Original Article

Is grayanotoxin directly responsible for mad honey poisoning-associated seizures

Abdülkadir GÜNDÜZ[1], Murat AYDIN[1], Metehan AKÇA[2,4], Süha TÜRKMEN[1],

Süleyman TÜREDİ[1], Umut ERYİĞİT[1], Ali CANSU[3,4], Mehmet YILDIRIM[2,4]

Aim: The aim of this study was to investigate the effects of grayanotoxin on epileptiform activity in rats.

Materials and methods: Forty-two male Sprague Dawley rats were equally divided into 1 of 7 groups. Thirty minutes after induction of epileptiform activity induced by penicillin injection, 0.5, 1, 2, 4, or 8 µg of grayanotoxin-III was intracerebroventricularly administered. Epileptiform activity spike frequency and amplitude were converted into numerical data using software following the experiment.

Results: Our results show that grayanotoxin reduces epileptiform spike frequency and amplitude in a dose-dependent manner. Five minutes postinjection, grayanotoxin significantly reduced epileptiform activity, especially at higher doses. This acute effect subsequently declined, but a dose-dependent decrease was observed through the end of the experiment. This suggests that the first observed effect of grayanotoxin on spikes probably consists of blocking voltage-gated sodium channel inactivation.

Conclusion: Grayanotoxin's suppression of epileptiform activity in this experimental study indicates that grayanotoxin is not directly responsible for mad honey poisoning-associated seizures observed in a clinical context.

Key words: Grayanotoxin, mad honey, seizure, experimental epilepsy, ECoG, rat

Introduction

Grayanotoxin is found in the leaves and flowers of rhododendrons, of which there are more than 850 varieties in the world, that grow in wet forests along Turkey's Black Sea coast (1,2). Since toxins taken up from rhododendron flowers by bees are not detoxified in their organisms, they cause poisoning by becoming directly mixed in with honey (mad honey poisoning) (3). Grayanotoxins exhibit toxic effects by binding to sodium channels in cell membranes. They increase the permeability of sodium ions in excited membranes. Experimental studies have shown that the toxin affects the skeletal muscles, heart muscle, respiratory system, central nervous system, and endocrine system (4,5).

Patients exposed to grayanotoxins generally exhibit mild symptoms such as nausea-vomiting and enhanced secretion or paresthesia, although potentially lethal findings such as full atrioventricular block or respiratory depression may also emerge (6). Grayanotoxin may also, albeit rarely, cause convulsions (7). Are seizures a direct effect of grayanotoxin on neuronal excitability or rather a result of hypotension-associated hypoxia? The literature contains no clinical or experimental studies capable of shedding light on this clinical problem and answering the questions arising from it.

This experimental study was intended to investigate the effect of 'mad honey' on epileptiform activity induced in rats with penicillin and to determine whether or not grayanotoxin, the toxic agent of mad honey, is directly responsible for epileptic seizures.

Material and methods

Animals

Experiments were performed on 42 adult male Sprague Dawley rats weighing 240-280 g. Adult male rats were obtained from the Karadeniz Technical University Research Center. Animals were housed under a 12:12 light-dark cycle (light on at 0700 hours) and at a room temperature of 20 ± 2 °C. They were given free access to food and water. Every effort was made to minimize animal suffering and the number of animals used. Experimental procedures were approved by the Animal Experimentation Ethics Committee of Karadeniz Technical University. All experiments were carried out according to local guidelines for the care and use of laboratory animals and the guidelines of the European Community Council for experimental animal care.

Drug and drug administration

Grayanotoxin-III, ethanol, and urethane were purchased from Sigma (St. Louis, MO, USA). Grayanotoxin-III was dissolved in ethanol to which was added sterile saline solution (final solution ethanol/saline, 2:98 v/v, respectively), and given in doses of 0.5, 1, 2, 4, or 8 µg/rat (2 µL) via the intracerebroventricular (i.c.v.) route 30 min after penicillin administration. The control and ethanol groups were injected via the i.c.v. route with 0.9% saline and 2% ethanol, respectively, 30 min after penicillin injection in a volume of 2 µL; injections were administered into the left lateral ventricle of each rat through a stereotaxic apparatus, 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.6 mm ventral to the surface of the cortex, based on the atlas of the rat brain (8).

Experimental design

Forty-two animals were equally divided into 7 experimental groups: 1) 500 units of penicillin (2.5 µL, intracortical (i.c.)) + 0.9% NaCl (2 µL, i.c.v.); 2) 500 units of penicillin (2.5 µL, i.c.) + 2% ethanol (2 µL, i.c.v.); 3) 500 units of penicillin (2.5 µL, i.c.) + 0.5 µg of grayanotoxin-III (2 µL, i.c.v.); 4) 500 units of penicillin (2.5 µL, i.c.) + 1 µg of grayanotoxin-III (2 µL, i.c.v.); 5) 500 units of penicillin (2.5 µL, i.c.) + 2 µg of grayanotoxin-III (2 µL, i.c.v.); 6) 500 units of penicillin (2.5 µL, i.c.) + 4 µg of grayanotoxin-III (2 µL, i.c.v.), and 7) 500 units of penicillin (2.5 µL, i.c.) + 8 µg of grayanotoxin-III (2 µL, i.c.v.). The 6 rats in each group were then further divided into 3 groups of 2 animals each.

Surgical procedure

The animals were anesthetized with an intraperitoneal (i.p.) injection of urethane (1.25 g/kg), additional doses being given as needed (9). The left cerebral cortex was carefully exposed by craniotomy. After incision of the skull, the head was placed in a stereotaxic apparatus. Body temperature was monitored using a rectal probe and maintained at 37 °C with a homeothermic blanket system (Harvard Homoeothermic Blanket, Harvard Instruments, South Natick, MA, USA).

Induction of epileptiform activity

Epileptiform activity was induced by i.c. injection of penicillin G potassium (500 units/2.5 µL) using a Hamilton microsyringe (Type 701N, Hamilton Co., Reno, NV, USA) after recording baseline activities within 10 min.

Electrophysiological recordings

Two Ag-AgCl ball electrodes were placed over the left somatomotor cortex (electrode coordinates: first electrode, 2 mm lateral to the sagittal suture and 2 mm anterior to the bregma; second electrode, 2 mm lateral to the sagittal suture and 5 mm posterior to the bregma). The common reference electrode was fixed on the right pinna. Recordings of electrocorticogram (ECoG) activity were carried out using a 16-channel data acquisition system (PowerLab 16/30, AD Instruments, Castle Hill, Australia). All recordings were made with animals under anesthesia. The signals from the electrodes were amplified and filtered (0.1-50 Hz bandpass) using BioAmp amplifiers (AD Instruments). The ECoG signal was then digitized at a sampling rate of 1024. The digitized brain signal was displayed and stored on a personal computer. The frequency and amplitude of epileptiform ECoG activity was analyzed offline (10).

Statistical analysis

Spike frequencies and amplitudes for each animal were automatically calculated and measured using LabChart Pro v7.1 (PowerLab software, AD Instruments). The results are given as means ± standard error of the mean (SEM). Statistical comparisons were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data analysis was performed using ANOVA and Tukey post hoc tests for comparisons. Data are expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

Results

Baseline activities of each animal were recorded before the administration of penicillin, having confirmed that none of the animals had spontaneous epilepsy (Figure 1). I.c. injection of penicillin (500 units) induced epileptiform activity within 3-5 min after administration (Figure 1). It reached a constant level in terms of frequency and amplitude in 30 min, and this lasted for 4-5 h.

Forty-five minutes after i.c.v. injection of saline solution, the mean spike frequency and amplitude
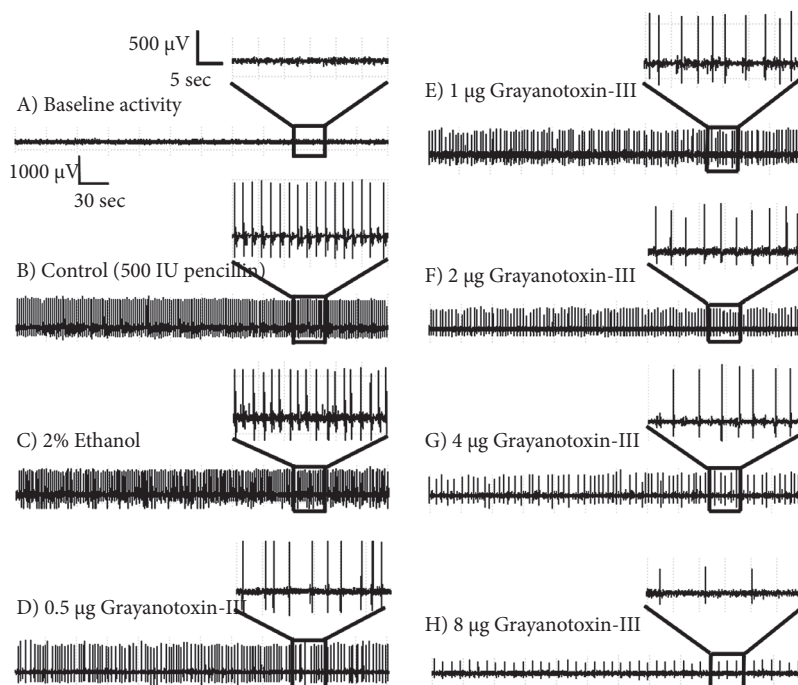

Figure 1. Representative ECoGs at 42-48 min after injections: A) baseline ECoG activity before the injection of penicillin or other substances; B) i.c. injection of penicillin (500 IU) induced epileptiform activity at ECoG, while 0.9% NaCl did not alter the frequency of penicillin-induced epileptic activity; C) 2% ethanol (i.c.v.) did not affect either the mean frequency or amplitude of penicillin-induced epileptiform activity; D) 0.5 µg of grayanotoxin-III (i.c.v.) did not affect either the mean frequency or amplitude of penicillin-induced epileptiform activity; E) 1 µg of grayanotoxin-III (i.c.v.) did not alter either the mean frequency or amplitude of penicillin-induced epileptiform activity; F) at 2 µg, grayanotoxin-III (i.c.v.) significantly reduced both the mean of frequency and the amplitude of epileptiform activity; G) at 4 µg, grayanotoxin-III significantly reduced both the mean frequency and amplitude of epileptiform activity; H) 8 µg of grayanotoxin-III (i.c.v.) significantly reduced both the mean frequency and amplitude of epileptiform activity.

were 32.4 ± 6 spikes/min and 1427 ± 323 μV, respectively. Control group epileptiform activity spike frequency and amplitude after the injection of 0.9% NaCl exhibited no great increase or decrease up to the end of the study, at 26-32 spikes/min and 1300-1500 μV, respectively (Figures 2 and 3). In the group administered ethanol (2%, i.c.v.), the mean spike frequency and amplitude 45 min after the injections were 31.5 ± 3 spikes/min and 1392 ± 320 μV. Compared with the control group, the injection of 2% ethanol had no statistically significant effect on epileptiform activity spike frequency or amplitude (Figures 2 and 3). Thirty minutes after the i.c. injection of penicillin, 0.5 μg of grayanotoxin-III was administered i.c.v. Forty-five minutes after the administration of 0.5 μg of grayanotoxin-III,

epileptiform activity spike frequency and amplitude were 19.8 ± 2 spikes/min and 1199 ± 221 μV, respectively. There was a slight reduction in mean spike frequency and amplitude after application of a 0.5 μg dose of grayanotoxin-III, but the difference was not statistically significant (Figures 2 and 3). Forty-five minutes after the i.c.v. administration of 1 μg of grayanotoxin-III, epileptiform activity mean spike frequency and amplitude were 21.6 ± 1 spikes/min and 996 ± 181 μV. There was again a slight decrease in mean spike frequency and amplitude in the group administered 1 μg of grayanotoxin-III, but this was also not statistically significant (Figures 2 and 3). Forty-five minutes after i.c.v. injection of 2 μg of grayanotoxin-III, epileptiform activity mean spike frequency and amplitude were 20.1 ± 4 spikes/
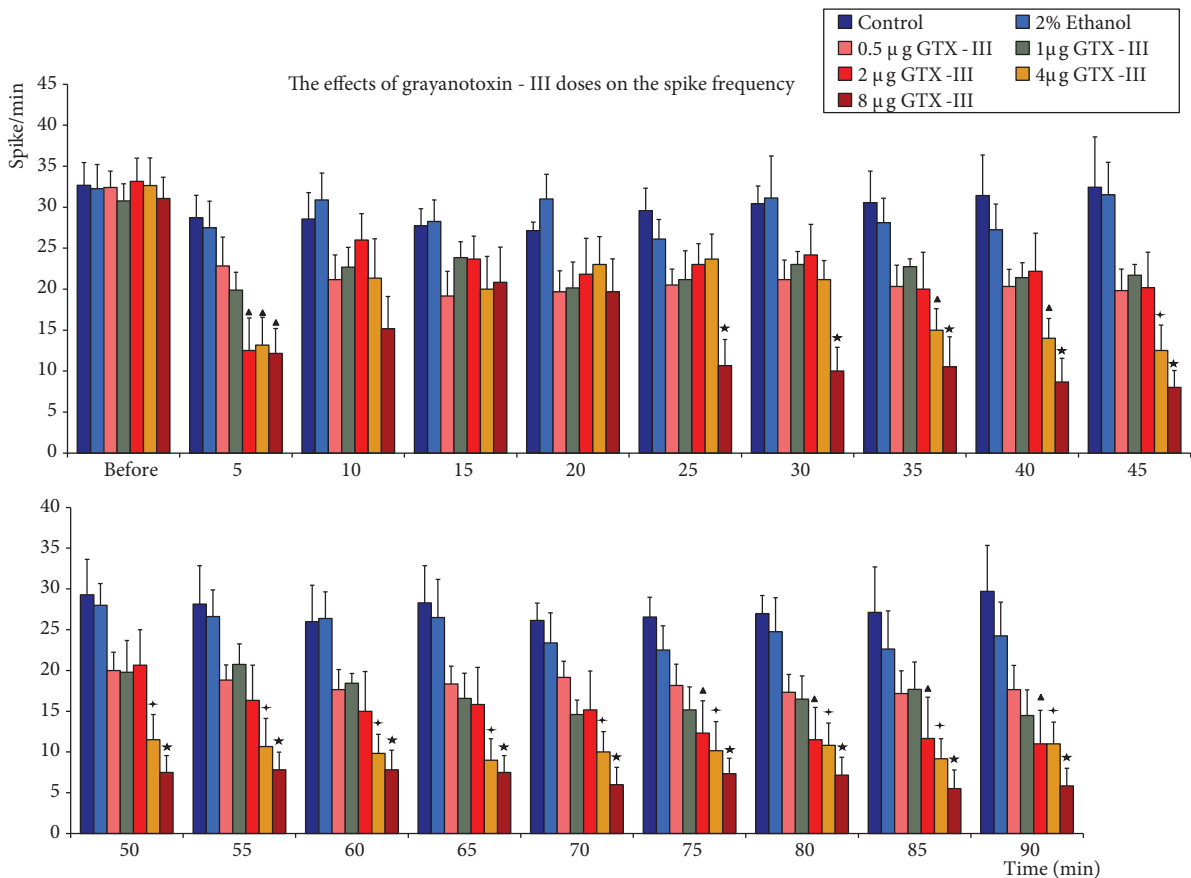

Figure 2. The effects of i.c.v. administration of grayanotoxin-III on the mean spike frequency of penicillin-induced epileptiform activity. At 0.5 and 1 μg/rat (i.c.v.), grayanotoxin-III did not affect the mean frequency of epileptiform activity. At 2, 4, and 8 μg/rat (i.c.v.), it significantly reduced the mean frequency of epileptiform activity (triangle, P < 0.05; 4-pointed star, P < 0.01; 5-pointed star, P < 0.001).
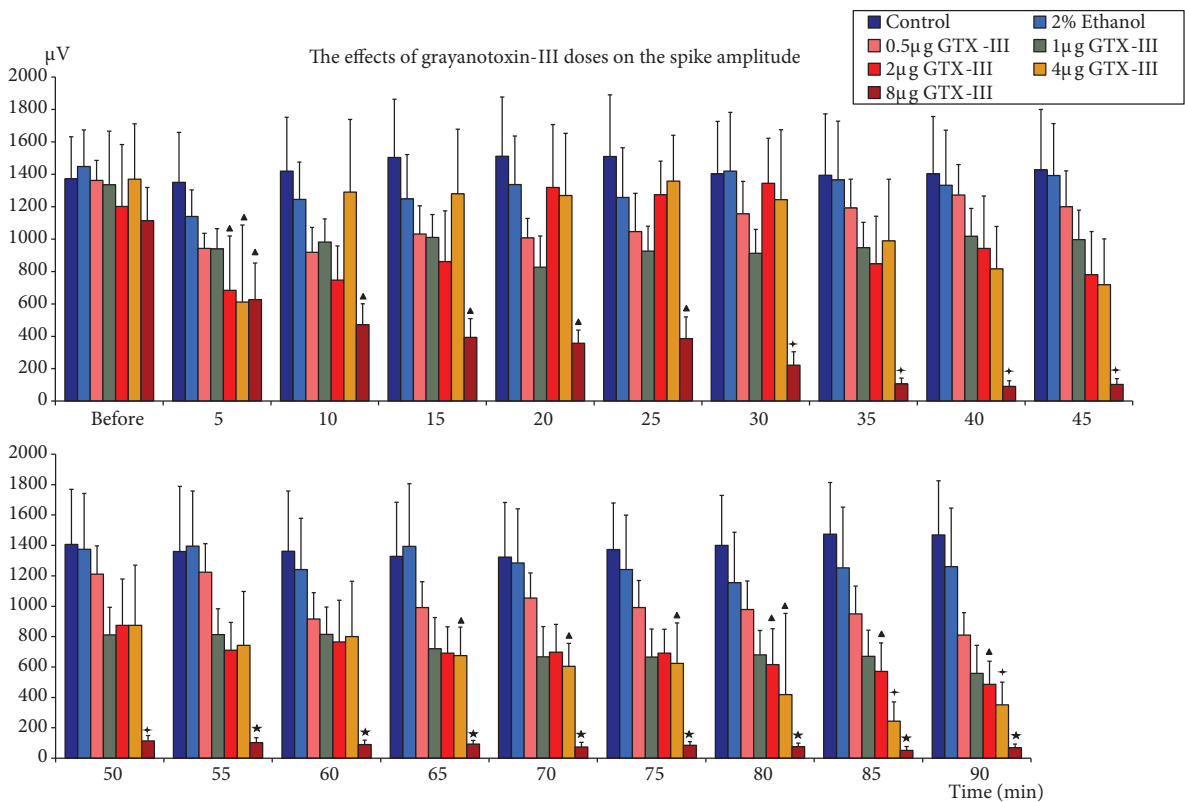
Figure 3. The effects of i.c.v. administration of grayanotoxin-III on the mean spike amplitude of penicillin-induced epileptiform activity. At 0.5 and 1 µg/rat, grayanotoxin-III (i.c.v.) did not affect the mean amplitude of epileptiform activity. At 2, 4, and 8 µg/rat (i.c.v.), it significantly reduced the mean amplitude of epileptiform activity (triangle, P < 0.05; 4-pointed star, P < 0.01; 5-pointed star, P < 0.001).

min and 780 ± 265 µV. Epileptiform activity spike frequency and amplitude decreased 5 min after the injection of 2 µg of grayanotoxin-III, but this effect was short-lived (P < 0.05). Toward the end of the experiment, average spike frequency in the group administered 2 µg of grayanotoxin-III again decreased significantly after 75 min (P < 0.05; Figures 2 and 3). Forty-five minutes after i.c.v. administration of 4 µg of grayanotoxin-III, epileptiform activity mean spike frequency and amplitude were 12.5 ± 3 spikes/min and 718 ± 282 µV. Epileptiform activity again temporarily decreased in terms of both spike and amplitude 5 min after injection of a 4 µg dose of grayanotoxin-III (P < 0.05). Mean spike frequency later decreased significantly as of 35 min, and mean spike amplitude decreased significantly as of 65 min (P < 0.05 and P < 0.01; Figures 2 and 3). Forty-

five minutes after i.c.v. administration of 8 µg of grayanotoxin-III, epileptiform activity mean spike frequency and amplitude were 8 ± 2 spikes/min and 103 ± 36 µV. Five minutes after injection of an 8 µg dose, mean spike frequency declined significantly (P < 0.05), and this persisted as of 25 min until the end of the experiment (P < 0.001; Figure 2). Statistical analysis of mean spike amplitude revealed significance at the P < 0.05 level between 5 min and 25 min postinjection, at the P < 0.01 level between 30 and 50 min, and at the P < 0.001 level between 55 and 90 min (Figure 3). At the end of the experiment all of the animals in the 0.9% NaCl, 2% ethanol, 0.5 µg grayanotoxin-III, and 1 µg grayanotoxin-III groups survived. Two rats in the 2 µg grayanotoxin-III group, 3 rats in the 4 µg grayanotoxin-III group, and 4 rats in the 8 µg grayanotoxin-III group died (Figure 4).
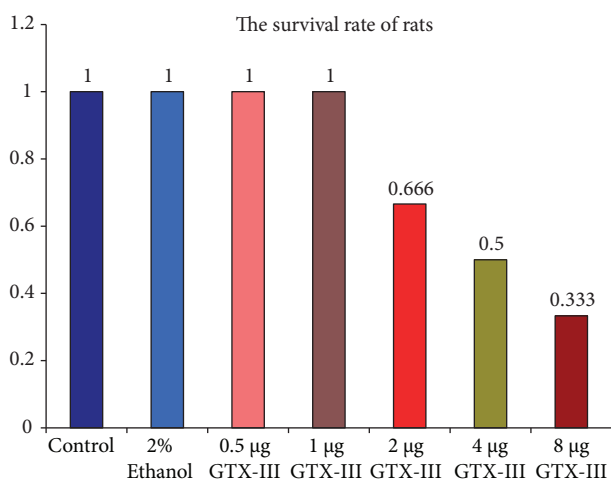
Figure 4. The effects of i.c.v. administration of grayanotoxin-III on rat survival rates.

Discussion

In this experimental epileptiform activity model, i.c.v. administration of grayanotoxin caused a decrease in epileptiform activity spike frequency and amplitudes. Grayanotoxin was administered in dosages from 0.5 µg to 8 µg. As dosages increased, there was a dose-correlated decrease in spike frequency and epileptiform activity was suppressed.

Following the i.c. administration of penicillin, there was a greater than 50% decrease in spike frequencies at 5 min after the grayanotoxin-III injections, especially in the 2, 4 and 8 µg toxin groups. Immediately afterward, there was a rise in spike frequencies, while from 25 min on there was a highly significant decrease in spike frequency, particularly in the 8 µg group (P < 0.001). The second decrease in spike frequencies increased from 35 min in the 4 µg group (P < 0.05) and acquired greater significance as of 45 min (P < 0.01). The decrease in spike frequencies becomes more significant as the dose of toxin administered increases and the time to appearance of dose-related decrease in spike frequencies shortens. Penicillin is known to cause epileptic activity by selectively blocking GABA-associated postsynaptic inhibition (11). Sitges reported that grayanotoxin increases the secretion of GABA, a powerful inhibitor neurotransmitter in presynaptic membranes (12). We cannot say, on the basis of our results in this study, that grayanotoxin

reduces penicillin-induced epileptiform discharges by affecting GABA receptors.

However, the effect of grayanotoxin, especially on early stage epileptiform discharges, may be associated with GABA discharges. There is therefore a need for studies demonstrating the effect of grayanotoxin on GABA receptors.

As with spike frequency, the same doses of toxin administered to the same groups also led to a significant decrease in spike amplitudes correlated to the increase in grayanotoxin dosage. In contrast, however, in the 8 µg group, spike amplitudes began decreasing very severely and very early (P < 0.05 at 5 min, P < 0.01 at 30 min, and P < 0.001 at 55 min). Spike amplitudes declining so fast and to such an extent, especially in the application of high doses, suggests that the toxin significantly suppresses the neuronal activity that establishes epileptiform activity. When the other groups were examined, the level and speed of decrease in amplitudes was less significant at lower dosages. Grayanotoxins are toxins dissolved in fat that increase channel activity and permeability of sodium ions in the cell membrane by binding to voltage-gated sodium channels in the membrane (13). In a study by Maejima et al. in 2003, grayanotoxin was reported to have a 3-stage effect on voltage-gated sodium channels (14). First, grayanotoxin binds to voltage-gated sodium channels during their opening phase. Once binding has taken place, the channel cannot be inactivated. The channels are subsequently modified. Finally, the modified sodium channels are hyperpolarized. This leads to voltage-dependent activation or inactivation in the cell membrane (14). The way that grayanotoxin caused a dose-dependent significant decrease in amplitude in epileptiform discharges by binding to voltage-gated sodium channels suggests that it thus blockades neuron excitability.

One of the factors that we considered in this study was rat survival rates. In the control group, the ethanol group and the 0.5 µg and 1 µg groups' survival rates were 100%, compared to 66.60% in the 2 µg group, 50.8% in the 4 µg group, and 33.3% in the 8 µg group. In conclusion, mortality rose with dosage. A decrease in rat respiratory rate was first observed, followed by a deceleration in heart beat and finally cardiac arrest. This finding shows that high doses in rats primarily block the respiratory center.

In light of the data from this study on significant changes in spike amplitudes in an experimental epilepsy model, the primary effect of grayanotoxin on spikes probably comes about by blocking voltage-gated sodium channel inactivation. However, considering the way that grayanotoxin increases GABA discharges, further studies illuminating the effect of grayanotoxin on GABA are now needed.

With high doses it probably leads to respiratory depression and death by reducing the excitability of all cortical and subcortical neurons. This pioneering, specific study is the first on the subject, and support from further wider experimental and clinical studies may reduce the number of deaths in poisoning with grayanotoxin (mad honey poisoning).

Acknowledgment

References

1. Avcı M. Orman gülleri (*Rhododendron* L.) ve Türkiye'deki doğal yayılışları. İstanbul Üniversitesi Edebiyat Fakültesi Coğrafya Bölümü Coğrafya Dergisi 2004; 12: 13-29.

2. Gunduz A, Turedi S, Russell RM, Ayaz FA. Clinical review of grayanotoxin/mad honey poisoning past and present. Clin Toxicol 2008; 46: 437-42.

3. Kurtoğlu S. Zehirlenmeler. Teşhis ve Tedavi 1992; 30: 569-70.

4. Yuki T, Yamaoka K, Yakehiro M, Seyama I. State-dependent action of grayanotoxin I on Na+ channels in frog ventricular myoctes. J Physiol 2001; 534: 777-90.

5. Öztaşan N, Altınkaynak K, Akçay FA, Göçer F, Dane Ş. Effects of mad honey on blood glucose and lipid levels in rats with streptozocin-induced diabetes. Turk J Vet Anim Sci 2005; 29: 1093-6.

6. Sütlüpinar N, Mat A, Satganoğlu Y. Poisoning by toxic honey in Turkey. Arch Toxicol 1993; 67: 148-50.

7. Dilber E, Kalyoncu M, Yarış N, Ökten A. A case of mad honey poisoning presenting with convulsion: intoxication instead of alternative therapy. Turk J Med Sci 2002; 32: 361-2.

8. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2nd ed. Sydney: Academic Press; 1986.

9. Kozan R, Ayyildiz M, Yildirim M, Agar E. The effects of ethanol intake and withdrawal on penicillin-induced epileptiform activity in rats. Brain Res Bull 2006; 71: 111-5.

10. Yildirim M, Marangoz C. Anticonvulsant effects of focal and intracerebroventricular adenosine on penicillin-induced epileptiform activity in rats. Brain Res 2007; 1127: 193-200.

11. Tsuda A, Ito M, Kishi K, Shiraishi H, Tsuda H, Mori C. Effect of penicillin on GABA-gated chloride ion influx. Neurochem Res 1994; 19: 1-4.

12. Sitges M. Characterization of the effect of monensin on gamma-amino-n-butyric acid release from isolated nerve terminals. J Neurochem 1989; 53: 442-7.

13. Catterall WA, Goldin AL, Waxman SG. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol Rev 2005; 57: 397-409.

14. Maejima H, Kinoshita E, Seyama I. Distinct site regulating grayanotoxin binding and unbinding to D4S6 of Nav1.4 sodium channel as revealed by improved estimation of toxin sensitivity. J Biol Chem 2003; 278: 9464-71.