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The Effects of Black Mulberry Fruit Extract, Sunflower Seed, and Pumpkin Seed With Exercise on Memory Function and Neural Activation Biomarkers among Healthy Young Adults

NOR AIJRATUL ASIKIN MOHAMAD SHALAN[1]*, NORHAZIRA ABDUL RAHIM[1] and NORAZALINA SAAD[2]

[1]Faculty of Sport Science and Coaching, Sultan Idris Education University, 35900 Tanjung Malim, Perak, Malaysia.
[2]UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Abstract

Neuronal survivability is fundamental for optimum memory performance and human wellbeing. This study aims to investigate the effect of black mulberry (*Morus nigra)* fruit extract, sunflower seed, pumpkin seed and exercise on memory span and neuroplasticity-related biomarkers. Study participants (n=40) were randomly assigned into four groups; 1) Negative control, 2) Treatment (100mg/kg body weight black mulberry + 50mg/kg body weight sunflower seed + 50mg/kg body weight pumpkin seed), 3) Exercise (30 min. slow run, heart rate monitored between 128-130bpm), and 4) Treatment and exercise (100mg/kg body weight black mulberry + 50mg/kg body weight sunflower seed + 50mg/kg body weight pumpkin seed + 30 min. slow run). All subjects in groups 2, 3, and 4 were required to complete cognitive task assessment for domain memory span on day 0 (baseline), 30, and 60 of experiments. Blood samples were collected at the end of the study and the concentrations of glucocorticoid receptor-α (GCR-α), glutamate dehydrogenase (GDH), and brain-derived neurotrophic factor (BDNF) concentrations were determined using ELISA kit. The results revealed that isolated treatment resulted in an improvement in the reaction time for the measure of memory span while the combination with exercise did not provide an additional effect. As for serum analysis, GCR-α level was significantly lower in all groups, whereas BDNF concentration was doubled in treatment and exercise group compared to the control group.

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CONTACT Nor Aijratul Asikin Mohamad Shalan aijratul@fsskj.upsi.edu.my Faculty of Sport Science and Coaching, Sultan Idris Education University, 35900 Tanjung Malim, Perak, Malaysia.

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concentration only increased significantly in the treatment group. These findings highlighted the potential of black mulberry fruit in combination with sunflower seed and pumpkin seed on improving memory function by enhancing the production of neuroplasticity marker and reducing the secretion of stress hormone.

Introduction

Enhancing memory ability is fundamental to sustain human wellbeing. The key mechanism for this lies within neuroplasticity, which is the brain capacity to reorganise synaptic connections in response to interactions with the environment. The ability of neurons to form synapses with other neurons and the strength of the synaptic connections will ensure the survival of the neuron. On the contrary, neurons that do not form synapse will undergo apoptosis or cell death.[1] To increase the survivability of the neuron, a few peripheral mechanisms need to work in concert, particularly the interaction between glutamate and brain-derived neurotrophic factor (BDNF). BDNF represents the main regulator in neurogenesis by forming short- or long-term synapses in many brain regions, including the hippocampus.[2] The production of BDNF is stimulated by glutamate, a major excitatory neurotransmitter that is responsible for synaptogenesis and thus neuron survival.[3]

Although the synaptogenesis in the hippocampus is fundamental for memory function, a few other factors such as oxidative stress and stress hormones may interrupt information flow and memory formation.[4,5] In response to stress, the expression of glucocorticoid receptors (GCR-α) would be elevated in the hippocampus. As a result, the production and survival of new hippocampal neurons may be affected. This response is linked to hippocampal atrophy whereby the synapse is altered and BDNF expression is reduced,[6] thus resulting in memory impairment.[7] Interestingly, BDNF has been shown to be capable of mediating the effects of environmental factors such as exercise[8] and nutrition[9] on hippocampal neurogenesis.

Black Mulberry (*Morus nigra*) has a long history in China for its medicinal functions. It contains a wide variety of phytochemical compounds such as anthocyanins and phenolics[10] which are linked to various medicinal properties, including neuroprotective properties.[11,12] Specifically, supplementation of anthocyanins has been found to increase the BDNF[13] and pro-BDNF levels in the hippocampus14, thus leading to improved learning and memory.[15,16] Anthocyanins also provide neuroprotection by inhibiting activated astrocytes and neuroinflammation via suppression of various inflammatory markers, and by improving deregulated synaptic proteins.[17]

In addition to that, studies have shown the ability of vitamin E as a potent antioxidant in protecting neuron cells against harmful oxidative agents.[18,19] A study by Dysken and colleagues showed 19% delayed of cognitive decline per year among patients with mild to moderate Alzheimer's disease who were supplemented with 2000IU per day of α-tocopherol.[20] The supplementation of δ-tocopherol enhanced neuronal differentiation and promoted maturation of neuron.[21] Vitamin E also improved BDNF concentration and increased the activity of antioxidant enzymes in the hippocampus, while diminishing the malondialdehyde (MDA).[22] However, most of the previous study used only one form of vitamin E, whereas supplementation of mixed form of vitamin E showed stronger effects compared to only one form.[23] Therefore, this current study used sunflower seed and pumpkin seed as natural sources of vitamin E as they contained multiple types of vitamin E isomers such as tocopherols[24,25] and tocotrienols.[26,27]

Furthermore, in recent years, there are increasing research exploring exercise as a potential technique to improve cognitive ability and brain health.[28] It has been found that running played a significant role in ameliorating brain blood flow and decreasing inflammation through the upregulation of the expression of BDNF and IGF-1.[29] Other research showed that voluntary exercise could enhance the generation of new hippocampal granule cells in aged brains via a process that involved the enhancement of neurogenesis and neuroprotective against inflammation.[30,31] Voluntary running also

enhanced spine density and dendritic branching in the adult dentate gyrus apart from inhibiting the early age-related grey matter loss. Collectively, this led to an increased volume of the hippocampus.[32,33]

To the best of our knowledge, the combined effect of functional foods and exercise on neuroplasticity-related biomarkers activity among healthy young adults has yet to be investigated. Therefore, this study aims to explore the neuroprotective effects of the black mulberry, sunflower seed, and pumpkin seed in combination with exercise among healthy young adults.

Materials and Methods

Materials

100% pure Dr Sweet™ black mulberry fruit extract was purchased from SteviaSugar Corporation (M) SDN BHD, Malaysia, whereas the raw sunflower and pumpkin seeds were purchased from Kuala Lumpur and Perak, Malaysia.

Participants

A total of 40 healthy young adults (females, age: mean 21.5 years, SD 1.87, range 19-24) were recruited into the study. All participants were undergraduate students at the Sultan Idris Education University. They reported themselves as being sedentary and declared themselves to be generally in a good health, free from drug, alcohol, prescription medication, herbal or vitamin supplement use, free from allergies or digestive problems. Participants who were regularly taking any supplement for brain performance, those who were excessive caffeine consumers (>6 cups of coffee/day) or current users of tobacco, had a history of cardiac injury, head injury, neurodegenerative disorder, sleep disorder, or currently pregnant were excluded from participation. All participants were required to provide informed consent. The study was approved by the International Islamic University of Malaysia Research Ethics Committee (IREC 2018-301).

Treatments

Participants were randomly assigned into four groups:

- Negative control
- Treatment: 100mg/kg body weight Black Mulberry + 50mg/kg body weight sunflower seed + 50mg/kg body weight pumpkin seed
- Exercise: 30 minutes slow run, heart rate monitored between 128-130 bpm
- Treatment and exercise: 100mg/kg body weight Black Mulberry + 50mg/kg body weight sunflower seed + 50mg/kg body weight pumpkin seed + 30 minutes slow run

Participants in groups 2 and 4 were provided with a daily mixture of black mulberry fruit extract, sunflower seed, and pumpkin seed. They were instructed to take the treatment orally, twice daily (after waking up in the morning and before sleep at night, one dose each time) for 60 days. Participants in groups 3 and 4 were assigned to complete 30 minutes of a slow run, 3 times a week for 8 weeks. The supplements were prepared fresh daily, and distributed to the subjects the night before. The subjects were given a form to track their daily intake of the supplements. The form was collected weekly by the researcher.

Cognitive Tasks

Participants were given training on each cognitive task for three days before the initial baseline data was collected. All participants were required to complete the tasks again on day 30 and day 60 of the experiment (30 days apart). Cognitive tasks on memory span were delivered on a laptop computer via *cognitivefun.net*. The detail for each task is as follow:

Visual Forward Digit Span

In this test, the participant must remember the five digits displayed on their computer screen within eight seconds, and type their answer in an empty box as soon as possible. The time taken to write the correct answer represents the participant's short-memory ability.

Visual Backward Digit Span

This task is similar to the forward digit span, except that the participants need to type their answer in reverse.

Auditory Forward Digit Span

This task is also similar to the visual forward digit span, except that auditory stimuli were used instead of visual stimuli.

Auditory Backward Digit Span

This task is similar to the visual backward digit span, except that the digit would be presented using audio stimuli.

Corsi Block Task

The participant must remember and reproduce the sequence of block positions, with five being the maximum number of blocks.

Working Memory Test

This test is a variant of the n-back task, where the participant needs to click on the target box when the current picture repeats what they have seen two pictures ago.

Serum Biochemical Analyses

At the end of study, blood samples were collected from each participant and centrifuged at 3500rpm for 15 minutes to obtain the serum. The serum was used to determine the concentrations of glucocorticoid receptor-α (GCR-α), glutamate dehydrogenase (GDH), and brain-derived neurotrophic factor (BDNF) using ELISA kit (Finetest, China), according to the manufacturer's instruction (www.fn-test.com).

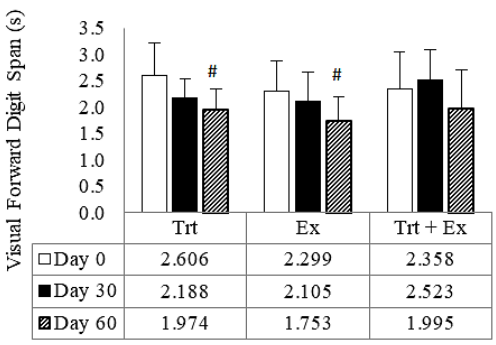
Statistical Analysis

All data were analysed using the one-way analysis of variance (ANOVA) and expressed as mean ± standard deviation. Differences between groups were evaluated by LSD post hoc test and statistical significance was taken as $p<0.05$. All statistical analysis was performed using SPSS 25 (SPSS Inc., Chicago, IL, USA).

Results

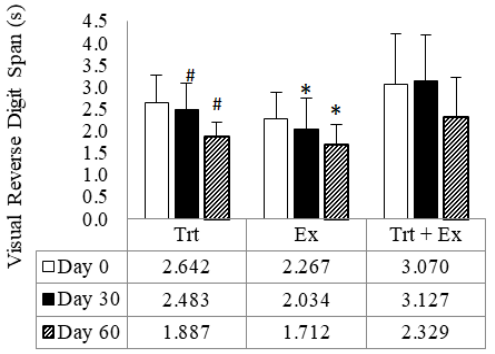
Effect on Memory Span Performance

As shown in Fig. 1a, the subjects in both the treatment and exercise groups showed a significant reduction in reaction time (Trt, p=0.008; Ex, p=0.05) during the assessment of the cognitive task at day 60 as compared to day 0. In Fig. 1b, significant changes in the reaction time at day 30 (p=0.006) and 60 (p=0.027) were only observed in the treatment group. The exercise group responded faster in the cognitive task on day 30 (p=0.012) and 60 (p=0.043) compared to the group receiving treatment and exercise.



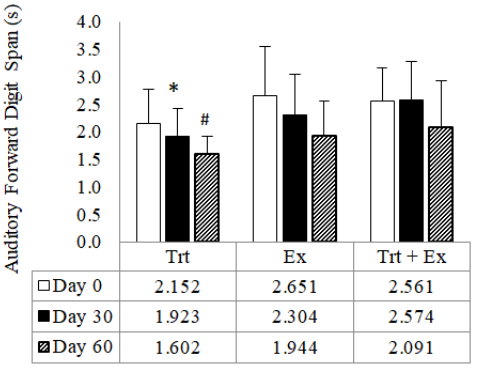
	Trt	Ex	Trt + Ex
Day 0	2.606	2.299	2.358
Day 30	2.188	2.105	2.523
Day 60	1.974	1.753	1.995

a)



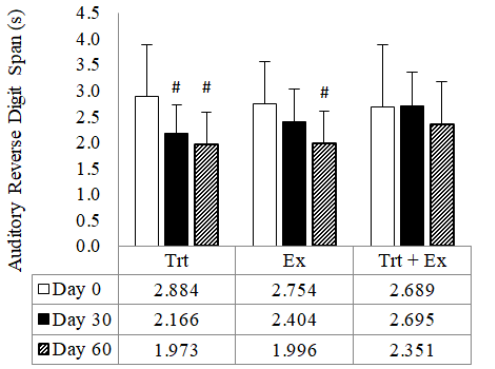
	Trt	Ex	Trt + Ex
Day 0	2.642	2.267	3.070
Day 30	2.483	2.034	3.127
Day 60	1.887	1.712	2.329

b)



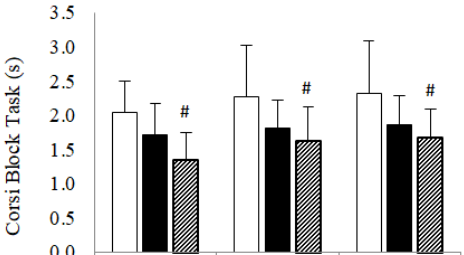
	Trt	Ex	Trt + Ex
Day 0	2.152	2.651	2.561
Day 30	1.923	2.304	2.574
Day 60	1.602	1.944	2.091

c)



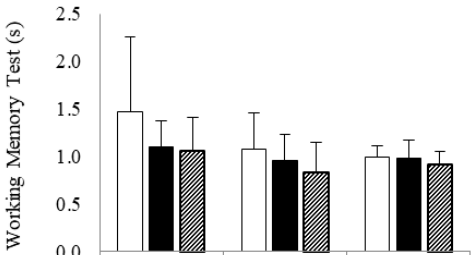
	Trt	Ex	Trt + Ex
Day 0	2.884	2.754	2.689
Day 30	2.166	2.404	2.695
Day 60	1.973	1.996	2.351

d)



	Trt	Ex	Trt + Ex
□Day 0	2.052	2.269	2.326
■Day 30	1.725	1.811	1.865
▨Day 60	1.361	1.640	1.682

e)



	Trt	Ex	Trt + Ex
□Day 0	1.472	1.078	0.995
■Day 30	1.109	0.956	0.979
▨Day 60	1.068	0.840	0.926

f)

Fig.1: Effects of Trt, Ex, and Trt+Ex on domain memory span assessment: (a) visual forward digit span, (b) visual backward digit span, (c) auditory forward digit span, (d) auditory backward digit span, (e) Corsi block task, and (f) working memory test

When auditory stimuli were used instead of visual, significant changes were observed in the treatment group on day 60 (p=0.019) and day 30 (p=0.045) compared to the group receiving treatment and exercise (Fig. 1c). Similar changes were shown by the treatment group (day 30, p=0.042; day 60, p=0.011) and exercise group (day 60, p=0.039), as presented in Fig. 1d. In Fig. 1e, all groups recorded better reaction time on day 60 compared to baseline data (Trt, p=0.001; Ex, p=0.02; Trt+Ex, p=0.016). However, in the working memory test, no significant difference was observed within the group and between groups (Fig. 1f). In short, subjects who were administered black mulberry, sunflower seed, and pumpkin seed without exercise showed better overall performance in memory span that the other groups.

Lower score denote greater memory span performance. Data are expressed as the mean ± SD. #p<0.05 (significant difference within group, compared with day 0), *p<0.05 (significant difference between group, comparison between each particular day). Trt, Treatment (black mulberry, sunflower seed and pumpkin seed); Ex, Exercise (30 minutes of slow run); Trt+Ex, Treatment with exercise (black mulberry, sunflower seed and pumpkin seed with 30 minutes of slow run).

Effect on Brain Activity Biomarkers

When compared to the control group, the serum concentration of stress hormone receptor marker (GCR-α) was significantly reduced (p=0.000) by 58%, 69%, and 61% in the treatment group, exercise group and group receiving treatment with exercise respectively (Fig. 2a). Remarkably, participants who were subjected to both treatment and exercise recorded doubled the concentration of serum BDNF (54%, p=0.01), whereas the treatment group showed s significant increased level of serum GDH by 40% (p=0.013) (Fig. 2b and 2c).

Shorter bar represent better therapeutic effect on GCR-α, whilst higher bar represent better effect on BDNF and GDH. Ctl, Control (no intervention); Trt, Treatment (black mulberry, sunflower seed and pumpkin seed); Ex, Exercise (30 minutes of slow run); Trt+Ex, Treatment with exercise (black mulberry, sunflower seed and pumpkin seed with 30 minutes of slow run).

Discussion

Memory performance is influenced by the generation of new hippocampal neurons and the strength of the synaptic connection between neurons. External factors, particularly dietary and physical activity, can protect existing neurons from malicious factors such as oxidative stress and stress hormone, hence facilitate the process of neurogenesis. The crucial mediator for this process is BDNF which modulates neuron proliferation, differentiation, and survival. The activity of BDNF is in turn regulated by glutamate.

In this study, the effects of black mulberry fruit extract, sunflower seed, and pumpkin seed with or without combination with exercise on memory span were assessed. The study approaches were designed

to explore the synergistic effect between dietary intervention and exercise. The results revealed that isolated intervention via nutritional supplementation resulted in an improvement in the reaction time for the measure of memory span, while a combination of treatment and exercise did not provide additional effect. This finding was in line with a recent systemic review in which none of the three human studies showed any additive effects from a combination of physical exercise and nutritional supplementation.[34]

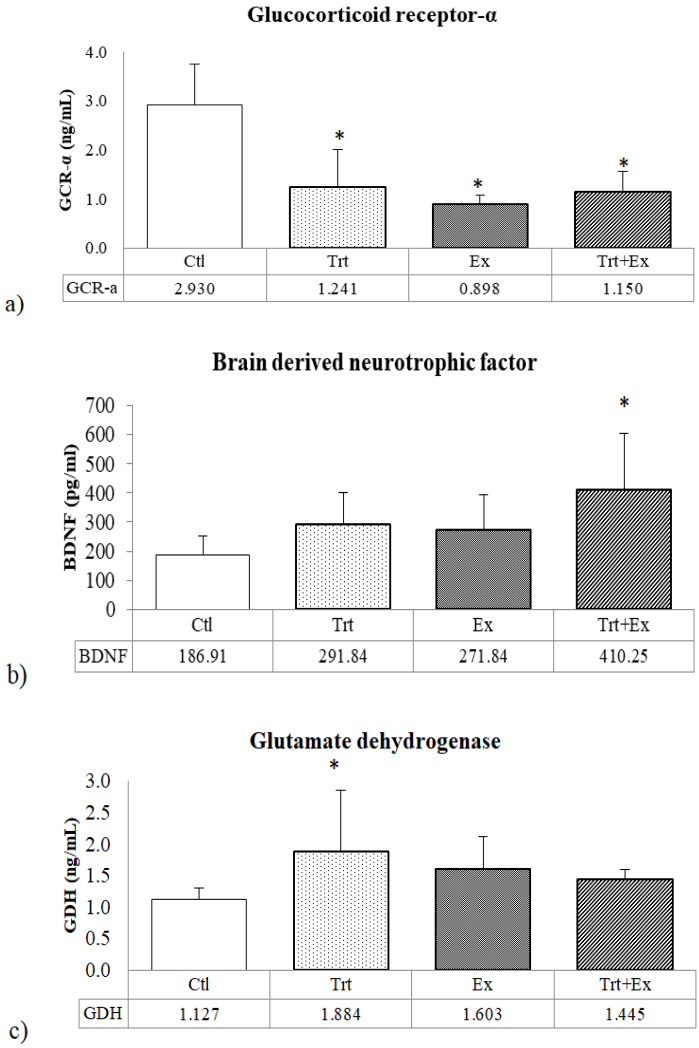


Fig. 2: Effects of Trt, Ex, and Trt+Ex on the concentration of serum GCR-α, BDNF, and GDH

A previous study has suggested that the effect of black mulberry fruit on memory performance can be attributed to the presence of high anthocyanins content.[35] Kent *et al.,* reported a significant improvement in verbal fluency, short-term memory and long-term memory in older adults with mild to moderate dementia that were supplemented with anthocyanin-rich juice for twelve weeks.[36] Interestingly, improvement memory functions were also detected in young rodents treated with anthocyanins, along with the inhibition of neuroinflammation and neurodegeneration. Specifically, the mechanism by which anthocyanins improved memory functions was via the reductions of neuronal apoptosis, increased the levels of memory-related pre- and post-synaptic proteins and improved the hippocampus-dependent memory.16 Furthermore, when administered with black mulberry

leaves extract for eight weeks, aging Balb-c showed significant improvement in learning dysfunction, memory retention, and oxidative status.[37]

In the human brains, the glucocorticoid receptors are widely distributed in the hippocampus, amygdala, and prefrontal cortex. The induction of glucocorticoid receptor activity is associated with memory impairment as it has been found to cause hippocampal neurons damage through neuroinflammation,[38] hyperphosphorylation of Tau in the hippocampus,[39] and reduction of the hippocampal BDNF level.[40] In this study, the supplementation of black mulberry fruit extract, sunflower seed and pumpkin seed with or without exercise, and exercise alone managed to reduce the serum glucocorticoid receptor-α level. This finding was consistent with previous reports41,42 in which prolonged physical activity and voluntary exercise led to a reduction in hippocampal glucocorticoid receptor activation in sedentary male and aged animal respectively. However, no previous report was found to support the effect of black mulberry fruit extract, sunflower seed, and pumpkin seed supplementation on the glucocorticoid receptor, thus making this the first study to provide the evidence.

The interaction between glutamate and neurotrophic factors including BNDF in regulating neurite outgrowth and synaptogenesis has been established.[3] In particular, glutamate and BDNF co-regulate each other in such a way that glutamate increases the transcription and secretion of BDNF and, conversely, BDNF enhances glutamate release. Dendritic growth of cortical neurons requires both the stimulation of cAMP response element-binding protein (CREB) phosphorylation by BDNF and the activation of the CREB-regulated transcription coactivator 1 (CRTC1) by glutamate.[43] In addition, BDNF plays several prominent roles in synaptic plasticity. In this study, the treatment group showed a higher level of serum glutamate dehydrogenase whilst group supplemented with both treatment and exercise showed a significant increase in the BDNF level. This finding is consistent with previous research that reported an increase in BDNF level following anthocyanin-rich fruit consumption,[13,44] acute high intensity exercise,45,46 and chronic exercise.[8,47]

Conclusion

Current study highlighted the first time evidence on the potential synergistic benefit of black mulberry fruit extract, sunflower seed, and pumpkin seed consumption on enhancing memory performance among healthy young adults, with or without exercise. The memory span test on day 0, 30 and 60 of the experiment showed that all approaches enhanced the memory performance either discretely or combined. The beneficial effects on memory can be associated with the decreased of the serum levels of the glucocorticoid receptor, and increased the glutamate dehydrogenase and brain-derived neurotrophic factor. Further research is required to establish the effect on other population including other age groups, individuals with cognitive impairment problems. Additional biomarkers should also be analysed to extend the understanding of the neurobiological mechanism.

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Conflict of Interest

The authors declare no conflict of interest.

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