Kaohsiung Journal of Medical Sciences (2016) 32, 446–451

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ORIGINAL ARTICLE

Rebaudioside A inhibits pentylenetetrazol-induced convulsions in rats

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Received 11 April 2016; accepted 1 July 2016
Available online 4 August 2016

KEYWORDS
Electroencephalogram;
Experimental epilepsy model;
Pentylenetetrazol;
Rebaudioside A

Abstract The safety of patients with epilepsy consuming sweetening agents, which is becoming increasingly prevalent for various reasons, is a topic that should be emphasized as sensitively as it is for other diseases. Patients with epilepsy consume sweetening agents for different reasons such being diabetic or overweight. They can occasionally be exposed to sweetening agents unrestrainedly through consuming convenience food, primarily beverages. This study aimed to investigate the effects of rebaudioside A (Reb-A), which is a steviol glycoside produced from the herb *Stevia rebaudiana* (Bertoni), on epileptic seizures and convulsions induced by pentylenetetrazole (PTZ). Forty-eight male rats were used. Twenty-four rats were administered 35 mg/kg PTZ to trigger epileptiform activity; the remaining 24 rats were administered 70 mg/kg PTZ to trigger the convulsion model. The epileptiform activity was evaluated by spike percentage, whereas convulsion was evaluated by Racine's Convulsion Scale and the onset time of the first myoclonic jerk. Statistical analysis revealed a statistically significant decrease in the Racine's Convulsion Scale score and increase in the latency of first myoclonic jerk in a dose-dependent manner for the rat groups in which PTZ epilepsy had been induced and Reb-A had been administered. For the groups that were administered Reb-A, the spike decrease was apparent in a dose-dependent manner, based on the spike percentage

Conflicts of interest: All authors declare no conflicts of interest.

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http://dx.doi.org/10.1016/j.kjms.2016.07.002

calculation. These results indicated that Reb-A has positive effects on PTZ-induced convulsions.

Introduction

Epilepsy is a disease that affects more than 50 million people worldwide [1]. Epilepsy frequently coexists with obesity, which is becoming a prevalent and serious public health problem [2]. The unrestrained increase in obesity and type-2 diabetes that began in the second half of the 20th century continues rapidly. An estimated 500 million obese and 1.5 billion overweight people exist worldwide [3]. More than two-thirds of the United States population is obese, and an estimated 86.3% of the adult population will be affected by the year 2030 [4]. It is estimated that diabetes mellitus affects approximately 382 million people globally. This number is expected to increase to approximately 592 million people by 2035 [5].

Obesity is sometimes comorbid with epilepsy, but some occurrences of obesity can also be explained by antiepileptic treatment [2]. The weight gain effect is common for certain antiepileptic agents frequently used among epilepsy patients such as valproic acid, carbamazepine, gabapentine, and vigabatrine [6]. The comorbidity of two ubiquitous diseases such as diabetes and epilepsy is also frequent.

Epilepsy patients consume sweetening agents for different reasons such as having diabetes or being overweight. They can occasionally be exposed unrestrainedly to such sweetening agents through consuming convenience food, primarily beverages. Rebaudioside A (Reb-A) is a steviol glycoside derived from the herb *Stevia rebaudiana* (Bertoni) [7]. Can the safety of these sweetening agents used by patients with epilepsy be determined by means of epileptic seizures? This study aimed to investigate the effects of Reb-A, which has not previously been evaluated from this point of view.

Materials and methods

Animals and laboratory

All experiments and protocols described in the present study were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by United States National Institutes of Health, and approved by the Medical Faculty Experimental Ethics Committee of Gaziosmanpasa University (Tokat, Turkey). This study used 48 male Sprague—Dawley rats—24 rats for electroencephalographic (EEG) recording and 24 rats for behavioral studies—each weighing 200—250 g. The rats were maintained under a 12 hour light/dark cycle (light from 07:00 to 19:00) in a quiet room with 22°C—24°C ambient temperature. They were provided free access to standard rat nutrients and purified drinking water *ad libitum*.

Drugs

Pentylenetetrazol (PTZ; Sigma—Aldrich, Interlab Inc., Istanbul, Turkey) and Reb-A (Candarel Stevia; Aris Inc., Istanbul, Turkey) were dissolved in 0.9% saline. Drug solutions were prepared fresh each morning.

Experimental procedures

Forty-eight rats were randomly divided into two groups: Group A for EEG recordings and Group B for behavioral assessment. For the EEG recordings, the rats were deeply anesthetized with 80 mg/kg ketamine HCL (Ketalar; Pfizer Pharmaceuticals, Istanbul, Turkey) and 4 mg/kg xylazine (Rompun, Bayer AG., Istanbul, Turkey) mixture administered intraperitoneally (i.p.). After inducing anesthesia, a small hole in the skull was opened stereotaxically with a drill. The electrodes (polyamide-coated stainless steel wires, 0.1-mm diameter, and electrical resistance $<1\Omega$/10 mm) were implanted on the dura of the left frontal cortex (2.0 mm lateral to the midline and 1.5 mm anterior to the bregma). The reference electrode was implanted over the cerebellum (1.5 mm posterior to the lambda on the midline). After the implantation, the electrodes were fixed by dental acrylic. Twelve days after electrode fixation, 24 rats were divided randomly into four groups (Group A1—Group A4; 6 rats per group).

Group A1 was the control group; this group received no medication. Group A2 received 1 mL/kg saline. Group A3 and Group A4 received 100 mg/kg Reb-A and 200 mg/kg Reb-A, respectively. All administrations were intraperitoneal. The drugs were administered 30 minutes before the PTZ injection. All groups, except Group A1, received 35 mg/kg PTZ and an EEG recording. The EEG recordings were obtained from awake rats in a special container 5 minutes after the PTZ administration.

All EEG recordings and behavioral assessment protocols were performed as previously described [8]. In summary, the EEG recordings were taken for 60 minutes, the signals were amplified 10 thousand times and filtered within a range of 1—60 Hz. The EEG records were obtained by the BIOPAC MP150 Data Acquisition System (Biopac System Inc., Santa Barbara, CA, USA) and the spike percentage was evaluated. Two clinical neurophysiologists scored the EEG data for the spike percentage (which is a reproducible way of quantifying epileptiform activity to quantify the percentage of 1-second bins with at least one spike-wave, called "spike-wave percentage" [8]). The onset and cessation of this complex were identified by a higher amplitude (at least two-fold), compared with the baseline values. The cumulative duration of spike-wave was detected within 2-minute intervals.

To perform the behavioral assessment, groups were formed with the remaining 24 rats (i.e., Group B). These rats were divided into four groups (Group B1—Group B4; 6 rats per group). Group B1 was the control group and received no medication. Group B2 received 1 mL/kg saline. Group B3 and Group B4 received 100 mg/kg Reb-A and 200 mg/kg Reb-A, respectively. All administrations were intraperitoneal. The drugs were administered 30 minutes before the PTZ injection (70 mg/kg, i.p.). Racine's Convulsion Scale (RCS) and the onset time of the first myoclonic jerk (FMJ) were used to evaluate the seizures (for the injection of PTZ [70 mg/kg] only) as follows: "0", no convulsion; "1", twitching of vibrissae and pinnae; "2", motor arrest with more pronounced twitching; 3, motor arrest with generalized myoclonic jerks (this duration was recorded for evaluating FMJ onset time); "4", tonic-clonic seizure while the animal remained on its feet; "5", tonic-clonic seizure with loss of the righting reflex; and "6", lethal seizure. Rats were observed for the onset times of the FMJ, as previously described [8]. The onset times were recorded in seconds. Nearly all animals that had tonic generalized extension died. The observation period for PTZ-induced seizures was limited to 30 minutes. After this period, the animals were euthanized.

Statistical analysis

The results are expressed as the mean ± the standard error of the mean. Data analysis was performed by running SPSS software (Version 15.0; SPSS Inc., Chicago, IL, USA) for Windows. The Shapiro—Wilk test was used to determine whether a population of values had a normal distribution. The Racine Convulsion scores were evaluated by the Kruskal—Wallis test. The FMJ onset times were evaluated by one-way analysis of variance. *Post hoc* Bonferronni and Mann—Whitney *U* tests were performed to identify differences between the experimental groups. The value of $p < 0.05$ was accepted as statistically significant.

Results

Evaluation of EEG recordings

Group-based samples of EEG recordings of the rats are shown in Figure 1. The averages of the spike percentages of each group are given in Table 1. The evaluations showed no spike indication in the EEG recordings of Group A1. However, the recordings of Group A2 indicated dense spike waves (i.e., high spike percentage) that reflect epileptiform activity after the 35 mg/kg PTZ injection (73.2% ± 3.7%). Because of Reb-A administration, the spike percentages of Group A3 and Group A4 were decreased at 56.5% ± 5.9% and 41.8% ± 4.1%, respectively, compared with Group A2 ($p < 0.05$ and $p < 0.0001$, respectively).

Evaluation of seizures

The RCS scores of the groups and the FMJ onset time averages are presented in Table 2. The evaluations showed no convulsive findings for the Group B1 rats; however, high RCS scores and FMJ values were detected for the Group B2 rats

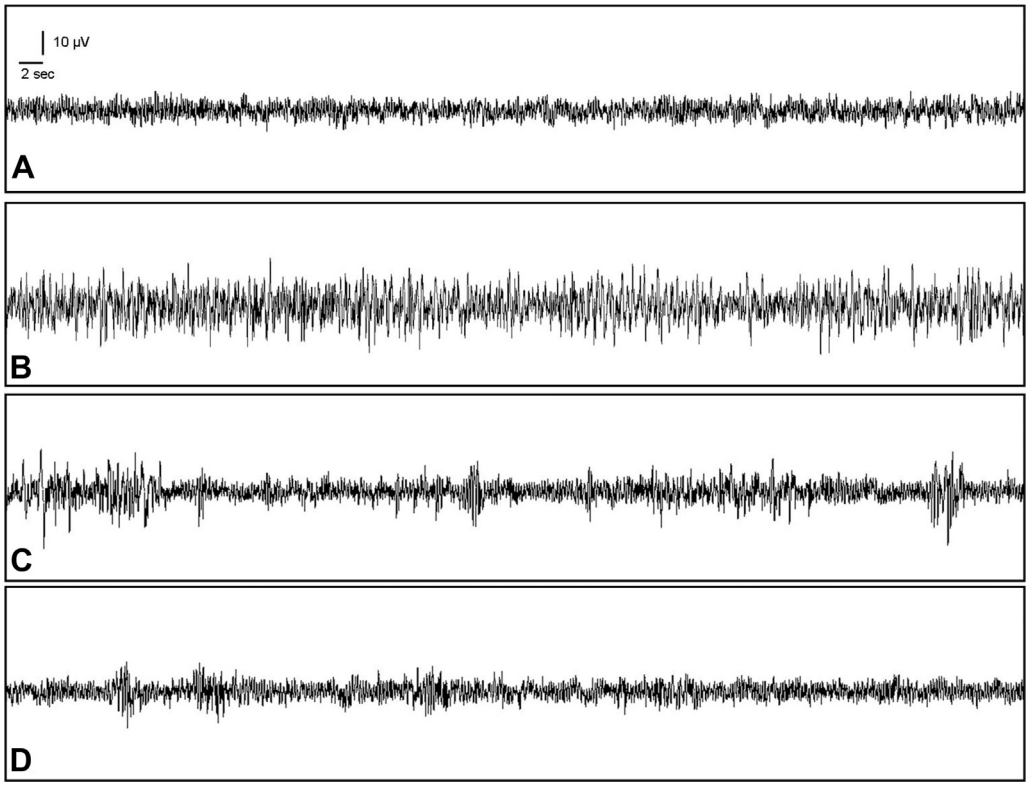

Figure 1. Electroencephalographic (EEG) recording. (A) Control group; (B) pentylenetetrazole and saline group; (C) pentylenetetrazole and rebaudioside A (100 mg/kg) group; and (D) pentylenetetrazole and rebaudioside A (200 mg/kg) group.

Table 1 Spike percentages by group.

Drug group	Spike percentage
Group A1	0%
Group A2	73.2% ± 3.7%
Group A3	56.5% ± 5.9% [a,*]
Group A4	41.8% ± 4.1% [a,**]

[a]Compared with Group A2.
*$p < 0.05$, ** $p < 0.0001$.
Group A1 = the control rats, which received no medication; Group A2 = the rats received 35 mg/kg pentylenetetrazol (PTZ) and 1 mL/kg saline; Group A3 = the rats received 35 mg/kg PTZ and 100 mg/kg rebaudioside A; Group A4 = the rats received 35 mg/kg PTZ and 200 mg/kg rebaudioside A.

after the 70 mg/kg PTZ injection (RCS, 5.60 ± 0.2; FMJ onset time, 67.80 ± 4.5 seconds). Because of Reb-A administration, the spike percentages of Groups B3 and B4 were decreased at 4.33 ± 0.3 and 3.50 ± 0.2, respectively, compared with Group B2 ($p < 0.05$ and $p < 0.0001$, respectively). However, the decline was greater in Group B4 than in Group B3 ($p < 0.0001$). On the other hand, when the onset times of groups were reviewed, the averages in Group B3 and Group B4 (85.60 ± 6.70, 115.20 ± 10.30, respectively) were significantly increased, compared with the averages in Group B2 ($p < 0.05$ and $p < 0.001$, respectively).

Discussion

Stevia rebaudiana is a very valuable herb that is specific to South America [7]. It is native to South America, although it is grown in Paraguay, Brazil, Colombia, Mexico, Uruguay, Guatemala, Peru, Japan, and South Korea [9,10]. The indigenous people used *S. rebaudiana* as a sweetening agent for centuries in South America [11]. Pure stevioside extract has been used in food since mid-1970s in Japan instead of as a defined synthetic sweetening agent. The leaf extract of this herb has been traditionally used for the treatment of diabetes [12].

Table 2 Comparison of groups by the Racine's Convulsion Scale (RCS) score and first myoclonic jerk (FMJ) latency.

Drugs group	Convulsion stage (RCS score)	FMJ onset time (s)
Group B1	0	0
Group B2	5.60 ± 0.2	67.80 ± 4.5
Group B3	4.33 ± 0.3[a*]	85.60 ± 6.70[a*]
Group B4	3.50 ± 0.2[a***,b**]	115.20 ± 10.30 [a***]

[a]Compared with Group B2.
[b]Compared with Group A3.
*$p < 0.05$.
**$p < 0.0001$.
***$p < 0.001$.
Group B1 = the control rats, which received no medication; Group B2 = the rats received 70 mg/kg pentylenetetrazol (PTZ) and 1 mL/kg saline; Group B3 = the rats received 70 mg/kg PTZ and 100 mg/kg rebaudioside A; Group B4, the rats received 70 mg/kg PTZ and 200 mg/kg rebaudioside A.

In contrast to other synthetic sweeteners, *S. rebaudiana* (Bertoni) contains a natural sweetening agent that is 250- to 300-fold sweeter than saccharose. Reasons for its preference among convenience food manufacturers include high temperature- and pH-stability [13]. Reb-A and stevioside are steviol glycosides derived from the *S. rebaudiana* herb [12,14–16].

In the literature, studies on stevioside, which is more frequently used than Reb-A, have a bias toward steviol glycosides [16]. Stevioside is a high molecular weight hydrophilic diterpenoid glycoside. Digestive enzymes cannot reduce it, and therefore it cannot be absorbed by the intestines. Stevioside is disintegrated with the aid of the intestinal bacterial flora. After absorption, it passes to the liver where it is transformed into steviol glucuronide. Excretion from the body occurs *via* bile and urine.

Intestinal microflora metabolize Reb-A into aglykon and steviol *in vitro* [17]. The only structural difference between Reb-A and stevioside is the additional glucose molecule in Reb-A [18,19]. The basic difference between the pharmacokinetic curves of Reb-A and stevioside occurs during the separation of the glycoside units by intestinal microflora from the aglykon unit before absorption. Reb-A and stevioside can be rapidly excreted in urine and, more frequently, in feces after being absorbed and metabolized in the liver as steviol [17,19].

Limited studies on the mechanism of action of Reb-A exist in the literature. The quality of current data is insufficient to explain the potential antiepileptic effect of Reb-A, although the existing data may provide the antiepileptic effect.

Several studies demonstrate the anti-inflammatory effect of stevioside [20–24]. Steviol glycosides have a strong anti-inflammatory effect induced by 12-O-tetradecanoylphorbol-13-acetate in mice [23]. *S. rebaudiana* has the highest antioxidant capacity among five different types of herbs (S.eupatoria, S. ovata, S.plummerae, S. salicafolia, S.serrata) [14].

The seizure-initiating roles of proinflammatory cytokines are accepted for epileptogenesis. By using the interleukin-1-beta (IL-1B) gene polymorphism, Kanemoto et al. [25] compared nonepileptic controls with temporal lobe epilepsy patients with or without cortical sclerosis. They found a strong coexistence of the IL-1B gene polymorphism and cortical sclerosis.

Febrile seizures of childhood exist among predisposing factors of temporal lobe epilepsy. The level of IL-1B level increases in the central nervous system and plasma in febrile seizures. Increased IL-1B activates the molecular pathways over astrocytic IL receptors and nuclear factor kappa B (NF-kappa B), thereby leading to calcium-dependent glutamate release [26]. Overexcited glutamate receptors have initiative effects for seizures and for easing the effects of spreading [26]. Stevioside suppresses proinflammatory cytokines and prevents activation of the NF-kappa B pathway for many inflammatory diseases and demonstrates a decreasing effect for inflammation [26].

Reb-A induces insulin secretion from pancreatic beta cells as a result of intracellular calcium increase by inhibiting adenosine triphosphate-dependent K channels. This action leads to cellular depolarization, and subsequently activation of calcium channels [26]. Stevioside causes vasodilatation by

slowing down the flow of calcium into vascular smooth muscle cells [27]. In addition, the effect of Reb-A on electrolytes in cells of the central nervous system has not been determined. The effect of Reb-A on intercellular calcium accumulation, which is responsible for epileptogenesis in the central nervous system, should be investigated [28].

The relationship of free radicals and reactive oxygen species with many conditions such as cancer, atherosclerosis, degenerative diseases, and resistant epilepsy is well known. The antioxidant capacity of *Stevia* has been proved by various methods in some studies [29,30]. This leads us to believe that the antioxidant activity of *Stevia* is also effective in its antiepileptic mechanism.

A substance with antiepileptic properties must cross the blood-brain barrier and then bind to the target point or points to exert an effect. Voltage-gated ion channels, neurotransmitter receptors, carriers or neurotransmitter release, and metabolic enzymes ensuring uptake and metabolism form these target points. In the literature, there is no study on whether Reb-A crosses the blood-brain barrier. However, experimental research investigating the genotoxic effects of stevioside and its metabolites indicate that stevioside is effective by passing the blood-brain barrier [31,32]. This finding emphasizes the need for advanced studies on this topic.

The effect of Reb-A on epileptic seizures has not been previously investigated in the literature. However, studies exist on a synthetic sweetening agent called aspartame, which was discovered in 1965. The results of studies of aspartame show that the sensitivity to seizures may be increased *via* an increase of phenylalanine levels [33,34]. Saccharine, one of the most frequently used low-calorie sweetening agents was discovered in 1878. Studies on saccharine have mostly been related to the gall bladder, and research investigating the relationship between saccharine and epilepsy has not been conducted [11].

As a result of our study of rats with PTZ-induced epilepsy, a decrease in the RCS score and an increase in the FMJ latency (which is dose-dependently more distinct) were statistically significant ($p < 0.05$, $p < 0.001$, $p < 0.0001$). For the groups that were administered Reb-A, an apparent decrease in spiking occurred in a dose-dependent manner ($p < 0.05$ and $p < 0.0001$). In conclusion, the results we obtained are a reminder that Reb-A could be safely used in patients with epilepsy.

Our study is just an introduction to this topic, which has very many uncertainties. Even though PTZ-induced convulsions are only an experimental model, not a real epileptic syndrome, our results showed very important data about probable anticonvulsant effects of Reb-A. The safety of using sweetening agents whose consumption by epilepsy patients is becoming increasingly prevalent for various reasons is a matter that should be emphasized as sensitively as it is for other diseases. We believe that more studies and data on this subject are required to reach conclusive results.

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