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Tumescent contravenom: murine model for prehospital treatment of *Naja naja* neurotoxic snake envenomation

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Abstract

Background Snake envenomation is a neglected global health problem. There is a need for a prehospital treatment of neurotoxic snakebite that prolongs survival and allows time for a victim to reach a hospital for antivenom therapy. Tumescent epinephrine consists of a large volume of dilute epinephrine (2 mg/l) injected subcutaneously. It functions as "contravenom" by causing capillary vasoconstriction and delaying venom absorption.

Methods A murine model of neurotoxic envenomation using lidocaine as a surrogate for neurotoxic snake venom was first developed in a pilot study. A lethal dose of lidocaine was injected subcutaneously into control and treatment groups. Mice in the treatment group were then treated with a tumescent infiltration of dilute epinephrine in saline, while control mice either received no treatment or tumescent infiltration with saline alone. The experiment was repeated using lethal doses of neurotoxic *Naja naja* cobra venom. The main end-points were survival rate and survival time.

Results None of the control mice survived a lethal (LD_{100}) dosage of subcutaneous lidocaine. Mice given an LD_{100} of subcutaneous lidocaine and treated immediately with tumescent epinephrine had 80% survival. Following LD_{50} doses of *Naja naja* venom, 50% of control mice survived, while 94% survived when treated immediately with tumescent epinephrine ($P < 0.01$). All animals died following LD_{100} doses of *Naja naja* venom, but survival was significantly prolonged ($P < 0.0001$) by immediate tumescent epinephrine.

Conclusions Tumescent epinephrine, when given immediately after toxin injection, improves survival rates in mice following neurotoxic doses of lidocaine or *Naja naja* cobra venom.

Introduction

Snakebite envenomation is a neglected tropical and dermatologic disease. The World Health Organization estimates there are over 5 million venomous snakebites each year resulting in more than 100,000 deaths globally and more than 400,000 amputations or permanent disabilities.[1,2] Antivenom, currently the only antidote to snake venom, is expensive, and each variant is directed against a specific species; there is no universal antivenom. Antivenom must also be administered in a hospital setting due to potential complications such as anaphylactic shock and serum sickness reactions. Victims of neurotoxic snake envenomation may not survive long enough to reach a hospital for antivenom treatment. The ideal snakebite therapy would be effective against a multitude of venoms, cheap, easy to use, portable, and able to be delivered in the field. Furthermore, though antivenom can address the systemic effects of snake venom, it may not prevent skin necrosis and local pain at the snakebite site.

We propose a dermatopharmacologic approach to treating both the local and systemic toxicity of snakebites. In this study, we repurpose a well-known dermatologic surgery technique, tumescent anesthesia, as a "contravenom" against the tissue necrosis, local pain, and systemic toxicity of snakebites. Contravenom is defined as any non-antivenom drug that treats animal envenomation.

The word *tumescent* is an adjective describing the state of being swollen and firm. Tumescent drug delivery refers to the subcutaneous infiltration of a relatively large volume of a dilute drug administered in a physiologic saline solution containing a low concentration of epinephrine (≤2 mg/l). A sufficient volume of a tumescent solution produces widespread subcutaneous distribution of the drug. The inclusion of epinephrine induces prolonged local vasoconstriction and delays the systemic absorption of any drug in the solution.

Tumescent lidocaine anesthesia (TLA) solution is a well-known and commonly utilized dermatopharmacologic example of tumescent drug delivery of lidocaine (1 g/l) and epinephrine

(1 mg/l) in saline. TLA produces local anesthesia lasting for at least 10 hours with profound surgical hemostasis.[3] The delayed drug absorption permits large dosages of tumescent lidocaine (28 mg/kg) with an estimated risk for systemic toxicity of 1 per 5,000,000.[4]

We hypothesize that subcutaneous tumescent epinephrine, infiltrated into and around the site of a subcutaneous lethal dose of a snake venom neurotoxin, will delay the systemic absorption of the toxin and prolong survival.

Here, we establish the first animal model for tumescent drug delivery. This model was then used to test the effectiveness of tumescent drug delivery for the treatment of subcutaneous neurotoxic doses of *Naja naja* venom. There is a need for a prehospital snakebite treatment that is effective for a multiple snake species, safe, inexpensive, easily administered by persons having no medical training, and which is capable of prolonging survival and reducing local pain and tissue damage. Our proposed technique, "contravenom," meets the aforementioned criteria and offers a new approach for this profound global health issue.

Materials and methods

Use of the mice in this study was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.[5]

Animals

Pathogen-free outbred female Swiss-Webster mice (4–6 weeks old) were obtained from a commercial source (Charles Rivers Labs, San Diego). Mice were housed in groups (5 mice/cage) on corncob bedding with cotton nesting material in individually ventilated cages in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited biocontainment facility. Mice were allowed to acclimate for 72 hours, were fed standard laboratory rodent chow, and were provided with *ad libitum* access to reverse-osmosis-purified acidified water. The room was maintained at 20–21 °C with relative humidity of 30–70%, 10–15 air changes/h, and a photoperiod of 12 hours of light to 12 hours of darkness.

Venom

Naja naja (Indian cobra) venom was obtained from Kentucky Reptile Zoo. In order to determine the median lethal dose (LD_{50}) of *Naja naja* venom in mice, we used an animal-sparing "up-and-down" methodology of estimating the LD_{50} that involved serial intradermal injection of predetermined mg/kg dosages of venom.[6] Our estimated LD_{50} value was 1 mg/kg.

Experimental procedures

Lidocaine pilot study

The pilot study used lidocaine as a model neurotoxin. Fifty-five mice were randomly selected and assigned to six treatment and control groups. Published snake venom studies using mice utilized $n = 5$, as such we proceeded with $n \geq 5$ for the initial pilot studies.[7] All injections were given in the scruff of the neck.

Group 1 (epinephrine control): 5 mice treated with 3 ml of a tumescent solution containing epinephrine (2 mg/l) in saline.

Group 2 (LD_{50} control): 10 mice given a subcutaneous injection of "concentrated" commercial 2% lidocaine (2 g/l) at a dosage of 0.5 milligram per gram body weight. This dose was determined from previously published LD_{50} data for subcutaneous dosing.[8,9] These control mice did not receive tumescent epinephrine.

Group 3 (saline control): 10 mice given a toxic dose of lidocaine (0.5 mg/g, subcutaneous) followed by immediate rescue with 2 ml of normal saline without epinephrine.

Group 4 (treatment): 10 mice given a toxic dose of lidocaine (0.5 mg/g, subcutaneous) followed immediately by 2 ml of tumescent epinephrine (2 mg/l).

Group 5 (treatment): 10 mice given a toxic dose of lidocaine (0.5 mg/g, subcutaneous) followed 1 minute later by 2 ml of tumescent epinephrine (2 mg/l).

Group 6 (treatment): 10 mice given a toxic dose of lidocaine (0.5 mg/g, subcutaneous) followed 3 minutes later by 2 ml of tumescent epinephrine (2 mg/l).

Snake venom studies

The two snake venom studies, LD_{50} and LD_{100}, used *Naja naja* venom. Mice were randomly selected and assigned to treatment and control groups.

Group 1 (epinephrine control): 5 mice injected subcutaneously with tumescent epinephrine containing 1 mg/l epinephrine in saline solution (0.9% NaCl). These mice did not receive *Naja naja* venom.

LD_{50} study. Group 2 (LD_{50} control): 12 mice injected subcutaneously with *Naja naja* venom at an LD_{50} dosage of 0.8 mg/kg. This group did not receive tumescent epinephrine rescue.

Group 3 (LD_{50} treatment): 17 mice injected subcutaneously with *Naja naja* venom at an LD_{50} dosage of 0.8 mg/kg. This was followed 60 seconds later by treatment with tumescent epinephrine (2 ml) containing 1 mg/l epinephrine in a saline solution (0.9% NaCl) (Fig. 1).

LD_{100} study. Group 4 (LD_{100} control): 12 mice injected subcutaneously with LD_{100} dosage of venom (1 mg/kg, 0.1 ml total injection volume) in the hind leg without tumescent rescue.

Group 5 (LD_{100} treatment): 12 mice injected subcutaneously with LD_{100} dosage of venom (1 mg/kg, 0.1 ml total injection volume) in the hind leg. This was followed by rescue treatment at 60 seconds with tumescent epinephrine (2 ml) containing 1 mg/l epinephrine in a saline solution (0.9% NaCl) (Fig. 1).

Mice were monitored after venom administration every 15–60 minutes for 24 hours. An observer, blinded to treatment

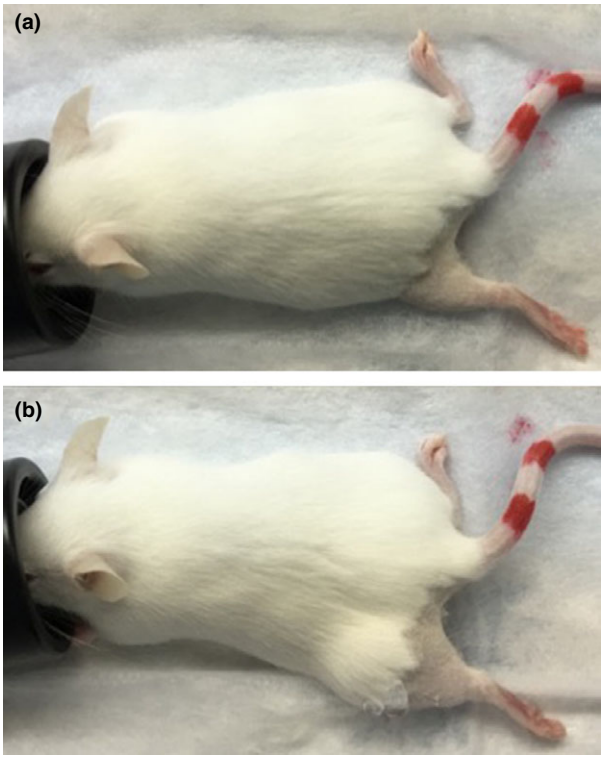

Figure 1 Left hindquarter before (a) and after (b) rescue treatment with 2 ml of contravenom (tumescent anesthesia)

status, assessed viability. Mice who were nonresponsive to stimuli, in marked respiratory distress (agonal breathing or intermittent gasping), or had a core body temperature <70% of baseline core temperature for >2 hours were euthanized. Surviving mice were euthanized 24 hours after venom administration. The main end-point measured was survival time.[6,10]

Statistical methods

Kaplan–Meier curves were plotted for both *Naja naja* venom LD_{50} and LD_{100}. Log-rank test was used to examine the difference of survival curves between control and treatment groups. Survival rate was also compared based on a two-sample proportion test for venom LD_{50}. Median survival time was calculated for both control and treatment groups in venom LD_{50}. Analyses were performed using R version 3.3.3.

Results

The duration of survival was documented following subcutaneous injections of tumescent epinephrine, neurotoxic doses of lidocaine, or *Naja naja* cobra venom.

Lidocaine pilot study

Table 1 shows the survival rates at 24 hours for control and treatment groups. All mice survived after being given tumescent epinephrine without lidocaine (Group 1). Only 1 of 10 control mice survived after being given LD_{100} lidocaine injection and no tumescent epinephrine rescue (Group 2). No mice survived an LD_{100} lidocaine injection followed by sham tumescent infiltration of saline alone (Group 3). However, 80% of mice survived an LD_{100} lidocaine injection followed by immediate tumescent epinephrine rescue. Moreover, 80% of mice survived an LD_{100} lidocaine injection followed by tumescent epinephrine with a 1 minute delay (Group 5). In Group 6, 30% of mice survived an LD_{100} lidocaine injection followed by tumescent epinephrine rescue with a 3 minute delay.

Table 1 Summary of survival data for mice challenge-exposed with lidocaine and rescued with tumescent anesthesia

Group	Treatment	Survival #
1	Tumescent epinephrine (2 mg/l)	5/5
2	Lido only (LD_{100})	1/10
3	Lido + saline only immediate	0/10
4	Lido + tumescent epinephrine immediate	8/10
5	Lido + tumescent epinephrine at 1 min	8/10
6	Lido + tumescent epinephrine at 3 min	3/10

Tumescent epinephrine = 2 ml of epinephrine (2 mg/l).
Lido: Lidocaine 0.5 mg/g (from published LD_{50} for subcutaneous dosing).

Naja naja studies

In Group 1 (epinephrine control), all mice survived.

LD_{50} study

In Group 2 (LD_{50} control), 50% of mice survived after an LD_{50} dosage of *Naja naja* venom without tumescent epinephrine rescue. Among Group 3 (LD_{50} treatment), 94% mice survived an LD_{50} dosage of *Naja naja* venom tumescent epinephrine rescue with a 1 minute delay ($P < 0.01$).

LD_{100} study

In Group 4 (LD_{100} control), all mice died with a mean survival time of 158 minutes after an LD_{100} dosage of *Naja naja* venom without tumescent epinephrine rescue. In Group 5 (LD_{100} treatment), all mice died with a mean survival time of 230 minutes after an LD_{100} dosage of *Naja naja* venom with tumescent epinephrine rescue at 60 seconds ($P < 0.0001$).

The results are displayed graphically by Kaplan–Meier survival curves in Figures 2 and 3. The survival curve for treatment and control group under venom LD_{50} were significantly different based on a Log-rank test with $P = 0.0039$. For LD_{50}, the percentage of mice surviving to 24 hours after venom injection was 94% (16 out of 17) in treatment group, while the survival rate in control group was only 50% (6 out of 12). The survival rate was also significantly different

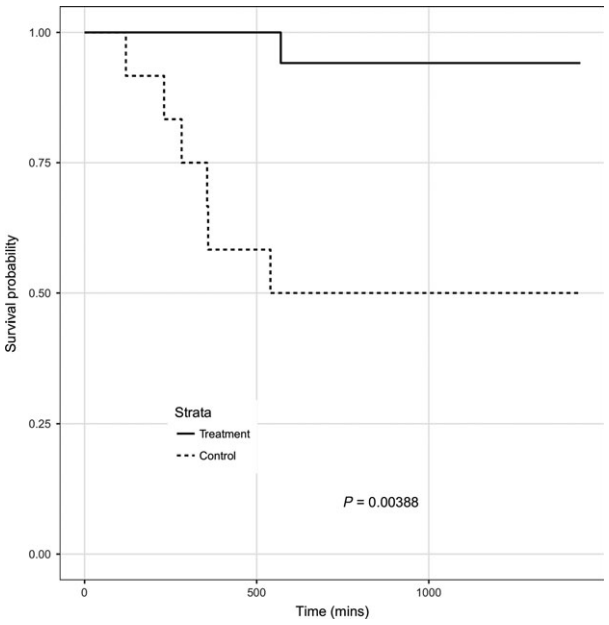

Figure 2 Kaplan–Meier curves for *Naja naja* venom LD_{50}

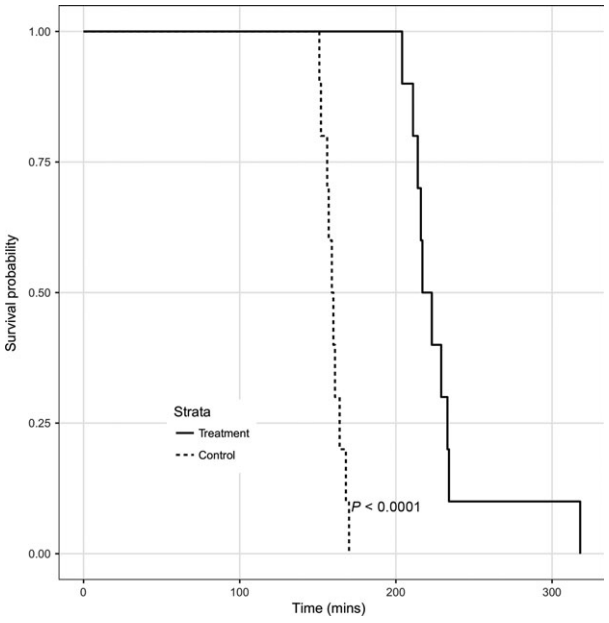

Figure 3 Kaplan–Meier curves for *Naja naja* venom LD_{100}

between two groups based on a two-sample proportion test with $P = 0.0062$. For $LD_{100,}$ all mice died within 24 hours after venom injection for both groups. The median survival time in the control and treatment group was 158 and 230 minutes, respectively. Log-rank test indicated that survival curves were significantly different between control and treatment ($P < 0.0001$) (see Fig. 3; Table 2).

Discussion

The results of this research support the hypothesis that tumescent epinephrine prolongs survival of mice after lethal neurotoxic subcutaneous doses of either lidocaine or *Naja naja* cobra venom.

The lidocaine arm of this research involved six experimental groups. All mice survived (5/5) in Control Group 1 after being given tumescent epinephrine alone. There was no evidence of epinephrine toxicity from the tumescent contravenom therapy. Only one mouse survived (1/10) in Control Group 2 after an LD_{100} dose of lidocaine, without any tumescent epinephrine or saline treatment. This established that the selected LD_{100} dose was in fact highly lethal. No mice survived (0/10) in Control Group 3 after an LD_{100} dose of lidocaine followed by normal saline alone without tumescent epinephrine. This suggests that epinephrine-induced vasoconstriction, rather than a dilutional effect, was responsible for the improved survival observed in Treatment Groups 4, 5, and 6.

Most mice survived (8/10) in Treatment Groups 4 and 5, when tumescent epinephrine was injected within 1 minute after an LD_{100} of lidocaine. Fewer mice survived (3/10) in Treatment Group 6, when tumescent epinephrine injection was delayed by 3 minutes after an LD_{100} dose of lidocaine. This suggests that lidocaine absorption after subcutaneous injection in mice is rapid and that tumescent epinephrine must be injected without delay to prevent lidocaine toxicity.

For the *Naja naja* arm of this research, the tumescent contravenom solution consisted of dilute epinephrine (1 mg/l) in saline. The principal outcome variables following *Naja naja* envenomation were survival rate and survival duration.

After an LD_{50} of *Naja naja* cobra venom, survival rate (number of survivors at 24 hours after envenomation) was the endpoint. LD_{50} doses of *Naja naja* venom followed by tumescent epinephrine resulted in a significant improvement in survival rate.

After an LD_{100} dose of cobra venom, survival duration after venom injection (number of minutes survived up to 24 hours) was the endpoint. LD_{100} doses of *Naja naja* venom resulted in uniform death of all mice, but tumescent epinephrine provided a significantly increased survival duration.

Slow systemic absorption

Tumescent epinephrine is effective as a treatment for snakebite because it induces capillary vasoconstriction that slows the rate of venom absorption into the systemic circulation. Other drugs might also slow venom absorption. Smaller snake venom molecules enter the circulation by both diffusion across capillary endothelium and by lymphatic transport. Larger venom molecules reach the systemic circulation mainly by lymphatic transport. Lidocaine (1 g/l) and verapamil at safe dosages and concentrations are known to impair lymphatic smooth muscle function and slow lymphatic transport of large protein molecules toward the thoracic duct and the systemic circulation.[11] Future

Table 2 Summary of survival data for mice challenge-exposed with *Naja naja* venom at LD_{50} and LD_{100} doses and then rescued with tumescent epinephrine

	***Naja naja* venom**			
	LD_{50}, 0.8 mg/kg		**LD_{100}, 1 mg/kg**	
	Control (*n* = 12)	**Tumescent epinephrine rescue (*n* = 17)**	**Control (*n* = 12)**	**Tumescent epinephrine rescue (*n* = 12)**
Survival at 24 h, *n* (%)	6 (50)	16 (94)	0 (0)	0 (0)
Survival time (min)				
Mean	1316	1389	158	230
Minimum	120	570	151	204
Maximum[a]	1440	1440	170	318
P value				
z-test for proportions	0.0062		n/a	
Log-rank analysis	0.0039		<0.0001	

n/a, not applicable because there were no surviving mice in either group.
[a]An endpoint of 1440 minutes (i.e., 24 hours) for survival was determined prior to the study. Despite the fact that some mice were expected to live >24 hours after venom injection, survival time was limited in this manner to avoid effects on reported mean survival times in surviving mice and is in accordance with commonly accepted practices for survival studies.[21]

studies are needed to investigate the efficacy of lidocaine and verapamil against lymphatic transport of larger snake venom molecules.

Neutralizing venom *in situ*

Contravenom offers the possibility of neutralizing venom *in situ* before it has been absorbed into the systemic circulation. Snake venom contains a wide variety of toxic peptides and proteins (both enzymatic and nonenzymatic) that produce systemic neurotoxic, hemotoxic, cardiotoxic, nephrotoxic, and myotoxic effects, as well as local skin necrosis.[12]

Numerous drugs other than antivenom have been reported to neutralize venom *in vitro*. There are a number of plant-derived compounds that have been shown to neutralize snake venom *in vitro*.[13] Such contravenom drugs might be effective if delivered subcutaneously in a tumescent solution at the site of snakebite.

Snake venoms typically contain hyaluronidase. Adding a hyaluronidase-inhibitor to a tumescent contravenom solution might limit the extent of venom diffusion beyond the snakebite site and delay systemic absorption of venom and limit the spread of venom within local tissues.[13–18]

Venom contains cytotoxic enzymes, such as phospholipase A2 and metalloproteinases, that aid in the digestion of prey and cause tissue necrosis at the site of a snakebite. Compounds that can neutralize snake venom phospholipase A2, for example, varespladib and synthetic nanoparticles, are candidates to become contravenom drugs.[7,19] Drugs that chelate the zinc in metalloproteinases are contravenom candidates.

Potential clinical applications

Our results suggest that human victims of a *Naja naja* snakebite might benefit from prompt tumescent epinephrine injection with delayed venom absorption and more time to get to hospital for antivenom therapy and life support.

For reasons of experimental design, we limited the composition of the experimental tumescent solution to epinephrine in saline. The addition of lidocaine (1gm/L) to the tumescent contravenom solution offers a safe means of treating the localized pain associated with venomous snakebites. The basic components (saline, epinephrine, and lidocaine) are cheap, portable, and storable. Tumescent contravenom is easy to administer by anyone, anywhere. As a field treatment, contravenom has the potential to treat pain, attenuate the systemic absorption of venom, and allow time to seek additional medical treatment.

Study limitations

Our study is based on a murine model of tumescent drug delivery. Murine skin is quite lax with a thin subcutaneous fat layer. Bulk flow of tumescent fluid in mice is quite different than in humans. A larger animal might provide a more realistic model for studying tumescent contravenom pharmacokinetics.

We only focused on subcutaneous neurotoxin envenomation.[20] Our studies did not consider intramuscular envenomation. Snake venom myonecrosis is more difficult to study than neurotoxicity. Intravenous delivery of traditional antivenom may not effectively treat intramuscular envenomation. Intramuscular tumescent delivery of antivenom or contravenom ought to be studied.

Only the distal hind quarters of the mice received venom. Venom delivered to the face or neck may not respond as well to tumescent contravenom treatment. Tumescent contravenom was only given as a single dose. Repeated administrations of tumescent contravenom might also be beneficial.

Conclusion

Tumescent infiltration with dilute epinephrine in normal saline solution prolongs survival in mice after envenomation with lethal neurotoxic doses of lidocaine and *Naja naja* venom. The success of the mouse model for tumescent epinephrine contravenom treatment suggests that further experimentation with a variety of potential contravenom drugs including chelating and neutralizing agents is warranted.

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Author contribution

Joy R. Makdisi, Dennis P. Kim, and Jeffrey A. Klein helped with the experimental design and prepared the manuscript. Paytra A. Klein helped with the experimental design and contributed to the manuscript. All authors approved the final manuscript and attest to the integrity of the original data and analysis reported in the final manuscript.

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