

The dose-dependent effect of grayanotoxin on the cardiovascular system

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Aim: Mad honey (grayanotoxin, GTX) poisoning is caused by a toxin binding to the sodium channels of a cell membrane. This study investigates the dose-dependent effect of GTX on the cardiovascular system.

Materials and methods: Twenty-four male Sprague Dawley rats were divided into 4 equal groups. Group 1 was the control group and was administered only saline. GTX-III was administered in doses of 200, 400, and 800 µg/kg for Groups 2, 3, and 4, respectively. After GTX-III injection, blood pressure and heart rate were recorded using an electrophysiological data acquisition system.

Results: After 200 µg/kg GTX-III injection, at 31–60 min, the blood pressure decreased significantly from the 84 ± 8 mmHg value of the control group to 53 ± 4 mmHg (P = 0.023). In the group to which 800 µg/kg GTX-III was administered, the blood pressure decreased to 50 ± 7 mmHg and 54 ± 5 mmHg in the postinjection periods of 31–60 and 61–90 min, respectively. These values were significantly different from the 70 ± 6 and 84 ± 8 mmHg values recorded for the control group. The heart rate in the group injected with 400 µg/kg GTX-III decreased significantly 40 min after injection. In the 800 µg/kg GTX-III group, the heart rate decreased significantly 20 min after injection.

Conclusion: According to our findings, the bradycardiac and hypotensive picture seen in patients applying to the clinic with mad honey poisoning also emerges clearly in experimental animals at the high dose of 800 µg/kg GTX-III.

Key words: Mad honey, grayanotoxin, poisoning, cardiovascular system

1. Introduction

Mad honey poisoning is seen in settlement areas along the Turkish Black Sea coast and generally involves a clinical picture of bradycardia, hypotension, and syncope (1,2). The toxic effect of mad honey stems from the grayanotoxin (GTX) it contains. GTX is present in rhododendron (forest rose) leaves and flowers. Rhododendrons grow in the damp forests that extend along the Black Sea coast (3). The toxins taken up from *Rhododendron* species plants by the bees in this region lead to poisoning from the honey made by these bees (4). The GTX binds to sodium channels on cell membranes. The binding unit is the group II receptor site, localized on a site of the sodium channel involved in the voltage-dependent activation and inactivation (1,5). These compounds prevent inactivation. Thus, excitable cells (nerve and muscle) are maintained in a state of depolarization, during which entry of calcium into the cells may be facilitated. This action is similar to

that exerted by the alkaloids of *Veratrum* and aconites (6). All the observed responses of skeletal and heart muscles, nerves, and the central nervous system are related to the membrane effects (7). It has been suggested that sites of cardiac and respiratory activity of GTX are within the central nervous system and that bradycardiac effects of GTX are mediated by vagal stimulation at the periphery (8,9). There have been no studies showing the dose-dependent effect of GTX on the cardiovascular system. Future experimental studies on this subject will help physicians see the correlation between the clinical picture and the amount of honey ingested by a patient presenting to the emergency department with such poisoning.

This study was intended to investigate the effect of systemically administered GTX-III in varying doses on the blood pressure, heart rate, and respiratory frequency of rats under anesthesia.

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2. Materials and methods

2.1. Study design and setting

Approval of the local animal experiments ethics committee was obtained for the procedures involved. All experiments and the care and use of laboratory animals were carried out in compliance with local guidelines and the European Union Guide to the Care and Use of Experimental Animals.

2.2. Selection of participants

This experimental study used 24 adult male Sprague Dawley rats weighing between 249 and 280 g (mean weight 260 g). Grayanotoxin III Hemi(ethyl acetate) adduct and pure ethanol were purchased from Sigma, ketamine HCl (Ketalar) from Pfizer, and xylazine HCl (Xylazine Bio 2%) from Intermed. GTX-III was first dissolved in pure ethanol and a 2% solution obtained with the addition of sterile saline (ethanol and saline, 2:98 volume). GTX-III was administered intraperitoneally to 3 experimental groups in dosages of 200 µg/kg, 400 µg/kg, or 800 µg/kg. The control group was injected with 0.9% NaCl (1 mL/kg). General anesthesia was administered before the experiment began with the intramuscular administration of 90 mg/kg ketamine and 10 mg/kg xylazine.

2.3. Interventions

The 24 experimental animals were randomized into 4 groups: Group 1, the control group, was given 1 mL/kg intraperitoneal 0.9% NaCl. Group 2 was given 200 µg/kg GTX-III intraperitoneally. Group 3 was given 400 µg/kg GTX-III intraperitoneally. Group 4 was given 800 µg/kg GTX-III intraperitoneally. Experimental animals were anesthetized with an injection of ketamine/xylazine and additional doses were given to those requiring it. Rats were placed face up on the surgical table for carotid cannulation. Skin, fatty tissue, and submaxillary gland excision was performed around the trachea in such a way as to expose both carotid and jugular veins. The operating area flaps were folded to the side and sutured. The trachea was exposed by excising the lower part of the sternohyoid and sternomastoid muscles in front of it. The left carotid was suspended with 2 silk sutures (5/0). A pediatric catheter (24 G) was carefully inserted inside the carotid and a knot was made with silk suture from outside the vessel and fixed.

2.4. Methods and measurements

Once cannulation was complete, animals were placed face up and a pressure transducer (MLT0670 Disposable BP (Blood Pressure) Transducer, AD Instruments, Castle Hill, Australia) was inserted into the other end of the catheter to measure blood pressure. The BP transducer was attached to a BP amplifier (ML117 BP Amp, AD Instruments), and that was attached to a 16-channel electrophysiological data acquisition unit (PowerLab, AD Instruments). For electrocardiogram (ECG) recording, gel was applied

to the animals' front right and left and rear left legs and crocodile clip-type ECG electrodes were attached. The cables of these electrodes were attached to an amplifier (ML136 Animal Bio Amp, AD Instruments) linked to the PowerLab unit. Respiratory frequencies were recorded with the help of a piezoelectric conductor attached to the animals' chests with an elastic FILE bandage (MLT1010 Piezo Electric Pulse Transducer, AD Instruments). Rats' body temperatures were monitored with a rectal probe throughout the experiment and maintained at 37 °C with a homeothermic blanket system (Harvard Homoeothermic Blanket, Harvard Instruments, South Natick, MA, USA). Rats were attached to the electrophysiological data acquisition unit and ECG, blood pressure, heart rate, and respiratory frequency were recorded for 120 min. Baseline ECG, pressure, and respiratory records were first taken for 20 min. Subsequently, rats were administered intraperitoneal doses of 0.9% NaCl or GTX-III. Data from the animals were monitored constantly in a computer environment using LabChart software (v7.3, AD Instruments) over 2 h. Data were recorded onto hard disk for analysis after the experiment. ECG, blood pressure, and respiratory frequency were converted to numerical data with LabChart.

2.5. Analysis

Statistical comparisons were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Conformity with normal distribution was tested using the Shapiro–Wilk test. Following one-way analysis of variance (ANOVA) for normally distributed blood pressure data, the Bonferroni post hoc test was used to determine statistical difference between groups. Since heart rate and respiratory frequency data did not conform to normal distribution, we used a nonparametric statistical technique. The Mann–Whitney U test with Bonferroni correction was applied to these data following Kruskal–Wallis analysis of variance. Intragroup comparisons were performed with repeated-measures ANOVA and the paired samples t-test for variables having normal distribution. The Friedman test and Wilcoxon test with Bonferroni correction were used for intragroup comparisons of variables with nonparametric distribution. Data are expressed as mean ± standard error of the mean. Statistical significance was set at $P < 0.05$.

3. Results

The effect of systemic administration of GTX-III on arterial blood pressure was monitored. On the basis of the data obtained we compared preinjection pressure levels and minimum blood pressure values at intervals of 0–30, 31–60, and 61–90 min after injection between the groups. Mean arterial pressures before injection in Group 1, Group 2, Group 3, and Group 4 were 87 ± 4 mmHg, 76 ± 4 mmHg, 79 ± 8 mmHg, and 80 ± 5 mmHg, respectively.

No statistically significant difference was seen between the groups in terms of blood pressure values recorded before injection ($P > 0.05$).

Blood pressure after 0.9% NaCl injection in the control group at intervals of 0–30, 31–60, and 61–90 min was 70 ± 6 mmHg, 84 ± 8 mmHg, and 83 ± 9 mmHg. No significant difference was determined between the time-dependent blood pressure values in the control group. In Group 2, a statistically significant fall to 53 ± 4 mmHg was observed at 31–60 min after injection compared to the control group ($P = 0.023$). In Group 3, while a partial decrease was determined in blood pressure values measured at the various time intervals, there was no statistical difference between this value and that of the control group. Blood pressure in Group 4 fell to 50 ± 7 mmHg and 54 ± 5 mmHg at 31–60 and 61–90 min after injection. Compared with blood pressure values for the same intervals in the control group, the decrease in Group 4 was statistically significant ($P = 0.011$ and $P = 0.011$). Statistical analysis was performed using the Bonferroni post hoc test after ANOVA.

Animals in the groups administered GTX-III underwent hypotensive attack throughout the experimental period after GTX-III injection. An average of 4 hypotensive attacks took place during the 90-min experimental period. There was no difference between the GTX-III groups in terms of number of hypotensive attacks, and attack numbers did not increase in a GTX-III dose-dependent manner (Figure 1).

Rats' heart rates before injection (0.9% NaCl or GTX-III) were 291 ± 30 /min, 258 ± 22 /min, 301 ± 13 /min, and 225 ± 8 /min in Group 1, Group 2, Group 3, and Group 4,

respectively. There was no statistically significant difference between the groups in terms of preinjection baseline heart rate values ($P > 0.05$).

In Group 3, heart rate decreased significantly 40 min after injection compared to the control group, and this significant fall persisted until the end of the experiment ($P < 0.01$ for 40–90 min). In Group 4, heart rate decreased significantly 20 min after injection compared to the control group, and this significant fall also persisted until the end of the experiment ($P < 0.05$ for 20–30 min; $P < 0.01$ for 40–90 min). Heart rate data were compared using the Mann–Whitney U test after Kruskal–Wallis analysis of variance (Figures 2 and 3).

A piezoelectric transducer was used to determine the effect of doses of GTX-III injected intraperitoneally on respiratory frequency, and frequency was monitored for each animal. Mean preinjection respiratory frequency was 44 ± 3 breaths/min, and there was no statistically significant difference between the groups ($P > 0.05$). Respiratory frequency rose in a time-dependent manner, especially in Group 4, but this was not significant compared to the control group ($P > 0.05$). Since the standard deviations of respiratory data were very high, frequency is expressed as percentage changes from preinjection values (Figure 4).

4. Discussion

While studies on this subject state that the severity of poisoning findings is correlated with the amount of honey ingested, there have been no studies showing a correlation between the level of toxin and the clinical picture (10). This study shows the correlation between severity of poisoning and amount of ingested toxin. Individual

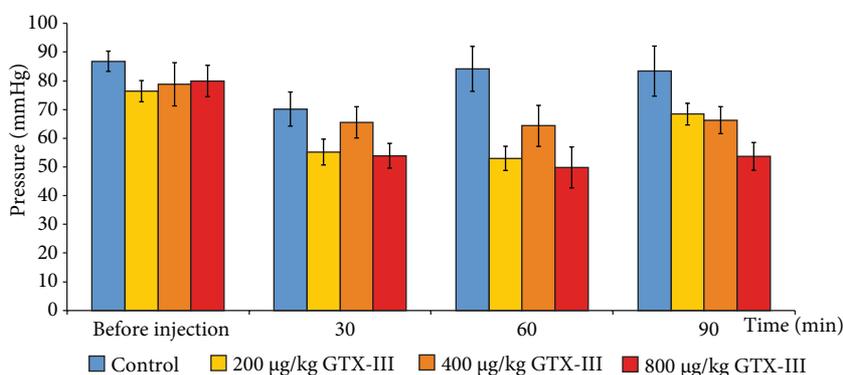


Figure 1. The effect of systemic administration of GTX-III on mean arterial pressure. *: Significant difference versus control group with ANOVA (Bonferroni post hoc test), $P < 0.05$; # and ##: significant difference versus preinjection time with repeated-measures ANOVA (paired sample t-test), $P < 0.05$ and $P < 0.01$, respectively; \$, \$\$, and \$\$\$: significant difference versus preinjection time with repeated-measures ANOVA (paired sample t-test), $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; && and &&& = significant difference versus preinjection time with repeated-measures ANOVA (paired sample t-test), $P < 0.05$ and $P < 0.01$, respectively.

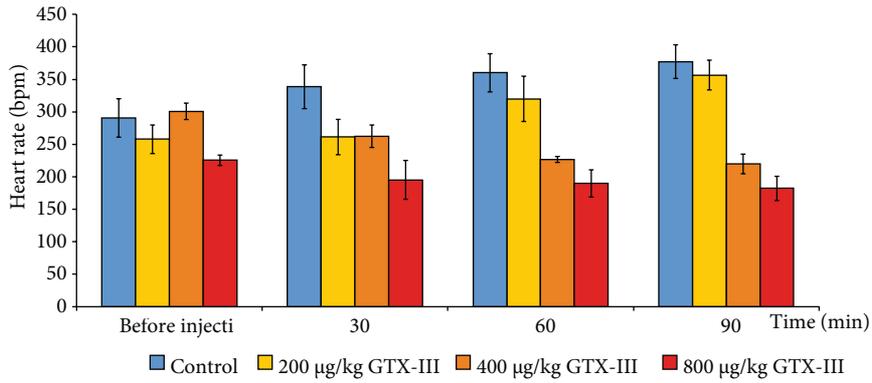


Figure 2. The effect of systemic administration of GTX-III on heart rate.

*: Significant difference versus control group with Mann–Whitney U test with Bonferroni correction, $P < 0.0167$.



Figure 3. Heart rate data of the 4 groups.

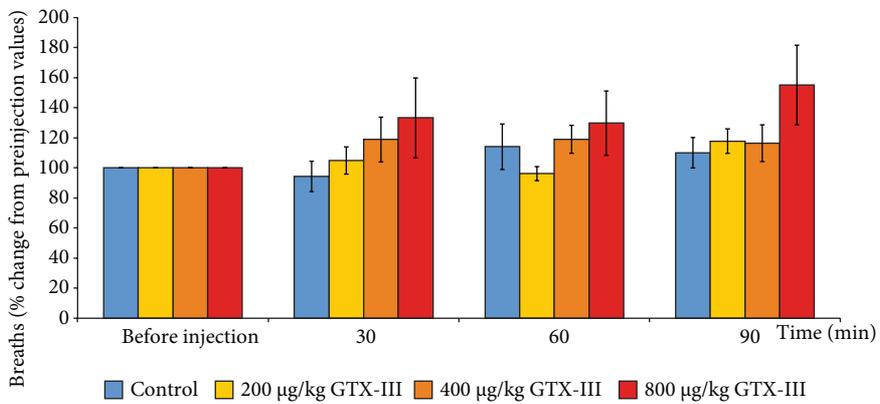


Figure 4. The effect of systemic administration of GTX-III on respiratory frequency.

differences can be seen in mad honey poisoning. Cases of poisoning with ingestion of 5–30 g have been reported (11). Some cases proceed with mild symptoms, such as nausea, vomiting, dizziness, and low blood pressure, but cases of complete block and asystole have also been reported (10,12). GTX can affect the central nervous system, skeletomuscular system, respiratory system, heart, and peripheral nervous system (8,13). In an experimental study, Onat et al. showed the effects of GTX on the respiratory system, central nervous system, and peripheral nervous system by inducing bradycardia and respiratory depression in rats. The same study also showed the bradycardiac effect of GTX with bilateral vagotomy. This shows that the effect of bradycardia is on the nervus vagus and its extensions. In another study, Onat et al. showed that bradycardia and respiratory depression in GTX poisoning improved with the administration of atropine, a nonspecific antimuscarinic agent. They also showed that bradycardia improved with the administration of AF-DX 116, a selective M2 muscarinic agent, but that this had no effect on respiratory depression (14). Studies have shown that GTX is a fat-soluble toxin and increases Na ion permeability by binding to voltage-dependent sodium channels in the cell membrane. Kim et al. showed that it increased Ca oscillation in inhibitory and excitatory nerve tips. In addition, it was suggested that GTX intoxications exhibit autonomic effects with an increase in GABA and glutamate oscillation from these tips (15). GTX and toxins such as tetrodotoxin, veratridine, and aconitine have been shown to exhibit effects with modification in specific canal functions (16).

GTX also has unique actions on Na⁺ channels, such as 1) causing a shift of Na⁺ channel activation to hyperpolarizing transmembrane potentials, 2) eliminating Na⁺ channel inactivation, and 3) binding to the Na⁺ channel in its open state, as established from the observation that these toxins require repetitive rather than single long-lasting, depolarizing stimuli to modify Na⁺ channels in excitable cells of vertebrates (17).

Many case series and reports have shown that patients presenting with GTX poisoning are subjected to cardiovascular system effects. As shown in some studies, this effect manifests itself as fluctuation in vital findings. The scale of this fluctuation may range as far as asystolic attack (18). Bradycardiac-tachycardiac and hypotensive attacks were clearly visible in the time-dependent tension and heart rate traces in our study. Baseline preinjection mean arterial blood pressure ranged from 76 to 87 mmHg in Group 1, Group 2, Group 3, and Group 4. Minimum blood pressure values between 31 and 60 min after GTX

injection decreased significantly in Group 2 and Group 4 compared to the control group ($P < 0.05$). On the other hand, the significant fall in minimum blood pressure value in Group 4 persisted ($P < 0.05$). Hypotensive attacks in Group 2 and Group 4 were significant compared to the control group. Although hypotensive attacks in Group 3 were lower than in the control group, the difference was not statistically significant. An average of 4 hypotensive attacks were observed over the 90-min recording period in all groups administered GTX-III. These data account for the hypotensive attacks observed clinically in grayanotoxin patients. Investigation of the effect on heart rate of 200, 400, and 800 µg/kg GTX-III showed that 200 µg/kg GTX-III had no effect. However, 400 µg/kg GTX-III decreased heart rate in a statistically significant manner 40 min after intraperitoneal injection, and this persisted until the end of the study ($P < 0.01$). The effect of 800 µg/kg GTX-III was manifested earlier compared to that of 400 µg/kg. A significant effect of the 800 µg/kg GTX-III dose commenced 20 min after injection and persisted until the end of the experiment ($P < 0.05$ for 20 and 30 min; $P < 0.01$ for 40 and 90 min). These data account for the bradycardiac attacks observed clinically in patients with grayanotoxin poisoning. While no effect was seen at a low dose, bradycardia became more evident as dosage increased. This finding is in agreement with the more severe cardiac findings in patients with a higher ingestion of honey in clinical series. Some experimental studies have reported that grayanotoxin also has a suppressive effect on respiratory functions (8,14). Although, in our study, respiratory frequency increased toward the end of the experiment in comparison to baseline values in animals administered high doses of GTX-III, this rise was not statistically significant. No decrease in respiratory frequency was observed in the study.

The bradycardiac and hypotensive picture seen in patients applying to the clinic with mad honey (GTX) poisoning also emerged clearly in experimental animals at the high dose of 800 µg/kg GTX-III. Our findings showed a statistically insignificant rise in respiratory frequency in experimental animals given high-dose GTX. With this preliminary study, the determination of an experimental GTX dosage needed to induce mad honey poisoning in experimental animals and a detailed revelation of the changes in blood pressure and heart rate at this dosage will pave the way for further studies intended to determine the extent of cardiovascular effects in GTX poisoning and alternative treatment options. The present study is the first animal study in which authors have used the GTX administration model.

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