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Title: Cross neutralization of Hypnale hypnale (hump-nosed pit viper) venom by polyvalent and monovalent Malayan pit viper antivenoms in vitro and in a rodent model

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Cross neutralization of *Hypnale hypnale* (hump-nosed pit viper) venom by polyvalent and monovalent Malayan pit viper antivenoms *in vitro* and in a rodent model. Choo Hock Tan, Poh Kuan Leong, Shin Yee Fung, Si Mui Sim, Gnanajothy Ponnudurai, Christeine Ariaratnam, Sumuna Khomvilai, Visith Sitprija, Nget Hong Tan.

Malayan pit viper antivenom and a hematoxic polyvalent antivenom effectively cross-neutralized the lethality, procoaguant, hemorrhagic and necrotic activities of *H. hypnale* venom.

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<th>Antivenom</th>
<th>mg <em>H. hypnale</em> venom per mL antivenom</th>
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<tr>
<td>Monovalent Malayan Pit Viper antivenom</td>
<td>0.89</td>
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<td>Hemato Polyvalent antivenom</td>
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Cross neutralization of Hypnale hypnale (hump-nosed pit viper) venom by polyvalent and monovalent Malayan pit viper antivenoms in vitro and in a rodent model

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Abstract

_Hypnale hypnale_ (hump-nosed pit viper) is a medically important venomous snake in Sri Lanka and Southwestern India. Bite of this snake may result in hemostatic dysfunction, acute kidney injury and death. Clinical studies indicated that the locally available polyvalent antivenoms produced in India are not effective against hump-nosed pit viper envenoming. Hence, there is an urgent need to search for effective antivenom. In this paper, we examined the ability of _Calloselasma rhodostoma_ (Malayan pit viper) monovalent antivenom and the Hemato polyvalent antivenom (both produced by Thai Red Cross Society, TRCS) to neutralize the lethality and toxic effects of _H. hypnale_ venom, as _C. rhodostoma_ is considered a sister taxon of _H. hypnale_. _In vitro_ neutralization studies showed that the Hemato polyvalent antivenom effectively neutralized the lethality of _H. hypnale_ venom (1.52 mg venom/ml antivenom) as well as the hemorrhagic, procoagulant and necrotic activities of the venom. The monovalent _C. rhodostoma_ antivenom could also neutralize the lethality and toxic activities of the venom, but the potency was lower. The Hemato polyvalent antivenom also effectively protected mice from the lethal and local effects of _H. hypnale_ venom in an _in vivo_ rodent model of envenoming. Furthermore, the polyvalent antivenom could also effectively neutralize the venom of _Daboia russelii_ (2.50 mg venom/ml antivenom), another common cause of snake bites in Sri Lanka and South India. These findings suggested that the Hemato polyvalent antivenom may be beneficial in the antivenom treatment of _H. hypnale_ envenoming.

Keywords: _Hypnale hypnale_ venom, hump-nosed pit viper, neutralization by commercial antivenoms.
1. Introduction

_Hypnale hypnale_ (hump-nosed pit viper) is widely distributed in Sri Lanka and southwestern coast of India (Ariaratnam et al., 2008). It is the major cause of venomous snake bites in Sri Lanka (de Silva et al., 1994). In India, the medical importance of _H. hypnale_ bite has been overlooked for many years perhaps because of misidentification of biting species mainly as _Echis carinatus_. Recently, Joseph et al. (2007) reported the first authenticated cases of life-threatening envenoming by _H. hypnale_ in southwestern India. de Silva et al (1994) and Ariaratnam et al. (2008) also reported that bites by this snake can cause debilitating local and fatal systemic envenoming. Victims of _H. hypnale_ bite may develop hemostatic dysfunctions including coagulopathy, thrombocytopenia or spontaneous systemic hemorrhage, as well as acute kidney injury, with an overall fatality rate of 1.7% in hospitalized patients (Ariaratnam et al., 2008; Joseph et al., 2007, Premawardena et al., 1996, 1998).

Previous clinical and laboratory studies of _H. hypnale_ venom demonstrated that the venom exhibits procoagulant and fibrinolytic activities (de Silva et al., 1994; Premawardena et al., 1998) and contains phospholipases A2 similar to those in the Malayan pit viper (Calloselasma rhodostoma) venom (Wang et al., 1999). Tan et al. (2010) also reported the presence of hyaluronidase, L-amino acid oxidase, thrombin-like enzymes, arginine esterase and proteases in the venom. To date, however, there is still no specific antivenom against the venom for envenoming treatment. The polyvalent antivenoms available in Sri Lanka and southwestern India, including Bharat polyvalent antivenoms and Haffkine polyvalent antivenoms, for examples, were found to be not effective in the treatment of _H. hypnale_ bite (Ariaratnam et al., 2008; Joseph et al., 2007). There is therefore an urgent need to search for effective antivenom against the venom.

The Malayan pit viper (Calloselasma rhodostoma) is the sister taxon of _H. hypnale_ (Parkinson et al., 1997; Vidal and Lecointre, 1998). Previously, both snakes were classified as members of the Agkistrodon genus. As such, the venom of _C. rhodostoma_ may possess toxic components similar to those in the _H. hypnale_ venom. In view of the close phylogenetic relationship between _H. hypnale_ and _C. rhodostoma_, the Thai Red Cross Malayan Pit Viper antivenom had been tested for neutralizing _H. hypnale_ venom using rodent assay (Ariaratnam et al., 2008). However, there was no effective protection found. In this paper, we report our studies on the in vitro cross-neutralizations of _H. hypnale_ venom by newly manufactured monovalent Malayan pit viper antivenom, and in vitro as well as in vivo (rodent model) cross-neutralizations of the venom by the newly developed Hemato polyvalent antivenom. Both antivenoms were produced by Thai Red
Cross Society (TRCS) in Thailand. The Hemato polyvalent antivenom was produced by immunization of horses with a mixture of the following venoms, all of Thai origins: *C. rhodostoma*, *Cryptelytrops albolabris* (Green pit viper) and *Daboia siamensis* (Russell’s viper).

2. Materials and methods

2.1 Venoms and antivenoms

*Hypnale hypnale* venom was a pooled sample obtained from the milking of >10 adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the snake farm at University of Colombo, Sri Lanka and were identified by Anslem de Silva, an expert herpetologist. *Daboia russelii* venom was from the same source and the snakes were captured in Sri Lanka (Anuradhapura, Ratnapura, Galle and Colombo regions). *Calloselasma rhodostoma* (Malayan pit viper, from Malaysia) venom and *Echis carinatus sochureki* (Pakistan) venom used in this study were obtained from Latoxan (France). Monovalent Malayan pit viper antivenom (MPVA) (Batch no. CR00909, exp. date 2/11/2014) and Hemato polyvalent antivenom (HPA) (Batch no. HP00108, exp. date 6/11/2013) were supplied by Queen Saobhva Memorial Institute, Thai Red Cross Society (TRCS). The antivenoms were freeze-dried F(ab’) 2 (90%) immunoglobulin fragments, obtained from hyperimmunized horses, refined by caprylic acid precipitation and pepsin digestion. The Bharat Polyvalent ASVS (anti-snake venom serum) (Batch no.A5309049, exp. date 03/2013) is a gift from Bharat Serums and Vaccines, Mumbai, India. This antivenom is a refined and concentrated preparation of F(ab’) 2 obtained by fractionating antisera from hyperimmununized horses, and is capable of neutralizing cobra, common krait, Russell’s viper and saw-scaled viper venoms. The antivenoms were reconstituted with sterile water according to instructions and stored at 4°C.

2.2 Reagents and animals

All reagents and chemicals were purchased from Sigma Chemical Company (USA) and were of analytical grade. Albino mice (ICR strain) were supplied by the Laboratory Animal Center, Faculty of Medicine, University of Malaya. The animals were handled according to the guidelines given by CIOMS on animal experimentation (Howard-Jones, 1995). Human citrated sera were a pooled sample from five healthy adult volunteers with consents.
2.3 Determination of lethality, hemorrhagic, procoagulant and necrotic activities

The LD$_{50}$ values of the venom were determined by intravenous (via caudal veins) as well as intramuscular injection into mice (16-25 g, n = 4) and the survival ratio was recorded after 48 h. The LD$_{50}$ (and the 95% confidence intervals, C.I.) values were calculated with the probit analysis method of Finney (1952), using Biostat Analysis software. MLD (minimum lethal dose) of the venom was the dose that killed all the animals tested.

Hemorrhagic activity was determined using mice, a method modified from Theakston and Reid (1983) where rats were used. The minimum hemorrhage dose (MHD) was determined by injecting 40 μL of varying amount of venom (dissolved in normal saline) intradermally into the shaved dorsal skin of lightly sedated mice (with diethyl ether). After 90 min, the animals (n=3 per dose) were killed by overdose of diethyl ether and the dorsal skin was removed. The mean diameter of the hemorrhagic lesion on the inner surface of the skin was measured immediately upon skin removal. Dose response curve between the mean diameter of the hemorrhagic lesion and venom dose was plotted. The MHD was the dose that caused a hemorrhagic lesion diameter of 10 mm.

Procoagulant activity was determined by the method adapted from Bogarin et al. (2000). Various amount of venom, dissolved in 0.1 mL of normal saline, were added separately into 0.2 mL of bovine fibrinogen solution (2 g/L) or 0.2 mL of human citrated plasma at 37°C. Coagulation times were recorded. The minimum coagulant dose (MCD) is defined as the minimal amount of venom (μg/mL) that clots a standard solution of bovine fibrinogen or human citrated plasma in 60 s.

Necrotic activity was determined by injection of the venom at varying amount intradermally into the shaved dorsal skin of lightly sedated mice (with diethyl ether), and the animals were kept with free access to water and feed ad libitum. After 72 h, the animals (n = 3) were killed by overdose of diethyl ether and the dorsal skin was removed. The mean diameter of the necrotic lesion was then measured immediately upon skin removal. The minimum necrotic dose (MND) is defined as the amount of venom that induces a necrotic lesion with a diameter of 5 mm.

2.4 In vitro neutralization of the venom lethality, hemorrhagic, procoagulant and necrotic activities by antivenoms

These were carried out as modified from Ramos-Cerrillo et al. (2008).
Neutralization of lethality: 5 LD_{50} of *H. hypnale* venom was mixed thoroughly with various dilution of the antivenom in phosphate-buffered saline, to give a total volume of 300 μL. The mixture was incubated at 37°C for 30 min with gentle shaking. The mixture was subsequently centrifuged at 10000 g and then injected intravenously into the caudal vein of mice, and the number of animals survived 48 h post-injection was recorded. Neutralization potency of the antivenom is measured by ED_{50} (μL antivenom /5 LD_{50} venom), which is defined as the amount of antivenom (μL) at which the survival ratio of the animals is 50%. The estimated amount of venom neutralized per ml of antivenom was then calculated based on the ED_{50} value, the LD_{50} value and weight of animals used.

Neutralization of the hemorrhagic activity: 2 MHDs of *H. hypnale* venom was mixed thoroughly with various dilution of the antivenom in phosphate-buffered saline, to give a total volume of 40 μL. The mixture was incubated at 37°C for 30 min with gentle shaking. The mixtures were subsequently injected intradermally into the dorsal areas of lightly sedated mice (with diethyl ether). After 90 min, the animals were killed by overdose of diethyl ether. The dorsal skin was removed immediately and the mean diameter of the hemorrhagic spot was measured. Neutralization potency of the antivenom is measured by ED_{50} which is defined as the volume of antivenom (μL) which reduced the activity of the challenge dose (2 MHDs) of the venom by 50%. For comparison purpose, it is also expressed in term of the ratio of μL antivenom/mg venom.

Neutralization of procoagulant activity: 2 MCDs of *H. hypnale* venom were mixed thoroughly with various dilution of the antivenoms in phosphate-buffered saline, to give a total volume of 100 μL. The mixture was incubated at 37°C for 30 min with gentle shaking. To these, 200 μL of bovine fibrinogen (2 g/L) or human citrated plasma, preincubated at 37°C, were added and the coagulation times were recorded. Neutralization potency of the antivenom is measured by effective dose (ED), which is defined as the volume of the antivenom (μL) at which coagulation time was increased three times when compared to coagulation time of the fibrinogen solution or citrated human plasma incubated with venom alone. For comparison purpose, it is also expressed in term of the ratio of μL antivenom/mg venom.

Neutralization of necrotic activity: 2.5 MNDs of the venom (100 μg) was mixed well with various dilutions of the antivenoms in phosphate-buffered saline to give a total volume of 50-80 μL. The mixtures were incubated at 37°C for 30 min under gentle agitation. The mixtures were subsequently injected intradermally into the dorsal areas of lightly sedated mice (with diethyl ether), and the animals were kept with free access to feed and water *ad libitum*. After 72 h, the animals were killed.
by an overdose of diethyl ether and following which, the skins were removed immediately to examine the mean diameter of the dermal necrotic lesion. Neutralization potency of the antivenom is measured by ED$_{50}$ which is defined as the volume of antivenom (μL) which reduced the necrotic activity of the challenge dose (2.5 MNDs) of the venom by 50%. For comparison purpose, it is also expressed in term of the ratio of μL antivenom/mg venom.

2.5 In vivo neutralization of the lethality of H. hypnale venom by Hemato polyvalent antivenom using a rodent model

This was carried out by intramuscular injection of the minimum lethal dose (20 μg/g) of H. hypnale venom into mice (n = 4) followed by intravenous injection of 200 μL of the Hemato polyvalent antivenom, 5 min after the venom inoculation. The number of animals survived 48 h post-injection was recorded. The local effects of the venom were also examined. Control group consisted of mice (n=4) challenged with intramuscular minimum lethal dose of the venom, followed by intravenous injection of 200 μL of normal saline.

2.6 Statistical analysis

Results for procoagulant, hemorrhagic and necrotic activities are presented as mean ± S.E.M., while the variability of lethality assays was expressed as 95% confidence intervals (C.I.). The significance of the differences of the means was determined by Student’s $t$-test. ED$_{50}$ (median effective dose) and the 95% confidence intervals (C.I.) were calculated using the probit analysis method of Finney (1952). Statistical analyses were carried out using the Biostat Analysis software.

3. Results and Discussion

3.1 Toxinological activities of H. hypnale venom

The intravenous LD$_{50}$ and intramuscular LD$_{50}$ of H. hypnale venom were determined to be 0.90 μg/g mouse (95% confidence interval of 0.42-1.84 μg/g mouse) and 13.7 μg/g (8.44-19.50 μg/g mouse), respectively. The i.v. LD$_{50}$ reported herein is slightly lower than an earlier one reported by Ariaratnam et al., (2008), who reported an intravenous LD$_{50}$ of 65.4 μg per mouse, or approximately equivalent to 3 μg/g. The difference could be due to either geographic or individual variation. The i.v. LD$_{50}$ of the venom is comparable to that of C. rhodostoma venom examined in this study (LD$_{50}$ of 1.48 μg/g, 95% C.I. 0.42-1.84 μg/g mouse).
The venom exhibited strong procoagulant, hemorrhagic and necrotic activities. This is consistent with the clinical observations in proven *H. hypnale* bite (Ariaratnam et al., 2008). The minimum coagulation dose (MCD) is 56.2 μg/ml for bovine fibrinogen, and 55.1 μg/ml for human citrated plasma. The minimum hemorrhagic dose (MHD) and minimum necrotic dose (MND) are 10.5 μg and 39.3 μg, respectively. These values are comparable to those of the *C. rhodostoma* venom, with MCDs of 27.3 μg/ml for bovine fibrinogen or 24.9 μg/ml for human citrated plasma; MHD of 24.0 μg and MND of 28.7 μg, respectively.

3.2 *In vitro* neutralization of *H. hypnale* and *C. rhodostoma* venoms

We examined the abilities of three commercial antivenoms to neutralize the toxic activities of *H. hypnale* venom *in vitro*. The neutralization was evaluated in assays involving incubation of venom and antivenom for 30 min prior to testing.

Bharat polyvalent antivenom (ASVS), the antivenom produced by immunizing horses with venoms from the ‘Big 4’ (*Naja naja, Bungarus caeruleus, Daboia russelii* and *Echis carinatus*) failed to protect against *H. hypnale* venom: all mice (n=4) injected with 5 LD₅₀ (i.v.) of the venom died despite receiving 200 μL/mouse of the antivenom. This is consistent with the clinical observations reported by Joseph et al. (2007) that administration of the Bharat polyvalent antivenom did not bring any benefits to patients envenomated by *H. hypnale*. Other authors also reported that the Haffkine polyvalent antivenom (also produced against the ‘Big 4’) was totally ineffective in the treatment of victims bitten by *H. hypnale* (Ariaratnam et al., 2008; Sellahewa et al., 1995).

The capability of the monovalent Malayan pit viper (MPV) antivenom to neutralize *H. hypnale* venom was then examined, as Malayan pit viper (*C. rhodostoma*) is considered a sister taxon to *H. hypnale* by mitochondrial DNA analysis (Parkinson et al., 1997). Ariaratnam et al. (2008) reported that (expired) monovalent MPV antivenom failed to neutralize 5 i.v.LD₅₀ of *H. hypnale* venom *in vitro*, even with 200 μL of the antivenom. A preliminary study, however, showed that when lesser amount of *H. hypnale* venom were used (2 LD₅₀, intraperitoneal injection), the newly manufactured monovalent MPV antivenom could neutralize the lethality of the venom in mice, though only moderately (Tan et al., 2010). The present study showed that the newly manufactured monovalent MPV antivenom indeed could effectively protect mice injected with 5 i.v. LD₅₀ of *H. hypnale* venom, and the ED₅₀ was determined to be 70.71 μL/5 i.v. LD₅₀, or equivalent of 0.89 mg venom neutralized per mL of antivenom. The discrepancies with the previous report by Ariaratnam et al. (2008) could be due to batch differences in the antivenom, as the batch of monovalent MPV
The antivenom used in this study was manufactured using a new process (caprylic acid precipitation) and appears to be more potent (Khomvilai, 2008). The monovalent MPV antivenom was also effective in the neutralization of the procoagulant effect on both bovine fibrinogen and human citrated plasma, with EDs of 432.1 μL/mg venom and 384.4 μL/mg venom, respectively. It also neutralized the hemorrhagic and necrotic activities of *H. hypnale* venom, with ED_{50}s of 472.3 μL/mg venom and 612.6 μL/mg venom, respectively. As expected, the monovalent MPV antivenom was much more potent in the neutralization of the lethality and toxicities (*p*<0.05) of *C. rhodostoma* venom, in particular the necrotic and hemorrhagic activities. The ED_{50} against *C. rhodostoma* venom lethal effect was 41.53 μL/5 i.v. LD_{50}, or equivalent of 3.23 mg venom neutralized per mL of antivenom. The neutralization ED/ED_{50}s are 209.2 μL/mg and 152.7 μL/mg for procoagulant activities against bovine fibrinogen and human citrated plasma, respectively; 151.7 μL/mg for hemorrhagic activity and 38.1 μL/mg for necrotic activity of the venom (See Table 1 and 2). Nevertheless, these results suggest that at least some of the venom toxins from the two snakes, *H. hypnale* and *C. rhodostoma*, are antigenically similar. It is known that the procoagulant enzymes (thrombin-like enzymes) and hemorrhagins from different venomous snakes can have very different antigenic properties. For example, thrombin-like enzyme from *Cryptelytrops purpureomaculatus* exhibited very little ELISA cross reactions with the thrombin-like enzymes from *C. rhodostoma* venom (Tan, 2010). Also, Fung (2002) reported that the major hemorrhagin of *C. purpureomaculatus* venom did not cross-react with *C. rhodostoma* venom at all when examined by double sandwich ELISA. The ability of the monovalent MPV antivenom to neutralize the lethality, procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom therefore supports the conclusion by Parkinson et al (1997) that *C. rhodostoma* is a sister taxon to *H. hypnale*. This is an interesting example of similarities in the immunological properties of the venom proteins support phylogenetic relationship derived from mitochondrial DNA analysis. The close phylogenetic relationship between *H. hypnale*, found in India subcontinent and *C. rhodostoma*, found in Southeast Asia, is a fascinating example of the existence of the ‘Malayan’ element in the fauna of Peninsular India. This phenomenon has been known since a long time, and the cause of these similarities have been explained by the Satpura Hypothesis (Hora, 1949), according to which fauna migration followed the Satpura trends of mountain through Peninsular India during the Pleistocene. The validity of Satpura Hypothesis, however, has been disputed recently (Karanth, 2003).

Recently, a new polyvalent antivenom against hematoxic snake venoms in Thailand has become available. This polyvalent antivenom, termed Hemato polyvalent antivenom, was produced from plasma of horses hyperimmunized by venoms from the three common viper and pit vipers in Thailand: Green pit viper (*Cryptelytrops albolabris*), Russell’s viper (*Daboia siamensis*) and
Malayan pit viper (*C. rhodostoma*). The Hemato polyvalent antivenom was found to be much more effective than the monovalent MPV antivenom in neutralizing the lethality, procoagulant, hemorrhagic and necrotic activities of *C. rhodostoma* venom: the ED$_{50}$ for neutralization of the lethality was determined to be 22.47 μL/5 i.v. LD$_{50}$, or 7.14 mg venom neutralized per mL of antivenom. The EDs for the neutralization of procoagulant activities against bovine fibrinogen and human citrated plasma are 133.9 μL/mg and 104.0 μL/mg venom, correspondingly; whereas the ED$_{50}$s for the neutralization of hemorrhagic and necrotic activities are 122.8 μL/mg venom and 29.7 μL/mg venom respectively (Table 1 and 2). It is therefore not surprising to find that the Hemato polyvalent antivenom is also very effective in the neutralization of the lethality, procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom (Table 1 and 2). Against 5 LD$_{50}$ (i.v.), the ED$_{50}$ of the polyvalent antivenom was determined to be 41.53 μL, which is equivalent to neutralization of 1.52 mg of *H. hypnale* venom per mL of the reconstituted Hemato polyvalent antivenom. In comparison, 1 ml of monovalent MPV antivenom neutralized only 0.89 mg of the venom. Comparison of the ED/ED$_{50}$s of neutralization of procoagulant, hemorrhagic and necrotic activities of the venom also showed that the Hemato polyvalent antivenom is far more effective (p<0.05) than the monovalent MPV in neutralizing the toxic activities of the *H. hypnale* venom. In fact, the ED$_{50}$s of the neutralization of the procoagulant, hemorrhagic and necrotic activities of *H. hypnale* venom by the Hemato polyvalent antivenom is comparable to the ED/ED$_{50}$s against *C. rhodostoma* venom (Table 1 and 2), though the antivenom is more effective against *C. rhodostoma* than *H. hypnale* in term of neutralization of lethality (7.14 mg venom/mL antivenom, versus 1.52 mg venom/mL antivenom, p<0.05).

The greater efficacy of the Hemato polyvalent antivenom than the monovalent MPV antivenom in the neutralization of *H. hypnale* venom suggested that the other two venoms (of *D. siamensis* and *C. albolabris*) used in the immunization scheme of preparation of the polyvalent antivenom may contain venom toxins that are immunologically similar to some *H. hypnale* venom toxins. Our preclinical studies therefore suggest that both the monovalent MPV and Hemato polyvalent antivenom may be useful in the antivenom treatment of systemic *H. hypnale* envenoming. The polyvalent antivenom might be the preferred one because of its greater efficacy, though the drawback is its higher cost (USD 60 per vial compared to USD 20 per vial for the monovalent antivenom). In view of the relatively low yield (average of 13 mg per milking) of venom from *H. hypnale* because of its relatively small size (Tan et al., 2010), and the strong neutralization capacity of the Hemato polyvalent antivenom, 1-2 vial (10-20 ml reconstituted antivenom) of the polyvalent antivenom would probably be sufficient in the treatment of most systemic *H. hypnale* envenomation. Nevertheless, while the results of the preclinical studies are promising, these studies
must be followed by a randomized controlled clinical trial in the relevant regions, which is the final criterion for assessing the clinical efficacy and safety of the antivenom.

3.3. In vitro neutralization of the lethality of Daboia russelii and Echis carinatus sochureki venom by the Hemato polyvalent antivenom

The ability of the Hemato polyvalent antivenom to neutralize the other two common vipers (D. russelii and E. carinatus sochureki) in Sri Lanka and India subcontinent was also investigated, as these two vipers are also common causes of snake bites in the region, and it is not always possible to identify the biting species in these snake bites. Our results showed that the Hemato polyvalent antivenom could neutralize the Sri Lankan D. russelii venom effectively, with an ED$_{50}$ of 7.52 µL/5 LD$_{50}$ (i.v.), or equivalent to 2.50 mg venom per mL antivenom. This is not surprising as the Hemato polyvalent antivenom was raised against a mixture of 3 venoms, including the Thai D. siamensis venom, which presumably contains some common antigens with the Sri Lanka D. russelii venom. The polyvalent antivenom, however, was not effective in the neutralization of E. carinatus sochureki venom: all 4 mice that were injected with 5 LD$_{50}$ (i.v.) died despite receiving a maximum of 200 µL of the antivenom. This result is not surprising as the toxinology of E. carinatus sochureki venom is very different from the three venoms used in the production of the Hemato polyvalent antivenom. Nevertheless, the ability of the Hemato polyvalent antivenom to neutralize D. russelii venom increases the potential benefit of the use of Hemato polyvalent antivenom in management of viper/pit viper bites in Sri Lanka and Southern India, as D. russelii is the second commonest cause of snake bite in the region, next to H. hypnale (Ariaratnam et al., 2009). Since clinically it is not always possible to distinguish between envenomation by H. hypnale, D. russelii and E. carinatus, double-sandwich ELISA should be developed to assist in biting species identification in future clinical trials of the antivenoms.

3.4 In vivo neutralization of H. hypnale venom using a rodent model of envenoming

To further evaluate the potential of the application of the Hemato polyvalent antivenom in the treatment of H. hypnale bites, we also carried out an in vivo neutralization experiment, using a rodent model. In this experiment, four mice received intravenous injection of 200 µL of Hemato polyvalent antivenom 5 min after i.m. injection of minimum lethal dose (400 µg per mouse) of H. hypnale venom. All four mice survived, and observations of the treated animals indicated that in addition to neutralization of the venom lethality of the venom in vivo, the antivenom also prevented or largely reduced the occurrence of venom-induced local tissue damage (myonecrosis and...
bleeding) of the venom. This observation is consistent with the result of in vitro neutralization of the necrotic and hemorrhagic effects of H. hypnale venom by the polyvalent antivenom (Table 2). Ariaratnam et al. (2008) reported in the series of humped-nose pit viper bites, 117 (39%) were systematically envenomed, all with hemostatic abnormalities and 30 with acute renal failure. We at this moment have not been able to assess the ability of the antivenoms to neutralize the nephrotoxicity due to the lack of sufficient amount of H. hypnale venom to develop a reliable in vivo nephrotoxicity assay. Nevertheless, coupled with our in vitro findings, the current in vivo results demonstrate further the potential benefits of the polyvalent antivenom in the treatment of H. hypnale bite.

4. Conclusions

Our results showed that both the monovalent MPV antivenom and Hemato polyvalent antivenoms are effective in the neutralizations of the lethality and major toxic activities induced by H. hypnale venom when using in vitro and in vivo rodent assay protocols, in spite of the fact that the H. hypnale venom is not included in the immunizing mixture used in the production of the antivenom. Hence, this indicates that there are substantial immunological cross-reactivities between toxic components present in H. hypnale venom and the viperid venoms used in the production of the Hemato polyvalent antivenom. Extensive cross-neutralization between several Bothrops venoms and antivenoms has been described (Rojas et al., 2005; Bogarin et al., 2000). Our results therefore support the hypothesis that some antivenoms can be effective against heterologous venoms in other countries and may be helpful in situations where locally produced antivenoms are not available, as demonstrated by Otero et al (1996).

Acknowledgements

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12
REFERENCES


Table 1: Neutralization of lethality of *Hypnale hypnale, Calloselasma rhodostoma* and other venoms by the monovalent Malayan pit viper antivenom and Hemato polyvalent antivenom.

<table>
<thead>
<tr>
<th>Antivenom</th>
<th>Venom</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (i.v.) μg/g</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; (μL antivenom / 5 i.v. LD&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>mg venom neutralized per mL antivenom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent Malayan Pit Viper antivenom</td>
<td><em>C. rhodostoma</em></td>
<td>1.48 μg/g (0.78-2.06)</td>
<td>41.53 μL (20.4-88.4)</td>
<td>3.23</td>
</tr>
<tr>
<td></td>
<td><em>H. hypnale</em></td>
<td>0.90 μg/g (0.42-1.84)</td>
<td>70.71 μL (33.7-148.4)</td>
<td>0.89</td>
</tr>
<tr>
<td>Hemato Polyvalent antivenom</td>
<td><em>C. rhodostoma</em></td>
<td>1.48 μg/g (0.78-2.06)</td>
<td>22.47 μL (14.8-34.1)</td>
<td>7.14</td>
</tr>
<tr>
<td></td>
<td><em>H. hypnale</em></td>
<td>0.90 μg/g (0.42-1.84)</td>
<td>41.53 μL (20.4-88.4)</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td><em>D. russelii</em></td>
<td>0.24 μg/g (0.19-0.62)</td>
<td>7.52 μL (3.53-15.3)</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td><em>E. carinatus sochureki</em></td>
<td>2.08 μg/g (1.02-4.42)</td>
<td>&gt;200 μL</td>
<td>Not effective</td>
</tr>
</tbody>
</table>

Values in range for LD<sub>50</sub> and ED<sub>50</sub> indicated 95% confidence intervals. For neutralization experiments, mice (n=4) were challenged with 5 i.v. LD<sub>50</sub> of the various venoms.
Table 2: Neutralization of the procoagulant, hemorrhagic and necrotic activities of *Hypnale hypnale* and *Calloselasma rhodostoma* venoms by the monovalent MPV antivenom and Hemato polyvalent antivenom

<table>
<thead>
<tr>
<th>Toxic Activity</th>
<th>Minimum Dose</th>
<th>Neutralization by MPV Antivenom (ED or ED_{50})</th>
<th>Neutralization by HP Antivenom (ED or ED_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Procoagulant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. hypnale</em></td>
<td>MCD</td>
<td>ED = 4.8 ± 0.1 μL{\textsuperscript{*}} (432.1 ± 10.9 μL/mg venom)</td>
<td>ED = 1.3 ± 0.0 μL{\textsuperscript{*}} (114.2 ± 2.7 μL/mg venom)</td>
</tr>
<tr>
<td></td>
<td>56.2 ± 1.3 μg/mL{\textsuperscript{*}}</td>
<td>ED = 4.2 ± 0.1 μL{\textsuperscript{*}} (384.4 ± 11.2 μL/mg venom)</td>
<td>ED = 1.3 ± 0.0 μL{\textsuperscript{*}} (121.3 ± 0.7 μL/mg venom)</td>
</tr>
<tr>
<td><em>C. rhodostoma</em></td>
<td>55.1 ± 1.4 μg/mL{\textsuperscript{*}}</td>
<td>ED = 1.1 ± 0.0 μL{\textsuperscript{*}} (209.2 ± 0.2 μL/mg venom)</td>
<td>ED = 0.7 ± 0.0 μL{\textsuperscript{*}} (133.9 ± 0.3 μL/mg venom)</td>
</tr>
<tr>
<td></td>
<td>27.3 ± 0.3 μg/mL{\textsuperscript{*}}</td>
<td>ED = 0.8 ± 0.0 μL{\textsuperscript{*}} (152.7 ± 3.7 μL/mg venom)</td>
<td>ED = 0.5 ± 0.0 μL{\textsuperscript{*}} (104.0 ± 1.2 μL/mg venom)</td>
</tr>
<tr>
<td></td>
<td>24.9 ± 0.4 μg/mL{\textsuperscript{*}}</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hemorrhagic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. hypnale</em></td>
<td>MHD</td>
<td>ED_{50} = 9.9 ± 0.3 μL (472.3 ± 13.9 μL/mg venom)</td>
<td>ED_{50} = 1.4 ± 0.1 μL (67.4 ± 5.1 μL/mg venom)</td>
</tr>
<tr>
<td><em>C. rhodostoma</em></td>
<td>10.5 ± 0.6 μg</td>
<td>ED_{50} = 7.3 ± 1.4 μL (151.7 ± 29.3 μL/mg venom)</td>
<td>ED_{50} = 5.9 ± 0.2 μL (122.8 ± 3.9 μL/mg venom)</td>
</tr>
<tr>
<td></td>
<td>24.0 ± 0.9 μg</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Necrotic</strong></td>
<td>MND</td>
<td>ED_{50} = 61.2 ± 1.7 μL (612.6 ± 17.5 μL/mg venom)</td>
<td>ED_{50} = 5.3 ± 0.1 μL (53.8 ± 0.59 μL/mg venom)</td>
</tr>
<tr>
<td><em>H. hypnale</em></td>
<td>39.3 ± 1.6 μg</td>
<td>ED_{50} = 2.7 ± 0.1 μL (38.1±1.9 μL/mg venom)</td>
<td>ED_{50} = 2.1 ± 0.0 μL (29.7 ± 0.4 μL/mg venom)</td>
</tr>
<tr>
<td><em>C. rhodostoma</em></td>
<td>28.7 ± 2.6 μg</td>
<td></td>
<td></td>
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</tbody>
</table>

Neutralization of hemorrhagic and procoagulant activities were determined by challenge with 2 MHDs and incubation with 2 MCDs, respectively, of the venom. For neutralization of necrotic activity, mice were challenged 2.5 MNDs of venom intradermally. Values are expressed as mean ± S.E.M. (n=3 for procoagulant, hemorrhagic and necrotic activities)

{\textsuperscript{*}} Procoagulant activity tested on bovine fibrinogen

{\textsuperscript{*}} Procoagulant activity tested on human citrated plasma