METHODS FOR TREATMENT OF MOTOR AND COGNITIVE FUNCTIONS USING SODIUM NITRITE

Various aspects provide a method for treatment of motor and cognitive functions, comprising administering a composition comprising sodium nitrite and a pharmaceutical excipient to a subject.
FIELD OF INVENTION

The present disclosure relates, generally, to compositions and methods for the treatment of motor and cognitive functions using sodium nitrite.

GOVERNMENT RIGHTS

This invention was made with government support under grant numbers AG013038 and HL107105 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Advancing age is associated with systemic physiological dysfunction including impairments in motor function that can lead to disability and chronic disease. Because older adults comprise the fastest growing segment of the population, the incidence of physical impairment and associated costs are projected to increase dramatically in the absence of effective intervention. Implementing interventions that reverse or slow physiologic dysfunction until a later age (compression of disability) would extend "healthspan," i.e. the portion of lifespan during which function is sufficient to maintain function, independence, productivity and well-being.

The mechanisms underlying age-related physical dysfunction are complex and systemic. One recognized mechanism is the loss of muscle mass and cross-sectional area. However, given the dissociation between loss of muscle mass and strength, and the numerous upstream pathways essential for preservation of both muscle size and functional capacity, identification of systemic processes that contribute to multifactorial physiological impairments with aging is paramount. Examples of such upstream targets are inflammation and nitric oxide (NO) bioavailability. Advancing age is accompanied by an increase in chronic low-grade inflammation characterized by increases in pro-inflammatory mediators. IL-6 produced in the skeletal muscle may act as a myokine signaling molecule (Munoz-Canoves P, et al, Febs J, 2013; 280(17):413-48) whereas circulating IL-6 is strongly related to frailty (Ko F, et al, Age (Dordr), 2012; 34(3):705-15) and physical dysfunction (Ferrucci L, et al, J Am Geriatr Soc,
Inflammation is associated with functional ability and disability in older adults, and mortality and a frail mouse model. In addition, reduced production, bioavailability, and signaling of the critical signaling molecule, NO, and its oxidation products, nitrite and nitrate, appear to be linked to numerous expressions of physiological dysfunction of aging.

One intervention to target multiple domains of age-associated dysfunction is the nitrite anion, inorganic nitrite (Kevil CG, et al., Free Radic Biol Med., 2011; 51(3):576-93; Lundberg JO, et al., Nat Chem Biol, 2009; 5(12):865-9). Previously considered an inert byproduct of NO metabolism, nitrite now is recognized as a physiological important storage form of NO. Sodium nitrite administration is known to improve vascular endothelial dysfunction and arterial stiffness in old animals, and can restore tissue specific inflammatory cytokine levels to that of young animals thereby reversing age-related physiological dysfunction. Moreover, treatment with NO donors and nitrates has been shown to increase skeletal muscle mass when coupled with exercise in old animals, and to improve electrically stimulated muscle force in the extensor digitorum longus (EDL) of young animals.

It has been shown that 7-day treatment with dietary nitrate in young C57BL/6 male mice was sufficient to induce a leftward shift in the force-frequency curve in the EDL, such that greater contractile forces could be exerted for a given frequency of stimulation (Hernandez A, et al., The Journal of physiology, 2012; 590 (Pt 15):3575-83). Further, 6 weeks treatment of 18-mo old female C57BL/6 with an NO donor, isosorbide dinitrate, demonstrated improved vascular density and sarcolemma integrity in skeletal muscle particularly when coupled with voluntary exercise (Leiter JR, et al., Am J Physiol Cell Physiol, 2012; 302(9):C1306-15).

It has been demonstrated that nitrite concentrations are reduced with aging in multiple tissues and plasma, and that identical concentration of sodium nitrite supplementation in the drinking water increased nitrite concentrations in old mice to levels not significantly different from, or even greater than young control mice (Sindler AL, et al, Aging Cell, 2011; 10(3):429-37).

An increase in nitrite levels could affect physiological functions, including motor functions, in numerous ways. Dietary nitrates and inorganic nitrites consumed in the diet are reduced to NO, which exerts biological effects in numerous systems, including vascular, neural, and motor/skeletal muscle.

Accordingly, novel compositions and methods of use of sodium nitrite treatment as described herein are believed to be advantageous.
SUMMARY OF THE INVENTION

The present invention provides compositions and methods for treating declining motor and/or a method for improving cognitive function. In various aspects, methods for treating declining motor and/or a method for improving cognitive function in a subject are provided, administering an effective amount a pharmaceutical composition comprising sodium nitrite and a pharmaceutical excipient, wherein the composition comprises about 0.1 mg to about 5.0 mg of sodium nitrite per kg body weight per day. In various embodiments, the methods further comprise assessing an indicator comprising motor and/or cognitive function in the subject, wherein such assessment is conducted prior to administration of the pharmaceutical composition. In further embodiments, the indicator of motor and/or cognitive function comprises measuring muscle strength and force development, grip strength, balance, fatigability, mobility and dexterity, and wherein the step of administration is conducted only in response to an indicator at a threshold value. In further embodiments, an indicator of motor and/or cognitive function comprises measuring at least one metabolite from lipid, amino acid, peptide, nucleotide, xenobiotic, inflammatory cytokines and/or cofactor metabolic pathways, and wherein the step of administration is conducted only in response to an indicator at a threshold value. In further embodiments, the at least one metabolite comprises 11B-PGF2A isopropyl ester, PGF2a, sphingolipid, glycan, glycerophospholipid, fatty acyl, polyketide, choline, L-arginine, arachidonic acid, tryptophan, lysine, phenylalanine, leucine, dehydroepiandrosterone, cholesterol, LTB4, LTC4, PGE2, RVD1, lipoxin A4, and pro-inflammatory cytokines. In further embodiments, the pro-inflammatory cytokines comprise at least one selected from IL-1β, IL-6, IFN-γ and TNF-a. In further embodiments, the composition is administered over at least one of about 30 days, about 3 months, about 6 months, about 12 months, about 18 months, about 2 years, about 5 years, about 7 years, about 10 years, about 15 years, about 20 years, about 25 years, about 30 years, about 35 years, about 40 years, or over the lifetime of the subject.

In further aspects, a method of improving motor and/or cognitive function in a subject is provided comprising determining a decrease in motor and/or cognitive function in a subject; and administering an effective amount a pharmaceutical composition comprising sodium nitrite and a pharmaceutical excipient, wherein the composition comprises 0.1 mg to about 5.0 mg of sodium nitrite per kg body weight per day and wherein the composition is administered...
to said subject. In further embodiments, an improvement of motor and/or cognitive function is associated with an increase in sodium nitrite concentration in the subject.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The subject matter of the present invention is particularly pointed out and distinctly claimed in the concluding portion of the specification. A more complete understanding of the present invention, however, is best obtained by referring to the detailed description and claims when considered in connection with the drawing FIGS, wherein like numerals denote like elements and wherein:

FIG. 1 illustrates grip strength (g) (FIG. 1A), open field distance (m) (FIG. 1B) and endurance run (min) (FIG. 1C).

FIG. 2 illustrates cytokine expression (pg/mL) in mice.

FIG. 3 illustrates knee extension rate of torque development (Nm/s) (FIG. 3A) and RTD (Rate of Torque Development) (% change) (FIG. 3B), and individual rate of torque development (FIG. 3C), and knee flexion rate of torque development (Nm/s) (FIG. 3D) and individual rate of torque development (FIG. 3E).

FIG. 4 illustrates rapid step test errors (FIG. 4A) and rapid step test time (s) (FIG. 4C), and rapid step test (% change) (FIG. 4C).

FIG. 5 illustrates grip strength (kg) (FIG. 5A) and heelrise time (s) (FIG. 5B), and treadmill endurance (s) (FIG. 5C).

FIG. 6 illustrates executive function TMT-B (s) (Trail Making Test) (FIG. 6A), and TMT-B (% change) (FIG. 6B), and processing speed TMT-A (s) (FIG. 6C) and TMT-A (% change) (FIG. 6D), and individual TMT-A (s) (FIG. 6E), and individual TMT-B (s) (FIG. 6F).

**DETAILED DESCRIPTION**

The following description is merely exemplary in nature and is not intended to limit the present invention, its applications, or its uses. It should be understood that throughout the drawings, corresponding reference numerals indicate like or corresponding parts and features. The description of specific examples indicated in various embodiments of the present invention are intended for purposes of illustration only and are not intended to limit the scope of the invention disclosed herein. Moreover, recitation of multiple embodiments having stated features is not intended to exclude other embodiments having additional features or other embodiments incorporating different combinations of the stated features.
Furthermore, the detailed description of various embodiments herein makes reference to the accompanying drawing FIGS, which show various embodiments by way of illustration. While the embodiments are described in sufficient detail to enable those skilled in the art to practice the invention, it should be understood that other embodiments may be realized and that logical and mechanical changes may be made without departing from the spirit and scope of the present invention. Thus, the detailed description herein is presented for purposes of illustration only and not of limitation. For example, steps or functions recited in descriptions any method, system, or process, may be executed in any order and are not limited to the order presented. Moreover, any of the step or functions thereof may be outsourced to or performed by one or more third parties. Furthermore, any reference to singular includes plural embodiments, and any reference to more than one component may include a singular embodiment.

As used herein, a "pharmaceutically acceptable excipient" refers to any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Some examples of pharmaceutically acceptable excipients include water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Additional examples of pharmaceutically acceptable substances include wetting agents or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the composition. Pharmaceutical compositions of the present invention and methods for their preparation will be readily apparent to those skilled in the art. Such compositions and methods for their preparation may be found, for example, in Remington's Pharmaceutical Sciences, 19th Edition (Mack Publishing Company, 1995). Pharmaceutical compositions are preferably manufactured under GMP conditions. A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses.

As used herein, a "unit dose" is a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. In some embodiments, one or more active ingredients may be present in the composition in addition to sodium nitrite. The amount of the active ingredient is generally equal to the dosage of the active ingredient which
would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

As used herein, a "therapeutically effective amount" or "effective amount" of a composition refers to an amount effective in the prevention or treatment of a disorder for the treatment of which the composition is effective. A "disorder" refers to any condition that would benefit from treatment with the composition. In some embodiments, a composition of the invention is effective in the treatment of declining motor and cognitive functions.

As used herein, "treated," "treating" or "treatment" refers to the diminishment or alleviation of at least one symptom associated or caused by the state, disorder or disease being treated. For example, treatment can be diminishment of one or more symptoms of a disorder or complete eradication of a disorder. In embodiments, the disorder may be declining or decreasing motor and/or cognitive functions, or a disorder associated with declining or decreasing motor and/or cognitive functions.

In various aspects, without being bound to any theory, the administration of sodium nitrite leads to an increase in sodium nitrite levels that result in an improvement or increase in motor and/or cognitive function. In various embodiments, an increase in sodium nitrite levels improves or increases one or more motor and/or cognitive functions. In embodiments, motor and cognitive function includes, but is not limited to, grip strength, muscle strength, force development, balance, fatigability, mobility and dexterity. In aspects a decline or decrease in motor and/or cognitive function may be associated with one or more indicators. In various embodiments, a threshold value for each of the one or more indicators may be determined by any suitable technique, whereas such technique may be now known or further developed. In aspects, an indicator may be determined by visual observation of a subject's physical condition, using a blood or sample based assay or test, or by any other means available as known to those of ordinary skill in the art. In various embodiments, a subject's physical condition may be observed using one or more physical tests to determine a subject's motor and/or cognitive function. In aspects, a decline or decrease in motor and/or cognitive function may be determined by measurement of at least one metabolite. In embodiments, metabolites include but are not limited to lipid, protein, amino acid, carbohydrate, nucleic acids, nucleotide, xenobiotic, inflammatory cytokines and cofactor. In embodiments, at least one metabolite comprises 11B-PGF2A isopropyl ester, PGF2a, sphingolipid, glycan, glycerophospholipid, fatty acyl, polyketide, choline, L-arginine, arachidonic acid, tryptophan,
lysine, phenylalanine, leucine, dehydroepiandrosterone, cholesterol, LTB4, LTC4, PGE2, RVD1, lipoxin A4, and pro-inflammatory cytokines. In embodiments, the pro-inflammatory cytokines comprise at least one selected from IL-1β, IL-6, IFN-γ and TNF-a. In further embodiments, metabolites may be measured from a sample, for example, a plasma sample, wherein the sample is extracted from a subject.

In various aspects of the invention, sodium nitrite may be administered as an active ingredient in therapeutic compositions, for treating a decline or decrease in motor and/or cognitive function, among others. Generally, sodium nitrite is suitable to be administered in association with one or more pharmaceutically acceptable excipient(s). The term 'excipient' is used herein to describe any ingredient other than the active ingredient. The choice of excipient(s) will to a large extent depend on factors such as the particular mode of administration, the effect of the excipient on solubility and stability, and the nature of the dosage form.

Actual dosage levels of the active ingredient(s) (for example, sodium nitrite) in pharmaceutical compositions and formulations may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the composition found in the formulation, the route of administration, the time of administration, the rate of excretion of the particular composition being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular composition employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician having ordinary skill in the art, can readily determine and prescribe the effective amount of the pharmaceutical composition of the present invention required. For example, the physician could start doses of the composition of the invention employed in the pharmaceutical formulation at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In various aspects, the concentration of the active ingredient is between about 10 mg to about 6000 mg of sodium nitrite per ml of liquid formulation. In embodiments, the concentration of sodium nitrite is about 100 mg, about 125 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 500 mg, about 750 mg, about 1000 mg, about 1200 mg.
about 1500 mg, about 2000 mg, about 2500 mg, about 3000 mg, about 3500 mg, about 4000 mg, about 4500 mg, about 5000 mg, about 5500 mg, or about 6000 mg per ml of liquid formulation.

In various aspects, the concentration of sodium nitrite may be calculated based on a subject's body weight. In embodiments, the concentration of sodium nitrite is between about 0.01 mg to about 10 mg, about 0.5 mg to about 5.0 mg and about 0.1 mg to 1.0 mg per kg body weight. In embodiments, the concentration of sodium nitrite is about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.75 mg, about 1 mg, about 1.5 mg, about 2.0 mg, about 2.5 mg, about 3.0 mg, about 3.5 mg, about 4.0 mg, about 4.5 mg, or about 5 mg per kg body weight. In embodiments, the concentration of sodium nitrite is about 0.84 mg to about 2.8 mg of sodium nitrite per kg, for body weight of about 56.2 kg to about 95.1 kg, per day. In embodiments, the concentration of sodium nitrite administered to a subject is about 80 mg dose to about 160 mg dose per day of sodium nitrite to a subject weighing about 150 lbs (-68 kg).

In various aspects, a composition of the invention may be administered as a daily dose over a period of time to a subject. In embodiments, a composition of the invention may be administered as a single dose or as multiple doses, for example a daily 80 mg dose of sodium nitrite may be administered to a subject as a 40 mg dose of sodium nitrite given twice a day. In embodiments, a composition of the invention may be administered chronically or long-term. In embodiments, the composition may be administered for a period of days, weeks, months, years or continued therapy over the lifetime of a subject. In embodiments, the composition may be administered for a period of about 30 days, about 3 months, about 6 months, about 12 months, about 18 months, about 2 years, about 5 years, about 7 years, about 10 years, about 15 years, about 20 years, about 25 years, about 30 years, about 35 years, or about 40 years. In embodiments, a treatment regime may be determined for an individual subject dependent on various factors. In some embodiments, a factor may include, but not be limited to, a determination of the change in motor and/or cognitive function in response to administration of the composition of the invention. In embodiments, a change in motor and/or cognitive function may be an improvement or increase in motor and/or cognitive function. In embodiments, a subject exhibiting an immediate response to the composition, for example, an immediate increase in motor and/or cognitive function, may require less frequent doses than a subject exhibiting a response to the composition at a later time or after several doses.
Any method for administering pharmaceutical or nutriceutical-like compounds in the art may suitably be employed in accordance with the invention.

EXAMPLES

Example 1

Treatments that improve or reverse physical function via systemic mediators of inflammation and NO bioavailability in middle/older age may prevent the increase in chronic disease and disability with aging.

Oral sodium nitrite treatment for age-related decline and its ability to improve motor function in old mice was assessed. Specifically in this example, nitrite-induced improvements in motor function in old mice related to skeletal muscle mass, size and inflammation were determined.

Methods

Animal husbandry and experimental groups

C57BL/6 mice were obtained from the National Institute on Aging rodent colony. All mice were housed in an animal care facility at the University of Colorado at Boulder on a 12:12 light/dark cycle. Upon arrival, mice were ear punched for identification and housed in groups (~3-4 per cage). Mice were acclimated to an existing colony in the animal care facility for 4 weeks under a 12-hr light/dark schedule (7am to 7pm light cycle) with normal rodent chow and water ad-libitum. Following baseline motor function testing, mice were organized into treatment and control subgroups (control n = 40, sodium nitrite treated n = 22) for 8-week intervention periods spanning 20-22 months of age (n = 38, 12 treated) or 24-26 months of age (n = 24, 10 treated). The control animals continued drinking regular drinking water and the treated animals were administered sodium nitrite-supplemented drinking water (50 mg/L). Drinking water intake was monitored 4 times per week for nitrite-treated and control animals.

Behavioral motor function tests were administered in all animals before and after the 8-week intervention period. An additional cohort of young C57BL/6 mice were obtained from Charles River at 2 months of age (n = 87) to serve as young reference animals and were administered the same battery of behavioral tests (10). A subset of animals from each cohort was sacrificed (20-22 month n = 8, 24-26 month n = 12, equal numbers nitrite-treated and controls per cohort, Young n = 6) for skeletal muscle and tissue collection. All animal procedures conformed to
the Guide to the Care and Use of Laboratory Animals (NIH publication n. 85-23, revised 1996) and were approved by the UCB Animal Care and Use Committee.

Experimental measurements

Motor Function and Home Cage Activity

A battery of behavioral motor function tests was administered over three days. Forelimb grip strength (day 1), open field locomotor activity (day 1), accelerating (day 2) and endurance rota-rod run ability (day 3) were assessed. Briefly, grip strength was measured as the average force recorded at forepaw release over five trials using a custom grip strength device that included a force transducer attached to a trapeze grip. Open field distance (locomotor activity) was determined as the total distance traveled during 5 minutes in a novel arena, quantified offline from a video-recorder using multi-arena video tracking software (EthoVision XT; Noldus Information Technology, Leesburg, VA, USA). Latency to fall from a five-station accelerating rota-rod (Ugo Basile, Comerio, Italy) over three trials performed on a single day, was used as a functional outcome (average across three trials) but was primarily administered to determine the speeds at which the endurance rota-rod trial would be set (maximal latency to fall from the three trials). In the endurance rota-rod protocol, the time and distance run until falling off the rota-rod at speeds relative to accelerating maximum determined endurance run ability assessed during a single session. The test comprised four consecutive phases corresponding to 25% (refresh, 2 min), 50% (warm-up, 5 min), 75% (endurance 1. 10 min or until fall) and 100% (endurance 2, 20 min or until fall) of group maximum, and the latency to fall was recorded during the endurance phases.

Home cage activity in old animals was determined by telemetry in a subset of treated and control animals (n = 12 per group). Transmitters were surgically implanted in the intraperitoneal cavity via ventral incision to detect core body temperature and gross home cage activity (TA-F10 transmitters, Data Sciences International (DSI), St. Paul, MN U.S.A.). Home cage activity and body temperature were determined over 24-hour periods following 8-weeks of sodium nitrite or drinking water.

Muscle mass and size

Following 8 weeks of sodium nitrite supplemented or regular drinking water, the quadriceps and gastrocnemius muscles were dissected and weighed; mass was recorded as absolute and normalized to body mass. Quadriceps muscles were cut in half across the muscle belly, and halves were either placed in Eppendorf tubes and snap frozen in liquid nitrogen and
stored at -80 °C until extraction of protein, or fixed in formaldehyde, paraffin embedded, and sectioned mid-belly. Sections were stained with hematoxylin and eosin (H&E; Fisher, NH) and imaged using a photomicroscope (TS100 Nikon Eclipse) to determine quadriceps cross-sectional area across groups.

5 Skeletal muscle protein expression

Quadriceps were excised, cleared of surrounding tissues, and frozen in liquid nitrogen before storage at -80 °C. The tissue was homogenized in ice-cold Radioimmunoprecipitation Assay (RIPA) lysis buffer containing protease and phosphatase inhibitors [Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN, USA) and 0.01% phosphatase inhibitor cocktail (Sigma, St Louis, MO, USA)]. Equal amounts of protein (15 µg) were used to determine concentrations of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF-a) by multiplex ELISA (Searchlight Mouse Inflammatory Cytokine Kit; Auslion Biosystems; Billerica, MA, USA) following manufacturer’s instructions. TNF-a levels were also determined separately by ELISA (Mouse TNF-a ELISA Max™ Deluxe Sets, BioLegend, San Diego, CA, USA) per manufacturer’s instructions.

Statistics

Data are presented as mean ± SEM (see FIGS) or mean ±SD (see tables and text). Statistical analysis was performed with SPSS software (v21, IBM, Somers, NY, USA). The two age groups of old animals (20-22 mo and 24-26 mo) were combined to maintain statistical power. Prior to primary analysis of the motor function outcomes, normality of each variable was assessed with the Kolmogorov-Smirnov test. No outliers (>3 SD) were identified. Group difference in motor function with sodium nitrite treatment or drinking water was determined by mixed-model ANOVA (Analysis of Variance; within factor, time; between factor, treatment). Differences in young, old control and old treated mice (post treatment only) for motor function, body mass, muscle mass, and pro-inflammatory cytokines were compared by analysis of variance (ANOVA) followed by post-hoc Tukey’s HSD (Honest Significant Difference) means-comparison. Differences in home-cage activity and temperature between old treated and old control mice were determined by t-test. The contribution of pro-inflammatory cytokines and treatment group on the change in motor function outcomes was determined by stepwise, linear regression equations, with separate models run to predict grip strength, open
field distance, and endurance run. An absence of multicollinearity for the explanatory variable was verified by variance inflation factor (VIF) and tolerance.

**Results**

**Animal characteristics**

Characteristics of the groups are illustrated in Tables 1 and 2 provided herein. Body mass, muscle mass and quadriceps cross sectional area were not statistically different between old control and old nitrite treated animals (Table 1, p > 0.05). Sodium nitrite treatment had no effect on home cage activity or core body temperature in old animals as measured by surgically implanted telemetry devices (Table 2, p > 0.05).

**Table 1.** Skeletal muscle size and mass

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body mass (g)</strong></td>
<td>33.8 ± 2.6</td>
<td>34.5 ± 2.8</td>
</tr>
<tr>
<td><strong>Muscle Mass (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadriceps</td>
<td>145 ± 18</td>
<td>173 ± 30</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>140 ± 21</td>
<td>138 ± 15</td>
</tr>
<tr>
<td><strong>Cross Sectional Area (mm²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadriceps</td>
<td>17.4 ± 4.12</td>
<td>19.2 ± 3.50</td>
</tr>
</tbody>
</table>

**Table 2.** Telemetry acquired home cage activity and body temperature

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Home cage activity (avg count/day)</strong></td>
<td>20.9 ± 3.1</td>
<td>21.5 ± 3.8</td>
</tr>
<tr>
<td><strong>Body temperature (°C)</strong></td>
<td>36.2 ± 1.50</td>
<td>36.1 ± 0.95</td>
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</table>

**Sodium nitrite improves motor functions in old mice**

Motor function was impaired in old compared to young control mice, but eight weeks of nitrite treatment improved function in old animals (FIG. 1A, B and C, multivariate p < 0.001). Prior to treatment, age-related declines in motor function were observed between young and old animals for grip strength (FIG. 1A, Y: 130 ± 14 g; O: 96.1 ± 15.1 g, p < 0.001), grip strength per body mass (Y: 5.58 ± 0.55; O: 2.75 ± 0.45, p < 0.001), distance traveled in a novel open arena (FIG. IB, Y: 1859 ± 265 cm; O: 1525 ± 516 cm, p < 0.001), latency to fall from a constantly accelerating rota-rod (Y: 240 ± 57 s; O: 168 ± 61 s, p < 0.001), and rota-rod endurance run time (FIG. 1C, Y: 14.4 ± 5.9 min; O: 10.9 ± 7.2 min, p < 0.001) and distance (Y: 24.4 ± 9.9 m; O: 14.9 ± 7.9 m, p < 0.001). There were no significant statistical differences between old treated and old control animals prior to treatment with sodium nitrite (p > 0.05, all). Nitrite treatment improved grip strength in old animals (p < 0.05), grip strength per body mass, and endurance run.
mass (p < 0.05), and endurance rota-rod distance (p < 0.01) and attenuated further age-related decline in open field distance (p < 0.001) in old animals (Table 3). Endurance run time (p < 0.01) was not only improved in old animals following 8-weeks sodium nitrite treatment, but was restored to that of young animals (p > 0.1 young vs. old treated). There was no significant effect of nitrite treatment on accelerating rota-rod latency in old animals (p > 0.05, Table 3).

**Table 3.** Motor function outcomes in young, old control and old sodium nitrite treated animals.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Pre</th>
<th>Young</th>
<th>Old Control</th>
<th>Old Nitrite</th>
<th>Post</th>
<th>Old Control</th>
<th>Old Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grip strength (g)</td>
<td>« = 87</td>
<td>« = 40</td>
<td>« = 22</td>
<td>« = 40</td>
<td>« = 22</td>
<td>« = 40</td>
<td>« = 22</td>
</tr>
<tr>
<td>Grip strength per mass</td>
<td>130 ± 14</td>
<td>95.5 ± 14.4</td>
<td>99.4 ± 14.7</td>
<td>97.0 ± 16.6</td>
<td>111.3 ± 16.9*</td>
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<tr>
<td>Endurance time (min)</td>
<td>5.58 ± 0.55</td>
<td>2.75 ± 0.43</td>
<td>2.81 ± 0.45</td>
<td>2.83 ± 0.44</td>
<td>3.25 ± 0.45*</td>
<td></td>
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<tr>
<td>Endurance distance (m)</td>
<td>14.4 ± 5.9</td>
<td>10.7 ± 7.8</td>
<td>10.5 ± 5.6</td>
<td>8.4 ± 6.3</td>
<td>14.9 ± 5.2*</td>
<td></td>
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<tr>
<td>Open Field (cm)</td>
<td>24.4 ± 9.9</td>
<td>14.6 ± 8.5</td>
<td>14.5 ± 5.7</td>
<td>11.3 ± 5.9</td>
<td>17.8 ± 5.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accelerating rota-rod (s)</td>
<td>18.6 ± 2.7</td>
<td>16.4 ± 4.9</td>
<td>13.6 ± 5.2</td>
<td>11.8 ± 4.5</td>
<td>14.9 ± 4.8*</td>
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| Accelerating rota-rod (s)    | 240 ± 57  | 160.9 ± 61.3 | 175.8 ± 63.7 | 167.2 ± 55.4 | 184.6 ± 46.5 |

Significant multivariate treatment effect for all motor functions (p < 0.001). * indicates significant univariate time x treatment interactions (p < 0.05). ▲ indicates motor function restored to that of young animals (t-test, p > 0.1).

**Sodium nitrite treatment rescues inflammation in skeletal muscle of old mice**

Inflammation, as determined by protein expression of the inflammatory cytokines IL-1β, IL-6, INFγ, and TNFα was increased in quadriceps muscle of old compared with young mice. Treatment with sodium nitrite reduced skeletal muscle inflammatory cytokines IL-1β, INFγ, and TNFα selectively in old mice, normalizing the expression to levels observed in young controls (p < 0.05, FIG. 2). Sodium nitrite treatment ameliorates age-associated increases in skeletal muscle inflammation in old animals.

**Inflammatory cytokines predict improvements in motor functions in old animals**

The relative contribution of skeletal muscle inflammatory cytokines and treatment to motor function gains was assessed with stepwise linear regression models for grip strength (4 variables: $R^2 = 0.822$, p < 0.001, power = 100%, maximum VIF = 0.784, minimum tolerance =
1.275), open field distance (3 variables: $R^2 = 0.574$, $p < 0.01$, power = 99.9%, maximum VIF = 0.884, minimum tolerance = 1.132), and endurance run time (4 variables: $R^2 = 0.477$, $p < 0.05$, power = 91.2%, maximum VIF = 0.811, minimum tolerance = 1.234). The improvements in motor function were predicted by selective inflammatory cytokines, such that, greater improvements in function were negatively related to markers of inflammation, with the exception of IL-6, which was positively associated with endurance run time improvement. Each inflammatory marker contributed to the overall models describing the influence of inflammation on functional outcomes. IL-1$\beta$, and INF$\gamma$ were demonstrated to be predictive of function. Moreover, nitrite treatment was demonstrated as an important explanatory variable in describing functional gains in each model (Table 4).

Table 4. Regression models demonstrating relations between skeletal muscle cytokine expression and motor functions in old C57BL/6 mice.

<table>
<thead>
<tr>
<th>Grip strength ($R^2 = 0.822$, $p &lt; 0.001$)</th>
<th>Beta</th>
<th>Standard Error</th>
<th>Partial Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1$\beta$</td>
<td>-3.68</td>
<td>1.79</td>
<td>-0.481*</td>
</tr>
<tr>
<td>INF$\gamma$</td>
<td>-5.27</td>
<td>1.39</td>
<td>-0.711*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-13.0</td>
<td>8.76</td>
<td>-0.369</td>
</tr>
<tr>
<td>Treatment Group</td>
<td>19.8</td>
<td>3.70</td>
<td>0.819*</td>
</tr>
<tr>
<td>(0= control, 1 = Nitrite)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Open field distance ($R^2 = 0.574$, $p &lt; 0.01$)</th>
<th>Beta</th>
<th>Standard Error</th>
<th>Partial Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1$\beta$</td>
<td>-1.14</td>
<td>0.53</td>
<td>-0.484*</td>
</tr>
<tr>
<td>Treatment Group</td>
<td>3.10</td>
<td>1.17</td>
<td>0.447*</td>
</tr>
<tr>
<td>(0= control, 1 = Nitrite)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>-2.61</td>
<td>2.12</td>
<td>-0.302</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Endurance Run Time ($R^2 = 0.477$, $p &lt; 0.05$)</th>
<th>Beta</th>
<th>Standard Error</th>
<th>Partial Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>-5.20</td>
<td>4.30</td>
<td>-0.318</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.04</td>
<td>0.90</td>
<td>0.304</td>
</tr>
<tr>
<td>INF$\delta$</td>
<td>-1.08</td>
<td>0.84</td>
<td>-0.513*</td>
</tr>
<tr>
<td>Treatment Group</td>
<td>2.98</td>
<td>1.95</td>
<td>0.391</td>
</tr>
<tr>
<td>(0= control, 1 = Nitrite)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates partial correlation $p < 0.05$
Discussion

It was demonstrated that 8 weeks of sodium nitrite treatment in old male C57BL/6 mice improved motor functions and restored endurance run time to that of young animals. The preclinical findings, therefore, support treatment with sodium nitrite for the prevention of age-related motor impairment related to frailty and disability with aging in humans.

Age-related motor dysfunction

A dissociation between changes in skeletal muscle mass and functional behavioral outcomes was demonstrated (see for example, Tables 1-4), which indicates a more complex, and systemic, relation between age and motor output. The restoration of multiple subdomains of function by sodium nitrite suggests that NO bioavailability and subsequent signaling may be a potent mediator for the preservation of age-related motor functions.

Sodium nitrite treatment was demonstrated to improve multiple subdomains of motor function and restore endurance run time to those observed in young animals. Sodium nitrite treatment improved motor function in a setting of impaired baseline function.

The improvements in motor function demonstrated that sodium nitrite treatment increases nitrite levels systemically and leads to the observed improvements in motor functions. While not wishing to be bound by theory the results support that nitrite supplementation both boosts NO bioavailability, which could directly enhance blood flow and vascularization thereby improving systemic function, and alters gene and protein expression or activity, which would thereby feedback to reduce oxidative stress, and importantly, inflammation.

Inflammation

It was demonstrated that inflammation increases with aging as indicated by a marked increase in inflammatory cytokines in old compared with young control mice (FIG. 2). Sodium nitrite treatment reduced the expression of several pro-inflammatory cytokines, for example, IL-1β, IFNγ and TNFa, in skeletal muscle of old mice to concentrations observed in young controls.

It was demonstrated that the restoration of inflammatory cytokines was associated with functional performance. IL-6 was not significantly restored with sodium nitrite and reduced levels of IL-6 were not demonstrated as predictive of motor function (FIG. 2). The potent anti-inflammatory effect of sodium nitrite treatment in skeletal muscle was demonstrated, and this effect is associated with the functional improvements observed with 8 weeks treatment.
Late-life treatment with sodium nitrite improved multiple subdomains of motor functions in old C57BL/6 mice and restored endurance run time to that of young animals. The improvement demonstrated in motor functions are mediated by anti-inflammatory influences of sodium nitrite on skeletal. The improvement demonstrated in functional capability and inflammatory profiles were independent of changes in skeletal muscle mass, skeletal muscle cross-sectional area, and habitual activity.

Example 2

Methods

Subjects

A total of 87 middle-aged and older (age 50-80 years) adults were recruited from the community using local newspaper and radio advertisements, university email advertisements and direct mailings. All subjects were non-diabetic, had BMI <40 kg/m², were cognitive intact as indicated by a Mini Mental Status Exam score >25, and were free of clinical diseases as assessed by medical history, physical examination, blood chemistry and resting and maximal exercise ECG and BP (blood pressure). Subjects were not taking vasodilating drugs, drugs metabolized by the same liver enzymes as sodium nitrite, or CNS depressants or drugs affecting neurological health. Women were post-menopausal for at least 1 year and had not taken hormone replacement therapy for at least the previous 6 months. Subjects were not excluded based on physical activity, though this was used as a potential covariate in regression analyses. Following screening, 33 subjects were admitted to the study.

All study procedures complied with the Declaration of Helsinki and the informed consent and study documents were approved by the Institutional Review Board of the University of Colorado Boulder and the Scientific Advisory and Research Committee of the University of Colorado Denver. The nature, benefits and risks of the study were explained to the volunteers and their written informed consent was obtained before participation.

Study procedures

Measurements were performed at the University of Colorado Boulder Clinical Translational Research Center (CTRC) and the Neurophysiology of Human Movement Laboratory. The CTRC measures of subject characteristics and the metabolome were obtained following 12-h overnight fast and 24-h abstention from alcohol and exercise. Motor and cognitive measures were obtained 2-h after ingesting a small snack, and following 24-h abstention from alcohol.
Following screening and baseline measures subjects were randomized to low-dose (40 mg capsules, 2x/day), high-dose (80 mg capsules, 2x/day) of sodium nitrate or placebo (0 mg capsules, 2x/day) for 10 weeks. Adherence and safety were assessed at days one and two, and weeks four, eight and ten. Following 10-weeks treatment, baseline study procedures were repeated. During the 10-week intervention/control period, 1 subject dropped out of the treatment groups (x) and 1 dropped out of the control group. Therefore 31 subjects completed the study: 11 in the low-dose group, 10 in the high-dose group, and 10 in the placebo group.

**Subject characteristics, blood assays and dietary analysis**

BMI (body mass index) was calculated from height and weight to the nearest 0.1 kg. Total body fat, total lean mass, and regional lean mass were determined using dual energy X-ray absorptiometry (DEXA; GE/Lunar) (Kohrt WM., Preliminary evidence that DEXA provides an accurate assessment of body composition, J Appl Physiol, (1985), 1998;84(1):372-7). BP was measured after 15 min of supine rest at least three times and averaged using a semi-automated device (Dynamap XL; Johnson and Johnson). Diet composition was estimated from 3-day food intake records (The Food Processor 8.2; ESHA Research) analyzed by a CTRC bionutritionist to identify and control for nitrate-rich diet. Fasting serum concentrations of glucose, insulin and total cholesterol were determined using standard assays performed by the University of Colorado at Denver Health Sciences Center Adults CTRC core laboratory. VC\(^\text{max}\) (maximal oxygen consumption) was assessed during incremental treadmill exercise performed to exhaustion. Habitual physical activity was assessed at baseline from estimates of daily energy expenditure during leisure time and occupational activities. Usual daily activities before and after the treatment period were assessed via the CHAMPS (Community Healthy Activities Model Program for Seniors) Activities Questionnaire for Older Adults and estimated total kcals and total steps as measured by 7-day (5 week day, 2 weekend day) monitoring by Actigraph (wGT3X-BT monitor). The CHAMPS questionnaire assesses weekly frequency and duration of various physical activities typically undertaken by older adults.

All assays were performed by the University of Colorado at Denver Health Sciences Center Adults CTRC core laboratory. Fasting serum concentrations of total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triacylglycerols (triglycerides), glucose and white blood cell counts were determined using standard assays. Plasma concentrations of CRP (C-reactive protein) were measured using a
high-sensitivity Chemistry Immuno Analyzer (AU400e; Olympus). Plasma interleukin-6 (IL-6), tumor-necrosis factor-a (TNFa) (both R&D Systems), and oxidized LDL (Alpco) were measured by ELISA. Total antioxidant status was measured by a colorimetric assay (Randox Laboratories).

Acute and chronic levels of plasma nitrites and nitrates were assessed by HPLC. Acute levels were obtained 30 min following a single (initial) dose. Chronic levels were assessed at 10 weeks of sodium nitrite or placebo, 12 hours after the most recent dose.

**Motor and cognitive function**

Motor and cognitive function was assessed during a single, ~3-hour session in the Neurophysiology of Human Movement Laboratory on University of Colorado Boulder campus. Table 5 provides an overview of the motor/cognitive function testing session and outcomes. Briefly, motor function was assessed by measurement of muscle strength and force development, balance, fatigability, mobility and dexterity. Simple and time-effective tests of cognition, trail making tests part A and B, supplemented the motor battery. Additionally, questionnaires related to sense of fatigue and perceived exhaustion were incorporated. Subjects were allowed ample rest time between physical tests to ensure safety and adequate recovery from the previous task; the breaks times listed in Table 5 reflect minimal rest times after each test.

**Table 5.** Overview of motor function tests.

<table>
<thead>
<tr>
<th>Subdomain</th>
<th>Test</th>
<th>Equipment</th>
<th>Trials</th>
<th>Breaks</th>
<th>Variables</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Daily Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobility</td>
<td>400 m walk</td>
<td>Indoor track, timer</td>
<td>1</td>
<td>10</td>
<td>Time</td>
<td>Endurance walk time</td>
</tr>
<tr>
<td></td>
<td>Timed Up &amp; Go (TUG)</td>
<td>Standard height chair, timer</td>
<td>1</td>
<td>5</td>
<td>Time</td>
<td>&quot;TUG&quot; time</td>
</tr>
<tr>
<td><strong>Dexterity</strong></td>
<td>Pegboard</td>
<td>25-hole grooved pegboard</td>
<td>3</td>
<td>2</td>
<td>Time</td>
<td>Dexterity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Balance</strong></td>
<td></td>
<td>Tape, timer</td>
<td>3</td>
<td>2</td>
<td>Time</td>
<td>Step time</td>
</tr>
<tr>
<td></td>
<td>Rapid Step Test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Strength / Torque Development</strong></td>
<td>Knee Extension</td>
<td>Force transducer</td>
<td>3-5</td>
<td>5</td>
<td>Peak torque</td>
<td>Knee extension</td>
</tr>
</tbody>
</table>
MVC Knee Extension RTD
Force transducer 3-5 5 Rate of torque (N-m/s) strength Knee extension power
Knee flexion MVC Force transducer 3-5 5 Peak torque (N-m) Knee flexion strength
Knee flexion RTD Force transducer 3-5 5 Rate of torque (N-m/s) Knee flexion power
Hand grip MVC Hand dynamometer 3-5 5 Handgrip strength (kg) Grip strength

Cognition
Trail Making Timer 1 1 TMT-A time Processing speed
Test-A Time
Trail Making Timer 1 1 TMT-B time Executive function
Test-B

Fatigability
Heel-rise to Metronome, 1 10 Time Endurance
exhaustion Timer

Tests are listed in order of presentation. Inter-trial intervals of 60-s were used during strength trials. Breaks are reported as minimum subject rest time following test (min).

Daily Function
Daily function was quantified by tests of mobility and dexterity, which are known to decline with advanced age and may predict loss of independence and increased risk of severe age-related disability in older adults. These tests were included in the battery to demonstrate the high-functional capacity at baseline of the community-dwelling elders included in the pilot clinical investigation. Mobility was quantified with measures of endurance walk and Timed Up and Go. Mobility has been identified by the International Classification of Functioning, Disability, and Health as essential in classification of activity limitation (WHO 2001, available at the CDC Government website), and was measured to determine the extent to which sodium nitrite treatment may be related to tasks related to everyday activity in older adults. Walking was assessed as the time to walk 400 m. Subjects walked at a brisk pace for 2.5 laps around an indoor track; the instructions given to each subject immediately before testing were: "Please walk 2.5 laps at a brisk, but comfortable pace. Walk as quickly as you would if you were at an airport, and you were a few minutes late for your plane. This should be a brisk walk, but please, no running." Mobility was also quantified by a Timed Up and Go task in which the
time to stand unassisted, walk out 3-m and return to a seated position was assessed, a task that is related to loss of independence in older adults.

Dexterity, or hand function, was measured by the Grooved Pegboard test, a standardized test of manual dexterity that is related to decline in older adults compared with middle-aged and young adults. Subjects were instructed to place the 25 keyhole shaped pegs into a pegboard (Lafayette Instruments; Lafayette, IN) as fast as possible. All subjects completed three trials and the average and fastest time was recorded.

**Balance**

Balance during stepping was measured by the time and number of errors committed during a rapid step test. Prior to the rapid step test, the maximum step length was determined. Subjects began standing at a natural stance width (marked by tape forming a box) then with the arms folded across the chest they stepped as far as possible on one leg and then returning to the initial position in one step. Subjects were allowed one practice and three measured trials of maximal stepping in each leg-direction (right and left leg: front, side, back). Maximum step was quantified as the average step length over a series of three trials in each leg-direction, and was controlled between pre- and post-intervention conditions. Based on the maximum step length, taped targets were placed on the floor corresponding to 80% of the maximum step length for each leg and direction. The rapid step test required the subject to step past each target position with one leg as quickly as possible and return to the initial starting position. An investigator gave the command for each leg and direction (e.g., "left-front") in a random order that includes three steps in each of six leg-directions, for a total of 18 repetitions. The command was given as the subject returned to the starting position; the subject’s speed of stepping dictated the pace of the commands. An error was defined as failure to step beyond the target, loss of balance such that the experimenter must approach the subject to assist in preventing a fall; failure to return to initial position, multiple steps, noncompliance with leg or direction, or failure to keep arms crossed. Total time to complete 18 repetitions and total errors occurring during the 18-repetition trial was recorded. The rapid step test was repeated 3 times with a minimum of one minute of rest between trials.

**Muscle strength and force development**

Muscle strength was quantified as the peak torque (N·m) - recorded force (N) multiplied by the measured moment arm for each subject - during slow (maximal voluntary contraction, MVC), and the peak torque per unit time during fast (N·m/s, rate of torque
development, RTD) isometric contractions for the knee extensors, and knee flexors. It has been demonstrated that values achieved on these tests are often uncorrelated as the performance depends on both muscle size and the capacity of the nervous system to provide the requisite activation signals. The torque exerted by the limb during the strength and rate of torque development tasks was measured with a strain-gauge transducer (MLP-300, Transducer Techniques, Temecula, CA) (FIG. 3A-E). The force signal was low-pass filtered (0-50 Hz; Coulbourn Instruments, Allentown, PA), recorded on a computer, and digitized at 1,000 samples/s. Knee extension (FIG. 3A, 3B and 3C) and flexion (FIG. 3D and 3E) MVC torque was recorded during 3-5 maximal isometric contractions in which subjects were instructed to slowly increase their force over 3 counts and to maintain their maximal isometric contraction for 3 sec. During the RTD task subjects were instructed to perform 5-10 isometric contractions, with each contraction performed as quickly and forcefully as possible but without holding the contraction at maximum torque production. Strength, and most importantly RTD - an index of muscular power, for these lower body muscles is essential for mobility, and were measured as indices of functional ability. The knee extensor and knee flexor contractions were performed in a supine posture at set hip- and knee-joint angles (1.57 rad, FIG. 3A, 3B and 3D).

Subjects also performed grip strength MVC with a standard dynamometer (hydraulic hand dynamometer, 300# capacity, Baseline Evaluation Instruments). Grip strength correlated with total body strength and neurological health and may be predictive of future disability and mortality.

**Table 6.** Select motor functions do not change with treatment.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Low Dose</th>
<th>High Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 10</td>
<td>Baseline</td>
</tr>
<tr>
<td>MVC, Knee extension</td>
<td>54.2 ± 19.3</td>
<td>61.7 ± 18.1</td>
<td>72.5 ± 33.4</td>
</tr>
<tr>
<td>MVC, Knee flexion</td>
<td>107 ± 27.1</td>
<td>107 ± 30.5</td>
<td>103 ± 28.8</td>
</tr>
<tr>
<td>Rapid step</td>
<td>40.2 ± 6.6</td>
<td>38.1 ± 6.2</td>
<td>40.4 ± 4.8</td>
</tr>
</tbody>
</table>
time (s)  
400 mwt (s) 196 ± 2 1  184 ± 2 4  206 ± 2 0  209 ± 1 8  199 ± 2 0  195 ± 2 0  
Timed up 5.3 ± 1.1  5.5 ± 1.5  6.2 ± 0.9  6.1 ± 0.5  5.9 ± 0.5  5.5 ± 0.4  
& go (s)  
Pegboard 66.7 ± 17.3  63.3 ± 13.5  67.5 ± 10.4  64.3 ± 10.7  65.1 ± 9.4  65.3 ± 7.8  

Fatigability  
Fatigability was assessed as the time to failure during a heel-rise test. The heel-rise test 
is a single-leg test in which subjects performed plantar flexion contractions at a rate of one 
maximal effort heel-rise every 2 s until task failure. Prior to beginning the test, leg dominance 
was determined via the Waterloo Footedness Survey, and when unhindered by previous injury 
or training, tests were performed on the dominant leg. Displacement about the ankle was 
measured with an electrogoniometer (SGI 10 and K800, Biometrics Ltd, Cwmfelinfach, 
Gwent, UK). Subjects were positioned in front of a small clinical table with fingertips 
touching the table top for balance. Practice of the test was allowed with the non-dominant leg. 
The subjects were instructed to stand straight and to raise and lower the body on the ball of the 
dominant foot in rhythm with a metronome set to a rate of one heel-rise every two seconds. 
The test was terminated when the subject quit due to exhaustion or discomfort, or when, for 2 
consecutive heel raises, the subject leaned onto the table, used more than fingertips for balance, 
flexed the knee, or decreased the required plantar flexion range of motion by more than 50%.  

Sense of Fatigue  
Self-reported perception of fatigue in daily life was measured via two questionnaires: 
the Fatigue Severity Scale (FSS) and a Fatigue Questionnaire. The FSS questionnaire is a 
standardized questionnaire that contains nine statements that rate the severity of fatigue 
symptoms. Subjects read each statement and circled a number from 1 to 7, based on how 
accurately it reflected their condition during the past week and the extent to which they agree 
or disagree that the statement applies to them. Fatigue Severity Score (FSS score) was derived 
by summing the numerical responses. In addition, fatigue was dichotomously classified 
according to two questions evaluating whether participants felt that "everything was an effort" 
or they "could not get going" on three or more days in the past week. Subjects were asked to 
consider their experience in the past week related to two statements: 1) "I feel that everything I
did was an effort" and 2) "I could not get going". Possible answers are (a) rarely or none of the time (less than 1 day), (b) some or a little of the time (1-2 days), (c) occasionally or a moderate amount of time (3-4 days), (d) all of the time (5-7 days). Those reporting three or more days to either question were classified as being fatigued. These two questions were adapted from the Center for Epidemiologic Studies-Depression scale (CES-D) and are associated with functional impairment, limitation, and disability in older adults (Vestergaard S. et al., Characteristics of 400-meter walk test performance and subsequent mortality in older adults, Rejuvenation Res., 2009; 12(3): 177-84).

Metabolomics
5 Small molecules were extracted from plasma samples using an in-house developed technique that yields molecules from 4 major classes (lipid, protein, carbohydrate and nucleic acids). Samples were analyzed using Liquid Chromatography Mass Spectrometry (LCMS) and data analyzed using commercial software. The analysis involved the assessment of metabolites from major metabolic pathways including lipid, amino acid, peptide, nucleotide, xenobiotic and cofactor. Quality control included analysis of a pooled plasma sample and 16 labeled or exogenous negative or positive spike-in controls, resulting in false discovery rates <3%. Initial data analysis was conducted using commercial software (Mass Hunter, Agilent) or freeware (XCMS) to extract molecular "features" from raw data. Data were imported into Mass Profiler Professional (MPP, Agilent) and "features" were filtered to reduce noise.

Statistics
10 Data are presented as mean ± SEM (FIGS) or mean ±SD (tables and text). Statistical analysis was performed with SPSS software (v21, IBM, Somers, NY, USA). Prior to primary analysis of the motor function outcomes, normality of each variable was assessed with the Kolmogorov-Smirnov test. No outliers (>3 SD) were identified. Homogeneity of variance between groups was assessed by Levene's test and did not differ between treatment groups (p > 0.05). Group difference in motor function with sodium nitrite treatments compared with placebo was determined by mixed-model ANOVA (within factor, time; between factor, treatment), with contrast set to changes observed in placebo controls, and followed by post-hoc Tukey's HSD means-comparison. The contribution of baseline subject characteristics the change in motor (rate of torque development) and cognitive (TMT-B) function outcomes was determined by stepwise, linear regression equations.
To determine the effects of sodium nitrite treatment on the metabolome, normalized feature intensities were compared using ANOVA and data was visualized using PCA, K-means clustering and volcano plots. A final fold-change filter was applied following ANOVA. Features were initially identified using a database comprised of Metlin, HMDB, Lipid Maps and KEGG. Compounds were then further identified using molecular formula generation, MS" and a novel informatics program. Tentatively identified molecules were verified using purchased standards or at Medicinal Chemistry Core lab at UC Denver. To determine the relations between changes in motor and cognitive function and the fold changes in the metabolome, a weighted correlation network approach as implemented in the Weighted Gene Co-expression Network Analysis (WGCNA) R package was used. Briefly, using correlation network methodology, WGCNA identified clusters of highly correlated metabolites referred to as "modules". These modules were then related to treatment condition as well as to functional and mechanistic outcomes. Multiple linear regression was used to evaluate the association between changes in metabolites or change in metabolite module membership as a function of nitrite treatment status adjusting for covariates as necessary.

Results

Subject characteristics

Table 7. Subject characteristics for primary health measures at baseline and 10 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
</tr>
<tr>
<td></td>
<td>« = 10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.6 ± 8.3</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.7 ± 2.7</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>72.9 ± 7.9</td>
</tr>
<tr>
<td>Total lean mass (kg)</td>
<td>46.7 ± 13.8</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>30.8 ± 11.7</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>94.9 ± 5.2</td>
</tr>
<tr>
<td>Fasting insulin (µU/dL)</td>
<td>8.6 ± 3.3</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>180 ± 30</td>
</tr>
<tr>
<td>Activity (steps/wk)</td>
<td>60.7 ± 24.3</td>
</tr>
</tbody>
</table>
Activity (steps/wk) was determined by accelerometry and presented in 10,000 step increments. CHAMPS Activities Questionnaire for Older Adults was used to estimate total kCal expended per week based on self-reported frequency and duration of typical activities.

Characteristics of the groups are shown in Table 7. At baseline and following 10-weeks treatment or placebo, no significant group differences in body composition characteristics (total body mass, lean body mass, regional lean mass), basic blood panel (fasting glucose, insulin, and cholesterol) and habitual daily activity (step counts, and estimated kCal energy expenditure) were observed (p > 0.05, all). Furthermore, maximal and submaximal heart rate and oxygen consumption outcomes (VC^max, Rating of Perceived Exertion (RPE), Respiratory Exchange Ratio (RER), and Ventilatory Exchange) derived from modified Balke incremental treadmill protocol revealed no significant baseline differences or significant effects of nitrite treatment (Table 8; p > 0.05, all).

Table 8. Markers of voluntary effort at end exercise and VC^max were not altered by treatment.

<table>
<thead>
<tr>
<th>CHAMPS (kCal/wk)</th>
<th>Placebo</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4948 ± 2991</td>
<td>4776 ± 2662</td>
<td>5515 ± 2515</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10 weeks</th>
<th>Placebo</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 10</td>
<td>11</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.6 ± 2.8</td>
<td>24.6 ± 2.8</td>
<td>24.1 ± 3.2</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>72.6 ± 7.9</td>
<td>71.3 ± 9.2</td>
<td>69.0 ± 8.6</td>
</tr>
<tr>
<td>Total lean mass (kg)</td>
<td>48.4 ± 11.2</td>
<td>47.9 ± 8.4</td>
<td>47.0 ± 7.8</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>30.5 ± 11.9</td>
<td>29.4 ± 8.7</td>
<td>28.0 ± 10.8</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>94.1 ± 6.4</td>
<td>91.4 ± 5.1</td>
<td>92.1 ± 5.0</td>
</tr>
<tr>
<td>Fasting insulin (xx/dL)</td>
<td>9.3 ± 3.4</td>
<td>10.3 ± 2.9</td>
<td>7.8 ± 3.4</td>
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<tr>
<td>Total cholesterol</td>
<td>180 ± 22</td>
<td>194 ± 28</td>
<td>195 ± 26</td>
</tr>
<tr>
<td>Activity (steps/wk)</td>
<td>55.4 ± 18.3</td>
<td>67.4 ± 35.5</td>
<td>66.0 ± 36.2</td>
</tr>
<tr>
<td>CHAMPS (kCal/wk)</td>
<td>4960 ± 2224</td>
<td>3732 ± 1840</td>
<td>4200 ± 2769</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 10</td>
<td>Baseline</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>RPE (U)</td>
<td>18 ± 0</td>
<td>19 ± 0</td>
<td>18 ± 0</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>166 ± 5</td>
<td>163 ± 5</td>
<td>169 ± 5</td>
</tr>
<tr>
<td>RER (U)</td>
<td>1.17 ± 0.03</td>
<td>1.16 ± 0.02</td>
<td>1.16 ± 0.03</td>
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<tr>
<td>Ventilation (L/min)</td>
<td>68 ± 8</td>
<td>67 ± 7</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>$V_\text{O}_{2}\text{max}$ (ml/(kg·min))</td>
<td>30.5 ± 6.9</td>
<td>30.0 ± 5.7</td>
<td>31.6 ± 6.7</td>
</tr>
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**Plasma nitrite and nitrate**

As measured by HPLC, plasma nitrite concentrations averaged 0.30±0.16 μM at baseline. At 30 min following a single (initial) dose, concentrations were unchanged in the placebo group (0.21±0.11 μM), but increased by ~10-fold (to 3.18±0.73 μM) and ~20-fold (to 6.22±0.75 μM) after acute ingestion of the low (40 mg) and high (80 mg) sodium nitrite doses, respectively. These preliminary data demonstrate that ingestion of a sodium nitrite formulation, but not placebo capsules, acutely increases circulating nitrite concentrations. Moreover, despite a relatively short half-life (10's of min), plasma nitrite measured 12 hours after the most recent dose was ~115% and 215% greater after vs. before the 10-week intervention period in the low and high sodium nitrite groups, respectively, but was unchanged in the placebo control group (~8%). The data demonstrates that sodium nitrite treatment produces small, but consistent chronic increases in circulating nitrite concentrations..

**Baseline functional capacity**

The middle-aged and older adults demonstrated high functional capacity as indicated by baseline performance on tests of mobility (400 m walk time, Timed Up & Go), dexterity, grip strength, and cognition (MMSE and Trail Making Tests A&B). Performance on each of these tests revealed that the participants included in this study were not considered impaired based on clinical cut-points or national normative data.

**Acute and chronic nitrate and nitrite levels were assessed.**

Acute and chronic nitrate and nitrite levels were assessed in the placebo, low dose (40 mg) and high dose (80 mg) groups. Acute nitrite and nitrate levels were determined prior to initial dosage, and 30-min following first dose or placebo. Chronic nitrite and nitrate levels were determined 12 hours after ingesting final pill at week 10. Preliminary analysis
demonstrates that nitrite increases acutely (p < 0.001) and may be increasing chronically (p < 0.1) (Table 9).

Table 9. Acute and chronic plasma nitrite and nitrate levels

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Low Dose</th>
<th>High Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Week 10 Baseline Week 10 Baseline Week 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute (baseline and 30 min after 1st dose)</td>
<td>Nitrite 0.23 ± 0.24 0.20 ± 0.19 0.47 ± 0.47 3.34 ± 1.88† 0.27 ± 0.31 7.13 ± 3.36*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic (baseline and 24 hours final dose at week 10)</td>
<td>Nitrite 0.20 ± 0.21 0.26 ± 0.16 0.21 ± 0.17 0.47 ± 0.27† 0.15 ± 0.15 0.37 ± 0.21†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05; † p < 0.1

5 Sodium nitrite treatment improves motor function

Despite subjects demonstrating no functional impairments at baseline, 10 weeks of sodium nitrite treatment improved select motor functions, including muscle power (RTD) (FIG. 3A-E), balance (FIG. 4A-C), grip strength and heel-rise time (FIG. 5B). The rate of torque development in knee extension (FIG. 3A; 80mg: 964 ± 385 to 1204 ± 492 N-m/s; 160mg: 902 ± 281 to 1208 ± 429 N-m/s) and knee flexion (FIG. 3D; 80mg: 983 ± 249 to 1309 ± 416 N-m/s; 160mg: 1034 ± 320 to 1481 ± 410 N-m/s) were improved with low and high doses of sodium nitrite compared with placebo (FIG. 3A and 3D; 897 ± 354 to 905 ± 399 and 989 ± 368 to 839 ± 243 N-m/s, respectively, p < 0.01). A significant dose response such that RTD was greater with the highest dose was not observed (p > 0.05 between high and low doses), indicating that the significant changes in RTD are not improved with increased dosing. The changes in RTD are observed both as the mean changes across groups (FIG. 3B and 3E) and in percent change from baseline per individual, as reflected by the upward shift in individual data points with low (80 mg/d) and high (160 mg/d) sodium nitrite treatment (FIG. 3C and 3E). The improvements in RTD were observed in the absence of significant changes in MVC for knee extension or flexion (p > 0.05). The improvement in RTD but not MVC indicates a functional change in the skeletal muscle recruitment and neural activation, which may be reflective of an improvement in muscular power production.

The numbers of balance errors committed during the rapid step test (FIG. 4A; 80mg: 2.9 ± 1.7 to 2.0 ± 1.3; 160mg: 3.5 ± 2.4 to 2.4 ± 1.5) were also significantly improved.
compared with placebo (FIG. 4A; 3.2 ± 1.4 to 3.3 ± 1.0 respectively, p < 0.05) in the absence of changes in rapid step time (FIG. 4B) or maximum step length (p > 0.05 both). Additionally, nitrite treatment improved maximal exercise capacity in treadmill time to exhaustion during a modified Balke incremental treadmill exercise protocol (FIG. 5C; 80 mg/d: +54.7 ± 38.4 s; 160 mg: +18.6 ± 28.4 s) compared with placebo (FIG. 5C; 0.3 ±20.0 s, p< 0.05). When sodium nitrite groups were combined, treatment effects were observed in grip strength (FIG. 5A; Nitrite: 38.1 ± 7.2 to 39.9 ± 7.8 kg; Control: 38.3 ± 9.2 to 37.6 ± 9.2 p < 0.05) and heel-rise endurance (FIG. 5B; Nitrite: 54 ± 20 to 56 ± 21 s; Control: 92 ± 45 to 117 ± 68 s, trend, p < 0.10). Additional improvements were not observed in daily functional tasks such as mobility and dexterity.

**Sodium nitrite improves cognition**

Cognitive ability was improved with 10-weeks sodium nitrite, as indicated by improvements in time to complete the TMT-B and TMT-A (FIG. 6A-F). Executive function, as quantified by the time to complete TMT-B was improved with sodium nitrite (FIG. 6A and 6B; 80mg: 56 ± 11 to 46 ± 10 s; 160mg: 59 ± 19 to 51 ± 12 s), compared with placebo (FIG. 6A and 6B; PI: 59 ± 23 to 65 ± 28 s; p < 0.01). A trend was observed for an improvement in processing speed (TMT-A) (FIG. 6C and 6D; PI: 29.0 ± 10.9 to 30.0 ± 7.1 s; 80mg: 26.8 ± 6.7 to 22.3 ± 2.5 s; 160mg: 27.6 ± 7.2 to 25.0 ± 5.9 s, p < 0.08) that reached significance when treatment groups were combined (p < 0.05).

**Small metabolite signature differs with sodium nitrite treatment**

Baseline and post-treatment plasma samples were analyzed from 7 placebo, 8 low dose (80 mg/d) and 7 high dose (160 mg/d) sodium nitrite subjects. To establish feasibility and gain preliminary insight, small molecules initially were assessed in the non-aqueous lipid fraction of plasma. About ~4400 metabolites were detected, ~1200 of which were confirmed in 2/3 rds of the subjects using 0.05 and 1.5-fold cutoffs. Of these, ~40 molecules changed in response to both doses of sodium nitrite, but not with placebo. These included reductions in pro-inflammatory molecules in the arachidonic acid pathway (e.g., 11B-PGF2a isopropyl ester and PGF2a), as well as changes to molecules related to sphingolipid, glycan, glycerophospholipid, fatty acyl, polyketide and choline metabolism. Overall in the sample, metabolites were identified from several pathways related to NO production (for example, L-arginine), aging (for example, arachidonic acid), cognitive function (for example, tryptophan, lysine, L-arginine, phenylalanine, leucine), motor function (for example, dehydroepiandrosterone), CVD
(for example, cholesterol) and inflammation (for example, LTB4, LTC4, PGE2, PGF2a, 11B-PGF2a, RVD1, lipoxin A4).

**Table 10.** Functional capacity at baseline.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Baseline function</th>
<th>Reference Value</th>
<th>Reference age</th>
<th>Source</th>
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<tbody>
<tr>
<td>Age</td>
<td>n = 31</td>
<td>Age: 60.9 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 m walk (s)</td>
<td>204 ± 25</td>
<td>&gt;345cc</td>
<td>65-102 yrs</td>
<td>Vestergaard 2009</td>
</tr>
<tr>
<td>TUG (s)</td>
<td>5.8 ± 0.9</td>
<td>&gt; 12cc</td>
<td>65-80 yrs</td>
<td>Bischoff et al. 2003</td>
</tr>
<tr>
<td>Pegboard (s)</td>
<td>66.5 ± 12.2</td>
<td>65.7 ± 8.6</td>
<td>40-60 yrs</td>
<td>Marmon et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>88.9 ± 15.7</td>
<td>&gt;65 yrs</td>
<td>Marmon et al. 2011</td>
</tr>
</tbody>
</table>

**Grip strength**

<table>
<thead>
<tr>
<th>(kg)</th>
<th>Men</th>
<th>42.5 ± 7.0</th>
<th>47.9 ± 6.4</th>
<th>60-64 yrs</th>
<th>Werle et al. 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Women</td>
<td>30.5 ± 9.8</td>
<td>28.7 ± 5.5</td>
<td>60-64 yrs</td>
<td>Werle et al. 2009</td>
</tr>
<tr>
<td>MMSE</td>
<td>29.4 ± 0.4</td>
<td>26</td>
<td>_</td>
<td>Crum et al. 1993</td>
<td></td>
</tr>
<tr>
<td>TMT-A</td>
<td>27.8 ± 8.0</td>
<td>31.3 ± 7.0</td>
<td>60-64 yrs</td>
<td>Tombaugh 2004</td>
<td></td>
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<tr>
<td>TMT-B</td>
<td>57.9 ± 17.3</td>
<td>64.6 ± 18.6</td>
<td>60-64 yrs</td>
<td>Tombaugh 2004</td>
<td></td>
</tr>
</tbody>
</table>

CC = clinical cutpoint; mobility cut-off times that represent significant increase in risk of mortality or loss of independence. MMSE = Mini-Mental Status Exam; TMT-A = Trail Making Test part A (processing speed); TMT-B = Trail Making Test part B (executive function). Cognitive normative data (MMSE, TMT-A and TMT-B) reflect 12+ years of schooling.

**Discussion**

It was demonstrated that 10 weeks of sodium nitrite treatment in healthy middle-aged and older adults improved motor and cognitive functions compared with placebo controls. Age is associated with an increased risk of motor dysfunction and cognitive impairments that may lead to an increased risk of physical impairment, loss of independence, and disability. Sodium nitrite treatment improved an RTD, balance, and executive functions in already functionally
intact middle-aged and older adults. The results demonstrated treatment of age-related motor impairment associated with frailty and disability with aging in humans.

All references cited herein are hereby incorporated by reference in their entirety.

It is believed that the disclosure set forth above encompasses at least one distinct invention with independent utility. While the invention has been disclosed in the exemplary forms, the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense as numerous variations are possible. Equivalent changes, modifications and variations of various embodiments, materials, compositions and methods may be made within the scope of the present invention, with substantially similar results. The subject matter of the inventions includes all novel and non-obvious combinations and subcombinations of the various elements, features, functions and/or properties disclosed herein.

Benefits, other advantages, and solutions to problems have been described herein with regard to specific embodiments. However, the benefits, advantages, solutions to problems, and any element or combination of elements that may cause any benefit, advantage, or solution to occur or become more pronounced are not to be construed as critical, required, or essential features or elements of any or all the claims of the invention. Many changes and modifications within the scope of the instant invention includes all such modifications. Corresponding structures, materials, acts, and equivalents of all elements in the claims below are intended to include any structure, material, or acts performing the functions in combination with other claim elements as specifically claimed. The scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given above.
CLAIMS

1. A method for treating declining motor and/or a method for improving cognitive function in a subject, comprising:

   administering to said subject an effective amount of a pharmaceutical composition comprising sodium nitrite and a pharmaceutical excipient, wherein the pharmaceutical composition comprises about 0.1 mg to about 5.0 mg of sodium nitrite per kg body weight per day.

2. The method of claim 1, further comprising assessing an indicator comprising motor or cognitive function in the subject, wherein such assessment is conducted prior to administration of the pharmaceutical composition.

3. The method of claim 1, wherein the indicator of motor and/or cognitive function comprises measuring muscle strength and force development, grip strength, balance, fatigability, mobility and dexterity, and wherein the step of administration is conducted only in response to an indicator at a threshold value.

4. The method of claim 1, wherein an indicator of motor and/or cognitive function comprises measuring at least one metabolite selected from lipid, amino acid, peptide, nucleotide, xenobiotic, inflammatory cytokines and/or cofactor metabolic pathways, and wherein the step of administration is conducted only in response to an indicator at a threshold value.

5. The method of claim 4, wherein the at least one metabolite comprises 11B-PGF2A isopropyl ester, PGF2a, sphingolipid, glycan, glycerophospholipid, fatty acyl, polyketide, choline, L-arginine, arachidonic acid, tryptophan, lysine, phenylalanine, leucine, dehydroepiandrosterone, cholesterol, LTB4, LTC4, PGE2, RVD1, lipoxin A4, and pro-inflammatory cytokines.

6. The method of claim 5, wherein the pro-inflammatory cytokines comprise at least one selected from IL-1β, IL-6, IFN-γ and TNF-a.
7. The method of claim 1, wherein the composition is administered over at least one of about 30 days, about 3 months, about 6 months, about 12 months, about 18 months, about 2 years, about 5 years, about 7 years, about 10 years, about 15 years, about 20 years, about 25 years, about 30 years, about 35 years, about 40 years, or over the lifetime of the subject.

8. A method of improving motor and/or cognitive function in a subject comprising:
   determining a decrease in motor and/or cognitive function in a subject; and
   administering an effective amount of a pharmaceutical composition comprising sodium nitrite and a pharmaceutical excipient, wherein the composition comprises about 0.1 mg to about 5.0 mg of sodium nitrite per kg body weight per day and wherein the composition is administered to said subject.

9. The method of claim 8, wherein an improvement of motor and/or cognitive function is associated with an increase in sodium nitrite concentration in the subject.
**FIG. 1**

**A**

**Grip Strength (g)**

- Young
- Baseline
- 8 Weeks

**B**

**Open Field Distance (m)**

- Young
- Baseline
- 8 Weeks

*Significant difference
†Significantly different from baseline
∧Significantly different from young
FIG. 1
FIG. 3

A

KNEE EXTENSION

RTD (N·m/s)

BASE WK 10 PLACEBO
BASE WK 10 NITRITE (80 mg/d)
BASE WK 10 NITRITE (160 mg/d)

B

KNEE EXTENSION

RTD (% CHANGE)

BASELINE  WK 10
FIG. 3

C

PLACEBO

NITRITE (80 mg/d)

NITRITE (160 mg/d)

RTD (N•m/s)

BASE  WK 10
FIG. 3

Histogram showing RTD (N·m/s) for different conditions:
- BASE WK 10 PLACEBO
- BASE WK 10 NITRITE (80 mg/d)
- BASE WK 10 NITRITE (160 mg/d)

KNEE FLEXION

* indicates statistical significance.
FIG. 3

Example graph showing RTD (N•m/s) for PLACEBO, NITRITE (80 mg/d), and NITRITE (160 mg/d) at BASE and WK 10.
**FIG. 4**

**A**

Rapid Step Test Errors

- BASE WK 10 PLACEBO
- BASE WK 10 NITRITE (80 mg/d)
- BASE WK 10 NITRITE (160 mg/d)

**B**

Rapid Step Time (s)

- BASE WK 10 PLACEBO
- BASE WK 10 NITRITE (80 mg/d)
- BASE WK 10 NITRITE (160 mg/d)
FIG. 4
FIG. 5
FIG. 6
FIG. 6
FIG. 6
INTERNATIONAL SEARCH REPORT

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C01B 21/20; A61P 25/00; A61K 33/00 (2015.01)
CPC - C01B 21/20; C01B 21/50; A61K 33/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C01B 21/20; A61P 25/00; A61K 33/00 (2015.01)
CPC: C01B 21/20; C01B 21/50; A61K 33/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/718; 423/385

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar, PubWEST

C. DOCUMENTS CONSIDERED, TO BE RELEVANT

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<td>X</td>
<td>WO 2012/135623 A1 (GIORDANO et al.) 04 October 2012 (04.10.2012) pg 1, in 28 to pg 2, in 14; pg 7; ln 10-13; ln 24; pg 14; in 24-27; pg 14; in 30-31; pg 20, ln 8-12</td>
<td>1, 7-9; 2-6</td>
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<td>Y</td>
<td>US 6,067,986 A (KLUGER et al.) 30 May 2000 (30.05.2000) col 1, ln 31- col 2, ln 3; Fig 5</td>
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Date of the actual completion of the international search: 17 June 2015 (17.06.2015)

Date of mailing of the international search report: 10 JUL 2015

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PCT OSP: 571-272-7774

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