Long-Term Supplementation with EGCG and Beta-Alanine Decreases Mortality but does not Affect Cognitive or Muscle Function in Aged Mice

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ABSTRACT

We have previously shown that 6 weeks of a diet containing epigallocatechin gallate (EGCG) and beta-alanine (B-ALA) was not effective in improving either cognitive or muscle function in aged (18 month) mice (Gibbons et al. Behav Brain Res 2014). However, diet reduced oxidative stress in the brain, and previous studies using longer-term interventions have documented beneficial effects in cognitive, but not muscle, function. Therefore, we investigated the effect of 6 months of feeding on measures of cognitive and muscle function in mice. Mice (12 months, N=15/group) were fed AIN-93M containing 0.15% EGCG and 0.34% B-ALA or standard AIN-93M for 6 months, then underwent a battery of tests for cognitive and muscle function at 18 months. Interestingly, a higher percentage of mice receiving EGCG and B-ALA (E+B, 80%) survived to study end compared to control (Ctrl, 40%) mice (p=0.02). E+B did not affect arm preference in the Y-maze test (p=0.74, novel arm) and did not alter performance in an active avoidance test (p=0.16, avoidances per 50 trials). E+B increased rotarod performance (p=0.03), did not affect grip strength (p=0.91), and decreased time to exhaustion in a treadmill fatigue test (p=0.02) compared to Ctrl. In conclusion, E+B reduced mortality, had no effect on cognitive function and variable effects on muscle function. **Keywords**: epigallocatechin gallate, beta-alanine, mortality, cognition, muscle function, aging.

INTRODUCTION

Physiological aging includes several functional changes that lead to impairments in numerous organ systems. Notable among these is age-related loss of muscle function (sarcopenia) (Morley et al., 2011). Sarcopenia is associated with dysfunction of skeletal muscle mitochondria (Rooyackers et al., 1996) and increased skeletal muscle oxidative stress (Capel et al., 2005). In addition to sarcopenia, aging causes pathological changes in the brain, leading to decreased cognitive performance and memory (Amrein et al., 2011; Kuhn et al., 1996; Lucassen et al., 2010). Aging upregulates expression of pro-inflammatory cytokines and increases oxidative stress in the hippocampus, and this in turn can lead to decreased hippocampal neurogenesis (Fukui et al., 2002; Huang et al., 2012; Vallieres et al., 2002). Due to the frequency of age-associated impairments in cognition and muscle function, therapeutic interventions aimed at preventing or ameliorating these conditions are of major interest.

Nutritional strategies to counteract processes linked to the pathophysiology of aging, such as oxidative stress and inflammation, have been widely investigated. Green tea is of particular note, and green tea bioactive constituents include catechins, the most abundant of which is epigallocatechin-3-gallate (EGCG) at 60% of total catechin content (Cabrera et al., 2006). In the

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central nervous system, EGCG has been shown to reduce microglial activation (Li et al., 2004; Wu et al., 2012), oxidative stress (Mandel et al., 2005; Sutherland et al., 2006), and inflammation (Kim et al., 2007) and to increase neuronal survival (Ortiz-Lopez et al., 2016), and green tea consumption is associated with reduced cognitive impairment in aged humans (Kuriyama et al., 2006). In skeletal muscle, EGCG has been shown to decrease activity of antioxidant enzymes such as catalase and glutathione reductase (Senthil Kumaran et al., 2008), to modulate autophagy (Takahashi et al., 2017), and to prevent muscle loss or enhance muscle recovery after hind-limb suspension-induced unloading in rats (Alway et al., 2014; Alway et al., 2015). β-Alanine (β-Ala) is the non-proteinogenic β form of alanine and along with L-histidine is a precursor to carnosine. Carnosine is a cellular antioxidant and pH buffer (Boldyrev et al., 1993) and a potential muscle performance enhancer, as it increases calcium sensitivity and aids calcium ion release in skeletal muscle (Dutka et al., 2012). In aged adults, β-Ala supplementation has been shown to increase carnosine content in skeletal muscle and to improve physical capacity (del Favero et al., 2012), and these findings were positively correlated. Muscle carnosine content naturally decreases with age (Stuerenburg and Kunze, 1999), making β-Ala supplementation an attractive strategy to reduce or prevent age-related muscle dysfunction. There is additionally some limited evidence that β-Ala has positive effects on learning and memory. β-Ala has been shown to increase in the hippocampus of mice after performance of a probe trial in the Morris Water Maze task (Sase et al., 2013), suggesting β-Ala may be involved in spatial memory.

In previous work, we demonstrated that a short 6 week intervention of EGCG and β-Ala feeding had little effect on either cognitive or muscle function in aged (Gibbons et al., 2014; Pence et al., 2016) or young (Bhattacharya et al., 2015) mice (Balb/cByJ strain). However, the intervention, which consisted of EGCG and β-Ala given in the diet at respective concentrations of 0.15% and 0.343%, did show some promise by altering several molecular markers associated with both muscle and cognitive function in aged mice. Dietary intervention increased gene expression of several markers of mitochondrial biogenesis in skeletal muscle in our previous study (Pence et al., 2016), including Ppargc1a and Sirt1. As mitochondrial functional impairments are thought to be a major driver behind age-related muscle dysfunction (Rooyackers et al., 1996), this suggests that our feeding paradigm may have had a subclinical effect. Likewise, the dietary intervention also decreased oxidative stress in the cerebellum of aged mice (Gibbons et al., 2014), and oxidative stress has been linked to cognitive impairments in aging (Fukui et al., 2002).

Therefore, we considered an alternative strategy which might increase efficacy of our dietary intervention. Given the molecular evidence above, we considered that (a) the feeding period might have been too short to derive clinical effects, and (b) that our intervention might be better suited as preventative (e.g. starting earlier in life, prior to onset of cognitive and muscle dysfunction) rather than as therapeutic. Therefore, we elected to examine whether a longer-term feeding intervention (6 months) begun earlier in life (12 months) would show benefit in preventing age-related cognitive or muscle dysfunction. We hypothesized that dietary supplementation with EGCG and β-Ala would improve performance on several muscle and cognitive function tasks, and that these improvements would be associated with reduced inflammation and oxidative stress and increase growth factor and neurotrophic factor expression in the skeletal muscle and hippocampus.

METHODS

Animals

Male Balb/cByJ retired breeder mice (7-9 months old, N=30) 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 12 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^{o}C. All procedures used were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign.

Study Design

The study design is outlined in Figure 1. Mice were transferred to standard shoebox cages at the onset of the experiment (12 mo. of age). Mice were randomized equally (N=15/group) to receive either control diet or diet containing EGCG and β-Ala and maintained on these diets for 6 months prior to behavioral testing. After the feeding intervention, mice underwent a battery of behavioral tests to assess cognitive and muscle function. Mice were maintained on their respective experimental diets for the duration of behavioral testing and through euthanasia/tissue collection. Behavioral procedures are described below. During the behavioral testing period, mice underwent Y-maze testing on days 1 and 2 and muscle function testing on days 3 and 4. Following this, mice underwent active avoidance testing for 5 days on days 5-9 and, following a rest day on day 10, mice were euthanized for tissue collection on day 11 of the

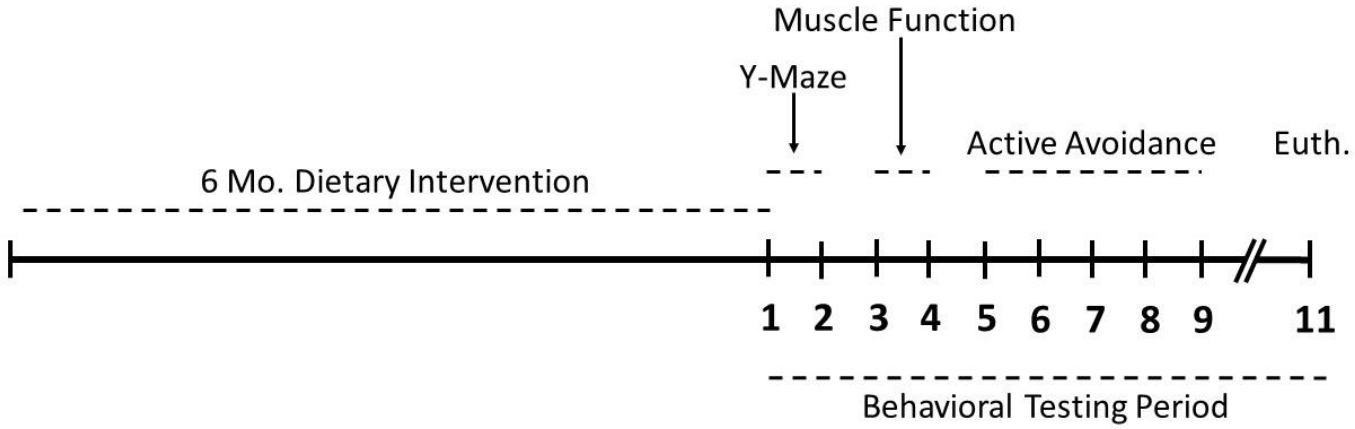

Figure 1. Study design. Euth: euthanasia and tissue collection day.

testing period. On the day of tissue collection, mice were euthanized by rapid CO_2 asphyxiation, and gastrocnemius and hippocampus samples were collected and immediately flash-frozen. Samples were stored at -80°C until processing for gene expression analysis (see below).

Diets

Ctrl and EGCG + ß-ALA diets were purchased from Research Diets, Inc. (New Brunswick, NJ) and were based on the purified AIN-93M mature rodent diet. Mice receiving Ctrl were given ad libitum access to the AIN-93M diet throughout the study. EGCG + ß-ALA was manufactured by Research Diets by mixing 1.7 mg Teavigo (90% EGCG, DSM Nutritional Products, Basel, Switzerland) and 3.43 mg β-Ala (NutraBio, Middlesex, NJ) per g of AIN-93M diet, which was then pelleted to match the consistency and appearance of Ctrl. Dietary constituents have been previously published (Gibbons et al., 2014). Target EGCG dosage was determined based on previous studies demonstrating beneficial cognitive effects of EGCG in mice (Li et al., 2009). As studies examining β-Ala supplementation and its effects on cognition or muscle function in mice are lacking, we calculated β-Ala dosage using the 2.4 $g \cdot day^{-1}$ dose that has been shown to improve physical work capacity in humans (Stout et al., 2008). For a 70 kg human, this equates to 34 $mg \cdot kg^{-1} \cdot day^{-1}$ and was adjusted for mice using the FDA-recommended conversion of 12.3 (Us, 2005). Thus, the target dose for β-Ala in this study was 418 $mg \cdot kg^{-1} \cdot day^{-1}$.

Y Maze Testing

The Y-shaped maze used is made of dark gray colored plexiglass with each arm being 15 inches in length, 5 inches in height, 3 inches 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 the movement of mice in the maze.

Animals were brought to the behavioral suite 10 minutes 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 that is made 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. An animal was placed in far end of the start arm and allowed to navigate both start arm and open arm for 15 minutes. The apparatus was cleaned with ethanol after each animal to remove any remnant of odor. Percent time durations in start arm, familiar arm, and center were measured. All four paws of animal must enter the area to be counted.

Two hours after acquisition trial, animal was returned to the maze. All three arms were opened and mice were allowed to explore for 5 minutes. The percent time duration and number of entries for start arm, familiar arm, center, and novel arm were measured to assess spatial working memory associated with preference for the novel arm. Y-maze was performed over 2 days (days 1 and 2 of the testing period), and testing was balanced by group.

Active Avoidance Testing

Following Kohman et al. (Kohman et al., 2012), animals were trained on the task for 50 trials per day for 5 consecutive days (days 5-9 of the behavioral testing period) using the GEMINI Avoidance System (San Diego Instruments, San Diego, Ca). Each animal was placed in an active avoidance chamber and allowed to acclimate for 5 minutes. A trial begins with presentation of a yellow cue light (conditioned stimulus) from the opposite side of where animal is present, for 5 seconds. If the mouse shuttles to the room containing the cue light within the 5 seconds, the program records the response as an active avoidance, and the trial will terminate right away. If the mouse fails to shuttle to the

room containing the cue light, the mouse receives a 0.50 mA foot shock (unconditioned stimulus) lasting up to 5 seconds. If the mouse moves to the other room while the shock is still delivered, the program records the response as an Escape, and the trial will end. Otherwise, if the mouse remains in the same chamber for the entire duration of the shock, the program will count the response as a No Response, and the trial will end after 5 seconds. Each trial is followed by a 20-second interval with no cue light or shock.

Body Composition

Body composition was analyzed on the final day of the pre-testing portion of the study (i.e. the day before behavioral testing began) by small animal magnetic resonance imaging (EchoMRI, Houston, TX). Total fat mass and lean mass were assessed for each mouse by this method. Data were expressed as % fat mass or % lean mass by dividing fat mass or lean mass by total body mass.

Muscle Function Testing

Forelimb grip strength was assessed using a commercially available force gauge (Columbus Instruments, Columbus, OH) and was assessed in 5 separate trials per day over 2 consecutive days during the muscle function testing period (days 3 and 4 of the behavioral testing period) by the same investigator. Grip strength was quantified as the average of the highest recorded grip force on each testing day at both time points and as the percent change in maximal grip strength from the beginning to the end of the study in each group. Grip strength is expressed as peak force in Newtons (N).

An exhaustive treadmill test was performed to assess fatigability (day 4 of the behavioral testing period). 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 seconds. The test ended at 120 minutes 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 acceleration to a maximum of 60 rpm over 180 seconds. Timing was controlled by photobeam, and timing for each mouse was stopped automatically by the system when the falling mouse broke the plane of the photobeam. Mice underwent 4 consecutive trials per day (days 3 and 4 of the behavioral testing period). Data were expressed as the average performance across all 8 trials at pre-intervention (pre) and the average performance across all 8 trials at post-intervention (post).

Gene Expression Analysis

Total RNA was isolated from frozen gastrocnemius and frozen hippocampus samples by Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, quantified by nanospectrophotometry, and stored at -80°C until reverse transcription. RNA was reverse-transcribed to cDNA using a commercially available high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The cDNA was stored at -20°C until gene expression analysis.

Gene expression analysis was performed by Taqman Low Density Array (TLDA, Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. A total of 1000 ng of cDNA was loaded per sample. Commercially-available, pre-validated primers (Applied Biosystems, Carlsbad, CA) were used for TLDA analysis (Supplementary Table S1). TLDA cards were run on a high-throughput real-time polymerase chain reaction system (7900HT, Applied Biosystems, Carlsbad, CA), and Ct values were determined using SDS 2.4 and RQ Manager 1.2.1 software packages (Applied Biosystems, Carlsbad, CA). All samples were run in duplicate, and gene expression was expressed relative to the housekeeping gene (*Gapdh*) and expressed as fold change from the referent Ctrl-Sed group by the $2^{-\Delta\Delta Ct}$ method.

Data Analysis

Body weight (pre- and post-intervention) was analyzed by repeated-measures (RM-) analysis of variance (ANOVA) in a 2 × 2 (time × diet) 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 independent-samples t-tests with Welch correction in the event of unequal variances. Active avoidance data were analyzed by RM-ANOVA in a 5 × 2 (day × diet) design. Treatment differences were assessed by Bonferroni-corrected post-hoc mean separation in the event of a significant main effect or interaction. Gene expression data were analyzed in R v. 3.2.5 (R Foundation for Statistical Computing, Vienna, Austria) by independent-samples t-tests with Benjamini-Hochberg correction for false-discovery rate. Mortality data were analyzed using the Log-rank (Mantel-Cox) test in Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA. All data (with the exception of survival curves and gene expression) 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 are expressed as mean ± SEM except where noted, and figures reporting individual data points are used for graphical communication of results wherever practical.

RESULTS

Descriptive Data

There was a time × treatment interaction for body weight ($F_{1,17}$=18.9, p=0.01), but no significant main effects for time ($F_{1,17}$=6.3, p=0.13) or group ($F_{1,17}$=0.05, p=0.83, Figure 2A). This may have been driven by pre-intervention weight differences between groups, although this was not significant ($t_{df=29}$, p=0.10). When body weight was expressed as percent change pre- to post-intervention, there was a significant difference between groups (p=0.019) such that Ctrl mice lost weight (post-intervention 92.2% of pre-intervention body weight), whereas EGCG/β-Ala mice showed a slight gain (post-intervention 102.5% of pre-intervention body weight).

There was a significant difference in food intake, such that mice in the EGCG/β-Ala group consumed less food per day than mice in the Ctrl group ($t_{(df=15.5)}$=3.1, p=0.02, Figure 2B). Additionally, EGCG/β-Ala mice had reduced percentage lean mass at the end of the intervention ($t_{(df=17)}$=3.8, p=0.001, Figure 2C) compared to Ctrl. Fat mass percentage was not significantly different ($t_{(df=17)}$=0.05, p=0.96, Figure 2D).

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 (Pence et al., 2017). There were no differences in percentage of total time spent in any arm between EGCG/β-Ala and Ctrl (start t(df=18)=-0.40, p=0.69; center t(df=18)=0.17, p=0.87; familiar t(df=18)=-0.83, p=0.42; novel t(df=18)=0.89, p=0.38; Figure 3A). 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 (t(df=18)=1.1, p=0.27, Figure 3B), and neither group

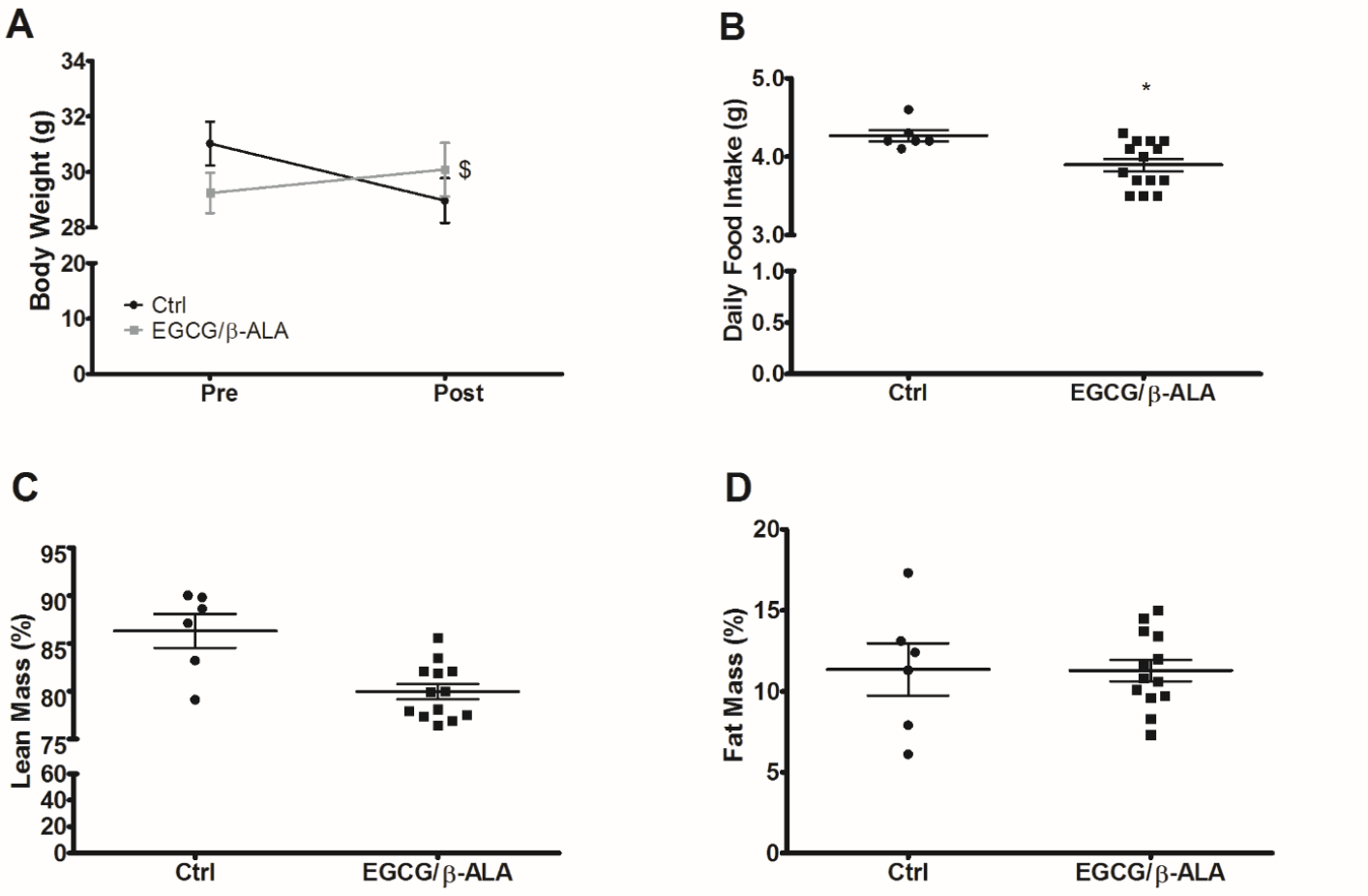

Figure 2. Descriptive data. **(A)** Body weight at pre-intervention (Pre) and at sacrifice (Post). $ Significant time × treatment interaction (p=0.013). When body weights were expressed as percent change pre-to-post, there was a significant main effect of treatment (p=0.019). **(B)** Daily food intake. **(C)** Percentage total lean mass. **(D)** Percentage total fat mass. * Significantly different than Ctrl (p<0.05). Ctrl: control group, N=6-7. EGCG/β-ALA: EGCG and β-alanine group, N=13-14.

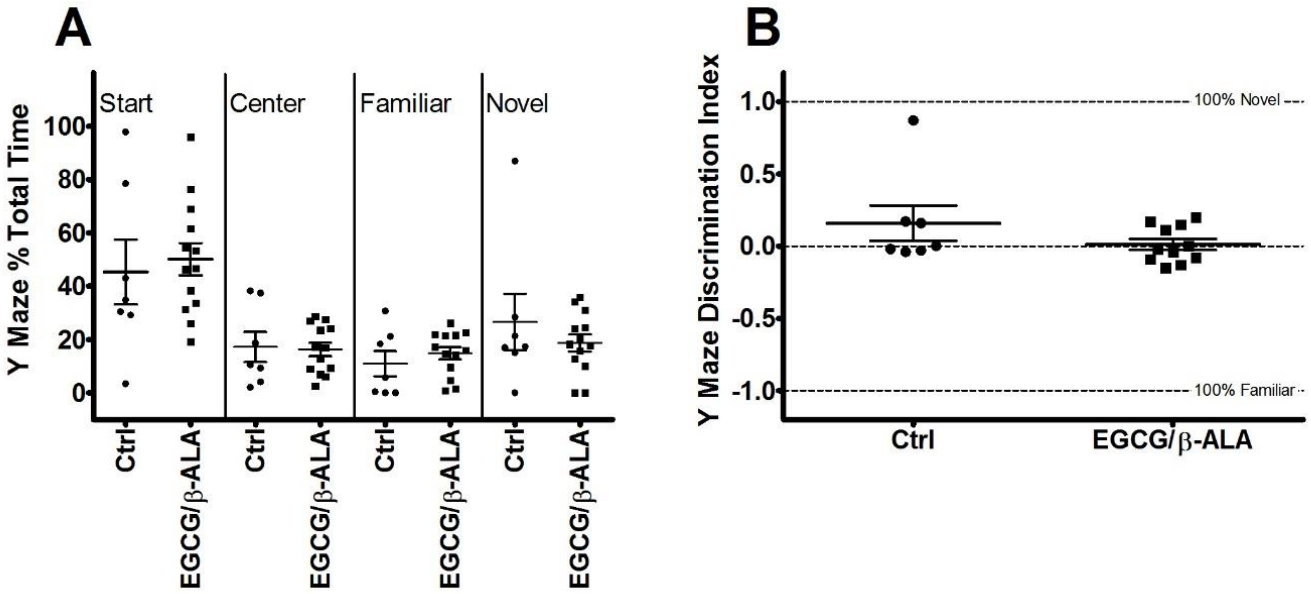

Figure 3: Y-maze. **(A)** Percentage of total test time spent in each area of the Y-maze. **(B)** Discrimination index, a comparison of time spent in the familiar arm to time spent in the novel arm, omitting time spent in the start arm or center. A value of 1.0 is equivalent to 100% preference for the novel arm, whereas as value of -1.0 is equivalent to 100% preference for the familiar arm. Ctrl: control group, N=7. EGCG/β-ALA: EGCG and β-alanine group, N=13.

showed a preference for novel arm versus familiar arm as indicated by 95% confidence intervals overlapping with 0 (Ctrl [-0.15, 0.49]; EGCG/β-Ala [-0.04, 0.12]).

Active Avoidance Testing

Active avoidance data are expressed as number of successful avoidances out of 50 trials per day for 5 days (Figure 4). Mice in both groups successfully learned the task, as indicated by a significant main effect of Day ($F_{4,68}$=47.2, p<0.001), but there was no significant main effect of Treatment ($F_{1,17}$=2.2, p=0.16) and no significant Day × Treatment interaction ($F_{4,68}$=0.13, p=0.97), suggesting that EGCG/β-Ala treatment did not affect performance on the active avoidance task.

Muscle Function Testing

Maximal forelimb grip strength did not differ between groups ($t_{(df=17)}$=-0.11, p=0.91, Figure 5A). However, EGCG/β-Ala consumption resulted in improved performance on the rotarod test ($t_{(df=17)}$=-2.4, p=0.03, Figure 5B) and decreased performance on the treadmill test to exhaustion ($t_{(df=16.2)}$=2.7, p=0.02, Figure 5C).

Gene Expression Analysis

Gene expression relative to Ctrl for isolated hippocampal and gastrocnemius samples is presented in Figure 6. After correction for the number of comparisons using the Benjamini-Hochberg procedure, there were no significant differences in expression of any gene between groups, suggesting that EGCG/β-Ala treatment did not alter gene expression of markers of inflammation, neurogenesis, oxidative stress, cell signaling, or other processes in the central nervous system or skeletal muscle.

Mortality

Interestingly, consumption of EGCG/β-Ala prevented mortality during the course of the study. Fewer mice in the EGCG/β-Ala treatment group (3/15) died during the course of the study than mice in the Ctrl group (9/15, χ^2=5.6, p=0.02, Figure 7).

DISCUSSION

We have previously shown that a diet containing EGCG and β-Ala did not alter cognitive (Gibbons et al., 2014) or muscle function (Pence et al., 2016) in aged mice when given over a short-term intervention

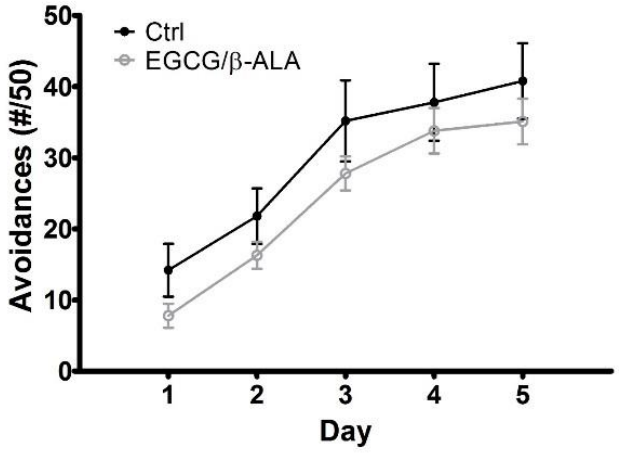

Figure 4: Active avoidance, number of successful avoidances per 50 trials per day. Ctrl: control group, N=7. EGCG/β-ALA: EGCG and β-alanine group, N=13.

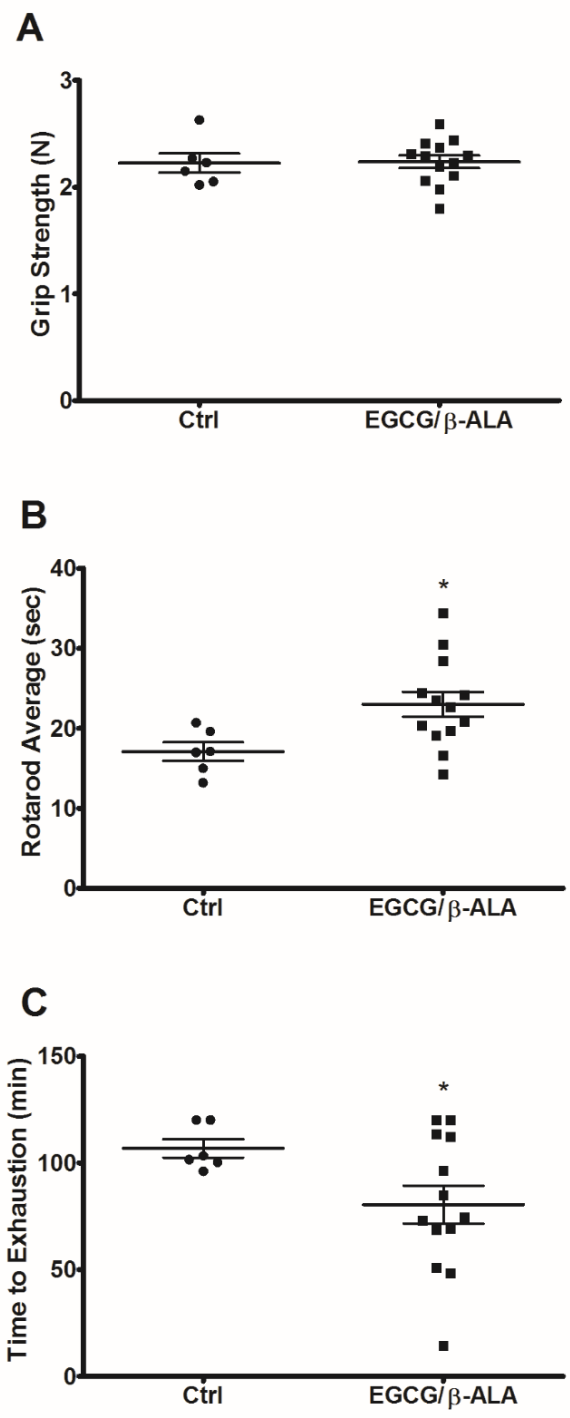

Figure 5: Muscle function. **(A)** Forelimb grip strength, average of best performance on each of two consecutive days. **(B)** Rotarod, average of 4 tests per day for two consecutive days. **(C)** Time to exhaustion on a graded treadmill test. Mice were stopped at 120 minutes. * Significantly different than control ($p<0.05$). Ctrl: control group, N=6. EGCG/β-ALA: EGCG and β-alanine group, N=13.

(6 weeks). However, because we saw some molecular changes that are known to be associated with improvements in our outcomes of interest, we hypothesized that our intervention may have been insufficient in length to demonstrate functional changes. Thus, we investigated the effects of a 6-month dietary intervention on cognitive and muscle function, using similar strategies to those in our previous published studies.

The most interesting finding arising out of this study is the reduction in mortality with EGCG/β-Ala supplementation in aged Balb/cByJ mice. As this was not considered a priori as a primary outcome of the study (and indeed was not hypothesized), we unfortunately did not collect data which could have suggested mechanisms as to how this effect occurred. For example, because this was an unexpected finding, we did not perform necropsies on these mice as they died, thus we do not have information as to the cause of mortality for mice which did not survive to the end of the study. It would be interesting in future research to examine mice by necropsy in this context to determine if there is a cause underlying mortality in these mice which might be prevented by EGCG and/or β-Ala supplementation.

As with our previous studies in aged mice (Gibbons et al., 2014; Pence et al., 2016), we combined EGCG and β-Ala and did not investigate the supplements separately. Because we found no significant effects of the combined treatment on our established outcomes of interest, it made little economic or scientific sense to pursue these supplements individually, as it is unlikely that either supplement would have a significant effect where none was detected with both supplements in conjunction. One limitation of this is that we are left unable to determine if one or both of the supplements was responsible for the decrease in mortality seen in this study. However, evidence from both our group and others suggests that the decrease in mortality seen in this study was most likely mediated by EGCG. For example, green tea consumption has been associated with reduced mortality in several larger studies in Japan (Mineharu et al., 2011; Saito et al., 2015), and a recent meta-analysis showed an inverse relationship between green tea consumption and both all-cause and cardiovascular-disease related mortality (Tang et al., 2015). Additionally, in a separate study we determined that EGCG alone reduces mortality in a dose-dependent fashion in aged mice (Pence et al., 2017).

Although EGCG and green tea are more well-established as longevity promoters in the experimental literature, β-Ala may also have a significant effect of extending lifespan. Carnosine supplementation has been shown in a few studies to be anti-senescent (possibly due to anti-oxidant activity), and carnosine supplementation has increased lifespan in Drosophila, although these effects are inconsistent (Hipkiss, 2009). Thus, it is possible that β-Ala mediated the longevity

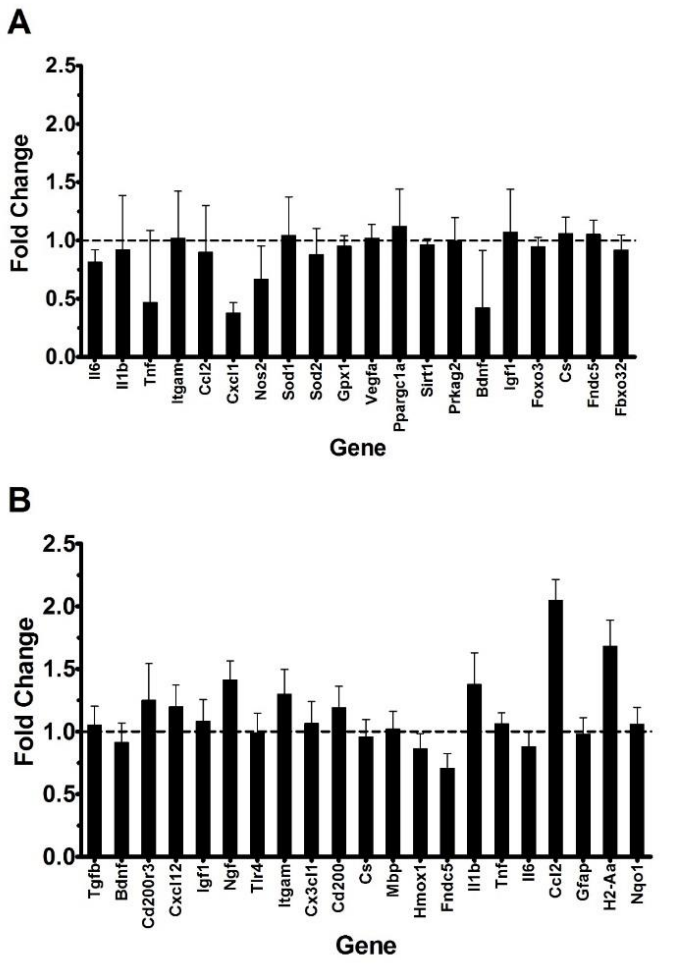

Figure 6: Gene expression. Data are shown for EGCG/β-ALA group with Ctrl group as referent (expression set to 1.0 for each gene and marked by the dotted line). **(A)** Gastrocnemius gene expression. **(B)** Hippocampus gene expression. Ctrl: control group, N=6. EGCG/β-ALA: EGCG and β-alanine group, N=13.

effect in this study, or that the combination of ingredients had an additive effect. Further experimentation is needed to determine the relative contributions of the supplements. However, as longevity was not the primary aim of the overall project, we did not pursue this line further.

Beyond the mortality data, we found little effect of the EGCG/β-Ala intervention on any outcomes of interest. In our cognitive function tests, mice given the dietary intervention did not perform better on the Y-maze or active avoidance tasks. Similarly, muscle function tests (forelimb grip strength, rotarod, treadmill exhaustive fatigue) also showed inconsistent effect of dietary intervention, with diet-based improvements in rotarod and impairments in treadmill performance. The significant negative effect of the dietary treatment on treadmill performance was unexpected, as neither beta-alanine nor EGCG are known to have ergolytic properties in the doses given in this study. Indeed, we had originally hypothesized that the dietary treatment would be ergogenic, although our previous shorter-term study found no effect of the dietary treatment on treadmill performance (Pence et al., 2016). This unusual finding may be explained by mortality-related bias as discussed below.

Contrary to our previous work, in this study we also found no effect of the dietary intervention on molecular markers related to cognitive and muscle function decline in aging. For example, we previously reported (Pence et al., 2016) that 40 days of EGCG/β-Ala increased skeletal muscle expression of Ppargc1a, the gene encoding PGC-1α, and Sirt1, the gene encoding SIRT-1. Both PGC-1α and SIRT-1 are important in mitochondrial biogenesis (Puigserver and Spiegelman, 2003; Sack and Finkel, 2012), and mitochondria are known to be dysfunctional in aging. Likewise, our previous work (Gibbons et al., 2014) showed that EGCG/β-Ala reduced expression of 4-hydroxynonenal, a marker of oxidative stress, in the cerebellum.

Here, we showed no effects of EGCG/β-Ala on gene expression of any markers measured. This included markers of inflammation, such as genes encoding IL-1β, TNF-α, IL-6, and others in both the hippocampus and gastrocnemius. Additionally, we found no diet-induced changes in expression of genes encoding markers of oxidative stress, such as NOS2, GPX1, SOD1, and SOD2 in the gastrocnemius and NQO1 in the hippocampus, nor were there diet-induced changes in expression of genes producing growth factors such as BDNF (hippocampus and gastrocnemius) and TGF-β (hippocampus). Gene expression for markers of mitochondrial biogenesis and content (PGC-1α and SIRT1 in the gastrocnemius, citrate synthase in the gastrocnemius and hippocampus) were also not differentially regulated by diet. We measured expression of a number of other markers, and no changes were found in this study.

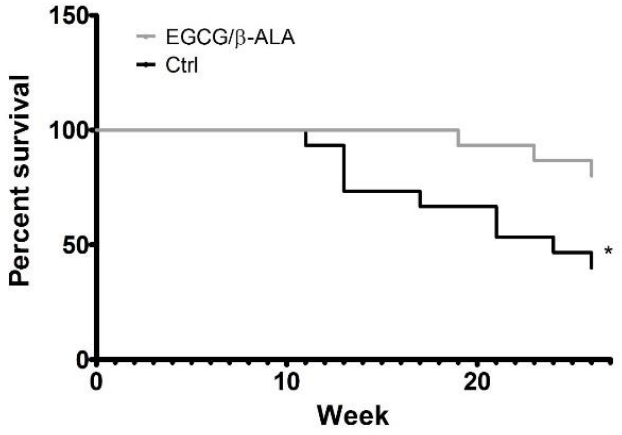

Figure 7: Percentage survival across time. * Significantly different than EGCG/β-ALA ($p<0.05$). Ctrl: control group, 6/15 survived. EGCG/β-ALA: EGCG and β-alanine group, 12/15 survived.

One potential limitation in this study is the difference in mortality rate itself. Most behavioral tests cannot be performed serially, as latent memory invalidates repeated tests. Thus, our analyses were limited to surviving mice remaining in the study at the end of the intervention, and the groups by this time were unbalanced due to differing mortality. Additionally, it is possible, although we have no direct evidence for this speculation, that the EGCG/β-Ala treatment caused poorer-performing mice to remain alive and thus to be tested, while only the best performers in the Ctrl group remained alive at study's end. This can best be visualized in Figure 5C. In the treadmill test for exhaustion, EGCG/β-Ala-treated mice performed significantly worse than control mice. However, the top 6 performers in the EGCG/β-Ala group performed qualitatively similarly to the 6 remaining mice in the Ctrl group, and the mean for the EGCG/β-Ala group was brought down by a number of worse-performing mice. It is possible (and perhaps even likely) that the mice which died prior to testing in the Ctrl group would have performed worse than the 6 tested mice, and thus the Ctrl mean might be artificially raised due to the mortality in that group. This remains speculative but may account for some of the unusual between-group differences seen in this study, especially in the treadmill time-to-exhaustion test and in the body composition (lean mass) data.

Finally, our study may be limited by our dosing strategy. Addition of these components in the diet causes the mice to receive "micro-doses" of each supplement across each day, rather than in one bolus as in many dietary intervention studies. However, we found in preliminary work (not shown) that mice did not tolerate addition of the EGCG supplement to their water, even when additives such as sucrose were used, and we further rejected repeated gavage feeding as too stressful for aged mice, especially in the latter portion of the study. Additionally, we preferred the natural oral route of delivery of the supplements, thus intraperitoneal injection was rejected on those grounds. Thus, it is possible that an alternate dosing strategy might be more efficacious in these mice, but incorporation of EGCG/β-Ala in the diet was chosen in this case as the best compromise given our research model and desired delivery route.

Conclusions

In conclusion, treatment with EGCG/β-Ala for 6 months reduced mortality but did not have any notable effects on muscle function or cognitive performance, nor on gastrocnemius or hippocampus expression of any genes related to these measures. Alternative dosing strategies or strategies to enhance bioavailability of the dietary supplements may be necessary in order for EGCG/β-Ala to effectively regulate muscle or cognitive function.

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CONFLICTS OF INTEREST

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Supplemental Table S1: Primers used for Taqman Low Density Array detection of gene expression.

Gene	Primer Set†	Tissue‡
Il6	Mm00446190_m1	G H
Il1b	Mm00434228_m1	G H
Tnf	Mm00443260_g1	G H
Itgam	Mm00434455_m1	G H
Ccl2	Mm00441242_m1	G H
Cxcl1	Mm04207460_m1	G
Nos2	Mm00440502_m1	G
Sod1	Mm01344233_g1	G
Sod2	Mm01313000_m1	G
Gpx1	Mm00656767_g1	G
Vegfa	Mm01281449_m1	G
Ppargc1a	Mm01208835_m1	G
Sirt1	Mm00490758_m1	G
Prkag2	Mm00513977_m1	G
Bdnf	Mm04230607_s1	G H
Igf1	Mm00439560_m1	G H
Foxo3	Mm01185722_m1	G
Cs	Mm00466043_m1	G H
Fndc5	Mm01181543_m1	G H
Fbxo32	Mm00499523_m1	H
Tgfb1	Mm01178820_m1	H
Cd200r3	Mm01343888_m1	H
Cxcl12	Mm00445553_m1	H
Ngf	Mm00443039_m1	H
Tlr4	Mm00445273_m1	H
Cx3cl1	Mm00436454_m1	H
Cd200	Mm00487740_m1	H
Mbp	Mm01266402_m1	H
Hmox1	Mm00516005_m1	H
Gfap	Mm01253033_m1	H
Gapdh	Mm99999915_g1	G H

† Catalog number for pre-validated commercial primers ordered from Applied Biosystems (Carlsbad, CA). ‡ Tissue assayed for expression of indicated gene. G: gastrocnemius; H: hippocampus.