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The effects of ketogenic diet on beta-hydroxybutyrate, arachidonic acid, and oxidative stress in pediatric epilepsy

Bita Poorshiri [a], Mohammad Barzegar [a], Mohammadreza Afghan [a], Siamak Shiva [a], Parviz Shahabi [b], Zahra Golchinfar [c], Hamid Reza Yousefi Nodeh [a], Sina Raeisi [a,*]

[a] Pediatric Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Department of Food Science and Technology, Faculty of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

The exact mechanism of a ketogenic diet (KD) as a suitable alternative therapeutic approach for drug-resistant epilepsy (DRE) in alleviating seizures is not yet fully understood. The present study aimed to evaluate the role of the KD in reducing oxidative stress (OS) by increasing the ketone body beta-hydroxybutyrate (BHB) and Arachidonic acid (ARA), an essential polyunsaturated fatty acid, as a possible mechanism in relieving seizure attacks in children with DRE. Forty children with refractory epilepsy were included in the present study. The serum levels of BHB, ARA, and OS markers, malondialdehyde (MDA), and 8-hydroxyl-deoxyguanosine (8-OHdG), were evaluated in children with DRE and compared before and after the three months of KD therapy. Thirty-four of 40 included children could complete the three-month KD therapy. Twenty-one (61.76%) patients had more than a 50% reduction in seizure frequency after the KD (responders). The remaining 13 children were considered non-responders to the diet. The serum levels of ARA and BHB significantly ($p < 0.05$) increased after the KD therapy. The serum levels of OS parameters MDA and 8-OHdG before the diet therapy were significantly ($p < 0.05$) higher than those after the administration. The serum levels of BHB and MDA after the KD therapy in the responders were respectively higher and lower than those in the non-responders ($p < 0.001$). Ketogenic diet might reduce brain OS by increasing BHB and ARA. The role of BHB in diminishing OS and seizure might be more remarkable than ARA.

1. Introduction

Epilepsy is one of the most common neurological diseases that consists of recurrent spontaneous seizure attacks caused by an imbalance between stimulation and inhibition of the brain [1]. Recurrent seizures in this disease may cause progressive nerve damage leading to reduced quality of life and even life-threatening episodes [1,2]. Most of the treatments applied for this disease are the use of various anti-seizure medications (ASMs). However, 20–30% of the patients have seizures that are not controlled by these drugs [drug-resistant epilepsy (DRE)] [1]. According to the definition by International League Against Epilepsy (ILAE), refractory epilepsy is defined as "failure of adequate trials of two tolerated, appropriately chosen and used ASM schedules (whether as monotherapies or in combination) to achieve the sustained seizure freedom" [3]. Surgical and dietary therapies are the most commonly used drug-independent treatment strategies for DRE management [1]. Epileptic surgery aims to eliminate or reduce seizure attacks by removing the seizure onset zone or limiting the spread of seizure activity without loss of normal brain function [2]. Due to the unavailability, high cost, and possible complications of surgical procedures, diet therapy may be a more appropriate alternative therapy for DRE management [1,2]. A ketogenic diet (KD) that has been used to treat patients with refractory epilepsy since the early 1920s includes a 4: 1 (3: 1 in young children) ratio of fat to carbohydrate plus protein. It means about 90 percent of daily calories come from fat [2,4].

The exact mechanism of the KD in alleviating seizures is not yet fully understood; however, various factors may be involved [2]. It has been revealed that ketone bodies and polyunsaturated fatty acids (PUFAs) have prominent roles in the anticonvulsant effect of KD [2,5,6]. These substances have been shown to reduce the production of reactive oxygen species [7,8]. Given that oxidative stress

* Corresponding author at: Pediatric Health Research Center, Zahra Mardani Azari Children Training, Research and Treatment Center, Mardani Azari Street, Khavaran Town, Tabriz, Iran.
E-mail addresses: sina_raeisi7007@yahoo.com, raeisis@tbzmed.ac.ir (S. Raeisi).

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(OS) can predispose to seizure [9], the present study aimed to assess whether KD could diminish OS and seizures by increasing ketone bodies and PUFAs. It was assumed as a possible antiseizure mechanism of KD.

2. Materials and methods

2.1. Patients and study design

A non-randomized, pre/post-intervention study was carried out among 73 children with DRE referred to Tabriz Children Hospital (Tabriz, Iran) from March 2019 to November 2020. Refractory epilepsy was considered according to the ILAE definition [3]. The inclusion criteria were the children aged 2 to 15 years, had seizures at least once a month, had not responded to at least two ASMs, and had not previously been treated with the KD. Hyperlipidemia, chronic diseases, progressive neurologic disorders, congenital metabolic disorders, liver diseases, systemic diseases, kidney disorders, and noncompliance with KD were considered as exclusion criteria. Of the 73 referred children, 21 patients did not meet the inclusion criteria, and 12 refused to participate. Forty patients were included in the study to receive a three-month KD therapy after obtaining informed consent from their parents. A flow chart of the study design is presented in Fig. 1.

2.2. Ketogenic diet

The diets were calculated on an individual basis by a dietitian according to each patient's regular diet and the recommendations for energy and calorie requirements [10]. A non-fasting induction protocol was used, and no changes in the children's daily normal diet were recommended before initiating the KD. The KD was introduced during a 5-day hospitalization at the trial center. The KD consisted of 1 (0.8–1.2) g/kg protein, 5–10 g/day carbohydrate, and high fat (90% of total daily calorie intake). However, calories were restricted to 75% recommended daily intake [11]. The lipid-to-nonlipid ratio was 4:1 for three months. The start of the diet was defined as the first change made to the patient's daily diet. Diets were fully supplemented with vitamins and minerals. The parents were made to be familiar with the implications of the KD and were prepared to be available for regular monitoring of the children at home. The children were monthly visited as outpatients and monitored by telephone calls between the clinic visits. Urine ketone levels were checked twice daily by the parents at home using keto strips. Manipulations in the diets were made if necessary to maintain proper ketosis. The end of the diet was considered the first step in down-titration to a regular diet. No changes were made to the children's ASMs medication during the three-month study period. The seizure frequencies, demographic and clinical characteristics of the patients were recorded at baseline at the hospital and during the three-month follow-up according to the parents' report.

2.3. Seizure evaluation

A seizure cluster (≥5 seizures in a short time) was calculated as one seizure for the seizure frequency calculations. The seizure reduction was calculated as the ratio of the average seizure number (frequency) in one month (30 days) before the time point to the average seizure number in the month before the diet initiation (baseline). Children with a ≥50% reduction in seizure frequency were considered responders and those with a <50% seizure reduction as non-responders. Based on the previous studies [12–14], not only complete seizure control but also a seizure reduction of 50% can be considered as an improvement in children with DRE.

2.4. Sampling and biochemical tests

Venous blood samples were collected from the children before and after the three months of the KD therapy. The children were

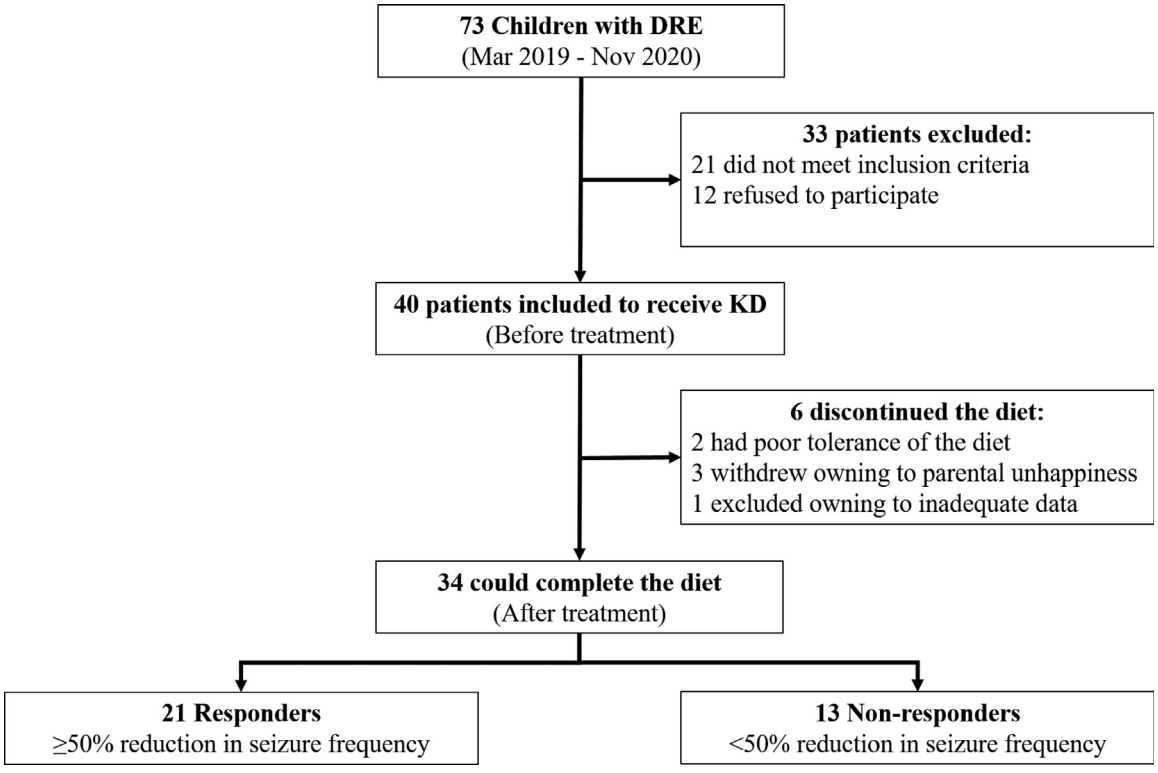

Fig. 1. Study flowchart.

clinically checked for any condition possibly affecting oxidative stress markers such as inflammatory or infectious disease immediately prior to the blood sampling. No inflammatory condition was diagnosed among the patients. All blood samples were collected in the morning following a 12-hour fast. Sera were subsequently separated after centrifugation at 3000 rpm for 10 min at room temperature and stored at −70 °C until analysis. Serum BHB was measured enzymatically by a biochemical autoanalyzer (MINDRAY, China) using a commercially available kit (assay range, 0.01–0.32 mmol/L; sensitivity, 0.01 mmol/L; intra-assay, CV < 6.6%; and inter-assay, CV < 10%; Biorex Fars; Shiraz, Iran) according to the manufacturer's instruction. Arachidonic acid was measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Fine Biotech Co., Ltd; China). Malondialdehyde (MDA) level as a lipid peroxidation marker was evaluated spectrophotometrically using a thiobarbituric acid reactive substances assay based on the method of Lapenna et al. [15]. 8-hydroxyldeoxyguanosine (8-OHdG), another marker of OS, was evaluated by an ELISA kit (Cusabio Biotech Co; China).

2.5. Ethics

The study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.1110) and registered in the Iranian Registry of Clinical Trials (https://www.irct.ir; IRCT Registration Number: IRCT20131012014988N4; 2018-05-15).

2.6. Statistical analyses

The sample size was calculated based on the previous studies [16,17]. Considering the 80% power and $\alpha = 0.05$, the sample size was calculated as 30 patients. Assuming a 20% dropout rate, a minimum sample of 38 children was required.

The data were analyzed by SPSS software (version 16.0; SPSS, Inc., Chicago, IL). Initially, the variables were statistically checked for normality by the one-sample Kolmogorov–Smirnov test. All variables had normal distributions and therefore were shown as mean ± standard deviation. Qualitative variables including sex, positive family history, and response to KD therapy, were shown as percentages. The paired-samples t-test and McNemar test were respectively applied to compare continuous and categorical variables before and after the treatment. Independent samples t-test and Chi-Square test were respectively used to compare quantitative and qualitative variables between the boys and girls. Independent samples t-test, Univariate analysis of variance, and Chi-square test were applied to compare the data between the responders and non-responders groups where it was appropriate. The correlations of the variables were analyzed by the Pearson correlation coefficient method. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

In the present study, 34 of 40 included children could complete the three-month of KD therapy. Six patients did not complete the trial due to poor diet tolerance (acute constipation and vomiting), parental unhappiness, and inadequate data.

The serum levels of BHB, ARA, and OS markers (MDA and 8-OHdG) were evaluated and compared before and after the three months of KD therapy. The demographic characteristics of the studied children were depicted in Table 1. The children consisted of 18 (52.94%) boys and 16 (47.06%) girls with a mean age of 4.2 1 ± 1.39 years. The mean age of seizure onset was 1.57 ± 0.46 years. Nine (26.5%) children had a positive family history of epilepsy. Of 34 DRE patients, 18 (52.94%), 14 (41.18%), and 2 (5.88%) children were diagnosed with symptomatic, cryptogenic, and idiopathic epilepsy, respectively. The seizure types were generalized tonic-clonic, myoclonic, epileptic spasm, focal without awareness, and focal in 10 (29.41), 9 (26.47), 6 (17.65), 3 (8.82%), and 1 (2.94%) patients, respectively. Four (11.76%) patients had both generalized tonic-clonic and myoclonic seizures, and one (2.94%) had both focal seizures and epileptic spasms.

The ASMs used by patients (as monotherapies or in combination) in the KD treatment period were phenobarbital (22, 64.71%), valproic acid (18, 52.94%), clobazam (17, 50%), vigabatrin (9, 26.47%), primidone (8, 23.53%), levetiracetam (5, 14.71%), phenytoin (5, 14.71%), carbamazepine (3, 8.82%), lamotrigine (2, 5.88%), nitrazepam (2, 5.88%), and topiramate (2, 5.88%). Among the 34 children on the diet, 16 (47.06%) experienced some mild side effects. The reported side effects during the diet therapy period were constipation [11 (32.35%)], vomiting [7 (20.59%)], hunger [6 (17.65%)], abdominal pain [4 (11.76%)], fatty diarrhea [4 (11.76%)], and kidney stone [1 (2.94%)].

Among included children, 21 (61.76%) patients had a ≥50% reduction in seizure frequency after the KD therapy and were considered as responders. The remaining 13 children with <50% reduction in seizure frequency were considered non-responders to the diet. No seizure-free patient was identified after the diet therapy.

Serum lipids, total cholesterol (TC), and triglyceride (TG) were measured and compared before and after the KD therapy. As

Table 1
Demographic characteristics of the studied children with DRE (n = 34).

Variables	
Age (year)	4.21 ± 1.39
Sex (F/M)	16/18
Age of onset (year)	1.57 ± 0.46
Positive family history of epilepsy [n (%)]	9 (26.5)
Epilepsy etiology	
Symptomatic [n (%)]	18 (52.94)
Cryptogenic [n (%)]	14 (41.18)
Idiopathic [n (%)]	2 (5.88)
Seizure type	
GTC [n (%)]	10 (29.41)
Myo [n (%)]	9 (26.47)
ES [n (%)]	6 (17.65)
GTC, Myo [n (%)]	4 (11.76)
FWA [n (%)]	3 (8.82)
ES, Foc [n (%)]	1 (2.94)
Foc [n (%)]	1 (2.94)
AEDs	
Phenobarbital (3–6 mg/kg/day) [n (%)]	22 (64.71)
Valproic acid (10–15 mg/kg/day) [n (%)]	18 (52.94)
Clobazam (0.5–1 mg/kg/day) [n (%)]	17 (50.00)
Vigabatrin (25–50 mg/kg/day) [n (%)]	9 (26.47)
Primidone (10–25 mg/kg/day) [n (%)]	8 (23.53)
Levetiracetam (10–30 mg/kg/day) [n (%)]	5 (14.71)
Phenytoin (8–10 mg/kg/day) [n (%)]	5 (14.71)
Carbamazepine (10–20 mg/kg/day) [n (%)]	3 (8.82)
Lamotrigine (4.5–7.5 mg/kg/day) [n (%)]	2 (5.88)
Nitrazepam (0.5–1 mg/kg/day) [n (%)]	2 (5.88)
Topiramate (5–9 mg/kg/day) [n (%)]	2 (5.88)
Side effects	
Constipation [n (%)]	11 (32.35)
Vomiting [n (%)]	7 (20.59)
Hunger [n (%)]	6 (17.65)
Abdominal pain [n (%)]	4 (11.76)
Fatty diarrhea [n (%)]	4 (11.76)
Kidney stone [n (%)]	1 (2.94)
Response to KD	
<50% Seizure reduction [n (%)]	13 (38.24)
≥50% Seizure reduction [n (%)]	21 (61.76)

AEDs, antiepileptic drugs; ES, epileptic spasm; DRE, Drug-resistant epilepsy; GTC, generalized tonic-clonic; F, female; Foc, focal; FWA, focal without awareness; KD, ketogenic diet; M, male; Myo, myoclonic.

shown in Table 2, the serum levels of TC (152.21 ± 35.82, mg/dL), and TG (99.21 ± 35.90, mg/dL) significantly ($p < 0.001$) increased after the diet (188.65 ± 57.48, 148.18 ± 63.42; mg/dL; respectively). The presence of high serum TC (>200 mg/dL) or high TG levels (>130 mg/dL) was considered dyslipidemia [18]. The prevalence of dyslipidemia, high serum TC or TG levels, after the diet therapy [11 (32.4%), 17 (50%); respectively] were significantly ($p = 0.031$, $p = 0.008$; respectively) more than those before the diet [5 (14.7%), 9 (26.5%); respectively].

A comparison of the main measured parameters, ARA, BHB, MDA, and 8-OHdG, in DRE children before and after the KD, was shown in Fig. 2. As shown in A and B, the serum levels of ARA (16.86 ± 6.04, μg/mL) and BHB (0.56 ± 0.34, mmol/L) significantly ($p < 0.001$) increased after the KD therapy (28.86 ± 16.53, μg/mL; 3.67 ± 1.78, mmol/L; respectively). The serum levels of OS parameters, MDA (8.54 ± 2.03, nmol/L) and 8-OHdG (13.83 ± 5.40, ng/mL) before the diet therapy were significantly ($p < 0.001$, $p = 0.021$; respectively) higher than those (5.61 ± 2.79, nmol/L; 12.29 ± 5.22 , ng/mL; respectively) after the administration (Fig. 2C and D).

The evaluated parameters before and after the administration were also compared between the responders and non-responders groups. As shown in Table 3, the mean age (year), sex distribution, mean age of onset (year), serum lipids, and frequencies of dyslipidemia were not significantly different between the groups ($p > 0.05$). However, the number of patients with a positive family history of epilepsy among non-responders [6 (46.2%)] was significantly ($p = 0.041$) higher than in the responders' group [3 (14.3%)]. The comparisons of serum BHB, ARA, and OS markers between the responders and non-responders groups were depicted in Fig. 3. As shown in Fig. 3A, the serum levels of ARA after the KD therapy in both responders (30.57 ± 17.04, μg/mL) and non-responders (26.10 ± 15.94, μg/mL) were significantly ($p = 0.001$, $p = 0.019$; respectively) higher than those (17.45 ± 6.49, 15.91 ± 5. 35; μg/mL; respectively) before the administration. The serum levels of BHB after the diet therapy in both responders (4.70 ± 1. 41, mmol/L) and non-responders (2.00 ± 0.75, mmol/L) were significantly ($p < 0.001$) higher than those (0.55 ± 0.35, 0.58 ± 0.33; mmol/L; respectively) before the diet. Also, the serum BHB levels after the administration among responders were significantly higher than in the non-responders group ($p < 0.001$) after adjusting for its baseline value (Fig. 3B). The serum MDA levels before the diet in both responders (8.10 ± 1.85, nmol/L) and non-responders (9.25 ± 2.18, nmol/L) were significantly ($p < 0.001$, $p = 0.005$; respectively) higher than those (4.28 ± 2.29, 7.75 ± 2.16; nmol/L; respectively) after the diet. Also, the serum MDA levels after the administration in the responders' group were significantly ($p < 0.001$) lower than in the non-responders after adjusting for MDA value before the diet (Fig. 3C). As depicted in Fig. 3D, the serum levels of 8-OHdG were significantly ($p = 0.024$) decreased after the diet therapy (13.18 ± 4.98 *vs.* 10.98 ± 4.22, ng/mL) only in the responders' group. Any other significant difference in the evaluated parameters before and after the diet was not found among the groups.

Table 2
Serum lipids and frequencies of dyslipidemia in DRE children before and after the KD.

Parameters	Before	After	p-value
TC (mg/dL)	152.21 ± 35.82	188.65 ± 57.48	<0.001[a*]
TG (mg/dL)	99.21 ± 35.90	148.18 ± 63.42	<0.001[a*]
TC > 200 mg/dL (n/%)	5 (14.7)	11 (32.4)	0.031[b*]
TG > 130 mg/dL (n/%)	9 (26.5)	17 (50)	0.008[b*]

DRE, drug resistant epilepsy; TC, total cholesterol; TG, triglyceride.
*Statistically significant ($p < 0.05$); [a] compared by Paired-samples t-test; [b] compared by McNemar test.

The evaluated parameters were also compared between boys and girls. As shown in Table 4, none of the evaluated parameters was significantly different between the male and female patients ($p > 0.05$).

The correlations between the measured parameters after the administration were also evaluated. As shown in Fig. 4, serum levels of ARA and BHB were negatively correlated with MDA ($r = -0.425$, $p = 0.012$; $r = -0.642$, $p < 0.001$; respectively) and 8-OHdG ($r = -0.360$, $p = 0.036$; $r = -0.474$, $p = 0.005$; respectively). The correlation between MDA and 8-OHdG was also significant ($r = 0.524$, $p = 0.001$). No other significant correlation was found among the serum-measured parameters after the KD therapy.

4. Discussion

In the present study, the serum levels of BHB, ARA, and the OS markers MDA and 8-OHdG were evaluated in children with DRE and compared before and after the three months of KD therapy. Ketogenic diet has long been used as an adjunct therapy for DRE, especially in nonsurgical patients.

This diet therapy has been used in DRE patients to simulate the metabolic state of fasting regarding its proven effect in reducing seizure frequency [2]. In the present study, 21 (61.76%) patients had a ≥50% reduction in seizure frequency after the KD therapy who were considered as responders. The remaining 13 children with <50% reduction in seizure frequency were non-responders to the diet. In a systematic review by Keene et al. [19], the results showed that 33% of DRE patients under KD gained >50% reduction in their seizure, and 15.6% of them gained total seizure control. In a previous study by our group [4], 45.8% of children with DRE had ≥50% response to the KD therapy.

Although KD is an effective treatment against pediatric DRE, it is not as safe as a normal diet. It may sometimes produce different side effects that among which gastrointestinal disturbances are suggested as its most frequent adverse effect [2]. In the present study, two included children who suffered from acute constipation and vomiting and therefore quit the diet. Other reported side effects including constipation, vomiting, hunger, abdominal pain, fatty diarrhea, and kidney stone during the administration period were mild and did not lead to diet discontinuation. Ketogenic diet may also cause dyslipidemia [hypercholesterolemia (>200 mg/dL), and hypertriglyceridemia (>130 mg/dL)] [2,18]. In the present study, the serum levels of TC and TG and the dyslipidemia prevalence were significantly increased after the diet.

Although all mechanisms associated with KD-reduced seizures have not been fully elucidated, it has been shown that ketone bodies and PUFAs, which can increase under KD, may play primary roles [2].

The present study results showed that serum levels of BHB increased after three months of KD in children with DRE. During the KD therapy, the metabolic efficiency of the tricarboxylic acid (TCA) cycle decreases, and the body's energy is generally obtained from the oxidation of fatty acids in the mitochondria, which leads to the production of large amounts of acetyl-CoA. Accumulation of acetyl-CoA leads to synthesizing two primary ketone bodies, BHB, and acetoacetate, mainly in the liver entering the bloodstream. Acetone, another ketone body, is a metabolite of acetoacetate that is eventually excreted by breathing and urine [20]. Some monocarboxylate transporters and specific mitochondrial enzymes are present in the blood-brain barrier and brain allowing the components of blood ketones to be extracted and used by the brain. This is why ketone bodies in glucose deficiency can be used as an alternative energy source in the brain [2,21]. In the brain, ketone bodies can be converted to acetyl-CoA and then enter the TCA cycle in the

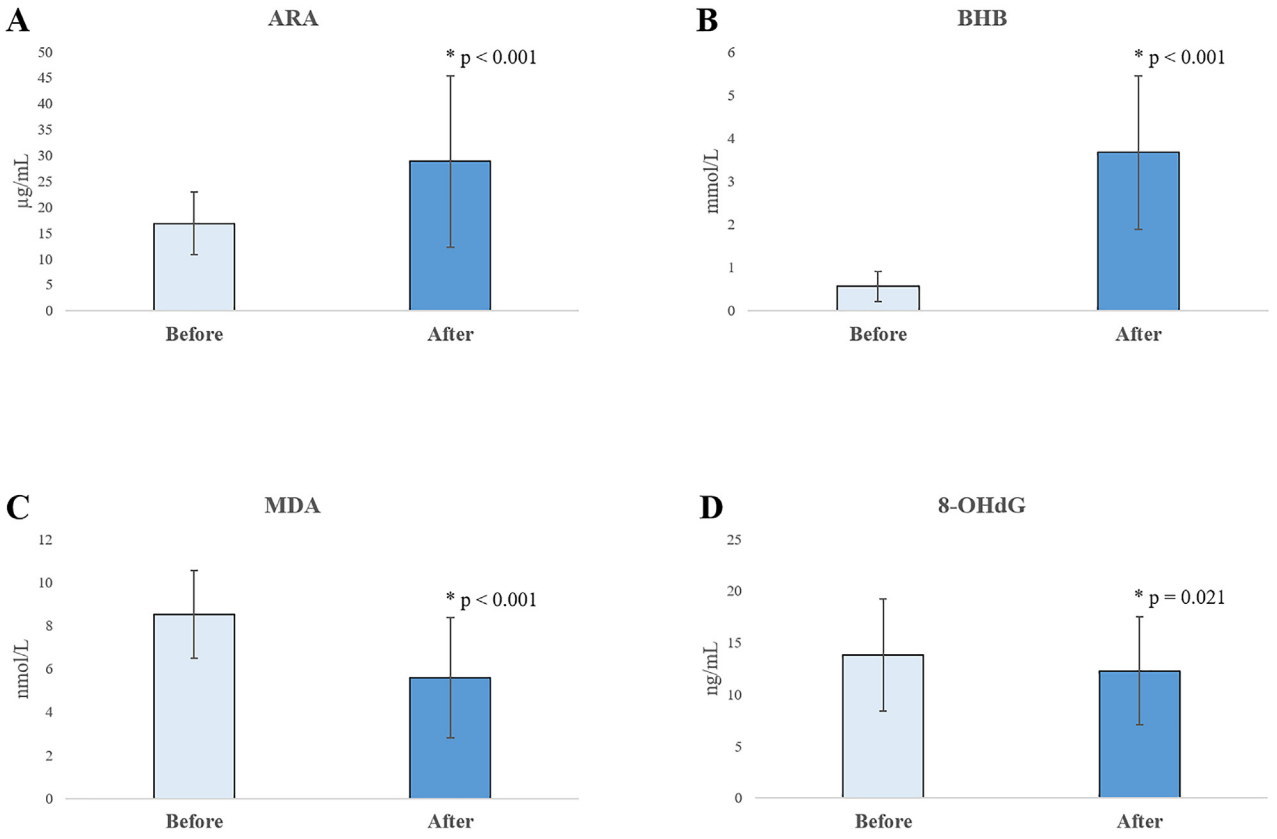

Fig. 2. Comparison of serum BHB, ARA, and oxidative stress markers in the studied children with DRE before and after the KD therapy. (A) The serum levels of ARA (28.86 ± 16.53, µg/mL) after the KD therapy were significantly ($p < 0.001$) higher than those (16.86 ± 6.04, µg/mL) before the administration. (B) The serum levels of BHB (3.67 ± 1.78, mmol/L) after the KD therapy were significantly ($p < 0.001$) higher than those (0.56 ± 0.34, mmol/L) before the administration. (C) The serum levels of MDA (8.54 ± 2.03, nmol/L) before the diet therapy were significantly ($p < 0.001$) higher than those (5.61 ± 2.79, nmol/L) after the diet therapy. (D) The serum levels of 8-OHdG (13.83 ± 5.40, ng/mL) before the diet therapy were significantly ($p = 0.021$) higher than those (12.29 ± 5.22, ng/mL) after the diet therapy. ARA, arachidonic acid; BHB, beta-hydroxybutyrate; DRE, drug-resistant epilepsy; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine. *Statistically significant ($p < 0.05$); compared by Paired-samples t-test.

Table 3
Demographic characteristics in responders and non-responders to KD.

Parameters	Non-responders (n = 13)	Responders (n = 21)	p-value
Age (year)	4.08 ± 1.32	4.29 ± 1.45	0.677[a]
Sex (F/M)	6/7	10/11	0.934[b]
Age of onset (year)	1.58 ± 0.45	1.57 ± 0.48	0.974[a]
Positive family history of epilepsy (n/%)	6 (46.2)	3 (14.3)	0.041[b*]
TC – before (mg/dL)	153.77 ± 35.38	151.24 ± 36.93	0.845[a]
TC – after (mg/dL)	181.54 ± 45.78	193.05 ± 64.34	0.578[a]
TG – before (mg/dL)	105.69 ± 37.88	95.19 ± 34.95	0.416[a]
TG-after (mg/dL)	138.15 ± 37.42	154.38 ± 75.43	0.477[a]
TC > 200 mg/dL – before (n/%)	2/15.38	3/14.29	0.930[b]
TC > 200 mg/dL – after (n/%)	4/30.77	7/33.33	0.877[b]
TG > 130 mg/dL – before (n/%)	5/38.46	4/19.05	0.212[b]
TG > 130 mg/dL – after (n/%)	6/46.15	11/52.38	0.724[b]

KD, ketogenic diet; TC, total cholesterol; TG, triglyceride.
*Statistically significant ($p < 0.05$); [a] compared by Independent-samples t-test; [b] compared by Chi-square test.

mitochondria. Eventually, it leads to the production of adenosine triphosphate (ATP) and energy [22].

In the present study, serum ARA was also detected to be increased after the KD therapy. Arachidonic acid is one of the essential PUFAs. These fatty acids have more than one double bond in their hydrocarbon chains [23]. The brain contains high amounts of PUFAs (25–30%) [24]. Polyunsaturated fatty acids are essential for the growth, maintenance, and function of the nervous system [25]. The endothelial cells of the brain capillary network may contain some specific fatty acid transporters facilitating the transfer of PUFAs into the brain [26]. Dietary PUFAs strongly influence PUFA content in all brain structures. Change in fatty acid content has been revealed to affect the function of brain cells [24]. The four double bonds in the ARA structure give membranes selective flexibility, fluidity, and permeability that affect specific membrane proteins involved in cellular signaling. These characteristics may explain the possible role of ARA in neuronal function [27]. It was also shown in the present study that 21 out of 34 (61.76%) children had a ≥50% reduction in seizures after KD treatment, which may be related to the BHB and ARA increase. In a study by Fraser et al. [28], serum BHB levels increased in all children after KD. Elevated serum levels in the majority of PUFAs after KD were also observed in children (ARA, 1.6 to 2.9-fold; docosahexaenoic acid, 1.5 to 4.0-fold). High serum ARA was associated with improved seizure control in children.

Various mechanisms have been proposed for the anticonvulsant properties of ketone bodies and PUFAs [2]. One important mechanism might be related to OS reduction [7,8]. The most consequential effect of elevated free radicals is lipid peroxidation, which causes cell membrane disruption, leading to their destruction. The brain's high oxygen consumption and PUFA content, make it susceptible to lipid peroxidation and oxidative damage [1]. The OS pathway can also cause oxidative damage to nucleic acids. MDA (high in PUFAs) and 8-OHdG are common end-products of lipid peroxidation and DNA oxidation, respectively. They are stable

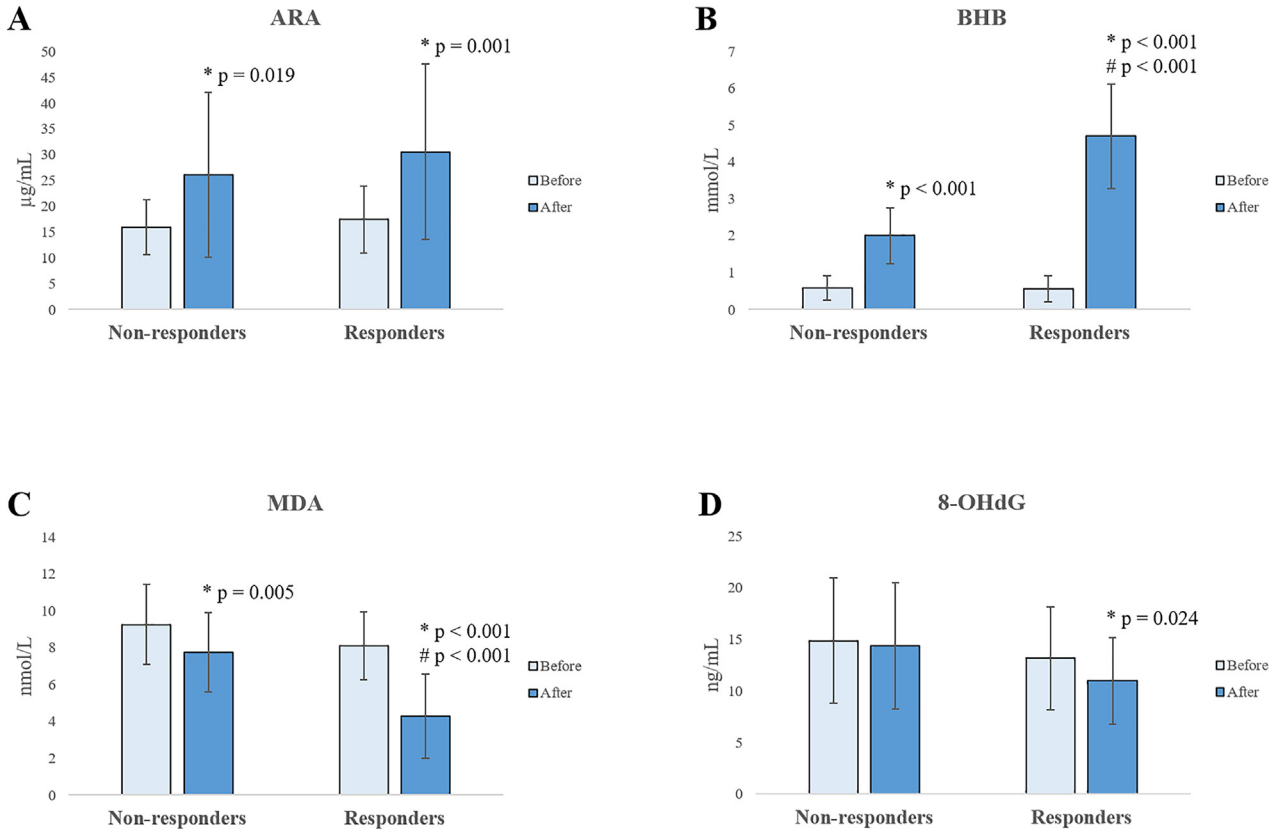

Fig. 3. Comparison of serum BHB, ARA, and oxidative stress markers between the responders and non-responders groups. (A) The serum levels of ARA after the KD therapy in both responders (30.57 ± 17.04, µg/mL) and non-responders (26.10 ± 15.94, µg/mL) were significantly (p = 0.001, p = 0.019; respectively) higher than those (17.45 ± 6.49, 15.91 ± 5.35; µg/mL; respectively) before the administration. (B) The serum levels of BHB in both responders (4.70 ± 1.41, mmol/L) and non-responders (2.00 ± 0.75, mmol/L) after the diet were significantly (p < 0.001) higher than those (0.55 ± 0.35, 0.58 ± 0.33; mmol/L; respectively) before the diet. Also, the serum BHB levels after the administration among responders were significantly higher than in the non-responders group after adjusting for its value before the diet (p < 0.001). (C) The serum MDA levels before the diet in both responders (8.10 ± 1.85, nmol/L) and non-responders (9.25 ± 2.18, nmol/L) were significantly (p < 0.001, p = 0.005; respectively) higher than those (4.28 ± 2.29, 7.75 ± 2.16; nmol/L; respectively) after the diet. The serum MDA levels after the administration in the responders' group were significantly lower than in non-responders after adjusting for MDA value before the diet (p < 0.001). (D) The serum levels of 8-OHdG after the administration (13.18 ± 4.98, ng/mL) were significantly (p = 0.024) lower than those before the diet (10.98 ± 4.22, ng/mL) in the responders' group. ARA, arachidonic acid; BHB, beta-hydroxybutyrate; DRE, drug-resistant epilepsy; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine. *Statistically significant (p < 0.05) compared to before KD (compared by Paired-samples t-test). #Statistically significant (p < 0.05) compared to non-responders after KD [compared by Univariate analysis of variance (ANCOVA)].

Table 4
The comparison of the parameters between boys and girls.

Parameters	Boys (n = 18)	Girls (n = 16)	p-value
Age (year)	4.33 ± 1.28	4.06 ± 1.53	0.578[a]
Age of onset (year)	1.58 ± 0.49	1.56 ± 0.44	0.898[a]
Positive family history of epilepsy (n/%)	5 (27.8)	4 (25.0)	0.855[b]
TC-before (mg/dL)	156.50 ± 31.94	147.38 ± 40.25	0.467[a]
TC-after (mg/dL)	193.28 ± 60.53	183.44 ± 55.32	0.626[a]
TG-before (mg/dL)	103.39 ± 34.70	94.50 ± 37.77	0.480[a]
TG-after (mg/dL)	140.06 ± 51.40	157.31 ± 75.40	0.437[a]
ARA-before (µg/mL)	17.50 ± 6.12	16.15 ± 6.06	0.526[a]
ARA-after (µg/mL)	26.31 ± 14.75	31.74 ± 18.38	0.346[a]
BHB-before (mmol/L)	0.54 ± 0.33	0.59 ± 0.36	0.691[a]
BHB-after (mmol/L)	3.66 ± 1.71	3.68 ± 1.92	0.980[a]
MDA-before (nmol/L)	8.67 ± 1.82	8.40 ± 2.29	0.708[a]
MDA-after (nmol/L)	5.99 ± 2.94	5.18 ± 2.64	0.404[a]
8-OHdG-before (ng/mL)	13.10 ± 4.02	14.65 ± 6.66	0.414[a]
8-OHdG-after (ng/mL)	12.02 ± 3.17	12.58 ± 6.95	0.760[a]
Responders/non-responders (n/%)	11(61.11)/7 (38.89)	10(62.50)/6 (37.50)	0.934[b]

ARA, arachidonic acid; BHB, beta-hydroxybutyrate; DRE, drug-resistant epilepsy; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; TC, total cholesterol; TG, triglyceride.
[a] Compared by Independent-samples t-test; [b] compared by Chi-square test.

during OS and can be detected in human body fluids or tissues [29–31]. In the present study, the levels of 8-OHdG and MDA were evaluated, and the results showed that these OS markers decreased in the studied children after the KD treatment. There were also significant negative correlations between BHB and ARA levels with these OS markers. Regarding the antioxidant effect of BHB, it has been shown that BHB is an endogenous and specific inhibitor of histone deacetylases (HDACs) [32]. In a study by Shimazu et al. [32] administration of exogenous BHB, calorie restriction, or fasting, conditions associated with BHB increase, leading to increased histone acetylation in rat tissues. Inhibition of HDAC by BHB was correlated with changes in expression of the OS-resistance genes, *Forkhead box class O3a* (*FoxO3a*) and *Metallothionein-2* (*MT2*). In the in-vitro model, BHB treatment of the cells induced *FoxO3a* and *MT2* by histone acetylation of their promoters. Both genes were activated by selectively reducing HDAC1 and HDAC2. Consistent with increased *FoxO3a* and *MT2* activity, BHB treatment provided significant protection against OS. Kong et al. [33] also investigated the effects of BHB on the suppression of OS by inhibiting HDACs in in-vivo and in-vitro models. The in-vivo model showed that the three-week administration of KD by increasing BHB lead to down-regulating the expression of nicotinamide adenine dinucleotide phosphate (*NADPH)* oxidase (*NOX)2*, and *NOX4* in rats. Also, *FoxO3a*,

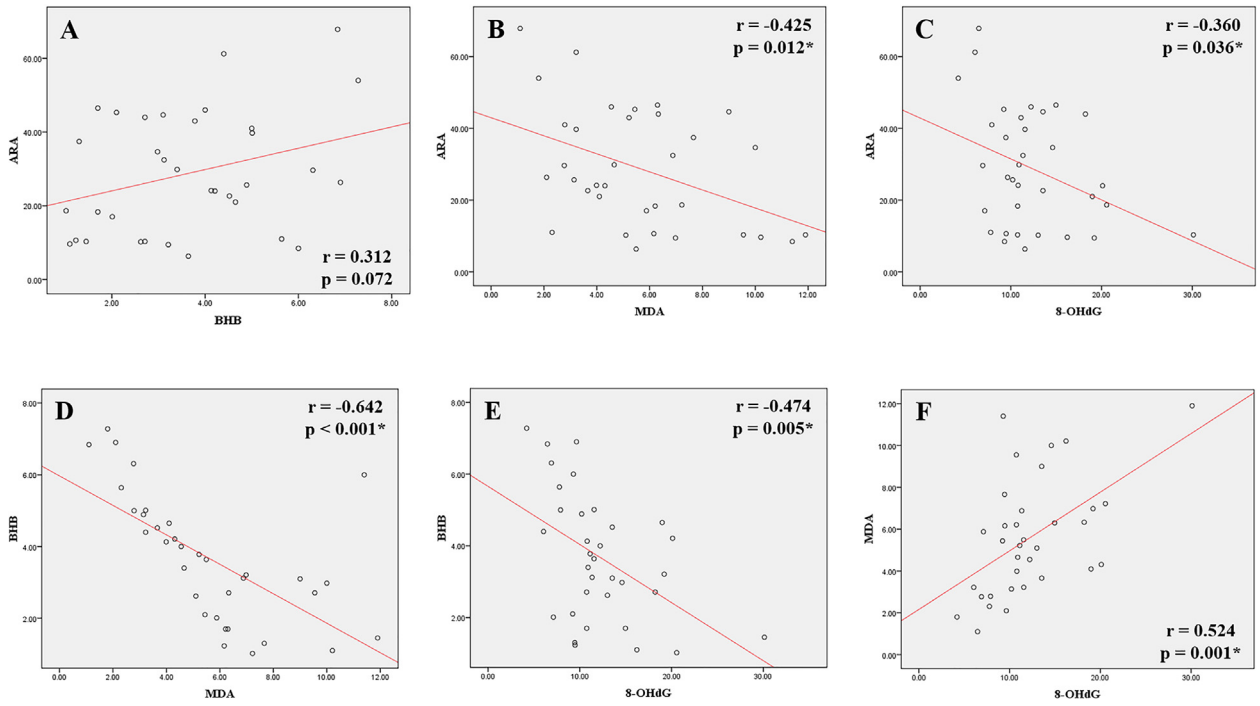

Fig. 4. The correlations between serum measured parameters after ketogenic diet therapy. (A) The positive correlation between serum levels of Arachidonic acid (ARA) and Beta-hydroxybutyrate (BHB) was not significant (r = 0.312, p = 0.072). (B) Serum levels of ARA and malondialdehyde (MDA) were negatively correlated (r = −0.425, p = 0.012). (C) Serum levels of ARA and 8-hydroxydeoxyguanosine (8-OHdG) were negatively correlated (r = −0.360, p = 0.036). (D) Serum levels of BHB and MDA were negatively correlated (r = −0.642, p < 0.001). (E) Serum levels of BHB and 8-OHdG were negatively correlated (r = −0.474, p = 0.005). (F) Serum levels of MDA and 8-OHdG were positively correlated (r = 0.524, p = 0.001). The correlations of the variables were analyzed by the Pearson correlation coefficient method.

mitochondrial superoxide dismutase (*MnSOD*), and *catalase* were upregulated after KD treatment. Therefore, it was proposed that KD could reduce spinal cord injury (SCI) by reducing OS. The in-vitro study showed that BHB could dose-dependently inhibit the H2O2-induced ROS production through downregulating *NOX2* and *NOX4*, and upregulating *FoxO3a*, *MnSOD*, and catalase in PC12 cells. It was also shown that BHB inhibited HDAC1, HDAC2, and HDAC3 activity, but not HDAC8 in SCI rats and PC12 cells.

Regarding the antioxidant effects of ARA, Wang et al. [34] showed that ARA enhanced the antioxidant enzymes, SOD and catalase, in the rat hippocampal slices in a dose-dependent manner through the Peroxisome proliferator-activated receptor gamma (PPARγ) pathway, thus protecting against glutamate and H2O2 damage. Qu et al. [35] showed that ARA reduced brain damage in a rat ischemia/reperfusion model by inhibiting the inflammatory response and OS. Arachidonic acid treatment increased SOD and Glutathione peroxidase (GSH-Px) while decreasing MDA levels. In the present study, the measured parameters were also compared between the responders and nonresponders groups. Serum levels of BHB and MDA among responders were significantly more and less than nonresponders, respectively, after KD therapy. This result could indicate that the role of BHB might be more remarkable than ARA in relieving seizures. Also, MDA might be a more sensitive indicator of OS than 8-OHdG in the nervous system.

The results also revealed that patients with a family history of epilepsy were more common in the nonresponders than respondents, raising the possibility of a lack of treatment response to the KD in familial forms of epilepsy. The results also showed that there was no significant difference in the evaluated parameters between the boys and girls, indicating that the anti-seizure effect of KD might not be affected by gender or sexual hormones. However, more accurate studies with larger sample sizes are needed.

5. Conclusion

In conclusion, it was shown in the present study that KD, as a suitable alternative therapeutic approach for DRE, might reduce brain OS by increasing BHB and ARA. The role of BHB in diminishing OS and seizure might be more remarkable than ARA. Also, MDA might be a more sensitive indicator of nervous system OS than 8-OHdG. Given that the increase in free radicals can predispose to seizure development, the reduction of OS can be considered as a possible mechanism in the anticonvulsant properties of KD. However, further studies with larger sample sizes are needed to confirm the results. Seizure intensity improvement as the decrease in the seizure duration was reported by some parents of children in the non-responders group. However, the given information seemed to be not reliable and therefore, they were not recorded in the questionnaires. It could be considered a study limitation. As other study limitations of the present study, there was no control group consisting of patients not under KD; the association of the therapeutic effect of KD with time and dose was not determined; and behavioral issues and cognitive function were not evaluated. Also, the possible effects of KD on other PUFAs and metabolites such as renal and liver function biomarkers, electrolytes, hydration status, and their relationship with OS were not investigated.

Response to KD therapy might be multifactorial, and therefore, it is imperative that other possible mechanisms related to the anticonvulsant properties of KD be evaluated in future studies.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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