Applied Physiology, Nutrition, and Metabolism, Vol. 42, No. 5, pp. 495-502, 2017.

Dose-Dependent Decrease in Mortality with No Cognitive or Muscle Function Improvements Due to Dietary EGCG Supplementation in Aged Mice.

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ABSTRACT

We have previously shown that a diet containing epigallocatechin gallate (EGCG) and beta-alanine is not effective in improving either cognitive or muscle function in aged (18 month) mice (Gibbons *et al*. *Behav Brain Res* 2014, Pence et al. *Appl Physiol Nutr Metab* 2016). However, this diet reduced oxidative stress in the brain, and previous studies using longer-term interventions and other doses have documented beneficial effects in cognitive and muscle function, especially with EGCG. Here we hypothesized that a different dose of EGCG or longer feeding period would be more efficacious in improving cognition. Aged (21-25 mo) Balb/cByJ male mice underwent 63 days of feeding with EGCG at 0, 0.091, or 3.67 mg/g AIN-93M diet and were then subjected to a battery of cognitive and muscle function tests. EGCG feeding at either of the two doses did not alter preference for novel versus familiar arm in the Y-maze test (p=0.29) and did not affect learning in the active avoidance test (p=0.76). Similarly, EGCG did not affect preference for novel versus familiar mice in a social discrimination test (p=0.17). Likewise, there was no effect of EGCG on muscle function by grip strength (p=0.16), rotarod (p=0.18) or treadmill test to exhaustion (p=0.25). EGCG reduced mortality in a dose-dependent fashion (p=0.05, log rank test for trend), with 91% of high EGCG, 72% of low EGCG, and 55% of control mice surviving to the end of the study. In conclusion, EGCG improves survival in aged mice but does not affect cognitive or muscle function. **Keywords:** Aging, dietary intervention, epigallocatechin gallate, cognition, mortality.

INTRODUCTION

Physiological aging leads to a number of functional changes in a variety of organ systems, including both the skeletal muscle and the central nervous systems. Age-related loss of muscle mass and muscle function (sarcopenia) is related to performance impairments, including movements associated with activities of normal daily living such as mobility and locomotion (Morley et al. 2011). Aging muscle additionally has dysfunctional mitochondria (Rooyackers et al. 1996) that is causally-linked to muscle oxidative stress in the aged (Wanagat et al. 2001).

Several aspects of cognitive function decline with age, even in an otherwise healthy state (Barrientos et al. 2010, Perry et al. 1993, Wynne et al. 2009). Increased central inflammation and oxidative stress and downregulation of neurotrophic factors impair neurogenesis and cognitive function in aging. One well-studied biomarker is brain-derived neurotrophic factor (BDNF), downregulation of which in aging lead to impairments in hippocampal neurogenesis (Amrein et al. 2011, Barnes and Thomas 2008, Kuhn et al. 1996, Sanchez et al. 2011) and reduced hippocampal volume in aging (Erickson et al. 2010), whereas increases in BDNF are associated with an improvement in a variety of cognitive functions (Leckie et al. 2014). Due to the increasing percentage of the worldwide population over age 60, safe and cost-effective therapies that might counteract age-related impairments in function of physiological systems such as the brain and skeletal muscle are of growing medical importance.

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This is a post-review version formatted by the author. A publisher's final version is available as Pence *et al. Appl Physiol Nutr Metab* 42(5): 495-502, 2017

Green tea is widely touted for its antioxidant benefits (Yang 1999). The major bioactive catechin in green tea is (-)-epigallocatechin-3-gallate (EGCG), which constitutes about 60% of total catechins (Cabrera et al. 2006). This EGCG component is anti-inflammatory and acts as a scavenger of free radicals (Aktas et al. 2004). EGCG is an antioxidant *in vivo* in both muscle (Senthil Kumaran et al. 2008) and brain (Mandel et al. 2005, Sutherland et al. 2006); reducing microglial activation (Li et al. 2004, Wu et al. 2012) and inflammation (Kim et al. 2007) in the latter. EGCG additionally reduces stress-related impairments in cognition and locomotor activity in rats (Soung et al. 2015) and has recently been shown to attenuate cognitive impairments in severe rodent models of conditions such as Alzheimer's disease (Chang et al. 2015) and chronic cerebral hypoperfusion (Han et al. 2016). Green tea catechins are thus considered to be both neuroprotective (Mandel et al. 2005) and anti-sarcopenic (Alway et al. 2014, Alway et al. 2015) in aged subjects, although direct evidence and potential mechanisms for these effects remain largely underexplored.

We previously established that 40 days of feeding a diet consisting of 0.15% EGCG (1.5 $mg \cdot g^{-1}$ diet) and 0.343% β-Alanine (β-Ala, 3.43 $mg \cdot g^{-1}$ diet) has no effect on muscle function (Pence et al. 2016) or cognitive performance (Gibbons et al. 2014) in aged Balb/cByJ mice as well as adult C57Bl/6J mice (Bhattacharya et al. 2015). However, in aged mice, the dietary intervention caused several molecular changes in both brain and muscle tissues. Feeding the combination of both EGCG and β-Ala increased expression of several genes in the gastrocnemius of aged mice (Pence et al. 2016), including *Ppargc1a* and *Sirt1*, both of which are involved in mitochondrial biogenesis (Jornayvaz and Shulman 2010). This dietary intervention also reduced oxidative stress, as measured by 4-hydroxynonenal, in the cerebellum of aged mice (Gibbons et al. 2014). Both increased mitochondrial content and reduced oxidative stress are associated with improved cognitive performance (Volobouevа and Giffard 2011) and muscle function (Carter et al. 2015) in aged animals. This led us to hypothesize that our nutritional strategy (i.e. dosing and exposure time) might have been insufficient to improve functional outcomes because of their capability to alter the underlying mechanisms relating to improved brain and muscle function.

In our our previous aging studies (Gibbons et al. 2014, Pence et al. 2016), mice ingested an average of 182 mg EGCG per kg body weight per day. Based on the generally negative findings in our previous work, we considered that a change in either dose of EGCG or duration of feeding might yield more positive outcomes. The dose of EGCG provided in rodent studies varies widely, especially in regard to skeletal muscle, with doses ranging between 1 $mg \cdot kg^{-1} \cdot d^{-1}$ (Chen et al. 2009) to 1500 $mg \cdot kg^{-1} \cdot d^{-1}$ (Friedrich et al. 2012). The latter study, with a maximum EGCG concentration in the diet of 1%, as well as a study by Sae-Tan et al. (Sae-Tan et al. 2011) that used a 0.32% EGCG concentration in the diet, are among a handful of studies that incorporated EGCG into a rodent diet and found positive effects in primary (skeletal muscle-related) outcomes. Both reports used higher EGCG concentrations than in our previous studies (0.135%). We also considered that our dose of EGCG may have been too high, although there is less support in the literature for this possibility.

The goal of the present experiments was to examine the effects of varying doses of EGCG, in conjunction with a longer nutritional intervention, on cognitive and muscle function outcomes in aged mice. For the purpose of this study, we chose doses of EGCG corresponding generally to the highest (Friedrich et al. 2012) and lowest (Chen et al. 2009) reported doses which were successful in modulating cognitive or skeletal muscle function in previous studies. Because the positive effects due to the dietary intervention in the previous studies (Gibbons et al. 2014, Pence et al. 2016) are more likely to be associated with EGCG than β-Ala, we examined only EGCG. We hypothesized that EGCG supplementation would improve both cognitive and muscle performance in aged mice, possibly in a dose-dependent fashion.

MATERIALS AND METHODS

Animals

Male Balb/cByJ retired breeder mice (7-9 months old, birth date range June to October 2012) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained singly-housed in ventilated cages on *ad libitum* Teklad 8640 chow diet (Harlan, Indianapolis, IN) and tap water until 21-25 months of age. Mice were maintained in an AAALAC-accredited facility on a 12-hour reversed light-dark cycle (dark period 1000 to 2200 U.S. CST) at a constant temperature of 24°C. All procedures used were approved by the Institutional Animal Care and Use Committee at the

University of Illinois Urbana-Champaign.

Study Design

At onset of the experiment (age 21-25 months), mice were moved to standard shoebox cages (non-ventilated), stratified by age, and randomized equally (n = 11/group) to one of three diet groups (0.367%/high EGCG, 0.0091%/low EGCG, control). We chose doses of EGCG corresponding generally to the highest (Friedrich et al. 2012) and lowest (Chen et al. 2009) reported doses which were successful in modulating cognitive or skeletal muscle function in previous studies. Mice were maintained on these diets for 9 weeks prior to behavioral testing. After the 9 week feeding intervention, mice underwent a battery of behavioral tests to assess cognitive function. Mice remained on their respective experimental diets during the duration of behavior testing until euthanasia.

Mice underwent Y-maze testing over a period of 3 days, followed by a 2-day rest period. Mice then underwent muscle function testing during two days, with grip strength and rotarod conducted in the first 3 h of the dark cycle on both days. Time to exhaustion on a treadmill was conducted starting at 5-6 h into the dark cycle on the first day. Following a rest day, mice then underwent active avoidance testing for 5 days and social discrimination testing for 2 days, with a 2-day rest period between these tests. Following a one-day rest period, mice were euthanized for tissue collection over two consecutive days (approximately 50% of mice in each group on each day). The study design is outlined in Figure 1.

At sacrifice, mice were euthanized by rapid CO_2 asphyxiation, and brains were collected and dissected to yield the following sections: hippocampus, striatum, cerebellum, hypothalamus. Samples were frozen on dry ice and stored at -80°C until later analysis.

Diets

Diets were produced by Research Diets (New Brunswick, NJ) using purified AIN-93M as a base. Mice in the control group (Ctrl) received AIN-93M. Mice in the EGCG groups received AIN-93M supplemented with Teavigo (90% EGCG, DSM Nutritional Products, Basel, Switzerland) at one of two doses. Mice receiving high-dose EGCG (Hi) received AIN-93M supplemented with 4.095 mg Teavigo per gram diet, yielding a concentration of 3.67 mg EGCG per gram diet (0.367%). Mice receiving low-dose EGCG (Lo) received AIN-93M supplemented with 0.1012 mg Teavigo per gram diet, yielding a concentration of 91.1 µg EGCG per gram diet (0.0091%). Supplemented diets were pelleted to match the consistency and appearance of Ctrl diet, with the exception of addition of Yellow Dye #5 and Red Dye #40 (Lo) or Blue Dye #1 (Hi) for easy diet identification. EGCG added to the diet is stable for at least 6 months, based on our previous experience with independent assay verification of dietary EGCG concentration (Pence et al. 2016).

Y Maze Testing

The Y-shaped maze was constructed of dark gray colored plexiglass with each arm 38 cm in length, 13 cm s in height, 8 cm in width, and 120 degrees apart from each other. The Y-maze was surrounded by external-maze visual cues to allow the rodents to orientate themselves in the environment. The behavioral suite was dimly lighted. The TopScan (CleverSys, Reston, VA) tracking system was used to track movement of mice in the maze.

Animals were brought to the behavioral suite 10 min prior to the experiment for habituation. For acquisition, only two arms were available for mice to explore: the start arm, and familiar arm. The third arm was blocked by a dark gray divider constructed of the same material as the maze. The start arm was kept consistent for all animals but the open arm was switched between animals to avoid any arm-specific preference. A mouse was placed in far end of the start arm and allowed to navigate both the start and open arm for 15 min. The apparatus was cleaned with ethanol after each animal to remove any remnant of odor. Duration in the start and familiar arms and center was measured and expressed as a percentage of total time in the maze. All four paws of animal must have entered the area to be considered as an arm entrance.

Two h following the acquisition trial, mice were returned to the maze. All three arms were opened and mice were allowed to explore for 5 min. The percent time duration and number of entries into the start arm, familiar arm, center, and novel arm were measured to assess spatial working memory associated with preference for the novel arm. The y-maze test measures hippocampal-dependent spatial memory (Conrad et al. 1996).

Active Avoidance Testing

As described by Kohman *et al.* (Kohman et al.

2007), mice were trained on the task for 50 trials per day for 5 consecutive days using the GEMINI Avoidance System (San Diego Instruments, San Diego, Ca). Each mouse was placed in an active avoidance chamber and allowed to acclimate for 5 min. A trial began with presentation of a yellow cue light (conditioned stimulus) for 5 seconds from the opposite side of where the animal was located. If the mouse shuttled to the side containing the cue light within the 5 sec period, the program recorded the response as an active avoidance and the trial terminated. If the mouse failed to shuttle to the side containing the cue light, the mouse received a 0.50 mA foot shock (unconditioned stimulus) lasting up to 5 sec. If the mouse moved to the other side while the shock was still delivered, the program recorded the response as an escape and the trial was terminated. If the mouse remained in the same chamber for the entire duration of the shock, the program considered the lack of activity as no response and the trial ended after 5 sec. Each trial was followed by a 20-sec interval with no cue light or shock. The outcome is reported as number of successful avoidances per 50 trials per day.

Social Discrimination Testing

This task assessed the cognitive ability of mice to discriminate between a novel and familiar mouse. It is based on the spontaneous preference of rodents for novelty and their ability to remember previously encountered mice (Wills et al. 1983). The day before testing, an 8 × 8 × 11.5 cm wire mesh protection box was placed into the home cage of each test mouse for acclimation to the boxes. Behavioral assessment was performed during the dark phase of the light cycle under red light illumination the following 2 days. During testing, a juvenile mouse (~ 4 wk old) was confined to a protection box which was placed at the end of a clean 21 x 21 x 43 cm empty mouse cage. A test animal was placed in the cage and a clear Plexiglas lid was used to cover the cage, preventing escape but permitting observation and recording. Behavior was recorded for 7 min. After this exploratory period, the protected mouse became familiar to the test animal. At predefined intervals (i.e. 1, 4, and 24 h later) the task was repeated in the presence of the same boxed familiar mouse but with the inclusion of a second protection box containing a new (novel) protected juvenile mouse. The familiar mouse was always located at the same end and side of the cage for all tests, and the novel mouse was placed at the other side of the cage. The placement in the same position avoided the familiar mouse from being considered novel based on positional cues. Behavior was recorded for 5 min. Time spent investigating each mouse was recorded by trained personnel blinded to treatment. A Social Discrimination Index was calculated as (time spent exploring the novel mouse)·(time spent exploring the familiar + novel mice)$^{-1}$.

Muscle Function Testing

Forelimb grip strength was assessed using a commercially available force gauge (Columbus Instruments, Columbus, OH) as determined in 5 separate trials per day over 2 consecutive days by the same investigator. Grip strength was quantified as the average of the highest recorded grip force on each testing day and expressed as peak force in Newtons (N).

An exhaustive treadmill test was performed to assess fatigability. Mice ran on an inclined (5%), motorized treadmill (Jog-a-Dog, Ottawa Lake, MI) using an incremental running velocity protocol as previously described (Martin et al. 2013). Fatigue was defined by an inability to continue running despite gentle prodding for at least 10 sec. The test ended at 120 min if mice had not reached fatigue. No electric shock was used. Data were expressed as time-to-exhaustion (min).

An automated rotarod unit (Accuscan, Columbus, OH) with a 30 mm diameter rotating dowel and a 63 cm fall height was utilized. Mice were placed on the dowel, and rotation started at 0 rpm with constant and continuous acceleration to a maximum of 60 rpm in 180 seconds. Timing was controlled by a photobeam, and timing for each mouse was stopped automatically by the system when the falling mouse broke the plane of the

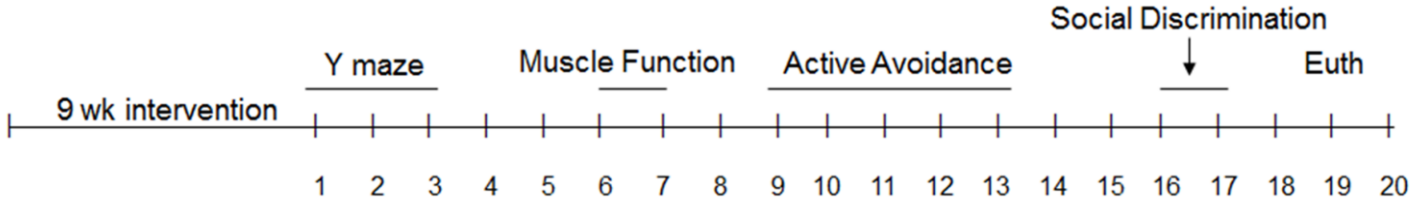

Figure 1: Study design. Diet feeding continued through behavioral testing. Euth: euthanasia and tissue collection.

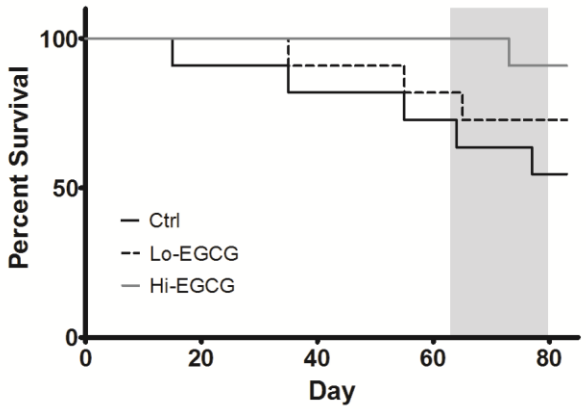

Figure 2. Survival across the study. The dose-response trend was significant by the Log-rank test for trend (p=0.05). Shaded area represents the behavioral testing period from day 63 to day 80. Ctrl: control mice (0 mg EGCG per gram diet, N=6/11 remained at study end). Lo-EGCG: mice given low EGCG dose (91.1 µg EGCG per gram diet, N=8/11 remained at study end). Hi-EGCG: mice given high EGCG dose (3.67 mg EGCG per gram diet, N=10/11 remained at study end).

photobeam. Mice underwent 4 consecutive trials per day over two days. Data were expressed as the average performance across all 8 trials over both days.

Data Analysis

Body weight (pre- and post-intervention) was analyzed by repeated-measures (RM-) analysis of variance (ANOVA) in a 2 × 3 (time × diet) experimental factorial design. Mean daily food disappearance, muscle function outcomes, Y maze discrimination index, and Y-maze percent total time in each maze area were analyzed by one-way ANOVA. Active avoidance data were analyzed by RM-ANOVA in a 5 × 3 (day × diet) design. Social discrimination data were analyzed by RM-ANOVA in a 3 × 3 (time point × diet) design. Treatment differences were assessed by Bonferroni-corrected post-hoc mean separation in the event of a significant main effect or interaction. Mortality data were analyzed using the Logrank test for trend in Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA) to compare the linear trend between dose of EGCG and survival. All data, with the exception of survival curves, were analyzed using SPSS software v. 22 (IBM, Armonk, NY) with significance set at $p \leq 0.05$. Figures were plotted in GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). All results were expressed as mean ± SEM except where noted.

RESULTS

Mortality

There was an EGCG dose-dependent effect on mortality during the study time course. Mice fed the Hi diet died (1/11) at a lower rate than Lo mice (3/11), which died at a lower rate than Ctrl mice (5/11). The dose-response effect was significant by the Log-Rank Test for Trend ($\chi^2_{(df=1)}=3.739$, p=0.05, **Figure 2**). Data presented below are from mice that survived to the end of the study. Mice that died during feeding were omitted from analysis as they had incomplete data sets. Mortality was by natural causes or based on veterinary

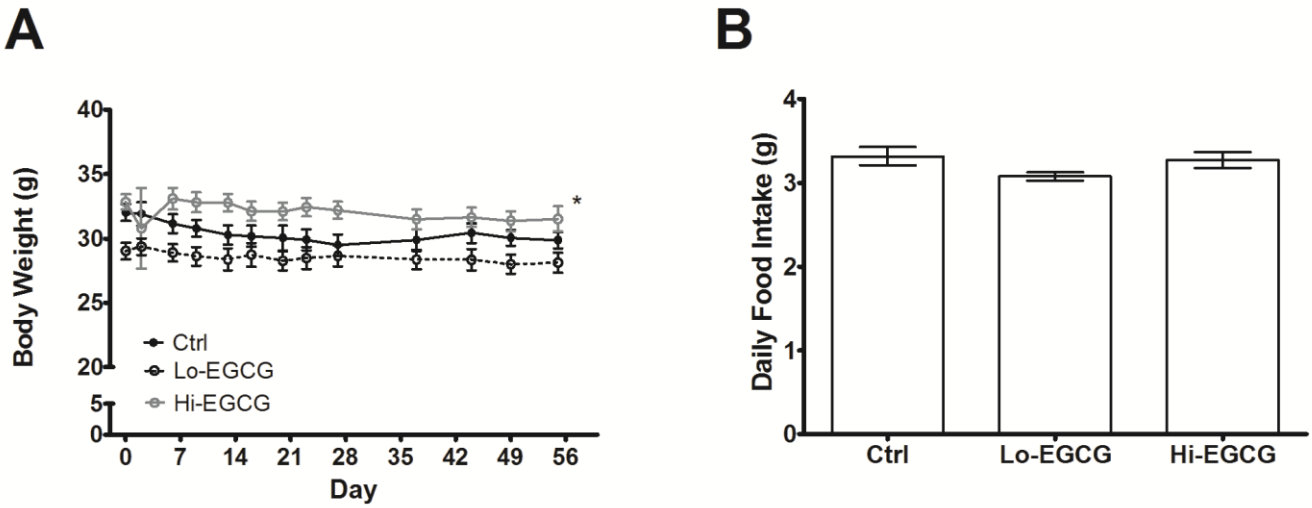

Figure 3: Body weight and average daily food intake. A) Body weight across the dietary intervention. * Hi-EGCG vs. Lo-EGCG across time (p<0.05). B) Average daily food intake. Ctrl: control mice (0 mg EGCG per gram diet, N=6). Lo-EGCG: mice given low EGCG dose (91.1 µg EGCG per gram diet, N=8). Hi-EGCG: mice given high EGCG dose (3.67 mg EGCG per gram diet, N=10).

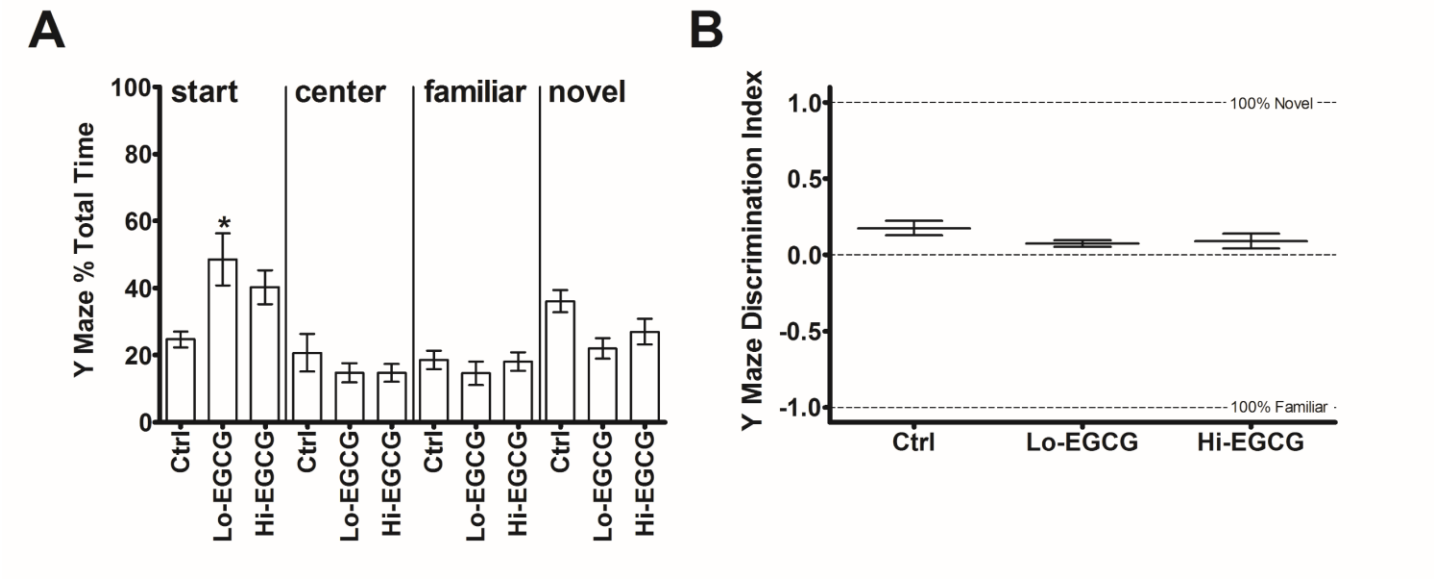

Figure 4. Y maze. A) Percentage time spent in each area (start arm, center, familiar arm, novel arm) by group. * Lo-EGCG vs. Ctrl ($p<0.05$). B) Discrimination index considering only time spent in familiar and novel arms. A value of +1.0 is 100% preference for the novel arm. A value of -1.0 is 100% preference for the familiar arm. Ctrl: control mice (0 mg EGCG per gram diet, N=6). Lo-EGCG: mice given low EGCG dose (91.1 µg EGCG per gram diet, N=8). Hi-EGCG: mice given high EGCG dose (3.67 mg EGCG per gram diet, N=10). Data expressed as mean ± SEM.

instruction due to morbundity. Weight loss cutoffs were not employed as euthanasia criteria.

Descriptive Data

By chance due to randomization, mice in the Hi group weighed more than mice in the Lo group at baseline and across the study ($F_{2,21}$=6.259, p=0.01, **Figure 3a**). Both the main effect of time ($F_{12,252}$=1.611, p=0.09) and the time × treatment interaction ($F_{24,252}$=1.241, p=0.21) were not significant. Expression of body weight data as percentage of initial body weight removed the treatment differences from the first to last time point ($F_{2,21}$=0.707, p=0.50). There was no difference in mean daily food intake (as measured by food disappearance) between groups ($F_{2,21}$=1.942, p=0.17, **Figure 3b**).

Y-Maze Testing

Data generated from Y-maze testing were analyzed in two different ways. Mice were assessed for percent of total test time spent in each of four areas of the maze: the start arm, the maze center, the familiar arm, and the novel arm. Higher percentage time spent in the novel arm is indicative of learning, as mice demonstrate a preference for exploring new areas (Wills et al. 1983). The overall ANOVA for the start arm was significant ($F_{2,21}$=3.544, p=0.05), and post-hoc analysis revealed that there was a significant preference for the start arm in Lo mice compared to Ctrl (p=0.05, **Figure 4a**). No other comparison yielded significant results, although there was a trend for the novel arm (overall ANOVA $F_{2,21}$=3.272, p=0.06, **Figure 4a**) in which Ctrl mice spent a greater amount of time compared to mice given the Hi and Lo diets.

Y-maze data were also analyzed for discrimination index, in which preference for familiar versus novel arms are compared and time spent in the start arm or the center of the maze is omitted. There was no effect of treatment on discrimination index (overall ANOVA $F_{2,21}$=1.306, p=0.29, **Figure 4b**). However, only Ctrl (95% CI 0.054 - 0.296) and Lo (95% CI 0.020 – 1.284) groups showed a significant (albeit slight and likely not meaningful) preference for the novel arm.

Active Avoidance Testing

Active avoidance data were expressed as number of successful avoidances out of 50 trials per day for 5 days (**Figure 5a**). There was a significant main effect of time ($F_{4,84}$=108.456, $p<0.001$), indicating that the mice learned the task sufficient to increase the number of successful avoidances on successive days. However, there was no significant main effect of diet ($F_{2,21}$=0.281, p=0.76) and no significant interaction ($F_{8,252}$=1.295, p=0.26). This finding indicates that EGCG was not

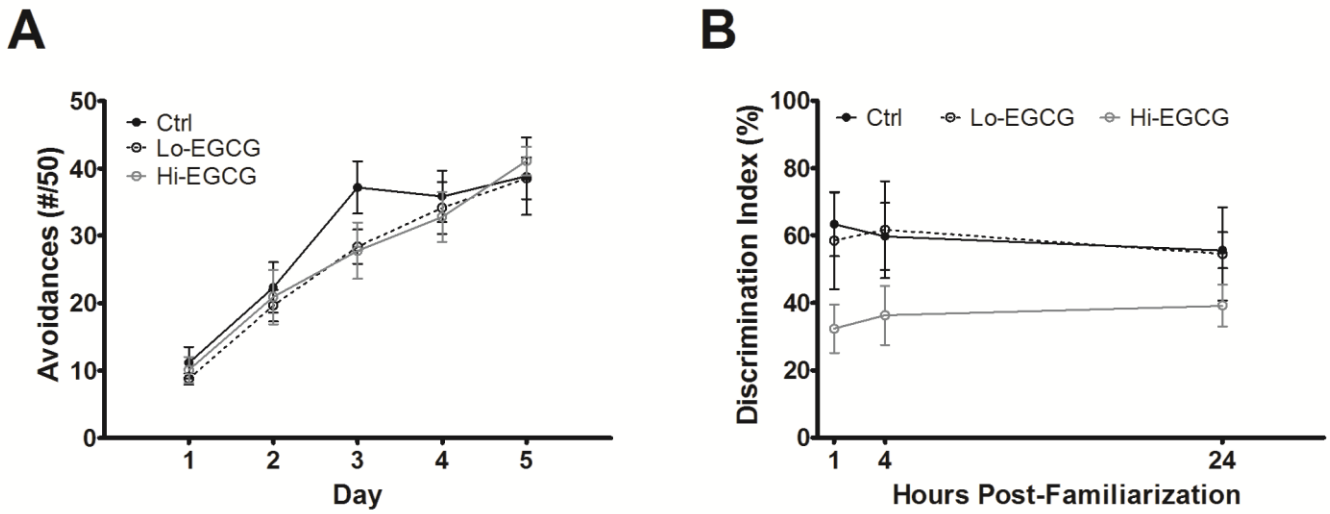

Figure 5. Active avoidance and social discrimination. A) Active avoidance, number of avoidances per day per 50 trials. B) Discrimination index on the social discrimination task. A value of 100 is 100% preference for the novel mouse. A value of 0 is 100% preference for the familiar mouse. Ctrl: control mice (0 mg EGCG per gram diet, N=6). Lo-EGCG: mice given low EGCG dose (91.1 µg EGCG per gram diet, N=8). Hi-EGCG: mice given high EGCG dose (3.67 mg EGCG per gram diet, N=10). All data are mean ± SEM.

effective at improving memory and performance in the active avoidance test.

Social Discrimination Testing

Social discrimination results were expressed as a discrimination index with a value of 100 being equivalent to 100% preference for the novel mouse and a value of 0 being equivalent to 100% preference for the familiar mouse at 1 h, 4 h, and 24 h post-familiarization. Although mice on the Hi diet displayed reduced preference for the novel mouse compared to those on the Ctrl and Lo diets, this effect was not significant (**Figure 5b**). There was no main effect of time ($F_{2,42}$=0.300, p=0.74) or treatment ($F_{2,21}$=1.950, p=0.17) and no significant interaction ($F_{4,42}$=0.929, p=0.46), indicating that diet did not alter preference for exploration of novel versus familiar mice. Additionally, 95% confidence intervals for all groups at all time points overlapped with 50, indicating no significant preference for novel vs. familiar mice in any group during the test.

Muscle Function Testing

Maximal forelimb grip strength did not differ between groups ($F_{2,21}$=2.020, p=0.16), indicating that the diet did not alter muscular strength in this test (**Figure 6a**). Additionally, rotarod ($F_{2,21}$=1.872, p=0.18, **Figure 6b**) and treadmill time to exhaustion ($F_{2,21}$=1.543, p=0.25, **Figure 6c**) did not differ between groups, suggesting that the dietary interventions did not alter balance or exercise capacity.

DISCUSSION

We previously reported that a diet containing EGCG in addition to beta-alanine was not efficacious in increasing either muscle function (Pence et al. 2016) or cognitive performance (Gibbons et al. 2014) in aged Balb/cByJ mice. However, other studies that reported significant changes due to dietary EGCG in mice used higher doses (Friedrich et al. 2012, Sae-Tan et al. 2011), and low-dose EGCG has also been reported to be effective in mice (Chen et al. 2009). Based on these heterogeneous reports, we investigated the effect of dietary incorporation of high dose (3.67 mg EGCG per gram diet) and low dose (91.1 µg EGCG per gram diet) EGCG on muscle and cognitive function in aged mice.
We demonstrated that dietary supplementation with EGCG resulted in a dose-dependent reduction in mortality in aged Balb/cByJ mice, but that there was no effect on cognitive or muscle function due to the dietary intervention. As mortality was not considered *a priori* as an outcome of interest, our experiment was not designed to collect certain relevant data, such as cause of death from necropsy that might help elucidate the underlying mechanism.

Consumption of green tea has been shown to be associated with reduced mortality in several large-scale studies, although these findings are not universal. A long-term prospective study of over 90,000 Japanese adults found that green tea consumption was inversely-related to heart, cerebrovascular, and respiratory

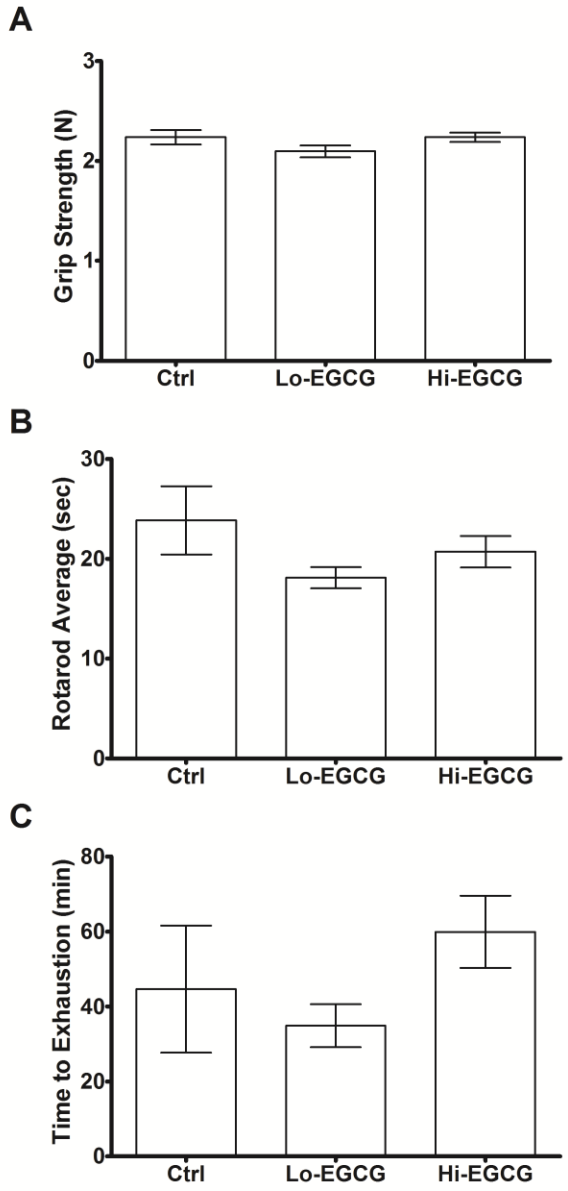

Figure 6. Muscle function testing. A) Grip strength, average of the maximum grip strength each day in 5 trials over 2 days. B) Rotarod, average of total of 8 trials, 4 trials per day over 2 days. C) Time to exhaustion on a treadmill test. Ctrl: control mice (0 mg EGCG per gram diet, N=6). Lo-EGCG: mice given low EGCG dose (91.1 μg EGCG per gram diet, N=8). Hi-EGCG: mice given high EGCG dose (3.67 mg EGCG per gram diet, N=10). All data are mean ± SEM.

disease-related mortality in men and heart disease-related mortality in women (Saito et al. 2015). Likewise, a study of over 75,000 Japanese adults found a reduction in cardiovascular disease-related mortality with increased green tea consumption (Mineharu et al. 2011). However, a similar study in over 50,000 Chinese adults found no association between green tea consumption and all-cause or disease-specific mortality (Odegaard et al. 2015). A recent meta-analysis of 18 prospective studies found a significant inverse relationship between green tea consumption and all-cause and cardiovascular disease-related mortality (Tang et al. 2015). Interestingly, although black tea consumption appears to be associated with reduced mortality to cancer, green tea has consistently been found to have no association with cancer-specific mortality (Tang et al. 2015). The underlying physiological mechanisms responsible for these findings are unclear, but it can be reasonably speculated that the antioxidant properties of green tea and its constituents, especially EGCG, may play a role in reducing risk of death due to cardiovascular disease.

Similar to our previous findings (Gibbons et al. 2014, Pence et al. 2016), neither high nor low-dose EGCG improved cognitive function compared to control mice, as assessed by Y-maze, active avoidance, and social discrimination tests, or muscle function as determined by exhaustive fatigue, forelimb grip strength, and rotarod tests. Several previous studies have demonstrated EGCG-mediated improvements in mechanisms related to cognitive (Mandel et al. 2005, Sutherland et al. 2006) and muscle function (Alway et al. 2014, Alway et al. 2015, Senthil Kumaran et al. 2008), although few have done so by incorporating EGCG into the diet (Friedrich et al. 2012, Sae-Tan et al. 2011).

We speculate that one potential cause of the lack of a positive effect of EGCG on muscle or cognitive function was the dosing strategy, which is a primary limitation of this study. By incorporating EGCG into the diet, mice receive small doses of EGCG throughout the active period rather than in one bolus dose, possibly reducing its efficacy. However, we found through experimentation that EGCG supplement (Teavigo) was unpalatable to the mice when added to the drinking water, even when supplemented with compounds such as sucrose that are commonly used to encourage mice to consume water quickly (unpublished findings). Other strategies to give an oral bolus dose of EGCG, such as gavage feeding, were rejected as too stressful for a long-term study in aged mice undergoing behavioral testing and who are already prone to mortality due to advanced age. Another minor limitation of this study is the serial nature of the behavioral tests. In particular, the active avoidance test may induce aversive behavior in the mice, leading to decreased performance in the social exploration test. However, given our lack of diet effect on behavior in this and previous studies, we deem it unlikely that the order in which the tests were

performed in this case would have masked a positive dietary effect.

Several other alternative conclusions are possible. EGCG may be more efficacious in preventing age-related decline in cognitive and muscle function rather than in reversing them. In this case, starting an EGCG intervention earlier in life may be more successful. Additionally, it is possible that by preventing mortality in the EGCG groups, mice that performed more poorly in those groups in behavioral testing at the post-intervention time points were spared, while mice in the control group which would have performed poorly compared to surviving mice died and were thus omitted from the analysis. This would have the effect of reducing the difference in means between groups and removing any potential dietary effect. More simply stated, it is possible that the EGCG intervention preserved more unhealthy mice, while more unhealthy mice in the control group died, thus biasing our behavioral results toward no effect. This is speculative as we collected no data that could either refute or support this hypothesis. Further research using alternative experimental designs amenable to repeated behavioral measures would be needed to verify this possibility.

Conclusion

Dietary treatment with EGCG for 9 weeks resulted in a dose-dependent reduction in mortality in aged Balb/cByJ mice. Because this was not considered *a priori* to be a primary outcome of interest, the mechanisms responsible for this finding were not explored. However, several large prospective studies examining green tea consumption in humans have demonstrated lower mortality. In this study, we found no effect of EGCG at 0.091 or 3.67 mg per g AIN-93M diet in altering either cognitive or muscle performance.

Funding

Funded by the Center for Nutrition, Learning and Memory, a partnership between the University of Illinois at Urbana-Champaign and Abbott Nutrition.

Conflicts of Interest

JAW, RWJ, and JSR are funded by the Abbott-University of Illinois partnership, the Center for Nutrition, Learning, and Memory. For the remaining authors none are declared.

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