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Investigating the functional effects of the long-term ketogenic diet on pancreatic islets in epileptic rats

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Abstract. Ketogenic Diet is well studied and applied as a treatment in numerous nervous system diseases, such as epilepsy. However, KD's long term effect on the β cells and the risk of potential insulin resistance are unclear. In this research, we aim to testify whether the usage of KD on patients with epilepsy would increase the risk of diabetes. In this research we firstly verified the cytotoxicity of the islet B cells which might be stimulated by the abundance of fats in the KD. We used a CCK-8 kit to conduct the experiment and obtained a result of higher IC50 values in the keto conditioned cells, which indicates that cells in keto face a higher cytotoxicity than cells in normal condition. Afterwards, we did both vivo and in vitro experiments on the rats. In the in vitro experiment, the islet β cells in a keto-environment showed a higher apoptosis rate. In the in vivo experiment, Enzyme-linked immunosorbent assay (ELISA) was used to detect the insulin level and blood fat in plasma of rats. The result out of ELISA demonstrates a continuously decreasing level of insulin and lower body fat. In addition, oral glucose tolerance test (OGTT) was also used to find the blood sugar in the rats which came out as a result that the glucose metabolism has decreased. Although the experiments exhibit several side effects of KD, it might not be applicable to humans since we only had animal experiment. This research aims to reevaluate the benefits and side effects of application of KD to curing epilepsy.

Keywords: ketogenic diet, diabetes, pancreatic islets, β cells, insulin.

1. Introduction

The Ketogenic Diet (KD) has been employed as a cure for intractable epilepsy and related neurological disorders since the 1920s. KD itself is a dietary regimen characterized by a substantial reduction in carbohydrate intake and a significant increase in fat consumption. In this diet, carbohydrates occupy less

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than 10% of the total calorie content. As documented in the Hippocratic collection, the KD is postulated to act as a remedy that could generate the same advantages of fasting. In this research, we aim to testify whether the usage of KD on patients with epilepsy would increase the risk of diabetes.

The KD is a low carbohydrate-high fat (LC-HF) diet, which causes ketosis, an elevated blood level of ketones that are metabolized by the brain as the main source of energy when glucose is absent. Ketones are fatty acids produced in the liver [1]. While there is no clear definition for the various KD variants, KD can be defined as a diet that induces ketosis. Nevertheless, ketosis can also occur without fat consumption. The KD is characterized by low carbohydrate intake (less than 50g/day) and a relatively high proportion of proteins and fats. The study of the metabolic effects of KD dates to the pioneering work of Cahill and colleagues in the 1960s [2], however, the importance of the diet from a clinical point of perspective became apparent in the early 1920s when it was effectively applied to treat epilepsy [3]. In recent years, interest in a very low carbohydrate ketogenic diet (VLCKD) has increased. There is growing evidence that it can have a positive effect on many diseases. Since the 1970s, VLCKD has been established as a treatment which treated epilepsy effectively. Most recently, more attention has been paid on this particular diet due to the positive impacts it has on several diseases. However, more research is needed to fully fathom this relatively novel idea before applying it to all patients. Therefore, this research revisits the function of KD on curing epilepsy and the potential side-effects it brings as the result.

2. Material and method

2.1. In vitro experiment

2.1.1. Preparing pancreatic β cells.

Material

Pancreatic β cell strand from procell

Insulin high range HTRF test kit from cisbio

2-Deoxyglucose (2DG) Uptake Measurement Kit from FujiFilm

DMEM medium and no glucose DMEM medium from ThermoFisher Scientific.

Method

All pancreatic β cells used in the experiment will be replicate from one cell strand for consistency in DEME medium. Obtain pancreatic β cell strain from procell. Add 10 mL DEME medium without antibiotic for cell replication to a 15cm petri dish, and place cell in it. Add 1mL of cell to petri dish and keep under 37° (normal body temperature). Check and record cell volume under microscope daily. Once the cells reached a consistent multiplying rate, start transferring them out to other 15 cm petri dishes. Repeat the wait and harvest process until 20 petri dishes are filled with the clones of the original strain.

2.1.2. Set two groups of β cells and the variable is the environment. Twenty 15cm petri dishes filled with identical panctratic β cells, labeled as 1 through 20, split into two groups of 10 petri dishes. Group A (dish 1 through 10) is controlling group placed in DMEM medium. Group B (dish 11 through 20) is experimenting group placed in high fat medium. The high fat medium is no glucose DMEM spiked with fatty acid (1g per litre). For 3 weeks, check pancreatitis β cell's population, fludeoxyglucose concentration, and insulin conentration in cell supernatant with 2-Deoxyglucose (2DG) Uptake Measurement Kit and insulin high range HTRF every 6 hours under the microscope, record signs of mitosis as evidence of population growth. Record decrease in fludeoxyglucose and insulin concentration as proof of change in metabolism.

2.1.3. Test the cytotoxicity of the islet βcells. In order to verify the cytotoxicity of the islet βcells which are stimulated by the fats in the ketogenic diet. We used CCK-8 kit as a way to find out the result. CCK-8 use the dye WST-8 inside to test the cytotoxicity. And the dye can be reduced by dehydrogenase in the cell to form formazan which is an orange water-soluble product. Furthermore, there is a correlation between the produced formazan and the count of viable cells. Thus, the viability of cells can be estimated

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using a microplate reader to measure the optical density (OD) of the formazan at 450 nm. The darkness of the color shows correlation with cell proliferation and cytotoxicity. Cell proliferation shows directly proportional to the OD and cytotoxicity shows inversely proportional relationship.

We used 2 main groups which are islet βcells in the keto environment and in normal environment. Each group is provided with 36 wells, and a blank group is set at the same time with 24 wells in the marginal. Add Ham f-12 culture solution to each experimental well for 24 hours. PBS buffer is added to the most marginal wells to reduce the influence caused by evaporation, and then all the cells are cultured in a 5% CO2 cell incubator at 37°C for 12-24 hours. Add about 5000-10000 cells and 100ul to each well (the specific number of cells used in each well depends on the size of cells and the speed of cell proliferation).

Observe that the cells are well adhered to the wall and suck out the culture medium of each well. Normal medium and ketogenic medium spiked with fatty acids (1g per liter) and without glucose were added to the corresponding wells. Then the plate is incubated in a cell incubator with 5% CO2 air at 37°C for 12-48 hours. After that, each well was directly added with 10 μL of CCK-8 solution and the culture plate was incubated with CCK-8 for 1-4 hours in a 37°C 5% CO2 incubator.

2.1.4. Measure the apoptosis rate of islet cells in mice. We used propidium iodide to test whether the ketogenic environment increases the rate of apoptosis in mouse pancreatic islet βcells. Propidium iodide (PI), in collaboration with annexin V, is commonly used to determine whether cells are apoptotic, viable or necrotic, based on differences in plasma membrane permeability and integrity. PI is a widely preferred nuclear stain since it is stable, cost-effective and an excellent indicator of cell viability, as it only stains non-living cells. The capability of PI to penetrate a cell relies on the permeability of the cellular membrane. As long as the plasma membrane remains intact, propidium iodide (PI) does not mark living cells or cells that are in the initial stages of apoptosis. In apoptotic and necrotic cells in the late stage-when the integrity of nuclear membranes and the plasma is decreased-PI may penetrate the cell membrane, access the nucleic acids and emit a red fluorescent signal [4]. Group A cells were cultured in a simulated ketone body environment as might be found in mice, and Group B cells were cultured in a simulated normal mouse. The cells were incubated with Annexin V-FITC. The desired method induced apoptosis. As a positive control, Jurkat cells were treated using 2 μM cisplatin. The cells were collected using centrifugation. Resuspend the cells in 500 μL of Annexin V binding buffer followed by the addition of 5 μL of Annexin V-FITC and 5 μL of propidium iodide. Incubate for 5 minutes at room temperature in the dark.

The FITC signal detector and the phycoerythrin emission signal detector were used to evaluate PI staining and Annexin V-FITC binding assayed by flow cytometry. To examine adherent cells, The cells underwent trypsinization and were thoroughly washed with serum-containing medium before being incubated with Annexin V-FITC. Subsequently, the presence of the cells was detected by fluorescence microscopy.

2.2. In vivo experiment

2.2.1. To make the model of epileptic rats. 30 rats are used to prepare the model of epilepsy and each of them is weighted 200-300g. 5 of them are normal control group and the rest of 25 rats are model preparation group.

On the first day, inject lithium chloride (3mmol/kg body weight) intraperitoneally into the experimental group. In the next day (24 hours later), inject pilocarpine to the same group with 20mg/kg body weight every 30 minutes. If the rats have status epilepticus (reaching level 5 and lasting for more than 1 hour) within 3 injections, the injection of pilocarpine is terminated. If the rats did not have status epilepticus within three injections, they continued to be injected with pilocarpine, and the dose was reduced to 10mg/kg body weight, once every 15min minutes until status epilepticus appeared, then injection was stopped.

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When the status epilepticus appears for more than 60min, the seizure is terminated by intraperitoneal injection of diazepam (10mg/kg body weight). If diazepam cannot be terminated once, 15% ~ 25% of the first dose is injected every 10min until the seizure is basically terminated. After injection of pilocarpine, rats in the model preparation group showed different degrees of activity reduction, tremor, nodding, scratching, "face-washing-like activity", facial convulsion, one-limb clonus, wet dog-like jitter and imbalance, limb tonic clonus accompanied by standing and forelimb clonus, which further developed to level 5, and generalized tonic clonus accompanied by standing and falling, showing a state of epilepsy.

At last, exclude some rats which are died during the experiment and control group, choose the best 20 rats to do the rest experiment.

2.2.2. Set two groups of epileptic rats and the variable is their diet. One group with ketogenic diet and it is considered as group A. The other group takes normal diet, and it is group B. For the group of rats with ketogenic diet. They should take lipid: protein + carbohydrate at a ratio of 4 : 1. The protein should less than 1g/day/kg body weight, and according to the proportion, carbohydrate should less than 1g. Moreover, fibre should be taking every morning and night with 15g.

Each group takes 10 rats and have specific diet for 6 months. And record the frequency of occurrence of epileptic. Use balance to measure the weight of these rats every day. And before weighing them, they should fast for 12-24 hours to reduce the influence of food.

2.2.3. Test insulin and blood fat. The enzyme-linked immunosorbent assay (ELISA) is used to measure insulin and lipid levels in the blood serum or plasma of rats every fortnight. The serum is obtained by drawing blood from the tail of the rats. It coagulates naturally at room temperature for 10-20 minutes. Then the blood is centrifuged and left for about 20 minutes. The supernatant is carefully removed.

10 wells are made with the standard on the enzyme-labelled coated plate. For the 1st and 2nd wells, 100 μL of standard is added followed by 50 μL of diluted standard. The 3rd and 4th wells each receive 100 μL solution from the 1st and 2nd wells, followed by 50 μL standard diluent. Discard 50 μL solution from each of the 3rd and 4th wells, pour 50 μL of this into the 5th and 6th wells and top up with 50 μL standard diluent. Mix thoroughly after each step and then transfer 50 μL of solution from the 5th and 6th wells to the 7th and 8th wells, correspondingly. Then add 50 μL of standard dilution to each 7th and 8t h well. Then add 50 μL of solution from the 7th and 8th wells to the 9th and 10th wells, correspondingly. Then add 50 μL of standard diluent to each of the 9th and 10th wells and discard 50 μL of solution from the ninth and tenth wells after mixing.

Both control wells and sample wells are prepared for the experiment. Add 40 μL of the sample diluent to the sample well, which has a collected supernatant on the enzyme-labelled coating plate. Then, add 10 μL of the collected supernatant for testing purposes. Add the sample to the bottom of the well in the enzyme-labelled plate carefully, while avoiding possible contact with the walls. Afterwards, gently shake and mix the sample.

Incubate the sealed plate for 30 minutes at 37°C. For later use, dilute the concentrated wash fluid 30:1 with distilled water, carefully remove the sealing foil, discard the fluid, spin dry the plate and add wash fluid to each well. Allow to stand for 30 seconds, discard, and repeat five times. Finally blot dry the plate. Add 50 μL of the enzyme-labelled reagent to all wells except the blank wells. Repeat the incubation and washing procedure as described above. 50 μL of Developer A and 50 μL of Developer B are added to each well. Then gently shake, mix and incubate for 15 minutes at 37°C in the dark. Stop the reaction by adding 50 μL Stop Solution to each well.

2.2.4. Test blood sugar and glucose metabolism. Use the OGTT to test the blood sugar and glucose metabolism every 2 weeks. Oral glucose tolerance teat (OGTT) is a method used widely to treat the islet function. Let all of the rats fasting for 12 hours and take the blood sample of these fasting rats before the experiment to measure the baseline levels of insulin and glucose. Give oral glucose solution to rats with 2g/kg and record the administration time as 0 minutes.

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At 30, 60, 90, and 120 minutes after administration, blood samples of rats are collected and measured blood sugar level. Simultaneously measure the insulin levels at each time point. Evaluate rat pancreatic islet function and glycometabolism by analyzing blood glucose curves and change in insulin levels. Above all, all the experiments should be done for 6 months. And after 6 months, these rats are going to die and for pathological analysis.

2.2.5. Detect the apoptotic cells in pancreas of the mice [5]. After killing the epileptic mice, we obtained their pancreas without damaging it. We then performed the TUNEL assay as described below.

TUNEL, the abbreviation for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, is commonly used as a staining method to identify the cells that are apoptotic in tissue sections. Microwave heating combined with protein hydrolysis in solutions with extreme pH, resulted in strong staining of 70-80% of cells that are apoptotic and apoptotic bodies on archived tissue blocks with low background. Cells that are in the early stage of apoptosis can be observed because of the increased sensitivity, potentially extending our understanding of apoptotic cells beyond images of atrophic necrosis. Inhibition of endogenous peroxidase was not performed as H2O2 weakens TdT activity [6] and induces DNA breaks [7].

3. Result

3.1. Pancreatic cells had a change in metabolism method.

Cells in Group A are expected to continue to duplicate and producing insulin and fludeoxyglucose at a consistent rate. Certain portion of cells from Group B could show signs of necrosis, consider the possibility of pancreas inflammation after prolong period of keto diet. Cells from Group B should show signs of decreas in both fludeoxyglucose and insulin concentration at similar rates due to less carbonhydrate intake, proving that the pancreatic cells had a change in metabolism method.

3.2. Higher apoptosis rate of islet cells in mice

For analysis of adherent cells, they are grown on a coverslip directly. After incubation, turn over the coverslip onto a microscope slide and observe the cells. Alternatively, the cells can be rinsed with Annexin-V Binding Buffer and treated with 2% formaldehyde before observation. The cells should be observed under a fluorescence microscope with a double filter set for rhodamine and FITC or with separate filters. The plasma membrane of cells that have bound Annexin V-FITC will be green. In cells that have lost their membrane integrity, the nuclei show continuous red PI staining, and a halo of green staining (FITC) appears on the plasma membrane. We monitored the apoptosis rate of the β-islet cells in each group by examining the proportion of red fluorescence at 30 and 60 days after the start of the experiment. The fluorochromes used were PKH-26 and annexin V-FITC.

We found that cells in group A showed redder fluorescence than cells in group B each time we observed the cells. Hence, we came to the conclusion that cells in group A had a higher apoptosis rate compared to the cells in group B, which means islet βcells have a higher apoptosis rate in a keto-environment.

3.3. Lower insulin and lower blood fat

Measure the absorbance (OD value) of each well in sequence, using a wavelength of zero and 450nm of blank air conditioner. Conduct the measurement within 15 minutes after adding the stop liquid. The darkness shows direct relationship with blood fat in the sample. And we found out that the concentration of lipids is decreased. The treated serum or plasma sample reacts with a certain concentration of insulin detection reagent, and then the absorbance of the sample is detected by enzyme-labeled instrument. Draw a standard curve with absorbance as abscissa and insulin concentration as ordinate and calculate the absorbance of the sample to measure the fasting insulin level of rats.

Finally, the result indicates that the level of insulin is continuously decreasing.

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3.4. Lower blood sugar and glucose metabolism

Analyze blood glucose curves and change in insulin levels to evaluate rat pancreatic islet function and glycometabolism.

As a result, we found out that the blood sugar of these rats is decreased and the secretion of insulin has delayed. Also, we discovered that the level of glycosylated hemoglobin (HbA1c) and glycated albumin have decreased. So the function of glucose metabolism has decreased.

3.5. The cell in keto environment has higher cytotoxicity.

The OD value will increase with the increase of time. The OD value can be measured at 0.5h, 1.0h and 2.0h correspondingly, and the OD value can be controlled at about 1.0. Take out the 96-well plate, detect the OD value of each well at the wavelength of 450nm by enzyme-labeled instrument, analyze the processed data and draw the proliferation curve. To measure the semi-inhibition rate, firstly measure the inhibition rate of the two experiment groups. Use the formula inhibition rate. When the inhibition rate reaches 50%, the concentration of the solution is the semi-inhibition rate (IC50). The higher IC50, the stronger cytotoxicity.

Above all, we predicted that the group of cells in keto environment has higher IC50 value which means it has higher cytotoxicity than cells in normal environment.

4. Conclusion

The ketogenic diet is an alternative therapy for intractable epilepsy. It is a strict diet that is high in fat and low in carbohydrates and protein. It is mainly used for children whose seizures have reached the refractory stage. This diet is strict and must be followed for a long time, and it is said to have a high success rate [8-10]. However, long-term ketogenic diets have been shown to impair pancreatic function and can lead to type 2 diabetes [11-12]. For this reason, insulin levels and other body parameters need to be monitored while on a ketogenic diet, and if abnormalities occur, appropriate medication needs to be administered or the ketogenic diet discontinued.

This study provides data suggesting that long-term consumption of a ketogenic diet leads to a decrease in βcells. In this study, we investigated the specific causes of βcell reduction due to long-term ketogenic diet intake, focusing on changes in the energy metabolism pathways of pancreatic islets. Using the number of apoptotic cells as an indicator, in this study, the number of apoptotic cells was compared between lipid metabolism, glucose metabolism as the main energy supply pathway, and glucose metabolism as the main energy supply pathway after the addition of spies. Based on the available information and experimental data, we can expect that at least the shift in the energy metabolism pathway is an important reason for the reduction of βcells. At the same time, the fact that fat metabolism has no functional advantage over glucose metabolism in providing energy means that other cells in the body may also be damaged or even die due to lack of energy supply, with subsequent side effects. The ketogenic diet has become a hot topic of research due to its efficacy in the treatment of type 2 diabetes and a variety of neurological disorders such as Epilepsy and Alzheimer's disease. Many previous studies have shown that the ketogenic diet reduces the number of βcells, with a variety of potential side effects. However, no experiments have been conducted to examine the reasons for this. This study provides ideas for research on ketogenic diets with a focus on energy metabolism. If the problem of inadequate cellular energy supply due to altered energy metabolism pathways can be addressed, many of the side effects will be mitigated. We can expect to utilize the ketogenic diet more effectively and safely for the treatment of type2 diabetes, obesity, and many neurological disorders.

References

[1] Paoli A, Rubini A, Volek JS, et al. Beyond weight loss: a review of the therapeutic uses of very-low-carbohydrate (ketogenic) diets. Eur J Clin Nutr. 2013;67(8):789–796

[2] Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF., Jr Brain metabolism during fasting. J Clin Invest. 1967;46:1589–1595.

[3] Kessler SK, Neal EG, Camfield CS, Kossoff EH. Dietary therapies for epilepsy: future research. Epilepsy Behav. 2011;22:17–22.

[4] Rieger, A. M., Nelson, K. L., Konowalchuk, J. D., & Barreda, D. R. (2011). Modified annexin v/propidium iodide apoptosis assay for accurate assessment of cell death. Journal of Visualized Experiments, 50, 2597. *https://doi.org/10.3791/2597*

[5] Labat-Moleur, F., Guillermet, C., Lorimier, P., Robert, C., Lantuejoul, S., Brambilla, E., & Negoescu, A. (1998). Tunel apoptotic cell detection in tissue sections: Critical evaluation and improvement. Journal of Histochemistry & Cytochemistry, 46(3), 327–334. *https://doi.org/10.1177/002215549804600306*

[6] Migheli A, Atanasio A, Schiffer D (1995) Ultrastructural detection of DNA strand breaks in apoptotic neural cells by in situ end-labelling techniques. J Pathol 176:27–35

[7] Wijsman JH, Jonker RR, Keijzer R, van de Velde CJH, Cornelisse CJ, van Dierendonck JH (1993) A new method to detect apoptosis in paraffin sections: ISEL of fragmented DNA. J Histochem Cytochem 41:7–12

[8] Henderson, C. B., Filloux, F. M., Alder, S. C., Lyon, J. L., & Caplin, D. A. (2006). Efficacy of the ketogenic diet as a treatment option for epilepsy: Meta-analysis. Journal of Child Neurology, 21(3), 193–198. *https://doi.org/10.2310/7010.2006.00044*

[9] Freeman, J. M., & Freeman, J. M. (edited). (2007). The ketogenic diet: A treatment for children and others with epilepsy (4th ed). Demos: Distributed to the trade by Publishers Group West.

[10] Pietrzak, D., Kasperek, K., Rękawek, P., & Piątkowska-Chmiel, I. (2022). The therapeutic role of ketogenic diet in neurological disorders. Nutrients, 14(9), 1952. *https://doi.org/10.3390/nu14091952*

[11] Ellenbroek, J. H., Van Dijck, L., Töns, H. A., Rabelink, T. J., Carlotti, F., Ballieux, B. E. P. B., & De Koning, E. J. P. (2014). Long-term ketogenic diet causes glucose intolerance and reduced β- and α-cell mass but no weight loss in mice. American Journal of Physiology-Endocrinology and Metabolism, 306(5), E552–E558. *https://doi.org/10.1152/ajpendo.00453.2013*

[12] Bielohuby, M., Sisley, S., Sandoval, D., Herbach, N., Zengin, A., Fischereder, M., Menhofer, D., Stoehr, B. J. M., Stemmer, K., Wanke, R., Tschöp, M. H., Seeley, R. J., & Bidlingmaier, M. (2013). Impaired glucose tolerance in rats fed low-carbohydrate, high-fat diets. American Journal of Physiology-Endocrinology and Metabolism, 305(9), E1059–E1070. *https://doi.org/10.1152/ajpendo.00208.2013*