQ to the combination of NADH, CoQ_{10}, magnesium, B vitamins, and L-acetyl-carnitine was combined to see if it would lead to synergy in supporting mitochondrial function and clinical improvement. Previous supplements designed to decrease fatigue have not evaluated mitochondrial function and biogenesis along with clinical evaluations in research studies. The goal for our research pilot study was to evaluate clinical reduction of fatigue and correlate the clinical symptoms with an improvement in inflammation status and mitochondrial function, with the goal of using data from this methodology to optimally plan future trials on this nutritional blend in comparison with its key ingredients.

METHODS

Study Design

An open-label study design was used to evaluate the effects of consumption of a nutraceutical product. The study was of 8 weeks' duration, with evaluation at baseline, 1, 4, and 8 weeks of product consumption. Following the procedure used in a previous clinical trial on a nutritional formula,[20,21] the current study applied a screening procedure involving the Piper Fatigue Scale,[22,23] where people who scored 5 or higher out of the total score of 10 were considered for the study. People were recruited for the study if they met the inclusion criteria of being 18-75 years of age, having a body mass index between 18.0–34.9 kg/m^2, and experiencing fatigue at a level that moderately to severely interfered with daily activities for the past 6 months. People were excluded from the study if they had known active cardiovascular health issues, cancer and/or chemotherapy in the past 12 months, active uncontrolled autoimmune illness, were prescribed prescription medications for blood thinning, had a cortisone injection within the past 3 months, or if they had surgery or trauma during the past 3 months. The clinical phase of the study was conducted over a period of 7 months, from March to October 2018, where study participants were enrolled for their 8-week participation in a staggered manner as they passed the screening process. The location was Klamath Falls, Oregon, which has a high-desert arid climate, and where people live and work at an altitude 4000–5500 feet above sea level. Twelve study participants were recruited after providing written informed consent (as approved by the Sky Lakes Medical Center Institutional Review Board FWA 2603) and passing the screening process. Study participants were instructed to maintain a constant diet and lifestyle during the study. Study participants were instructed to consume similar breakfasts on the morning of each clinic visit, and to avoid vitamins, nutritional supplements, and exercise the morning of a clinic visit. They were instructed to avoid coffee, tea, nicotine, and energy drinks for at least an hour before arrival. Eleven people completed 4 weeks of study participation, and 10 people completed the 8-week pilot study.

Consumable Nutritional Supplement

The consumable test product for the study was a nutraceutical energy formula (NEF), namely the dietary

supplement blend ATP 360, designed to support cellular energy production, mitochondrial function, and resilience to oxidative stress (Table 1). The supplement contains CoQ_{10}, pantethine (a dimeric and more active form of vitamin B_5), vitamin C, R-lipoic acid, NADH, and the nutrients acetyl L-carnitine and PQQ, known to increase the volume of mitochondria in cells.[24] It also contains lipids important for cellular and mitochondrial membrane health including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and tocotrienols. The test product was provided by the manufacturer, Researched Nutritionals, Los Olivos, California. Study participants consumed 3 capsules once daily. At the baseline and 4-week visits, study participants were given a supply of the test product to last until the 4- and 8-week visits respectively. They were instructed to consume the capsules with food. Study participants were instructed to return the bottles with any unused capsules. Capsule counts were used to document compliance with respect to consumption of test product. The average compliance was 91%.

Table 1. Ingredients in 1 Daily Dose of the Nutritional Energy Formula (NEF)

Vitamin C	100 mg
Thiamine HCL	50 mg
Riboflavin 5-phosphate	42 mg
Choline	27 mg
Magnesium malate	50 mg
Tocotrienols (delta and gamma)	86 mg
Coenzyme Q_{10} with NADH	173 mg
Lipid concentrate[a]	400 mg
Acetyl L-carnitine with PQQ	210 mg

[a]Blend of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and other phytoglycolipids

Blood Pressure

Blood pressure was monitored at all study visits, using an Omron BP742 monitor, after the person had remained seated for at least 5 minutes.

Questionnaire-based Data Collection

At each study time point, questionnaires were administered where the study participant would answer by use of an electronic survey with a Visual Analogue Scale (0-10). Fatigue levels were scored using the validated Piper Fatigue Scale[22,23] where the average total score, as well as the subscores for fatigue disrupting daily living, physical symptoms of fatigue, affective meaning of fatigue, and fatigue impacting mental and mood states, were calculated. The pain questionnaire asked about pain for each person's anatomical area for primary pain (the area where pain affected their activities of daily living the most), and their secondary area of pain. The subjective pain levels were scored both at rest and during physical activity using the Visual Analogue Scale.[25-27] A wellness questionnaire contained questions regarding general health and wellness, including physical functioning, mental functioning, emotional well-being, stress level, social functioning, sleep, and energy/vitality. The total wellness score, as well as the 7 subscales, were calculated such that higher scores indicate better health and wellness.[28]

Testing of Inflammatory Biomarkers

Levels of 17 cytokines and growth factors were quantified using protein arrays and microsphere arrays. The biomarkers included the following: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1 (MCAF), MIP-1β, and TNF-α.

Reagents

RPMI 1640 Medium, Penicillin–Streptomycin solution 100×, IL-2, phosphate-buffered saline, and lipopolysaccharide (LPS) from *Salmonella enterica* were purchased from Sigma-Aldrich Co. (St Louis, MO). Cal-Lyse whole-blood lysing solution was purchased from Thermo Fisher Scientific (Waltham, MA). CD69 fluorescein isothiocyanate, CD56 phycoerythrin, CD3 peridinin chlorophyll protein, and heparin vacutainer tubes were purchased from Becton-Dickinson (Franklin Lakes, NJ). Customized Bio-Plex Pro human cytokine arrays were purchased from Bio-Rad Labs.

Blood Draws

Peripheral venous blood samples were collected at baseline and after 4 and 8 weeks in the study. For each blood draw, one heparin vacutainer tube and one serum separator tube were collected. Heparin tubes were used for leukocyte purification (described below) to test for mitochondrial function. The serum separator tubes were kept at room temperature for 30-60 minutes to allow complete coagulation, after which the tubes were centrifuged at 1000 g for 15 minutes, the serum harvested and transferred to a second tube and spun a second time at 400 g for 10 minutes (refrigerated). Each serum sample was aliquoted and frozen at -80° C.

Leukocyte Purification

For testing of mitochondrial function in the study participants, peripheral venous blood was drawn into heparin vacutainer tubes, and the peripheral blood mononuclear cells (PBMC) and polymorphonuclear (PMN) cells were isolated using Lympholyte Poly (Cedarlane, Burlington, NC) by centrifugation for 35 minutes at 400 g. The PBMC and PMN cells were washed twice in PBS, counted, and pooled. The cell density was adjusted to establish cultures with a cell density at 10^6/mL, using RPMI 1640 Medium containing penicillin–streptomycin and 10% fetal bovine serum.

For the in vitro testing of mitochondrial function, peripheral venous blood was drawn from 11 human healthy donors upon written informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board, Federalwide Assurance 2603. The blood donors were not participants in the fatigue study and had not consumed the

NEF. The blood was drawn into heparin vacutainer vials, and the PBMC and PMN cells isolated as described above.

Mitochondrial Mass per Cell

Two independent sets of cultures were established from each blood draw in V-bottom 96-well culture plates (NUNC, Denmark), where one set was left unstressed (normal culture conditions), and the second set was treated with the highly inflammatory bacterial toxin, lipopolysaccharide (LPS) from Salmonella enterica (0.1 µg/mL). The cultures were incubated at 37° C, 5% CO_2, for 2 hours. The cells were washed twice in PBS by centrifugation at 400 g for 2.5 minutes and resuspended in RPMI with 2 µM MitoTracker Green (MTG), a fluorescent probe that stains mitochondria in proportion to mitochondrial mass per cell. Unstained control cultures were resuspended in RPMI without MTG. The cultures were incubated at 37° C, 5% CO_2, for 30 minutes. The cells were pelleted by centrifugation, the supernatant discarded, the cells resuspended in 100 µL PBS in the V-bottom plate and transferred to 0.5 mL/well 96-well plate with 100 µL PBS for a total sample volume of 200 µL for flow cytometry. The fluorescence intensity for MTG was measured by flow cytometry, using an Attune acoustic-focusing flow cytometer (Thermo Fisher Scientific) equipped with a 96-well plate autosampler. Data analysis utilized gating on forward/side scatter to evaluate the MTG mitochondrial fluorescence intensity for lymphocytes, monocytes/macrophages, and PMN cells within each sample.

Mitochondrial Membrane Potential

Three independent sets of cultures were established from each blood draw, following the same protocol as described above for evaluation of mitochondrial mass. Instead of staining with MTG in RPMI, the cells were resuspended in PBS and stained with JC-1, a cationic carbocyanine dye that accumulates in mitochondria. The dye exists as a monomer at low concentrations and yields green fluorescence similar to fluorescein. At higher concentrations, the dye forms J-aggregates that exhibit a broad excitation spectrum and an emission maximum at ~590 nm. These characteristics make JC-1 a sensitive marker for mitochondrial membrane potential. The cells were incubated with JC-1 at 37° C, 5% CO_2, for 10 minutes, after which they were resuspended in phosphate-buffered saline (PBS) and transferred to a 0.5 mL 96-well plate for flow cytometry. Data analysis utilized gating on forward/side scatter to evaluate the JC-1 orange versus green fluorescence intensity for lymphocytes, monocytes/macrophages, and PMN cells within each sample. Healthy mitochondria will fluoresce in the orange spectrum, whereas stressed mitochondrial will show a shift with increased fluorescence in the green spectrum. Flow cytometric analysis for the mean fluorescence intensity in both the orange and green spectrum was evaluated. An increase in the ratio of orange versus green fluorescence intensity was interpreted as protection of mitochondrial membrane potential.

For the in vitro testing, similar cultures of pooled PBMC and PMN cells were established, where untreated cultures were compared to cultures pre-treated with aqueous versus ethanolic extract of NEF, and subsequently either left untreated, or stressed with LPS for 2 hours prior to staining and flow cytometry.

Statistical Analysis

Group averages and standard error of the means (SEM) for each data set were calculated using Microsoft Excel. Statistical significance of changes from baseline to later assessments was evaluated by between-treatment analysis using 'within-subject' analysis using the two-tailed, paired *t* test.

RESULTS

Study demographics and compliance**.** Twelve study participants were recruited across a broad age and BMI range, since metabolic health, physical health, ability to absorb, digest, metabolize and excrete nutrients changes through life. Eleven people completed at least 4 weeks of study participation and 10 people completed the 8-week pilot study (Table 2). The data analysis includes comparison of data from 11 people at baseline to 4 weeks of study participation, and comparison of data from 10 people at 8 weeks. The average compliance for consuming the nutritional supplement NEF was 91%.

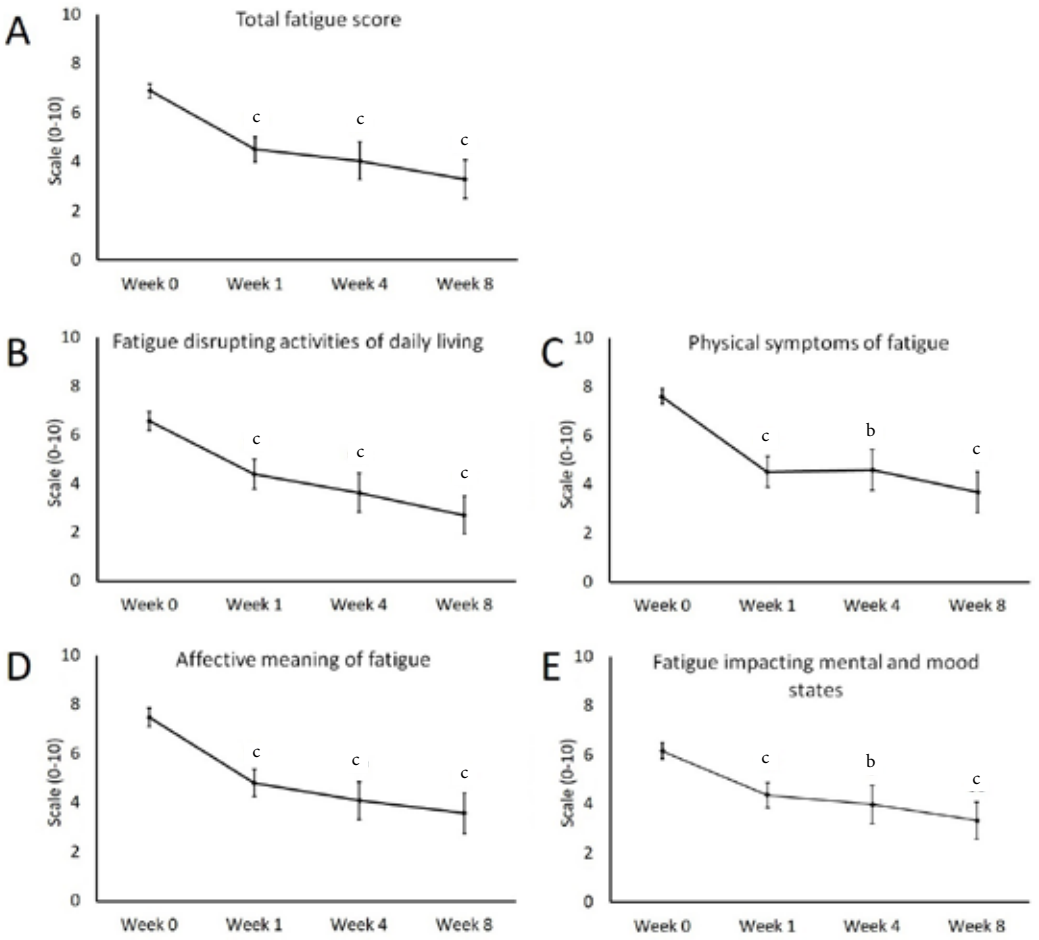
Fatigue levels were scored using the Piper Fatigue Scale at baseline and after 1, 4, and 8 weeks, where all study participants had scored 5 or higher (out of a maximum score of 10) during screening. Reduction in long-term fatigue was rapid and highly significant after 1 week of consuming NEF ($P < .01$) and remained highly significant throughout the study when compared to baseline scores (Figure 1A). The Piper fatigue subscales each showed significant reduction at 1 week and throughout the study, suggesting that the relief of long-term fatigue involved not only physical but also mental and mood improvements (Figure 1B-1E).

Table 2. Demographics of the Study Population

	Fatigue Study	Healthy Controls[a]
Females	8	7
Age average (years)[b]	47.0 ± 11.1	39.3 ± 21.7
Age range (years)	35.3 - 66.7	21.4 – 67.1
BMI average (kg/m^2)[b]	27.0 ± 3.7	23.4 ± 1.9
BMI range (kg/m^2)	20.8 – 34	20.2 - 25.1
Males	3	4
Age average (years)[b]	44.4 ± 21.3	41.2 ± 23.7
Age range (years)	25.3 - 67.4	22.0 - 71.1
BMI average (kg/m^2)[b]	23.7 ± 4.5	23.7 ± 1.8
BMI range (kg/m^2)	19.4 - 28.4	21.1 - 25.2

[a]Eleven healthy people with similar age and BMI ranges donated blood for comparative evaluation of mitochondrial function in non-fatigued subjects.
[b]The average ± standard deviation is shown.

Figure 1. Fatigue Scores



[a] $P < .1$
[b] $P < .05$
[c] $P < .01$

Note: Fatigue scores were collected using the Piper Fatigue Scale; total fatigue as well as 4 subscales were calculated. The average ± SEM is shown for each time point. The average total fatigue score (A), as well as the subscores for fatigue disrupting daily living and the affective meaning of fatigue, dropped rapidly reaching a high level of statistical significance at 1 week ($P < .01$), and remaining highly significant at 4 and 8 weeks when compared to baseline. The physical symptoms of fatigue (C) and fatigue impacting mental and mood states (E) also showed a rapid improvement at 1 week ($P < .01$), remained significant at 4 weeks ($P < .05$), and continued to show reduction, with a high level of statistical significance at 8 weeks ($P < .01$).

Pain levels were scored at baseline and after 1, 4, and 8 weeks, where study participants scored pain levels for their primary and secondary pain complaint for when at rest and when being physically active. Reduction in pain was rapid and measurable after 1 week of consuming NEF and reached statistical significance at 4 weeks when compared to baseline scores (Figure 2). Some fluctuations were seen in pain scores over time, likely associated with the decreased fatigue and increased activity levels.

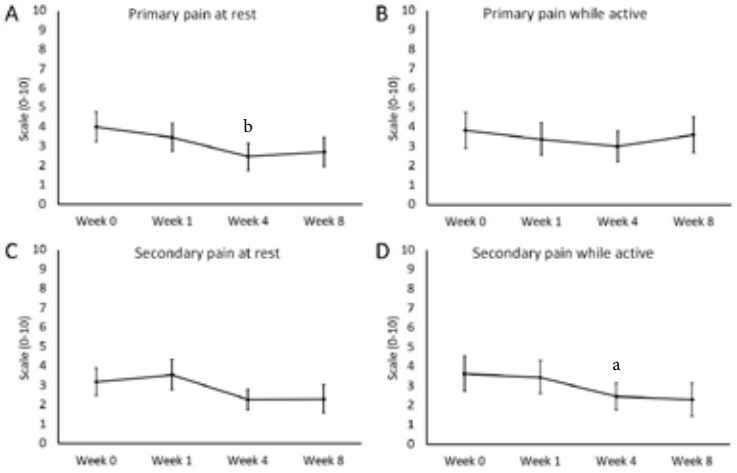
Wellness scores were collected (Figure 3), and improved overall wellness was seen, reaching a statistical trend at 8 weeks ($P < .1$, Figure 3A).

The improved wellness was driven by improvements in mental functioning, which was significantly improved at 4 and 8 weeks ($P < .05$ Figure 3C), along with improved emotional well-being and improved energy and vitality which reached statistical trends at 8 weeks ($P < .1$ Figures 3D, 3H).

A mild but insignificant reduction in stress and improvement in social functioning score was seen. Sleep improved noticeably, reaching statistical significance at 1 week ($P < .05$ Figure 3G) and a high level of significance at 4 weeks ($P < .01$), remaining significant at 8 weeks ($P < .05$) compared to baseline.

Blood Pressure. Consumption of NEF resulted in a significant decrease in diastolic blood pressure, along with a mild non-significant reduction in systolic blood pressure (Figure 4). The reduction in diastolic blood pressure reached a statistical trend already at 1 week ($P < .1$) and

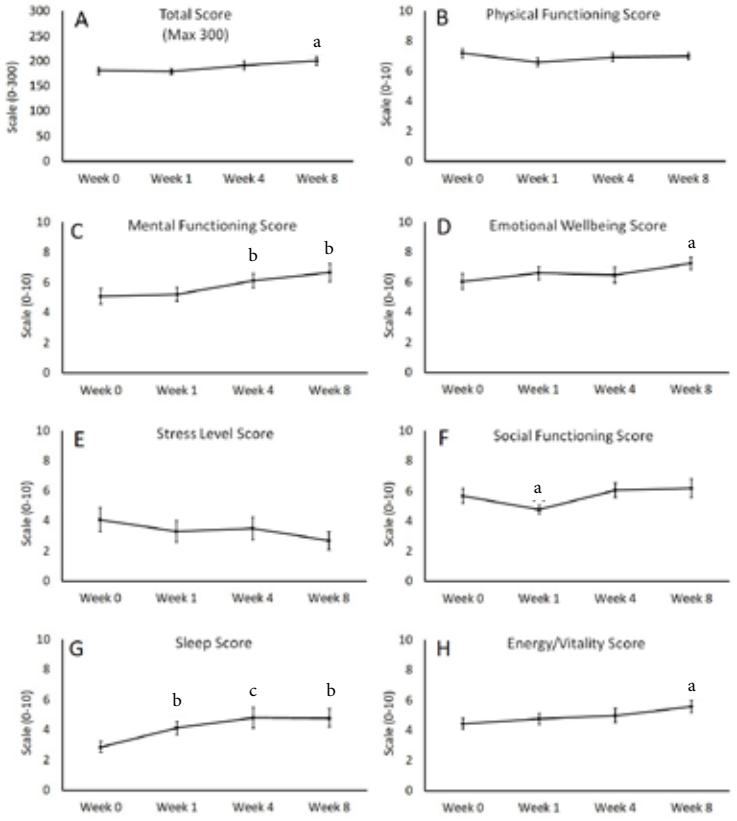
Figure 2. Pain Scores



[a] $P < .1$
[b] $P < .05$
[c] $P < .01$

Note: The pain scores were collected using Visual Analogue Scales. The average ± SEM is shown for each time point. Reductions in pain were seen for primary and secondary pain at rest and while active, and showed the clearest effect at 4 weeks where the average primary pain at rest (A) reached statistical significance ($P < .05$). Secondary pain while active reached a statistical trend at 4 weeks ($P < .1$).

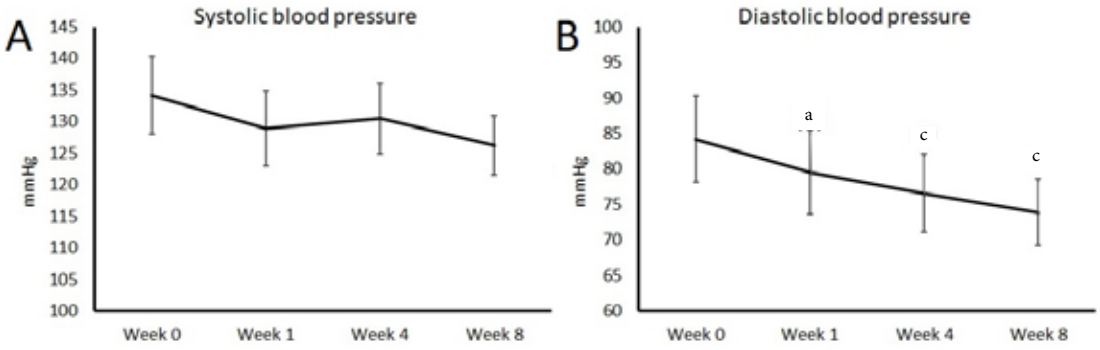
Figure 3. Wellness Scores



[a] $P < .1$
[b] $P < .05$
[c] $P < .01$

Note: The wellness scores were collected using Visual Analogue Scales; the total wellness score, as well as 7 subscales, were calculated. The average ± SEM is shown for each time point. Statistical significance is indicated by when $P < .05$, and when $P < .01$. A statistical trend is indicated when $P < .1$).

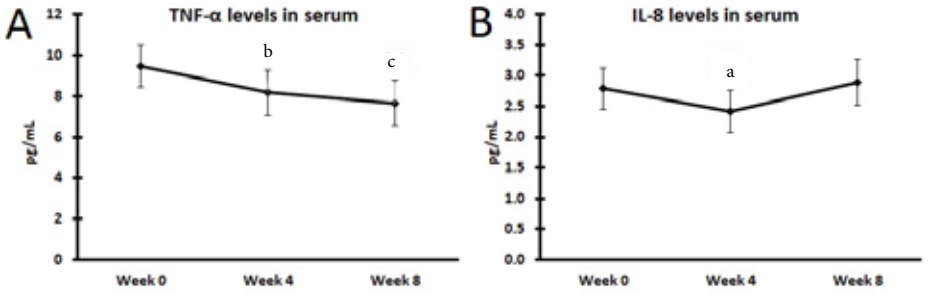
Figure 4. Systolic and Diastolic Blood Pressure



[a] $P < .1$
[b] $P < .05$
[c] $P < .01$

Note: The systolic (A) and diastolic (B) blood pressure readings are shown in mm Hg as the average ± SEM for each time point. An improvement was seen for both the systolic and the diastolic blood pressures, where the reduced diastolic blood pressure reached a statistical trend after 1 week ($P < .1$), and a high level of statistical significance at 4 weeks ($P < .01$), remaining highly significant at 8 weeks.

Figure 5. Serum Cytokine Levels



[a] $P < .1$
[b] $P < .05$
[c] $P < .01$

Note: The serum levels of tumor necrosis factor alpha (TNF-α) (A) and interleukin 8 (IL-8) (B) are shown in pg/mL as the average ± SEM for each time point. A statistically significant reduction was seen at 4 weeks for both cytokines ($P < .05$). For TNF-α the reduced levels at 8 weeks reached a high level of statistical significance when compared to baseline ($P < .01$).

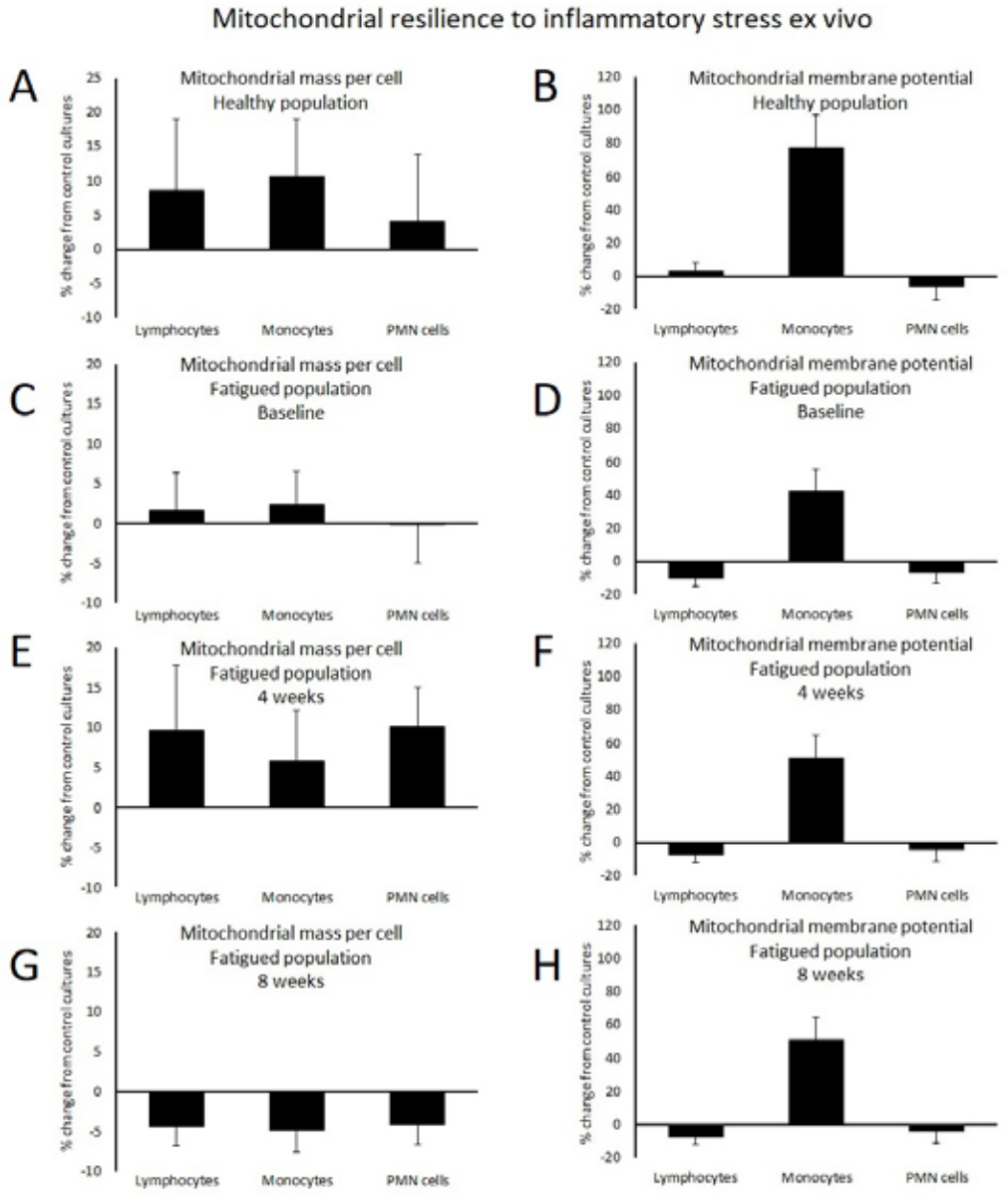
was highly significant at 4 and 8 weeks when compared to baseline ($P < .01$).

Cytokine Levels. Consumption of NEF resulted in the reduction of 2 inflammatory cytokines, where the reduction in tumor necrosis factor alpha (TNF-α) reached statistical significance at 4 weeks and was highly significant after 8 weeks ($P < .01$). The reduction in IL-8 reached a statistical trend at 4 weeks ($P < .05$) (Figure 5). In contrast, there was no significant change to IL-6 levels. Other cytokines, including IL-1β, IFN-γ, IL-4, IL-7, IL-10, and IL-17, were below the lower limit of detection at all time points measured, demonstrating that consumption of NEF for 8 weeks did not increase inflammatory blood biomarkers above levels seen at baseline. This also helped confirm that the study population was not in a diseased state at study start.

Mitochondrial Function. The effects of consumption of NEF on mitochondrial resilience under inflammatory conditions was tested ex vivo, where freshly drawn blood samples from the 11 study participants at baseline, 4 weeks, and 8 weeks, were exposed to the inflammatory endotoxin LPS for 2 hours, stained with mitochondrial reporter dyes, and analyzed by flow cytometry. As a control for this testing, 11 healthy non-fatigued adults in the same age range donated blood for the same test panel (Figure 6).

In the healthy non-fatigued population, the white blood cell subsets responded to inflammatory stress by increasing the mitochondrial mass per cell by an average of 11%. In addition, monocytes from healthy adults responded to inflammation by an average 77% increase in the mitochondrial

Figure 6. Mitochondrial Response to an Inflammatory Challenge Ex Vivo



Note: Data are shown as the average ± SD of triplicate measures from cells from 11 healthy, non-fatigued blood donors (top panels), compared to the 11 fatigued study participants at baseline, 4 weeks, and 8 weeks of consuming NEF.

membrane compared to untreated control cultures of cells from the same donors.

In contrast, the fatigued study participants entered the study with baseline measurements showing almost no change in mitochondrial mass per cell when the cells were challenged by an inflammatory insult, and the monocyte population responded to inflammation by an average of only 42% increase in mitochondrial membrane potential, in contrast to the higher increase seen in cells from healthy people. The difference between the ex vivo response to inflammation between monocytes from healthy and fatigued donors did not reach statistical significance ($P < .16$).

After 4 weeks' consumption of NEF, the white blood cell subsets from the fatigued population responded to inflammation ex vivo by a similar increase in mitochondrial mass per cell as the healthy non-fatigued population, and the monocyte population responded to inflammation by 51% increase in the mitochondrial membrane potential, a 21% increase from baseline. This increase was not statistically significant potential.

After 8 weeks' consumption of NEF, the white blood cell subsets responded to inflammation ex vivo by a mild reduction in mitochondrial mass per cell. The increase in the monocyte mitochondrial membrane potential returned to similar levels as baseline.

DISCUSSION

Fatigue is a frequent complaint for people seeking medical care for a wide range of illnesses. Finding adequate treatment methods to alleviate symptoms of fatigue can be difficult. Limited pharmaceutical options exist for effective symptom relief. The current research study examined a nutritional supplement, NEF, for targeting fatigue. Consumption of NEF resulted in highly significant improvements in fatigue scores ($P < .01$) including physical, mental, and emotional symptoms associated with fatigue, beginning as early as 1 week. The decreases in fatigue remained highly significant throughout the study, including results at 4 weeks and at 8 weeks. These decreases were concomitant with reduced levels of the inflammatory markers TNF-α and IL-8, where the reduction in TNF-α levels reached a high point of statistical significance at 8 weeks when compared to baseline. Overall wellness on quality of life questionnaires increased where improved energy, vitality, and mental functioning showed the primary areas of improvement.

Fatigue is often seen in chronic inflammatory states. Research has shown elevated levels of pro-inflammatory cytokines such at IL-6, IL-1, and TNF-α are associated with chronic fatigue.[29] In a human clinical trial with an experimental inflammatory challenge, a higher cytokine response was associated with more fatigue.[30] In addition, both pain and elevated blood pressure are associated with inflammation.[28,31] In the study reported here, we observed significant reductions in TNF-α at both 4 and 8 weeks. The decrease in TNF-α levels may be associated with the decreases in fatigue, pain reduction, and lowering of blood pressure markers, in part due to the anti-inflammatory properties of ingredients in NEF.

Inflammation can also be associated with mitochondrial dysfunction.[32] Mitochondrial dysfunction, through activating the innate immune system, can foster inflammation while inflammation and oxidative stress can then damage mitochondria creating a cycle seen in chronic disease.[34] Lowering inflammation by NEF may have contributed to the improvement in mitochondrial function in addition to supplying the nutrients needed for ATP formation.

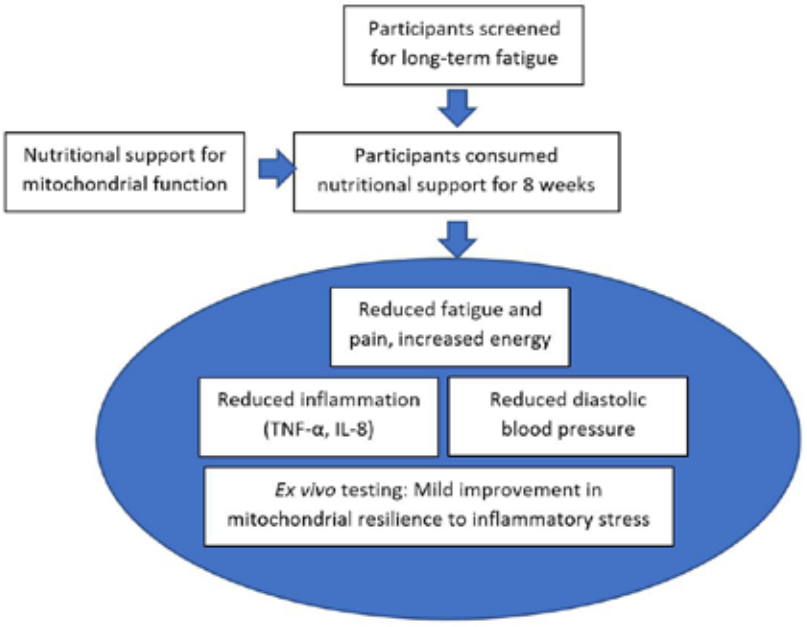
Other research studies have examined the use of nutritional supplements to improve fatigue. A meta-analysis review of randomized controlled trials utilizing various nutritional therapy found mixed results regarding improvement in fatigue levels with patients with chronic fatigue syndrome.[33] Only 4 out of 14 of these research trials showed improvement in fatigue levels with nutritional supplements that included NADH and CoQ_{10}, guanidinoacetic acid (GAA), pollen, and polyphenol-rich chocolate.[34-37] The cause of chronic fatigue syndrome is unknown, and multiple subgroups exist with different underlying etiology,[33,34] which may explain the lack of significant response to single-nutrient support. Most of these trials used 1 to 2 ingredients which may explain some of the negative findings. The single ingredient trials that did show positive decreases in fatigue included L-carnitine and CoQ_{10}.[39,40] In order to efficiently decrease fatigue, multi-ingredient formulations may be needed to specifically target multiple aspects of inflammation, mitochondrial function, cell membrane health, immune and nervous system function. A multi-ingredient nutraceutical blend was formulated combining many of the ingredients found to decrease fatigue in the literature, and evaluated in 2 clinical pilot studies. This multi-ingredient nutritional supplement combined several of the above ingredients including CoQ_{10}, carnitine, and NADH, and did find a positive reduction in fatigue scores in patients with chronic symptoms.[20,21]

The NEF formulation was designed to include targeted cofactors that are used in separate parts of mitochondrial metabolism, as well as phospholipids needed to support cell membrane formation and repair. Many of the ingredients also have antioxidant and anti-inflammatory properties. The results of NEF reported here showed faster improvements in fatigue reduction than what was seen in the previous multi-ingredient study.[20,21]

Because mitochondrial dysfunction can be a cause of fatigue and the NEF supplement was designed to support mitochondrial function, ex vivo testing of mitochondrial function was evaluated in PBMCs and PMN cells from blood samples at baseline, 4, and 8 weeks of consuming NEF. When cells from healthy non-fatigued people were exposed to an inflammatory insult ex vivo, a mild increase in mitochondrial mass was seen. This was accompanied by an average of 77% increase in the mitochondrial membrane potential in monocytes, which are expected to be the most active and immediate cell type to respond to the inflammatory insult, and as a result require more metabolic energy. In contrast, lymphocytes and PMN cells did not show increased mitochondrial membrane potential in response to inflammation, and the mild increase in mitochondrial mass did not reflect an improved functionality. At baseline, study participants with fatigue showed almost no increase in mitochondrial mass after exposure to an inflammatory insult, and the monocytes showed much less increase in mitochondrial membrane potential than in cells from healthy people, suggesting that the monocyte mitochondria in the fatigued population were not as capable as those in healthy individuals to produce sufficient cellular energy to support the monocyte responses to inflammation. After 4 weeks' consumption of NEF, participants showed improvement in mitochondrial mass to similar levels as seen in the control population. Furthermore, the participants with fatigue showed 21% improvement in the increased monocyte mitochondrial membrane potential in response to inflammation. Even though the increase was not statistically significant, we suggest there may be clinical relevance related to the observed reduction in fatigue scores.

At 8 weeks, there was an apparent return to baseline in the mitochondrial response to inflammation. When taking this in context to the reduced fatigue scores and improved energy and wellness, we suggest this may be a transient phenomenon that may be attributed to participants having less fatigue and being

Figure 7. Synopsis of the Clinical Pilot Study Where Participants Were Screened for Long-term Fatigue Lasting at Least 6 months Prior to Enrollment



Note: Nutritional support for cellular energy production via mitochondrial function was provided to 8 weeks. Rapid (1 week) improvement was seen in reduction of fatigue, pain, and diastolic blood pressure. Inflammatory markers were reduced after 4 weeks. The mitochondrial resilience to inflammatory stress was tested ex vivo in white blood cells from the study participants and showed mitochondrial impairment at baseline when compared to normal non-fatigued controls and showed a mild improvement at 4 weeks.

more active. This is often seen in studies on long-term chronic pain as well, where an initial rapid improvement allows people to become more physically active, leading to a higher burden on mitochondrial metabolism at a time where a person's physiology is still not stabilized at a new level of functioning. Therefore, we argue that the recovery of mitochondrial metabolic support of a person's energy expenditure may go through fluctuations before a new stable level is reached, and it is important to keep this in mind when supporting this recovery with nutritional methods. A longer-term study may be able to determine whether this was a short-term finding that would improve with study length.

The study with NEF showed highly significant improvements in fatigue, mitochondrial function, general well-being and decreased pro-inflammatory markers. The positive findings suggest that this study design has potential for establishing links between cellular energy production and the wellness of a person experiencing severe long-term fatigue. However, due to the small size of this study, conclusions must be made cautiously, and further studies are warranted. Since this was a small pilot study, follow-up studies with a larger number of participants including a randomized, placebo controlled, double blind trial, would help document the association between intake of the supplement and fatigue reduction and improvement of mitochondrial function. Comparison of NEF to single key ingredients would also help document to what extent the multifaceted multi-ingredient support of mitochondrial function is advantageous over and above single ingredients. A study should include wearable devices to help track activity levels and sleep quality. We also suggest that a longer consumption period may be necessary, to see whether the mitochondrial resilience can be brought closer to normal levels by consuming NEF. Such further investigation should examine the role of mitochondrial function and inflammation in situations of chronic fatigue, not limited to a formal diagnosis of chronic fatigue syndrome.

CONCLUSIONS

Fatigue is a common disabling symptom in chronic disease, and finding effective treatment methods for reducing fatigue is an important goal. Consumption of the NEF supplement blend was shown to be associated with reduced symptoms of moderate to severe fatigue, with alleviation of some of the symptoms within 1 week. NEF also decreased inflammation and pain while improving mitochondrial function (Figure 7). Inflammation and mitochondrial dysfunction contribute to the development and persistence of symptoms in chronic illness. NEF offers an option to improve the health of chronically ill patients by alleviating some of the most debilitating symptoms of fatigue and pain while improving the pathology of inflammation and mitochondrial dysfunction contributing to these issues.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Written informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board (Federalwide Assurance 2603), was obtained from the study participants.

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The clinical trial was conducted at NIS Labs, an independent contract research organization that specializes in natural products research and testing. The study was sponsored by Researched Nutritionals LLC, the manufacturer of the product tested in the clinical trial.

AUTHOR CONTRIBUTIONS

DH and GSJ designed the study. GSJ oversaw the trial and the biomarker testing. GSJ performed the data analysis. DH and GSJ wrote the manuscript. Both authors participated in final editing of the manuscript.

AUTHORS' DISCLOSURE STATEMENT

GSJ reports no conflicts of interest in this work. DH is employed as the Director of Physician Education and Clinical Trials for the study sponsor, Research Nutritionals, LLC.

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