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Short communication

Neutralization of lethality and proteolytic activities of Malayan pit viper (*Calloselasma rhodostoma*) venom with North American Virginia opossum (*Didelphis virginiana*) serum

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ABSTRACT

Malayan pit viper (*Calloselasma rhodostoma*) envenomation is a major health problem in South East Asia. During envenomation, venom components mainly affect the hemostatic system. The sera from the North American Virginia opossums (*Didelphis virginiana*) were able to neutralize the venom of the Malayan pit viper. These natural inhibitors could be explored as potential therapeutics against envenomations of a variety of venomous snake species in different geographical habitats.

The Malayan pit viper (*Calloselasma rhodostoma*) is a common snake found in most parts of South East Asia (Daltry et al., 1996). Its habitats include rubber and palm plantations, farms, rural villages, and rain forests. Snake envenomation is a serious medical problem in Thailand, especially in the agricultural areas where snakes are abundant (Pithayanukul et al., 2004). In 1992, a national survey of snakebites in Thailand showed that 70% of reported cases were caused by venomous snake species. The top four most common species responsible for snakebites in Thailand are *C. rhodostoma* (38%), *Trimeresurus albolabris* (27%), *Daboia russeli siamensis* (14%), *Naja atra* (10%), and others (11%) (Viravan et al., 1992). Malayan pit viper venom affects the hemostatic system causing hemorrhage (Lu et al., 2005; Marsh & Williams, 2005; Pithayanukul et al., 2004), and myonecrosis (Tan et al., 1986).

The Virginia opossum (*Didelphis virginiana*) is a marsupial animal living in Central and North America. Opossums show no signs of edema, ecchymosis, or necrosis when bitten by venomous snakes. These animals are naturally resistant to the proteolytic effects of Crotalid venoms (Pérez et al., 1978; Perez et al., 1979; Rodríguez-Acosta et al.,

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1995; Galán et al., 2004). They have metalloproteinase inhibitors in their sera that neutralize the hemorrhagic and other proteolytic activities caused by many snake venoms (Catanese & Kress, 1992; Domont et al., 1991; Jurgilas et al., 2003; McKeller and Perez, 2002; Perez et al., 1979; Sánchez et al., 1998). In light of these past discoveries, opossum sera were selected to test against Malayan pit viper venom.

The purpose of this research was to determine the inhibitory effects of *D. virginiana* sera on *C. rhodostoma* venom by utilizing a series of antiproteolytic assays and lethal doses. The toxicity of *C. rhodostoma* venom including lethal dose (LD_{50}) and proteolytic activities was also determined and compared with the activity of other North American snake venoms.

To date, there are no reports of any animal species in South East Asia having a natural resistance to the proteolytic activities of Malayan pit viper venom. The *D. virginiana* is the first marsupial species with antiproteolytic activity against Malayan pit viper venom. By nature, opossums and Malayan pit vipers exit in very distinct geographical locations; and therefore, opossums have never come in contact with Malayan pit vipers. Thus, it is assumed that the antiproteolytic activities observed in this study are not related to antibody neutralization. The neutralization is probably due to metalloproteinase similar to the results reported on the American continent (Menchaca and Perez, 1981; Perales et al., 1986; Pérez and Sánchez, 1999; Perales et al., 2005). According to a previous report, there was no evidence to support that opossums produce antibodies against venom molecules when immunized (McKeller and Perez, 2002). Opossum serum contains metalloproteinase inhibitors which neutralize toxins from Crotalid venoms (Menchaca and Perez, 1981; Perales et al., 1986; Perales et al., 2005).

Crude *C. rhodostoma* venom had fibrinolytic, hemorrhagic, and gelatinase activities (Fig. 1, Table 1). Minimal fibrinolytic dose (MFD) of the venom was tested by a method of Bajwa et al. (1980) resulting with MFD of 13 μg (Fig. 1A). The minimal hemorrhagic dose (MHD) of the venom, assayed by a method described by Omori-Satoh et al. (1972), resulted in an MHD of 15 μg (Fig. 1C) and the minimal gelatinase dose (MGD), followed by a method of Huang and Perez (1980), was 10 μg (Fig. 1E). A total of 0.175, 0.43 and 0.022 mg of *D. virginiana* sera completely inhibited one MFD, MHD and MGD, respectively (Fig. 1B, D and F; Table 1). The inhibition ratios of *D. virginiana* serum against *C. rhodostoma* venom are shown in Table 1. The most potent antiproteolytic activity was gelatinase (2.2:1). The antifibrinolytic and antihemorrhagic ratios were 13.5:1 and 29.2:1, respectively.

The LD_{50} and the ED_{50} for the venom and the sera, respectively, were calculated using the Spearman-Karber (1978) method. The LD_{50} for *C. rhodostoma* crude venom was 6.1 mg/kg body weight (Table 1). *D. virginiana* serum was used to neutralize the three LD_{50} of Malayan pit viper venom, with an ED_{50} of 546 mg/kg. The LD_{50} of Malayan pit viper venom was similar to those of Western Diamondback rattlesnake (5.10 mg/kg), Mojave rattlesnake Type B (5.10 mg/kg), the Southern copperhead (5.20 mg/kg), and Broad-banded Copperhead (6.80 mg/kg) (Sánchez et al., 2003). The most lethal venom indicated by LD_{50} value was from Mojave rattlesnake Type A (0.47 mg/kg), which was 12.97 times more toxic than Malayan pit viper venom. The MHD of Malayan pit viper venom (15 μg) was similar to

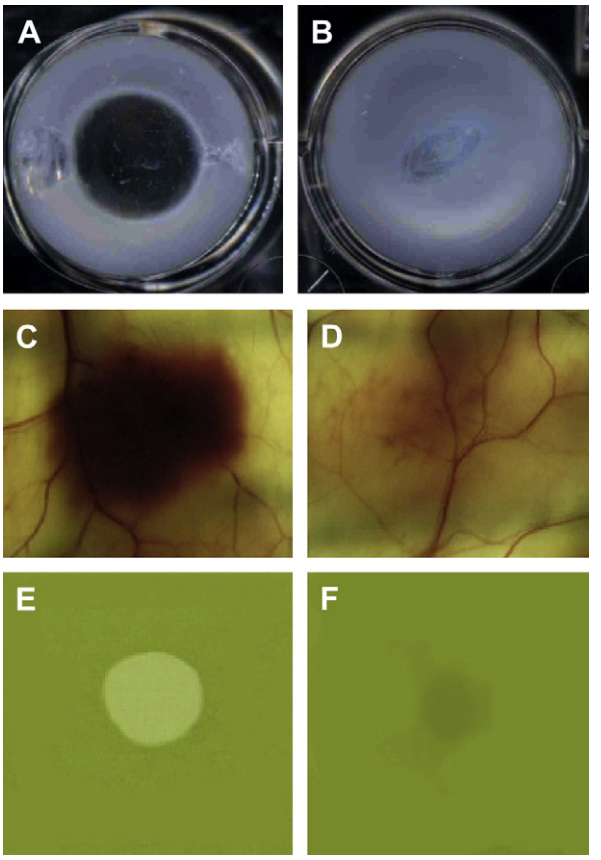

Fig. 1. Proteolytic activities of *C. rhodostoma* venom and antiproteolytic activity of *D. virginiana* sera. (A) Fibrinolytic; (B) antifibrinolytic; (C) hemorrhagic; (D) antihemorrhagic; (E) gelatinase and (F) antigelatinase.

Table 1
Minimal activity doses and lethal dose of Malayan pit viper venom and antiproteolytic activities and effective dose of the *D. virginiana* sera

Activity	Venom active dose (μg)[a]	Sera active dose (mg)[b]	Inhibition ratio[c]
MFD	13	0.175	13.5:1
MHD	15	0.438	29.2:1
MGD	10	0.022	2.2:1
LD_{50} (mg/kg)[d]	6.10		
ED_{50} (mg/kg)[e]		546	

[a] Venom minimal activity dose: MFD = minimal fibrinolytic dose, the amount of venom protein required to produce a 5 mm clearing zone on a fibrin plate; MHD = minimal hemorrhagic dose, the amount of venom protein required to produce a 10 mm hemorrhagic spot; MGD = minimal gelatinase dose, the amount of venom protein required to produce a 10 mm clearing zone on an X-ray film.

[b] The amount of opossum sera required to neutralize a minimal activity dose.

[c] The amount of sera protein in milligrams required to neutralize 1 mg of Malayan pit viper venom.

[d] The amount of venom that will kill 50% of a mouse population.

[e] The amount of sera that will protect 50% of a mouse population injected with three LD_{50} of venom.

those of Mojave rattlesnake Type B (12.2 μg) and Black-tailed rattlesnake (12.5 μg). The most hemorrhagic venom was from the Eastern Diamondback rattlesnake (0.3 μg), which was 50 times more hemorrhagic than that of the Malayan pit viper (Sánchez et al., 2003). From those comparisons, it can be conclude that most of North American snake species contain venom that is more potent than the venom in the Malayan pit vipers found in Thailand. On the other hand, the ED_{50} for the opossum sera was rather high compared to that of commercial antivenoms (Consroe et al., 1995; Sánchez et al., 2003). Unlike commercial antivenoms, which have been purified, the opossum sera contains many other non-neutralizing molecules, and it is likely that the ED_{50} will improve when the sera is purified.

This report provides supporting evidence of previous studies regarding opossums being resistant to snake venom. Opossum sera were capable of neutralizing metalloproteinases from Malayan pit viper venom including hemorrhagins, fibrinogenases, and gelatinases. Metalloproteinase inhibitors in opossum sera that are active against venom found in such a geographical diverse species of snake, raises a potential application for opossum sera to be used universally for treatment of envenomations. All venom molecules may not be equally important to neutralize and the hemorrhagins may be some of the most important to be neutralized. Hemorrhagins diffuse into the tissue, absorb on to vessels, and cause degradation of extracellular matrix and vascular basement membrane. The destruction of the vascular system can have a secondary effect of a quick release of other toxins (thrombin-like enzymes and procoagulants) into the circulation, which could cause coagulopathy problems. Anai et al. (2002) reported that JF-1 hemorrhagins from *Bothrops jararaca* venom played an important role in the development of coagulopathy by causing rapid spreading of thrombin-like enzymes into the circulation. Studies show that metalloproteases from snake venom (BaPI from *Bothrops asper* venom) have been neutralized by the synthetic inhibitor batimastat (Escalante et al., 2000). Furthermore, batimastat was able to reduce the local tissue damage induced by whole venom of *B. asper* (Rucavado et al., 2000). However, more studies should be considered to evaluate antivenom efficiency of opossum sera against a variety of snake venoms found in various parts of the world.

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