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Research Article

Exploring the Therapeutic Potential of β-Hydroxybutyrate (BHB) in Clear Cell Renal Cell Carcinoma: A Journey into Fat Browning, Autophagy, and Tumor Slimming

Roya Rezaei,[1] **Asra Abdali Larki**,[2] **Rosa Hosseinzadegan**,[3] **Zahra Dashti**,[4] **Saba Tarkashvand**,[5,6] **Reihaneh Akhoondi**,[6,7] **Morvarid Siri**,[8] **Mesbah Shams**,[9] **Alireza Monsef**,[10] **and Sanaz Dastghaib**[9,11]

[1]*Department of Microbiology, College of Science, Agriculture and Modern Technology, Shiraz Branch, Islamic Azad University, Shiraz, Iran*
[2]*Department of Biology, Faculty of Basic Science and Medicine, Islamic Azad University of Qom, Qom, Iran*
[3]*Department of Biology, Payame Noor University, Tehran, Iran*
[4]*Department of Genetics, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*
[5]*Sina Surgery Research Center, Tehran University of Medical Sciences, Tehran, Iran*
[6]*Universal Scientific Education and Research Network (USERN), Tehran University of Medical Science, Tehran, Iran*
[7]*Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran*
[8]*Autophagy Research Center, Department of Clinical Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[9]*Endocrinology and Metabolism Research Center, Shiraz University of Medical Science, Shiraz, Iran*
[10]*Fasa University of Medical Sciences, Fasa, Iran*
[11]*Autophagy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*

Correspondence should be addressed to Alireza Monsef; monsefalireza@yahoo.com and Sanaz Dastghaib; suny.respina@gmail.com

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This study delves into the therapeutic potential of β-hydroxybutyrate (BHB) in clear cell renal cell carcinoma (ccRCC), a cancer known for its complex pathogenesis and resistance to conventional treatments. The research specifically explores the impact of BHB on cell viability, autophagy induction, and lipid metabolism in Caki-1 cells. The findings reveal that BHB significantly reduces ccRCC cell viability, particularly under low-glucose conditions. The combination of glucose and BHB treatment activates autophagy pathways, as evidenced by increased expression of autophagy-related genes (Beclin-1, LC3IIβ, and ATG5) and decreased expression of P62 after 48 and 72 hours. Moreover, the combined therapy enhances lipid metabolism, as indicated by elevated expression of PGC-1α and UCP-1, along with upregulation of ACSL3 and CPT1A, which are associated with lipid droplet formation and facilitate lipid breakdown within cells. The study concludes that BHB holds promise as a therapeutic agent for ccRCC, targeting abnormal lipid metabolism, inducing autophagy-mediated cell death, and promoting fat browning. The results suggest potential avenues for precision-guided nutritional therapies in ccRCC treatment, highlighting the innovative role of BHB in addressing the challenges posed by this cancer.

1. Background

Renal cell carcinoma (RCC) represents over 85% of all renal malignancies and poses a significant challenge in the realm of urological cancers [1, 2]. The reprogramming of tumor metabolism, particularly through fatty acid oxidation (FAO), is crucial in promoting tumor growth, aggressiveness, metastasis, and resistance to therapies. Research on

clear cell renal cell carcinoma (ccRCC), which originates from renal tubular epithelial cells, has unveiled specific lipid alterations. These include an increase in acylcarnitine (CAR), cholesteryl ester (CE), and diacylglycerol (DG), coupled with a reduction in bile acid (BA) and lysophosphatidylserine (LPS) [3, 4]. The precise mechanisms underlying abnormal lipid metabolism in clear cell renal cell carcinoma (ccRCC) remain incompletely understood, presenting an attractive target for treatment in tumors characterized by such lipid abnormalities. Ketone bodies, particularly β-hydroxybutyrate (BHB), play a vital role as an alternative fuel source during periods of starvation. These molecules are integral to crucial metabolic pathways such as β-oxidation, gluconeogenesis, tricarboxylic acid cycle (TCA), de novo lipogenesis, and mammal sterol biosynthesis. In cancerous cells, substituting glucose with ketone bodies (BHB) as the primary energy source can attenuate the Warburg effect and inhibit tumor growth. While studies have explored the antitumor and antiangiogenic effects of ketogenic diets and BHB on various cancers, limited data are available regarding their impact on ccRCC [5–8].

In cancer cells, the beta-oxidation pathway is a crucial source of ATP essential for tumor growth. A potential therapeutic strategy involves targeting lipid breakdown without generating ATP. Brown and beige adipocytes, during cold-induced thermogenesis, convert lipid droplets (LDs) into heat rather than ATP [9]. UCP-1 in brown adipocytes disrupts the usual ATP production and proton transfer in mitochondria, redirecting energy to produce heat instead of ATP—an essential aspect of nonshivering thermogenesis [10, 11]. A novel approach to treating tumors with abnormal lipid metabolism involves inducing lipid browning. In clear cell renal cell carcinoma (ccRCC), oxygen deficiency (hypoxia) during rapid cancer cell growth reduces mitochondrial respiration and beta-oxidation by suppressing PPAR-γ coactivator-1α (*PGC-1α*) expression. *PGC-1α*, which normally enhances the transcription of mitochondrial uncoupling protein-1 (UCP-1), is reduced due to hypoxia, hindering fat browning [12, 13]. The reduced breakdown of fatty acids in the context of ccRCC can result in the accumulation of lipid droplets. Activating *PGC-1α* and UCP-1 can induce autophagy and lipid browning, facilitating the elimination of accumulated lipids in tumor cells without generating ATP. Autophagy, a cellular process that degrades unnecessary or dysfunctional components, including proteins and organelles, reduces the buildup of fatty deposits [14–18]. Its impact on cancer is intricate and context-dependent, as autophagy can both inhibit and promote tumor progression depending on the stage and environmental factors [17, 19, 20]. In ccRCC, enhancing autophagy activity may be beneficial, while defective autophagy could disrupt the balance of lipid storage and initiate ccRCC [21–23]. Furthermore, the synthesis of fatty acyl-CoA esters, which is essential for cellular fatty acid metabolism, is facilitated by the enzyme Acyl-CoA synthetase long-chain family member 3 (ACSL3). Increased expression of ACSL3 has been linked to the development and progression of several malignancies, such as pancreatic cancer, fibrosarcoma, leiomyosarcoma, rhabdomyosarcoma, breast cancer, and melanoma [24]. Contrary to what is seen in other cancer types, ACSL3 expression is lower in ccRCC than in normal kidney tissue. This implies that ACSL3 may have a tumor-suppressive function and that it can be used as a predictive biomarker for colorectal cancer [25]. A protective role in the onset and course of the illness is suggested by the correlation between higher ACSL3 expression and better survival rates in individuals with colorectal cancer [26]. Carnitine palmitoyltransferase 1 (CPT1), which is located on the outer mitochondrial membrane and is essential for the movement of fatty acids into mitochondria for oxidation, is required for the transport of acyl-CoA into mitochondria. When compared to normal kidney tissues, CPT1A expression and activity are markedly reduced in human malignancies. A higher risk of cancer and worse patient outcomes are linked to this decrease in CPT1A expression. Reduced CPT1A levels promote the development of lipid droplets, which aid in the progression of kidney cancer (ccRCC) [27]. Research has shown that although elevated CPT1A expression might hamper tumor growth, inhibiting CPT1A is essential for the creation of tumors [3]. This study aims to investigate whether BHB administration during glucose deprivation may initiate the autophagy process, which can result in enhanced fat browning and lipid decrease, which, in turn, can cause cell death in ccRCC.

2. Materials and Methods

2.1. Cell Culture. The National Cell Bank of Iran, affiliated with the Pasteur Institute in Tehran, provided the Caki-1 cell lines. The cells were grown in a growth medium made up of 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) from Gibco Life Technologies, UK. The growth medium also included 100 U/mL of penicillin and streptomycin as antibiotics. Moreover, 37°C was the incubation temperature, and the atmosphere had 5% CO_2 in it.

2.2. Cell Viability Assay. The MTT colorimetric test was used to evaluate the viability of Caki-1 cells. Concisely, 5,000 cells were placed in each well of a 96-well plate and subjected to β-hydroxybutyrate (obtained from Sigma-Aldrich) at doses ranging from 1 to 200 mM for 48 and 72 hours. After the treatment, 200 μL of MTT solution (5 mg/mL, Thermo Fisher Scientific) was introduced into each well, and the plates were placed in an incubator at a temperature of 37°C for a duration of 4 hours. Afterwards, the violet MTT formazan crystals were dissolved by introducing 100 μL of dimethyl sulfoxide (DMSO) solution into each well. The optical density of each well was measured at a wavelength of 570 nm.

Concentrations of 10 or 25 mM β-hydroxybutyrate were chosen for 48 and 72 hours based on the MTT findings. The cells were subsequently subjected to different glucose concentrations ranging from 0 to 4.5 g/L, with or without β-hydroxybutyrate, for a duration of either 48 or 72 hours.

Subsequently, a previously published MTT experiment was performed. The data are shown as the percentage of vitality or proliferation compared to the control cells grown with 4.5 g/L glucose.

2.3. RNA Isolation and cDNA Synthesis. The RNX-Plus RNA Extraction Kit from CinnaGen, Iran, was used to extract cellular RNA from Caki-1 cells in accordance with the manufacturer's instructions. The 260/280 ratios were evaluated with a spectrophotometer to evaluate the purity of the RNA. Using the cDNA Synthesis Kit from CinnaGen, Iran, 1 μg of RNA was converted into cDNA, yielding a final amount of 20 μL, for the synthesis of first-strand cDNA. The cDNA from each sample was then used in equal proportions as the substrate for qRT-PCR amplification.

2.4. Real-Time PCR (qRT-PCR). Gene expression levels of *Beclin-1*, *LC3IIβ*, *ATG5, P62, PGC-1α, UCP-1, CPT1A, and ACSL3* were assessed using quantitative real-time PCR. The PCRs were conducted on the ABI 7500 Real-Time PCR System, and the amplifications were carried out with the SYBR Green PCR Master Mix from Ampliqon, Denmark. The thermal cycling protocol involved 40 cycles, starting with a 10-minute holding period at 95°C, followed by 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C. Each data point was examined in duplicate.

The PCR mixture consisted of 1 μl of cDNA, 1 μl of forward and reverse primers (5 pM each), and 12.5 μl of SYBR Green DNA PCR Master Mix for a total volume of 20 μl. The delta-delta Ct ($2^{-\Delta\Delta CT}$) technique was used to calculate fold changes in gene expression, normalized to GAPDH as an internal reference. The primer sequences are presented in Table 1.

2.5. Statistical Analysis. GraphPad Prism 8 (GraphPad Prism, RRID: SCR_002798) was used for all data analysis. Tukey's post hoc test was used after a one-way ANOVA. The data are displayed as mean ± standard deviation (SD), with a P-value <0.05 indicating statistical significance.

3. Results

3.1. β-Hydroxybutyrate Suppressed Cell Proliferation in Low-Glucose Condition in Long Term. The MTT assay was utilized to assess the impact of BHB on the cell viability of Caki-1 cells. Following treatment with various concentrations of BHB (ranging from 1 to 200 mM), the cell viability was examined. According to Figure 1(a), a significant reduction in cell viability was observed when cells were exposed to concentrations exceeding 50 mM, at both 48 hours and 72 hours ($P < 0.0001$ for concentrations ≥100 mM at 48 hours and $P < 0.0001$ for concentrations ≥50 mM at 72 hours). Notably, there was no substantial effect on cell viability when BHB concentrations were less than 25 mM, after both 48 hours and 72 hours. Based on the MTT assay results and considering the highest concentration typically found in ketogenic diets, 10 mM and 25 mM BHB were selected for subsequent experiments.

As depicted in Figures 1(b) and 1(c), there was a significant decrease in the proliferation of Caki-1 cells under low-glucose conditions when compared to proliferation in a 4.5 g/L glucose environment, at both 48 and 72 hours ($P < 0.001$ for 1.5 g/L glucose and $P < 0.0001$ for concentrations ≤0.75 g/L glucose). In glucose-deprived conditions, the viability of Caki-1 cells was found to decrease when supplemented with either 10 mM or 25 mM of BHB, as compared to Caki-1 cells supplemented with BHB in a normal glucose environment (4.5 g/L) at both 48 and 72 hours (as shown in Figures 1(b) and 1(c)).

3.2. Supplementing with BHB Triggered Autophagy in Caki-1 Cells. Real-time PCR was used to evaluate the mRNA expression of Beclin-1 (A and B), LC3IIβ (C and D), ATG5 (E and F), and P62 (G and H). After treating Caki-1 cells with 1.5 g/l glucose or BHB (10 and 25 mM) alone, we found a considerable upregulation in Beclin-1 as compared to control ($P < 0.001$) after 48 and 72 hours, as shown in Figures 2(a) and 2(b). Furthermore, during 48 and 72 hours, Beclin-1 expression levels were higher in 1.5 g/l glucose +10 or 25 mM BHB than in 1.5 g/l glucose. Furthermore, after 48 and 72 hours, LC3IIβ was significantly increased in comparison with control when cells were treated with 1.5 g/l glucose or BHB (10 and 25 mM) alone ($P < 0.001$). Additionally, after 48 and 72 hours, we saw a considerable upregulation in the amount of LC3IIβ mRNA expression in 1.5 g/l glucose +10 or 25 mM BHB (Figures 2(c) and 2(d)).

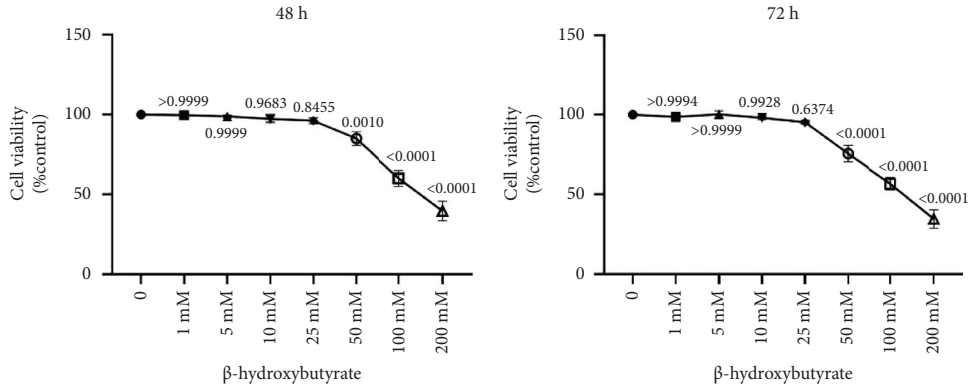
Figures 2(e) and 2(f) show that after 48 and 72 hours of incubation with 1.5 g/l glucose or BHB (10 and 25 mM) alone, ATG5 significantly increased ($P < 0.001$) in comparison with control cells. In comparison with low-glucose conditions (1.5 g/l glucose), treatment with 1.5 g/l glucose + 10 or 25 mM BHB significantly increased ATG5 after 48 and 72 hours.

As shown in Figures 2(g) and 2(h), Caki-1 cells were incubated with 1.5 g/l glucose or BHB (10 and 25 mM) alone, and after 48 and 72 hours, there was a substantial downregulation in the mRNA expression of P62 ($P < 0.0001$). Furthermore, at both time points, the mRNA level of P62 was lower in 1.5 g/l glucose +10 or 25 mM BHB than in 1.5 g/l glucose alone. It is noteworthy that following a 72-hour exposure to a mixture of 25 mM BHB and 1.5 g/L glucose, the mRNA expression of Beclin-1, LC3IIβ, ATG5, and P62 showed a reduction ($P < 0.0001$) in comparison with the mixture of 10 mM BHB and 1.5 g/L glucose.

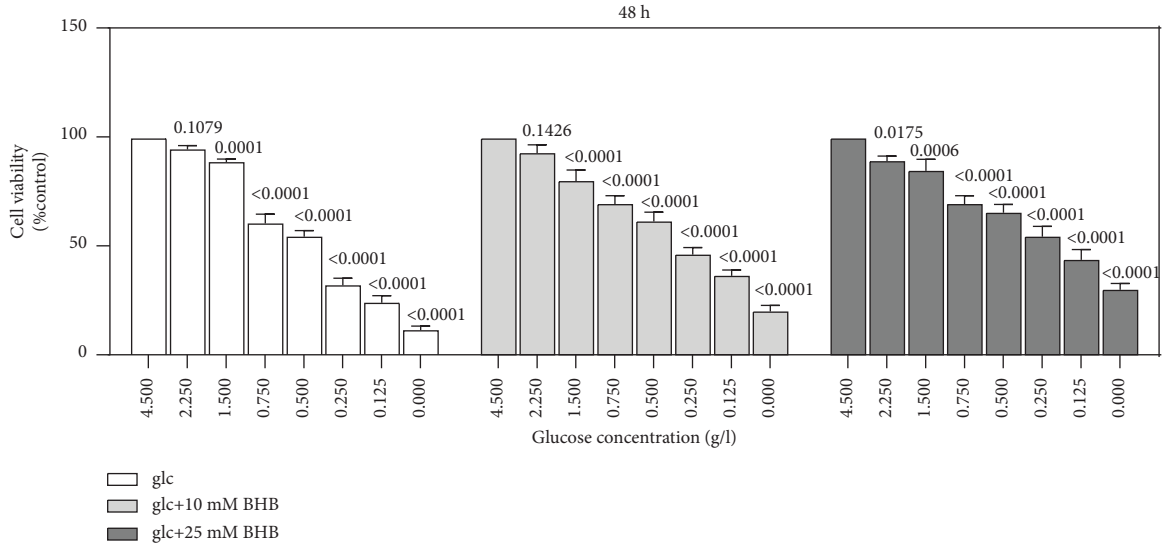
3.3. β-Hydroxybutyrate Provoked Tumor Suppressor Genes CPT1A and ACSL3 in Caki-1 Cells. Figures 3(a) and 3(c) show that following 48 hours of treatment of Caki-1 cells with BHB (10 and 25 mM) or 1.5 g/l glucose alone, there was a considerable upregulation in the expression levels of CPT1A and ACSL3. Furthermore, at 48 hours, the mRNA levels of CPT1A and ACSL3 were higher in 1.5 g/l glucose +10 or 25 mM BHB than in 1.5 g/l glucose alone. Figures 3(b)

Table 1: The primer sequences.

Primer sequences	Forward	Reverse
UCP-1	AGTTCCTCACCGCAGGGAAAGA	GTAGCGAGGTTTGATTCCGTGG
PGC-1α	CCAAAGGATGCGCTCTCGTTCA	CGGTGTCTGTAGTGGCTTGACT
Beclin-1	CTGGACACTCAGCTCAACGTCA	CTCTAGTGCCAGCTCCTTTAGC
LC3IIβ	GAGAAGCAGCTTCCTGTTCTGG	GTGTCCGTTCACCAACAGGAAG
ATG5	GCAGATGGACAGTTGCACACAC	GAGGTGTTTCCAACATTGGCTCA
P62	AATCAGCTTCTGGTCCATCG	TTCTTTTCCCTCCGTGCTC
CPT1A	GATCCTGGACAATACCTCGGAG	CTCCACAGCATCAAGAGACTGC
ACSL3	CATCGCCATCTTCTGTGAG	CGTGGCTTTCCATCAACAG

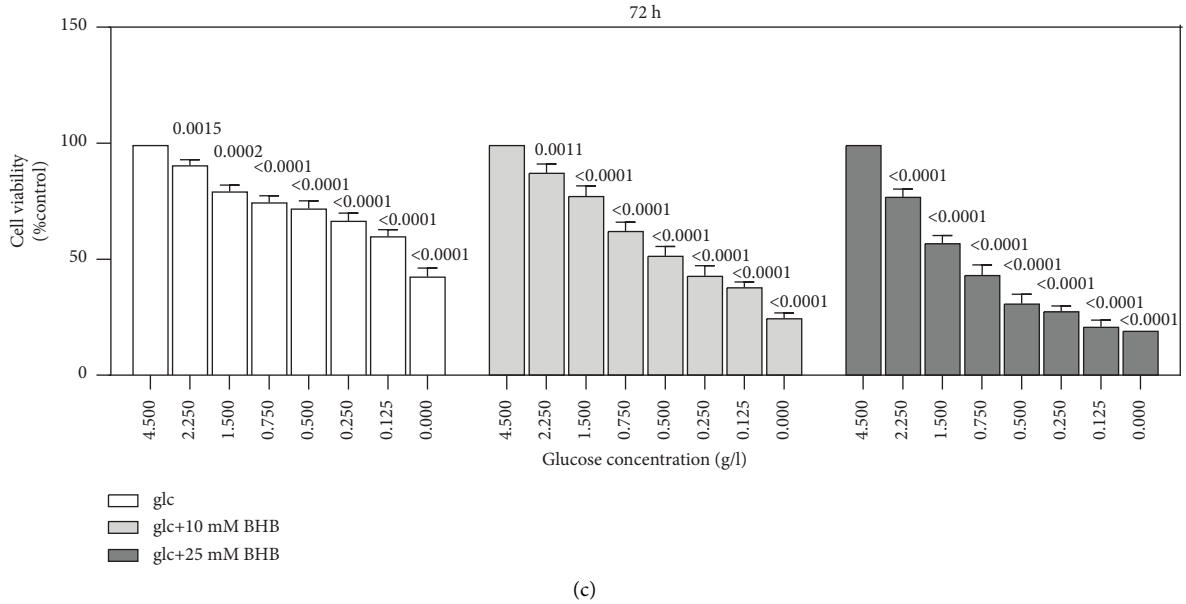


(a)



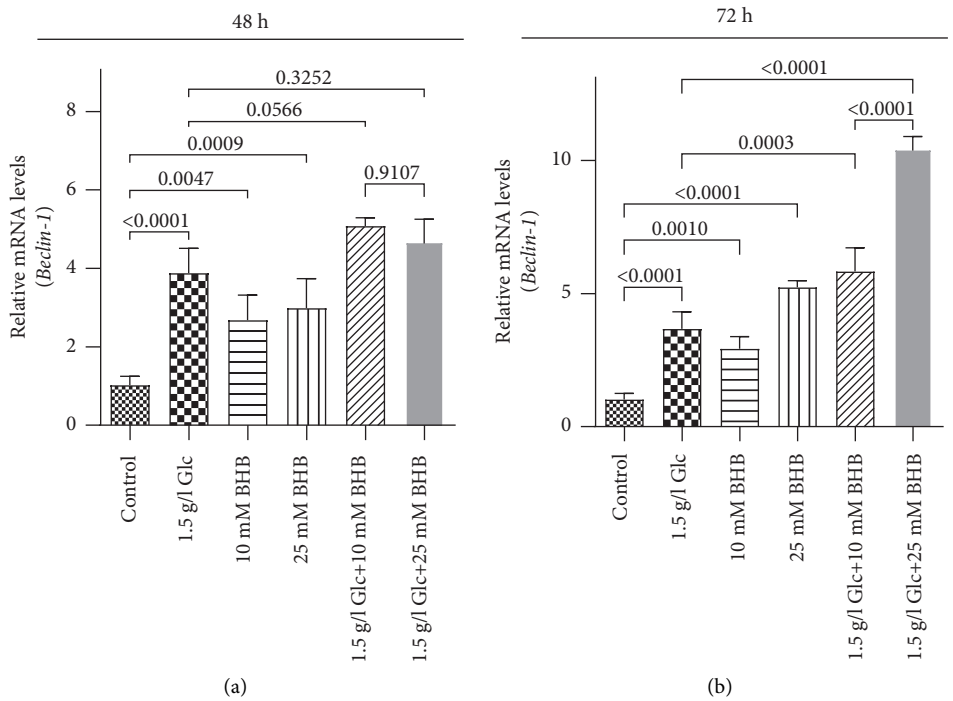
(b)

Figure 1: Continued.



(c)

Figure 1: Caki-1 cell viability as the concentration of glucose decreases. (a) A 48-hour and 72-hour treatment with BHB was evaluated to measure cell viability in Caki-1 cells. The cells were exposed to different glucose concentrations for (b) 48 and (c) 72 hours, either in isolation or in combination with 10 mM or 25 mM BHB. The MTT test was used to calculate the viability of the cells. The mean ± SD ($n = 3$) is used to display the results. $P < 0.05$ is considered statistically significant.



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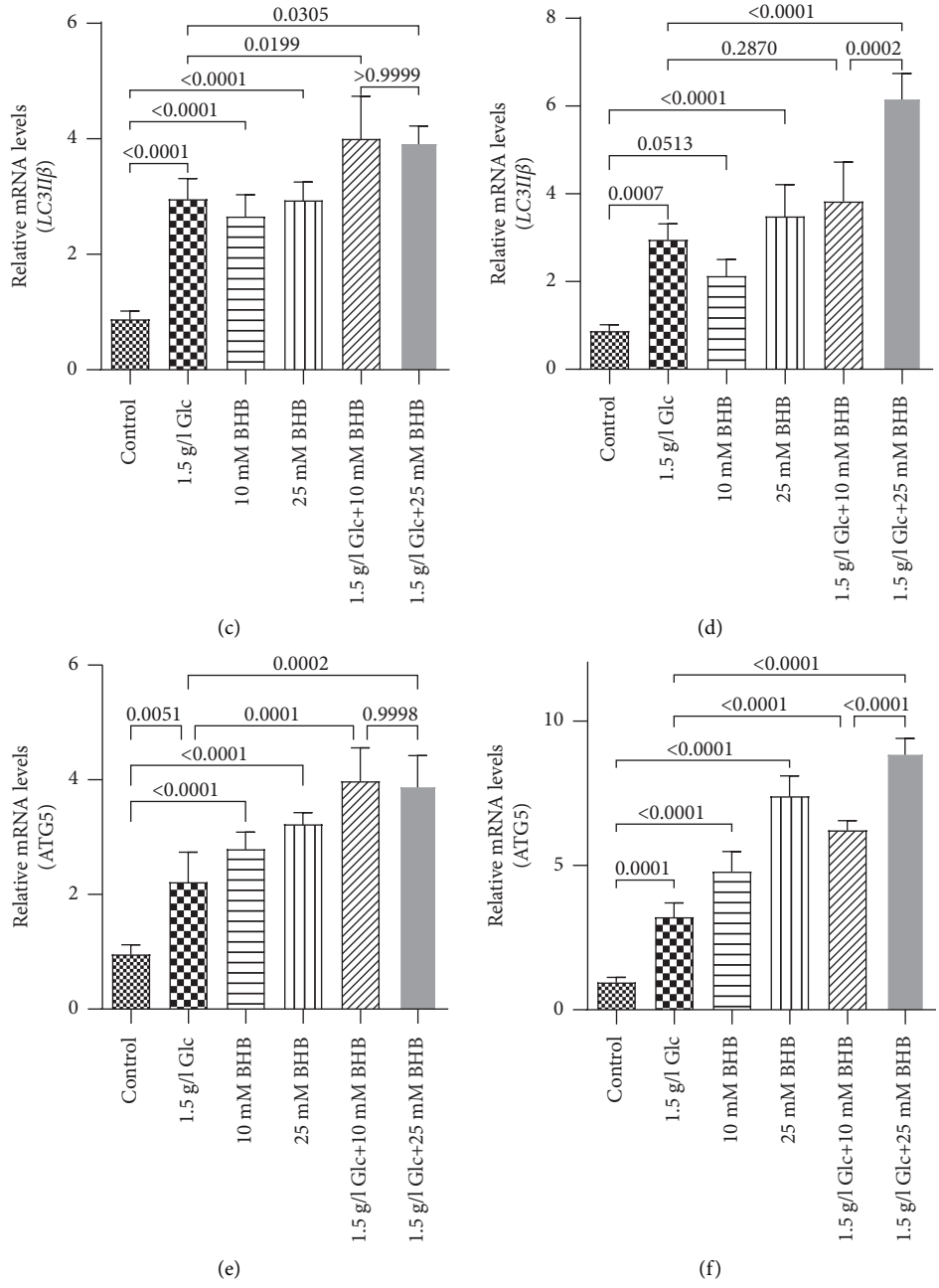

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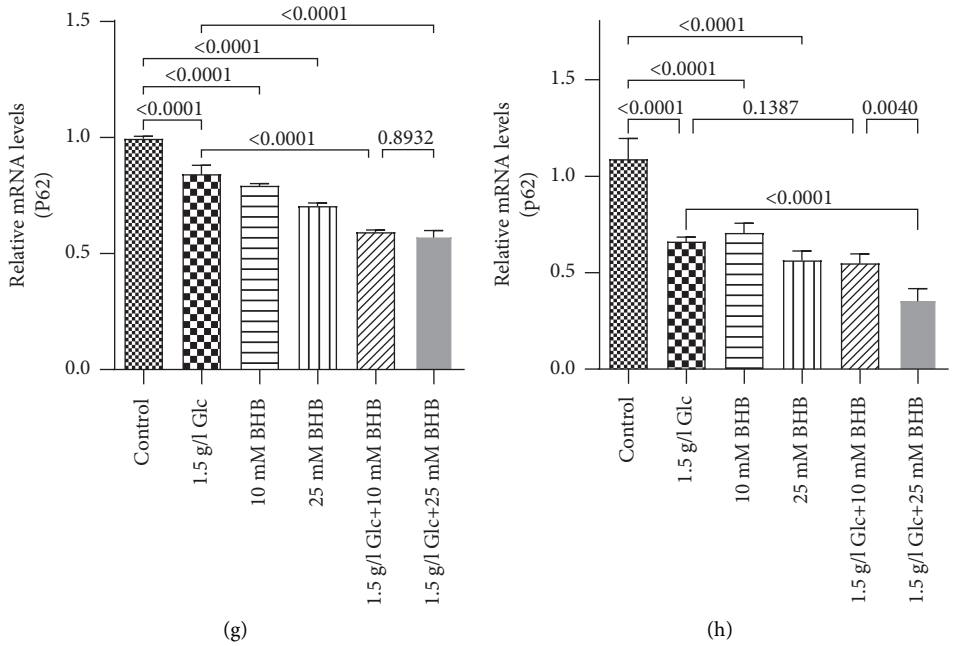

Figure 2: Supplementing with β-hydroxybutyrate increased autophagy in Caki-1 cells. Caki-1 cells were treated with decreasing glucose levels, either alone or in combination with 10 mM or 25 mM BHB, for 48 hours and 72 hours. RT-PCR was employed to assess the mRNA expression of (a and b) Beclin-1, (c and d) LC3IIβ, (e and f) ATG5, and (g and h) P62. The results are expressed as the mean ± SD ($n = 3$). $P < 0.05$ is considered statistically significant.

and 3(d) demonstrate the significant rise in CPT1A and ACSL3 expression following treatment with BHB (10 and 25 mM) or 1.5 g/l glucose alone, as measured at 72 hours, in comparison with the control. Following a 72-hour period, the combined treatment groups showed an increase in *CPT1A* and *ACSL3* mRNA expression when 25 mM BHB and 1.5 g/l glucose were combined, as opposed to when 10 BHB and 1.5 g/l glucose were combined ($P = 0.011$ and $P < 0.001$, respectively).

3.4. β-Hydroxybutyrate Enhanced Fat Browning in Caki-1 Cells. After incubating Caki-1 cells with BHB (10 and 25 mM) or 1.5 g/l glucose alone for 48 hours, we observed a substantial increase in *PGC-1α* and *UCP-1* expression levels compared with control (Figures 4(a) and 4(c)). Furthermore, combined treatment with 1.5 g/l glucose+ (10 or 25 mM) BHB significantly elevated *PGC-1α* and *UCP-1* mRNA expression compared with 1.5 g/l glucose alone.

Following a 72-hour period, there was no significant variation in *PGC-1α* mRNA expression between the groups that were given 1.5 g/L glucose alone (Figures 4(b) and 4(d)). However, *UCP-1* expression significantly increased following a 72-hour period of consuming 1.5 g/L glucose alone. Furthermore, after a full 72 hours, cells treated with BHB alone (10 mM or 25 mM) showed significantly higher *PGC-1α* mRNA expression than the control group. Moreover, *UCP-1* mRNA expression was higher in the 25 mM BHB group than in the control group after 72 hours.

Following a 72-hour incubation period, there was a significantly higher level in the expression of *PGC-1α* and UCP-1 mRNA in the treatment groups that combined 25 mM BHB with 1.5 g/L glucose, as compared with a combination of 10 mM BHB and 1.5 g/L glucose ($P = 0.003$ and $P = 0.009$, respectively).

4. Discussion

Indeed, in ccRCC, there is a disturbance in the body's processing of fatty acids, cholesterol, cholesteryl esters, and neutral lipids (triglycerides). This aberrant lipid metabolism has been identified as a crucial factor in the advancement of ccRCC and mirrors a prevalent trait in obesity characterized by excessive lipid production [2, 28]. Consequently, substances capable of modulating this abnormal lipid metabolism in ccRCC may present promising prospects as potential therapies for this form of kidney cancer [29].

The current study demonstrated that BHB exerts a notable influence on reducing the viability of Caki-1 cells when subjected to low-glucose conditions for both 48 and 72 hours of treatment. Moreover, the study revealed a more pronounced antiproliferative effect after 72 hours of BHB treatment. Concurrently, the assessment of autophagy markers revealed a significant impact of BHB on the expression of autophagy-related genes (Beclin-1, LC3IIβ, ATG5, and P62), signifying the activation of the autophagy pathway. Considering the cell viability results and additional data concerning apoptosis markers from the previous experiment, it becomes evident that the autophagy pathway plays a substantial role in triggering cell death in Caki-1 cells in response to BHB treatment.

Interestingly, the dose-dependent impact of BHB became evident only after 72 hours of treatment. Numerous studies have extensively documented the antitumorigenic

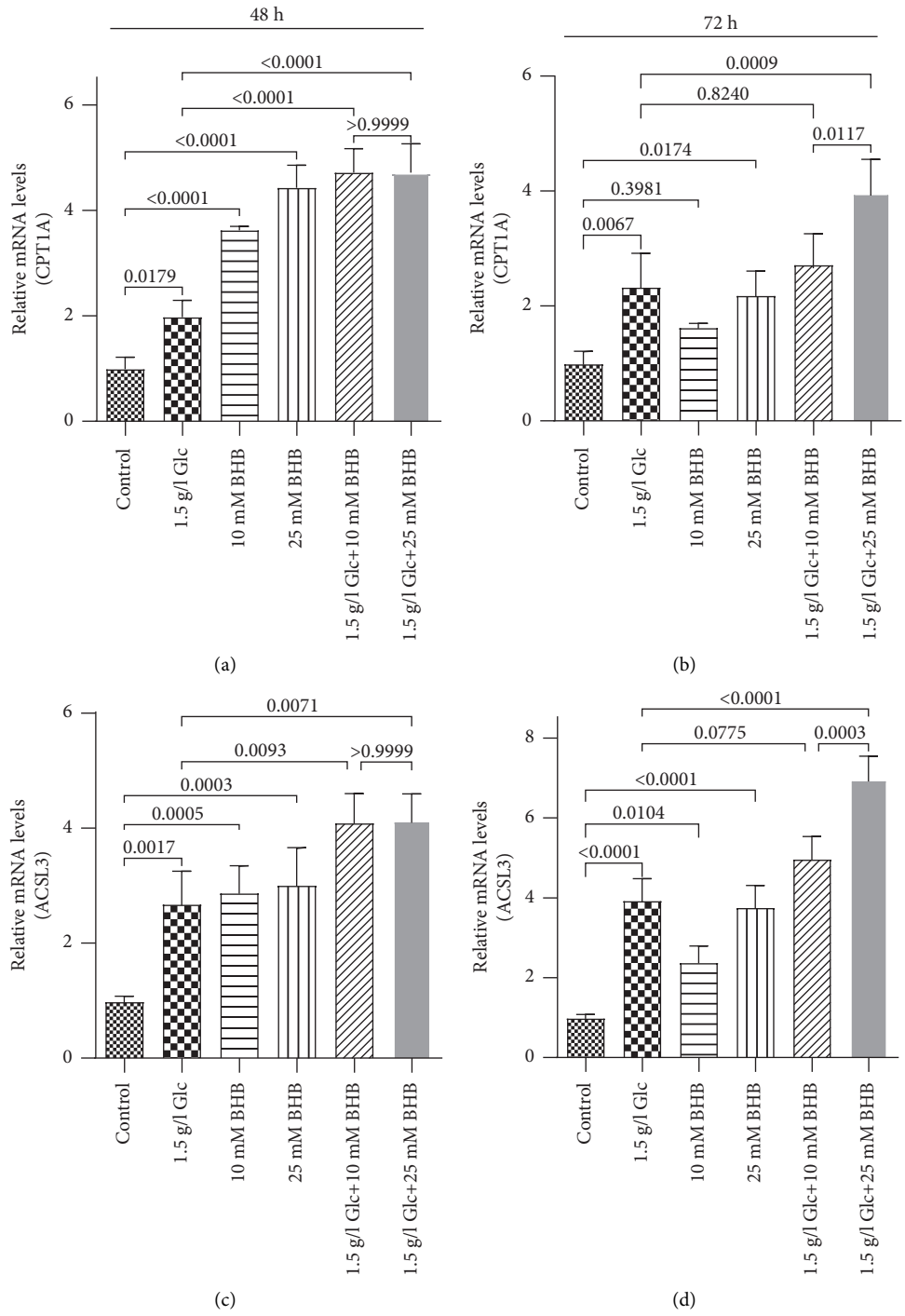

Figure 3: In Caki-1 cells, β-hydroxybutyrate upregulated the tumor suppressor genes CPT1A and ACSL3. For 48 and 72 hours, Caki-1 cells were treated with lowering glucose concentrations, either on their own or in conjunction with 10 mM or 25 mM BHB. The mRNA expression of (c and d) ACSL3 and (a and b) CPT1A was assessed using RT-PCR. The data are displayed as mean ± SD ($n = 3$). $P < 0.05$ is considered statistically significant.

effects of BHB, operating through various programmed cell death pathways such as apoptosis, unfolded protein response (UPR), ferroptosis, and autophagy-mediated apoptosis [7]. Previous research has indicated that BHB can induce autophagic flux and prevent cell death in glucose-starved neurons [30]. However, in the context of cancer cells, particularly ccRCC cells, BHB may stimulate autophagy and subsequently induce cell death, aligning with the current study's findings. This could be attributed to deficiencies in key enzymes involved in ketone body metabolism, including acetyl-CoA acetyltransferase (ACAT), HMG-CoA lyase (HMGCL), and/or β-hydroxybutyrate dehydrogenase (BDH) [31]. Certainly, lipophagy is a process where lipid droplets are transported to lysosomes for hydrolysis,

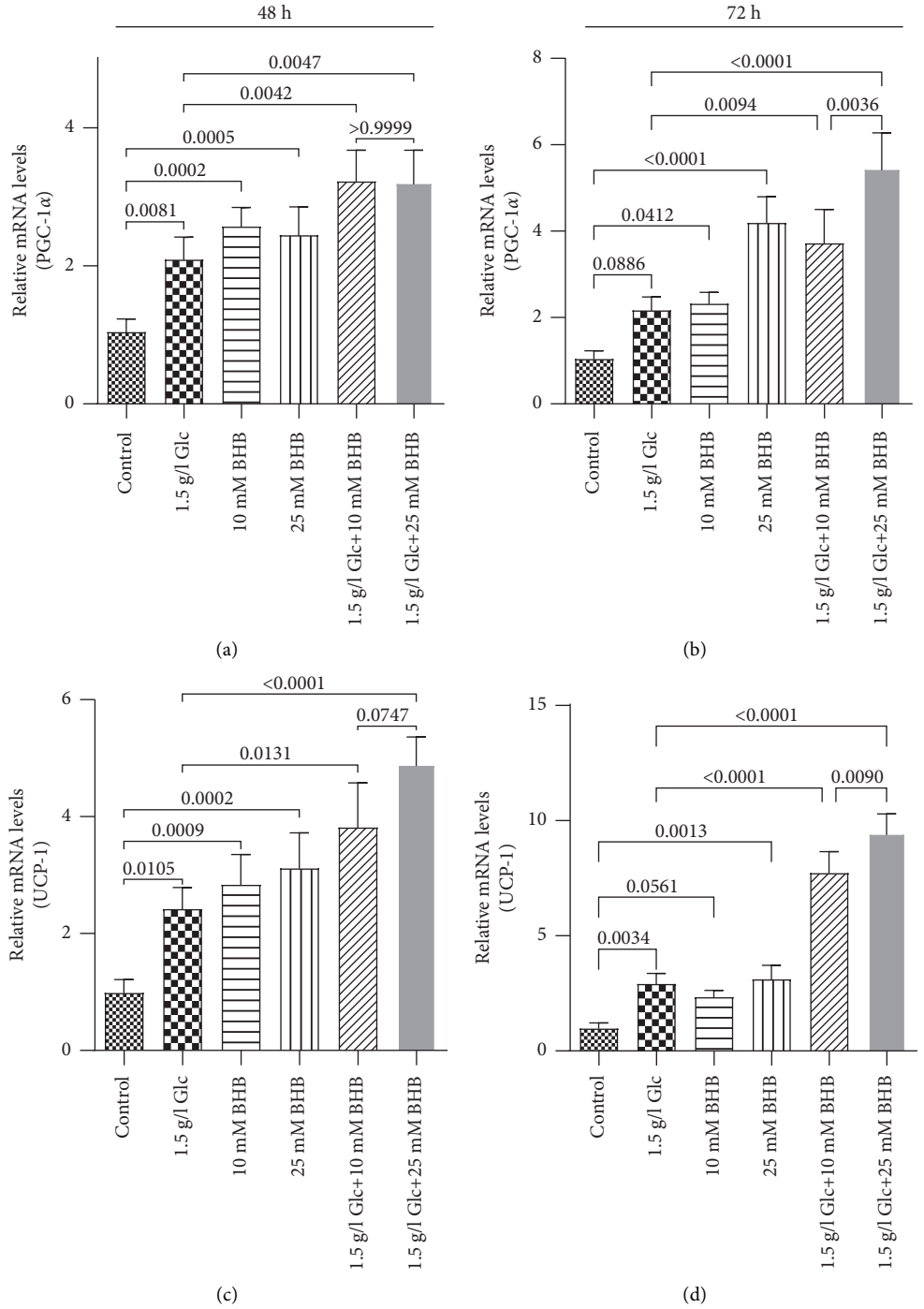

FIGURE 4: β-hydroxybutyrate increased fat browning in Caki-1 cells. Caki-1 cells were subjected to decreased glucose concentrations during 48 and 72 hours, either alone or in conjunction with 10 mM or 25 mM BHB. RT-PCR was employed to evaluate the mRNA expression of (a and b) PGC-1α, (c and d) UCP-1. Values are presented as mean ± SD ($n = 3$). $P < 0.05$ is considered statistically significant.

marking the first described functional role for autophagy in lipid metabolism. Lipophagy is a crucial link connecting autophagy and intracellular lipolysis [32, 33]. The autophagy process plays a role in breaking down lipids, and inhibited autophagy has been linked to forming and accumulating lipid droplets (LDs) in ccRCC cells [34]. Under conditions of glucose deprivation, LDs undergo catabolism, producing free fatty acids (FFAs), which are then subjected to β-oxidation through the autophagic/lysosomal pathway [35]. Yet, limited research is available on the effects of BHB on autophagy in ccRCC. Radovanovic et al. found that lower P62 expression levels and an increased autophagic pathway are associated with improved patient survival, lower tumor stage, and reduced metastasis in ccRCC [36]. Another study

demonstrated that melatonin induced autophagy by reducing P62 levels and increasing the expression of LC3IIβ, leading to the suppression of ccRCC cell proliferation [22]. Thus, enhancing the autophagy pathway by BHB may potentially serve as a mechanism to impede tumor progression and metastasis. The current study showed that BHB treatment could increase the expression of *PGC-1α* and UCP-1 in Caki-1 cells, potentially aiding in the restoration of lipolysis.

Prior studies have indicated that the role of *PGC-1α* varies depending on the specific tumor type [37]. In the context of ccRCC disease, characterized by a VHL mutation, *PGC-1α* is typically suppressed, reducing the number of mitochondria and decreasing lipid accumulation through the downregulation of fat browning [14]. Additionally, the inhibition of *PGC-1α* has been shown to disrupt the autophagy process induced by AMPK, causing a disturbance in phagosome fusion [38]. It is worth noting that the induction of autophagic flux has been reported to elevate *PGC-1α* and UCP-1 expression, promoting mitochondrial biogenesis and the lipid browning process in tumor cells [16]. PGC-1α has also been implicated in upregulating UCP-1 and activating autophagy, facilitating the breakdown of lipid droplets (LDs), and leading to increased lipid mobilization [30, 39]. Numerous investigations have demonstrated a favorable correlation between PGC-1α levels and UCP-1 expression in patients with ccRCC [16]. Furthermore, ccRCC tissues had much lower *PGC-1α* mRNA and protein levels than normal tissues did. Interestingly, *PGC-1α* expression was shown to be lower in patients with metastases than in those without metastases [14].

The elevation in UCP-1 expression results in reduced ATP production and energy dissipation in the form of heat, potentially contributing to decreased survival, proliferation, and metastasis of tumor cells [16, 40]. Consistent with our findings, a study by Srivastava et al. revealed that a ketogenic diet (KD) increased mitochondrial proteins in brown adipose tissue and UCP-1 levels in mice. The study also showed elevated PPAR-γ, *PGC-1α*, and Sirtuin 1 (SIRT1) levels in the KD group compared with those on a normal diet [39]. Another study indicated a significant rise in *PGC-1α* levels after caloric restriction and BHB administration in aged rats [41]. These results support the idea that BHB acts as an inducer of *PGC-1α*, highlighting its significant role in promoting fat browning. The current study demonstrated a significant upregulation of ACSL3 and CPT1A genes in the Caki-1 cell line when glucose levels were reduced. BHB was administered for 48 and 72 hours, and this effect was dose-dependent. ACSL3 is an enzyme that activates long-chain fatty acids, a process often dysregulated in tumors. Controversial information exists regarding the effects of ACSL3 in ccRCC. For instance, a study by Klasson et al. suggests that ACSL3 plays a crucial role in regulating lipid droplet formation in ccRCC. They found that ccRCC cells accumulate necessary substrates for lipid droplets by metabolizing exogenous lipids from serum rather than through de novo lipogenesis. Additionally, they revealed that metabolizing exogenous fatty acids into lipid droplets depends on the enzyme ACSL3. They proposed that genetic or pharmacological inhibition of ACSL3 could be cytotoxic to ccRCC. Conversely, another study suggests that inhibiting ACSL3 reduces the susceptibility of ccRCC cells to ferroptosis, a form of cell death associated with lipid peroxidation [25]. Furthermore, a different study found that the expression level of ACSL3 in ccRCC tissues had significantly decreased. Furthermore, renal cancer cell lines had significantly reduced levels of mRNA and protein expression. Additionally, this study demonstrated a strong association between ACSL3 and immune-cell infiltration, DNA methylation, and clinical stage [42]. According to Zhang et al.'s research, it is possible to successfully block the migration, invasion, and proliferation of ccRCC cells by overexpressing ACSL3. Moreover, it decreased aberrant lipid buildup and encouraged apoptosis [43]. To sum up, ACSL3 shows promise as a ccRCC biomarker and as a possible therapeutic target for the treatment of ccRCC malignancies. By identifying CPT1A as a direct HIF target gene, Du et al. shed light on the mechanism of lipid accumulation in ccRCC. CPT1A is the rate-limiting component of mitochondrial fatty acid transport. HIF1 and HIF2 suppress CPT1A, which reduces the amount of fatty acids that are transported into the mitochondria and causes lipid droplets to accumulate [44]. The current study demonstrated that BHB significantly increased the expression of CPT1A and ACSL3. This suggests that BHB has the potential to stimulate beta-oxidation without generating ATP, aligning with its role in promoting fat browning and lipophagy. Hui Yang and colleagues discovered that the overexpression of CPT1A can reduce lipid storage through the PPAR-α/CD36 signaling pathway and decrease cell proliferation in ccRCC. In summary, this study showcased the ability of BHB to upregulate the expression of genes associated with lipid browning, including PGC1A and UCP-1. Additionally, BHB increased the expression of ACSL3 and CPT1A, indicative of lipid droplet (LD) formation. Moreover, BHB influences autophagy, promoting the turnover of LDs and inhibiting their accumulation within cells. These combined effects contribute to the induction of a catabolic state, commonly known as "tumor slimming," ultimately suppressing tumor progression.

The study emphasizes the importance of conducting more research, such as in vivo studies and clinical trials, to confirm and expand on these findings. A future study might investigate BHB's translational potential as a therapeutic agent for ccRCC, with the possibility of incorporating it into precision-guided nutritional therapy or combination treatments. Furthermore, knowing the influence of BHB on additional aspects of ccRCC, such as metastasis and interactions with the tumor microenvironment, will help provide a more complete assessment of its therapeutic use. Overall, the study provides a foundation for future research targeted at focusing on BHB's potential role in treating ccRCC-related complications. While the study provides useful insights into the possible therapeutic benefits of BHB in ccRCC, overcoming these limitations and undertaking more research will be critical in determining BHB's clinical relevance and safety as a therapeutic agent for clear cell renal cell carcinoma. The work comprehensively investigates gene expression changes associated with autophagy and lipid

metabolism using real-time PCR. Western blot analysis may give further insights into protein expression levels, which is critical for confirming detected alterations at the protein level. Furthermore, the study focuses on a single cell line (Caki-1), which limits the generalizability of the results. Additional research incorporating several ccRCC cell lines or perhaps in vivo models would improve the reliability and usefulness of the findings. Furthermore, the study relies only on MTT tests to determine cell viability, and it would be beneficial to include other assays, such as flow cytometry or clonogenic assays, to offer a more thorough assessment of cell death processes and long-term impacts.

5. Conclusion

In this study, BHB emerged as a potential therapeutic option for ccRCC, a challenging urological cancer characterized by abnormal lipid metabolism. BHB demonstrated the ability to reduce cell viability, induce autophagy, and promote lipid browning, collectively contributing to the inhibition of ccRCC growth. These results underscore the promising role of BHB in targeting abnormal lipid metabolism and call for further exploration as a potential strategy for ccRCC treatment.

Abbreviations

RCC: Renal cell carcinoma
ccRCC: Clear cell renal cell carcinoma
CAR: Acylcarnitine
CE: Cholesteryl ester
DG: Diacylglycerol
BA: Bile acid
LPS: Lysophosphatidylserine
BHB: β-hydroxybutyrate
TCA: Tricarboxylic acid cycle
PGC-1α: PPAR-γ coactivator-1α
UCP-1: Uncoupling protein-1
LDs: Lipid droplets
ACSL3: Acyl-CoA synthetase long-chain family member 3
CPT1: Carnitine palmitoyltransferase 1
UPR: Unfolded protein response
ACAT: Acetyl-CoA acetyltransferase
HMGCL: HMG-CoA lyase
BDH: β-hydroxybutyrate dehydrogenase
FFAs: Free fatty acids
KD: Ketogenic diet
SIRT1: Sirtuin 1.

Data Availability

This article encompasses all the data generated and scrutinized during this investigation. The corresponding author is available to provide datasets that were used or analyzed in the current study upon receiving reasonable requests.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

RRAA, RH, and MS designed experiments, involved in statistical analysis, and prepared the figure. ZD, ST, and RA wrote the initial draft of the manuscript. AM coordinated test setups, and MS and SD crafted the second draft of the manuscript. AM and SD approved the final version of the manuscript and also contributed additional funds to the project. All authors have read and agreed to the published version of the manuscript. RR and AA contributed equally.

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