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Short communication

Nephrotoxicity of hump-nosed pit viper (*Hypnale hypnale*) venom in mice is preventable by the paraspecific Hemato polyvalent antivenom (HPA)

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ABSTRACT

Mice experimentally envenomed with *Hypnale hypnale* venom (1× and 1.5× LD_{50}) developed acute kidney injury (AKI) principally characterized by raised blood urea and creatinine. Prolonged blood clotting time and hemorrhage in lungs implied bleeding tendency. Pallor noted in most renal cortices was suggestive of renal ischemia secondary to consumptive coagulopathy. Intravenous infusion of Hemato polyvalent antivenom following experimental envenoming effectively prevented death and AKI in all mice, supporting its potential therapeutic use in envenoming cases.

Bites by hump-nosed pit vipers (*Hypnale hypnale*) have been identified as a leading and potentially fatal cause of snake envenomation in Sri Lanka as well as southwestern region of India (Kerala) (Ariaratnam et al., 2008; Joseph et al., 2007). The majority (90%) of envenomed patients suffered from local tissue damages, e.g., hemorrhagic blistering and extensive tissue necrosis, while approximately 40% of them developed concurrent systemic syndrome characterized by hemostatic derangement. Acute kidney injury (AKI), a severe systemic complication associated with the bite, was responsible for most deaths with an overall fatality rate of 1.8%, even though it occurred infrequently (10% of total bite cases, $n = 302$) (de Silva et al., 1994; Ariaratnam et al., 2008). The mechanism of kidney injury by *H. hypnale* venom was postulated to be a complication of consumptive coagulopathy secondary to the venom's procoagulant and fibrinolytic actions (Kanjanabuch and Sitprija, 2008), and the venom at a sublethal dose (1/3 intramuscular LD_{50}) did not appear to induce direct kidney damage in a rat model (Tan et al., 2011b). Nevertheless, in view of the venom's prominent proteolytic and cytotoxic activities (Tan et al., 2011b; Maduwage et al., 2011), a direct tissue-damaging effect on the kidney or indirect immunological responses induced by the venom at higher doses cannot be completely ruled out.

Although antivenom is the definite treatment for snakebite envenoming (Chippaux and Goyffon, 1998), there are currently no effective antivenoms clinically available to treat *H. hypnale* envenoming. A paraspecific antivenom (Hemato polyvalent antivenom, HPA, raised against venoms of Thai *Calloselasma rhodostoma*, *Cryptelytrops albolabris* and *Daboia siamensis*), previously shown effective to neutralize the venom's hematoxic, necrotic and lethal effects in mice (Tan et al., 2011a), is nonetheless a potential therapeutic treatment for the endemic medical urgency.

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Clinically, although most cases of AKI complicating *H. hypnale* bite were reversible with acute care (demanding dialysis), some surviving patients eventually progressed to chronic kidney disease with ensuing death within a year, simply because of the lack of affordable renal replacement therapy in a developing country that is socioeconomically constrained (Ariaratnam et al., 2008). Therefore, the antivenom therapy proposed for the treatment of *H. hypnale* bite needs to address the issue of venom nephrotoxicity in addition to its neutralization of other major toxic effects, and above all, lethality. In this study, the HPA was tested for its ability to prevent the development of AKI and death using an *in vivo* nephrotoxic animal model.

Albino mice of ICR-strain used in the current study were supplied by the Laboratory Animal Center, University of Malaya. The animals were handled according to the guidelines given by CIOMS on animal experimentation, and the experimental protocol was approved by the Animal Use and Care Committee of the institution [ethics clearance number: PM/03/03/2010/FSY(R)]. The mice were divided into one control group and two challenge groups (Group A and Group B). Mice in the control group ($n = 4$) each received 200 μL normal saline, while mice in Group A ($n = 9$) each received *H. hypnale* venom at 1× intraperitoneal LD_{50}, and those in Group B ($n = 4$) each received the same venom at 1.5× intraperitoneal LD_{50}. The venom (a pooled sample obtained from the milking of >10 snakes captured in Gamapha, Kelaniya, Avissawela and Colombo regions of Sri Lanka) was dissolved in normal saline and the volume of injection was adjusted to 200 μL. Both venom and vehicle (normal saline) were injected intraperitoneally. The dose used in this study was estimated from the intraperitoneal LD_{50} value of *H. hypnale* venom in rodents (6 μg/g) obtained in an earlier study (Tan et al., 2011b) from the same laboratory. Following injections, the mice were housed individually in standard metabolic cages with access to food and water *ad libitum*. Hourly observations on the mice's behaviors and toxic signs were charted over 48 h. Mice that showed features suggestive of impending death (e.g., labored breathing with pauses, feeble heartbeat, loss of righting reflex, inability to walk, and lack of response to manipulation) were more closely monitored until spontaneous death. Upon death, blood was collected immediately from the heart, and the major organs were then harvested for gross and microscopic examinations. At 48 h, all surviving mice were sedated with a mixture of xylazine and ketamine (0.1 and 1.0 mg, respectively, per 20 g mouse; chemicals supplied by Troy Laboratories, Australia), and blood was collected via cardiac puncture. Subsequently, the mice were euthanized by cervical dislocation, and the major organs were harvested. Blood collected in plain tubes were observed for clotting time before processing for urea and creatinine analysis. The urine samples collected throughout the study period were analyzed for specific gravity, hematuria and proteinuria using urine test strips (Roche Diagnostics, Germany). The harvested organs were fixed in 10% formalin in phosphate-buffered saline, followed by paraffin wax-embedding, sectioning of tissue, dewaxing, rehydration, and staining with hematoxylin and eosin. Kidney tissues were specifically sectioned at 1.5 μm thin layer.

The experimental envenoming with 1× and 1.5× LD_{50} was repeated in Group C ($n = 9$) and Group D ($n = 4$), respectively. Intervention was carried out 5 min following the venom challenge, where each mouse from both groups received an injection of 200 μL of Hemato polyvalent antivenom (reconstituted as 1 vial of HPA: 10 mL normal saline; antivenom was supplied by Queen Saovabha Memorial Institute, Bangkok) via tail vein. Animal housing, monitoring of signs, collection of blood and urine, processing of tissues/organs of the mice and further analyses were performed essentially as described in the nephrotoxic model above. The significance of the differences in the means of the results was determined by one-way ANOVA and Tukey's post hoc test.

H. hypnale venom at median lethal dose (LD_{50}) (Group A) caused serum biochemical derangement suggestive of acute renal failure, most prominently uremia (and observably increased blood creatinine level) that affected all challenged mice (Fig. 1a). Elevated blood urea level (directly proportionate to blood urea nitrogen) generally indicates a moderate-to-severe degree of renal failure. The degree of hematuria and proteinuria correlated with the deterioration of renal function and signs of toxicity observed in mice that were dead by 24 h (Table 1). In mice challenged with 1.5× LD_{50} (Group B), severe azotemia (with blood creatinine increased higher than urea) was observed in all mice (Fig. 1b), indicating that the renal function was further compromised. While significant proteinuria and prolonged clotting time were observed, there was however no hematuria detected, probably because glomerular involvement was not extensive in view of the short period of time to death. On autopsy, patches of pallor noted on the renal cortices were suggestive of renal ischemia, although histopatholgy study did not reveal obvious abnormal findings under light microscopy within the study period (48 h maximum). This suggests that electronic microscopic examinations may be necessary for investigating subcellular ultrastructural changes in the mouse model. Additional features implying possible bleeding disorder included prolonged clotting times (Table 1) and hemorrhagic spots in the lungs. Urine specific gravity was noted to be marginally higher in the challenge group with 1.5× LD_{50} ($p = 0.04$; Table 1), a peripheral finding suggestive of pre-renal azotemia as in reduced renal perfusion secondary to altered hemodynamics.

The result indicated that death in mice experimentally envenomed with *H. hypnale* venom was associated with AKI, consistent with clinical reports (Joseph et al., 2007; Ariaratnam et al., 2008). This likely cause of death was effectively prevented by the Hemato polyvalent antivenom (HPA) in the current *in vivo* rodent model, evidenced by normalization of blood and urine biochemistry of the envenomed animals (Fig. 1a: significant amelioration of hematuria ($p \leq 0.001$), proteinuria ($p \leq 0.001$) and uremia ($p \leq 0.05$); Fig. 1b: significant amelioration of proteinuria ($p \leq 0.05$), uremia ($p \leq 0.001$) and azotemia ($p \leq 0.001$)). Moreover, in the intervened groups, urine specific gravity was improved significantly ($p \leq 0.05$), along with normal anatomy of the kidneys and lungs as well as 100% survival. The HPA neutralizing efficacies on *H. hypnale* venom has been established previously by Tan et al. (2011a): against lethality, median effective dose at 1.52 mg venom/mL;

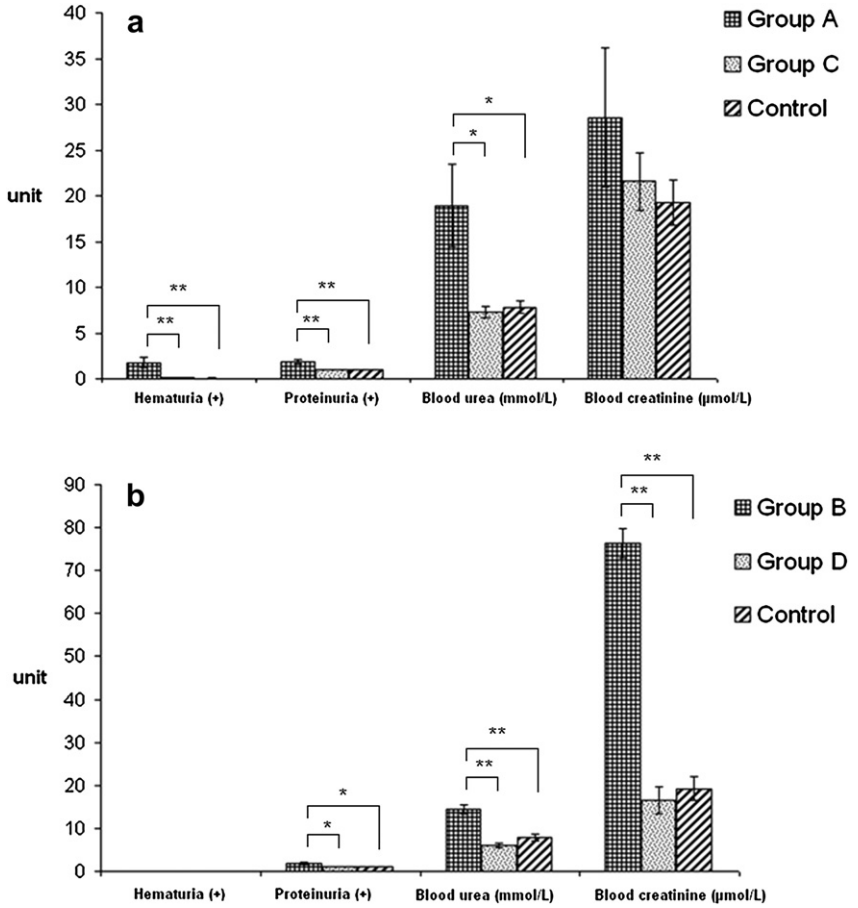

Fig. 1. Urine and blood biochemical parameters of mice: (a) Group A was challenged with 1× i.p. LD_{50} ($n = 9$, 55% death); (b) Group B was challenged with 1.5× i.p. LD_{50} ($n = 4$, 100% death). Group C and Group D were the intervened groups which received i.v. 200 μL of antivenom (HPA) 5 min after envenomation for each mouse. Control was treated with normal saline instead of venom and antivenom. Data were expressed as means ± SEM. Significant differences of mean between the challenged group and the intervened and/or control groups were indicated as * ($p \leq 0.05$) or ** ($p \leq 0.001$) over the bars, determined by one-way ANOVA and Tukey's post hoc test. No significant difference of means was noted between the intervened group and the control.

against procoagulant, hemorrhagic and necrotic effects, effective/median effective doses at 114.2, 67.4, and 53.8 μL/mg venom, respectively. The mechanism of nephrotoxicity neutralization likely involved the HPA cross neutralizing the procoagulant and hemorrhagic effects of *H. hypnale* venom, hence preempting AKI, a complication associated with consumptive coagulopathy (Sitprija, 2008). The cross neutralization observed was also recently supported by immunological cross reactivity study (Tan et al., 2012), which reflected similarity of antigenic properties between the venoms of *H. hypnale* and *C. rhodostoma*. However, venom-induced defibrinogenation complicating AKI is not a known feature in *C. rhodostoma* envenoming (Ho et al., 1986), hence implying subtle differences in the biochemical aspects of the procoagulant enzymes from these two crotalid venoms, possibly at the fibrinogenolytic

Table 1
Effects of *Hypnale hypnale* venom on urine and blood parameters of mice challenged with 1× LD_{50} (Group A) and 1.5× LD_{50} (Group B).

Group	Test						
	Time to death (h)	Urine			Blood		
		Specific gravity	Hematuria (+)	Proteinuria (+)	Clotting time (s)	Urea (mmol/L)	Creatinine (μmol/L)
Control ($n = 4$)	Not observed	1.023 ± 0.002	0.0 ± 0.0	1.0 ± 0.0	12.5 ± 2.9	7.8 ± 0.6	19.3 ± 3.1
A ($n = 9$)	18.4 ± 2.1	1.026 ± 0.001	1.8 ± 0.6[a]	1.9 ± 0.3	28.9 ± 6.5[b]	19.0 ± 4.5	28.6 ± 7.6
B ($n = 4$)	7.4 ± 1.3	1.029 ± 0.001[a]	0.0 ± 0.0[a]	1.8 ± 0.3	45.0 ± 2.9[b]	14.4 ± 1.0	76.3 ± 3.3[c]

Venom was dissolved in normal saline and volume was adjusted to 200 μL for intraperitoneal injection. Data were expressed as means ± SEM. Fatality in Group A = 56%; Group B = 100%.
Means (for Groups A and B) indicated with letters showed significant difference from the corresponding means of control for the test, as determined by one-way ANOVA and Tukey's post hoc test: [a] $p \leq 0.05$; [b] $p \leq 0.01$; [c] $p \leq 0.001$.

mechanism. Essentially, the current findings based on *in vivo* animal experiments complement that of the cross neutralization study reported previously on the venom hematoxic and lethal effects, hence supporting the ability of HPA to confer paraspecific protection, and justifying the suggestion for its clinical trial in the future.

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Conflict of interest

None declared.

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