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Neuromuscular and Microvascular Changes Associated with Chronic Administration of an Extract of *Teucrium stocksianum* in Mice

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Abstract

This work examines the effect on the weights of vital body organs, on blood biochemical variables, on neuromuscular coordination and on cerebral microcirculation of aqueous extracts of *Teucrium stocksianum*, given to mice in drinking water at concentrations of 2 and 4% for 56 days.

The treatment caused progressive impairment of neuromuscular coordination, as evidenced by the time spent on the rota-rod. After photochemical challenge, the time for first observable platelet aggregation in arterioles was shorter than for the control group by 22 and 45% in the 2 and 4% *T. stocksianum*-treated groups, respectively. Platelet aggregation on the venular side was not affected by the treatment nor were microvascular diameters. Treatment with the plant extract produced no statistically significant effect on the plasma biochemical variables that are considered indices of liver and kidney function. Histologically, brains obtained from mice treated with *T. stocksianum* showed loss of cerebellar Purkinje cells.

Although it is likely that the accelerated platelet aggregation might have contributed to an ischaemic effect which could, at least in part, have caused the cytotoxicological changes, this does not exclude the possibility of a direct cytotoxicological effect of the plant extract. Further pharmacological and toxicological investigations on *Teucrium* species seem warranted.

Previous work (Tanira et al 1996) has investigated the toxicity of *Teucrium stocksianum* Boiss (Labiatae) in rats after chronic administration. Treatment of rats with *T. stocksianum* was associated with neurological toxicity and with occasional apoptosis in the liver. In particular, a loss in the Purkinje cell layer in the cerebellum was observed. These cells play a significant role in coordinating muscular movement in the body by helping to turn on the agonist muscles with simultaneous turning-off of the antagonist muscles (Guyton 1991). Because neither the physiological implication nor the mechanism of the reported neurotoxicity of the plant was pursued in our previous work, in this work we investigated the effect of administration of an aqueous extract of *T. stocksianum* on muscular coordination in mice during and at the end of a 56-day period. We also tested the possibility of central ischaemia-induced cytotoxicity (as a potential mechanism of action) caused by the plant extract at the end of the treatment period by studying changes in the animals' pial microcirculation.

Materials and Methods

Animals

The study was performed on 30 albino male mice (TO strain), 25–30 g, obtained from a colony maintained at our animal facility. Animals were housed in groups in a controlled environment: temperature 22 ± 2°C, 12 h/12 h light/dark cycle, lights on at 0700 h. They were fed a standard pelleted diet (Abu Dhabi Flour and Animal Feed Factory, UAE) and had free access to water. Mice were distributed at random into three equal groups.

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Plant material and preparation of extract

The aerial parts of *T. stocksianum* were collected from the Khor Fakkan area in the eastern part of the United Arab Emirates (UAE) in April 1995. The plant material was botanically authenticated and herbarium specimens were deposited at the Herbarium of the Desert and Marine Environment Research Center, UAE University.

The plant extract was prepared by adding distilled water (350 mL) to coarsely powdered aerial parts of *T. stocksianum* (12 g) and the macerated material was left to stand for 12 h. The volume of the filtrate was adjusted to 300 mL with distilled water to yield a solution of 4% concentration; this was further diluted (1 : 1) to give a 2% solution. The extraction procedure adopted resembles that conventionally used in the preparation of folk medicine.

Experimental design

Freshly-prepared 2 and 4% aqueous extracts of *T. stocksianum* were received daily by two groups of mice (groups: 2% and 4%, respectively) in lieu of drinking water and a third group (control) received tap water only. The experimental period lasted for 56 days. Animals were weighed and subjected to a rota-rod test weekly. Certain plasma constituents (see below) were determined bi-weekly. On the last day of the experimental period, all animals were subjected to photochemical induction of platelet aggregation. Mice were then swiftly decapitated and the wet weights were recorded for the brain,

liver, heart, lungs, stomach and duodenum, spleen, testes, kidneys, flexor muscle, digitos muscle and soleus muscle.

Rota-rod test

The method used was adopted from that of Kuribara et al (1977) which employed a rota-rod treadmill for mice (7600 Ugo Basile, Italy). A plastic rod (diameter 30 mm, length 30 cm), with a non-slippery surface, was used 15 cm over the base of the cage. The rod was divided into five equal sections by six discs, thus enabling 5 animals to walk on the rod at the same time. The rod was rotated at a speed of 33 rev min^{-1}. The interval between the animal mounting the rod and falling off was recorded by means of a built-in timer; this was considered as the performance time.

Biochemical determinations

Blood was collected from the tail tips of all mice into heparinized capillary tubes. Plasma was separated by centrifugation for 10 min at 900 *g* and 5°C. Biochemical analyses were determined by use of a Cobas Fara II autoanalyser (Roche, Switzerland), using appropriate kits supplied by the manufacturer. The variables measured were: concentrations of glucose, creatinine, urea, cholesterol, triglycerides, bilirubin and total protein, and the activities of aspartate aminotransferase (AST) and acid phosphatase (AP).

Photochemical challenge

Mice were anaesthetized with urethane (1–2 mg g^{-1}, i.p.) and the trachea was intubated. A craniotomy (3 × 3 mm) was performed, always on the left side of the animal, with the aid of a micro-drill and the dura was carefully stripped open (Rosenblum & El-Sabban 1977). The mouse was then placed on the stage of a fluorescence microscope (BH2, Olympus), which was a component of a microscope-television circuit with a VCR recorder/player. The instrumentation was similar to that described by El-Sabban et al (1994). The mouse was also placed on a heating mat, its core body temperature was monitored by means of a rectal thermoprobe, until it reached 37°C (within 30 min). The pial preparation was kept moist with artificial cerebrospinal fluid of composition (mM): NaCl 124, KCl 5, NaH_2PO_4 3, $CaCl_2$ 2·5, $MgSO_4$ 2·4, $NaHCO_3$ 23 and glucose 10; pH 7·3–7·4.

Pial (brain surface) arterioles and venules were examined and any preparation showing a level of trauma either to vessels or to underlying brain tissue was discarded. During the period from performing craniotomy until the time of irradiation, a 25–50 mm diameter field containing both arterioles and venules was chosen. The observed field was recorded on video tape before and throughout the duration of the photochemical challenge. Tapes were re-played for measurement of vessel diameter and for any further analysis.

Platelet aggregation in pial microvessels was induced photochemically. An injection of sodium fluorescein (5% solution; 0·1 mL (25 g)$^{-1}$ was given via the tail vein and was allowed to circulate for 30–40 s. The cranial preparation was then exposed to a stabilized and intense (44 000 lux candle cm^{-2}) mercury light. The combination of light and dye causes injury to the endothelial cell layer of the vessel to which platelets adhere and then aggregate in response. The time for the first aggregate to appear and the time for full occlusion were measured for both arterioles and venules. Diameters before irradiation and after full occlusion were measured from the video recordings and changes were calculated. All microscopic observations were made using a 4× objective lens and a 10× eye-piece.

Histopathological evaluation

At the end of the experimental period, brains from the animals were immersed in 10% buffered formalin solution. Representative sections were processed in Shandon 2LE and Miles VP 1000 tissue processors. These were paraffin embedded, sectioned (5-μm thick), heated at 60°C for 1–2 h and stained with haematoxylin and eosin.

Statistical analysis

Statistical significance of rota-rod test results was assessed by a series of paired Student *t*-tests. A P value higher than 0·05 was considered insignificant. Platelet-aggregation data were analysed by a series of Mann–Whitney tests. All reported values are means ± s.e.m. (n = 10 per group).

Results

Body weight and organ weight changes

Throughout the 56-day experimental period the body weight of animals of the control, 2 and 4% groups increased by 9·6, 11·3 and 11·3 g (35, 41 and 40·5%), respectively (Table 1). No changes were noticed among weights of organs and tissues obtained from all groups, although livers obtained from treated groups weighed more (approximately 25%) than those from control animals without reaching statistical significance (Table 2).

Rota-rod test

Results from the rota-rod test are shown in Fig. 1. The time the control animals stayed on the rota-rod treadmill increased progressively throughout the experiment. In contrast, the test scores of mice treated with 2 or 4% *T. stocksianum* extract were lower by 44% ($P < 0·001$) and 85% ($P < 0·001$), respectively.

Platelet aggregation

Table 3 summarizes data on platelet aggregation. Chronic treatment with the 2 or 4% extracts caused a pro-aggregatory effect on platelets in arterioles. The time to first platelet aggregate was significantly ($P < 0·05$) shorter for the 4% group

Table 1. Body weight changes (g) in mice given *T. stocksianum* extract in drinking water for 56 days.

Day of experiment	Control group (tap water)	*T. stocksianum* group	
		2%	4%
0	27·46 ± 0·46	27·43 ± 0·43	27·80 ± 0·31
7	28·17 ± 0·80	30·20 ± 0·45	30·14 ± 0·40
14	31·13 ± 0·26	31·78 ± 0·56	30·91 ± 0·51
21	31·48 ± 0·34	33·16 ± 0·53	31·87 ± 0·71
28	33·21 ± 0·51	35·17 ± 0·46	33·80 ± 0·73
35	36·13 ± 0·46	37·70 ± 0·67	36·90 ± 0·86
42	36·46 ± 0·94	38·08 ± 0·71	37·50 ± 0·93
49	36·90 ± 0·51	38·62 ± 0·63	37·75 ± 0·81
56	37·00 ± 0·44	38·69 ± 0·71	39·07 ± 1·11
% Change: final compared with initial	35·0	41·0	40·5

Values in the table are means ± s.e.m. (n = 10).

Table 2. The effect on organ and tissue weight (g) of treating mice with *T. stocksianum* extract for 56 days.

Organ	Control group (tap water)	*T. stocksianum* group	
		2%	4%
Brain	0·47 ± 0·02	0·46 ± 0·03	0·46 ± 0·02
Liver	2·06 ± 0·29	2·55 ± 0·18	2·62 ± 0·34
Heart	0·20 ± 0·02	0·22 ± 0·02	0·19 ± 0·01
Spleen	0·10 ± 0·02	0·10 ± 0·02	0·12 ± 0·02
Lungs	0·24 ± 0·03	0·23 ± 0·02	0·23 ± 0·04
Kidneys			
Right	0·28 ± 0·04	0·30 ± 0·04	0·28 ± 0·03
Left	0·28 ± 0·03	0·28 ± 0·04	0·28 ± 0·03
Testes			
Right	0·13 ± 0·02	0·12 ± 0·01	0·12 ± 0·01
Left	0·12 ± 0·01	0·12 ± 0·01	0·11 ± 0·02
Muscles			
Flexor			
Right	0·08 ± 0·01	0·08 ± 0·01	0·08 ± 0·01
Left	0·08 ± 0·03	0·08 ± 0·02	0·09 ± 0·01
Digitos			
Right	0·02 ± 0·01	0·02 ± 0·01	0·02 ± 0·00
Left	0·02 ± 0·01	0·02 ± 0·01	0·02 ± 0·00
Soleus			
Right	0·01 ± 0·01	0·01 ± 0·00	0·02 ± 0·01
Left	0·01 ± 0·01	0·01 ± 0·01	0·02 ± 0·01

Values in the table are means ± s.e.m. (n = 10).

than for the control. In contrast, platelet aggregation data on the venular side did not differ among the three groups. In none of the animals did arteriolar or venular diameters change dramatically during photochemical challenge.

Plasma constituents

A summary of the plasma constituents determined is presented in Table 4. At the end of the experiment, creatinine and AST activity tended to be higher in all experimental groups when compared with initial values. Although cholesterol concentrations in the 2 and 4% groups increased by 77%, these values were not different from those for the control group. For the 4% group, AP activity and bilirubin level were 16 and 22% higher, respectively, than their initial values, but total protein was 12% lower. The 2% treatment did not affect the levels of these three variables.

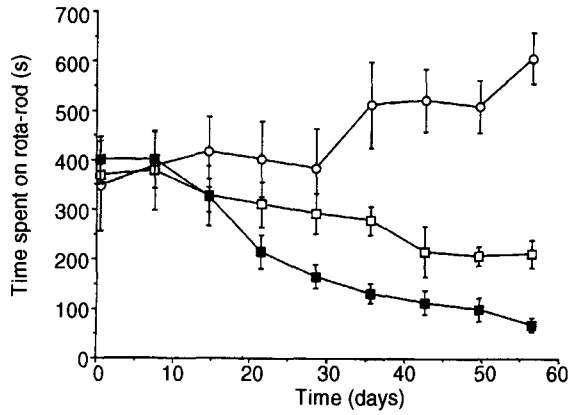

FIG. 1. The effect on motor control (as judged by the time spent on a rota-rod) of treating mice with aqueous extracts of *Teucrium stocksianum* in drinking water. The animals were given the extract at concentrations of 2% (□) and 4% (■) for 56 consecutive days; control animals (○) were given tap water. Each point and vertical bar represents the mean ± s.e.m. from 10 animals.

Histopathological findings

Tissue sections obtained from the brains of *Teucrium*-treated mice revealed focal loss of Purkinje cells from the cerebellum (Fig. 2).

Discussion

No mortality resulted from chronic treatment with *T. stocksianum*, suggesting the plant extract was non-lethal at the concentrations used. Likewise, no significant changes in body weight were noticed between the control mice and those of the *T. stocksianum*-treated groups. All animals gained comparable weight throughout the experimental period, probably indicating that there were no gross changes in food intake or in general body metabolic functions. Similarly, individual body organ and tissue weights were very similar among the animals of the three experimental groups. Although mice in the treated groups tended to have heavier livers than those of the control group, the difference in weight did not reach statistical significance.

Table 3. Photochemically-induced platelet aggregation in pial microcirculation in mice treated with *T. stocksianum* extract for 56 days.

	Control group (tap water)	*T. stocksianum* group	
		2%	4%
Arteriole diameter (μm)			
Before irradiation	46 ± 6	46 ± 7	46 ± 7
After irradiation	44 ± 8	47 ± 6	46 ± 6
% change	−6 ± 11	4 ± 10	1 ± 4
Arteriole platelet aggregation times (s)			
To 1st observed	85 ± 67*	66 ± 43	47 ± 29*
To flow stop	169 ± 76	174 ± 74	170 ± 61
Aggregate growth	84 ± 21	108 ± 43	123 ± 60
Venule diameter (mm)			
Before irradiation	51 ± 11	51 ± 11	48 ± 8
After irradiation	52 ± 12	52 ± 10	48 ± 8
% change	1 ± 2	2 ± 4	0 ± 0
Venule platelet aggregation times (s)			
To 1st observed	24 ± 5	24 ± 3	22 ± 3
To flow stop	192 ± 58	160 ± 34	198 ± 61
Aggregate growth	168 ± 58	136 ± 33	177 ± 61

Values are means ± s.e.m. (n = 10). *These values are significantly different from each other ($P < 0.05$).

Table 4. The effect on some plasma constituents of treating mice with extracts of *T. stocksianum*.

Days	Glucose (mM)	Creatinine (μM)	Urea (mM)	Cholesterol (mM)	Triglyceride (mM)	Bilirubin (mM)	Aspartate amino transferase (units L^{-1})	Acid phosphatase (units L^{-1})	Total protein (g per 600 mL)
Control group									
0	6·2 ± 0·4	34·1 ± 3·9	8·3 ± 0·6	2·0 ± 0·1	0·24 ± 0·03	0·8 ± 0·07	150·2 ± 14·3	24·0 ± 3·2	5·7 ± 0·31
14	5·9 ± 0·6	40·2 ± 3·8	6·3 ± 0·6	1·7 ± 0·3	0·22 ± 0·02	0·9 ± 0·05	149·2 ± 15·1	25·1 ± 2·1	5·8 ± 0·21
28	6·3 ± 0·5	39·2 ± 4·2	4·9 ± 0·5	2·2 ± 0·4	0·20 ± 0·02	0·7 ± 0·06	157·3 ± 16·2	23·3 ± 3·1	5·9 ± 0·37
42	5·8 ± 0·5	50·0 ± 6·1	5·7 ± 0·8	1·9 ± 0·5	0·23 ± 0·03	0·7 ± 0·05	171·3 ± 20·2	30·7 ± 4·1	5·6 ± 0·35
56	5·9 ± 0·4	48·3 ± 4·3	7·7 ± 0·7	2·3 ± 0·3	0·24 ± 0·05	0·8 ± 0·08	167·4 ± 15·7	23·1 ± 3·2	5·7 ± 0·20
T. stocksianum group (2%)									
0	6·7 ± 0·7	43·7 ± 5·5	9·2 ± 0·8	1·3 ± 0·2	0·25 ± 0·02	0·9 ± 0·08	163·2 ± 15·3	25·2 ± 2·3	6·0 ± 0·51
14	5·8 ± 0·5	41·7 ± 4·7	8·3 ± 0·7	1·9 ± 0·3	0·24 ± 0·02	0·9 ± 0·10	170·2 ± 14·9	26·5 ± 3·0	5·7 ± 0·52
28	6·9 ± 0·6	39·8 ± 5·6	9·1 ± 0·8	1·7 ± 0·4	0·24 ± 0·02	0·8 ± 0·11	170·3 ± 18·8	27·3 ± 3·1	6·1 ± 0·60
42	5·8 ± 0·6	42·8 ± 3·9	7·3 ± 0·5	2·2 ± 0·2	0·26 ± 0·04	0·9 ± 0·08	139·5 ± 14·2	25·1 ± 2·7	5·8 ± 0·51
56	6·0 ± 0·7	48·7 ± 5·2	6·9 ± 0·8	2·3 ± 0·3	0·23 ± 0·03	0·8 ± 0·07	182·7 ± 19·9	24·1 ± 2·4	6·0 ± 0·70
T. stocksianum group (4%)									
0	6·0 ± 0·7	46·3 ± 4·7	8·3 ± 0·7	1·3 ± 0·1	0·25 ± 0·02	0·1 ± 0·08	163·3 ± 17·1	25·2 ± 3·2	5·9 ± 0·57
14	5·8 ± 0·6	50·7 ± 6·1	9·0 ± 0·5	1·7 ± 0·2	0·27 ± 0·03	1·1 ± 0·09	179·2 ± 18·3	22·7 ± 3·1	6·2 ± 0·63
28	5·8 ± 0·5	49·7 ± 5·1	8·7 ± 0·8	2·0 ± 0·2	0·28 ± 0·02	1·2 ± 0·09	182·5 ± 17·9	25·1 ± 3·2	5·7 ± 0·43
42	5·9 ± 0·6	52·1 ± 6·3	7·9 ± 0·7	1·9 ± 0·2	0·22 ± 0·02	0·9 ± 0·10	153·7 ± 20·1	28·7 ± 3·2	5·2 ± 0·62
56	6·0 ± 0·5	56·7 ± 7·0	8·3 ± 0·9	2·3 ± 0·3	0·23 ± 0·02	1·1 ± 0·10	184·9 ± 19·2	29·2 ± 3·1	5·2 ± 0·70

Values are means ± s.e.m. (n = 10). *T. stocksanium* extract was given in the drinking water for 56 days.

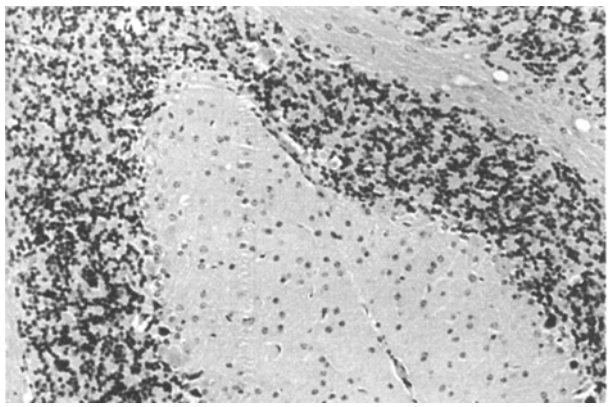

FIG. 2. Photograph showing loss of cerebellar Purkinje cells in a histological section obtained from the brain of a mouse treated with *T. stocksianum* (4%, 56 days).

Plasma analysis showed a rise in cholesterol concentration in the 2- and 4%-treated groups. In the 4%-treated group bilirubin concentration was 22% higher at the end of experiment than at the start. Other biochemical parameters were similar for all groups. The loss of Purkinje cells from the mouse (Fig. 2) confirmed our previous histopathological findings in the rat (Tanira et al 1996).

One main objective of this study was to investigate the physiological implication of the loss in the cerebellar Purkinje cells. The rota-rod test used as a measure of motor coordination clearly demonstrated that *T. stocksianum* treatment caused dose- and time-related impairment of muscular coordination (Fig. 1). The test showed that the performance of the control group improved with time (almost 50% increase at the end of the experimental period), presumably because of training of the animals. The performance time of the treated animals declined steadily. The maximum reduction observed for the 4%-treated group (at the end of the experiment) was 20% of that at the start. These data illustrate the probable physiological consequence of the loss of cerebellar Purkinje cells and add further evidence to the possibility of neurotoxicity of *T. stocksianum* in man.

The mechanism of *T. stocksianum*-induced neurotoxicity might have resulted from direct cytotoxic action of the plant, or might have been secondary to ischaemia, or both. The data obtained from the microcirculatory part of this study showed that there was no marked changes in the diameters of arterioles and venules during photochemical irradiation, although platelet aggregation in the arterioles of the 2 and 4% treated groups was initiated in a shorter time than in those of the control group, indicating the pro-aggregatory effect of the plant extract. Blood flow might have been impaired or completely restricted as a result of platelet aggregation, either by thrombosis or as a consequence of any subsequent embolism, or both. It is, therefore, likely that accelerated platelet aggregation in pial microcirculation observed after treatment with *T. stocksianum* might have contributed to an ischaemic effect which, in turn, could have, at least in part, caused the cytotoxicological changes. This does not, nevertheless, exclude the possibility of the direct cytotoxicological effect of the plant extract. Further pharmacological and toxicological investigations on *Teucrium* species seem warranted.

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