Original Research

β-hydroxybutyric acid attenuates oxidative stress and improves markers of mitochondrial function in the HT-22 hippocampal cell line

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Ketone bodies have been the topic of research for their possible therapeutic neurotropic effects in various neurological diseases such as Parkinson's disease, dementia, and seizures. However, continuing research on ketone bodies as a prophylactic agent for decreasing the risk for various neurodegenerative diseases is currently required. In this paper, hippocampal HT-22 cells were treated with β-hydroxybutyric acid at different doses to elucidate the neurotropic effects. In addition, markers of oxidative stress, mitochondrial function, and apoptosis were investigated. As a result, the ketone body (β-hydroxybutyric acid) showed a significant increase in hippocampal neuronal viability at a moderate dose. Results show that β-hydroxybutyric acid exhibited antioxidant effect by decreasing prooxidant oxidative stress markers such as reactive oxygen species, nitrite content, and increasing glutathione content leading to decreased lipid peroxidation. Results show that β-hydroxybutyric acid improved mitochondrial functions by increasing Complex-I and Complex-IV activities and showing that β-hydroxybutyric acid significantly reduces caspase-1 and caspase-3 activities. Finally, using computational pharmacokinetics and molecular modeling software, we validated the pharmacokinetic effects and pharmacodynamic (N-Methyl-D-aspartic acid and acetylcholinesterase) interactions of β-hydroxybutyric acid. The computational studies demonstrate that β-hydroxybutyric acid can interact with N-Methyl-D-aspartic acid receptor and cholinesterase enzyme (the prime pharmacodynamic targets for cognitive impairment) and further validates its oral absorption, distribution into the central nervous system. Therefore, this work highlights the neuroprotective potential of ketone bodies in cognitive-related neurodegenerative diseases.

Keywords

β-hydroxybutyric acid; Apoptosis; Mitochondrial function; Oxidative stress; Neuro-protection; Cognitive enhancer; Computational analysis

1. Introduction

Caloric restriction can delay or prevent several age-related disorders, including neurodegenerative diseases like Alzheimer's disease (AD) [1–5]. The most prominent metabolic alteration due to caloric restriction is the induction of ketosis (a phenomenon characterized by increased levels of ketone bodies in blood circulation), decreased oxidative stress, and apoptosis. Ketones, also called ketone bodies (KBs), are mainly formed by the catabolism of lipids and comprises β-hydroxybutyrate (BHB), acetoacetate, and acetone. The brain utilizes ketone bodies as an alternate fuel during energy hypometabolism, reducing its glucose requirement [6]. Moreover, ketone bodies, compared to glucose, generate more extensive energy due to changes in mitochondrial ATP production [7].

Given that impaired brain energy metabolism promotes CI/dementia, dietary or therapeutic intervention to improve energy regulation in the brain would be a promising approach [8]. As indicated earlier, since energy hypometabolism refers explicitly to glucose, either a ketogenic diet or administration of β-hydroxybutyrate may overcome the reduced glucose uptake and metabolism, thereby improve energy deficits in the brain [6, 9]. Additionally, the ketogenic diet is beneficial in epilepsy, amyotrophic lateral sclerosis, traumatic brain injury, multiple sclerosis Parkinson's disease, and other neurological diseases [10–12].

Molecular docking by utilizing structure-based drug design is the most widely used computational method to validate drug action. Moreover, this drug design technique is suitable to elucidate protein-ligand interactions and pre-

dict the binding poses of hit compounds within the active site of macromolecules such as enzymes and receptors [13, 14]. Molecular mechanics Generalized Born Surface Area (MM/GBSA) is another commonly used technique to calculate the free binding energy and predict binding poses and affinities of ligands [15, 16]. Therefore, follow-up docking studies and MM/GBSA analysis with the crystal structures of N-Methyl-D-aspartic acid (NMDA) receptor and acetylcholinesterase (AChE) can identify the potential critical interactions of β-hydroxybutyric acid within the catalytic binding pocket. Currently, the molecular mechanisms by which ketogenic diet or ketone body administration improve energy regulation and cognitive impairment have not been well characterized [17, 18]. Consequently, we sought to investigate the *in vitro* neuroprotective effects of β hydroxy-butyric acid on hippocampal neurons (HT-22) and the molecular docking on the current therapeutic target for cognitive impairment, NMDA receptor AChE. Furthermore, we also elucidated the effects of β hydroxy-butyric acid on the markers of oxidative stress (prooxidants/antioxidants), mitochondrial function, and apoptosis because these markers play a vital role in hippocampal neuronal proliferation or neurodegeneration.

2. Materials and methods

2.1 Chemicals and reagents

Thiazolyl Blue Tetrazolium Bromide (MTT), Dulbecco's Modification of Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), and Penicillin-Streptomycin solution were purchased from Corning® (Corning, NY). Griess reagent, Sodium nitrite Phosphate buffer saline (PBS), Dimethyl sulfoxide (DMSO), β-hydroxybutyric acid, Nicotinamide adenine dinucleotide (NADH), 2′,7′-dichlorofluorescein diacetate (DCFH), Hydrogen Peroxide (H_2O_2), Phosphoric acid, O-phthalaldehyde (OPT), Glutathione (GSH), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), and EDTA were purchased from Sigma Aldrich (St. Louis, MO). Caspase substrates AC-YVAD-AMC and AC-DEVD-AMC were purchased from Enzo Life Sciences (Farmingdale, NY). Thermo Fisher Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rock-ford, IL) for protein quantification.

2.2 HT-22 mouse hippocampal neuronal cell line

HT-22 mouse hippocampal neurons purchased from ATCC (accession number: CVCL_0321) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L Glucose, 4 mM L-Glutamine, 10% Fetal Bovine Serum, 100 units/mL Penicillin and 50 μg/mL Streptomycin. For the neuronal proliferation studies, an MTT assay was used. HT-22 neurons were seeded into 96 healthy plates at a density of 1×10^5 cells/well. Cells were used within 3–10 passages after they were received.

2.3 Treatment design

To evaluate the effect of β-hydroxybutyric acid on the HT-22 cell proliferation, different concentrations (0 μM–10 mM) were incubated for 24 h. β-hydroxybutyrate (250 and 500 μM) significantly increased the HT-22 neuronal proliferation. Hence, to establish the effect on oxidative stress, mitochondrial function, and apoptosis, the HT-22 neurons were treated with two different doses (250 and 500 μM) of β-hydroxybutyric acid for 12 h. For MTT assay: 24 h cell growth + 24 h incubation (0 μM–10 mM β-hydroxybutyric acid). Biochemical assays: 24 h cell growth + 12 h incubation (250 and 500 μM β-hydroxybutyric acid).

2.4 Effect of β-hydroxybutyric acid on HT-22 neuronal proliferation

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye-based cell proliferation assay was performed to study the effect of β-hydroxybutyric acid on the hippocampal neurons compared to the controls as described by our earlier publications [19]. Additionally, for effect on the morphological changes, the control and β-hydroxybutyric acid-treated cells were imaged using an Axiovert 25 inverted microscope equipped with a Nikon Coolpix 4500 camera. As a result, the control and β-hydroxybutyric acid-treated cells were observed for morphological changes.

2.5 Effect of β-hydroxybutyric acid on reactive oxygen species (ROS)

2′,7′-dichlorofluorescein diacetate (DCF-DA) (DCF, Calbiochem, 287810, 0.5%) dye-based fluorometric assay was performed to study the effect on ROS in the control and β-hydroxybutyric treated HT-22 neurons [20].

2.6 Effect of β-hydroxybutyric acid on nitrite content

A colorimetric assay using Griess reagent (Enzo life sciences, ALX-400-004-L050) was used to quantify the nitrate content in the control and β-hydroxybutyric treated HT-22 neurons [21].

### 2.7 Effect of β-hydroxybutyric acid on glutathione (GSH) content

Fluorometric-based OPT condensation method was used to quantify the glutathione content in the control and β-hydroxybutyric treated HT-22 neurons [22].

2.8 Effect of β-hydroxybutyric acid on catalase activity

A spectrophotometric method using hydrogen peroxide as a substrate was used to assess the catalase activity in the control and β-hydroxybutyric treated HT-22 neurons [23].

2.9 Effect of β-hydroxybutyric acid on Lipid peroxide content

Colorimetric method using thiobarbituric acid (TBA) (Sigma, T5500, 1%) was used to measure the thiobarbituric acid reactive substances (TBARS, lipid peroxide) content in the control and β-hydroxybutyric treated HT-22 neurons [24].

A

B
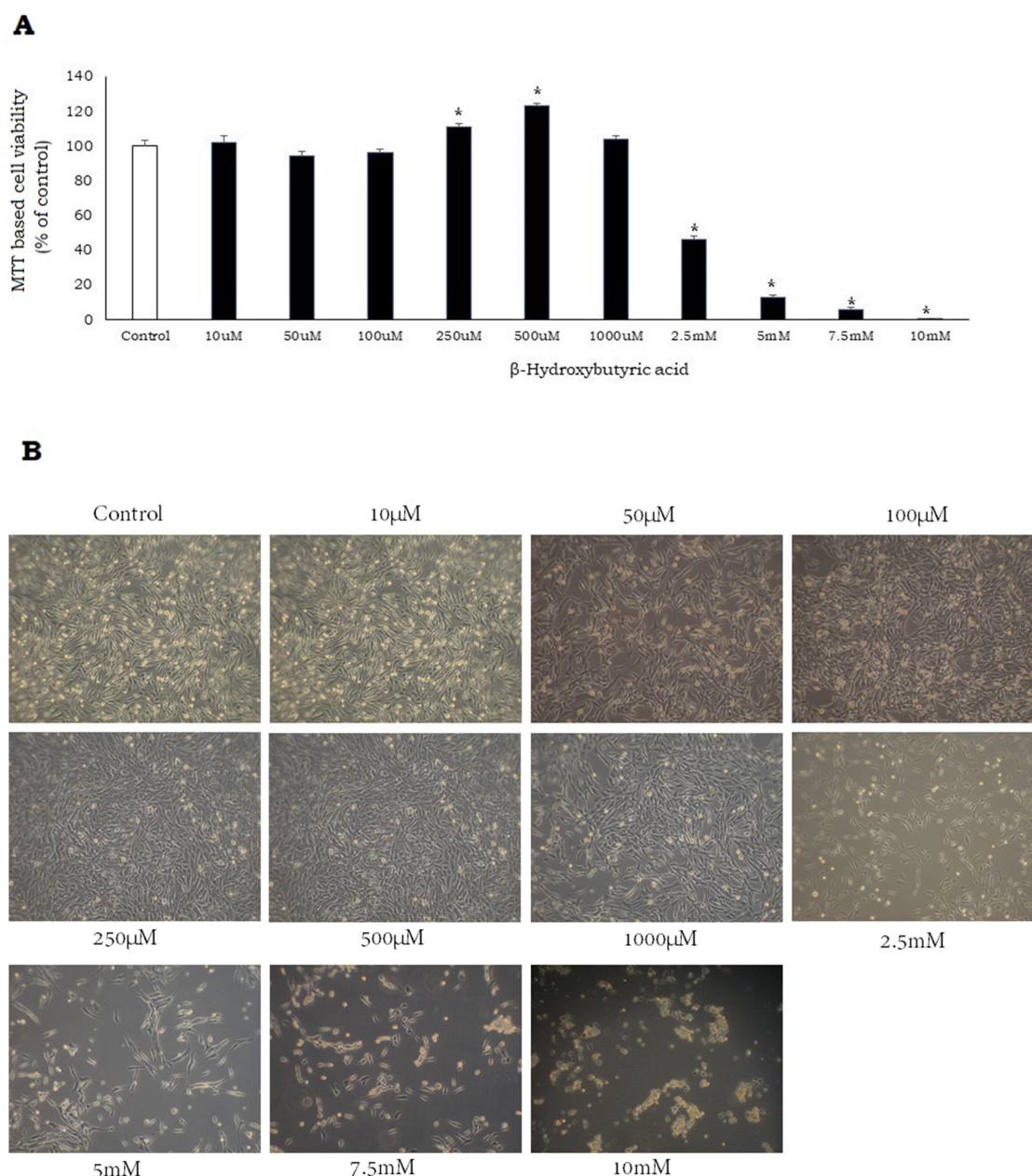
Fig. 1. Effect of β-hydroxybutyric acid on HT-22 cell viability and morphology. (A) Formazan formed due to the reduction of tetrazolium dye-MTT was measured colorimetrically at 540 nm. Low dose (10–100 μM) β-hydroxybutyric acid did not significantly affect the HT-22 cell viability. Moderate doses (250 and 500 μM) of β-hydroxybutyric acid significantly increased the HT-22 cell viability as compared to the controls at 24 h ($p < 0.001$, n = 12). A high dose (2.5–10 mM) of β-hydroxybutyric acid significantly decreased the HT-22 cell viability as compared to the controls at 24 h ($p < 0.0001$, n = 12). (B) Influence of various concentrations of β-hydroxybutyric acid (0–10 mM) on the morphology of HT-22 cells as observed after 24 hours of treatment: the control, 10 μM, 50 μM, 100 μM, 250 μM and 500 μM β-hydroxybutyric acid group displayed spindle or multipolar shaped cells with transparent cell body with significant growth, whereas from 2.5 mM β-hydroxybutyric acid group showed cell shrinkage and a significant reduction in neurons.

2.10 Effect of β-hydroxybutyric acid on caspase-1 and caspase-3 activities

Spectrofluorimetric method using Ac-Tyr-Val-Ala-Asp-7-amino-4-Trifluoromethlcoumarin (AC-YVAD-AMC, Enzo-260-024-M005)and N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Methylcoumarin (AC-DEVD-AMC) (Enzo-260-031-M005) as substrates were used to assess the caspase-1

and caspase-3 activities in the control and β-hydroxybutyric treated HT-22 neurons [21].

2.11 Effect of β-hydroxybutyric acid on Complex-I and Complex-IV activity

Colorimetric methods using NADH H (VWR, 0384, 1mM) and cytochrome-C (Cytochrome C oxidase, Sigma

C7752) as substrates were used to assess the Complex-I and Complex-IV activities in the control and β-hydroxybutyric treated HT-22 neurons [22, 25].

2.12 Protein quantification

Protein in the control and β-hydroxybutyric treated HT-22 neurons were quantified using Thermo Fisher Scientific Pierce 660 nm Protein Assay reagent kit.

2.13 Molecular docking

Computational analysis for understanding the pharmacokinetic and pharmacodynamic properties of β-hydroxybutyric acid (Absorption, distribution, metabolism, elimination (ADME) and Molecular docking profile).

Ligands were sketched in a 2D structure and converted into their 3D structure, and energy minimization was performed using LigPrep with OPLS3e Force field in Schrödinger software. The NMDA crystal structure (PDB ID 5U8C) and the acetylcholine esterase (PDB ID 2X8B) were used for docking and molecular mechanics energies combined with generalized Born and surface area continuum solvation (MM/GBSA) analysis. Protein Preparation Wizard utility tools structurally prepare both proteins in the Schrödinger Release 2019-2, Schrödinger, LLC, New York, NY. All hydrogen atoms were added to the selected proteins to optimize H-bonding interactions, missing atoms were added, and missing side chains and loops were filled. N-and C-terminal residues were specified and charged. Some histidine residues were either flipped or tautomerized.

In contrast, some residues were only flipped to improve H-bonding and avoid H-H clashes. Similarly, waters with less than 3 H-bonds to non-waters were removed. Finally, energy minimization of the protein hydrogens, water, and side chains was performed by utilizing the OPLS3e Force field. Glide docking (Schrodinger software) protocols were applied; in this docking program, the flexibility of the ligands is considered while the protein is considered a rigid structure. The 3D coordinates of the active site were identified using grid generation. Standard precision (SP) was selected, and all other parameters were left at the default settings. The binding free energies (ΔGbind in kcal/mol) were calculated for each ligand from the pose view file from glide docking scores using the MMGBSA (Schrodinger software) and applied in the OPLS3e Force field. The binding free energy of MMGBSA was predicted for each lig-and-protein complex, as follows: ΔGbind = G complex - G-protein - Gligand, where ΔGbind is the binding free energy and G complex, G-protein, and Gligand are the free energies of complex, protein, and ligand, respectively.

2.14 Statistical analysis

All data are expressed as means $\pm$ SEM. Statistical analyses were performed using Kruskal Wallis non-parametric test followed by an appropriate post-hoc test, including Dunn's method ($p < 0.05$ was considered to indicate statistical significance). All statistical analyses were performed using the Prism-V software (La Jolla, CA, USA).

3. Results

3.1 Effect of β-hydroxybutyric acid on cell viability

The effect of β-hydroxybutyric acid on HT-22 cells was determined by MTT assay and by microscopic images. It was found that β-hydroxybutyric acid at a low dose (10–100 μM) had no significant effect on the HT-22 cell viability. Interestingly, β-hydroxybutyric acid (250 and 500 μM) increased the HT-22 cell viability significantly as compared to the controls at 24 h (Fig. 1A, $p < 0.001$, n = 12, as seen by the image, Fig. 1B). However, β-hydroxybutyric acid at a high dose (2.5–10 mM, Fig. 1B) significantly decreased the HT-22 cell viability (Fig. 1A, *$p < 0.0001$, n = 12). To elucidate the effects of β-hydroxybutyric acid on oxidative stress and mitochondrial functions in HT-22 cells, we used 250 and 500 μM of β-hydroxybutyric acid.

3.2 Effect of β-hydroxybutyric acid on oxidative stress markers

Lipid peroxidation occurs do the increase in prooxidants and diminished antioxidants level and/or activity. Hence, we measured different prooxidants (ROS & nitrite content) and antioxidants markers (glutathione and catalase activity) to understand their role in altering lipid peroxide formation. β-hydroxybutyric acid decreased the ROS levels at 250 μM by 29.6% (statistically nonsignificant). At 500 μM by 41.9% (Fig. 2A), non-significantly decreased nitrite content at 250 μM by 11.14% and 500 μM by 21.56% (Fig. 2B), significantly increased the glutathione content at 250 μM by 267.3% and 500 μM by 357.36% (Fig. 2C) and significantly increased catalase activity at 250 μM by 42.64% and 500 μM by 153.24% (Fig. 2D). Due to the increased antioxidant effects and decreased prooxidant contents, β-hydroxybutyric acid significantly decreased the formation of lipid peroxide at 250 μM by 38.42% and at 500 μM by 43.54% as seen by the decrease in the formation of TBARS (Fig. 2E).

### 3.3 Effect of β-hydroxybutyric acid on apoptotic markers

Concerning the effect of β-hydroxybutyric acid on apoptotic action, caspase-1 and caspase-3 activity was elucidated. β-hydroxybutyric acid at 250 μM and 500 μM significantly decreased caspase-1 by 43.78% and 53.65% (Fig. 3A) and with respect to caspase-3 activity by 44.77% and 49.4% (Fig. 3B).

### 3.4 Effect of β-hydroxybutyric acid on mitochondrial functions

To study the effect of β-hydroxybutyric acid on mitochondrial functions, Complex-I and Complex-IV activities were measured. β-hydroxybutyric acid at 250 μM non-significantly increased Complex-I activity by 34.4% and at 500 μM significantly by 68.58% (Fig. 4A). Similarly, concerning Complex-IV, β-hydroxybutyric acid at 250 μM, non-significantly increased Complex-IV activity by 46.97% and at 500 μM significantly by 81.98% (Fig. 4B).

3.5 Molecular docking

To determine the pharmacokinetic parameters concerning the number of metabolites formed, CNS activity, and blood-brain barrier permeability, we performed a computational analysis of β-hydroxybutyric acid using a QikProp
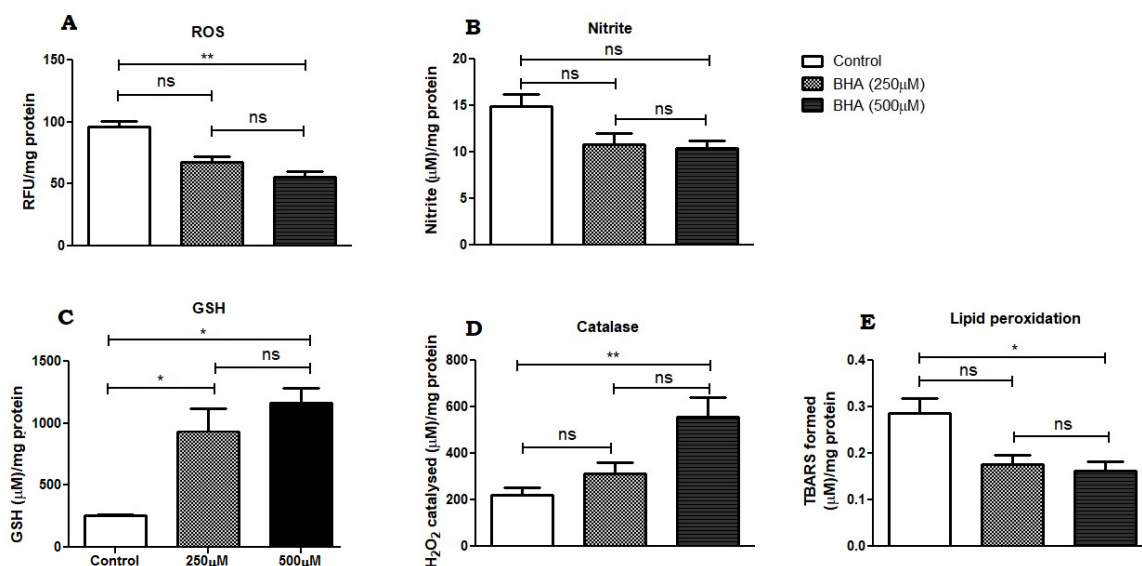
Fig. 2. Influence of β-hydroxybutyric acid on oxidative stress. (A) β-hydroxybutyric acid significantly reduced the generation of ROS content: ROS was quantified spectrofluorimetrically using non-fluorescent DCF dye and measuring the fluorescent DCF at RFU 492/527 nm. Results are expressed as change in RFU/mg protein, Mean + SEM, $**p < 0.01$, $***p < 0.001$, n = 5. (B) β-hydroxybutyric acid non-significantly decreased the nitrite con-tent: Nitrite content was quantified colorimetrically using Griess reagent at 545 nm. Results are expressed as nitrite (μM)/mg protein, Mean + SEM, $*p < 0.05$, n = 5. (C) β-hydroxybutyric acid significantly augmented the formation of GSH content: GSH was measured spectrofluorimetrically using OPT condensation method at 327/423 nm. Results are expressed as GSH (μM)/mg protein, Mean + SEM, $**p < 0.01$, $***p < 0.001$, n = 5. (D) β-hydroxybutyric acid significantly increased the catalase activity: Catalase activity was measured spectrophotometrically using hydrogen peroxide as substrate at 240 nm. Results are expressed as hydrogen peroxide catalyzed (μM)/mg protein, Mean + SEM, $*p < 0.05$, $**p < 0.01$, n = 5. (E) β-hydroxybutyric acid significantly reduced lipid peroxide content: TBARS formed was calculated spectrophotometrically using TBA at 532 nm. Results are expressed as TBARS formed (μM)/mg protein, Mean + SEM, $*p < 0.05$, n = 5.
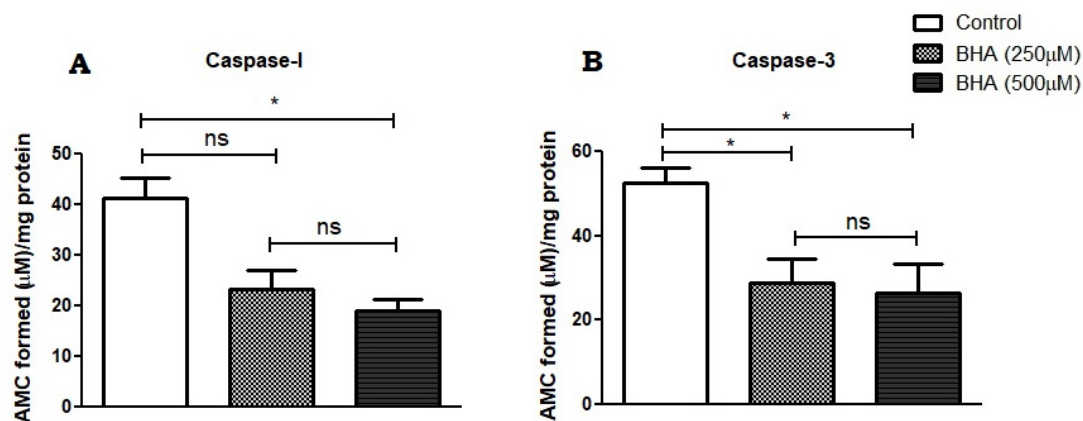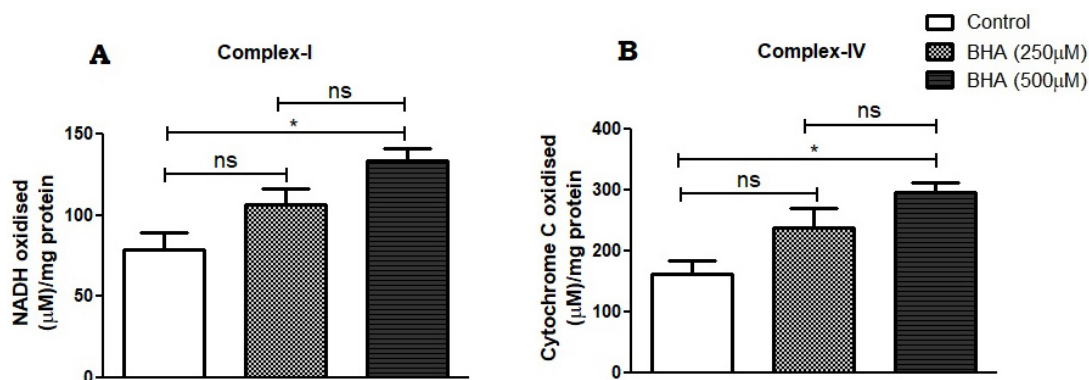

Fig. 3. β-hydroxybutyric acid significantly reduced the caspases' activity. (A) Caspase-1 activity was measured spectrofluorimetrically using AC-YVAD-AMC as a substrate at 326/460 nm. Results are expressed as AMC formed (μM)/mg protein, Mean + SEM, $**p < 0.01$, n = 5. (B) β-hydroxybutyric acid significantly reduced the caspase-3 activity: Caspase-3 activity was measured spectrofluori-metrically using AC-DEVD-AMC as a substrate at 326/460 nm. Results are expressed as AMC formed (μM)/mg protein, Mean $\pm$ SEM, $*p < 0.05$, n = 5.

filter from Schrö-dinger software. Our in-silico molecular docking results showed a good absorption, distribution, metabolism, and elimination profile of β-hydroxybutyric acid (Tables 1,2).

Solvent accessible surface area (SASA) of a molecule is its surface area in contact with the solvent in the biological sys-tem. Lower SASA scores indicate that more molecules inter-act with a biomolecule like a protein or a membrane. There-fore, most of it will likely remain in the unionized form, hence, higher absorption and bioavailability. On the other hand, higher SASA scores indicate that more of the molecule interacts with the solvent, such as the aqueous medium of the

Fig. 4. *β*-hydroxybutyric acid significantly increased mitochondrial function as seen by increased Complex-I and Complex-IV activities. (A) *β*-hydroxybutyric acid significantly increased the Com-plex-I activity: Complex-I activity was measured spectrophotometrically using NADH as a substrate (340 nm). Results are expressed as NADH oxidized (μM)/mg protein, Mean + SEM, *$p < 0.01$, n = 5. (B) *β*-hydroxybutyric acid significantly increased the Complex-IV activity: Complex-IV activity was measured spectrophotometrically using cytochrome-C as a substrate (550 nm). Results are expressed as cytochrome C oxidized (μM)/mg protein, Mean + SEM, **$p < 0.01$, n = 5.

Table 1. SASA, FOSA, FISA, PISA #metab, CNS, and QPlog BB values for *β*-hydroxybutyric acid.

Compound	SASA(Å)	FOSA(Å)	FISA (Å)	PISA (Å)	#metab	CNS	QPlog BB
9,12,15-octadecatrienoic_acid	702.9	549.8	108.06	45.09	5	−2	−1.4
9,12-octadecadienoic_acid	656.08	531.3	98.1	26.6	4	−2	−1.2
β-Hydroxybutyric acid	285.7	141.6	144.1	0	2	−2	−0.7

Note: The acceptable ranges are as follows: Area is (300–1000), FOSA: Hydrophobic components of the SASA (0.0–750.0), FISA: Hydrophilic components of the SASA (7.0–330.0), PISA: π (carbon and attached hydrogen) components of the SASA (0.0–450.0), #metab: Number of likely metabolic reactions (1–8) CNS: −2 (inactive) to +2 (active), QPlog BB: (−3.0 to −1.2) polar compounds have large negative values.

stomach (stomach acid), and most of it will likely stay ionized; thus, lower absorption and bioavailability.

Table 1, *β*-hydroxybutyric acid shows a lower SASA value consistent with what has been noted elsewhere for *β*-hydroxybutyric acid's favorable bioavailability profile. Other parameters like hydro-phobic components of the SASA (FOSA), hydrophilic components of the SASA (FISA), and π (carbon and attached hydrogen) components of the SASA (PISA) values for *β*-hydroxybutyric acid are all within the acceptable range. In terms of metabolic reaction predictions (#metab), *β*-hydroxybutyric acid has a lower number of metabolic reactions. Lesser negative QPlog BB values indicating accessibility into the blood-brain barrier and better CNS activity. Other parameters, including % Human oral absorption and Lipinski's rule of five, are within the acceptable range of drug bioavailability (Table 2).

We performed computational molecular docking to determine if *β*-hydroxybutyric acid has any interactions with receptors involved in neurotransmission. Docking score and free binding energy values of *β*-hydroxybutyric acid exhibit a possible interaction with NMDA receptor and with acetyl-cholinesterase to exhibit a potent pharmacological effect to enhance cognitive functions (Table 3 and Table 4). The results are comparable to some known agonists and antagonists of NMDA receptors and AChE enzyme activators and in-

hibitors. However, since this is an in-silico prediction model, further mechanistic studies need to be performed to validate our preliminary findings.

4. Discussion

We established the effects of *β*-hydroxybutyric acid, a ketone, on HT-22 hippocampal neurons to mitigate oxidative stress and improve mitochondrial functions. Accordingly, *β*-hydroxybutyric acid, at a moderate dose of 250 and 500 μM, increased the HT-22 cell viability, which could be attributed to the decreased oxidative stress due to decreased prooxidants and increased antioxidants content/activity (dose-dependently decreased ROS, decreased nitrite content, increased glutathione content, and increased catalase activity) leading to reduced neuronal damage as seen by decreased neuronal lipid peroxidation [17, 18]. Furthermore, the apoptotic pathway reduced caspase-1 and caspase-3 activity by *β*-hydroxybutyric acid, resulting in improved neuronal viability. Additionally, enhanced mitochondrial functions attributed to Complex-I and Complex-IV activities demonstrated by *β*-hydroxybutyric acid can cause increased HT-22 cell viability. The valid values such as FOSA-Hydrophobic components of the SASA, FISA-Hydrophilic components of the SASA, PISA-π carbon and attached hydrogen components of the SASA, number of metabolites, number of likely

Table 2. Basic pharmacokinetic data of β-hydroxybutyric acid.

Compound	Mol wt.	Donor HB	Accept HB	cLogP	% Human oral absorption	Rule of 5
9,12,15-octadecatrienoic_acid	278.43	1	2	5.6	89.4	1
9,12-octadecadienoic acid	280.45	1	2	5.5	90.4	1
β-Hydroxybutyric acid	104.10	1	2.7	0.8	68	0

Note: The permissible ranges are as follows: Mol wt.: (130–725), Donor HB: (0.0–6.0), Accept HB: (2.0–20.0), cLogP: (–2.0 to 6.5), % Human oral absorption: >80% high, <25% low, Rule of five (maximum 4).

Table 3. Docking score and free binding energy (MM/GBSA) of β-hydroxybutyric acid, Glutamate, Quinolinic acid, and Riluzole with NMDA.

Compound	NMDA	
	Docking Score	MM/GBSA Binding energy (kacl/mol)
β-hydroxybutyric acid	−5.5	−26.2
Glutamate [a]	−6.4	−30.6
Quinolinic acid [b]	−6.1	−26.6
Riluzole [c]	−4.4	−19.2

[a], Natural ligand; [b], Agonist; [c], Antagonist.

Table 4. Docking score and free binding energy (MM/GBSA) of β-hydroxybutyric acid, Acetylcholine, Tyrosine, and Rivastigmine with Acetylcholinesterase (AChE).

Compound	Acetylcholinesterase (AChE)	
	Docking Score	MM/GBSA Binding energy (kacl/mol)
β-hydroxybutyric acid	−3.3	−3.4
Acetylcholine [a]	−4.4	−24.3
Tyrosine [b]	−6.9	−40.7
Rivastigmine [c]	−4.2	−43.8

[a], Natural ligand; [b], Activator; [c], Inhibitor.

metabolic reactions, CNS action, Log P values, and percentage of human oral absorption favored the bioavailability of β-hydroxybutyric acid (Tables 1,2) for prophylactic and/or therapeutic use.

Several studies utilizing β-hydroxybutyric acid to improve human cognition have been conducted with varying results [8]. Jensen *et al.* [26] investigated the effect of β-hydroxybutyric acid on cognition in Type 2 diabetes mellitus patients. It improved working memory performance without any change in global cognition. Similarly, Alzheimer's patients showed improvements in cognition in response to acute elevations of β-hydroxybutyric acid [27]. The improvement in cognitive functions is attributed to ketones acting as an alternative fuel for neurons in MCI or AD patients. In patients with MCI or AD, defects in brain glucose utilization occur, attributed to amyloid deposition, inflammation, or altered lipid homeostasis [28].

The neuroprotective effects of β-hydroxybutyric acid have drawn additional interest due to the current hypothesis of energy deficiency in various neurodegenerative disorders. Most of the neurons with high energy demand do not efficiently produce high-energy phosphates from fatty acids. Still, β-hydroxybutyric acid can undergo oxidation during deficiency of glucose/carbohydrates leading to increased energy regulation [8, 29, 30]. Thus, the neuroprotective effects of β-hydroxybutyric acid are considered notably essential for future prophylactic and therapeutic treatment [31, 32].

Mitochondria are critical for several neuronal functions such as synaptic plasticity, neurotransmission, and energy regulation of neurons [33]. We found improvement in mitochondrial complex I and IV with β-hydroxybutyric acid treatment. Similarly, β-hydroxybutyrate has been shown to improve mitochondrial biogenesis and bioenergetics in cultured rat hippocampal neurons [34]. In addition, previous studies have shown to inhibit mitochondrial ROS production following glutamate excitotoxicity predominantly with acetoacetate [35, 36] and alleviate oxidative stress by decreasing ROS and malondialdehyde in an animal model of epilepsy [37].

Oxidative stress has been predominantly associated with all neurodegenerative disorders. Oxidative stress is a condition in which there is an increase in the generation of intracellular ROS, hydroxyl radicals, superoxide anion, and hydrogen peroxide responsible for damage to lipids, mainly leading to lipid peroxides associated with neuronal membrane damage [38, 39]. To counteract the negative impact of oxidative stress, there is a valid molecular defense mechanism consisting of enzymes, proteins and low molecular weight molecules. These antioxidants molecular defense mechanisms can catalytically remove the prooxidants. For instance, superoxide dismutase dismutases superoxide anions into hydrogen peroxide, which in turn is broken down by catalase. Glutathione (tripeptide), by scavenging the ROS, neutralizes the neurotoxic effect and reduces the ROS's impact to participate in any form of chemical reaction to complete or partial destruction of DNA, proteins and lipids leading to neurodegeneration. Interestingly, β-hydroxybutyric acid has been shown to exhibit neuronal solid antioxidant activity [40, 41].

Increased oxidative stress and decreased mitochondrial functions can lead to heightened expression and/or activity of several pro-apoptotic markers. Caspases are mainly associated with DNA damage resulting in decreased neuronal proliferation. Caspase-1 and caspase-3 are cysteine proteases that have been shown to increase the apoptotic cascade in mostly all neurodegenerative disorders. We showed a reduction in both caspase-1 and caspase-3 activity with β-hydroxybutyric acid. β-Hydroxybutyric acid has been shown to offer hippocampal neuron proliferative effects through

its anti-apoptotic effects [37]. Caspase inhibitors have been shown to reduce the initiation and/or progression of neurodegeneration [42]. β-hydroxybutyric acid, like the other existing neuroprotectants, also exhibits increased mitochondrial functions and decreased apoptosis, which might lead to cognitive enhancement.

Despite the beneficial effects of β-hydroxybutyric acid on cognition, several studies have reported the adverse effects of ketones [43]. For instance, long-term adverse effects include hepatic steatosis, hypoproteinemia, renal stones, vitamin and mineral deficiencies [44]. Furthermore, hypoglycemia is a common manifestation, especially in diabetic patients on treatment. Additionally, ketones are contraindicated in patients with pancreatitis, liver failure, lipid and fat metabolism disorders, and porphyria, limiting their utility.

Only limited studies have investigated the dose of β-hydroxybutyric acid required to confer neuroprotection. The uptake of β-hydroxybutyric acid by brain cells occurs either by diffusion or a carrier-mediated process. Furthermore, there is a high likelihood of a saturable dependent mechanism for carrier-mediated transport that could alter the amount of β-hydroxybutyric acid reaching the brain. For instance, the affinity of the carrier transporters is lowest for β-hydroxybutyric acid [45], which indicates that sufficient (50 uM) concentrations are required to attain sufficient concentrations in the brain [46]. One of the limitations is we investigated the effects of β-hydroxybutyric acid under normal conditions *in vitro* cell culture and found a concentration of 250–500 μM to offer beneficial effects. However, this concentration might not be sufficient to reach adequate levels in the brain due to the saturable dependent mechanism of transporters. Further studies in animal models to investigate the pharmacokinetics of blood-brain barrier permeability and concentrations attained in the brain tissue need to be performed.

Although the specific neuron proliferative mechanisms of β-hydroxybutyric acid in the treatment of neurodegenerative diseases are still uncertain, it is predictable that all neurodegenerative disorders can affect human health through oxidative damage mitochondrial dysfunction, and apoptotic pathways. Furthermore, neurodegenerative diseases often involve multiple mechanisms; hence β-hydroxybutyric acid may also exhibit multipotent neuron proliferative molecular processes. In summary, most of the previous reports associated protective effects of β-hydroxybutyric acid have been validated. However, our work specifically targeted the hippocampal neuroprotective effects of β-hydroxybutyric acid. Hence, either β-hydroxybutyric acid can play a prophylactic, supportive, or facilitate the therapeutic effects in various neurodegenerative diseases to improve the symptoms and quality of life in patients. Thus, β-hydroxybutyric acid has excellent clinical application potential, which further requires immediate exploration of the neuroprotective effects of invalid cognitive deficient animal models and humans.

5. Conclusions

Although ketone bodies have been previously studied to evaluate its neuroprotective effects, our work has validated the role of the β-hydroxybutyric acid at a dose of 250 and 500 μM concentration in neuroprotection. The hippocampal neuron proliferative role can reduce oxidative stress, maintain energy metabolism, improve mitochondrial function, and modulate apoptosis. Although the specific mechanisms of β-hydroxybutyric acid in treating neurological diseases are still uncertain, it is predictable that most neurogenerative diseases could affect human health through oxidative damage, energy metabolism disorders, mitochondrial dysfunction, and impaired apoptosis. Therefore, β-hydroxybutyric acid can be used as an adjunct facilitating the disease's therapy, improving patients' symptoms and quality of life. Future studies are necessary further to specify the roles of various components in ketone bodies, identifying therapeutic targets and related molecular signaling pathways to improve the approach and effectiveness of ketone bodies.

Author contributions

MM, SR, MA, MG, JS, MAI, FS, TT, MD performed experiments, statistical analysis, computational docking. In addition, VS, MR, TM, MD were involved in planning, funding, reviewing, editing, and writing the manuscript.

Ethics approval and consent to participate

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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