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 microbiolally synthesized polyhydroxybutyrate (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.

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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 socio-economic 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
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. HBME was a gift from Tianjin GreenBio Materials Co., Ltd. (Tianjin, China). Goat anti-mouse Ig G (TRITC conjugated), PI (Propidium iodide) and Calcein-AM were purchased from Dojindo Co., Ltd. (Japan). (Princeton, New Jersey, USA). IDE (IDE forward 5'-GGCGAATTGGAGATGAAC-3', reverse 5'-TGGATAAACCCTCCCCCAGTCTAGACA-3'). The internal reference primer had the same forward primer as APP, and the reverse primer of internal reference is 5'-AAAGGCGGAAAAGCTGGGGTGGGAA-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 diet), Aβ, 3HB, HBME, AXONA/C210, AXONA/C14. For testing of amyloidosis in mice brain, 5-months-old male mice were divided from 17–19 days ICR fetal mice brains, then cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 10% horse serum and 1% penicillin and streptomycin in a CO2 incubator (5% CO2 and 95% air). The mice were treated once daily through intragastric administration for 2.5 months prior to the immunohistochemical experiments. At the same time, we found that its anti-AD mechanism may be mediated by mitochondria protection.

2.2. Animals and treatments

C57/BL6 mice which are double transgenic mice expressing Mo/HuAPP95swe and PSEN1–E9 were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The double transgenic mouse model exhibits age-dependent Aβ plaque 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 primer sequences. APP forward 5’-TCCTCAGCCTCCAGATGGTCG-3’ and APP reverse 5’-GTGCTGGGATGGCTCTTC-3’. The internal reference primer had the same forward primer as APP, and the reverse primer of internal reference is 5’-AAGGCGGAAAAGCTGGGGTGGGAA-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 of HBME, and positive control (AD model mice treated with 80 mg/kg of AXXONA). AXXONA is an FDA approved healthy food against AD [36] and may have a similar mechanism as HBME. Due to its similarity to HBME, AXXONA 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 of HBME, and positive control (AD model mice treated with 1.6 mg/kg of 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 neurons [37]. Donep was used as a positive control to investigate the impact of HBME on amyloid-β deposition. The mice were treated one 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 on 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 1% 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 7.0 T ultra-high field animal MRI scanner (70/30-7.0T ClinScan, Bruker, Germany). 12 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. MT assay

Cell viability was assayed using the MT 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 HBME for 24 h. The cell viability rate was determined with the MTT cell proliferation and cytotoxicity assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). The survival rate was expressed by OD ratios of glucose-free groups to the high glucose group.

Table 1: Primer for quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gen bank accession no.</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
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<tr>
<td>ACHE</td>
<td>NM_009599</td>
<td>Forward: CGCAGACTTGGCTGAAGAC&lt;br&gt;Reverse: CGGCTGATACACCATCAGCAAG</td>
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<tr>
<td>APOE</td>
<td>NM_008696</td>
<td>Forward: AGCCGTCTTCCGATGATACC&lt;br&gt;Reverse: CTCACTTTCCTTCTTCTC</td>
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<tr>
<td>APP</td>
<td>NM_001198823</td>
<td>Forward: GCACGCAACAGGATGATAC&lt;br&gt;Reverse: GATTGGTCTAGAACACTTG</td>
</tr>
<tr>
<td>BACE1</td>
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<tr>
<td>Rax</td>
<td>NM_007527</td>
<td>Forward: GCAGAAATGGCACTAAC&lt;br&gt;Reverse: AAGTAAGAAGGGCCAAC</td>
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<tr>
<td>Bcl2</td>
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<tr>
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<td>Forward: ATGAGAACCCGTCATATAAGTAC&lt;br&gt;Reverse: TCTCCTGCGTATGAACT</td>
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<tr>
<td>Capp3s</td>
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<td>Forward: TTGCTGATGCTGGGACAG&lt;br&gt;Reverse: GAGAACAGGACTGATGAC</td>
</tr>
<tr>
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<td>NM_009891</td>
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<tr>
<td>GSK3β</td>
<td>NM_019827</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>PUMAG</td>
<td>NM_030701</td>
<td>Forward: CGCAAGGCTGCTGCTTC&lt;br&gt;Reverse: ATGCTGCTTCTGCTGCTTC</td>
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Fig. 1. 3-hydroxybutyric acid methyl ester (HBME) ameliorated spatial learning and memory impairments in AD model mice. Normal or AD mice were treated with water, HBME (20, 40 or 80 mg/kg/d) or 80 mg/kg/d AXONA™ for 2.5 months, followed by Morris water maze experiments. (A) Columns show average escape latencies of every day training for each treatment. During the testing, the platform was removed, three parameters were measured including platform spans (B), the time mice stayed in the platform quadrant (C) and thigmotaxis (D). (E) Long term memory was represented with the escape latency performed by mice after seven days without training. Values represent the mean ± SEM (n = 8–10). Data were analyzed by two-way ANOVA (A) and t-Tests (Unpaired t-Test) (C, D and E), #p < 0.05, ##p < 0.01 versus negative control; *p < 0.05, **p < 0.01 versus AD mice treated with water.
2.6. PI/Calcine-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 (Dojinbo 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 treated in the regular medium supplemented with 20 μM NaCl. Two of the treatment mediums were regular medium containing 5 μM 3HB or HBME, and another two used regular medium containing 5 μM 3HB or HBME accompanied with 20 μM NaCl. 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 μM Tris–HCL and 1 mM EDTA and 1 mM PMSF, followed by heating at 95 °C. Cell lysis including 1 × 10^6 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 (∆ψm) of PC12 cells. Cells with higher mitochondrial membrane potential predominantly contain JC-1 in aggregated form, and they should show red fluorescence. When the ∆ψm dissipates, JC-1 staining show predominantly a monomeric form emitting green fluorescent at 529 nm. The cells were co-treated with CCCP (carbonyl cyanide m-chlorophenyl hydradzone) and 3HB or CCCP and HBME for 6 h, and then incubated with JC-1 working solution for 20 min. The treated cells were rinsed twice with PBS, then visualized via the laboratory-graded Olympus confocal fluorescence microscope (BX61, Olympus, Japan) equipped with an argon laser.

2.10. Measurement of ROS level

The redox-sensitive green fluorescent proteins, Lenti-roGFP (GFP with mutations in C485, S147C, and Q204C), were utilized to visualize the oxidation state of the cells [39]. The 293T cells were transfected with Lenti-roGFP, and then incubated with 3HB or HBME for 36 h. Confocal laser scanning microscope (A1RSi, Nikon, Japan) was used to detect their fluorescent intensities at 405 nm and 488 nm exciting wavelengths and 515 nm emitting wavelength, respectively. Data was analyzed using Image J software (Wayne Rasband, National Institution of Health, USA).

2.11. Immunohistochemistry

Mice were deeply anaesthetized with chloral hydrate (400 mg/kg body weight) and were sacrificed by intracardiac injection of PBS followed by brain tissue fixation using 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 and 4 °C [40]. Brains were removed from the skull and post-fixed in the same fixative agent overnight before being transferred into 30% sucrose solution for dehydration. Subsequently, the brains were frozen sectioned into many 40 μm thickness layers in the coronal plane. The sections were then incubated in 3% normal goat serum in PBS for 30 min followed by another incubation with primary antibodies 6E10 (1:10000) overnight at 4 °C. Tissue sections were washed three times for 10 min in 0.3% phosphate buffered saline tween-20 (PBST) at pH 7.4, then were incubated with secondary antibody goat anti-mouse Ig G (TRITC conjugated) for 1 h, followed by staining with DAPI for additional 10 min. Finally, the sections were washed three times and then covered with enthanol. The images were captured by the laboratory-graded Olympus confocal fluorescence microscope (Olympus BX60, Tokyo, Japan). The quantitative analysis was carried out using the Image J software.

2.12. Quantitative real-time PCR

Wild-type mice were treated with water while AD model mice treated with water or HBME for 10 days. Then total RNA was prepared with TRizol reagent (Invitrogen, Temecula, CA, USA) and the Primer Express Software (Allele ID, PREMIER Biosoft International, Palo Alto, CA) was used to design the oligonucleotide primers for the housekeeping genes. The primer sequences are given in Table 1. Quantitative real-time PCR was performed by the SYBR Green (TANGene Co., Ltd, Beijing, China) detection method using the Mx3000p Quantitative PCR system (Agilent Co., Ltd, USA). Data was analyzed by the comparative Ct method [41].

2.13. Measurement of 3HB concentration

In each group, 3 mice were treated with 150 μl water, 40 mg/kg HBME or 30 mg/kg 3HB, and then sacrificed at specific time point for sample collections. Mice blood samples from ventriculus sinister stood at room temperature for 30 min. After 4 °C centrifugation, the serum from the transparent supernatants was obtained. Brain tissue samples were taken from cranial cavity after a heart perfusion process with PBS, the samples were grinded in Tris–HCL and then centrifuged to obtain the supernatant as the brain samples. The concentrations of β-hydroxybutyrate acid (3HB) in brain samples were assayed using the 3HB assay kits (Cayman Co. Ltd, Michigan, USA) and the absorption at 450 nm was detected using VersaMax ELISA Microplate Reader (Molecular Devices, LLC, Sunnyvale, California, United States).

Fig. 2. HBME treatment reduced the deposition of amyloid-β in mice brain. (A) After intragastric administration for 2.5 months, Aβ plaques (red) in cortex and hippocampus of mice brain detected using 6E10 antibody by immunohistochemistry. (B) Statistical results showed the area of Aβ plaques. Columns show mean values ± SD (n = 7). Data were analyzed by Student’s t-test, *p < 0.05, **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3. Results

3.1. The promotion of spatial learning and working memory in AD model mice

Morris water maze [42] was used to assess the effect of HBME on spatial learning acquisition of the AD model mice. When AD model mice were treated with HBME via intragastric administration, significant lower escape latencies were found compared with the water-treated ones (Fig. 1A). The escape latency of mice in all groups decreased with elapsed time (Fig. 1A). Notably, after 5 days of training, the AD model mice treated with 40 mg/kg/d HBME spent the least time to locate the platform, which were almost as fast as the control group consisting of normal mice treated with water, and were much faster than those AD model mice treated with water ($p < 0.05$). While the AD model mice groups treated with 20 and 80 mg/kg/d HBME did not perform well as expected (Fig. 1A). After the platform was removed, the group of AD model mice treated with 40 mg/kg/d HBME performed the best among all the AD model mice groups (Fig. 1B–C), as they spent most of the

Fig. 3. HBME inhibited the asymmetrical change of ventricle shown from mice brain Magnetic Resonance Imaging (MRI). Panel (A), (C) and (E) are mouse coronal sections, and (B), (D) and (F) are axial sections. The highlighted white regions are cerebrospinal fluid (CSF) in the ventricle, and the hippocampus is just around. In (A) and (B), the normal mice were treated with water. (C) and (D) were AD model mice treated with water. (E) and (F) were AD model mice treated with 40 mg/kg/d HBME.
time around the area where the platform locates. Thigmotaxis recording the time mice spent near the maze walls, which was considered an index of anxiety in mice [43]. Data showed mice treated with 40 mg/kg/d HBME behaved the calmest among all the six groups (Fig. 1D). Results of long term memories demonstrated that AD model mice treated with 40 mg/kg/d HBME were able to locate the platform the fastest, they behaved similarly with the control group (Fig. 1E).

All these results indicated that HBME improved learning and working memory ability of AD model mice. 40 mg/kg/d seemed to be the most suitable dose among tested HBME concentrations.

3.2. The reduction on deposition of amyloid-β in mice brain

After different treatments by intragastric administration for 2.5 months, Aβ plaques in cortex and hippocampus of mice brain were detected by immunochemistry studies.

As expected, Aβ plaque was not observed in normal mice (Fig. 2A), this control group was therefore not included for further analysis. AD model mice at 7.5 months of age were found to develop Aβ plaques in hippocampus and cortex (Fig. 2A). This observation agreed well with previous study [44].

Donepezil (Donep) and HBME treatments reduced the areas of Aβ plaques both in cortex and hippocampus compared with the AD mice treated with water. Interestingly, HBME inhibitory effect on plaque formation was significantly better than Donep in hippocampus, although Donep showed a better effect on the cortex region than HBME (Fig. 2B). These results demonstrated the ability of HBME to inhibit the Aβ plaque deposition in brains of AD mice.

3.3. The inhibition on brain atrophy of AD mice

After the Morris water maze studies, mice were randomly chosen to be subjected to intravital scanning using magnetic resonance imaging (MRI). Coronal and axial sections (Fig. 3A–F) of the mouse brains were studied. The sizes of ventricle in both brain sides were symmetric in a normal mouse brain, with their edges being clear and sharp (Fig. 3A and B). In contrast, an AD model mouse treated with water revealed ventricles asymmetry with a much larger size on the left than the right side with unclear edges, indicating an obvious brain atrophy (Fig. 3C and D). While an AD model mouse treated with HBME was found to have an obvious symmetrical ventricle (Fig. 3E and F).

3.4. The inhibition on cell apoptosis

The relative number of primary neuron in glucose-free medium was much less than those cultured in a high glucose medium. However, when 3HB or HBME was added to the glucose-free medium, cells grew more in numbers compared with their growth in...
the glucose-free medium only (Fig. 4A–B). HBME seemed to better support the cell survival than 3HB did reflected by more viable primary neurons detected when provided with HBME (Fig. 4A). A double dye consisting of PI and Calcein-AM confirmed the apoptosis inhibition effect of HBME. The absence of glucose led to significant cell death. However, the red spots associated with cell death significantly reduced when the cells were treated with HBME or 3HB for 4 h in the absence of glucose (Fig. 4B). The cell apoptosis rate after HBME or 3HB treatment decreased 67% or 33% (Fig. 4C). HBME treated cells showed a more normal morphology than those treated with 3HB. The studies showed that HBME is an energy source for the cells and could prevent cell from apoptosis in the absence of glucose.

3.5. The protection on mitochondrial functions

NaNO₃, a specific respiratory chain complex IV inhibitor, decreased the cellular ATP content remarkably (Fig. 5A). On the other hand, when NaNO₃ treatment was accompanied with 3HB or HBME, the ATP levels increased significantly, demonstrating a protective function of HBME and 3HB. HBME alone could also increase the cellular ATP content, it showed a better function than 3HB did (Fig. 5A).

The respiratory chain complex I oxidizes NADH to NAD. Rotenone, a specific complex I inhibitor, reduced the ratio of NAD/NADH, and HBME treatment restored this ratio to the normal level (Fig. 5B).

The cells transfected with lenti-roGFP became green when treated with 3HB or HBME, indicating that they were in a reductive state. In contrast, cells emitted more red light without the treatment (Fig. 5C), revealing that they were in a more oxidative state. HBME was better than 3HB to create a reductive state for the cells (Fig. 5D).

The dissipation of mitochondrial membrane potential (MMP) was closely related to the dysfunction of mitochondria [45]. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), an agent that can cause an uncoupling of the proton gradient and dissipation of MMP [37,46], was used as a positive control. CCCP treatment decreased MMP significantly, resulting in green light emission (Fig. 5C). A 6 h pretreatment using HBME or 3HB stabilized MMP, cells emitted more red light indicating a high MMP (Fig. 5D). This revealed that HBME or 3HB can stabilize MMP and protect mitochondria from damage. HBME showed a more positive effect than 3HB did.

3.6. Down-regulation on transcriptional level of apolipoprotein E and caspase-3

Transcriptional levels of two genes encoding apolipoprotein E (ApoE) that promotes Aβ aggregation, and caspase-3, which plays an important role in cell death, were found changed after HBME treatment. In the brains of AD model mice treated with water, transcriptional levels of the two genes were high. While the treatment with HBME reduced their transcriptional levels significantly (Fig. 6).

3.7. The pharmacokinetics of HBME and 3HB

To compare the permeability of 3HB and HBME for crossing the blood brain barrier (BBB), contents of 3HB in serum and brain at different time points after intragastric administration of 3HB and HBME were investigated. In serum, 3HB treatment led to an increase of 1.6 fold than the basal level, while HBME treatment led to a 7.5 times higher than the basal level (Fig. 7A). As for their concentration in brains, 3HB treatment led to a 1.5 times elevation, while HBME treatment increased the 3HB concentration 3 times than the resting state and this higher level of 3HB concentration sustained for 4 h (Fig. 7B). The pharmacodynamics showed that HBME had a better bioavailability than 3HB in brain.

4. Discussion

The Aβ aggregation [5,7], tau hyperphosphorylation [8,11], and the decline of acetyl cholinergic neuron [12] were widely recognized as closely related to AD development. However, whether these are the primary pathogenesis or just the symptoms of AD have been debated for a long time [43]. Current treatments do not solve the essential issues leading to AD, they just alleviate a few symptoms. Researchers have been focusing on a potential link between AD and the abnormal pathophysiologic events in mitochondria [47]. The changes in tricarboxylic acid cycle, redox state and mitochondria membrane potential for instance, initiate the late-onset and sporadic AD [22].

Ketone diet was reported to have the neuroprotective effects against epilepsy [48], amyotrophic lateral sclerosis (ALS) [49] and other neurodegenerative diseases such as AD and Parkinson disease [19], as well as the mitochondrial disease [50]. 3-Hydroxybutyric acid (3HB), the major ketone body, was found to be the vital functional component for this protective function [51]. However, as a charged compound, the therapeutic potential of 3HB could be enhanced by reducing its polarity through esterification of 3HB into 3-hydroxybutyrate methyl ester (HBME), allowing an easier BBB crossing. This was an expected result of this study.

HBME was reported to improve the learning and memory in normal mice [52]. And our in vivo experiments demonstrated that intragastric administration of HBME for 2.5 months to the Tg2576 double transgenic AD model mice [53] ameliorated their spatial learning, memory impairments represented by the more quickly location to the target quadrant of the pool by most of the mice treated with HBME (Fig. 1). And the reduced time swimming near the periphery of the pool indicated that HBME can relieve the anxiety of mice, which is a common symptom in AD. A dose dependent response was observed in mice treated with HBME, treatments of 40 mg/kg/d HBME produced the best response compared with that of the 20 or 80 mg/kg/d. Due to the lower concentration, the 20 or 80 mg/kg/d group showed a relative unsatisfactory performance. And as for the 80 mg/kg/d, the poor performance was not the result of the toxicity, but consequence of the reduced metabolism of glucose.

Fig. 5. HBME protects mitochondria through rescuing the respiratory chain complexes and stabilizing the membrane potential as well as reducing the oxidative damage. (A) Cellular ATP level. Columns show mean values ± SD. Data were analyzed by Student’s t-test, *p < 0.05 versus control, #p < 0.05, ##p < 0.01 versus NaNO₃ treatment. (B) Cellular reduction power. P2C2 cells were treated with Rot (rotenone) only or with 3HB and HBME for 4 h, respectively. Columns show mean values ± SD. Data were analyzed by Student’s t-test, *p < 0.05, **p < 0.01 versus control, #p < 0.01 versus rotenone treatment. (C) Cellular ROS level. 293T cells were transfected with Lenti-roGFP and incubated with 3HB or HBME for 36 h, followed by confocal laser microscopy observation. (D) The oxidative and reductive ratios. Columns show mean values (more than 500 cells) ± SD. Data were analyzed by Student’s t-test, *p < 0.01 versus control, or between 3HB and HBME. (E) Mitochondrial membrane potential (MMP). The red spots represented the higher MMP, while green spots the dissipated MMP. (F) Statistical results show the ratio of red/green indicating the level of mitochondria membrane potential. Columns show mean values (more than 500 cells) ± SD. Data were analyzed by Student’s t-test, *p < 0.05 versus CCCP alone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Therefore, the evidences showed in our study that HBME reduced ROS, which further perturbs mitochondrial functions [47,57]. HBME protects these complexes from damages, so that it can improve the redox state and increase the cellular ATP production. HBME protects these complexes from damages, so that it can improve the redox state and increase the cellular ATP production. The activity changes of these complexes affect proton gradient across the inner mitochondrial membrane, leading to the alteration of mitochondrial membrane potential (MMP) [63], which is widely considered as an indicator of mitochondrial functionality. The MMP maintains its regular oxidative phosphorylation state for the continuous production of ATP [64] and its dissipation is regarded as the early landmark of cell apoptosis [65]. We found that HBME treatments prevented the decrease of MMP and maintained it at almost the same level as that of the control group (Fig. 5E and F). The stabilization of MMP by HBME can prevent neuronal apoptosis at the very early stage.

However, the ventricle of HBME treated mice was symmetrical with clear edge. Since the hippocampus is just around the ventricle, we had the hypothesis that HBME may have some effects in preventing the hippocampus from atrophy. However, further confirmation of this hypothesis is needed.

The glucose metabolism is impaired in mitochondria of AD patients [25], resulting in an “energy crisis”, so an alternative energy source is needed. 3HB, the major ketone body, and its derivative HBME, can be an alternative energy source in the absence of glucose for neurons (Fig. 4). Both 3HB and HBME increased the cell survival rate and inhibited the cell apoptosis under the condition of glucose deprivation. The presence of HBME led to an increased viable cell number and better cellular morphology compared with results of 3HB or glucose free medium, showing the better effect of HBME. The "in vivo" degradation products of both 3HB and HBME are acetoacetate and then further to acetyl-CoA, this substrate enters and restores the impaired tricarboxylic acid cycle (TCA cycle) normally depending on glucose metabolism [62]. Thus, HBME can ameliorate the function of mitochondria and, providing energy for cellular activities. Meanwhile, HBME can penetrate cell membrane easier than 3HB, resulting in a better cellular uptake as well as a better effect in elevating cell survival rate. The down-regulation of caspase-3 after administered with HBME can explain this apoptosis inhibition effect from another aspect: HBME reduces the transcriptional level of caspase-3 and prevents the apoptosis from initiation.

The mitochondrial respiration chain complexes I and IV, the activities of which were reduced in AD [19], can be protected by HBME. This results in the elevation of ATP content and NAD/NADH level (Fig. 5A and B). The damaged complex I leads to the reduction of NAD/NADH ratio, leading to a change of the intracellular redox state [19]. On the other hand, a reduced complex IV activity decreases intracellular ATP levels, initiating the cell death process. HBME protects these complexes from damages, so that it can improve the redox state and increase the cellular ATP production. The activity changes of these complexes affect proton gradient across the inner mitochondrial membrane, leading to the alteration of mitochondrial membrane potential (MMP) [63], which is widely considered as an indicator of mitochondrial functionality.

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Aβ deposition within the brain induces neuronal apoptosis [54] and associated with spatial memory deficit, which is one of the classic pathological markers of AD [55]. The synaptic mitochondria are more susceptible to the Aβ toxicity [14]. An ability to reduce Aβ deposition is considered as a positive indication of an anti-AD drug [56]. This β-sheet protein, Aβ, is the response of a cell to the elevated b-sheet protein, Aβ, which is one of the classic pathological markers of AD [55]. However, the ventricle of HBME treated mice was symmetrical with clear edge. Since the hippocampus is just around the ventricle, we had the hypothesis that HBME may have some effects in preventing the hippocampus from atrophy. However, further confirmation of this hypothesis is needed.

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A high ROS level affects the permeability of mitochondria inner membrane, leading to a decrease on mitochondrial membrane potential [66]. It is generally accepted that mitochondria is both a generator and targets of ROS [67]. ROS generation is an important mechanism accounting for oxidative damage, leading to cellular injury in many neurodegenerative disorders [21]. Since HBME can reduce the ROS production (Fig. 5C and D), it is considered to be able to repair the oxidative damages.

We have found that ApoE and caspase-3 were the target genes of HBME. The transcriptional levels of these two genes in AD model mice were much higher than that of normal mice, they decreased after given appropriate dose of HBME. The ApoE gene on chromosome 19 encodes apolipoprotein E, which is associated with lipoprotein metabolism, cardiovascular disease and also the major genetic risk factor for AD [68]. The fragments of ApoE were also reported to accumulate in mitochondria, which affect mitochondrial function [69]. There are three ApoE alleles throughout the population and ApoE 4 produces more toxic fragments than other alleles [70], meanwhile it is the widely known genetic risk factor for late-onset and sporadic AD [71]. In ApoE 4, the ability to mitigate oxidative stress minimizes [71] and the variation affects cholesterol transport, which indirectly impact amyloidosis [72]. It is mostly believed that ApoE 4 expression reduced the quantity of mitochondrial respiratory complexes I and IV, lowering mitochondrial respiratory capacity [73].

Caspase-3 enriched at synapses plays an important role in synapse degeneration during AD progression [74]. D’Amelio et al. [75] identified the caspase-3-dependent mechanism that drives synaptic failure and contributes to cognitive dysfunction in AD. Since both ROS and Aβ can lead to the activation of caspase-3 [74], it is not surprised to see that HBME can reduce its transcription (Fig. 6). And this may help to inhibit the synaptic dysfunction and neuronal death. Thus, the down-regulation of these two genes led to a reduced toxicity of ApoE, resulting in improved synaptic function of neurons.

Due to the small molecular weight and amphiphilicity of HBME, it has a better cell membrane permeability. Meanwhile, a potential drug candidate for neuronal disease should have a good bioavailability and be able to cross the blood brain barrier (BBB) easily, so that it can maintain a high and effective concentration in brain for a longer time. Luckily, HBME meets these criteria like many successful drugs such as Cefuroxime axetil, Irinotecan, and Cilazpril etc., which elevate bioavailability via esterification.

5. Conclusion

In this study, HBME had shown profound effects in brain protection, the inhibition on Aβ aggregation and brain atrophy. Meanwhile it recued the cognitive defects in AD model mice. The mechanism of this positive anti-AD effect was found to be mainly mitochondrial mediated, and in this way, HBME is promised as a potential anti-AD drug.

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