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Ameliorative Processes of Beta-Carotene in Streptozotocin-Induced Diabetic Vascular Dementia in Rats

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Abstract: Beta-carotene (BC) is a precursor of vitamin A and an excellent antioxidant. It protects the vascular system. Vascular dementia (VaD) is one of the aging disorders causing memory dysfunction. The available medicines for the management of VaD are limited. The present study aimed to evaluate the ameliorative effect of BC in streptozotocin (STZ)-induced diabetic VaD in rats. Diabetic VaD was induced through the administration of nicotinamide (NA, 50 mg/kg; *i.p.*) and STZ (50 mg/kg; *i.p.*). The test compound BC (50 and 100 mg/kg; *p.o.*) and reference compound donepezil (1 mg/kg; *p.o.*) were administered for 15 consecutive days. Cognitive changes were assessed by transfer latency (TL) using the elevated plus maze (EPM) test. The changes in acetylcholinesterase (AChE) activity were estimated in the septohippocampal system of rat brains. The administration of STZ caused significant changes in cognitive functions (increased TL) as compared to the normal group. BC ameliorated the anxiety-related cognitive behavior and neurotransmitter (elevated AChE) changes provoked by diabetic VaD. Therefore, BC could be a potential therapeutic candidate in the management of VaD.

Keywords: acetylcholinesterase; beta-carotene; diabetic vascular dementia; hippocampus; septum

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1. Introduction

Dementia is a spectrum of neurological disorders that describes the general syndrome of loss of memory, inability to think, and difficulty in making a decision and performing daily activities [1]. The spectrum of neurodegenerative diseases is associated with progressive and irreversible loss of neuronal tissue. The types of dementia include Alzheimer's disease (AD), vascular dementia (VaD), Lewy body dementia, frontotemporal dementia, mixed dementia, Parkinson's disease dementia, Huntington's disease, and Creutzfeldt–Jakob disease [2]. VaD is the second most common type of dementia. The pathophysiological mechanisms of VaD are heterogeneous and complex. VaD usually occurs as a result of a reduction in cerebral blood flow (ischemia) in various parts of the brain [3,4]. The cerebrovascular lesions with ischemia in the septohippocampal region can cause the cognition deterioration and progression of VaD [5,6]. Moreover, VaD does not only cause memory loss; VaD patients also experience deterioration of attention and compromised executive function, such as difficulty in problem-solving, impairment of task execution, inability to plan, disorientation, and poor judgment [7].

The worldwide epidemiological survey revealed that more than 55 million people suffer from dementia. The number of cases of dementia is expected to rise to 78 million and 139 million in the years 2030 and 2050, respectively [8]. Moreover, COVID-19 infection and vaccination also showed that brain fog syndrome is similar to symptoms of VaD [9]. Moreover, 60% of dementia cases reside in low- and middle-income countries [10]. In Malaysia, the prevalence of dementia is predicted to be 6.3, 12.6, and 45.4 million people in the years 2005, 2020, and 2050, respectively [11,12]. A nationwide survey of dementia reported that the prevalence is higher in individuals above 60 years old (8.5%), and in females especially, who did not receive formal education and live in rural areas [13]. Furthermore, the available FDA-approved medications for VaD are very limited. Commonly, donepezil, rivastigmine,

and galantamine are used for VaD-associated cognitive disorders. These drugs act by inhibiting acetylcholinesterase. Nevertheless, the efficacy of these drugs reduces as dementia becomes more severe due to the low level of acetylcholine [14]. These drugs also cause undesirable side effects, poor compliance, and a lack of therapeutic potential [15]. On the other hand, herbal medicines were documented to show beneficial effects against the VaD without serious side effects. Natural medicines such as *Daucus carota*, *Gingko biloba*, and *Ocimum sanctum* are reported to be able to ameliorate memory dysfunction in the elevated plus maze (EPM) test via regulation of brain anticholinesterase activity levels [16–18]. Furthermore, carotenoid compounds, such as crocin, were also found to reduce neuronal oxidative stress and improve spatial memory in diabetic rats [19]. Moreover, the active metabolite of vitamin A, i.e., trans-retinoic acid, was shown to produce the attenuation of AD in a triple transgenic mouse model [20]. Another carotenoid, lycopene, ameliorates the β-Amyloid$_{1–42}$ peptide-induced AD in rats [21]. It is also demonstrated to produce poly-targeted actions and synergistic effects with herbal combinations [22].

Beta-carotene (BC) is a major natural carotenoid compound, and it is a precursor for vitamin A. BC also possesses a strong antioxidant effect and free radical scavenging actions due to the presence of abundant unsaturated bonds [23]. Moreover, BC has diverse ameliorative effects against various disorders due to its ability to reduce free radical generation, increase the expression of antioxidant enzymes, lower cholesterol levels, reduce amyloid-β protein levels, and normalize mitochondrial blood–brain barrier dysfunction [24]. A randomized controlled clinical trial (RCT) also showed that BC attenuates cognitive impairment [25]. However, the evidence of BC on diabetes (streptozotocin (STZ) toxin)-induced VaD and cognitive impairment is lacking. Hence, the present study is designed to investigate the effect of BC on diabetic VaD in rats.

2. Materials and Methods

2.1. Animals

Male Sprague Dawley rats 12 months old with weight range 220 ± 20 g were used in this study. They were obtained from Lab-rat Breeders Farm PVT Ltd., Selangor, Malaysia. Upon receiving the rats, they were housed in the central animal house of AIMST University, Malaysia. The animals were maintained at a temperature of 22 ± 1 °C, a humidity of 60%, and a 12 h light/dark cycle with free access to food and water. The rats were kept for a period of 7 days before the commencement of the experiment for adaptation. The behavioral assessment was performed during the daytime.

2.2. Induction of Diabetic VaD in Rats

The induction of type-2-diabetes-mellitus-associated VaD in rats was completed by the administration of streptozotocin and nicotinamide, as described by Masiello [26]. Briefly, freshly prepared nicotinamide (NA; 50 mg/kg) solution was injected via the intraperitoneal (*i.p.*) route into the rats. Then, freshly prepared streptozotocin (STZ) (50 mg/kg; *i.p.*) was injected after 15 min [26,27]. The day of induction was considered day 0. Random blood glucose was measured using the tail vein on day 3 and day 7. Rats with blood glucose level greater than 11.1 mmol/L (≈200 mg/dL) were considered diabetic [27,28].

2.3. Experimental Protocol

60 male Sprague Dawley rats were distributed into 5 groups, each having 12 rats. Group 1: Normal disease-free healthy male Sprague Dawley rats. Group 2: NA (50 mg/kg; *i.p.*) + STZ (50 mg/kg; *i.p.*) treated rats. Group 3: NA (50 mg/kg; *i.p.*) + STZ (50 mg/kg; *i.p.*) + BC (50 mg/kg; per oral (*p.o.*)) for 15 days. Group 4: NA (50 mg/kg; *i.p.*) + STZ (50 mg/kg; *i.p.*) + BC (100 mg/kg; *p.o.*) for 15 days. Group 5: NA (50 mg/kg; *i.p.*) + STZ (50 mg/kg; *i.p.*) + Donepezil (1 mg/kg; *p.o.*) for 15 days. The details of this protocol are elucidated in Figure 1. The dose of BC was chosen based on the fact that rodents have a good capacity to convert BC into vitamin A. Hence, they can be fed a large amount of BC. In a study that developed a novel BC formulation, BC at a dose of 50 mg/kg was administered to rats

for 7 days [29]. To the best of our knowledge, there is no research article published on the investigation of BC in the rat model of VaD.

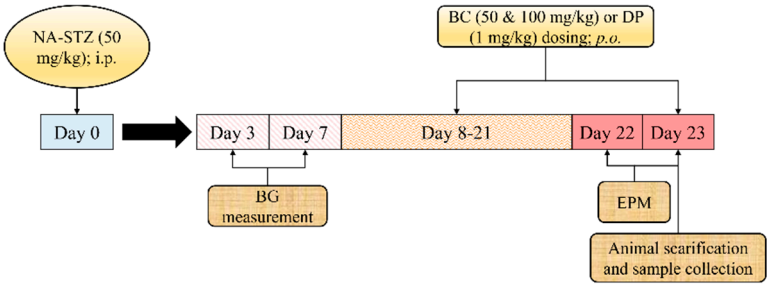

Figure 1. Experimental protocol of the study.

The induction day was considered day 0. Random blood glucose was measured on day 3 and day 7. Oral dosing of BC and donepezil was performed from day 8 to day 21. Thereafter, EPM was conducted on days 22 and 23. All rats were sacrificed for sample collection on day 23.

2.4. Collection of Biological Samples

All rats were anesthetized using diethyl ether. Then, they were sacrificed using the cervical dislocation method. The skull was cut open to obtain the brain. Each brain was dissected at bregma 0.00 mm, lambda 7.60 mm and bregma 3.80 mm, lambda 3.80 mm to isolate the medial septum and entire hippocampus, respectively. Hippocampus and septum were isolated [30,31]. The collected samples were homogenized with 10 parts of phosphate-buffered saline (pH 7.4). Then, the homogenate was centrifuged for 15 min at 3500 rpm (1720 g) at 4 °C [32]. The supernatant obtained was used for biochemical assessment.

2.5. Assessment of Rat Cognitive Functions by Elevated Plus Maze (EPM) Test

EPM test was performed as described by Pellow et al. [33] and Rishitha and Muthuraman [34]. EPM has two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) intersected (right angle). The EPM test apparatus (Stoelting Co., Wood Dale, IL, USA) was raised above the floor at the height of 50 cm. EPM test was performed by 2-trial assessment, i.e., training phase and retrieval phase. It was conducted on day 22 and day 23. During the training phase (day 22), each rat was placed on one end of the open arm with its face pointing away from the center square. Each rat was allowed to explore the maze for 120 s. The time taken for each rat to enter and reach the end of the closed arms is known as the transfer latency (TL). The same procedure was repeated on day 23 [35].

2.6. Estimation of Acetylcholinesterase (AChE) Activity

Rat brain AChE activity level was assessed in hippocampal and septum tissue homogenates as described by Ellman [36]. Briefly, 0.5 mL of brain supernatant was added with 2.5 mL of phosphate-buffered saline (pH 7.4), followed by 0.1 mL of 5,5–dithio–bis–(2–nitrobenzoic acid) (DTNB) solution. The changes in absorbance were measured using Shimadzu UV–1800 UV/Visible Scanning Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 412 nm wavelength. A total of 20 µL of acetylthiocholine iodide was added. The changes in absorbance were measured immediately and every 2 min until the absorbance became constant. The acetylcholinesterase activity was calculated using the following formula:

$$\text{Acetylcholinesterase activity} = \frac{\Delta A}{13,600} \times \frac{1}{\left(\frac{\text{VBs}}{\text{TVts}}\right)\text{P}} \quad (1)$$

where ΔA, changes in absorbance/min; P, protein content (mg/mL); VBs, volume of brain supernatant (VBs = 500 µL); and TVts, total volume of test samples (TVts = 3120 µL).

The AChE activity results were expressed as μM of acetylthiocholine hydrolyzed/mg of protein/minute.

2.7. Statistical Analysis

The data collected for both the EPM test and AChE activity estimation were expressed as mean ± standard deviation (SD). The Shapiro–Wilk test was first conducted to assess the normality of the data. Data for the EPM test were analyzed by two-way analysis of variance (ANOVA), while the data for AChE activity were analyzed using one-way ANOVA, correspondingly. Post hoc analysis was performed using Tukey's honestly significant difference test. Statistical analysis was conducted using Statistical Package for the Social Sciences software (SPSS version 25). The alpha value was set at 0.05.

3. Results

3.1. Effect of BC on VaD-Associated Changes of Transfer Latency (TL) in the EPM Test

There is a significant difference of TL between groups ($F(4,25) = 23.407$; $p < 0.001$) and between days (days 22 and 23) ($F(1,25) = 52.697$; $p < 0.001$). Further, the interactions between groups and days ($F(4,25) = 4.197$; $p = 0.01$) is also significant. The TL of the normal control group was significantly shorter than that of all other groups on day 22. Normal control rats showed intact memory as their TL was lower on day 23 when compared to day 22. On the contrary, the TL of the VaD group did not show improvement from day 22 to day 23. Treatment with BC (50 and 100 mg/kg) and donepezil (1 mg/kg) showed significant ameliorative effects against the diabetic VaD condition in the EPM test, as indicated by the shorter TL when compared to that of the VaD control group (Figure 2). There was no statistically significant difference in TL levels between BC and donepezil groups on day 23.

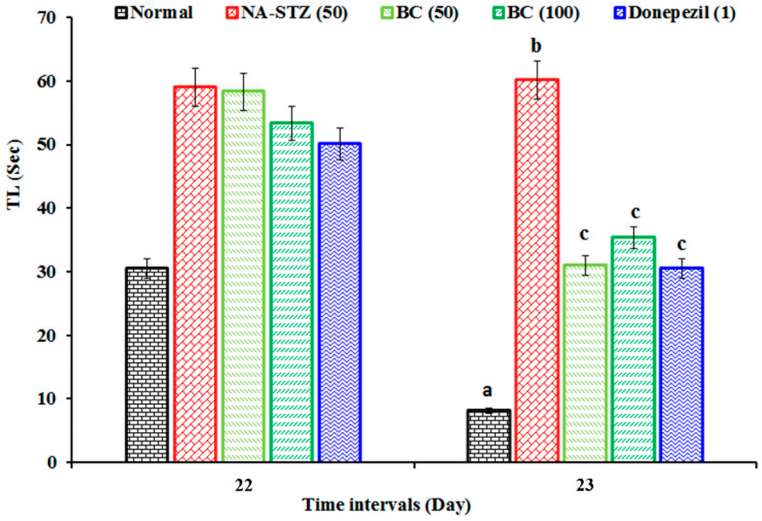

Figure 2. Effect of BC on TL in EPM. Digits in parentheses indicate dose in mg/kg. Data are expressed as mean ± SD; $n = 12$ rats. [a] $p < 0.05$ vs. normal group on day 22; [b] $p < 0.05$ vs. normal group on day 23, [c] $p < 0.05$ vs. VaD group on day 23.

3.2. Effect of BC on VaD-Associated Changes of Tissue AChE Activity

The one-way ANOVA of AChE activity showed statistical significant difference between groups (hippocampus: $F(4,25) = 30.277$; $p < 0.001$; septum: $F(4,25) = 15.415$; $p < 0.001$). The AChE activity of both hippocampus and septum in the VaD control group was higher when compared to the normal control group (Table 1). The treatment of BC (50 and 100 mg/kg) and DP (1 mg/kg) resulted in significantly lower AChE activity compared to diabetic VaD. In the septum, the administration of BC resulted in lowered AChE activity close to that of the normal control. Furthermore, BC was shown to be as effective

as donepezil in the regulation of AChE activity, as there was no statistically significant difference in AChE activity between animals treated with BC and donepezil.

Table 1. Effect of BC on VaD-associated changes of tissue AChE activity.

Groups	AChE (μmol of AChI/min/mg of Protein)	
	Hippocampus	Septum
Normal	159.73 ± 29.05	0.12 ± 0.04
NA-STZ	311.21 ± 26.58 [a]	0.33 ± 0.11 [a]
BC (50)	227.11 ± 18.82 [a,b]	0.18 ± 0.04 [b]
BC (100)	234.56 ± 20.97 [a,b]	0.15 ± 0.03 [b]
Donepezil (1)	231.29 ± 22.60 [a,b]	0.11 ± 0.02 [b]

Digits in parenthesis indicated dose in mg/kg. Data are expressed as mean ± SD; n = 12 rats. [a] $p < 0.05$ vs. normal group; and [b] $p < 0.05$ vs. NA-STZ (VaD) group.

4. Discussion

Our study demonstrated that rats treated with BC achieved shorter TL in the EPM test on day 23 when compared to the VaD group. This indicated that BC has the potential to ameliorate STZ-induced spatial memory dysfunction. The EPM test is commonly used for the assessment of anxiety in rats [33] and mice [37]. However, it is also used for the assessment of memory function in experimental animals [38]. The TL in EPM is influenced by anxiety and locomotion. Nevertheless, the effect of anxiolytics and anxiogenics agents do not interfere with the assessment of the learning and memory functions of rats [39]. Hence, it is also one of the methods for assessing spatial working memory [40]. The experimental data of the EPM test, e.g., a reduction in TL on the second trial, reflected the learning capacity of animals and also revealed the ability to retain memory [41]. Additionally, lycopene, a carotenoid compound, improved the β-Amyloid$_{1–42}$ peptide-induced AD and resulted in an improvement of TL in the EPM test in rats [21,42]. In this study, the administration of BC at a dose of 50 and 100 mg/kg; *p.o.* for 15 consecutive days ameliorated STZ-induced type 2 diabetic VaD, as evident by the EPM test in the rats.

The memory function is mainly regulated by AChE activity in the hippocampus [43,44]. The hippocampus is the most sensitive region of the brain and most susceptible to ischemia and hypoxia, particularly the CA1 region [45]. Various brain areas work in coordination for different types of cognitive functions [46]. Other than the hippocampal area, the entorhinal cortex, prefrontal cortex, cerebellum, septum, amygdala, and pons play a crucial role in memory function. It is evident that the septohippocampal loop largely contributes to spatial working memory [47,48]. Abundant cholinergic and GABAergic nerve fibers pathways can be found in this area. These pathways and the neurotransmitters are proven to play a crucial role in the regulation of cognitive function via bidirectional self-regulating cholinergic input [47,49]. Furthermore, the medial septum of the basal forebrain cholinergic system modulates hippocampal synaptic plasticity via the septal cholinergic inputs system [50]. The main source of hippocampal ACh comes from septohippocampal pathway interaction, which is assessed by electroencephalogram for brain theta waves activity [51,52]. The optogenetic suppression of rats′ medial septum is important for spatial memory [53].

The septohippocampal loop with cholinergic synapses and cholinergic transmission function are important for neuroplasticity and neurodegeneration [54]. The literature revealed that BC has a high binding capacity toward AChE, and it attenuated cognitive dysfunction in a mice model of AD [42]. There are mainly two types of cholinergic receptors, i.e., nicotinic and muscarinic cholinergic receptors. The hippocampal nicotinic receptors play an essential role in memory formation and the regulation of neuroinflammation [55]. Experimentally, the administration of AChE inhibitors, e.g., physostigmine and neostigmine, are known to ameliorate neuroinflammation via downregulation of interleukin-1 and tumor necrosis factor-α in activated microglia [56]. The present experimental data also revealed that BC attenuated AChE activity levels in hippocampal and septum tissues

of the rat brain [57]. It is evident that the regulation of AChE activity by BC enhanced spatial working memory functions via suppression of septohippocampal and hippocampal–entorhinal cortex pathways, which are responsible for the memory-consolidation process in interneuron connection of oriens lacunosum moleculare [58].

Furthermore, the presence of conjugated double chains in BC confers strong antioxidant potential [59]. It can chelate ~1000 singlet oxygen molecules and reduce the quantity of reactive oxygen species generated in neuronal tissue via physical interaction [60]. Chemically, the formation and cleavage of bonds transfer the energy from reactive oxygen molecules to BC molecules [61]. Moreover, BC inhibits the expression of transcription factors such as nuclear factor-kappa β and inflammatory cytokines pathways and subsequently reduces the peroxidation process in the cell membrane [62]. BC also reduces insulin resistance in experimental diabetic animals [63]. Hence, BC improves cognitive function in the type-2-diabetes-induced VaD rat, probably via a reduction in AChE activity levels in the septohippocampal loop area of the brain. In our study, there was a lack of a dose-dependent effect of BC, probably due to the dose being 50 mg/kg, which is more than what is required to produce the maximal therapeutic effects. This warrants further studies to determine the optimal dose of BC in rats for diabetic VaD, as there are limited studies on BC administration in rat models of VaD.

5. Conclusions

Our study showed that BC improves the cognitive performance of rats in EPM and attenuates elevated AChE activity. This indicates that BC could potentially be a naturally occurring compound to reverse cognitive changes due to VaD. However, more precise mechanisms of action and the regulatory pathways of BC in VaD are yet to be discovered. Thus, more extensive studies are required to explore the full therapeutic potential formulation of BC in different pathological conditions, including diabetic VaD.

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Institutional Review Board Statement: The animal study protocol was approved by the AIMST University Animal Ethics Committee of AIMST University (No: AUAEC/FOP/2020/13) on 9 November 2020.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

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Abbreviations

AChE, acetylcholinesterase; AChI, acetylthiocholine iodide; BC, beta-carotene; BG, blood glucose; DP, donepezil; EPM, elevated plus maze; *i.p.*, intraperitoneal; mg/kg, milligram per kilogram; NA, nicotinamide; *p.o.*, peroral; STZ, streptozotocin; TL, transfer latency.

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