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Original Research Article

Vitamin B2 blocks development of Alzheimer's disease in APP/PS1 transgenic mice via anti-oxidative mechanism

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Abstract

Purpose: *To study the effect of vitamin B2 (VB2) on the development of Alzheimer's disease (AD).*
Methods: *Memory and learning abilities were assessed in amyloid precursor protein/presenilin-1 (APP/PSI) transgenic mice aged 3 months, and C57BL/6 mice, using Morris water maze test. Brain tissue levels of key antioxidant enzymes, malondialdehyde (MDA), and reactive oxygen species (ROS) were assayed using ELISA kits. Western blot assay was used to monitor the expressions of Nrf2 and Keap1 proteins.*
Results: *Treatment with VB 2 significantly improved cognitive function of APP/PS1 mice with respect to number of crossings, duration of stay in target quadrant (TQ), and escape latency time ($p < 0.01$). Moreover, VB2 also significantly reduced the levels of MDA and ROS. It also brought about significant increases in brain activities of CAT, SOD, and GSH-Px in APP/PS1 mice, relative to the control group ($p < 0.01$). Moreover, VB2 up-regulated Nrf2 expression but down-regulated the expression of Keap1 in brain tissue of transgenic mice.*
Conclusion: *These results indicate that VB2 protects the brain from ROS-induced AD damage, most likely due to its potential anti-oxidant property and activation of the Nrf2 pathway.*

Keywords: *Alzheimer's disease, Cognitive function, Vitamin B2, Reactive oxygen species, Superoxide oxide dismutase, Anti-oxidation*

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INTRODUCTION

Alzheimer's disease (AD) is caused by degeneration of neurons, and manifests in patients in the form of progressively increasing memory loss, spatial disorientation, and dramatic slowdown in intellectual potency. These are the primary causes of dementia and disabilities (in old age) which impose enormous socio-economic burdens on many countries with rapidly growing elderly populations [1]. Although the pathogenesis of this disease has not been fully elucidated, several competing hypotheses have been proposed. These include amyloid-beta (Aβ) protein accumulation, cholinergic neurotransmitter deficiency, neurofibrillary tangles (NFTs) of tau proteins, increased inflammatory response, elevated reactive oxygen free radicals, and disordered ion dynamic equilibrium [2].

Increasing evidence indicate that oxidative stress is a key factor in the etiology of neurodegenerative diseases [3]. During the development of AD, oxidants are liberated in the brain, leading to excessive oxidative stress [4]. As products of aerobic metabolism, the levels ROS are controlled by the antioxidant and intracellular enzymes CAT, SOD and GSH-Px [5-7]. Under normal physiological conditions, ROS as signaling molecules are present at low levels in a transitory manner. However, excess accumulation of ROS leads to peroxidation, which does serious damage to DNA, phospholipids, and proteins. These damages are able to decrease the oxidase potency of mitochondrial cytochrome C and result in metabolic disturbance and cell apoptosis [8]. Thus, considerable research interest has been associated with the suppression of oxidative stress as a novel strategy for prevention and treatment of AD [9,10]. However, the current anti-oxidative drugs used for AD patients have not achieved satisfactory efficacies in clinical trials. As a result, there is need for new anti-oxidative drugs for treating AD. At the present, a lot of attention has been devoted to natural bioactive products as likely candidates for treating AD or ameliorating its symptoms [11,12].

Vitamin B2 (VB2, Figure 1), also known as riboflavin, is widely distributed in animals and vegetables. It is of benefit to human health, through its diverse functions in biological oxidation, metabolism of vitamin B6, energy and cell growth. It is also associated with the absorption, storage and mobilization of iron in the body [13]. Moreover, VB2 has proven antioxidant activity [14,15].

The present study was aimed at investigating the influence of VB2 on the development of AD pathologies using an animal (mouse) AD model.

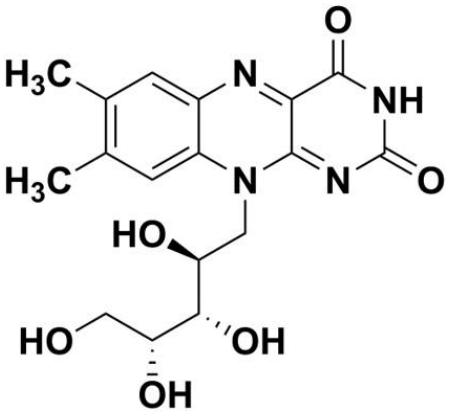

Figure 1: Structure of vitamin B2

EXPERIMENTAL

Animals and chemical reagents

Male APP/PS1 double transgenic mice (32) aged 3 months old and weighing 26 – 28 g (Nanjing Biomedical Research Institute, Nanjing, China, license No. SYXK (Su) 2017009) were used as the experimental group. Eight (8) male C57BL/6 mice of the same age (Animal Center of Yangzhou University, China) were used as controls. The experimental procedures used in this investigation were approved by the Animal Experiment Committee of Jiangsu University of China (approval no. 2017–012). The experimental procedure followed the US NIH Guide for the Care and Use of Laboratory Animals [16].

A stock suspension (3 mg/ml) of VB2 (Sigma-Aldrich, USA) was prepared in 0.5 % carboxymethyl cellulose sodium (CMC-Na), and administered via the intra-gastric route at a dose of 10 mL/kg body weight. The activity of lactate dehydrogenase (LDH), catalase (CAT), GSH-Px, SOD, and levels of MDA were determined with appropriate assay kits provided by Jiancheng Institute of Bioengineering (Nanjing, China).

Drug treatment

The mice were randomly assigned to five groups (12 per group) and treated as indicated below:

Group I: CMC-Na (0.5 %; 10 mL/kg) was administered to C57BL/6 male mice.

Group II: CMC-Na (0.5 %; 10 mL/kg) was administered to APP/PS1 transgenic mice.

Group III: Memantine-HCl was dissolved in CMC-Na (0.5 %) and given to the APP/PSI double transgenic mice at a dose of 5 mg/kg body weight.

Groups IV and V: APP/PS1 mice received daily injections of VB2 at two doses (10 and 30 mg/kg). All treatments were given intra-gastrically daily for 70 days, and the animals were assessed for behavioral parameters as from day 64.

Morris water maze test

Memory and learning were evaluated in the mice using the Morris water maze test, but with some modifications [17,18]. The water maze comprised a cylindrical tank brimming with water maintained at a temperature of 23 ± 1 °C. It was partitioned equally into 4 quadrants labeled east, west, north and south and east, and a 10-mm diameter 2 cm

beneath water within quadrant in the east. This was designated the target quadrant (TQ). Training was conducted for 5 days, at the rate of 4 trials per day, and the escape latency was recorded from day 64 to day 68 of the study. On day 68, the number of crossings and the time spent in TQ were noted and recorded according to a previously defined procedure [19].

Assay of CAT, SOD, and GSH-Px activities, and determination of ROS and MDA

Mice were sacrificed by decapitation 60 min after the final behavioral tests. The brain samples were carefully excised and dried with filter paper. Nine volumes of cold saline were used for preparation of 10 % brain homogenate which was subjected to centrifugation for 10 min at 2500 rpm. The supernatant was preserved at 4 ^{o}C for determination of ROS and MDA levels, as well as CAT, SOD, and GSH-Px activities using appropriate ELISA kits as per manufacturers' protocols.

Western blot assay

The procedure for Western blotting was based on the method reported previously [20]. Protein estimation in brain tissue of APP/PSI mice was done using BCA method. The proteins were separated on a gradient SDS-PAGE and then transferred to a PVDF membrane, and blocked for 1 h with 5 % skim milk in TBST buffer (Tris-HCl, 10 mM, pH 8.0, containing 0.1 % Tween 20 and 150 mM NaCl) (to block non-specific binding). This was followed by overnight incubation of the membrane with the primary antibody at 4 °C. After this, the membrane underwent washing thrice with TBST buffer, followed by incubation for 2 h with secondary antibody at room temperature. After washing thrice, the protein bands were detected using enzyme-linked chemiluminescence kits according to the kit instructions. β-actin was used as the internal reference. The intensities of the resultant bands were quantified using Quantity One software. The expression of protein was depicted in terms of the ratio of the intensity of target protein band to the intensity produced by the β-actin band.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). They were analyzed with one-way analysis of variance (ANOVA), and Tukey's multiple comparisons. All analyses were carried out with GraphPad Prism 6.0. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Effect of VB2 on cognitive function of APP/PS1 mice

Treatment with VB2 led to a significant reduction in escape latency time on day 68, when compared with day 64 in group I, indicating normal spatial learning capacity. However, the APP/PS1 mice with impaired learning had a significantly longer escape latency time on day 68, when compared with group I. Daily VB2 administration significantly and dose-dependently cut down the escape latency time of APP/PS1 mice on day 68 (Figure 2 A).

Moreover, when compared with the other groups, the time spent by Group 1 mice in TQ was significantly longer, relative to the other quadrants on day 68, suggesting normal memory. In contrast, APP/PS1 mice displayed a significant decrease in time spent in TQ on day 68 and showed fewer crossings in the platform area than mice in group I (Figure 2 B−C). However, daily VB2 administration led to significant and dose-dependent improvement in the time spent in TQ on day 68 and the number of platform area crossings by APP/PS1 mice in groups III and IV, to extents comparable to the positive group III.

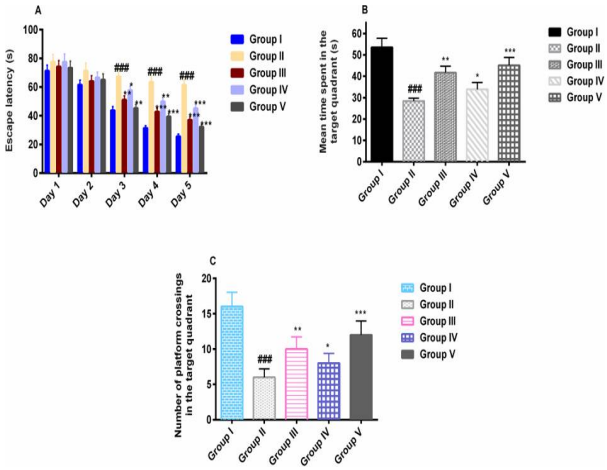

Figure 2: Effect of VB2 on memory and learning abilities of APP/PS1 mice. (A) Escape latency time during an acquisition trial in the Morris water maze. (B) Mean time spent in TQ on day 68. (C) Frequency platform crossings in TQ on day 68 during a retrieval trial in the Morris water maze. Values are mean ± SD (n = 12); $^{###}p < 0.001$, compared to group I; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, relative to group II

Effect of VB2 on ROS and MDA levels

The level of ROS in group II was significantly increased, relative to the control group (Figure 3). However, the VB2-treated groups had

significant and dose-dependent reduction in ROS levels, relative to group II. Besides, the ROS levels in groups IV and V were similar to that in the positive group (group III). A similar trend was seen in MDA levels.

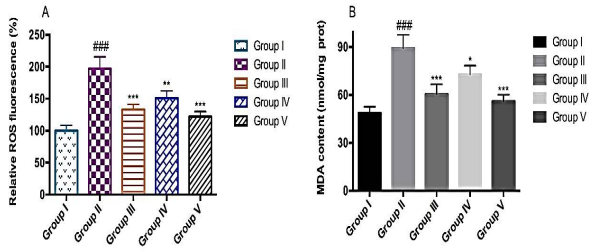

Figure 3: Effect of VB2 on ROS and MDA levels in brain tissues of APP/PS1 mice. (A) Relative ROS fluorescence; (B) MDA content. Values are mean ± SD (n = 12); $^{###}p < 0.001$, compared to group I; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared to group II

Effect of VB2 on the activities of GSH-Px, CAT and SOD

The results on Figures 4A – 4C show that in group 11 mice, there were significant decreases in GSH-Px, CAT and SOD levels, relative to their activities in group I. However, the activities of these antioxidant enzymes were significantly and dose-dependently increased by daily VB2 administration, when compared with corresponding activities in group II ($p < 0.05$) (Figures 4A - 4C).

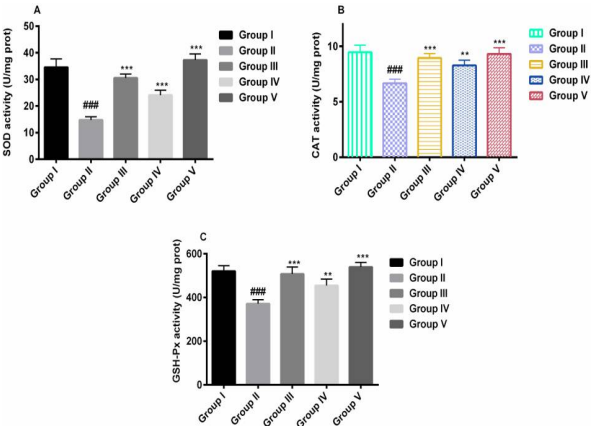

Figure 4: Effect of VB2 on the levels of SOD, CAT and GSH-Px in APP/PS1 mice, as assessed by ELISA. Values are mean ± SD (n = 12); $^{###}p < 0.001$, compared to group I; $*p < 0.05$, $**< 0.01$, $***p< 0.001$, compared to group II

Effect of VB2 on Nrf-2 and keap-1 expressions

As indicated in Figure 5, Nrf2 expression in group II was significantly lower than in group I, while Keap1 showed the reverse result. Compared with group II, the Nrf2 expression in low or high dose-treated mice increased significantly, while Keap1 expression decreased significantly.

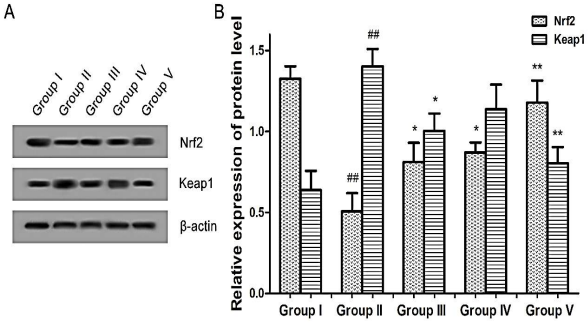

Figure 5: (A) Effect of VB2 on expression of Nrf2 and Keap1 in the brain tissues of APP/PS1 mice. (B) Relative expression of protein with respect to that of β-Tubulin. Values are mean ± SD; $^{##}p < 0.01$, relative to group I; $*p < 0.05$, $**p < 0.01$, compared to group II

DISCUSSION

In this study, the experimental APP/PS1 mice had significantly damaged abilities of learning and memory characterized by increased levels of hippocampal amyloid beta, tau protein and oxidative stress, relative to age-matched C57BL/6 mice. These mice are ideal models for studying the mechanistic processes involved in AD [22,23]. Increasing attention has been focused on the beneficial effect of fighting oxidative stress on AD development, especially the effect of diverse natural nutrient products with memory- and cognition-promoting effects. Thus, the effect of VB2 on AD mice was investigated in this study. The results showed that daily VB2 administration at two different doses can significantly increase the number of crossings in the target platform, and reduce escape latency in APP/PS1 transgenic mice, suggesting that VB2 could ameliorate AD-induced impairment in spatial learning and memory. Increased production of free radicals and decreased endogenous antioxidants may accelerate membrane phospholipid breakdown, leading to lipid peroxidation and cellular dysfunction.

Excess ROS are considered as a common characteristic of AD. In addition, increased levels of MDA in APP/PS1 mice relative to wild-type C57 mice have been reported as indicators of higher levels of oxidative stress [24]. The major lipid peroxidation indicator, MDA can seriously damage the structure of cell membranes leading to cell damage in a manner similar to the effect produced by ROS. Therefore, the contents of ROS and MDA indirectly reflect the severity of free radicals. In this study, the ROS and MDA

levels were markedly reduced by treatment of transgenic mice with VB2, indicating that VB2 ameliorated oxidative stress in the APP/PS1 mice.

The brain neurons are protected from ROS-induced oxidative damage by the antioxidant enzymes SOD, CAT and GSH-Px [25]. The results obtained in this study showed that SOD activity was significantly increased by VB2 administration in APP/PS1 mice, suggesting that VB2 exerts anti-oxidative stress effects through elevation of antioxidant enzyme activity.

Nrf2 is a nuclear transcription factor that regulates antioxidant activity *in vivo*, and is vital in the prevention and treatment of diseases of the cardiovascular, central nervous and digestive systems [26]. Under normal conditions, Nrf2 is combined with the inhibitory factor Keap1 in the cytoplasm, which keeps it in the inactive state thereby preventing it from playing its signal transmission function [27]. However, under oxidative stress or oxidative phosphorylation *in vivo*, Nrf2 in the cytoplasm is activated, and then enters the nucleus and binds to related antioxidant response factor, activating Nrf2 pathway and facilitating the expression of downstream antioxidant genes for CAT, SOD, GSH-Px [28-30].

In this study, VB2 administration led to significant increases in Nrf2 expression and significant decreases in the expression of the inhibitory factor Keap1 in brain tissues of transgenic mice. Thus, VB2 promoted the expressions of downstream antioxidant genes and increased antioxidant activities, which suggests that the anti-AD effect of VB2 is dependent on activation of the Nrf2 pathway.

CONCLUSION

Administration of VB2 to APP/PS1 transgenic mice confers protection against ROS-induced damage and mitigates impaired cognitive and memory capacities in AD. This protective effect may be attributed to its potential anti-oxidant property and activation of the Nrf2 pathway. These findings indicate the potential for clinical application of VB2 in the management of Alzheimer's disease.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Rong Zhao and Kai Zhao conceived and designed the study, Huajun Wang collected and analyzed the data, Chen Qiao wrote the manuscript. All authors read and approved the manuscript for publication.

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