Ketogenic diet protects against epileptogenesis as well as neuronal loss in amygdaloid-kindling seizures

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ABSTRACT

Ketogenic diets (KD) have shown beneficial effects in terms of anticonvulsant and anti-epileptogenic properties in several experimental models. However, few studies have investigated the consequences of KD with regards to the anti-epileptogenic and neuroprotective effects in kindling-induced seizures. Here, postnatal day 28 male Sprague-Dawley rats received one of two experimental diets for 4 weeks: (a) a ‘classic’ 4:1 KD; and (b) a normal regular rodent Chow diet (ND). Fully-kindled seizures were achieved by daily electrical stimulation in the amygdala. Seizure stage and after-discharge duration (ADD) were assessed daily. The after-discharge threshold (ADT) was measured every 5 days. The effects of the two diets on neuronal loss were observed before kindling and 20 days after stimulation by Nissl staining. We found that the progression of seizure stage and ADD was delayed by KD. KD prevented the ADT decrease on day 5. The incidence of generalized seizures was lower in the KD group compared to the ND group. The neuronal density was decreased in the ipsilateral hilus of the dentate gyrus (DG) and CA1 area, as well as the contralateral CA1 area before kindling in the KD group. However, KD prevented neuronal loss in the ipsilateral CA1 area 20 days after stimulation. Our data suggest that KD can protect against epileptogenesis by preventing both after-discharge generation and propagation in kindling seizures. In addition, KD also possesses a neuroprotective function during kindling although it changes hippocampal development in early life.

1. Introduction

Refactory epilepsy is a chronic disorder in which repetitive seizures produce a cascade of events that lead to the progressive and permanent modification of cortical neuronal networks [4,17]. Most current antiepileptic drugs are primarily symptomatic therapies that suppress seizures rather than correct the underlying brain abnormalities that cause epilepsy or alter the natural history and long-term prognosis of epilepsy [19].

The ketogenic diet (KD) is a high fat, adequate protein, and low carbohydrate dietary treatment that is commonly used to treat drug-resistant epilepsies, in particular intractable pediatric epilepsies including infantile spasms, Lennox–Gastaut syndrome, and certain inherited metabolic disorders [3,5,7]. Traditionally, ketogenic ratios (grams of fat: grams of carbohydrate plus protein) of 4:1 in older children and 3:1 in infants have been used. It has been accepted as a safe and effective alternative treatment option for various seizure types and syndromes [3,5]. The long-term benefit of KD has been observed not only after a few months of use, but also after discontinuation of the diet, which may indicate a potential anti-epileptogenic activity besides its antiepileptic function [3,5]. However, the anti-epileptogenic function can hardly been proved clinically since KD has never been applied for seizure prophylaxis.

The anti-epileptogenic effect of KD remains unknown because of inconsistency in data from different epileptic models. KD exhibits neuroprotective effects in the hippocampus but fails to prevent epileptogenesis in lithium-pilocarpine model [8]. In contrast, KD decreases the risk of developing chronic epilepsy in kainic acid (KA) models [11,21]. Therefore, whether KD plays a role in anti-epileptogenesis still require further investigation.

In the model of amygdaloid-kindling seizures, repeated administration of initially subconvulsive electrical stimulus eventually results in focal after-discharges which spread and ultimately cause secondarily generalized seizures [15]. Epileptogenesis can be studied at various defined points in its evolution. Here we used this model to observe the effect of KD on epileptogenesis and neuronal loss.

2. Materials and methods

2.1. Animal and diets

Postnatal day 28 male Sprague-Dawley rats (Grade II; provided by the SLAC laboratory animal company, Shanghai, China) were
used. The animals were housed individually and kept at 21 ± 1 °C under a normal 12 h light/dark cycle (lights on from 8:00 am to 8:00 pm). Experiments were performed between 10:00 am and 5:00 pm. All experiments were approved by the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used in the study and their suffering in the experiment. Rats were matched for weight and divided into two groups. Control rats received a normal regular rodent chow diet (Slac-MO1, Slacom, Shanghai, China), which is a nutritionally balanced low-fat, high-carbohydrate diet that delivers 3.52 kcal/g of gross energy. KD rats received ketoCal, a nutritionally balanced soy oil-based high-fat, low-carbohydrate KD which delivers 7.3 kcal/g of gross energy and has a ketogenic ratio of 4:1 (Nutricia, Shanghai, China). The foods were freshly prepared each day. Both food and water were provided to the animals ad libitum. All animals were fasted for a period of 8 h prior to initiation of the KD or normal control diet (ND). The animals continued to receive their specified diets (KD or ND) throughout the experimental surgery and electrophysiological testing.

2.2. Measurement of body weight and ketonemia

Body weights were measured every three days. The serum concentrations of beta-hydroxybutyrate (BHB) were taken as a measure of ketonemia. Blood samples (0.5 ml) in each group were collected from the orbital venous plexus at the same time of day (10:00 am–11:00 am) once a week for 8 weeks. The tubes were centrifuged at 3000 × g for 15 min, and the serum was then separated. BHB concentrations were measured using colorimetric method with the Beta-Hydroxybutyric Acid Test Kit (Ruiyuan, Ningbo, China).

2.3. Surgery

Surgery commenced on postnatal day 56 following 4 weeks of diet treatment. After deep chloral hydrate anesthesia (400 mg/kg, i.p.), rats were mounted on a stereotaxic apparatus (512600; Stoelting, USA). Electrodes were implanted into the right basolateral amygdala (coordinates from the bregma: AP = −2.4 mm, L = −4.8 mm, and V = −8.8 mm). The electrodes were made of twisted stainless steel wires with a diameter of 0.2 mm (A.M. Systems, USA) and Teflon-coated except for 0.5 mm at the tip. The tip separation was 0.7–0.8 mm. The electrodes were connected to a miniature receptacle, which was embedded in the skull with dental cement. Animals were allowed to recover from surgery over a 10-day period.

2.4. Procedure for kindling and threshold measurement

Electroencephalograms (EEGs) of the right amygdala were recorded with a digital amplifier (RM-6240, Chengyi, China). On the first day of stimulation, the after-discharge threshold (ADT) of each subject was determined by a constant current stimulator (YC-2; Chengyi, China). An initial current of 50 μA was used and current was then increased in a stepwise manner using 20 μA steps. Consecutive trials were separated by at least 30 min. The ADT was defined as the lowest current required to elicit an after-discharge lasting at least for 5 s on EEGs. All animals were subjected to kindling stimulation with the same current intensity as their own ADT once daily for 20 days. The daily kindling stimulus consisted of a 1 s train of monophasic, 1 ms square-wave pulses at 60 Hz. On days 5, 10, and 15, the ADTs were re-determined using the ascending-series procedure described above. Animals that exhibited three consecutive stage 5 seizures were considered to be fully kindled.

Seizure severity was classified according to a modification of the classification by Racine (1972), as follows: (1) facial movement; (2) head nodding; (3) unilateral forelimb clonus; (4) bilateral forelimb clonus and rearing; and (5) bilateral forelimb clonus, rearing and falling [15]. Stages 1–3 were considered to be focal seizures, while stages 4 and 5 were considered generalized seizures (GS) [17].

2.5. Histology

All animals were sacrificed 2 h after the last stimulation. The rats were deeply anesthetized with chloral hydrate and perfused transcardially with 100 ml of 0.1 mol/l phosphate-buffered saline, followed by 100 ml of 4% buffered paraformaldehyde for 10 min. The brain was then carefully removed and fixed at 4 °C in 4% paraformaldehyde and 30% buffered sucrose for a period of 3 days. Coronal brain sections of 10 μm thickness sections were collected for the Nissl protocol. After cresyl violet staining, neuronal counting was completed using a standardized two-dimensional technique (Image-Pro Plus analysis software 6.0, Media Cybernetics, USA). Neuronal density was measured in both the hippocampus and parahippocampal cortices, including the bilateral entorhinal and piriform cortices.

We also stained with cresyl violet to confirm the positions of the electrodes. Only those animals with correct placement at the right basolateral amygdala were included in the data analysis. In total, 20 out of the 28 rats fulfilled this criteria.

2.6. Statistical analysis

Body weight, mean β-hydroxybutyrate and group differences in kindling acquisition were assessed by a two-way analysis of variance (ANOVA) for repeated measures. The chi-square test was used for comparison between groups of the GS incidence in fully kindled rats. In the other comparisons, a nonparametric Mann–Whitney U-test was used.

The ADT measured on day 0 was normalized as 100%, and the change from this on days 5 and 10 was then calculated. Neuronal loss was defined as the percentage of neuron density decrease in hippocampal cell layers between day 0 and day 20.

All data are presented as mean ± SEM. For all analyses, the tests were two-sided and p < 0.05 was considered to be statistically significant.

3. Results

3.1. Body weight and ketosis

In both groups, body weight increased through the course of diet treatment (Fig. 1A). But the weight gain was significantly less in KD-fed rats than that in ND-fed rats. That is, rats on KD had a significantly slower growth development than those fed on ND (p < 0.01). Except for an oily appearance of their fur, molting and being slightly lower in weight, the KD-fed rats showed no difference in their behavior and health compared with the ND-fed rats.

Basal blood BHB levels were compared between ND and KD-fed animals (Fig. 1B). KD induced persistent ketonemia 1 week later as the BHB levels in KD-fed rats were significantly higher than that in the ND-fed group (p < 0.001).

3.2. Effect of KD on kindling acquisition

KD significantly delayed the progression of seizure stages (p < 0.01; Fig. 2A) and shortened the corresponding after-discharge duration (ADD) during kindling acquisition compared with ND group (p < 0.05; Fig. 2B). KD prevented the ADT decrease on day
Fig. 1. (A) Levels of body weight and (B) beta-hydroxybutyrate (BHB) of rats fed with either KD \((n = 5)\) or ND \((n = 5)\). ** \(p < 0.01\), *** \(p < 0.001\) compared with controls.

Fig. 2. Effects of KD on amygdaloid kindling acquisition \((n = 8\) for KD, \(n = 10\) for ND). (A) Behavioral stage. (B) After-discharge duration. (C) ADT decrease between KD and ND. * \(p < 0.05\), ** \(p < 0.01\) compared with controls. Abbreviations: AD, after-discharge; ADT, after-discharge threshold.

Fig. 3. Effects of KD on amygdaloid kindling acquisition \((n = 8\) for KD, \(n = 10\) for ND). (A) Number of stimulations in each stage during kindling acquisition. (B) Number of stimulations required to reach each stage. (C) Number of stimulations in stages 1–3 (focal seizures) and stages 4–5 (generalized seizures) during kindling acquisition. (D) Incidence of GS and (E) latency to seizure onset during stage 5. * \(p < 0.05\), ** \(p < 0.01\) compared with control.
5 compared to ND (p < 0.05; Fig. 2C). However, there were no group differences in the ADTs on day 10 or day 15. In the KD group, rats stayed in both stage 0 and stage 2 for a longer duration (p < 0.05 for both; Fig. 3A) compared with the ND group. KD also increased the number of stimulations required to reach stages 2–5 (Fig. 3B). In the KD group, the rats stayed in stages 1–3 for longer than the ND group (p < 0.05; Fig. 3C). KD decreased the incidence of GS (p < 0.05; Fig. 3D) compared to ND, but did not prolong the latency period to the onset of GS (Fig. 3E).

3.3. Histology

After 4 weeks of diet administration (before kindling), rats in the KD group showed a lower density of neurons than the ND group in the bilateral hippocampal CA1 and hilus of the ipsilateral DG (p < 0.05 for all; Fig. 4A and B). However, the Neuron density in entorhinal and piriform cortices showed no difference between two groups.

In the ND group, the neuron density significantly decreased in the hippocampus and parahippocampal cortices during kindling. The neuronal loss percentage in the ipsilateral hippocampal CA1, CA3 and DG areas was 34.1 ± 5.3% (p < 0.001), 26 ± 3.9% (p < 0.05), and 18 ± 4.5% (p < 0.05), respectively (Fig. 4D). The neuronal loss percentage in the ipsilateral piriform cortex, the ipsilateral and contralateral entorhinal was 15.6 ± 5.8% (p < 0.05), 13.4 ± 6.1% (p < 0.05) and 17.1 ± 5.9% (p < 0.05), respectively (Fig. 4C). After 20 stimulations, KD attenuated the neuronal loss in the ipsilateral hippocampal CA1 region in the KD group (p < 0.05, Fig. 4D), but not in other areas (Fig. 4C and D).

4. Discussion

4.1. Diet, weight gain and ketosis

KD is often an effective intervention for drug-resistant epilepsies in childhood. Therefore weaning rats were used in our research at the start of the experimental diet administration. We did not adopt the calorie restriction/limited-access feeding paradigm as used by other researchers [2,16] in order that the effect of fasting with the KD could be excluded more effectively.

Our research indicated that the ND was well tolerated with an absence of any significant health concerns despite the lower body weights in the KD-fed animals, was consistent with prior work [18,20]. A plateau level of ketosis was reached within 1 week in the KD-fed animals. This level was maintained as long as the diet was continued.

4.2. Epileptogenesis

Our data showed that KD significantly delayed seizure progression induced by amygdaloide kindling. This antiepileptogenic function of KD may help to prevent secondary epileptogenesis in intractable epilepsy in addition to the inhibition of ictogenesis. The rats in the KD group required more kindling stimuli to attain a fully kindled motor seizure. This finding was consistent with the effect of KD in a PTZ kindling model [6]. KD significantly decreases the incidence and severity of seizures, and increases the latency period before clonic convulsions occur in the PTZ kindling model. In addition, KD also elevates the seizure thresholds induced by intravenous PTZ infusion, but cannot reduce seizure severity once the seizure
has started [16]. On the other hand, KD fails to prevent epileptogenesis in terms of incidence, latency, or clinical expression of recurrent seizures in the lithium-pilocarpine model [8]. In the KA model, KD decreases the frequency and duration of spontaneous recurrent seizures, but does not affect the onset or severity of spontaneous recurrent seizures [11]. The damage observed in the SE model is more severe and extensive. In contrast, epileptogenic circuit develops in the absence of gross severe morphological damage in amygdaloïd-kindling seizure [4].

Amygdaloid kindling seizures enable us to analyze epileptogenesis in graded stage. The KD shows anti-epileptic action mainly in partial seizure (stages 1–3) stages. At the same time, it attenuated the decrease in ADT on day 5 of stimulation, but not on day 10 and day 15. The anti-epileptogenic action of KD is inherently related to the inhibition of cerebral glucose metabolism and production of ketone bodies [7]. Recent studies suggested these changes further inhibit seizure generation and propagation by different pathways [12] and finally promote the inhibitory function of γ-aminobutyric acid (GABA)ergic system [14]. On the other hand, there is accumulating evidence that the GABA<sub>3</sub>-receptor undergoes excitatory transformation during epileptogenesis, which contribute to hyper-excitability and abnormal synchronization on neuronal networks [1,13]. In this circumstance, GABA<sub>3</sub> receptor activation induces cellular depolarization and increases neural excitability. It is possible that the transformation of GABA<sub>3</sub>-receptor play a role in lack of antiepileptogenic function of KD in late stage of kindling.

4.3. Neuroprotection

The neuronal loss in amygdaloid kindling seizures is not widely distributed as there is a mild to moderate neuronal loss in the hippocampal and parahippocampal cortices. Our data is similar with Cavazos’s study, in which neuronal loss is mainly located in the hilus of the DG and CA1 after three generalized tonic–clonic seizures [4]. It is reported that hippocampal CA1 supports epileptogenesis in kindling model [10]. An inhibition of neuronal activity of CA1 neuron by local lidocaine injection was reported to decrease seizure severity and retard amygdaloid kindling. The data seems contradictory to our finding that KD retarded kindling and protected against CA1 neuronal loss. Our finding suggests that the KD’s anti-epileptogenic function and neuroprotection is independent.

Parahippocampal structures have dense connections with the amygdala and hippocampus. They play a crucial role in seizure pathways, especially in the secondary generalization of limbic seizures [9]. We found a mild neuronal loss in the parahippocampal cortices during kindling. KD did not exhibits significant neuroprotective effects in parahippocampal cortices in our study. It is possible that the neuronal loss in the parahippocampal cortices is too mild to reveal the neuroprotective effect of KD in our study. Similarly, KD also protects against the neuronal loss in the hippocampus, but not in entorhinal and piriform cortices in lithium-pilocarpine model [8]. It should be noted that KD-fed rats had a lower neuron density in the hippocampus before the kindling procedure, indicating that KD alters the development of hippocampus neurons in rats during the early period of life. Our finding is in line with research conducted by Zhao et al. [22], that young rats treated with the KD for one month showed deficient spatial learning and memory, regardless of whether they experienced SE or not.

5. Conclusions

We have demonstrated that KD has anti-epileptogenic functions in amygdaloid kindling seizures, which are more prominent in the stage of partial epileptic stages. KD also has neuroprotective effects on hippocampal neurons although the neuronal loss was found to be mild in this model. There are no spontaneous seizures occur in our study. Additional studies are required to address whether KD have long-term anti-epileptogenic and neuroprotective effect, which is clinically important to gain seizure control and prevent epileptic encephalopathy.

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References


