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Research Article

Impact of the Dietary Supplement CLOCK® on BDNF and Other Biochemical Measures Related to Cognitive Function and Health in Men and Women

Richard J Bloomer[1]*, Matthew Butawan[1], Judi Quilici Timmcke[2] and Bolin Qin[2,3]

[1]*Center for Nutraceutical and Dietary Supplement Research, School of Health Studies, University of Memphis, Memphis, Tennessee, USA*

[2]*IN-Ingredients, Columbia, Tennessee, USA*

[3]*Department of Radiation Oncology, School of Medicine, University of Maryland, Baltimore, Maryland, USA*

Abstract

Background: We have previously reported improvements in selected biochemical measures of health, as well as self reported well-being in men and women using the dietary supplement known as CLOCK®. In this follow up investigation, we evaluated CLOCK® at two different dosages using a randomized, placebo controlled, cross-over design in men and women with self reported low energy, impaired sleep quality and impaired well-being.

Methods: 30 subjects (14 men and 16 women) were randomly assigned in double blind manner to ingest a botanical agent (CLOCK®, containing *Rosmarinus officinalis* and *Hemerocallis fulva* at either 1gram or 1.5grams daily) or a placebo for two week periods with a two week wash out period after each condition assignment. Blood samples were collected pre and post each two week period in an overnight fasted state and analyzed for acetylcholine (ACh), choline, Brain Derived Neurotrophic Factor (BDNF), IGF-1, irisin, melatonin and serotonin. Heart rate and blood pressure were measured, as were subjective measures of well-being. The Leeds Sleep Evaluation Questionnaire was used as a measure of sleep quality and related variables.

Results: ACh (22%), choline (19%), BDNF (44%), irisin (23%) and melatonin (8%) all increased significantly from pre to post intervention following intake of CLOCK® (with a greater increase noted with 1gram vs. 1.5grams) but not placebo. Heart rate and blood pressure were unaffected by treatment ($p>0.05$). Time effects were noted for all well-being measures ($p<0.05$), except for anxious, depressed and tense, with improvements noted in all variables from pre to post intervention. When considering both supplement dosages together, improvements of 25% (attentiveness), 21% (alertness), 37% (focus) and 34% (energetic) were observed, with slightly less improvement noted for placebo. Although, not significant, CLOCK® reduced the feeling of grogginess (25%), lethargy (14%) and sluggishness (18%); whereas there were minimal changes with the placebo. No differences were noted between conditions or across time in measures of sleep or related variables ($p>0.05$).

Conclusion: CLOCK® significantly impacts biochemical variables related to health, while having little impact on resting heart rate or blood pressure.

Keywords: Daylily; Mood; *Rosemary*; Well-being

Background

Dietary supplements designed for the purposes of improved well-being and healthy aging are increasing in popularity, with many products being marketed to adults in the Western world. We have recently reported that the dietary supplement known as CLOCK®, a combination of *Rosmarinus officinalis* (*Rosemary*) and *Hemerocallis fulva* (*Daylily*), improves selected measures of subjective well-being, while resulting in favorable changes in biochemical measures of health [1]. Additional anecdotal reports using this agent have noted positive outcomes related to improved cognition and mood (e.g., increased perceived energy and enthusiasm), as well as the quality of nightly sleep.

Both *Rosemary* and *Hemerocallis* extracts have been demonstrated to favorably impact key biochemical variables important to health. Our initial work using CLOCK® at a daily dosage of 1000 mg noted a 27% increase in Brain Derived Neurotrophic Factor (BDNF), which may have been associated with the noted improvement in well-being measures [1]. Moreover, other outcomes that are known to be influenced by *Rosemary* and *Hemerocallis* such as serotonin and melatonin may have an influence on sleep quality. It was our purpose in the present study to expand on our initial work with CLOCK® with regards to its impact on selected biochemical variables known to impact mood and associated measures.

Impaired sleep quality is common among adults, with approximately 70 million Americans claiming poor quality of sleep and almost 60% of Americans noting chronic sleep problems [2]. Of these, many seek pharmaceutical assistance in terms of sleep aids, hoping that the improvement in sleep may lead to improved performance in daily tasks [3]. Selected dietary supplements may help in this regard. Specifically, both *Rosemary* (*Rosmarinus officinalis*) and *Daylily* (*Hemerocallis fulva*) have been reported to offer benefits.

Rosemary (*Rosmarinus officinalis* L.) is an evergreen herb native to the Mediterranean. This plant is used around the world for its culinary and medicinal uses, including use in both the food and fragrance industries. *Rosemary* is known for its powerful antioxidant properties and biologically active medicinal value [4]. The leaves are commonly used as a food additive. The plant has been used in traditional medicine, but also as an herbal treatment for inflammation, improving cognition, having anti-inflammatory and antidiuretic activity and

***Corresponding author:** Richard J Bloomer, Center for Nutraceutical and Dietary Supplement Research, School of Health Studies, 106 Roane Fieldhouse, The University of Memphis, Memphis, TN 38152, Tennessee, USA, Tel: +1 9016785638; Fax: +1 9016783591; E-mail: rbloomer@memphis.edu

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protecting the liver [4,5]. *Rosemary* has been studied in animals and humans as a nonprescription aid for anti-anxiety and depression [6]. In the form of tea, *Rosemary* was used in an animal study demonstrating anti-anxiety and anti-depressant properties [6]. Also, evidence indicates that *Rosemary* has an anti-insomnia effect in individuals suffering with opium withdrawal [7]. Further study is needed to examine the possibility of these effects, as well as others, in individuals with non addiction related sleep difficulties.

The *Hemerocallis fulva* species of *Daylily* is a perennial plant naturally found in China, Japan and Korea. Another name is Wang-You Cao, which in Chinese means "forget-one's sadness" plant [8]. *Daylily* has been used to treat a wide variety of conditions such as depression, jaundice and insomnia. More recently, *Daylily* has been shown to have neuroprotective properties, which were demonstrated by significantly reversing corticosterone and glutamate-induced neurotoxicity in a dose-dependent manner [8]. *Daylily* buds have also been shown to have a powerful effect as a Nitric Oxide (NO) scavenger and suppressor in cases where NO is elevated to supraphysiological levels, which was suggested to be useful in reducing inflammation in humans [9]. One type of *Hemerocallis* species showed an antidepressant effect in an animal study where neurotransmitters were measured in the frontal cortex and hippocampus of the brain [10]. The study demonstrated an antidepressant effect due to an increase in serotonin, dopamine and noradrenaline levels along with their particular receptors systems.

Collectively, it is possible that both *Rosemary* and *Daylily* could have a positive impact on biochemical measures of health, as well as related variables. This was noted in our prior work and the present study was designed to expand on our initial findings. Specifically, we evaluated CLOCK® at two different dosages (1000 mg and 1500 mg) using a randomized, placebo-controlled, cross-over design in men and women with self-reported low energy and impaired mood, as well as poor sleep quality. We evaluated several biochemical markers of health, those that we have included in our past work with CLOCK®, as well as those thought to impact mood and associated variables. In addition, we included measures of subjective well-being and variables specific to sleep quality, following two-week treatment periods with CLOCK® and placebo. Both the 1000 mg and 1500 mg dose of CLOCK® was included in the design, as we were interested in knowing how subjects responded to both dosages.

Methods

Subjects and screening

A total of 30 subjects (14 men and 16 women) with self-reported low energy, impaired well-being, and impaired sleep quality were enrolled in the study. Sample size was adequate based on our prior work with the supplement, specific to our primary biochemical measures. Although not a criterion for inclusion, nor was this medically diagnosed, many subjects self-reported having anxiety. The criteria below were used for study inclusion/exclusion: Subjects needed to have a total score of ≥8 on the Insomnia Severity Index and report having low energy during the day and impaired well-being (i.e., poor outlook on life) [11]. It should be noted that our subjects were not clinically diagnosed with any illness. Subjects could not have a BMI >40kg/m^2, since morbid obesity is known to negatively impact sleep quality and quality of life. While it was possible that subjects had sleep apnea, we did not specifically inquire about this; hence, this may be considered a limitation of this work. Subjects could not be using dietary supplements designed to improve sleep quality or dietary supplements designed to increase energy. They could not be using off the shelf or prescription medications for purposes of improving sleep quality, nor could they be using any other sleep aids. Subjects needed to be non-smokers and women could not be pregnant or nursing. Subjects could not be consuming alcoholic beverages at a quantity greater than 3 drinks per week. Finally, they could not consume more than 300 mg of caffeine daily and none after 2:00 pm each day. Subjects were recruited by use of recruitment flyers posted in the Memphis area.

A health history questionnaire was completed by all subjects to gather descriptive information. Women were required to take a urine pregnancy test to confirm that they were not pregnant. Prior to participation, each subject was informed of all procedures, potential risks, and benefits associated with the study through verbal and/or written form in accordance with the procedures approved by the University of Memphis Institutional Review Board for Human Subjects Research (#3961). Subjects provided written informed consent prior to being admitted to participate.

Initial laboratory visit: screening visit

During the initial visit to the laboratory, subjects completed the informed consent form, health history and physical activity questionnaires. Subjects' heart rate and blood pressure, height, weight, waist and hip circumference were measured. Upon completion of the screening, subjects were scheduled for their initial testing visit.

Conditions

Subjects were randomly assigned in a double-blind manner to the following 1) Placebo (rice bran powder); 2) CLOCK® at 1000 mg; 3) CLOCK® at 1500 mg. There was a two-week placebo lead-in period, and then each of the three conditions was taken for a period of two weeks. A two-week wash-out period was included between each treatment. Three capsules were taken nightly to provide the correct dosage. This same three capsule amount was taken while on the placebo. IN-Ingredients, (Columbia, TN, USA) provided the supplement (CLOCK®), which contains a 1:1 blend of *Rosemary* (*Rosmarinus officinalis*) and *Daylily* (*Hemerocallis fulva*). Both supplement and placebo capsules were produced by a contract manufacturer and were of near identical appearance. Capsules were distributed to subjects in unlabeled bottles, with a known quantity of vegetable-based capsules in each bottle. It should be noted that the capsule coating masked any odor that may have been given off by the botanical itself. Subjects were instructed to ingest the capsule(s) one hour prior to bedtime and at least two hours following their last meal. Subjects returned their capsule bottles at the end of each two-week period to allow for compliance to be determined.

Assessment

Subjects reported to the lab on eight different days over the course of the study on day one of each two-week period (day 1) and on the day following each period (day 15). Subjects reported in the morning hours (e.g., 6:00 am - 8:00 am) and the time of day was standardized for each subject across the days of testing. Following a 10-minute rest period, heart rate and blood pressure were measured using an automated unit and a blood sample was collected from an antecubital vein. Subjects then completed a questionnaire related to well-being. The words included in the questionnaire were provided to subjects and anchored with a 0 (none) and 10 (extreme) and subjects indicated the degree to which they "felt" each of these descriptors.

Subjects also supplied open ended comments pertaining to their sleep quality (e.g., ease of falling to sleep, frequency of waking at night,

tossing and turning during the night, ease of arising in the morning, and energy level upon rising). The Leeds Sleep Evaluation Questionnaire was completed on the morning of days 3, 4, 5, 6 and 7 of weeks two of each treatment period [12]. Rather than simply having subjects complete the questionnaire in reference to one night, we included five consecutive nights in an attempt to better capture subjects' overall sleep quality and related measures. The average values across days 3-7 for each week were then used in the data analysis.

Dietary intake and other variables

All subjects were instructed to consume their usual diet throughout the study period and to record all food and drink consumed during the 5 days prior to each test day. This was done to make certain that dietary intake was similar across time for subjects. In the event that significant differences were noted, dietary data could then be considered in statistical modeling of the outcome analysis. These records were analyzed for macro and micro nutrient intake using computer software (Food Processor Pro, Esha Research; Salem, Oregon). Subjects were also instructed not to consume caffeine after 2:00 pm each day of the study period. Subjects were instructed to continue with their usual physical activity throughout the study period. We also instructed subjects not to make changes in their sleep conditions throughout the study period.

Biochemical analyses

Following blood collection, samples were processed accordingly and the plasma was stored at -70°C until analyzed. The human ELISA kits for acetylcholine, choline, IGF-1 and serotonin were obtained from ABCAM (Cambridge, MA); BDNF was obtained from BOSTER Biological Technology (Pleasanton CA); Melatonin was obtained from LifeSpan BioSciences (Seattle, WA); and Irisin was obtained from Phoenix Pharmaceuticals (Burlingame, CA).

Procedures followed the manufactures' guidelines and all samples were assayed in duplicate.

Statistical analysis

The data were analyzed using a condition x time Analysis of Variance (ANOVA). Tukey post hoc tests were performed as needed. Percent change values were calculated for subjective well-being measures. Statistical significance was set at $p \leq 0.05$.

Results

Thirty subjects were enrolled in this study and all but one completed all aspects of the protocol. One subject only completed two conditions and then needed to cease participation due to relocation from the Memphis area. Additionally, a total of six of the potential 236 remaining blood samples were unavailable due to inability to collect blood on that particular day. Compliance to capsule intake was not different (p>0.05) for CLOCK® 1.5g (99±1%), CLOCK® 1g (98±2%), or placebo conditions (96±2% and 97±1%). One adverse outcome was noted during the protocol. Specifically, one female subject who reported having serotonin syndrome eight years prior to beginning the study indicated to investigators that she believed she had a seizure while participating in the study. This was not medically documented. It was noted that this occurred while on the placebo condition; however, this incident was filed with the IRB and processed accordingly. It was also reported to the study sponsor. Aside from this incident, we observed no adverse events. All other subjects tolerated the conditions well and none reported adverse reactions to use of the supplement or placebo. Subject characteristics are presented in table 1 and as expected, differences existed between men and women for selected variables (p<0.05). We present men and women separately to provide additional information regarding our subject population.

Variable	Men	Women	P value
	N=14	N=16	
Age (yrs)	27.1±10.6	25.0±3.9	0.47
Height (cm)	179.2±9.6	165.0±6.7	0
Weight (kg)	88.1±15.7	69.4±10.4	0
BMI ($kg \cdot m^{-2}$)	27.4±3.8	25.4±2.7	0.11
Waist (cm)	88.1±13.3	76.9±6.6	0.01
Hip (cm)	108.1±8.2	103.7±6.6	0.11
Waist: Hip	0.81±0.07	0.74±0.03	0
Heart Rate (bpm)	68.0±6.3	80.4±11.3	0
Systolic Blood Pressure (mmHg)	125.6±8.9	117.9±11.6	0.06
Diastolic Blood Pressure (mmHg)	73.4±10.5	76.2±8.5	0.43
Years anaerobic exercise training	4.1±5.4	1.9±1.9	0.14
Hours per week anaerobic exercise	1.9±1.8	1.5±1.6	0.58
Years aerobic exercise training	4.4±5.3	3.2±3.2	0.47
Hours per week aerobic exercise	2.3±3.9	2.6±2.9	0.8
Daily caffeine intake (mg)	100.5±91.1	78.3±78.4	0.48
Insomnia Index	14.4±3.9	17.2±4.3	0.08

Table 1: Characteristics of men and women assigned to CLOCK® or a placebo. Data are mean±SD.

Data for all biochemical markers are presented in table 2. Both CLOCK® 1gram and 1.5gram dosages increased plasma acetylcholine levels (p<0.05 and 0.01, respectively), although the CLOCK® 1gram dose was slightly but insignificantly higher than the CLOCK® 1.5gram dose. CLOCK® 1gram dose enhanced plasma choline levels (p<0.05); however the 1.5gram CLOCK® did not do so in a significant manner (p=0.42). Both CLOCK® dosages increased plasma BDNF levels (p<0.01, respectively). Plasma BDNF levels in the 1gram group were significantly higher in comparison to the 1.5gram group, while there was no significant differences between both dosage groups after CLOCK® consumption. CLOCK® increased irisin levels (p<0.05 and 0.01, respectively), with greater values observed for 1gram. CLOCK® at 1gram improved plasma melatonin levels compared with the pre visit (p<0.05); and the 1.5gram CLOCK® was also effective but failed to reach significance (p=0.09). There was a trend showing that both CLOCK® dosages enhanced plasma IGF levels, but there was no significant difference compared with baseline (p=0.44, 1.5g ; p=0.18, 1g). There was a trend showing that CLOCK® consumption of both dosages increased plasma serotonin levels, but there was no significant difference compared with baseline (p=0.43, 1.5g ; p=0.30, 1g).

Data for heart rate and blood pressure are presented in table 3. No interaction or effects for condition or time were noted for any variable (p>0.05).

No interaction or condition effects were noted for any variable related to well-being (p>0.05). However, time effects were noted for the following variables: attentive (p=0.0004), tired (p=0.0005), alert (p=0.006), groggy (p=0.0004), focused (p=0.0003), sluggish (p=0.003), energetic (p=0.0002), lethargic (p=0.003), enthusiastic (p=0.0001), well rested (p<0.0001) and fatigued (p=0.0008). All

Variable	CLOCK® 1.5g	Placebo 1	CLOCK® 1g	Placebo 2
Acetylcholine (pMol/mL)				
Pre	528 ± 151[a]	535 ± 57[a]	537 ± 64[a]	521 ± 70[a]
Post	616 ± 149[b]	538 ± 37[a]	656 ±127[bc]	534 ± 102[a]
Choline (nMol/mL)				
Pre	8.30 ± 2.74[a]	8.57 ± 2.54[a]	8.31 ± 2.51[a]	8.49 ± 2.56[a]
Post	9.17 ± 2.67[a]	8.80 ± 2.55[a]	9.91 ± 2.70[b]	8.57 ± 2.56[a]
BDNF (pg/mL)				
Pre	141.0 ± 52.4[a]	141.9 ± 55.9[a]	138.0 ± 52.9[a]	151.6 ± 52.4[a]
Post	180.8 ± 54.6[c]	143.9 ± 54.9[a]	199.4 ± 65.3[c]	149.4 ± 47.1[a]
IGF-1 (ng/mL)				
Pre	159 ± 49[a]	163 ± 50[a]	161 ± 49[a]	160 ± 50[a]
Post	170 ± 54[a]	164 ± 49[a]	178 ± 47[a]	164 ± 51[a]
Irisin (ng/mL)				
Pre	40.3 ± 9.9[a]	40.0 ± 9.6[a]	40.2 ± 9.1[a]	41.3 ± 8.8[a]
Post	47.3 ± 10.8[b]	41.8 ± 9.7[a]	49.3 ± 10.5[bc]	41.5 ± 9.5[a]
Melatonin (pMol/L)				
Pre	244.3 ± 25.5[a]	245.5 ± 26.9[a]	243.0 ± 29.0[a]	239.9 ± 29.8[a]
Post	252.5 ± 26.8[a]	242.9 ± 27.9[a]	263.1 ± 28.8[b]	243.4 ± 27.7[a]
Serotonin (ng/mL)				
Pre	152 ± 53[a]	159 ± 53[a]	156 ± 52[a]	157 ± 51[a]
Post	164 ± 57[a]	160 ± 53[a]	170 ± 51[a]	158 ± 52[a]

Table 2: Biochemical markers of men and women assigned to CLOCK® or a placebo for two weeks.

Values are mean±SD. Pre: pre-intervention; Post: post-intervention.

Different letters indicate significant differences between the placebo and CLOCK® groups ([a] vs [c] $p < 0.01$; the other differences $p < 0.05$ at least).

Placebo 1: lead-in period.

Variable	CLOCK® 1.5g	Placebo	CLOCK® 1g	Placebo
Heart Rate (bpm)				
Pre	72.8±2.3	72.1±2.0	72.4±2.0	70.5±2.0
Post	73.3±2.6	71.7±2.4	72.1±2.3	73.1±2.6
Systolic Blood Pressure (mm Hg)				
Pre	118.5±1.8	117.9±2.0	116.1±1.8	118.3±2.1
Post	117.6±2.5	117.8±2.2	118.3±2.2	117.3±2.4
Diastolic Blood Pressure (mm Hg)				
Pre	72.2±1.8	73.4±1.7	70.9±1.7	73.6±2.2
Post	72.1±2.2	73.1±1.9	73.1±1.8	73.1±2.0
Rate Pressure Product				
Pre	8610.1±286.0	8500.7±284.8	8400.2±252.0	8343.2±267.2
Post	8605.5±334.4	8458.0±335.7	8534.2±305.2	8590.9±365.4

Table 3: Heart rate, blood pressure and rate pressure product of men and women assigned to CLOCK® or a placebo for two weeks.

Values are mean ± SE.

No differences of statistical significance were noted for any variable ($p > 0.05$).

variables improved from pre- to post-intervention. Data are shown in table 4. When considering both supplement conditions together (combining the 1.5gram and 1gram dosage conditions), improvements of 25% (attentiveness), 21% (alertness), 37% (focus), and 34% (energetic) were observed, with slightly less improvement noted for placebo. Feeling of grogginess (25%), lethargy (14%) and sluggishness (18%) were reduced with supplement but remained relatively unchanged with placebo.

No interaction or time effects were noted for any variable of the Leeds Sleep Questionnaire ($p > 0.05$). However, a condition effect was noted for "quality of sleep" ($p = 0.01$), with values higher for placebo than for CLOCK® 1.5grams. Data for the Leeds Sleep Questionnaire and dietary intake are not presented. No findings of statistical significance were noted for any dietary variable ($p > 0.05$).

Discussion

This is the second study to evaluate the combination of *Rosemary* (*Rosmarinus officinalis*) and *Daylily* (*Hemerocallis fulva*) in relation to selected biochemical measures of health and related variables in men and women with self-reported difficulty sleeping. The supplement had no impact heart rate or blood pressure measures. Although positive changes were noted in many of the well-being measures from pre- to post-intervention with the supplement, similar changes were noted for placebo, highlighting the well-described placebo effect, which may be applied to dietary supplement/pharmacological studies [13-15]. This issue of a potential placebo effect may have impaired our ability to detect differences between conditions in our subjective measures.

Aside from the subjective measures, the main focal point of this work was to determine potential changes in biochemical measures related to health between the two dosages of supplement. The biochemical measurements showed a significant increase in ACh, choline, BDNF, melatonin and irisin following CLOCK® treatment, in particular at a dosage of 1gram daily. It is unknown why the 1gram dose was more effective than the 1.5gram dose. Indeed, many botanical agents appear more effective at lower dosages than at higher dosages, including such agents as polyphenol antioxidants. This may be due to the potential for greater oxidation with higher dosing. Future study is needed to determine why a lower dosage of CLOCK® appears more effective in a sample of men and women. These measurements may have implications for healthy aging, cognitive function, memory and mood. Comments provided by subjects at the end of each treatment period indicated that subjects' subjective mood was improved following treatment with CLOCK®. For example, subjects commented that: "over the past 2 weeks I believe this has been the easiest I have fallen asleep. Usually within 20 minutes of my lying down I fall asleep. I sleep thru the night without waking up. When I get out of bed I feel well rested and I don't have to take extra naps". Another subject reported "Slept so well through the night. No weird dreams. Woke up normal. Increased energy". Also one subject stated, "The last 2 weeks have probably been the best I have slept in a few months. I have fallen asleep quickly and rarely (never woke up in the middle of the night). Ready to start my day more quickly than before" and "saw increased productivity at work, could work longer and harder! More optimistic.

Variable	CLOCK® 1.5g	Placebo	CLOCK® 1g	Placebo
Anxious				
Pre	3.6±0.4	4.0±0.5	3.9±0.4	4.0±0.5
Post	3.2±0.4	3.5±0.4	3.7±0.5	3.3±0.4
Attentive*				
Pre	5.5±0.3	5.2±0.3	5.4±0.3	5.8±0.2
Post	6.1±0.3	6.5±0.3	6.4±0.3	6.0±0.4
Tired*				
Pre	5.8±0.4	5.7±0.4	5.6±0.4	5.5±0.4
Post	5.0±0.4	4.4±0.4	4.3±0.4	4.7±0.5
Alert*				
Pre	5.3±0.3	5.2±0.2	5.4±0.3	5.8±0.3
Post	6.0±0.2	6.1±0.3	6.2±0.3	5.7±0.4
Groggy*				
Pre	4.2±0.4	4.8±0.4	4.7±0.4	4.1±0.4
Post	3.1±0.4	3.7±0.4	3.2±0.4	3.7±0.4
Focused*				
Pre	5.2±0.3	5.2±0.3	5.1±0.3	5.6±0.3
Post	5.9±0.3	6.3±0.3	6.2±0.3	6.1±0.4
Sluggish*				
Pre	4.7±0.4	4.9±0.3	4.3±0.4	4.1±0.4
Post	3.6±0.4	3.7±0.4	3.2±0.4	4.0±0.5
Energetic*				
Pre	4.8±0.3	4.6±0.2	4.9±0.3	5.2±0.4
Post	5.6±0.3	5.8±0.4	5.9±0.4	5.9±0.4
Lethargic*				
Pre	3.9±0.4	4.2±0.4	4.0±0.4	3.9±0.4
Post	2.9±0.4	3.6±0.4	2.7±0.4	3.3±0.4
Enthusiastic*				
Pre	4.8±0.3	4.4±0.2	5.2±0.2	5.1±0.3
Post	5.7±0.3	5.5±0.3	6.0±0.3	5.5±0.4
Depressed				
Pre	2.8±0.5	3.0±0.5	3.0±0.5	2.8±0.5
Post	2.2±0.4	2.8±0.4	2.2±0.4	2.1±0.4
Well Rested*				
Pre	4.3±0.3	4.1±0.3	4.4±0.3	4.6±0.4
Post	5.4±0.3	5.4±0.3	5.4±0.4	5.6±0.4
Fatigued*				
Pre	4.7±0.4	4.9±0.4	4.4±0.4	4.4±0.4
Post	4.0±0.4	3.6±0.4	3.1±0.4	3.6±0.4
Tense				
Pre	3.7±0.4	4.2±0.5	4.1±0.5	3.8±0.5
Post	3.4±0.4	3.4±0.5	3.1±0.4	3.6±0.5

Table 4: Well-being measures of men and women assigned to CLOCK® or a placebo for two weeks.

Values are mean ± SE.

*Time effects noted for the following variables: attentive (p=0.0004), tired (p=0.0005), alert (p=0.006), groggy (p=0.0004), focused (p=0.0003), sluggish (p=0.003), energetic (p=0.0002), lethargic (p=0.003), enthusiastic (p=0.0001), well rested (p<0.0001) and fatigued (p=0.0008).

No other differences of statistical significance (condition or interaction effects) were noted for any variable (p>0.05).

Slept better". Besides these observations from subjects, the biochemical markers noted to be elevated following CLOCK® intake appear important to overall health, as described below.

Acetylcholine (ACh) is an essential brain neurotransmitter that is involved in sleep, cognition and muscle control. It is the most populated neurotransmitter found in the peripheral and central nervous systems and has been shown to decline with aging [16]. In the brain, loss of ACh and cholinergic tone occurs during senile dementia, which is believed to be the cause for decreased cognitive function [17]. The CLOCK® 1gram and 1.5gram dosages increased plasma ACh levels, which may help contribute to the improvement of cognition.

Choline is the precursor to ACh and is a key methyl-group donor needed, which is required for homocysteine metabolism. Loss of cholinergic neurons is linked to impaired cognition, especially memory loss [18]. Plasma choline has been studied in different age groups especially because of its role in the central nervous system, and in mental function and memory. Consumption of CLOCK® demonstrated a cholinergic effect since plasma levels of both ACh and choline levels increased. This may provide support to the central nervous system, which includes the brain, especially through the aging process.

BDNF is a neurotrophin with dominant expression in the hypothalamus, hippocampus, cerebral cortex and cerebellum in the Central Nervous System (CNS). It is stored in human platelets and circulates in plasma. It has been shown to influence brain function and the peripheral nervous system and declines with age [19]. In all ages, BDNF is involved in promoting the growth and maintenance of several neuronal systems as well as its involvement in functions of neuronal plasticity. The hippocampus and prefrontal cortex are most susceptible to aging, which causes the problems with cognitive function and memory [20]. BDNF, as a neurotrophic factor has been shown to be severely affected in the aging process and is associated with the decline of cognitive function [21]. Loss of mental function is related to changes in the levels of BDNF and other factors [21]. Our data showing a significant increase in BDNF is important and may have implications for improving/maintaining cognitive function. Future work is needed to determine the extent to which the CLOCK® associated increase in BDNF is associated with cognitive function through functional testing.

Irisin, a hormone discovered in 2012, has been shown to increase with physical activity has also been shown to decline with age [22,23]. Irisin has been evaluated in young adults and it has been reported that as this hormone increases, there is an elevation in BDNF expression and activation of genes involved in learning and memory [24,25]. Evidence demonstrates that irisin effects brain function [26]. Increased peripheral irisin levels stimulate the hippocampal genes related to neuroprotection, learning and memory [26]. Telomeres are important DNA protein complexes that are located at chromosome tips to protect genes. The length of telomeres has been correlated with life span and a measurement of cellular aging. Irisin levels may predict chromosome telomere length in healthy adults [27]. The results of a recent clinical study indicate that irisin levels had an effect on peripheral blood mononuclear cell telomere length in healthy, non-obese subjects [27]. The study's data indicate that CLOCK® increases plasma irisin levels, which may provide cognitive support, as well as neuroprotection over the course of a lifespan.

Melatonin, a hormone secreted by the pineal gland in the brain generates circadian rhythms. Circulating melatonin levels decline with age [28,29]. Plasma levels of melatonin are higher at nighttime

throughout the life span, although it is the absolute concentrations that decline with age [30,31]. It has been shown that when melatonin is given, it provides neuroprotection [32,33] and improved cognitive function [34]. It is believed that age-related decline in melatonin levels may be associated with increased neurodegenerative disease and insomnia. Melatonin has sleep-promoting actions linked to its feedback to the Suprachiasmatic Nucleus (SCN). Melatonin has an effect on circadian rhythms phase and amplitude, thereby helping to synchronize them [28]. Melatonin is believed to be important to brain health protecting it from oxidative damage and neurodegeneration [29]. The human body's circadian rhythms have an effect on the whole body including metabolic and physiologic functions and behavior. Research shows that melatonin is a biomarker of circadian dysregulation [35]. Melatonin is directly correlated to circadian rhythms. Research has not been determined the best time to measure melatonin levels to evaluate and determine its relationship on health, circadian rhythms and sleep. Although, one way is to observe individual melatonin changes over a study period. Research has shown that measurements taken before 9 am. are useful in determining effects [36]. This study's measurements were taken between 6 to 8 am. CLOCK® showed a significant increase in melatonin levels, which would have a positive effect on circadian rhythms and neuroprotection, possibly providing support with the advancement in age, and with sleep.

Collectively, ACh, choline, BDNF and irisin levels are important to cognition. In the present study, CLOCK® has shown a positive effect on these biomarkers, which may provide a preventative effect for individuals as they advance in age. That said, since our subject sample was relatively young, additional study of the CLOCK® involving a group of older adults would be helpful in providing more information on how this supplement can impact health and performance within this population.

In the first double blind, placebo controlled clinical trial published in 2016, CLOCK® 1g group noted increases in alertness, focus, attentiveness, feeling energetic, alertness, enthusiasm, which supports the current findings [1]. In addition, we observed an increase in feeling well rested, as well as a reduction in feeling depressed and sluggish. These markers were not improved in the placebo group. The biomarkers that increased in this study also improved in the previous study. BDNF significantly increased in the CLOCK® 1g group by 27% with the largest effect size was seen for irisin (d=1.36), whereas the placebo condition did not change. ACh and melatonin were also increased in the CLOCK® 1g group. Having data now from two clinical investigations reinforces the benefits related to mental function.

The compounds in CLOCK® have been studied for their health related therapeutic roles for the Central Nervous System, including the brain. A recent review of *Rosemary* explains the therapeutic role it may play to aid those with Alzheimer's disease [4]. Various studies have demonstrated that *Rosemary's* bioactives support brain health and may have a role in enhancing cognitive function [37]. As mentioned, *Hemerocallis* has anti-depressant effects, as well as being neuroprotective. Both *Rosemary* and *Hemerocallis* extracts have been reported to significantly modulate the expression of circadian clock proteins in C6 animal brain glioma and IPEC-1 cells [38].

The biochemical markers were significantly influenced by CLOCK®, particularly BDNF, irisin and melatonin. There is a substantial amount of research that shows that aerobic exercise increases baseline circulating BDNF levels in the hippocampus and other areas of the brain [39]. In the present study, CLOCK® but not the placebo, increased BDNF levels by 44%, which is higher than what has been observed after aerobic exercise [39]. We are unaware of any research pertaining to other dietary supplements that elevate BDNF levels over a two week period. Irisin levels, which were significantly increased in CLOCK® subjects, have been shown to increase in middle-aged, older individuals after 8 weeks of endurance training [40].

Conclusion

CLOCK®, a botanical dietary supplement composed of *Rosemary* and *Daylily* has an impact on biochemical measures of health and aging. There appeared no association between these measures and reductions in blood pressure, perhaps due to the fact that subjects had normal resting blood pressure to begin with and little need for a blood pressure reduction. Future studies may focus on additional biochemical parameters that might elucidate the potential of CLOCK® to improve overall health, possibly inclusive of a homogenous sample of older men and women.

Competing Interests

Financial support for this work was provided in part by IN-Ingredients. JQT and BQ are employees of IN-Ingredients. None of the other authors have a financial interest in this company. RJB has received research funding or acted as consultant to other nutraceutical and dietary supplement companies. MB declares no competing interests.

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Authors' Contributions

RJB was responsible for the study design, statistical analyses, and manuscript preparation. MB was responsible for subject recruitment and retention, data collection, data entry and assistance with manuscript preparation. JQT was responsible for assistance with the study design and manuscript editing. BQ was responsible for performing the biochemical analyses, statistical analyses and manuscript editing. All authors read and approved of the final manuscript.

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