Tropical Biomedicine 27(3): 366–372 (2010)

Protective effects of *Mucuna pruriens* seed extract pretreatment against cardiovascular and respiratory depressant effects of *Calloselasma rhodostoma* (Malayan pit viper) venom in rats

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Received 30 November 2009; received in revised form 31 May 2010; accepted 5 June 2010

Abstract. The protective effects of *Mucuna pruriens* seed extract (MPE) against the cardio-respiratory depressant and neuromuscular paralytic effects induced by injection of *Calloselasma rhodostoma* (Malayan pit viper) venom in anaesthetized rats were investigated. While MPE pretreatment did not reverse the inhibitory effect of the venom on the gastrocnemius muscle excitability, it significantly attenuated the venom-induced cardio-respiratory depressant effects ($p<0.05$). The protection effects may have an immunological mechanism, as indicated by the presence of several proteins in the venom that are immunoreactive against anti-MPE. However, we cannot rule out the possibility that the pretreatment may exert a direct, non-immunological protective action against the venom.

INTRODUCTION

Calloselasma rhodostoma (Malayan pit viper) is a medically important snake indigenous to Malaysia. The major toxins of the pit viper venom are thrombin-like enzymes, platelet-aggregation inducers and inhibitors; as well as hemorrhagic proteases (Tan, 1991). In recent years, there has been a growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. In almost any part of the world, where venomous snakes occur, numerous plant species are used as folk medicine to treat snake bite (Mors, 1991; Martz 1992; Houghton & Osibogun, 1993). Mors (1991) stated that 578 species of higher plants from 94 families have been cited in the literature as being active against snake bite.

Velvet beans (*Mucuna pruriens*. Also known as Cowhage seed) are found in Asia, America and Africa. The plant is widely used as traditional medicine (Sathiyanarayanan & Arulmozhi, 2007). Acute and subacute toxicity studies on a polyherbal formulation that include *M. pruriens* suggested that the plant is safe for treatment purpose (Chandra *et al*., 2007), though it has been reported that consumption of the unprocessed raw seed is often accompanied by toxic symptoms (Sathiyanarayanan & Arulmozhi, 2007). The beans have been prescribed by traditional practitioners in Nigeria as an oral prophylactic for snakebites. The protective effect of the aqueous *M. pruriens* seed extract (MPE) has been demonstrated in mice against the lethal effect of venoms from *Echis carinatus* (saw-scaled viper) (Aguiyi *et al*., 1999, Guerranti *et al*., 2002),

Naja (cobra) and, to a lesser extent, *C. rhodostoma* (Tan *et al*., 2009). In this paper, we report the protective effect of *M. pruriens* extract pretreatment against the cardiovascular and respiratory depressant effects of *C. rhodostoma* venom.

MATERIALS AND METHODS

Materials

Male *Sprague Dawley* rats and rabbits used were supplied by the Central Animal House, Faculty of Medicine, University of Malaya. All animals were handled according to guiding principles given by the Council for International Organization of Medical Sciences (CIOMS) on animal experimentation (Howard-Jones, 1985). Lyophilized *C. rhodostoma* crude venom was obtained from Latoxan (Rosans, France). *Mucuna pruriens* (family: Fabaceae, subfamily: Papilionoideae, genus: *Mucuna*, species: *pruriens*) seeds were collected from Rukuba area in Jos, Nigeria, with the aid of a traditional healer. They were authenticated by Prof. S.W.H. Hussini of the Department of Botany, University of Jos. Voucher specimen Number A102 is deposited in the Pharmacy Herbarium of the University of Jos. Molecular weight markers were obtained from GE Healthcare (Singapore). All chemicals and reagents used in this study were of ACS grade and obtained from Sigma (USA).

Preparation of *Mucuna pruriens* seed extract (MPE)

Sun-dried seeds of *M. pruriens* were ground to a paste of uniform consistency. 50 g of which were soaked in 100 ml of deionized distilled water for 24 hrs at 4ºC, and centrifuged at 10,000 x g for 20 min. The supernatant was lyophilized to powder known as *M.pruriens* extract (MPE) which was stored at -4ºC and prepared freshly for use.

Production of antibodies against *Mucuna pruriens* seed extract

Rabbit was first bled from the marginal ear vein for pre-immune sera. One rabbit (approximately 2 kg) was used for immunization. Rabbit was injected intramuscularly with MPE at a dosage of 18 mg/kg (Aguiyi *et al*., 1999). The powdered extract was dissolved in phosphate buffered saline, pH 7.2. The homogeneous aqueous was injected intramuscularly into the left and right thighs of the rabbit alternatively at 0, 3rd, 6th and 9th week. A booster injection was given on week 12. A week from the booster injection, the rabbit was anesthetized and bled by cardiac puncture. The pooled blood from the immunized rabbit was allowed to clot for 1 hr at 37ºC, after which it was transferred to 4ºC and kept overnight, in order to contract the blood clot. The serum was then removed from the clot by centrifugation at 15,000 rpm for 20 min. IgG antibodies were purified using protein A affinity chromatography as described by Hudson & Hay (1980).

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis was conducted according to Studier (1973). iBlot™ Gel Transfer Device (Invitrogen) was used. Polyacrylamide mini gel was removed from the gel cassette and assembled on the iBlot™ Gel Transfer Device with the iBlot™ Gel Transfer Stacks and transferred according to manufacturer's instructions. The membrane was handled according to immuno-detection protocol of the Western Breeze (Invitrogen®).

Determination of the lethality of *Calloselasma rhodostoma* venom

Lethality was determined by intravenous injection of various amount of the venom sample into the caudal veins of *Sprague Dawley* rats (220 ± 10g). Five rats were used at each dose level and the effect of the

venom was observed for 48 hours. Lethality in rats was expressed in terms of LD_{50} (*i.v.*) and was calculated according to the Spearman-Karber method (WHO, 1981). LD_{50} of *C. rhodostoma* was determined to be 6.0 ± 0.1 mg/g body weight.

Pretreatment of rats with *Mucuna pruriens* seed extract

The pretreatment of rats with *M.pruriens* seed extract was carried out according to Guerranti *et al.* (2002). Rats were injected with the extract at a dose of 21 mg/kg (*i.p.*), once a week for three weeks (pretreatment scheme) (Day 0, 7, 14). The untreated group was injected with saline of a similar volume.

Pharmacological studies (effects on blood pressure, heart rate, respiratory rate and muscle twitch tension) using anesthetized rats

Pretreatment group consists of 9 rats injected with whole seed extract at a dose of 21 mg/kg (*i.p.*), once a week for three weeks. The control (or untreated group) was injected with saline of a similar volume. After 21 days, the animals were anesthetized and prepared for the pharmacological experiments.

The rat was anesthetized with urethane (1.4 g/kg) injected intraperitoneally. Its trachea was cannulated to facilitate spontaneous respiration. A lamp was used to keep the animal warm. The common carotid artery was cannulated (PE 25 tubing) for the monitoring of the systemic blood pressure via a Statham pressure transducer connected to a MacLab Data-Acquisition system. The heart rate (HR) was estimated from the blood pressure (BP) recording. A syringe filled with heparin (50 units/ml) was connected to one outlet of the pressure transducer so as to prevent clotting in the polyethylene cannula and in the barrel of the pressure transducer. The external jugular vein was cannulated (PE 25 tubing) for the administration of normal saline and venom. The maximum volume used for intravenous injection was 0.1 ml/100 g body weight of rats.

A thread was tied to the skin just below the diaphragm to monitor the respiratory rate (RR) via a Grass-compatible isometric force transducer connected to a MacLab Data-Acquisition system. The gastrocnemius was separated from other adjacent muscles. A thread was tied onto its Achilles tendon which was then cut to free the isolated gastrocnemius. The thread tying the tendon was connected to another channel of the physiograph recorder via another isometric force transducer for the recording of skeletal muscle contractions. The sciatic nerve innovating the gastrocnemius was detected and carefully hooked to an electrode. The contractions of the gastrocnemius were elicited through the stimulation of the sciatic nerve at a frequency of 0.1 Hz, a pulse width of 0.5 milliseconds and at supramaximal voltage of 3-8 V. A resting tension of 3-6 g was applied to the gastrocnemius before stimulation commenced.

The systemic blood pressure, respiratory movement and gastrocnemius muscle contractions were monitored simultaneously for at least 30 minutes to ensure stabilization of these parameters. After stabilization, *C. rhodostoma* venom (3.0 mg/g) was administered intravenously into the rats through the external jugular vein. The blood pressure, heart rate and the skeletal muscle contractions were measured at stipulated time. The normal arterial blood pressure of the anesthetized rat was calculated as the mean blood pressure (mBP) using the formula:

$$\text{Mean BP} = \text{diastolic BP} + \frac{1}{3}\ (\text{systolic BP} - \text{diastolic BP})$$

Anesthetized rat experiments were usually carried out on a pair of animals (a treated and an untreated rat) simultaneously each time.

After venom injection, the blood pressure, heart rate, respiratory rate and muscle twitch tension were measured at an interval of 10 seconds for the first 5 mins and finally every min thereafter up to 30 mins after venom injection or until the rat died.

Statistical analysis

All biological data are presented as mean S.E.M. of (n) experiments. Differences in the means between groups were analyzed using unpaired Student's t test with $P < 0.05$ considered as significant. The analysis were carried out using a Biostat Analysis package.

RESULTS

Western blotting studies of the immunological cross reactions of *Calloselasma rhodostoma* venom with anti-*Mucuna pruriens* IgG

Anti- *M. pruriens* IgG cross reacted with various protein components of the *C. rhodostoma* venom. The immunoreactive protein components range from high molecular weight proteins (175,000 Da) to low molecular weight proteins (7,000 Da) (Fig. 1). The identity of cross-reacting proteins was not identified.

Effects of MPE pretreatment on the cardiorespiratory depressant effects and neuromuscular paralysis induced by *Calloselasma rhodostoma* venom

The effects of *C. rhodostoma* venom on blood pressure, heart rate, respiratory rate and muscle twitch tension of the MPE-treated and untreated rats are shown in Figure 2A-2D.

a. Protection against venom-induced cardiovascular depression

Calloselasma rhodostoma venom at half-LD50 caused a significant cardiovascular depression in the control (untreated) group of anesthetised rats (Fig. 2A and 2B). There was a marked reduction in the heart rate (from 390.0 ± 10.0 to 183.3 ± 38.9 beats/min) and blood pressure (from 102.1 ± 5.2 mmHg to 47.8 ± 9.1 mmHg) within the first half to one minute. After this transient marked fall in the cardiovascular activities, the animals recovered partially for a brief moment (about 1-2 min) before further progressive decrease of both heart rate and blood pressure from then on until the animals died or the end of the 30-min monitoring period (2 animals survived). Marked fluctuations of heart rate were also observed during this monitoring period. Pretreatment with MPE conferred a significant, protective effect against the cardiodepressant effects of the venom; the protection being more significant on the heart rate than on the blood pressure. At 30 min, the blood pressure and heart rate of the treated rats remained at 54.3 ± 11.2 mmHg and 156.3 ± 50.3 bpm (beats per min), respectively. In comparison, the average BP and HR of the untreated animals

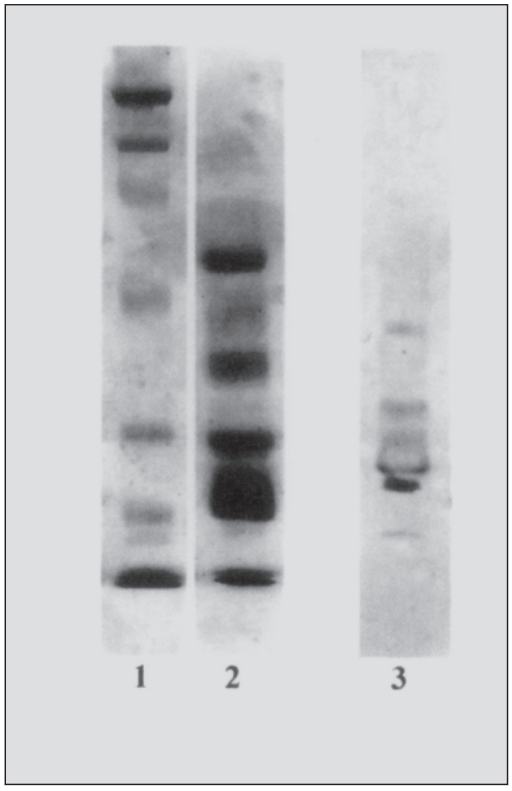

Figure 1. Cross reactions of proteins from *Calloselasma rhosdostoma* venom with anti-MPE IgG by Western blotting analysis

Lane 1: SDS-PAGE of broad range protein standards (from top): myosin (200, 768 Da); β-galactosidase (115, 281 Da); bovine serum albumin (96, 190 Da); ovalbumin (51, 783 Da); carbonic anhydrase (37, 659 Da); soybean trypsin inhibitor (29, 054 Da); lysozyme (20, 461 Da) and aprotinin (7,100 Da). Lane 2 : SDS-PAGE of *Calloselasma rhodostoma* venom. Lane 3: Western blotting analysis. The immunoreactive bands have molecular weights ranging from 7 kDa to 175 kDa

dropped to 21.6 ± 9.6 mmHg and 12.5 ± 3.2 bpm, respectively.

b. Protection against venom-induced decline in respiratory rate

The crude *C. rhodostoma* venom caused a significant decline in the respiratory rate of the rats, even though at the beginning there was a transient, slight increase in the respiratory rate (Fig. 2C). At 30 min, the average respiratory rate dropped to 19.3 ± 2.5 per min. MPE-pretreatment conferred significant protection against the venom-induced respiratory depression: at 30 min post injection of venom, the respiratory rate remained at 73.4 ± 11.2 per min, only about 30% lower than control.

c. Protection against venom-induced neuromuscular paralysis

Upon injection of the venom, blockade of nerve-evoked muscle twitches occurred promptly and worsened progressively until the animals died or the end of the 30-min monitoring period (Fig. 2D). However, unlike the effects observed with venom-induced cardiovascular and respiratory activities, MPE-pretreatment did not protect against the venom-induced neuromuscular paralysis (16.9 ± 7.5% versus 43.5 ± 11.5%, $p>0.05$, at 30 min) (Fig. 2D).

DISCUSSION

Tan *et al.* (2009) reported that pre-treatment with *M. pruriens* seed extract (MPE) conferred effective protection against the lethality of *Naja* (cobra) venoms and moderate protection against *C. rhodostoma* venom. Indirect ELISA studies showed that there were cross-reactions between anti-MPE IgG and *C. rhodostoma*

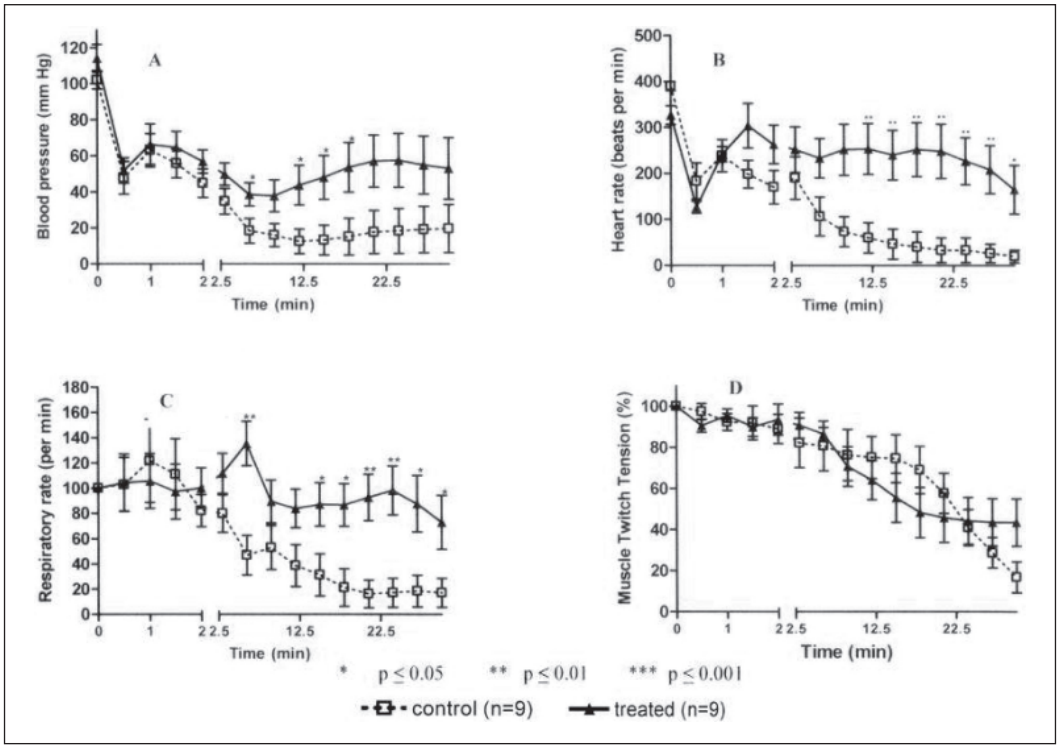

Figure 2. Effect of MPE-pretreatment on the cardio-respiratory depressant effects and neuromuscular paralysis induced by *Calloselasma rhodostoma* venom in rats

Rats (MPE-pretreated and controls) were injected with ½ LD_{50} (3.0 mg/kg, *i.v.)* of crude *Calloselasma rhodostoma* venom. The effects on blood pressure (A), heart rate (B), respiratory rate (C) and muscle twitch tension (D) (mean ± S.E. n=9) were measured

venom, and *in vitro* neutralization studies suggested that the protective action of MPE probably involves immunological mechanism. In the present studies, result of Western blotting confirmed that there were indeed cross-reactions between anti-*M. pruriens* IgG and many protein components of the *C. rhodostoma* venom. The reactive proteins include proteins with molecular weight ranging from 7 kDa to 200 kDa. The identity of the immunological reactive venom proteins, however, has not been established. These results are consistent with the studies by Guerranti *et al.* (2002) who demonstrated that the prophylactic protective action of MPE against *E. carinatus* venom is due to the production of specific antibodies in the treated animals that could neutralize certain *E. carinatus* venom components.

Injection of ½ LD_{50} dose of *C. rhodostoma* venom into the anesthetized rats induced marked depression of cardiovascular, respiratory and neuromuscular activities in the animals. Since there have been no reported presence of cardiotoxins or neurotoxins in the *C. rhodostoma* venom (Tan, 1991), these venom-induced cardiovascular, respiratory and neuromuscular changes may be secondary to the venom's multiple effects on hemostasis via its thrombin-like enzymes, hemorrhagins, platelet aggregation inducers and inhibitors (Tan 1991), which result in extensive systemic bleeding internally followed by circulatory shock. The decreased blood perfusion to the various tissues and organs concerned (e.g. heart, lungs and skeletal muscles) may indirectly reduce their activities as a result of tissue ischemia. It is of interest to note that the neuromuscular paralytic effect caused by *C. rhodostoma* venom occurred more gradually following cardiovascular depression and often worsened much more rapidly after the death of animals. This suggests that the neuromuscular paralysis observed in experimental *C. rhodostoma* envenomation is the consequence of poor muscle perfusion caused by marked circulatory failure. It is, however, also conceivable that the phospholipase A_2 enzymes present in *C. rhodostoma* venom may have contributed to some of the observed venom-induced depressant effects on the heart and skeletal muscles.

Our results showed that while MPE-pretreatment did not reverse the inhibitory effect of *C. rhodostoma* venom on the gastrocnemius muscle excitability, it significantly attenuated (albeit partially) the cardiorespiratory depressant effects of the venom. The protection against cardiorespiratory depressant effects may have an immunological mechanism, as earlier *in vitro* neutralization studies indicated that anti-MPE was able to neutralize *C. rhodostoma* venom partially (Tan *et al.*, 2009), and our Western blotting studies did demonstrate the presence of several proteins in the venom that are immune-reactive against anti-MPE. However, we cannot rule out the possibility that the pretreatment may exert a direct, non-immunological protective action against *C. rhodostoma* venom.

In conclusion, our studies suggest that injection of MPE confers protection against *C. rhodostoma* venom, as the pretreatment attenuated (albeit only partially) the cardio-respiratory depressant effects of *C. rhodostoma* venoms in anesthetized rats. Together with the results of Guerranti *et al.* (2004) and Tan *et al.*(2009), our results suggest that pretreatment with *M. pruriens* seed extract may confer protective action against the toxic effects of a wide spectrum of snake venom.

Acknowledgements. We would like to acknowledge our appreciation to Ms. Noor Shafila Shafie for her skillful technical assistance. This study was supported by research grants from the Ministry of Science, Technology and Innovation, Government of Malaysia (IRPA 02-01-03-SF 0153, Science Fund 12-02-03-2084) and Research University Grant RG022/09HTM.

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