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3-Hydroxybutyrate methyl ester as a potential drug against Alzheimer's disease via mitochondria protection mechanism

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ABSTRACT

Alzheimer's disease (AD) is induced by many reasons, including decreased cellular utilization of glucose and brain cell mitochondrial damages. Degradation product of microbially synthesized poly-hydroxybutyrate (PHB), namely, 3-hydroxybutyrate (3HB), can be an alternative to glucose during sustained hypoglycemia. In this study, the derivative of 3HB, 3-hydroxybutyrate methyl ester (HBME), was used by cells as an alternative to glucose. HBME inhibited cell apoptosis under glucose deprivation, rescued activities of mitochondrial respiratory chain complexes that were impaired in AD patients and decreased the generation of ROS. Meanwhile, HBME stabilized the mitochondrial membrane potential. *In vivo* studies showed that HBME crossed the blood brain barrier easier compared with charged 3HB, resulting in a better bioavailability. AD mice treated with HBME performed significantly better ($p < 0.05$) in the Morris water maze compared with other groups, demonstrating that HBME has a positive *in vivo* pharmaceutical effect to improve the spatial learning and working memory of mice. A reduced amyloid-β deposition in mouse brains after intragastric administration of HBME was also observed. Combined with the *in vitro* and *in vivo* results, HBME was proposed to be a drug candidate against AD, its working mechanism appeared to be mediated by various effects of protecting mitochondrial damages.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, which is globally the fourth largest fatal disease [1]. It is characterized by loss of recent memory and, as the disease progresses, symptoms develop including irritability, aggression, problems with speaking and writing [2,3]. With the deprivation of body functions, the disease ultimately results in death. The estimated annual worldwide funds to Society of Dementia totaling US$ 604 billion highlights the enormous impact that dementia has on socioeconomic conditions worldwide [4].

The cause and progression of AD are not yet well understood [5]. There have been many hypotheses on AD, including Aβ (amyloid-beta plaque) aggregation [5–7], hyperphosphorylation of tau protein [8–11], and reduced synthesis of the neurotransmitter acetylcholine [12]. Therapeutic drug developments for AD mainly based on the acetylcholine hypothesis or the antibody of Aβ, but none of the drugs could stop or reverse the progression. Therefore, more and more researchers turn to the dysfunction of mitochondria [13–16] and the impairment of energy metabolism [17,18], which are now believed to have close relationship with AD. For instance, when the activity of mitochondrial respiration chain complex IV is decreased [19], the redox state in mitochondria will also be changed, resulting in the accumulation of ROS (reactive oxygen species), which leads to oxidative damage to the cell especially to mitochondria [20–22]. One common form of impairments on energy metabolism is the weakened glucose based TCA cycle, which is harmful to the cells [23,24]. Both in sporadic and family AD, glucose

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metabolisms in brain are impaired [25]. In this case, ketone bodies are the only alteration of energy supplement for the brain [26]. 3-hydroxybutyrate acid (3HB), a degradation product of microbial, natural and biocompatible biomaterial polyhydroxybutyrate [27–29], also, as the major component of ketone bodies, has been reported to have neuroprotective effects [19,30–34]. But due to its charged nature and acidity, 3HB may not be an ideal drug candidate. Yet the esterification product of 3HB, namely, 3-hydroxybutyrate methyl ester (HBME), has a lower polarity with a neutral pH, therefore it is expected to have a better bioavailability and enter the brain easier than 3HB. Thus, HBME could be a better drug candidate for developing central nervous system drugs, especially anti-AD drugs.

In this study, we investigated the potential of HBME as an AD drug by *in vitro* and *in vivo* experiments. At the same time, we found that its anti-AD mechanism may be mediated by mitochondria protection.

2. Materials and methods

2.1. Cell culture and reagents

PC12 cell line was kindly donated from Professor Lijun Du (Tsinghua University, Beijing, China) and cultivated in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 10% horse serum and 1% penicillin–streptomycin in a CO_2 incubator (5% CO_2 and 95% air) (Thermo electron corporation, USA) at 37 °C. Primary neurons were divided from the 17–19 days ICR fetal mice brains, then cultured in Neurobasal® medium with B27® serum free supplement, 1% glutamate and 1% penicillin. HBME was a gift from Tianjin GreenBio Materials Co., Ltd. (Tianjin, China). 3-hydroxybutyrate acid (3HB) was purchased from Sigma Chemical Co. (USA). Beta amyloid (Aβ) monoclonal antibody, 6E10, was purchased from Covance Inc. (Princeton, New Jersey, USA). Goat anti-mouse Ig G (TRITC conjugated), PI (Propidium Iodide) and Calcein-AM were purchased from Dojindo Co., Ltd. (Japan).

2.2. Animals and treatments

C57/BL6 mice which are double transgenic mice expressing Mo/HuAPP695swe and PSEN1-dE9 were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The double transgenic mice model exhibits age-dependent Aβ plaques aggregation as well as a distribution of Aβ plaques in the cerebral cortex and the hippocampus, and they show declining learning and memory ability [35].

We identified the double transgenic mice by crossing the mutation site of APP gene. Genotyping was performed by PCR on genomic DNA using specific transgenic primers (APP forward: 5′-CTGACCACTCGACCAGGTTCTGGGT-3′, APP reverse : 5′-GTGGATAACCCCTCCCCCAGCCTAGACCA-3′). The internal reference primer had the same forward primer as APP, and the reverse primer of internal reference is 5′-AAGCGGCCAAAGCCTGGAGGGTGGAACA-3′.

All experiments were approved by the Institution on Animal Care and Use, Committee of the Tsinghua University under the experimental plan number 2010-ChenGQ-AD-3HBME. For Morris water maze test, these 7.5-months-old male mice were divided into six groups with ten mice in each group: Negative control (normal mice treated with deionized water), AD model mice treated with deionized water, 20, 40 and 80 mg/kg/d HBME, and positive control (AD model mice treated with 80 mg/kg/d AXONA®). AXONA® is an FDA approved healthy food against AD [36] and may have a similar mechanism as HBME. Due to its similarity to HBME, AXONA® was used to serve as a positive control for the *in vivo* study. The mice were treated once daily through intragastric administration for 2.5 months prior to the experiments.

For testing of amyloid-β deposition in mice brain, 5-months-old male mice were divided into groups with ten mice in each group: Negative control (normal mice treated with deionized water), AD model mice treated with 40 mg/kg/d HBME, and positive control (AD model mice treated with 1.6 mg/kg/d Donepezil). Donepezil (Donep), an acetylcholinesterase inhibitor, is an approved drug for treatments of mild to moderate AD [35] which can slow amyloid plaque deposition and protect neuron [37]. Donep was used as a positive control to investigate the impact of HBME on amyloid-β deposition. The mice were treated once daily through intragastric administration for 2.5 months prior to the immunohistochemical experiments.

2.3. Morris water maze test

The Morris water maze is a circular pool measured 120 cm in diameter and 60 cm in height with white bottom and wall. A white circular platform (diameter 6 cm; height 30 cm) was submerged 1 cm beneath the water surface. 250 mL milk was poured into the tank to whiten the water (maintained at 24 ± 1 °C) so that the platform was not easily and visually recognized by the mice. On each of the walls of the four quadrants, a distinct colored paper was pasted as a visual positional hint. A closed-circuit television camera was mounted onto the ceiling directly above the center of the pool to monitor subject-swimming parameters.

Mice were trained a two-trial-per-day regime for seven consecutive days before testing. In the every day training, mice were allowed swimming in water for 1 min. If a mouse could not find the platform, it would be guided there and allowed to stay there for 30 s to remember the platform location. Mice were provided with two opportunities, their average time to find the platform was calculated. The time mice consumed to find the platform (escape latency) every day was recorded. The tests were carried out when most of the mice could find the platform within 30 s. During the testing, the platform was removed, three parameters were measured including the platform spans (the number of a mouse crossing the exact platform location), the time mice stayed in the platform quadrant, and thigmotaxis defined as the behavior that mice display when swimming close to the walls of the water maze. The parameters were analyzed by EthoVision 3.1 analyze system.

2.4. Mice brain MRI (magnetic resonance imaging)

After being treated by intragastric administration with water or HBME for 2.5 months, mice were scanned by MRI. The experiment was conducted in Beijing Neurosurgical Institute of Capital Medical University, China. Anaesthetized by 10% chloral hydrate-saline, the mice were placed in the prone position, their chests connected to a life signal detector (Model 1025L Monitoring and Gating System, SA Instruments, Inc., USA) to observe their physiological situation. The heads of the mice were scanned by 7.0T ultra-high field animal MRI scanner (70/30-7.0T ClinScan, Bruker, Germany). T2 weighted images on the mouse heads were taken from sagittal, axial and coronal directions under following conditions: TR/TE = 3200/54 ms, field of view (FOV) = 22 × 22 mm, slice thickness = 0.5 mm and scan encoded matrix of 384 × 384. Data from these images were collected for analysis.

2.5. MTT assay

Cell viability was assayed using the MTT assay. Primary neurons were pretreated for 36 h on regular media containing 10 mM 3HB or HBME, respectively. Then the medium was replaced by a high glucose medium or glucose-free medium supplemented with 10 mM 3HB or HBME for 24 h. The cell viability rate was determined with the MTT cell proliferation and cytotoxicity assay kit (Beyotime Institution of Biotechnology, Jiangsu, China). The survival rate was expressed by OD ratios of glucose-free groups to the high glucose group.

2.6. PI/Calcein-AM dyeing

Double staining with PI and Calcein-AM was employed to investigate the PC12 cell apoptosis rate after glucose deprivation. PI entered the nucleus of dead cells to emit red light. In contrast, Calcein-AM entered the living cells emitting green light. PC12 cells were cultured in high glucose DMEM medium or glucose-free DMEM medium, glucose-free medium supplemented with 3HB or with HBME for 4 h after their seeding in 35 mm dish containing the regular medium for 24 h. Subsequently, the double dyeing was performed following the manufacturer's instruction (Dojindo Co., Ltd, Japan).

2.7. Cellular ATP measurement

PC12 cells in 100 μl medium were plated to at least three 96-well plates. The blank control used only the regular medium, while the negative control cultured in the regular medium supplemented with 20 mM NaN_3. Two of the treatment mediums were regular medium containing 5 mM 3HB or HBME, and another two used regular medium containing 5 mM 3HB or HBME accompanied with 20 mM NaN_3. Each group contained four parallel samples. After incubation 12 h, the ATP contents were assayed using Adenosine 5′-Triphosphate (ATP) Bioluminescent Somatic Cell Assay Kit (Sigma). The fluorescence intensity was detected by Centro XS3 LB 960 Microplate Luminometer (Berthold Technologies, GmbH & Co. KG, Germany).

2.8. Measurement of [NAD+]/[NADH+] ratio

PC12 cells in 500 μl medium were plated in the wells of at least three 24-well plates, and incubated with rotenone alone or co-incubated with rotenone and 3HB or HBME for 4 h. After the treatment, cells were digested by trypsin followed by three times washing with PBS. The cells were centrifuged at 3000 rpm for 5 min. The precipitates were lysed for 30 min on ice with 50 μl lysis solution consisting of 150 mmol/NaCl, 10% Triton X-100, 50 mM Tris–HCl, 1 mM EDTA and 1 mM PMSF, followed by heating at 95 °C. Cell lysate including 1×10^4 cells were assayed their [NAD+]/[NADH+] ratio as described [38].

2.9. Assessment of mitochondrial membrane potential

Fluorescent probe JC-1 (Beyotime, Jiangsu, China) was used to study the mitochondrial membrane potential ($\Delta\Psi m$) of PC12 cells. Cells with higher mitochondrial membrane potential predominantly contain JC-1 in aggregated form, and they

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