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Blueberry Supplementation Improves Memory in Middle-Aged Mice Fed a High-Fat Diet

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ABSTRACT: Consuming a high-fat diet may result in behavioral deficits similar to those observed in aging animals. It has been demonstrated that blueberry supplementation can allay age-related behavioral deficits. To determine if supplementation of a high-fat diet with blueberries offers protection against putative high-fat diet-related declines, 9-month-old C57Bl/6 mice were maintained on low-fat (10% fat calories) or high-fat (60% fat calories) diets with and without 4% freeze-dried blueberry powder. Novel object recognition memory was impaired by the high-fat diet; after 4 months on the high-fat diet, mice spent 50% of their time on the novel object in the testing trial, performing no greater than chance performance. Blueberry supplementation prevented recognition memory deficits after 4 months on the diets, as mice on this diet spent 67% of their time on the novel object. After 5 months on the diets, mice consuming the high-fat diet passed through the platform location less often than mice on low-fat diets during probe trials on days 2 and 3 of Morris water maze testing, whereas mice consuming the high-fat blueberry diet passed through the platform location as often as mice on the low-fat diets. This study is a first step in determining if incorporating more nutrient-dense foods into a high-fat diet can allay cognitive dysfunction.

KEYWORDS: *high-fat diet, obesity, blueberry, recognition memory, spatial memory*

INTRODUCTION

Long-term consumption of a high-fat (HF) diet has been shown to increase morbidity and mortality in humans and other animals.[1] The metabolic syndrome that can result from consuming a HF diet may result in neuronal dysfunction through a multitude of mechanisms.[2] HF diet consumption has been shown to increase inflammation and oxidative stress[3,4] and reduce neurogenesis and nerve growth factors in the brains of rodents[5] in ways that parallel some of the brain alterations associated with aging. HF diet consumption may also result in similar behavioral deficits as observed in aging animals.[3,6] Studies have demonstrated that a HF diet can impair rodent probe trial performance in a spatial maze[6] and retention in a T-maze.[3] Studies have also shown that mice fed a HF diet make more errors in the Stone T-maze[4] and have impaired object location memory.[7]

Flavonoid- and anthocyanin-rich foods have been shown to be effective in reversing age-related deficits in learning and memory.[8] Furthermore, it has been demonstrated that diets supplemented with anthocyanin-rich berries, such as blueberries (BB), can mitigate behavioral deficits and brain inflammation and oxidative stress associated with aging.[9−12] Given that many of the alterations observed in the brain and on behavior in HF diet-fed animals may be similar to those observed in aging, it stands to reason that supplementation of a HF diet with BB may offer protection against these putative HF diet-related declines. Dietary studies typically employ a single food supplementation, but not many have used a combination of a HF diet with possible beneficial foods such as fruits or vegetables. One study found that BB can decrease mouse adipose tissue production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα) and interleukin (IL)-10, elicited by HF diet,[13] but few have examined the effects of this type of intervention on the central nervous system. One recent study demonstrated that mice fed a HF diet with 200 mg/kg resveratrol (a stilbenoid found in small amounts in BB) daily for 20 weeks had shorter escape latencies during acquisition and spent more time in the target quadrant during probe trials in the Morris water maze (MWM) than mice fed a HF diet without resveratrol supplementation.[14] However, it is not clear if whole foods, such as BB, will be able to allay the behavioral alterations induced by a HF diet.

This study will begin to determine if healthy foods that have been demonstrated to forestall aging and other central nervous system dysfunction might have an impact on the brains of those consuming HF diet. The aim of this study is to determine the potential benefit of consuming BB to improve the cognitive impairment associated with HF diet consumption and to further elucidate specific impairments associated with HF diet consumption.

MATERIALS AND METHODS

Chemicals. The diets were purchased from Teklad Lab Animal Diets, Madison, WI, USA. The diets were low-fat (LF) diet (TD.08806, 10% calories from fat, Table 1), LF + 4% BB (LFBB, freeze-dried powder, Tifblue cultivar, U.S. Highbush Blueberry Council), HF diet (TD.06414, 60% calories from lard fat, Table 1), and HF + 4% BB (HFBB). The blueberry powder added to the diet

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Table 1. Macronutrient Composition of Low-Fat and High-Fat Lard-Based Diets[a]

% kcal from	10 kcal% fat diet[b]	60 kcal% fat diet[b,c]
protein	20.5	18.4
carbohydrate	69.1	21.3
fat (lard)	10.4	60.3
kcal/g	3.6	5.1

[a]Values are calculated from ingredient analysis or manufacturer data. The LF diet (10 kcal% fat) is TD.08806, which is a Teklad Custom Research Diet from Harlan. The HF diet (60 kcal% fat) is TD.06414, and it also from Harlan. [b]For the 4% BB diets, 40 g of Tifblue powder replaced 25 g of starch, 6 g of cellulose, and 9 g of corn starch. [c]Approximate fatty acid profile (% of total fat): 37% saturated, 47% monounsaturated, 16% polyunsaturated.

was made by freeze-drying whole Tifblue blueberries, and it was supplied by the U.S. Highbush Blueberry Council (Folsom, CA, USA).[15] Tifblue is a rabbiteye variety of BB (*Vaccinium ashei*) and phenolic acid, flavonol, anthocyanin profiles, as well as the nutritional composition of fresh and frozen BB, have been quantified previously.[16−18]

Animals. Sixty-four 9-month-old male C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were individually housed and maintained on a 12 h light/dark cycle. The mice were given food and water ad libitum and given 2 weeks to adjust to their new environment, after which time they were weight-matched (no initial difference in body weight between the four groups: $F_{3,56} = 0.071$, $p = 0.97$, one-way ANOVA) and placed on one of four diets (Teklad Lab Animal Diets) for 5 months ($n = 16$/diet): LF, LFBB, HF, or HFBB. Food intake was assessed over two 24 h periods. All animals were weighed at least weekly and observed daily for clinical signs of disease. Animals were utilized in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. This study was approved by the Jean Mayer Human Nutrition Research Center on Aging at Tufts University Institutional Animal Care and Use Committee. During the 5 month period on the diets, the animals underwent behavioral tests designed to examine object recognition learning and memory and spatial learning and memory. Behavioral testing was performed on $n = 14−16$ mice per group because 1 mouse died ($n = 1$ LF) and 4 mice were removed from the study because of rapid weight loss ($n = 2$ HF, $n = 1$ HFBB) and cataracts ($n = 1$, HFBB).

Novel Object Recognition Test. Mice were tested with the novel object recognition test[19] after 2, 3, and 4 months on the diets (test sessions 1, 2, and 3, respectively). The novel object recognition test measures the ability to remember an object by observing reaction to the introduction of a novel object compared to a previously viewed object; animals with intact recognition memory will spend more time exploring a novel object than a familiar one. The test was performed similarly to the methods of Carey et al.[20] Animals were habituated to an empty testing chamber for 10 min (plastic rectangular cage, 16 × 24 × 12 cm). Next, mice were given three 10 min trials with 10 min intertrial intervals (Figure 3A). In trials 1 and 2 (training trials), mice explored the testing chamber containing two identical objects that were centered across from each other, 2 cm away from the walls. In trial 3 (testing trial), one of the objects was replaced with a novel object. For every trial, the time that the mice spent attending to each object was recorded with stopwatches. Attending to the object was defined as the duration of time the mouse spent in physical contact with the object using any body part other than the tail or whenever it was engaged in active exploration (e.g., sniffing or manipulating). In test session 1 (when mice had been on diets for 2 months), the objects used in testing were dice and marbles, in test session 2 (when mice had been on diets for 3 months) Eppendorf tubes and binder clips were used, and in test session 3 (when mice had been on diets for 4 months) chess pawns and 15 mL tube caps were used. The novel and familiar objects were counterbalanced within a test session so that, for example, in test session 1 half the mice had a marble as the novel object and dice as familiar objects and the other half of the mice had the die as the novel object and marbles as familiar objects.

Morris Water Maze. Mice were tested for 4 days in the MWM after 5 months on the diets similar to previous methods.[21] This paradigm requires the mouse to use spatial memory and distal cues in the testing room to find a hidden white platform 1 cm below the surface of a circular pool of water (colored opaque with white nontoxic paint) and to remember its location from the previous trial. The mice were given 60 s to find the hidden platform during each acquisition trial. There were five acquisition trials on day 1 and four acquisition trials on days 2 and 3. The platform remained in the same position during acquisition trials, but start locations were varied. On day 4, a reversal test was performed during which the platform was moved to the opposite quadrant, thereby requiring the mouse to retain the learned escape strategy but to quickly learn the new escape position. A 60- s probe trial was performed during the fifth and last trial on days 2−4, during which the platform was removed. Performance was monitored with image tracking software (HVS Image, UK).

Data Analysis. Data are represented as the mean ± SEM and were analyzed using IBM SPSS Statistics 21 (IBM, Armonk, NY, USA) and Systat (Systat Software, Inc., Chicago, IL, USA). Significance was set at $p \leq 0.05$. Food intake, caloric intake, and percent increase in weight were analyzed with two-way ANOVA (factors: fat percentage and presence of blueberry). The novel object recognition test data are presented as a percent recognition index (RI) for object B: RI = (time attending to object B/time attending to object A + B) × 100 (Figure 3A). As there were no differences between the two training trials within each diet group, those data were combined for further analysis ($p > 0.05$, training trial 1 versus training trial 2, Wilcoxon signed ranks test). Novel object recognition was analyzed by comparing the RI of the training trial to the RI of the testing trial with the nonparametric Wilcoxon signed ranks test for paired samples. Testing trial performance was further analyzed by comparing RIs to chance performance (50% RI) with one-sample t tests. Morris water maze latency was analyzed with univariate ANOVA (factors: fat, BB, and day for acquisition learning or trial for reversal learning), and probe trial data were analyzed with independent sample t tests.

RESULTS

Mouse Weights and Food Intake. An average of two 24 h food intake measurements indicated that the mice that consumed HF diets ate fewer grams of food than the mice that ate LF diets ($F_{1,58} = 4.00$, $p \leq 0.05$, main effect of fat percentage, two-way ANOVA, Figure 1A). There was no effect of BB on food intake ($F_{1,58} = 0.003$, $p = 0.96$). Although the mice on HF diets ate less, they consumed more calories on average. An analysis of caloric intake based on the food intake measurements and calories in the diets (from Table 1) indicated that mice that consumed the HF diets consumed more calories than mice on the LF diets ($F_{1,58} = 12.41$, $p = 0.001$, main effect of fat percentage, Figure 1B). Furthermore, after 5 months on the diets, mice consuming HF diets gained significantly more weight than the mice on LF diets ($F_{1,56} = 60.32$, $p < 0.001$, main effect of fat percentage, Figure 2).

Novel Object Recognition Test. Analysis of novel object RI indicated that after 2 months on the diets, mice on LF and LFBB diets showed a significant increase in time spent on the novel object (object B, Figure 3A) in the testing trial compared to time on object B in the training trial ($p < 0.05$ Wilcoxon signed ranks test, Figure 3B). The mice on HF and HFBB diets did not spend more time on the novel object (object B) in the testing trial (versus training trial, $p = 0.59$ and $p = 0.72$, respectively). After 3 months on the diets, mice on LF and LFBB diets continued to demonstrate intact novel object recognition (training versus testing trial RI, $p < 0.05$, Figure

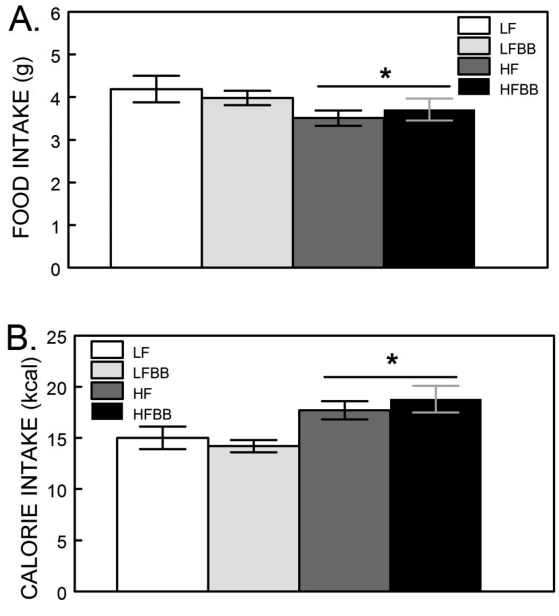

Figure 1. Food (A) and caloric (B) intake. An average of two 24 h food intake measurements indicated that overall the mice that ate HF diets consumed less than the mice that ate LF diets. However, mice on HF diets consumed significantly more calories than those on LF diets. (* $p < 0.05$, significant difference from LF diets, two-way ANOVA.)

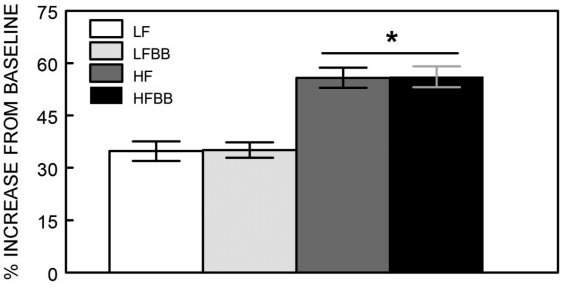

Figure 2. Percent increase in weight after 5 months on diets. Mice on HF diets gained significantly more weight than mice on LF diets. Blueberry had no effect on weight. (* $p < 0.05$, significant difference from LF diets, two-way ANOVA.)

3C), whereas mice on the HF diet continued to show impaired performance ($p = 0.65$). However, mice on the HFBB diet demonstrated a trend toward a difference between RIs in the training and testing trials ($p = 0.056$). After 4 months on the diets, mice on LF and LFBB diets demonstrated intact novel object recognition (testing versus training trial, $p < 0.05$, Figure 3D), whereas mice on the HF diet did not spend more time on the novel object in the testing trial ($p = 0.92$). Mice on the HFBB diet showed a significant increase in time spent on the novel object in the testing trial compared to object B in the training trial ($p < 0.05$).

Another method of examining performance in the novel object recognition task is to compare testing trial performance to chance performance (50% RI). A mouse with intact novel object recognition will spend >50% of its time on the novel object in the testing trials. The LF and LFBB diet-fed mice performed at levels greater than chance during all test sessions ($p < 0.05$, one-sample t test, Figure 4). Mice fed the HF diet did not perform above chance in any test session ($p > 0.05$). The HFBB-fed mice did not perform above chance after 2 months on the diet ($p = 0.23$), but after 3 months on the diet there was a trend ($p = 0.15$), and after 4 months on the diet, the mice demonstrated performance that was significantly greater than chance ($p = 0.02$).

There were no differences between diet groups in the overall amount of time mice explored the objects during novel object recognition testing, indicating that differences in RI were not influenced by differences in general exploration of the objects (effect of diet: $F_{3,164} = 0.930$, $p = 0.93$; diet × test session: $F_{6,165} = 0.507$, $p = 0.80$, two-way ANOVA, data not shown).

Morris Water Maze. There was a trend toward a main effect of fat percentage on the average acquisition day latencies in the MWM ($F_{1,165} = 3.53$, $p = 0.062$, univariate ANOVA), but no effect of BB or interaction of fat percentage × BB on acquisition latency (data not shown). There was no effect of diet on reversal learning latency (data not shown). However, there were differences in passes through the platform location during the probe trials. On the day 2 probe trial, mice that were on the HF diet passed through the platform location significantly less often than the mice on the LFBB diet ($p < 0.05$, t test, Figure 5) and there was a trend from the LF diet (HF versus LF, $p = 0.14$). On the day 3 probe trial, there was a trend that suggested fewer passes through the platform by mice on the HF diet compared to mice on both LF diets (LF, $p = 0.16$; LFBB $p = 0.13$). On all probe trials, there were no differences in the number of passes through the platform location between the HFBB-fed mice and either group of LF-fed mice. There were no differences in probe trial performance during the reversal day between any groups.

DISCUSSION

In this study, middle-aged mice were fed HF or LF diets with and without BB supplementation for 5 months. Mice that were fed HF diets gained significantly more weight than those fed LF diets. This is congruent with the fact that the mice on HF diets were consuming more calories on a daily basis. BB supplementation did not prevent or augment weight gain. This corroborates previous research. BB has been shown to decrease weight gain induced by a HF diet when combined with defatted soybean flour.[22] However, whole BB in the diet has not been shown to prevent weight gain from a 45 or 60% HF diet, although feeding isolated anthocyanins from BB has been demonstrated to decrease weight gain and body fat when fed in conjunction with a HF diet.[23]

Mice fed the HF diet showed impaired novel object recognition performance when tested after 2, 3, and 4 months of consuming the diet. A recent study demonstrated that a HF diet, similar to the diet employed in this study (60% calories from fat), did not impair novel object recognition memory in mice that were approximately 8.5 months old.[7] The mice in our study were 11−13 months of age during novel object recognition testing; thus, the impaired memory demonstrated by the mice in the current study may be due to an interaction of HF diet and age. Mice that were fed the HFBB diet initially showed impaired performance, but their performance improved over subsequent test sessions and, after 4 months on the diet, their performance was no longer impaired. This suggests that over time BB may be able to reverse some of the impact a HF diet may have on certain types of learning and memory. It is unclear why the effect of BB in the current study is time dependent and why the BB did not prevent the initial impairment of novel object recognition associated with consumption of a HF diet. One possible explanation is that there is a threshold level of fat-soluble antioxidants in BB, such

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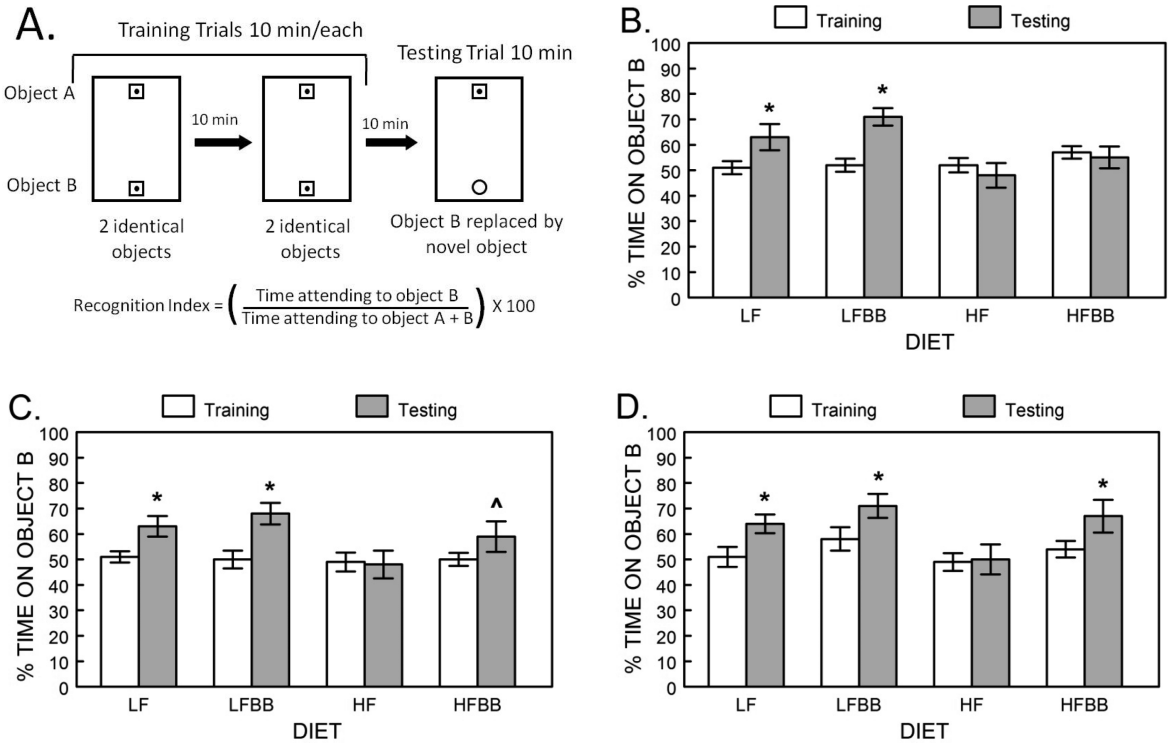

Figure 3. Novel object recognition performance after 2 (B), 3 (C), and 4 (D) months on the diets. (A) The schematic represents testing cages as they were arranged in each trial of testing. (B) After 2 months on the diets, mice on LF and LFBB diets showed a significant increase in RI in the testing trial compared to the training trial, suggesting intact object recognition learning/memory. Mice on HF and HFBB diets did not demonstrate an increase in RI in the testing trial, suggesting impairment. (C) After 3 months on the diets, mice consuming HFBB diet showed a trend toward an increase in RI in the testing trial ($\hat{p} = 0.056$). (D) After 4 months on the diets, mice on LF, LFBB, and HFBB diets showed a significant increase in RI in the testing trial compared to the training trial, whereas the mice on the HF diet did not. ($* p < 0.05$, testing trial is different from training trial, Wilcoxon signed ranks test.)

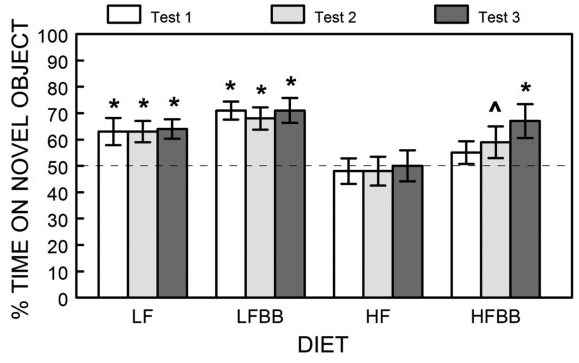

Figure 4. Testing trial performance in the novel object recognition task over three test sessions. The LF- and LFBB-fed mice, but not the mice fed the HF diet, performed at levels greater than chance when tested after 2, 3, and 4 months on the diets (chance is 50% time on the novel object and is indicated by the dotted line). The HFBB-fed mice demonstrated a trend ($\hat{p} = 0.15$) after 3 months on the diet, and after 4 months on the diet, the mice demonstrated performance that was significantly greater than chance. ($* p < 0.05$, one-sample t test compared to a mean of 50%.)

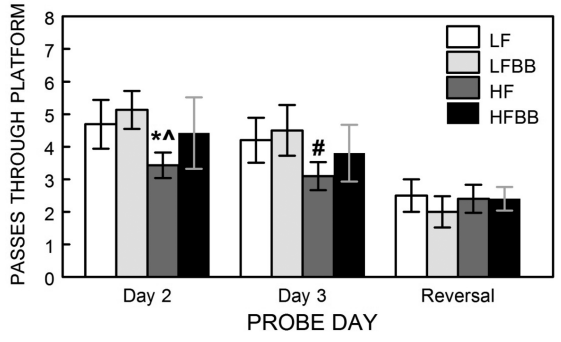

Figure 5. Morris water maze probe trials. On days 2 and 3 of the probe trial, mice that were on the HF diet passed through the platform location less often than the mice on the LF diets. On all probe trials, there were no differences in the number of passes through the platform location between the HFBB-fed mice and either group of LF diet-fed mice. ($* p < 0.05$ from LFBB diet, $\hat{p} = 0.14$ trend from LF, # trend from LF ($p = 0.16$) and LFBB ($p = 0.13$), t test.)

as vitamin A, that must accumulate in the brain to alleviate memory impairment, similar to the "threshold hypothesis" discussed by Malin and colleagues.[24] The time-dependent manner in which novel object recognition memory is rescued by BB supplementation in mice on a HF diet may be attributed to the time course of accumulation of BB fat-soluble antioxidants in the brain, although this remains to be directly tested.

The diets did not have considerable effects on the performance of mice in the MWM; however, during the probe trials, the mice on the HF diet showed deficits in performance. Notably, the probe trial performance of the mice on the HFBB diet did not differ from the mice on the LF diets. The lack of profound effects on latency to find the hidden platform during acquisition learning or reversal learning could potentially be due to a floor effect in which the middle-aged

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mice showed age-related deficits that were not significantly worsened by the HF diet or prevented by the BB diet. A recent study found a decline in MWM performance (i.e., path length to the hidden platform) in middle-aged rats, but no further decline due to being on a HF diet.[25] Previous research has demonstrated that BB is able to prevent or reverse age-related performance deficits in spatial mazes such as the MWM,[26] which may suggest that we should have observed improved performance in the LFBB mice. However, the study by Joseph et al.[26] tested old rats in the MWM, whereas our study tested middle-aged mice, so differences in rodent, age, or both, could be a reason for the discrepancy in results.

It is more likely that the lack of more significant differences between groups in this test is due to the possible lack of sensitivity of the MWM to alterations in mouse learning and memory or the diets having different effects on different types of memory (object recognition versus spatial). Water-based mazes such as the MWM were initially developed for rats, which swim in their natural environment, but may not be as ecologically valid for mice that evolved on dry land.[27] For example, mice are more likely to passively float, which could obscure deficits or enhancements in learning and memory. Furthermore, research suggests that object recognition memory may be largely mediated by the perirhinal cortex in the medial temporal lobe,[28] whereas spatial memory is associated with the intact functioning of the hippocampus.[29] Thus, the behavioral results may suggest possible differential effects of the diets in different brain regions.

The mechanisms by which blueberry can modulate the effects of a HF diet were not evaluated in this study. One possibility is that the memory impairment is due to HF diet-related oxidative stress. Morrison et al. found that a HF diet fed to aged mice impaired memory retention in the 14-unit T maze, increased oxidative stress in the brain, and impaired NF-E2-related factor 2, which may play a role in the body's endogenous antioxidant response.[3] BB has been found to reduce oxidative stress in vivo[26] and in vitro.[30,31] Also, BB may have reduced the inflammation elicited by consumption of a HF diet; consumption of a HF diet has been demonstrated to increase the inflammatory cytokines TNFα and IL-6.[4] In studies of aging, BB has been shown to reduce overexpression and overproduction of these inflammatory factors.[32,33] It has also been shown that resveratrol, a component of blueberry, reduces indices of oxidative stress (4-hydroxynonenal) and inflammation (TNFα and ionized calcium binding adaptor molecule 1 expression) in the hippocampus of mice fed a HF diet.[14] A HF diet also has been demonstrated to decrease neurogenesis in the mouse hippocampus,[5] whereas BB has been demonstrated to increase neurogenesis in aged rodents.[34] Furthermore, HF diet consumption has been linked to reductions in brain-derived neurotrophic factor (BDNF) in both the cortex and hippocampus of mice,[5,35] areas crucial for recognition and spatial memory. In contrast, BB has been known to increase BDNF, and this increase was correlated with spatial memory improvements.[36] BDNF is important for hippocampal synaptic plasticity because it can promote morphological changes that support synaptic efficiency and thus influence hippocampus-mediated behaviors, such as spatial learning and memory.[37] There is also the possibility that BB and fat acted synergistically, with dietary fat enhancing the absorption of fat-soluble vitamins such as vitamin A. Vitamin A and its derivatives have been shown to improve memory deficits in aged mice,[38] but the possible interaction between fat and BB intake in the absorption of nutrients necessitates further study.

The Tifblue powder used in this study is made from a rabbiteye variety of BB (*Vaccinium ashei*). Rabbiteye BB has been shown to reduce DNA damage in mouse hippocampus and cortex, areas important for recognition and spatial memory.[39] HPLC analysis of rabbiteye BB has shown it to contain high levels of flavonoid anthocyanidins and anthocyanins.[40] In fact, Li and colleagues found nine different anthocyanins in rabbiteye BB using HPLC-DAD-MS^n, including delphinidin-3-glucoside and petuidin-3-glucoside.[41] Flavonoids have been shown to modulate neuronal signaling pathways that influence synaptic plasticity and neurogenesis, which ultimately can have an effect on learning and memory.[42,43] Flavonoids also can promote neuronal viability and suppress inflammation by inhibiting c-jun N-terminal kinase, apoptosis signal-regulating kinase-1, and p38 pathways, thus preventing neurodegeneration.[42] Therefore, it is likely that the effects of BB are attributable, at least in part, to the biological activities of flavonoids. Flavonoids have been shown to cross the blood–brain barrier,[44] and specifically anthocyanins have been shown to accumulate in the brains of aged rats fed a BB-supplemented diet.[9] A recent study found that aged rats fed diets supplemented with anthocyanins in amounts similar to that found in BB showed increased BDNF in the hippocampus as well as improvements in cross-maze spatial memory performance.[8] Recently, delphinidin-3-glucoside, a component of rabbiteye BB, has been found to reduce the release of proinflammatory mediators from brain microglial cells;[45] thus, the anthocyanin components of BB may be reducing inflammation induced by a HF diet. Furthermore, another component of BB, cyanidin-3-glucoside, has been shown to reduce spatial recognition memory impairment by possibly reducing oxidative stress.[46]

This study supports that adding BB to a HF diet could reverse some of the behavioral deficits associated with consumption of a HF diet, specifically deficits in object recognition memory. It is important to find ways to prevent or reverse the effects that a HF diet may have on the brain and behavior, as the incidence of obesity is increasing worldwide, and consumption of a HF diet has been implicated as a factor in the development of obesity and obesity-related diseases. The purpose of this study was not to encourage consumption of a HF diet, but rather to suggest that making small changes to a putative unhealthy diet, such as incorporating more nutrient-dense foods, could provide some level of protection against cognitive deficits attributed to that diet. This is an important finding that requires further study in both animal models and humans. Furthermore, it is not known the exact mechanism(s) by which BB may be modulating the effects of a HF diet, but research suggests a multitude of probable mechanisms; however, this topic also calls for further study.

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Notes

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