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ORIGINAL PAPER

Experimental *Tityus serrulatus* scorpion envenomation: age- and sex-related differences in symptoms and mortality in mice

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Abstract: Among the various methods for evaluating animal venom toxicity, the calculation of the median lethal dose (LD_{50}) is the most widely used. Although different protocols can be used to calculate the LD_{50}, the source of the venom and the method of extraction, as well as the strain, age, and sex of the animal model employed, should be taken into consideration. The objective of the present study was to evaluate the influence of sex and age on the toxicity of *Tityus serrulatus* scorpion venom in Swiss mice. Although the symptoms of envenomation were similar in male and female animals, female mice proved to be more resistant to the venom. In females, age had no impact on the susceptibility to scorpion envenomation. Male mice were more sensitive to *T. serrulatus* venom. Moreover, in males, age was an important parameter since sensitivity to the venom increased with age.

Key words: scorpions, LD_{50}, stings, age factors, gender.

INTRODUCTION

Scorpion envenomation is a public health problem in various countries worldwide. Data from the Brazilian Ministry of Health show that, in 2009, there were 45,721 registered cases of scorpion envenomation, resulting in 103 deaths in the country. Most of the cases occurred in the north-eastern region of the country, followed by the south-eastern, northern, central-west, and southern regions. In the state of São Paulo, 5,547 cases were registered in the year 2009 alone (1, 2). The yellow scorpion, *Tityus serrulatus*, is considered the most dangerous species in Brazil, being responsible for most of the cases of scorpion envenomation (3, 4). This might be due to the fact that *T. serrulatus* proliferates easily, through parthenogenesis, meaning that there is no sexual reproduction and that all *T. serrulatus* individuals are therefore female (5).

The venom of the yellow scorpion is composed of insoluble mucus, mucopolysaccharides, oligopeptides, nucleotides, low molecular weight molecules (such as serotonin and histamine), protease inhibitors, histamine releasers, amino acids, and other organic compounds, as well as numerous low molecular weight proteins that have a neurotoxic effect (6-10). These proteins act mainly on specific sodium channel sites, leading to depolarization of postganglionic neural elements, affecting the sympathetic and parasympathetic systems, as well as promoting the release of adrenaline, noradrenaline, and acetylcholine in victims (11). The victims present with a variety of clinical manifestations, including tachycardia, hypertension, sweating, mydriasis, irritability, hyperthermia, salivation, vomiting, tremor, convulsion, increased gastric/pancreatic secretion, release of neurotransmitters, and intestinal motility disorder leading to diarrhea. A

common characteristic of scorpion envenomation is the rapid onset of signs and symptoms after the venom has been inoculated due to the rapid distribution of scorpion venom components. Death from scorpion envenomation occurs as a result of cardiorespiratory failure. Victims may die within 1 to 6 hours after envenomation, and late deaths can also occur (12-14).

Individual differences in susceptibility to scorpion venom have been observed among patients from the same areas, as well as in individuals who have been stung more than once (15). Genetic polymorphism, age, and sex might be involved in this variation (16).

The evaluation of the median lethal dose (LD_{50}) is an important parameter in the study of animal venom toxicity. However, the LD_{50} varies widely in the literature, since it depends on various factors, such as the site of sting, scorpion diet, weight of the victim, route of venom administration, age, and sex, as well as on the animal model employed (17). Therefore, the present study investigated the variations in *T. serrulatus* venom toxicity by determining the LD_{50} in male and female Swiss mice of various ages.

MATERIALS AND METHODS

Animals

In an adaptation of the model proposed by Padilla *et al.* (16), who used 4-, 6-, and 10-week-old mice to evaluate the toxicity of *Centruroides limpidus limpidus* venom, we used 4-, 6-, and 10-week-old male and female Swiss mice. The animals were obtained from the Central Laboratory Animal Facility of the University of São Paulo at Ribeirão Preto School of Medicine, located in the city of Ribeirão Preto, Brazil.

T. serrulatus Venom

T. serrulatus venom was extracted by electrical stimulation of the telson and obtained from the serpentarium of the University of São Paulo at Ribeirão Preto School of Medicine, Ribeirão Preto, Brazil. Venom was extracted from approximately 50 scorpions from different localities in the state of São Paulo, Brazil, in order to obtain the venom pool.

T. serrulatus Venom Electrophoresis

The protein levels in soluble venom were determined by the Lowry method (18) modified

by Hartree (19). In a few words, soluble venom is venom without mucus, which is obtained from the supernatant of venom diluted in saline and centrifuged at 11,600 g for 15 minutes. Then, the precipitate that corresponds to the mucus is discarded. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in accordance with Laemmli (20). The sample (2 μg) was boiled for three minutes and subsequently applied to a gradient (8-20%) polyacrylamide gel, which was then stained with silver nitrate.

Determination of the LD_{50} and Evaluation of Venom Toxicity

The LD_{50} was determined in accordance with the method described by Kärber (21). We emplyed male and female non-syngeneic Swiss mice, weighing 18 to 25 g, with 4, 6, and 10 weeks of age. Animals were maintained in autoclaved polypropylene boxes (30 x 20 x 13 cm) with filtered air and the temperature was adjusted to 23 ± 2°C. Animals were fed with standard rodent food (Purina) and autoclaved water, *ad libitum*. A total of six experiments were performed, organized by age/sex groups. The soluble venom was diluted in 0.9% sterile saline solution and injected subcutaneously into the dorsum at doses of 1.0, 1.5, 2.0, 2.5, and 3.0 mg of venom per kilogram (n = 6 animals/dose), in a final volume of 0.250 mL. An equivalent volume of sterile saline solution was injected into control animals (n = 6). The animals were observed for 24 hours.

The LD_{50} was calculated by the proportion of deaths (number of dead animals after 24 hours divided by the total number of animals in the group; for all groups, n = 6). The LD_{50} was calculated by Bayesian analysis. Toxicity was also evaluated by the classic signs of envenomation, including ptosis, hyperactivity, sweating, and tachycardia. The experiment lasted 24 hours and the signs during this period were registered.

RESULTS

T. serrulatus Venom Electrophoresis

As shown in Figure 1, electrophoresis of the *T. serrulatus* venom pool showed a profile that was similar to that found by other authors (22).

Variations in the LD_{50}

The LD_{50} of *T. serrulatus* venom was determined in 4-, 6-, and 10-week-old male and female Swiss

Figure 1. Electrophoretic profile of *Tityus serrulatus* venom. SDS-PAGE on a gradient (8-20% w/v) gel under non-reducing conditions. Lane 1: standard molecular weight markers (#SMO661; Thermo Fisher Scientific, USA). Lane 2: *T. serrulatus* venom (2 µg). Silver staining.

mice, resulting in a total of six experiments. The LD_{50} values and the differences are shown in Table 1. Figure 2 displays the statistical differences among the groups in terms of the LD_{50}.

At six weeks of age, male Swiss mice were more sensitive to *T. serrulatus* venom than females. Likewise, 10-week-old male Swiss mice were more sensitive to *T. serrulatus* venom than were 10-week-old female Swiss mice. There were no significant differences between 4-week-old male mice and 4-week-old female mice in terms of the LD_{50}. There were no significant differences among female mice in terms of the LD_{50}. However, among the male mice, 4-week-olds were more resistant to the venom than were 6-week-olds and 10-week-olds.

Variations in the Signs of Envenomation

The signs of *T. serrulatus* envenomation were registered for 24 hours in all groups. Table 2 shows the main signs of envenomation in 6-week-old mice, and Table 3 shows the major signs of envenomation in 10-week-old mice, both challenged with 1.0 mg of *T. serrulatus* venom per kilogram. The signs of envenomation in 4-week-old animals are not shown, as there were no significant differences between genders in terms of the LD_{50}. In general, the symptoms analyzed were similar in males and females.
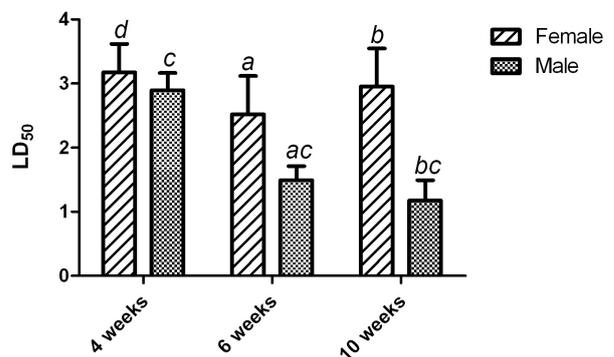

Figure 2. Median lethal dose of *Tityus serrulatus* venom. The figure represents six different experiments ± standard deviation. Identical letters indicate no significant difference by post test of Tukey ($p < 0.005$).

Table 1. Median lethal dose of *Tityus serrulatus* venom by age and sex

Age (weeks)	4		6		10	
Gender	M	F	M	F	M	F
LD$_{50}$ (µg/g)	2.90	3.15	1.49	2.64	1.23	2.83
95%CI	2.62-3.16	2.74-3.63	1.27-1.71	1.87-3.05	0.84-1.46	2.43-3.60

CI: confidence interval, M: male, F: female.

Table 2. Signs observed in 6-week-old female and male mice experimentally envenomed with *Tityus serrulatus* venom

	Female (n = 6)/Male (n = 6)									
	Time									
Signs	10 min.	30 min.	1 h	2 h	3 h	4 h	5 h	6 h	12 h	24 h
Hyperactivity	5/6	6/6	6/2	5/2	2/2	3/2	3/1	1/1	1/0	0/0
Ptosis	1/1	0/1	2/4	3/3	5/5	5/5	5/5	5/5	5/0	0/0
Sweating	1/0	1/1	1/3	2/1	1/1	1/1	1/0	1/0	1/0	0/0
Tachycardia	0/0	0/0	2/2	6/3	5/4	5/6	5/6	6/5	3/0	0/0

Table 3. Signs observed in 10-week-old female and male mice experimentally envenomed with *Tityus serrulatus* venom

	Female (n = 6)/Male (n = 6)									
	Time									
Signs	10 min.	30 min.	1 h	2 h	3 h	4 h	5 h	6 h	12 h	24 h
Hyperactivity	2/6	6/6	6/1	6/2	2/2	2/2	0/2	0/2	0/0	0/0
Ptosis	2/1	5/2	2/6	4/4	3/2	4/2	4/2	1/2	0/0	0/0
Sweating	0/1	5/2	4/2	4/2	4/3	5/3	0/5	0/5	0/0	0/0
Tachycardia	0/0	0/0	6/3	5/5	5/5	5/5	6/5	5/5	0/0	0/0

DISCUSSION

The evaluation of the toxicity of any given venom is an important parameter for the characterization of the components of the venom, as well as for the determination of the actual neutralization of any given anti-venom. Different methods have been used to determine the LD$_{50}$. Although various animal models can be used to determine the LD$_{50}$ – rats, rabbits, guinea pigs, cockroaches, chicks, houseflies, and blowflies – that most widely used is the murine model (23-29). It is known that animal venom toxicity is influenced by geographic origin, sex, age, diet, and weight, as well as by the method used for venom extraction (30-35). The animal model employed and the route of administration also play a role in animal venom toxicity (16). Therefore, it is no surprise that the LD$_{50}$ of different types of animal venoms, including that of *T. serrulatus* venom,

varies widely in the literature (36-38). In 1993, Ismail (39) showed that the lowest reported LD_{50} value for *Leiurus quinquestriatus* scorpion venom was 26 to 28 times more lethal than the highest reported values. In addition, previous observed that the LD_{50} values for manually extracted venom are nearly twice or three times lower than those for venom extracted by electrical stimulation (35, 40, 41).

In the present study, we determined the LD_{50} of *T. serrulatus* venom in 4-, 6-, and 10-week-old male and female Swiss mice. A comparison between female and male mice revealed that 6- and 10-week-old female mice were more resistant than were 6- and 10-week-old male mice, the LD_{50} for the female mice being nearly twice as high as that for male mice. This was expected, since males are more susceptible to infections than females, which is mainly due to differences in steroid hormones (42-44). In addition, studies investigating inflammatory diseases – such as atherosclerosis, rheumatoid arthritis, and periodontitis – indicate that the female sex hormones modulate the inflammatory response (45). Estrogen, particularly 17β-oestradiol, is the principal factor responsible for this process. The binding of estrogen to the estrogen receptor-alpha (OR-α), OR-β, or both causes the OR protein to dissociate from the inhibitory protein complex, causing the OR-estrogen complex to form dimers and bind to regulatory proteins or transcription factors, which regulate cellular gene activity. Although this pathway remains unclear, it is known that estrogen-induced OR-α and OR-β are anti-inflammatory mediators (46, 47). The clinical manifestations of and the mortality from *T. serrulatus* envenomation are directly related to the inflammatory response (48). The acute phase response, which includes fever, metabolic changes, leucocytosis, increased corticotropin, increased cortisol, changes in the concentration of various cytokines, and acute phase proteins, is the principal factor responsible for the signs of envenomation (49). These findings explain why, in the present study, female mice were more resistant to *T. serrulatus* envenomation than male mice, since female mice presented reduced inflammation and decreased acute phase response due to the presence of estrogen.

In humans, babies and children are more susceptible to envenomation; this is commonly attributed to the differences in the dose/body weight ratio and to a greater sensitivity of young victims (16). Contrary to what would therefore be expected, sensitivity to the toxic effect of the venom increased with age in the male mice analyzed in the present study. Other authors reported similar results in studies involving BALB/cAnN and C57B1/6J mice or Charles Foster rats and Swiss mice (16, 23). It is possible that the greater sensitivity of children and babies to envenomation is principally related to the concentration of venom in the victim and not to a greater sensitivity to the venom (23). Further studies should be conducted in order to increase the understanding of this issue, since basic toxicology principles such as the dose/body weight ratio do not always apply. In the present study, there were no significant differences among female mice in terms of the LD_{50}, regardless of age. Unlike what occurs in human females, anovulatory female rodents maintain a baseline secretion of gonadal steroid hormones even after reaching maturity, which they do at 12 months of age (45). Therefore, the protection that estrogen confers continues to exist even at a more advanced age, which means that the results of the present study probably would not have changed even if we had evaluated anovulatory female mice.

There were no significant differences among the mice observed in the present study in terms of the signs of *T. serrulatus* envenomation (hyperactivity, sweating, ptosis, and tachycardia). Hyperactivity, sweating, and ptosis were observed immediately after the venom had been inoculated (within ten minutes). In contrast, tachycardia was observed later, approximately 60 minutes after the beginning of the experiment. The cardiovascular toxicity caused by the *T. serrulatus* venom, as well as that caused by the venom of other species, such as *Mesobuthus tamulus* and *Buthus quinquestriatus*, is believed to be related to the ability of the venom to release neurotransmitters from sympathetic and parasympathetic nerve terminals (6, 50-52). The venom stimulates the release of catecholamines in the blood, initially causing mild cholinergic stimulation, which can lead to vomiting, sweating, bradycardia, and hypotension. Subsequently, there is a prolonged effect caused by adrenergic hyperactivity, which can lead to hypertension, tachycardia, and myocardial failure (51). Although the female mice were more resistant than male mice, the signs of scorpion envenomation were no less intense in

the females. This finding can be explained by the types of signs observed, which resulted from the neurotoxic effect of the venom. However, if we had measured fever or performed biochemical and immunological analyses, we would probably have found significant differences between male and female mice, as have been demonstrated in other studies (48, 52, 53). Although other symptoms, such as tremor and convulsion, were also observed, they were rare (data not shown).

Most of the studies cited evaluated venom toxicity in syngeneic mice, which facilitates the reproduction of the experiments but disregards the natural variability among individuals, and the results obtained are often fallacious. In addition, one of the problems of using syngeneic mice is that all genetic loci are homozygous, meaning that there is a genetic defect that can affect homeostasis (54, 55). With the objective of simulating a more realistic situation, we decided to use Swiss mice, a non-syngeneic strain of mice that presents polymorphism among animals. Therefore, the present study demonstrated the toxicity of *T. serrulatus* venom in male and female mice of different ages.

CONCLUSIONS

The present study demonstrated that the toxicity of *T. serrulatus* venom differs between male and female mice, possibly due to the presence of estrogen in females. Moreover, the present study showed that age is an important variable when male animals are employed. Therefore, the present study underscores the idea the LD_{50} of any given animal venom can be standardized only on a model-by-model basis.

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SUBMISSION STATUS

CONFLICTS OF INTEREST

There is no conflict.

FINANCIAL SOURCE

ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee on Animal Experimentation of the Ribeirão Preto School of Medicine, USP, under the protocol number 109/2010. Moreover, it follows the ethical principles in animal research adopted by the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA).

CORRESPONDENCE TO

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