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ORIGINAL ARTICLE

Investigation Of Mutagenic Effects Of Grayanotoxins II And III On Cultured Human Lymphocytes

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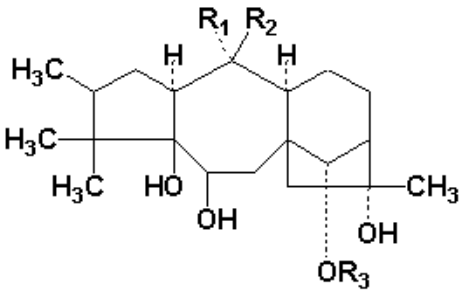
Abstract: In order to detect the mutagenic effects of Gryanotoxins (GTX) II and III on human lymphocytes, two lymphocyte culture sets, one for micronuclei and other for chromosome analysis, were prepared. GTX-II and GTX-III at 3.52 mg/ml and 0.352 mg/ml concentrations were added to the lymphocyte cultures of ten female and ten male subjects by standard protocols. Then, the obtained data were compared with that of positive and negative control groups. Consequently, mean MN and chromosomal aberration values of twenty subjects were not considerably higher than those of negative control group ($p > 0.05$). Only positive control group showed higher MN and chromosomal breakage values than the other groups ($p < 0.001$). In conclusion, the usage of the GTX-II and GTX-III containing mad honey as a folk remedy may not cause chromosomal damage.
Key Words: Grayanotoxins, GTXs, micronucleus, mutagenicty, chromosome damage.

Introduction

Grayanotoxins (GTXs) are toxic compounds contained in the leaves, flowers, pollen and nectar of some species of the Rhododendron genus (Ericaceae) and the toxic honey produced from the floral nectar of these plants [1-2]. The toxin content in R. ponticum is particularly high [3]. But, R. ponticum is widely used as an analgesic in northern Anatolia (Turkey) for the treatment of rheumatic or dental pain, common colds, and edema, both internally and externally [4]. Other species of Rhododendron and other members of the botanical family Ericacae, to which Rhododendrons belong, may produce the toxins but are not often linked to the disease. Mountain laurel (Kalmia latifolia) and sheep laurel (Kalmia angustifolia) are probably the other most important sources of the toxin [5]. GTXs are sodium channel toxins similar to Batrachotoxin, veratridine and aconitine. These toxins depolarize the sodium dependent excitable membranes through a specific increase in resting permeability to sodium ions [6]. GTXs have a wide range of systemic effects including hypotension, arrhythmias, respiratory depression, nausea, vomiting, dizzines, torsion spasm and reduction in spontaneous movement indicating a central nervous system effect [7-8]. Their mutagenic effects have not been studied, but honey from the nectar of the Rhododendron species is used traditionally in north eastern Turkey, in quantities of one teaspoonful to two tablespoonfuls, to heal gastrointestinal disorders such as gastritis and peptic ulcers [8]. Extracts of Rhododendron species are consumed in Japan as a folk remedy for hypertension [9]. The chemical structure of GTX compounds involve OH, CH2 and CH3 groups, and their molecular structures are resembling the structures of methylating and intecalating agents (Figure-1). Therefore, they might have potential of being mutagenic agents. For this reason, we aimed to investigate if these compounds have mutagenic effects on cultured human

lymphocytes [10-14]. To our knowledge, there is a lack of studies on the possible genotoxic effects of GTX II and III. For this reason, we aimed to investigate the MN stimulating and clastogenic effects of GTX II and III on cultured human lymphocytes.



GRAY	R_1	R_2	R_1R_2	R_3
GRAY1	OH	CH_3	-	H
GRAY2	-	-	$=CH_2$	H
GRAY3	OH	CH_3	-	Ac

Figure 1. Molecular structures of GTXs (10,15).

Materials and Methods

Material: The compounds GTX-II and GTX-III, which kindly provided by Dr. Tadamasa Terai (Department of Applied Chemistry, Osaka Institute of Technology, Osaka , Japan).

Sampling: Heparinized blood samples were taken at sterile conditions from 25-46 years old normal, healthy volunteers. These volunteers were not drug users or smokers.

Addition of the GTXs to the culture media: The compounds GTX-II and GTX-III were added into the related culture sets with final concentrations of 0.3525 mg/ml 3.525 mg/ml, at 24 hour of the culture (these doses were equal to the doses of GTX II: $1x10^{-2}$ mM and $1x10^{-3}$ mM, and of GTXIII**:** 8,492 X 10^{-3} mM ve 8,492 X 10^{-4} mM). Because of the lack of the previous genotoxicity studies in the literature the doses were determined according to the fatal doses reported by The Beddgelert Rhododendron Management Group [15] and Kisch-Volders et al [16]. Since the limited amounts of the compounds were available, only two possible highest doses with ten fold range were searched.

Cell Culture: Lymphocyte cultures were performed according to Ascioglu et al [17] in double set (one for MN test and other for chromosomal breakage analysis) by adding 8 drops of whole blood into sterile, disposable culture tubes which contained 5 mls of Ham's F10 medium (Catalog no. 01-090-1B, Biological Industries, kibbutz beth haemk-Israel, briefly: BI) supplemented with 25% Fetal calf serum (BI), 2% Sodium heparine (Roche), Antibiotic 5000 IU Penicillin (BI), 0.5 mg Streptomycin (BI) and PHA-M at final concentration 100 µg/ml (Difco lab-Bacto-Phytohemagglutinin M Cat.No:0528-56-6) and incubated at 5% CO_2 and 5% humidity, 37°C, for 72 hours. The whole blood sample of each volunteer was

separately cultured in media with 3.525 mg/ml and 0.3525 mg/ml of GTX-II and GTX-III for experiment groups, for 72 hours. In addition, we performed cultures without GTXs or Mitomycin C (MMC) as negative control, and with 50 µg/ml of 6.25 µg/ml final stock MMC solution (final concentration adjusted to 0.19µM) of Mitomycin C as positive control.

Harvestig for methaphases and the analysis for the chromosomal breaks: 50 µl of colchicine (Carl Roth, Carlsruhe) solution from the stock of 1/25000 (W/W) was added to each culture of chromosome analysis set (final concentration of 0, 04µg/ml), at 70 hour (2 h before harvesting) of the culture. After hypotonic treatment with KCl 0.075 M; fixation with methanol (Merck): acetic acid (Merck) 3:1, the cells spreading on slides and air drying at room temperature were done. Then, the slides were stained in 5% Giemsa (Merck), for 7 minutes. A hundred metaphase plaques from each sample were examined for chromosomal breakages.

Harvesting and analysis for the micronucleus assay: To observe the micronuclei, according to the cytokinesis block method of Fenech and Morley [18], Cytochalasin-B (Sigma) was added to each culture of MN set, at a final concentration of 3.0 µg/ml, at 44^{th} h of incubation, and culture tubes were covered with aluminium foil against the photolytic effect. Harvesting was performed by usual steps including the mild hypotonic treatment with KCl solution (0.1 M) at 72 hour of the culture, and the material kept at $4\,^{o}C$ for at least 24 hours. Then, slides were prepared and stained in 5 % Giemsa, for 7 minutes. The MN analyses were randomized and scored by a single observer. From each individual, 2000 cytokinesis blocked (binucleated: BN) cells were examined under X400 magnification and the cells with MN were examined in detail using X1000 magnification. Following the criteria suggested by Sarto et al [19] dead or degenerating cells and nuclear blebbings were excluded from the evaluation, while only micronuclei equal to or smaller than one-fifth of the main nucleus were considered.

Statistical Analysis Data were analyzed using SPSS v.9.0 software. Statistical differences between groups were examined by Post Anova Turkey test, Student's t test. Data are presented as mean $\pm$ SD.

Results

The sex, age and MN ratio of each individual are shown in Table 1. The mean MN values of negative control, positive control, 3.52 mg/ml GTX-II, 0.352 mg/ml GTX-II, 3.52 mg/ml GTX-III and 0.352 mg/ml GTX-III concentration groups were 0.69 ± 0.63 %, 4.61 ± 0.96 %, 1.00 ± 0.57 %, 0.80 ± 0.56 %, 1.15 ± 0.62 % and 0.99 ± 0.55 % respectively. According to the SPSS variance analysis, except the positive control group ($p< 0.001$), there was no difference among the mean percentages of MN cells for all other groups ($F=103.308$, $p > 0.05$). When the percentages of MN cells from concentration and control groups of female volunteers were compared with those of male volunteers, using Student's t-test, the significant difference was not detected (all $p>0.05$).

Table 1: The distribution of the analyzed MNBN lymphocytes (%)

Subject	Sex	Age (yrs)	(-) Control	MMC (+C) (0.0625 µg/ml)	GTX II (3.52 mg/ml)	GTX II (0.352 mg/ml)	GTX III (3.52 mg/ml)	GTX III (0.352 mg/ml)
1	F	25	0.13	4.22	0.49	0.36	1.38	1.29
2	F	26	0.14	3.88	0.70	0.20	1.90	1.30
3	F	27	0.16	3.59	0.42	0.21	0.29	0.18
4	F	28	0.20	4.02	0.61	0.42	0.42	0.37
5	F	31	0.32	4.16	0.83	0.47	1.81	0.89
6	F	35	0.48	5.00	0.66	0.50	0.71	1.66
7	F	36	0.53	4.97	0.96	0.59	0.76	1.72
8	F	39	0.80	4.84	0.97	0.82	0.69	0.68
9	F	45	1.63	5.53	1.84	1.64	1.36	1.40
10	F	45	1.96	6.11	1.98	1.68	1.72	1.60
11	M	23	3.11	0.05	0.69	0.22	0.91	0.72
12	M	24	0.07	3.62	0.20	0.38	0.14	0.10
13	M	28	0.22	3.12	0.38	0.30	0.42	0.36
14	M	34	0.46	4.48	0.76	0.61	0.69	0.50
15	M	34	0.34	4.39	1.09	0.96	1.14	0.51
16	M	38	0.58	4.06	0.67	0.60	0.80	0.76
17	M	42	1.00	5.98	1.42	1.08	1.00	1.10
18	M	43	1.31	5.25	1.54	1.35	2.01	1.28
19	M	44	5.92	1.45	1.91	1.66	2.00	1.53
20	M	46	1.90	6.00	1.98	1.90	1.98	1.80
Mean (±SD)		34.6± 7.83	0.69± 0.63	4.61± 0.96	1.00± 0.57	0.80± 0.56	1.15± 0.62	0.99± 0.55

The sex, age and chromosomal aberration frequency of each individual are shown in Table 2. The mean chromosomal breakage values of negative control, positive control, 3.52 mg/ml GTX-II, 0.352 mg/ml GTX-II, 3.52 mg/ml GTX-III and 0.352 mg/ml GTX-III and 0.352 mg/ml GTX-III concentration groups were 0.15±0.36 %, 4.75±0.91 %, 0.01±0.30 %, 0.15±0.36 %, 0.55±0.22 % and 0.05±0.22 % respectively. According to the variance analysis, only the positive control group value was higher ($p<0.001$) than those of other groups ($F=334.872$ and all p values >0.05).

Table 2: The distribution of the analyzed cells with chromosomal breaks (%)

Subject	Sex	Age (yrs)	(-) Control	MMC (+C) (0.0625 µg/ml)	GTX II (3.52 mg/ml)	GTX II (0.352 mg/ml)	GTX III (3.52 mg/ml)	GTX III (0.352 mg/ml)
1	F	25	0.00	3.00	0.00	0.00	0.00	0.00
2	F	26	0.00	5.00	1.00	0.00	0.00	0.00
3	F	27	0.00	5.00	0.00	0.00	0.00	0.00
4	F	28	0.00	4.00	0.00	0.00	0.00	0.00
5	F	31	0.00	4.00	0.00	0.00	0.00	1.00
6	F	35	0.00	4.00	0.00	0.00	0.00	0.00
7	F	36	0.00	4.00	0.00	0.00	0.00	0.00
8	F	39	0.00	6.00	0.00	1.00	0.00	0.00
9	F	45	1.00	6.00	0.00	0.00	0.00	0.00
10	F	45	1.00	5.00	0.00	0.00	0.00	0.00
11	M	23	0.00	4.00	0.00	0.00	0.00	0.00
12	M	24	0.00	5.00	0.00	1.00	0.00	0.00
13	M	28	0.00	4.00	0.00	0.00	1.00	0.00
14	M	34	0.00	4.00	1.00	0.00	0.00	0.00
15	M	34	0.00	5.00	0.00	0.00	0.00	0.00
16	M	38	0.00	5.00	0.00	0.00	0.00	0.00
17	M	42	0.00	4.00	0.00	0.00	0.00	0.00
18	M	43	1.00	6.00	0.00	0.00	0.00	0.00
19	M	44	0.00	6.00	0.00	1.00	0.00	0.00
20	M	46	1.00	6.00	0.00	0.00	0.00	0.00
Mean (±SD)		34.6± 7.83	0.15± 0.36	4.75± 0.91	0.10± 0.30	0.15± 0.36	0.05± 0.22	0.00± 0.22

Discussion

GTXs are diterpenoid compounds consisting of two hexameric and one pentameric cycle. Although they have no nitrogen atoms, because of their basic structure, they look like purin bases which consist of one hexameric and one pentameric cycle [11-12]. On the other hand, the GTXs have CH_2 and CH_3 groups that are linked the cyclic structure provide these compounds a possible mutagenic character, such as alkylating agents, for example as Mitomycin-C. Thus, GTXs might probably intercalate between DNA strands instead of purine bases during DNA replication and may form cross linkings between two DNA strands and may lead to breakages or methylation [13-14]. Terpenoids are hydrocarbon polymers of isoprene units, some of which can initiate or promote cancer [20]. Some others are known to have anti-cancer chemo-preventive and/or chemotherapeutic aspects [21-22], while remains show no relationship with mutagenesis or carcinogenesis [23]. Most of the effects of these substances generally occur at molecular level, affecting posttranslational proteins that are products of oncogenes (proto-oncogenes) or related enzymes [20-22]. The micronucleus test is a simple and sensitive short term assay for the detection of environmental genotoxicants [24]. Data showed that 3.52 mg/ml and 0.352 mg/ml concentrations of GTX II and III had not caused an increase in (in vitro) MN cell frequency. When volunteer age was taken as a parameter, MN cell frequency in all groups, including controls, showed positive correlation with the age. Thus, the percentage of MN cells was affected by volunteer age rather than GTXs. Our chromosomal analysis data also showed that the GTXs were not of the agents cause chromosomal damage. Most of the previous studies are related with the chemical properties of GTXs, and the others are biological studies about effects of various types of GTXs on neuronal activity or developmental toxicity [7, 25]. Although the toxic honey which contains GTXs is consumed as a traditional folk remedy, as far as we know, there is a lack of information about the effects of GTXs on genetic material. The only report which has genetic aspects is our previous article on effect of GTX II on in vitro mitotic activity of human lymphocytes [17]. For this reason, our data could not have been compared to those from other studies. In conclusion, our preliminary data showed that the in vitro usage of mad honey, containing GTX-II and GTX-III may not cause chromosomal breakage risk on cultured human lymphocytes. Further studies should be performed related to the genetic effects of GTXs and their metabolites on human cells.

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