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β-hydroxybutyrate Does Not Influence Viability and Clonogenicity of A549 Lung Cancer Cells

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ABSTRACT

Background/Purpose: The metabolic shift from catabolism of carbohydrates to lipids results in production of ketone bodies leading to a state called ketosis. Ketosis via ketone supplement or ketogenic diet has been proposed as a non-toxic therapeutic option for a broad range of malignancies. Although the clinical impact of ketogenic diet is well-documented, the effect of ketone bodies on cancer cell biology is not clear for some cancers including non-small-cell lung cancer (NSCLC). In this study, we aimed to demonstrate the effects of the most prominent ketone body, β-hydroxybutyrate, on a NSCLC cell line, A549.

Methods: A549 cell line was utilized as the in vitro model in this study. The effects of different β-hydroxybutyrate concentrations on cell viability were measured via sulphorodamine-B (SRB) viability assay. Long term effects of ketosis were evaluated via colony formation assay. Finally, the effect of β-hydroxybutyrate on cell migration was determined via scratch assay.

Results: Our results suggest that introduction of β-hydroxybutyrate in physiologically relevant concentrations into the cell culture media does not influence cell viability, clonogenicity or migration.

Conclusion: β-hydroxybutyrate has been previously demonstrated to induce, inhibit or does not influence the viability of different cell lines but there is no report regarding its effects on NSCLC cells. Here we report that physiologically relevant concentrations of β-hydroxybutyrate have no effect on viability, clonogenicity and migration of A549 cells.

Keywords: ketosis, beta-hydroxybutyrate, cancer, non-small-cell lung cancer

Ketoz A549 Akciğer Kanseri Hücrelerinin Canlılığını ve Klonojenitesini Etkilememektedir

ÖZET

Giriş/Amaç: Metabolizmanın karbohidrat katabolizmasından lipid katabolizmasına geçişi ketoz adı verilen bir duruma yol açan keton cisimlerinin üretimine neden olur. Keton cismi takviyesi veya ketojenik diyet yoluyla tetiklenen ketoz, çok çeşitli maligniteler için toksik olmayan bir tedavi seçeneği olarak önerilmektedir. Ketojenik diyetin klinik etkisi iyi belgelenmiş olsa da, küçük hücreli dışı akciğer kanseri de dahil olmak üzere bazı kanserler için keton cisimlerinin kanser hücresi biyolojisi üzerindeki etkisi net değildir. Bu çalışmada en önemli keton cismi olan β-hidroksibütiratın küçük hücreli dışı akciğer kanseri hücre hattı A549 üzerindeki etkilerinin gösterilmesi amaçlanmıştır.

Yöntemler: Bu çalışmada in vitro model olarak A549 hücre hattı kullanılmıştır. Farklı β-hidroksibutirat konsantrasyonlarının hücre canlılığı üzerindeki etkileri, sülforhodamin-B (SRB) canlılık testi ile ölçülmüştür. Ketozun uzun vadeli etkileri, koloni oluşum testi ile değerlendirilmiştir. Son olarak, β-hidroksibutiratın hücre göçü üzerindeki etkisi, çizik testi ile belirlenmiştir.

Bulgular: Çalışmada elde edilen veriler, fizyolojik konsantrasyonlarda β-hidroksibutiratın hücre kültürü ortamına dahil edilmesinin hücre canlılığını, klonojenisitesini veya göçünü etkilemediğini göstermektedir.

Sonuç: β-hidroksibutiratın farklı hücre hatlarında canlılığı artırdığı, azalttığı veya etkilemediği daha önceki çalışmalarla gösterilmiştir ancak küçük hücreli olmayan akciğer kanseri hücreleri üzerindeki etkilerine dair bir veri bulunmamaktadır. Bu çalışmada fizyolojik β-hidroksibutirat konsantrasyonlarının A549 hücrelerinin canlılığı, klonojenitesi ve göçü üzerinde hiçbir etkisinin olmadığı belirlenmiştir.

Anahtar kelimeler: ketoz, beta-hidroksibütirat, kanser, küçük hücreli dışı akciğer kanseri

During fasting, starvation or strenuous exercise, as a response to decreasing blood glucose levels, energy metabolism shifts from carbohydrate catabolism to lipid oxidation. Increased β-oxidation rate in liver enabled by this shift results in production of excessive amounts of acetyl-CoA which is then utilized to synthesize a group of small water-soluble metabolites called ketone bodies. Ketone bodies including acetoacetate, β-hydroxybutyrate (BHB), and acetone can be oxidized via Krebs cycle to produce energy when they are transported to extrahepatic tissues. Amongst ketone bodies BHB is the most prominent one in the blood during ketosis (1) and BHB is the most common ketone body supplement (2).

The state of ketosis which is characterized by elevated serum levels of ketone bodies occurs if the rate of ketone body production exceeds their utilization. Ketosis may be a physiological condition if the levels of ketone bodies in circulation are between 0.5 and 3.0 millimolar (mM) (2). Higher levels (≥10 mM) indicate ketoacidosis, a potentially dangerous form of metabolic acidosis, and require medical intervention (2).

Mild (physiological) ketosis, generally achieved via nutritional alterations such as ketogenic diet and ketone supplementation, has been demonstrated to offer therapeutic potential in various medical conditions including epilepsy, Alzheimer's disease, Parkinson's disease and metabolic syndrome (3). Recent preclinical and clinical studies suggest that ketogenic diet may also be a strong candidate as an adjuvant cancer therapy for a spectrum of malignancies of brain, breast, colon, lung and others (2,4). Ketogenic diet has been shown to slow tumor growth, prolong survival rate, and enhance drug response in mice with cancer (4) especially for brain malignancies including glioma (5), glioblastoma multiforme (6) and astrocytoma (7). Meta-analysis of clinical data for different cancers suggest that the ketogenic diet is safe and beneficial for cancer patients (8). On the other hand, there are many pre-clinical and clinical studies demonstrating that neither ketogenic diet nor ketone supplementation alone has any effect on viability of cancer cells and/or tumor progression for brain, pancreas, breast, liver (9–14) and other cancers (4).

Even though the data is more limited for lung cancer, the leading cause of cancer-related deaths (15), current evidence suggest that similar to many others, ketosis suppresses tumor growth (13,16) and according to the results of one clinical study from Turkey, ketogenic diet improves survival and treatment response in metastatic non-small-cell lung cancer patients (NSCLC) (17). There is currently no data regarding the effects of ketone bodies on lung cancer cells. In this study we aimed to reveal the effects of β-hydroxybutyrate, the most prominent ketone body, on A549 non-small-cell lung cancer cells.

Material and Methods

Cell Culture

Non-small-cell lung cancer cell line A549 (CCL-185, ATCC) cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% (2 mM) L-glutamine (L-Gln) and 1% penicillin/streptomycin (complete medium) at 37°C in a humid incubator with 5% CO_2 pressure.

Cell Viability

β-Hydroxybutyrate (BHB) (Sigma, #166898) was dissolved in absolute Ethanol (EtOH) to obtain 50 mg/mL solution. Subsequently, different concentrations were prepared in complete medium. EtOH equivalent of the highest concentration was prepared in complete medium and was used as vehicle control.

The sulphorodamine B (SRB) test was performed to determine the effect of BHB on cell viability of A549 lung carcinoma cells (18). $5x10^3$ cells/well were seeded in 100 µL medium to the wells of 96-well plates. After overnight incubation, increasing concentrations of BHB in 100 µL medium were applied to the cells as 6 replicates (N=6) in the test wells. Final BHB concentrations were 1 µM, 1 mM and 3 mM representing physiological ketosis (2). After 48 hours of incubation following administration of the solutions, 50 µL of 50% trichloroacetic acid (TCA) solution was added to the wells and fixed at 4°C for 1 hour. TCA was then removed by sequential washes and 50 µL of the SRB solution was added to each well and incubated for 30 min at room temperature in the dark. The wells were washed to remove unbound dye and the plate was dried. The protein-bound dye was dissolved with 150 µL/well of 10 mM tris base (pH 10) on a shaker at approximately 150 rpm for 10 min. Then, spectrophotometric reading was performed at 564 nm/690 nm. After subtraction of absorbance values at 690 nm from at 564 nm, the resulting signals were compared between wells equivalent to cell viability using the GraphPad Prism V9 program and visualized as dose-response curves.

Migratory Abilities

Migratory abilities of A549 cells were determined by scratch assay. Cells were trypsinized and resuspended in complete medium. Cells were then counted and seeded in 3 replicates, 300,000 cells in 0.5 mL per well of 24 well plates. Cells were incubated in 37°C humidified CO_2 incubator overnight. The next day, scratches in the form of crosses were made using sterile 200 µL pipet tips. Wells were rinsed with PBS to remove detached cells. Subsequently sample wells were treated with 3 mM BHB. Experimental steps were performed according to previous literature (19). Images were acquired under inverted light microscope (Nikon, Eclipse TS2) and analyzed using FIJI (ImageJ) with the "Wound healing assay" macro by Kees Straatman. Images are also uploaded as a supplementary document. Calculations were performed to obtain percentage of migration against untreated cells and graphs were plotted using GraphPad Prism v9.

Clonogenicity

Assessment of clonogenicity of A549 cells was carried out by colony formation assay (CFA). Cells were trypsinized, resuspended in complete medium and counted on hemocytometer. 50 cells per well were seeded in 24 well plates in triplicate on the 1st day. Plates were incubated in 37°C humidified CO_2 incubator. 3 mM BHB was applied on the 2nd day. Media were replaced on 4th day. Cells were fixed and stained by 0.2% crystal violet (Sigma-Aldrich) solution in 2% EtOH for 10 min in dark at room temperature. Then the plates were washed with water, dried overnight and visualized by inverted light microscope (Nikon, Eclipse TS2) on the 8th day. Colonies were then counted; colony formation percentages were calculated against untreated wells and graphs were plotted using GraphPad Prism v9.

Data Analysis

All graphs were plotted using GraphPad Prism v9. All columns represent mean values of all samples and error bars represent standard deviation. In SRB data analysis, untreated cell viability was considered as 100% and all the others were calculated accordingly.

Results

Short-term BHB treatment does not influence A549 cell viability.

Cells were either left untreated, treated with vehicle control of with increasing concentrations of BHB (1 µM, 1 mM and 3 mM) for 48 hours. We tested these concentrations to mimic physiological blood concentrations (2) of BHB during pre-ketosis (1 µM) and mild ketosis (1-3 mM) states. We measured cell viability via SRB assay. According to our findings, BHB treatment failed to alter cell viability compared to untreated and vehicle controls (Figure 1A).

Long-term BHB treatment caused a slight decrease in A549 clonogenicity compared to untreated cells.

Since there was no viability inhibition after 48-hours of treatment, we decided to observe effects of BHB on longer periods of time. We carried out a colony formation assay by which we counted the number of formed colonies after the treatment for 8 days. Cells were either left untreated, treated with vehicle control or 3 mM BHB, and formed colonies were counted after the treatment period. Our findings suggest that the BHB treatment decreased the number of colonies formed only slightly compared to untreated and there was no difference compared to the vehicle control (Figure 1B).

BHB does not affect migratory abilities of A549 cells.

After short- and long-term effects were determined we moved on to investigate the effects of BHB on A549 cell migration. Cells were treated with 3 mM BHB for 3 days. Cell migration was evaluated each day via scratch assay. BHB treatment decreased cell migration compared to vehicle control on the first day, but the difference was not apparent on following days (Figure 1C).

Discussion

Ketosis achieved via ketone supplementation or ketogenic diet has been proposed as a safe adjuvant therapeutic option for cancer (20). However, its efficiency in reducing tumors and inhibiting cancer cell viability is up to debate as there are many contrasting reports including anti-tumor activity (60% of studies), ineffectiveness (17%) and pro-proliferative effects (10%) (4). The effect of ketosis on cancer progression may depend on the cancer type and it can indirectly influence tumor growth via enhancing chemo/radiotherapy response without directly influencing cancer cell viability (12,21).

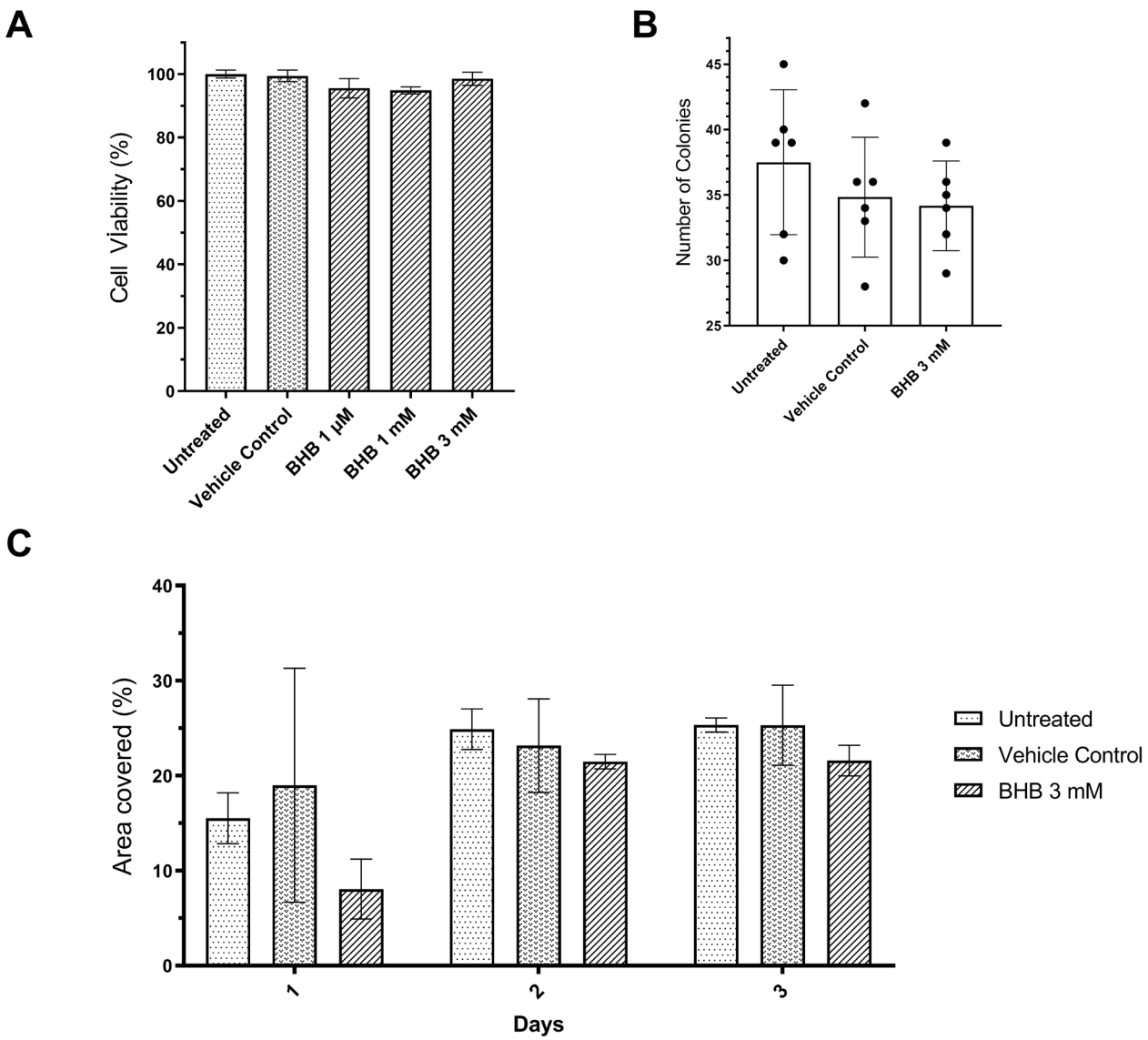

Figure 1. Effects of BHB treatment on A549 cells. A. Mean cell viability percentage of different samples according to the SRB assay. Untreated cell viability was considered as 100% and all the others were calculated accordingly. Vehicle control is ethanol (0.6%), N=6 and error bars represent standard deviation. B. Number of colonies formed according to the CFA analysis. Dots represent separate measurements (N=6) and the columns represent mean values, vehicle control is ethanol (0.6%) and error bars represent standard deviation. C. Mean area percentage covered by migrating cells according to the scratch assay. Columns represent mean of 3 separate samples (N=3), error bars represent standard deviation.

In case of NSCLC, limited data suggest that ketosis, achieved via ketogenic diet, suppresses tumor growth, enhances chemo/radiotherapy response and reduces angiogenesis in mice models (13,16). One clinical study support this by providing evidence of improved survival and enhanced treatment response in patients with metastatic NSCLC (17). However, currently there is no data regarding the direct effects of ketone bodies on cancer cells and whether the anti-cancer effects of ketosis are due altered cancer cell viability. Here we report that the physiologically relevant concentrations of BHB does not have any short- or long-term effects on viability of A549 cancer cells. Therefore, previously reported anti-tumor activity of ketogenic diet in NSCLC may be due a mechanism other than inhibition of cancer cell viability.

Another proposed anti-cancer effect of BHB was the inhibition of cancer cell migration (22,23). However, we demonstrated that BHB does not influence cancer migratory ability of A549 cells in vitro.

Since we focused on a single cell line, our results should not be generalized to NSCLC. Cell-line dependent effects of BHB may be investigated in future studies. Moreover, further studies are needed to identify the anticancer mechanism of ketogenic diet on NSCLC, focusing on previously proposed alternative mechanisms such as enhancing treatment response, altering cellular metabolism or remodelling tumor microenvironment (13,16,24–26).

Conclusion

Here we report ineffectiveness of BHB treatment on viability, clonogenicity and motility of A549 NSCLC cells. Since both clinical and in vivo data suggest an anti-cancer effect for ketogenic diet, further studies are needed to investigate the mechanism of such effect.

Declarations

Funding

Internal funds of the institutions.

Conflicts of Interest/Competing Interests

Authors declare no conflict of interest.

Ethics Approval

Not applicable (Cell culture study).

Availability of Data and Material (Data Transparency)

All data has been presented.

Authors' Contributions

All authors contributed to this work in accordance with the ICMJE authorship criteria.

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