



The Effect of Different Patterns of Intermittent Fasting Diet on the Convulsive Behaviors: the Possible Role of Glutamic Acid Decarboxylase Enhancement

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ABSTRACT

Introduction: Intermittent fasting diet (IFD) has been known as a supplementary therapy for epilepsy. The main mechanisms involved in the anti-epileptic effect of IFD have not been well understood. This study has investigated the effect of IFD on hippocampal glutamic acid decarboxylase enzyme (GAD65) expression as a critical enzyme to fast modulation of GABA level.

Method: Male adult rats were divided into 4 groups of sham, seizure, fasting & seizure, and pre-seizure fasting. Seizures were induced by pentylenetetrazol (PTZ) injection every other day for 4 weeks. The protocol of IFD was alternate-day feeding (24 hours of access to food every 48). In the pre-seizure fasting group, rats were put on the alternate-day feeding schedule for weeks 1–8 and PTZ was injected every other day in weeks 5–8. Hippocampal level and distribution of GAD65 have evaluated using western blotting and immunofluorescence analysis respectively.

Result: Study findings revealed a significant reduction of seizure behavior scores in the pre-seizure fasting group on days 10, 16, 20, and 22. In the CA3 area, expression of GAD65 decreased in the seizure group compared to the sham group. In the CA1 area, expression of GAD65 increased significantly in both fasting groups compared to the seizure group. Moreover, the hippocampal protein level of GAD65 increased significantly in both fasting groups compared to the seizure group.

Conclusion: The IFD before seizure induction has more potential to modulate the development of seizure behaviors, compared to IFD simultaneously with seizure.

Keywords: GAD65; Caloric restriction; Hippocampus; Seizure; GABA; Pentylenetetrazol

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INTRODUCTION

Epilepsy is known as one of the common neurological disorders with more than 50 million confirmed cases in the world [1]. Despite numerous antiepileptic drugs, some patients are resistant to treatment and have to resort to

supplementary therapies such as surgery, vagal nerve stimulation and dietary control [2].

Diet therapies including the ketogenic diet, Atkins diet, enriched diet in polyunsaturated fatty acids and calorie restriction has been well established as supplementary therapies for epilepsy [3, 4]. The effects of intermittent

fasting diet (IFD) as a kind of caloric restriction have been investigated in several studies. IFD decreased seizure severity in some animal models of epilepsy but increased epileptic activity induced by electrical stimulations [5, 6]. It has been indicated IFD in combination with the ketogenic diet, suppressed seizures successfully in pediatric patients [6]. IFD could reduce seizure severity in the chemical induction of tonic-clonic seizures [7]. IFD diminished excitotoxicity in the hippocampal neurons in the kainic acid model of seizure induction [8]. IFD postponed the process of epileptogenesis in the model of temporal lobe epilepsy induced by pilocarpine [9]. On the contrary, IFD had no effect on the seizures severities were induced by intraventricular administration of pentylenetetrazol (PTZ) in rats [5]. Controversies effect of IFD in the different types of seizures in the animal models or patients might be depended on different pathophysiology of seizures.

An imbalance between the excitatory glutamate-mediated and inhibitory GABA-mediated neurotransmission has been considered as the main pathophysiology of epilepsy [10, 11]. Gamma-aminobutyric acid (GABA) is a fundamental inhibitory neurotransmitter in the brain and prevents the neurons from being overexcited in neuronal networks [12]. GABAergic neurons were more vulnerable during the epileptic status and GABA transmission depressed in some models of generalized tonic-clonic epilepsy [13, 14]. Glutamic acid decarboxylase enzymes (GAD65 and GAD67) are required as a major factor to synthesize and regulate of steady-state concentration of GABA [15]. GAD65 proteins are highly expressed in the axon terminals and regulate GABA level in the fast modulation which is necessary to balance between inhibition and excitation [15, 16]. GAD and GABA receptor expression in the brain is closely associated with epileptic conditions [17, 18].

Considering the critical roles of GAD65 and GABA in the brain during epilepsy, the aim of this study was to assess the effect of different patterns of IFD on the alteration of hippocampal GAD65 expression in an animal model of seizure.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 220–250 g, were housed in the standard environment with light/dark cycle at 22 ± 1 °C and carried out according to the protocol approved by the animal ethics of Iran University of Medical Sciences, Tehran, Iran. They were divided into four groups containing 9 rats.

Sham group: rats were injected with normal saline intraperitoneally (i.p.) every other day for 4 weeks.

Seizure group: Pentylenetetrazol (PTZ) was injected (35 mg/kg, i.p.) every other day for 4 weeks and had unrestricted access to food and water.

Fasting & seizure: PTZ was injected in the protocol the same as PTZ group and simultaneously were put on an alternate-day feeding schedule (24 hours of access to food every 48h) for 4 weeks.

Pre-seizure fasting: rats were put on the alternate-day feeding schedule for weeks 1–8 and PTZ was injected every other day in weeks 5–8.

Scoring of seizure behavior

All rats were monitored for epileptic behaviors for 30 minutes after PTZ injection. Epileptic behaviors were scored as followed: 1 for immobility, 2 for rigid posture, 3 for repetitive scratching, circling, head bobbing, 4 for forelimb clonus, rearing and falling, 5 for repeated occurrence of level four behaviors and 6 for severe tonic-clonic behaviors [7].

The labelled cells with GAD65 antibody were counted by Image Tool software (version 3.0) in the hippocampal CA1 and CA3 areas in each specimen (five visual fields/specimen; $\sim 1\text{mm}^2$).

Western blot analysis

Three animals of each group were decapitated under deep sedation and the hippocampus manually dissected. The tissues were homogenized in the buffer solution (50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 $\mu\text{g}/\text{ml}$ leupeptin). Protein concentrations were measured by the Bradford test. Samples were diluted at 0.5 $\mu\text{g}/\mu\text{l}$

in 0.25 M Tris-HCl pH 6.8, 10% (v/v) glycerol, 10% (wt/vol) sodium dodecyl sulphate (SDS), 10 mM dithiothreitol. Protein separation was carried out by SDS-polyacrylamide gel electrophoresis (12%) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore). The PVDF membranes were incubated overnight in the blocking buffer (100mM Tris-HCl; 0.9% NaCl, 0.1% Tween 20, 5% non-fat dry milk with pH = 7.4). The membranes were incubated overnight by anti-GAD65 antibody and mouse monoclonal anti- β actin antibody (1:1000, Sigma, St. Louis, MO). After washing with Tris buffer, membranes were incubated for 90 minutes with a secondary antibody. Immunoreactivity was revealed by ECL (Amersham Biosciences, Freiburg, Germany). The blots were exposed to X-ray film sensitive to blue light for 5–30 s. The developed films were scanned densitometrically on a Bio-Rad scanner. Quantitative analysis was carried out by the monomeric band's data with Image J software.

Statistical analysis

All data are given as mean $\pm$ S.E.M. The normality of the data distribution was assessed by the Kolmogorov - Smirnov test, which indicated that the data were normal. Therefore, parametric tests were performed. Data were

analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance was considered in the probability values less than 0.05. The PASW Statistics 20 was used for statistical analysis.

RESULTS

The effect of fasting on seizure severity

Seizure behaviors were scored following PTZ injection. The mean of behavior scores reduced significantly in the pre-seizure fasting group in days 10 ($p < 0.01$), 16 ($p < 0.001$), 20 ($p < 0.01$), 22 ($p < 0.001$) after PTZ injection compared to seizure and fasting & seizure groups (Fig. 1). There was no significant difference in the behavior scores between the fasting & seizure and seizure groups.

Hippocampal protein level of GAD65

The hippocampal protein level of GAD65 was assessed by western blot (Fig. 2). The total protein level of GAD65 significantly decreased in the seizure group compared to the sham group ($p < 0.01$). IFD regimen had significantly increased the total protein level of GAD65 in both fasting groups compared to the seizure group ($p < 0.001$).

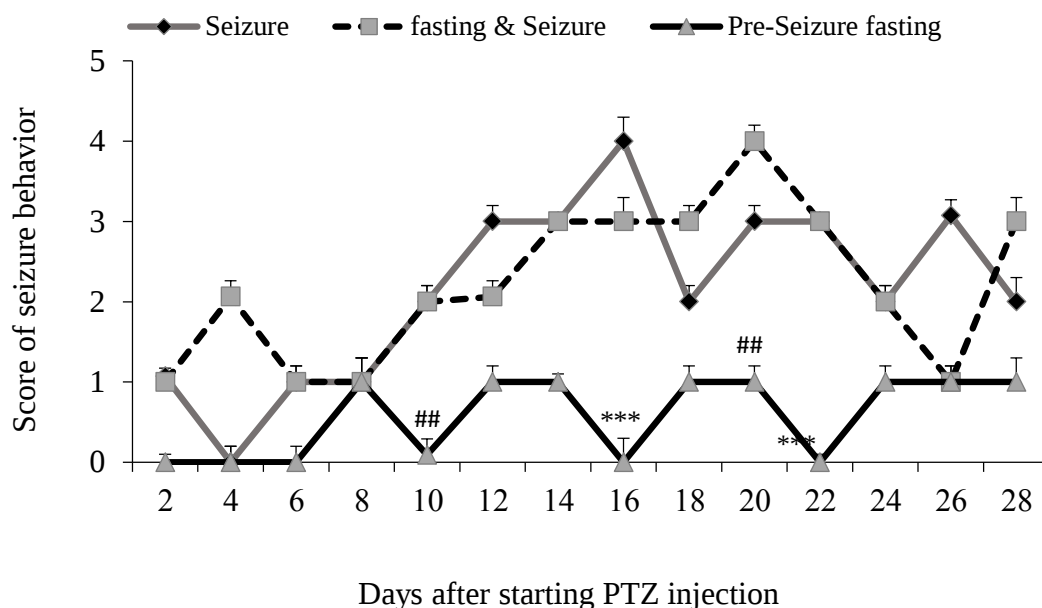


Figure 1. The behavior score of seizure-induced by PTZ injection. The significant reduction of seizure behavior scores in the pre-seizure fasting group has been shown on days 10, 16, 20, 22. Values are means $\pm$ SEM. ## $p < 0.01$ (vs fasting & seizure), *** $p < 0.001$ (vs seizure).

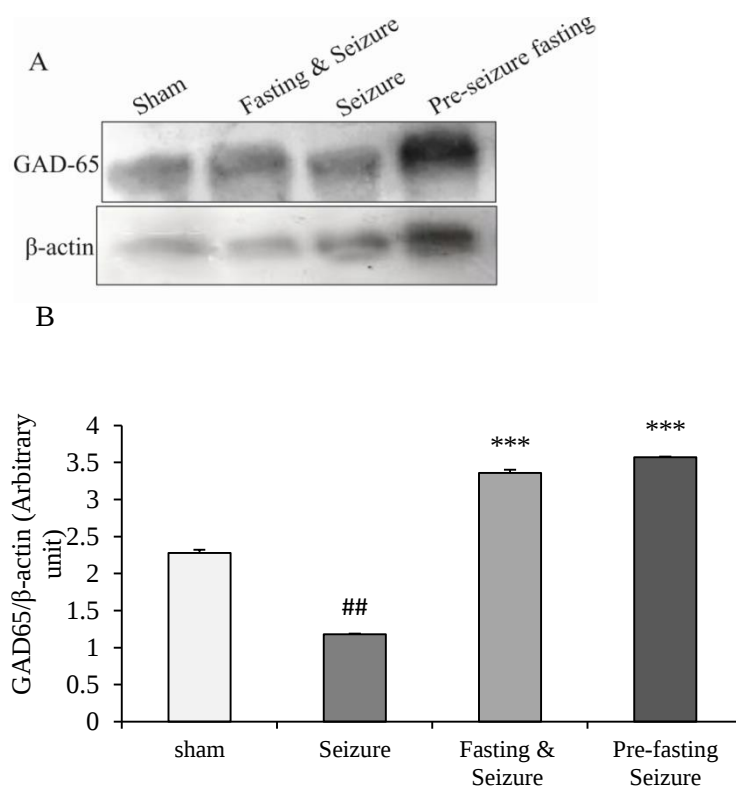


Figure 2. Fig. 2. Results of the IFD effect on the total protein level of GAD65 in the hippocampus. A: The representative western blot images of GAD65 protein in the hippocampus. B) The bar graph indicates the quantitative results (mean $\pm$ SEM). ## $p < 0.01$ (vs sham) and *** $p < 0.001$ (vs seizure).

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DISCUSSION

In this study, we showed that IFD started 4 weeks before seizure induction decreased seizure severity, but IFD simultaneously with the seizure induction did not have a significant effect on the seizure severity.

The effect of caloric restriction on the feature of seizure has been evaluated in different kinds of epilepsy. PTZ-induced seizures is one of the most reliable animal models used to study the neurobiology of epilepsy, seizure mechanisms, and evaluation of novel treatments [19, 20]. The decrease in the activity of the GABA, as a main epileptogenesis factor, after the development of PTZ-induced seizure have been shown in previous investigations [21-23]. meanwhile, prior studies have indicated that the doses of PTZ to induce seizures were increased in rats and mice which were put on caloric restriction [24, 25]. Moreover, restricted diet decreased the duration of status epilepticus in the pilocarpine model of epilepsy and the severity of seizures induced by kainic acid and PTZ injections [5, 7,

has been reported that mild to moderately restricted diet reduced seizure susceptibility in a genetic model of idiopathic epilepsy [25].

Cell energy management has a critical role in the antiepileptic effect of a caloric restriction diet. Both levels of glucose and ketone bodies have been considered as the main source of energy for the brain's cells [26]. Reduction of blood glucose levels was one of the assumptions mechanisms involved in the antiepileptic effect of caloric restriction regimen including IFD. [25]. In addition, it has been suggested a low level of blood glucose increased blood ketone levels and resulted in more efficacy of the ketogenic diet to suppress seizures [26]. Ketones could regulate neurotransmitters expression. Elevation of ketones level reduced aspartate formation as an excitatory neurotransmitter and triggered GAD activity that required for GABA synthesis [27, 28]. It has been reported caloric restriction increased GAD65 and 67 in the inferior and superior colliculi and in the cerebellar cortex [29]. A low level of blood

glucose enhanced GAD protein expression in contrast to hyperglycemia in the rats' brain [29].

Previously we have shown different patterns of IFD have different outcomes on the seizure features [7]. In this experiment, although in both IFD groups, GAD65 increased in the hippocampus (especially in CA1 region), seizures suppressed in the animals that have experienced fasting several weeks before seizure induction. GABA in the brain is a critical preventive factor in epileptogenesis [30]. In line with our findings, prior studies were shown that a long-term increase of GAD expression could improve GABA inhibitory transmission [31]. But it was shown elevation of brain GABA levels beginning two days after status epilepticus does not prevent epileptogenesis in rats [32]. Therefore, probably an increase in GAD65 concomitantly the onset of epileptic seizures couldn't sufficiently increase the GABA needed for compensation the defect in GABAergic circuitries, so, starting a diet at the same time as epileptic seizures could not reduce seizure behaviors.

Epileptoid activity in the hippocampal CA1 field may be due to the weakening of GABA-inhibition [33, 34], and the reduction of GABA-inhibition leads to an excessive increase in the excitation of principal hippocampal neurons [35]. As an imbalance between inhibitory GABA-mediated and excitatory glutamate-mediated neurotransmission result in hyperexcitable neurons and is the key mechanism of the development of epileptic seizures [36, 37], so an increase in the GABA transmission, mediated by GAD65, could lead to sedative and anticonvulsant effects.

The assessment of GABA level as well as GABA receptors was the limitation of our study and more investigations have been suggested.

CONCLUSION

In conclusion, our findings suggested the direct and indirect role of hypoglycemia in the anti-epileptic effect of IFD. A low level of glucose might trigger GAD expression directly and had the potential to increase the hippocampal level of GABA neurotransmitter. A low level of glucose-stimulated ketone bodies, and resulted in GAD increase indirectly. Furthermore, it seems that

the restricted diet in epileptic patients who have not experienced fasting should be used with caution.

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ETHICAL APPROVEMENT

This experimental study was approved by the Ethical Committee of Iran University of Medical Sciences based on NIH Guide for the Care and Use of Laboratory Animals and was carried out in accordance with its guidelines for animal use.

DECLARATIONS

The authors have no conflict of interest in this research.

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