รายงานวิจัยฉบับสมบูรณ์

โครงการ เภสัชจลนศาสตร์และเภสัชพลศาสตร์ของพิษต่อกล้ามเนื้อและพิษต่อไตจากงูแมวเซาในหนูขาวที่หมดสติ

Pharmacokinetics and pharmacodynamics of the myotoxic/nephrotoxic venom of *Daboia siamensis* (Russell's viper) in the anesthetized rat

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พฤษภาคม 2562

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และ
วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า

Abstract

Project Code: TRG6080009

Project Title: Pharmacokinetics and pharmacodynamics of the myotoxic/nephrotoxic venom of *Daboia siamensis* (Russell's viper) in the anesthetized rat

Investigator: Lt. Col. Assistant professor Janeyuth Chaisakul

Background: Snakebite is a life threatening neglected tropical disease that predominately affects impoverished individuals that inhabit rural regions of the tropics, particularly sub-Saharan Africa and South and Southeast Asia. *Daboia siamensis* (the Eastern Russell's Viper) is a medically important snake species that is widely distributed in Southeast Asia, including China and Taiwan. Envenomings by this species can result in systemic bleeding disorders, local tissue injury and/or renal failure.

Objective: To investigate the time course of myotoxicity and nephrotoxicity following envenoming of *D. siamensis* venom and to determine the effectiveness of antivenoms against nephrotoxicity.

Methods: Anesthetized rats were administered *D. siamensis* venom (Russell's viper) either through i.v., i.m. or s.d. route, including a range of dose (250-700 µg/kg). Serial blood samples were collected for measurement of creatine kinase, blood urea nitrogen and creatinine. Antivenom was administered before and 1 h after venom administration to investigate its effect on renal and muscle injuries.

Findings: There was a significant dose-dependent increase in BUN and serum creatinine, but not for creatine kinase. The intramuscular administration of *D. siamensis* venom (700 µg/kg) significantly increased plasma creatinine and BUN levels ($p < 0.05$) in anaesthetised rats, consistent with the early signs of venom-induced nephrotoxicity. The intravenous administration of *D. siamensis* monovalent antivenom at three times higher than the recommended scaled therapeutic dose, prior to and 1 h after the injection of venom, resulted in reduced levels of markers of nephrotoxicity, although lower doses had no therapeutic effect. Histological examination of kidney displayed extensive tubular injury with glomerular and interstitial congestion. However, indication of muscle injury was not observed.

Conclusions: This finding indicates that nephrotoxicity following envenoming by *D. siamensis* can be prevented by the early administration of high concentration of monospecific antivenom.

Keywords: venom; snake; antivenom; Russell's viper; nephrotoxicity

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ชื่อโครงการ: เภสัชจลนศาสตร์และเภสัชพลศาสตร์ของพิษต่อกล้ามเนื้อและพิษต่อไตจากงูแมวเซาใน หนูขาวที่หมดสติ

ชื่อนักวิจัย และสถาบัน : พ.ท. ผศ.เจนยุทธ ไชยสกุล

ภาควิชาเภสัชวิทยา วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า

บทนำ การถูกงูกัดเป็นปัญหาทางสาธารณะสุขที่ถูกละเลยอย่างหนึ่ง ซึ่งมักพบในถิ่นทุรกันดารในแถบประเทศเขตร้อน เช่น ในทวีปแอฟริกา เอเชียใต้และเอเชียตะวันออกเฉียงใต้ งูแมวเซาเป็นงูพิษชนิดหนึ่งที่เป็นสาเหตุของการบาดเจ็บและเสียชีวิตในหลายประเทศในเอเชียตะวันออกเฉียงใต้ รวมทั้งจีนและไต้หวัน การบาดเจ็บที่พบหลังถูกงูชนิดนี้กัดได้แก่ การแข็งตัวและการเกาะกลุ่มกันของเลือดผิดปกติ มีการทำลายของเนื้อเยื่อ และภาวะไตวายฉับพลัน

วัตถุประสงค์งานวิจัย ศึกษาความสัมพันธ์ของการเกิดพิษต่อกล้ามเนื้อลายและไตหลังการฉีดพิษเข้าไปในหนูขาวที่หมดสติ รวมถึงการทดสอบประสิทธิภาพของเซรุ่มต้านพิษงูแมวเซาในการป้องกันภาวะไตวายฉับพลัน

วิธีการศึกษา หลังฉีดพิษงูแมวเซาขนาด 250 และ 700 มิลลิกรัมต่อกิโลกรัมไปยังหนูขาวที่หมดสติ จะทำการเก็บตัวอย่างเลือดในช่วงเวลาที่กำหนดเพื่อทำการตรวจวัดระดับ blood urea nitrogen: creatine kinase และ creatinine หนูทดลองบางกลุ่มจะได้รับการฉีดเซรุ่มต้านพิษงูก่อนการฉีดพิษงูและหลังการฉีดพิษงูเป็นระยะเวลา 1 ชั่วโมง เมื่อครบกำหนดเวลาการศึกษาจึงทำการเก็บกล้ามเนื้อลายและเนื้อเยื่อไตสำหรับการศึกษาการเปลี่ยนแปลงทางพยาธิวิทยาต่อไป

ผลการทดลอง หนูขาวที่ได้รับการฉีดพิษงูขนาด 700 มิลลิกรัมต่อกิโลกรัมเข้าทางกล้ามเนื้อ จะมีระดับ BUN และ plasma creatinine เพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.05$) เมื่อเปรียบเทียบกับกลุ่มทดลองซึ่งเป็นข้อบ่งชี้ถึงการเกิดภาวะไตวายฉับพลัน อย่างไรก็ตามพบว่าการฉีดพิษงูแมวเซาเข้าทางกล้ามเนื้อลายของหนูขาวจะไม่ส่งผลถึงการเพิ่มขึ้นของระดับ creatinine kinase ในพาสม่าอย่างมีนัยสำคัญ นอกจากนี้การฉีดเซรุ่มต้านพิษงูแมวเซาในปริมาตรที่มากกว่าปริมาตรที่ผู้ผลิตแนะนำสามเท่าสามารถป้องกันการเพิ่มขึ้นของระดับ blood urea nitrogen และ creatinine หลังการฉีดพิษงูแมวเซาได้อย่างมีนัยสำคัญ ($p < 0.05$) การศึกษาทางพยาธิวิทยาของเนื้อเยื่อไตพบการบาดเจ็บและอุดตันของท่อไตหลังการฉีดพิษงู อย่างไรก็ตามไม่พบว่ามีการบาดเจ็บต่อกล้ามเนื้อลายจากการฉีดพิษงูแมวเซาซึ่งจากผลการศึกษาทางพยาธิวิทยานี้มีความสอดคล้องกับผลตรวจระดับเอนไซม์ที่เปลี่ยนแปลงหลังการได้รับพิษงูแมวเซา

สรุปผลการทดลอง พิษงูแมวเซามีผลต่อการเกิดภาวะไตวายฉับพลันอย่างชัดเจน แต่ทำให้เกิดพิษต่อกล้ามเนื้อลายได้น้อยกว่า การรักษาภาวะไตวายฉับพลันสามารถรักษาได้โดยการฉีดเซรุ่มต้านพิษงูแมวเซาในขนาดที่สูงกว่าขนาดที่ผู้ผลิตได้ระบุ (3 เท่า) และต้องได้รับการฉีดเซรุ่มอย่างรวดเร็วหลังถูกงูกัด จึงสามารถลดการเกิดการบาดเจ็บได้อย่างมีประสิทธิภาพ

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Introduction to research problem and its significance

Snakebite is responsible for considerable mortality and morbidity throughout the world. The highest burden exists in South Asia, Southeast Asia, South America and sub-Saharan Africa [1]. Obtaining reputable data to establish firm number of snakebite statistic is almost impossible given the fact that many victims reside in underdeveloped countries where reporting of snakebite is not mandatory and/or record keeping is extremely poor. However, recent WHO funded modeling estimates that between 1,200,000-5,500,000 snake bites occur worldwide annually, with 421,000-1,841,000 envenoming and 20,000-94,000 deaths [1].

In Thailand, there has been a dramatic decrease in fatal snake envenoming during the 21st century. Over the last 5 years, both incidence and case fatality have declined to 8,000-10,000 bites/year (12-18/100,000/year) with an admirably low case fatality of 0.5%. From this mortality rate, *Calloselasma rhodostoma* (Malayan pit viper) was responsible for the most attributable bites while Russell's viper caused 2% of fatality. Indeed, *Daboia siamensis* (Russell's viper) and *Bungarus candidus* (Malayan krait) are classified as the venomous snakes of category 1 which cause high levels of morbidity, disability or mortality [3].

Russell's viper envenoming generally causes renal toxicity (i.e. acute kidney injury) [4], bleeding/clotting disturbances including haemorrhagic infarction of the anterior pituitary resulting in Sheehan's-like syndrome [5]. Moreover, neurotoxic activities causing flaccid paralysis of skeletal muscle as well as myotoxicity involving myoglobinuria (dark brown urine) and myocardial infarction are also observed following Russell's viper envenomation [6].

In fact, myotoxicity can be classified as (1) local myotoxicity from muscle surrounding bite site and (2) systemic myotoxicity which often be referred as rhabdomyolysis due to systemic myotoxins that cause widespread muscle injury and lead to hyperkalaemia and acute kidney injury. Skeletal muscle breakdown will result in increased plasma level of creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase and myoglobin. However, the CK appears to the commonly indicator in evaluation of myotoxicity in envenomed victims.

In clinical approach, the knowledge of snake venom pharmacokinetics and pharmacodynamics including the relationship between the two is very important for the prediction of toxicity following envenomation. For myotoxicity, the systemic myotoxins will reach the circulation then distribute and target muscle entire the body. Severe systemic myotoxicity is commonly found in Australasian elapid envenomation and was demonstrated in several animal studies [7, 8]. However, the onsets of myotoxicity and nephrotoxicity as well as time course of CK changes are less well investigated.

Antivenoms are mainstay therapeutics for the treatment of systemically envenomed patients although previous studies have questioned *in vivo* effectiveness and *in vitro* efficacy of snake antivenom [9, 10]. Large quantity and multiple administrations of antivenom have been recommended to neutralize the symptoms of snake envenoming. In fact, the common treatment for snake venom-induced myotoxicity is to wait until the CK becomes abnormal levels before administration of antivenom [11]. However, recent study suggests that early administration of antivenom prior myotoxicity manifestation is more useful for prevention of myotoxicity [12]. Therefore, it is necessary to understand the time course of CK in relation to venom concentrations and the time window in which antivenom may be effective.

The objective of this study is to investigate the pharmacokinetic of venom by measuring plasma venom concentrations and the pharmacodynamics of venom by evaluation of plasma CK level in rat models. We also aim to study the relationship between antivenom administration timing and the progression of myotoxicity as well as the relationship between myotoxicity and nephrotoxicity following envenoming by Russell's viper.

Literature review

Global Epidemiology of Snakebite

Snake envenoming is an occupational hazard in many countries throughout the world. The highest burden of snakebite is in tropical regions of Asia (i.e. South Asia and Southeast Asia), Papua New Guinea, almost all African countries, and Latin America [13]. A study funded by the World Health Organization estimated that over 440,000 snake envenoming cases and 20,000 deaths occur globally each year [1]. Interestingly, Thailand is home to many venomous snakes whose venom is 'ranked' in the top 25 for lethality based on LD_{50} values (i.e. 'dose' of venom required to kill 50% of a population of mice) [14]. Fetal envenomation in Thailand is relatively low compared to other developing countries. This is most likely due to developed first aid procedures, an extensive collection of high quality antivenoms and relatively easy access to clinical assistance from most parts of the country. However, morbidity and mortality caused by snakebite are still the significant problem for healthcare system in many tropical countries.

In certain remote areas of Laos, most snakebite victims received treatment by traditional healer or self-treatment at home and nobody went to hospital due to adequate therapies are not available in hospitals [15]. In Papua New Guinea, fetal envenomation was reported in Port Moresby General Hospital where 12% of patients admitted following snake envenoming died from severe respiratory complications and/or intracerebral hemorrhage [16].

In Myanmar, Russell's viper (*Daboia siamensis*) bite was once the fifth and is now the twelfth leading cause of death in this country. In 1991, there were 14000 bites with 1000 deaths and 1997, 8000 bites with 500 deaths. From 2005 until 2008, 8994-111172 bites were reported annually with 748-794 death. The average case fatality is 7.9%. 90% of bites are caused by Russell's vipers (*Daboia siamensis*) [17]. Other important species are cobras (*Naja kaouthia*), kraits (*Bungarus* spp.) and green pit viper. Annual antivenom production by Myanmar Pharmaceutical Factory is 46000 vials of Russell's viper and 6000 vials antivenom. This is inadequate for national needs and so currently 3869 vials of Thai Red Cross Russell's viper antivenom are imported each year [3].

In Sri Lanka, reported snakebite number increased from 12175 per year in 1991 to peak at 37244 in 2002 and 36861 in 2005. In hospital, case fatality decreased from 3.5% in 1985 to 0.2 % in 2006. Most fetal cases were caused by *Daboia russelii* (30%). In Thailand, Mortality has declined from an average of 178/year in the 1950s to fewer than 10/year recently. *Calloselasma rhodostoma* causes 40% of attributable bites, *Cryptelytrops* (*Trimeresurus*) *albolabris* causes 37%, *Naja* spp. 16% and *Daboia siamensis* induces 2% of envenoming [3].

Few attempts have been made to clarify the factors responsible for death in cases of bites. Factor indentified as contributing to a fatal outcome included problems with antivenom use (e.g. inadequate dose or use of a monospecific antivenom or inappropriate specificity), delayed hospital treatment resulting from prolonged visit to traditional medicine and problems with transportation, death on the way to hospital, inadequate artificial ventilation or failure to attempt e.g. treatment, failure to treat hypovolaemia in shocked patients, airway obstruction, complicating infections, and failure to observe patients closely after they were admitted to hospital [3].

Venomous Snakes of Southeast Asia

There are three families of venomous snakes in South-East Asia i.e. **Elapidae**, **Viperidae** and **Colubridae**. Snake of family Elapidae have relatively short fixed front fangs with long, thin, uniformly-coloured snakes with large smooth symmetrical scales (plates) on the top of their heads. Those elapids of South-East include cobras, king cobras, kraits, coral snakes and sea snakes. Russell's viper and saw-scaled viper are classified as snakes of family Viperidae. These snakes have long fangs which are normally folded flat against the upper jaw but, when the snake strikes, they are erected (Figure 1). Viperidae are relatively short, thick-bodies snakes with many small rough scales on the top of the head and characteristic pattern of coloured marking on dorsal surface of the body. Other medically important venomous snakes include snakes of family Colubridae and those in sub-family Crotalinae.

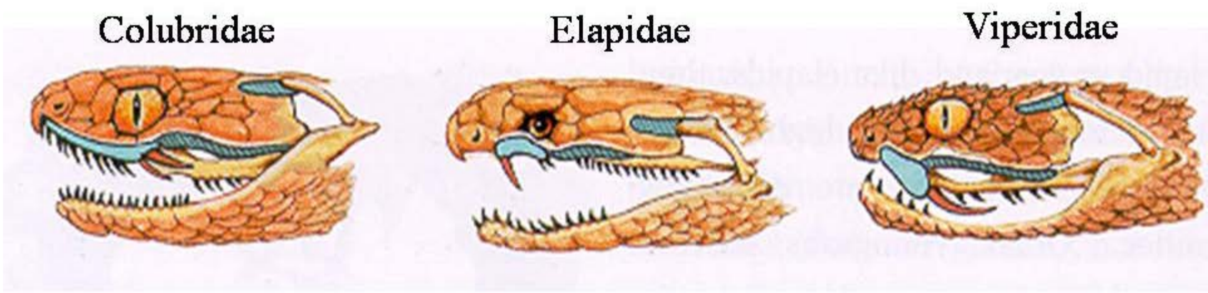


Figure 1: Differentiation of Snake

In 2010, the most important snake species of Asia were classified based on medical point of view as

Category 1; Highest medical importance: highly venomous snakes which are common or widespread and cause numerous snakebites, resulting in high levels of morbidity, disability or mortality. Venomous snakes of Thailand in this category include *Bungarus candidas, Naja kaouthia, Naja siamensis, Calloselasma rhodostoma* and *Daboia siamensis*.

Category 2; Secondary medical importance: Highly venomous snakes capable of causing morbidity, disability or death, but (1) for which exact epidemiological or clinical data are lacking or (2) are less frequently implicated because of their behavior, habitat preferences or occurrence in areas remote to large human population. Thai venomous snakes of category 2 such as *Bungarus fasciatus, Ophiophagus hannah* and *Cryptelytrops macrops* [3].

Snake venom components and symptoms following envenoming

Venom has evolved for the immobilization and digestion of prey, as well as a defensive weapon. Variation in the composition of venom or the toxicity of snake venoms is dependent on the species, geographical location, habitat, season, sex and diet of the snake [18-22]. Snake venom components which induce toxic effects on important physiological systems are described below.

Phospholipase A_2 (PLA_2)

Phospholipases are classified into 4 main groups (phospholipase A_1, A_2, C or D), based on their sites of hydrolysis at ester bonds of 3-*sn*-phosphoglycerides (Figure 2) [23]. PLA_2 (EC

3.1.1.4) enzymes are the most common phospholipase found in animal venoms. They have been identified in mammalian tissues such as the pancreas, synovial fluid and platelets (as inflammatory-type $sPLA_2$) as well as in venoms [24]. Secreted PLA_2 are categorized into three classes. Elapid or hydrophid (sea snakes) venoms contain class I $sPLA_2$, while class II$sPLA_2$s are found in crotalid and viper venoms [23]. Class III $sPLA_2$s are abundant in the venoms of beaded lizards [23] and bee venom [25].

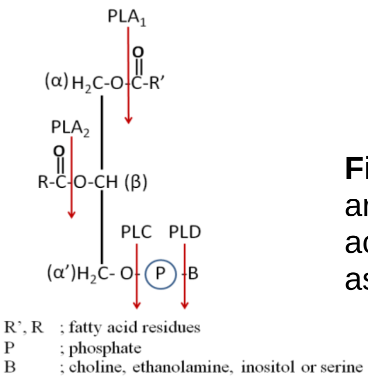


Figure 2: The site of hydrolysis (red arrow) of 3-sn-phosphoglycerides according to different phospholipase as type A_1, A_2, C or D [2].

The pharmacological effects of PLA_2 may be produced either as a consequence of enzymatic activity of PLA_2 or as a consequence of binding activity of PLA_2 enzyme without catalytic activation [26]. Thus, interaction between 'specific target binding sites' (membrane lipids or glycoproteins) on the surface of cells and 'pharmacological sites' on the PLA_2 molecule (independent of the catalytic site) can result in activation of the target site with pharmacological outcomes [27].

For the catalytic-dependent pharmacological effects, hydrolytic activity of the PLA_2 enzyme at the phospholipid membrane induces the release of lysophospholipids (LysoPL) and fatty acids (FAs). These products generate pharmacological effects within cells, including membrane damage, resulting in disruption of membrane-bound protein and functional disturbances [26].

Neurotoxicity

Neurotoxicity is the most common symptom following envenoming by elapids. This is due to inhibitory effects at the skeletal neuromuscular junction and is an important mechanism of immobilizing the prey.

Snake neurotoxins interrupt signal transduction at the neuromuscular junction either presynaptically, at the motor nerve terminal or postsynaptically, at nAChRs on the plasma membrane of skeletal muscle cells. This results in a disturbance of transmitter release or disruption of binding activity between ACh and nAChRs, respectively. Venoms from Thai venomous snakes (elapids and vipers) contain potent neurotoxins that cause extraocular muscle weakness (ptosis), flaccid paralysis and respiratory failure due to respiratory muscle weakness.

Snake pre-synaptic neurotoxins (β-neurotoxins)

Most snake pre-synaptic neurotoxins display PLA_2 activity which interrupts neurotransmitter release, synthesis, storage or turn over on the synaptic nerve terminal [28]. Nevertheless, a direct relationship between PLA_2 activity and β-neurotoxin-induced neurotoxicity is not proven [29]. The effects of pre-synaptic neurotoxins is difficult to neutralize by administration of antivenom or washing out with fresh medium unless performed within a short time period after envenoming [30].

Snake post-synaptic neurotoxins (α-neurotoxins)

Snake post-synaptic neurotoxins or α-neurotoxins display a similarity of action to d-tubocurarine, a competitive nicotinic receptor antagonist [31]. Thus, they are also known as "curaremimetic toxins". Post-synaptic neurotoxins bind to nAChR at the NMJ blocking the binding of ACh, and preventing muscle contraction. The pharmacological activity of some post-synaptic neurotoxins occurs quite rapidly in comparison to the effects of pre-synaptic neurotoxins.

Myotoxic activity

Myotoxicity is generated by small molecules which can induce direct cytotoxicity in skeletal muscle causing the release of myoglobin and creatine kinase (CK). Snake myotoxins have been classified into three different types [32]. They are (1) myotoxin 'a' and crotamine or 'small myotoxins' from rattlesnake venoms [33], (2) "cytolysins" or "cardiotoxins" , polypeptides found in cobra venom belonging to three finger toxin (3FTx) family [34] and (3) PLA_2s which appear to be the most abundant myotoxic compounds in snake venom.

Snake myotoxins can either induce local and systemic myotoxicities. Local myotoxicity causes necrosis and inflammation around the bite site and can be observed in viper envenomation, such as *Bothrops* spp. and *Crotalus* spp. [35, 36]. Systemic myotoxicity is often referred to ‘rhabdomyolysis’ which cause widespread muscle damage, hyperkalaemia and kidney injury [12, 37]. Systemic myotoxicity is observed following envenoming by sea snake, Australian elapid and Asian viper envenomations.

The characteristics of snake venom-induced skeletal muscle cell damage are hypercontraction of myofilaments, disruption of the plasma membrane, release of CK and necrosis [38]. PLA_2 myotoxins evoke their myotoxicity rapidly at the plasma membrane of the muscle cell (approximately 3-4 min) causing membrane depolarization [38]. The mechanism behind myotoxin-induced muscle degeneration can be attributed to the binding of the myotoxin to specific plasma membrane receptors. Binding causes phospholipid membrane hydrolysis resulting in the generation of ionic pores for cations such as Na^+ and Ca^{2+} [39]. Cationic influx depolarizes the cell, opening voltage operated calcium channels (VOCCs), which causes Ca^{2+} release from the sarcoplasmic reticulum (SR) and local hypercontraction of skeletal muscle cells [40].

Myotoxins applied directly to muscle rapidly cause irreversible effects and only the pre-administration of antivenom or pre-mixing the antivenom with the myotoxins appears to abolish myotoxic activity [41]. However, previous studies have shown that antivenom was not effective for local myotoxicity following snake envenoming [42]. Animal models of myotoxicity provide information of the time course of envenomation to estimate the period for which antivenom may be beneficial. Administration of antivenom to mice receiving intramuscular or intravenous injections of myotoxins displayed the protective effect on myotoxicity in vivo [43]. It has been shown that antivenom also prevents the rise in CK following snake envenomation [42]. In mulga snake (*Pseudechis australis*) envenomation, administration of antivenom prior to venom, 1h after or 6 h after venom prevented the rise in plasma CK concentration significantly [8]. This is in agreement with clinical observations from mulga snake envenoming in humans where early administration of venom protected myotoxicity while the late administration failed to abolish the increase in CK levels [12].

Renal damage

Renal damage (i.e. nephrotoxicity) can result following viper and elapid envenomings, and involves mechanisms related to myotoxic activity and elevated CK levels. Nephrotoxicity can be attributed to several mechanisms: (1) Impairment of glomerular perfusion as a result of intravascular coagulation [44]; (2) a decrease in renal vascular resistance, glomerular filtration rate and urinary flow [45] and (3) Obstruction of released myoglobin from damaged smooth muscle in renal tubules which can lead to renal ischemia and acute renal failure [43]. Kidneys of animals treated with the venoms of coral snakes displayed extensive necrosis of tubular epithelial cells, rupture of basal membrane and tubule thickening. These lesions resulted from glomerular damage by myoglobin deposits [46].

Cell death and cytotoxic activity

The terms cytotoxin, cardiotoxin, direct lytic factor or membrane-disruptive polypeptide have been used to describe a snake toxin causing cellular degeneration. Effects of snake cytotoxins include membrane depolarization and Ca^{2+} release from SR [47], muscle contraction [48], hemolysis [49] and cytolysis. Numerous venoms and toxins from elapids (e.g. cobras (*Naja* spp.), kraits (*Bungarus* spp.)) and vipers (e.g. *Bothrops asper*, *Bothropsatrox*) induce marked cytotoxicity which can be due to either apoptosis or necrosis [50].

The mechanisms mediating β-bungarotoxin-induced cytotoxicity have been elucidated in mice neurons. β-bungarotoxin induces a rise in intracellular calcium $[Ca^{2+}]_i$, leading to the activation of nitric oxide synthase (NOS) which promotes the production of nitrogenous species and nitric oxide (NO) [51]. Neuronal cell death was blocked by BAPTA-AM and EGTA (Ca^{2+} chelators), MK801 (NMDA receptor antagonist) and diltiazem (L-type Ca^{2+} channels blocker) suggesting that the elevation in $[Ca^{2+}]_i$ and neurotoxicity result from Ca^{2+} influx through NMDA receptors as well as via L-type VOCCs [52].

Hypotension and sudden cardiovascular collapse

A number of mechanisms have been postulated to explain the sudden collapse following snakebite. Many hypotensive agents from snake venoms were identified and reported to have profound effects on cardiovascular system. These include the presence of (i) bradykinin potentiating peptides which have currently only been isolated from viper and crotalid venoms, (ii) natriuretic peptides and (iii) L-type calcium channel blockers.

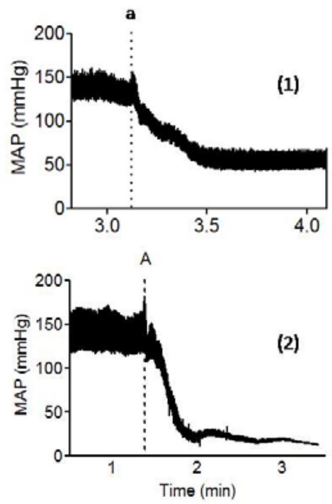


Figure3. Data from our laboratory show hypotensive effect and sudden collapse following administrations of (1) snake PLA_2 OSC3 and (2) *Pseudonaja textilis* venom in the anaesthetized rats (Chaisakul et. al., 2013)

Sudden cardiovascular collapse following envenoming by some Australasian elapids has been suggested to be due to coronary occlusion via the action of some snake prothrombin activators [53-55]. However, this is controversial since thrombus formation at multiple sites would be expected to result from prothrombin activator activity.

Another mechanism suggested to be responsible for the transient systemic hypotension producing cardiovascular collapse is venom-induced authopharmacological release of endogenous vasoactive compounds (e.g. histamine, bradykinin) which results in hypotension and may appear similar to anaphylaxis [5].

Snake Venom Antivenins

Antivenoms appear to be the only effective remedy for envenomed patients. Snake antivenoms are produced by repetitive injection of venom into animals (usually horses or sheep). Therefore, they are polyclonal antibody mixtures with an affinity for the different antigenic components in the venom [9]. Antivenoms are classified as (1) monovalent antivenoms, which are prepared by the injection of venom from only one species of snake or (2) Polyvalent antivenoms which are produced by the injection of different snake venoms.

Monovalent antivenoms have a low volume of specific antibodies for the snake species involved. Polyvalent antivenoms are more cost-effective to manufacture and are a better option for snakebite patients in order to minimize the problem of incorrect antivenom application due to diagnostic error [56]. However, polyvalent antivenom requires a larger volume and may increase the risk of adverse reactions [56]. Antivenom administration is necessary when the presence of systemic envenoming (e.g. coagulation, paralysis) is observed.

In Thailand, Queen Saovabha Memorial Institute (QSMI) of Thai Red Cross is a manufacturer of snake antivenoms for many countries in Asia. A number of monovalent and polyvalent snake antivenoms are manufactured and distributed all over Southeast Asia for snake envenoming treatment.

Snake venom pharmacokinetic studies

In clinical approach, it is very important to understand the pharmacokinetics of individual snake venom and its component, especially the venom's distribution, elimination half-lives, systemic clearance and bioavailability. Indeed, snakebite induces subcutaneous (small-fanged elapids) or intramuscular (large-fanged viperids) deposition of venom after which there is absorption of venom components from distal bite site into the central circulation, followed by distribution of venom to target organs. Nowadays snake venom pharmacokinetics have been demonstrated in many animal models [8, 57], however it is well observed that venom are highly variable from species to species and the pharmacokinetics parameters cannot be overall generalized across all snake venoms [58]. Previous studies have reported the pharmacokinetics of *Naja sumatrana* [57], *Brothrops erythromelas* [59], and *Vipera russelli* [60] venoms in conscious animals. In anesthetized rats, the pharmacokinetics of *Pseudechis australis* venom and the relationship between the timing of antivenom administration and development of myotoxicity was elucidated [8]. This study reported the delayed relationship between venom absorption and the increase in CK, consistent with the delayed onset of myotoxicity in envenomed patients [8]. The good understanding of snake venom pharmacokinetics will indicate the time course of envenomation pathophysiology which is essential for clinical monitoring and treatment strategy of snake envenoming.

Russell’s Viper (*Daboia* sp.) Envenomation

Cardiovascular disturbances including collapse, shock, hypotension and arrhythmias were reported in Russell’s viper victims in India, Sri Lanka [6] and Myanmar [5]. Neurotoxicity appears to be an important clinical manifestation following Russell’s viper envenomation which characterized by drowsiness, external opthalmoplegia and paralysis of facial muscles as well as other muscles innervated by cranial nerves. In addition, acute renal failure is a significant cause of death following Russell’s viper envenoming. Moreover, Russell’s viper venom also contains a number of potent prothrombin activators which associate to the clinical features of acute disseminated intravascular coagulation (DIC).

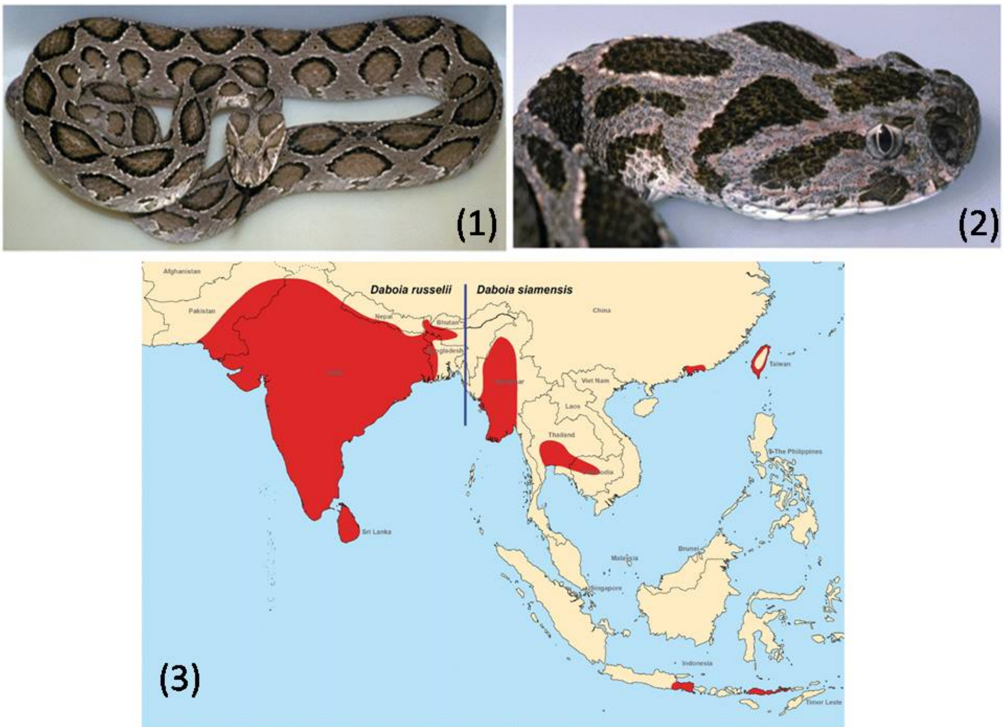


Figure 4. (1) Sri Lankan Russell’s viper (*Daboia russelii*). (2) Burmese Russell’s viper (*Daboia siamensis*). (3) Distribution of *Daboia russelii* and *Daboia siamensis* [61]

Renal vasculature of envenomed patients frequently demonstrated fibrin deposits in patho-histological studies. Thus acute renal failure induced by Russell's viper envenoming is usually involved bleeding, hypotension and haemolysis. Spontaneous systemic bleeding from gum, epitaxis bleeding into the tears and fetal cerebral haemorrhage were reported in a victim of Russell's viper envenoming. Some victims also displayed haemorrhagic infraction of the anterior pituitary resulting in 'Sheehan's-like syndrome' [3]. Hypopituitarism following *Daboia russelii* envenomation was reported in Sri Lanka with the presence of acute kidney injury, hypoglycaemia and hypotension [61].

Clinical relevance of venom research

To optimize outcomes, it is essential that research into the pharmacological effects of venoms and possible treatment strategies is influenced by clinicians. For a mechanistic understanding of venoms to be useful it must reflect the clinical envenoming syndromes. Venoms contain large numbers of toxic components, but only a few appear to be important in human envenoming. Thus, focusing on the effects of the clinically important components will allow a better understanding of human envenoming, testing currently available treatments such as antivenom, and the development of potential new therapies. The onus is partly on clinicians to properly define the clinical envenoming syndromes so that basic research can focus on the correct organisms that cause them.

Research Objectives

Russell's viper envenomation appears to be a significant health issue for many Asian countries. Although many investigations have shown the mechanisms behind significant outcomes of Russell's viper venom e.g. nephrotoxicity, neurotoxicity and haemotoxicity, the aspect of pharmacokinetic and myotoxicity of Thai Russell's viper (*Daboia siamensis*) venom have not been fully elucidated.

Therefore, the objectives of this project are to:

1. investigate pharmacokinetics of Russell's viper (*Daboia siamensis*) venom by measuring serial venom concentration in anaesthetized rats.
2. investigate pharmacodynamics of venom by measuring serial CK concentration in rats.
3. determine the relationship between timing of antivenom and development of myotoxicity following Russell's viper envenomation.
4. investigate pathology of kidney and the relationship between the timing of antivenom and development of nephrotoxicity following Russell's viper venom administration

This study will provide insight into the relationship between the venom concentrations and CK level, and whether Thai Red Cross snake antivenom will diminish the increase in CK. The outcomes of this study will contribute to a better understanding of snake venom pharmacokinetics and the development of myotoxic and nephrotoxic activities following Russell's viper envenoming.

Methodology

Venom preparation and storage

Freeze-dried Russell's viper (*Daboia siamensis*) venom (DSV) is obtained from Queen Saovabha Memorial Institute of Thai Red Cross (Bangkok). Venom is dissolved in distilled water and stored at -20°C until required. Thawed solutions were kept on ice during experiments. Venom protein content was determined via a BCA Protein Assay Kit (Pierce biotechnology; Illinois, USA) as per manufacturer's instructions.

Antivenom

Monovalent antivenoms for *D. siamensis* (DSAV; Lot NO: WR00117, expiry date 11/2022) was purchased from QSMI of Thai Red Cross Society, Bangkok, Thailand. The freeze-dried antivenom was dissolved with pharmaceutical grade water supplied by the manufacturer. The dissolved antivenom was then stored at 4 ºC prior to use. The protein concentration was measured using a Nanodrop (ThermoFisher) and BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

Animals

Male Sprague-Dawley rats were purchased from Nomura-Siam International Co. Ltd., Bangkok, Thailand. Rats were housed in stainless steel containers with access to food and drinking water *ad libitum*. Approvals for all experimental procedures were obtained from the Subcommittee for Multidisciplinary Laboratory and Animal Usage of Phramongkutklao College of Medicine and the Institutional Review Board, Royal Thai Army Department, Bangkok, Thailand (Documentary Proof of Ethical Clearance no: IRBRTA 1130/2560) in accordance with the U.K. Animal (Scientific Procedure) Act, 1986 and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Anaesthetized rat preparation

Rats will be anaesthetized with pentobarbitone sodium (40-60 mg/kg, i.p., supplemented as required). Cannulae are inserted into the trachea, jugular vein and carotid artery, for artificial respiration (if required), administration of venom (for i.v. administration) and measurement of blood pressure, respectively. Arterial blood pressure is recorded using a Gould Statham P23 pressure transducer connected to a MacLab system. Blood pressure measurement is only to monitor the well-being of the animals. The rats are kept under a heat lamp and a rectal thermometer is used to take hourly temperatures to prevent hypothermia. Venom is administered via the jugular vein for intravenous route. Intramuscular (i.m.) administration is done as an injection into the gastrocnemius muscle of the left hind-limb. Subcutaneous (s.c.) administration is performed above the gastrocnemius region of the left hind-limb, approximately 1 cm from the heel of the rat. A 50 µL syringe with a 27-gauge needle will be used for i.m. and s.c. administrations. The venom dose is between 250-700 µg/kg for concentration response examination. Venom is dissolved and injected in normal saline in a volume of 30-40 µL. At the conclusion of the experiment animals are killed by an overdose of pentobarbitone (i.v.).

As indicated, the Thai Red Cross monovalent Russell's viper antivenom will be administered intravenously via the jugular vein at an infusion rate of 0.25 mL/min over 5 min using a syringe pump. In control rats will be given the same volume of normal saline. Antivenom will be administered 10 min prior, 1 h after, or 3 h after DSV administration.

At the end of experiment, the right gastrocnemius muscle and both kidneys were removed and preserved for further histological study.

Blood collection for the measurement of serum venom concentrations

Blood samples of approximately 0.3 mL are taken via the carotid artery and collected in MiniCollect Z serum separation tubes. For i.v. venom administration, samples are collected at 5, 10, 15, 30, 60, 120, 240 and 360 min after venom injection. For s.c. and i.m. venom administration, samples were taken at 15, 30, 60, 120, 240 and 360 min after injection. Immediately after collection, the sample are spun in a centrifuge at 6500 rpm for 15 min and frozen. The supernatant was stored at - 80 ºC until required for the venom enzyme immunoassay.

Preparation of IgG

The saturated ammonium sulfate was used to separate IgG. Hyperimmune horse plasma was obtained from horse farm of QSMI (Queen Saovabha Memorial Institute), then added 40% and 30% ammonium sulfate for precipitation IgG and dissolved the precipitate with PBS buffer.

Selection of specific venom component from Russell's viper snake venom and separation of specified Horse IgG

Russell's viper snake venom loaded into 12.5% SDS-PAGE and separated the specific venom component. After that affinity chromatography was used to isolate IgG which recognized the specific venom component of Russell's viper. The precipitated horse IgG were loaded into affinity column which had the specific component of the venom. The unbound and bound fractions were eluted with PBS and Gly/HCl buffer, respectively.

Preparation of peroxidase-conjugated specific IgG of Russell's viper snake venom

Peroxidase enzyme was used to label the venom specific horse IgG. Briefly, 10 mg of peroxidase enzyme were dissolved in 0.3 M sodium bicarbonate pH 8.1, then added 0.2 ml of 1 %DNFB (2,4-Dinitrofluorobenzene) in ethanol, stirring for 1 hr. After that, 2 ml of 0.06 M sodium metaperiodate were added and stirred for 1 hr. Next, 2 ml of 0.16 M ethylene glycol were added and stirred for 1 hr. The mixture was dialyzed 3 times with 0.01 M sodium carbonate buffer pH 9.5 at 4 ºC. 10 mg of specific horse IgG were dissolved in 1 ml of 0.01 M sodium carbonate buffer, and added into the prepared mixtures. After that, 5 mg of sodium borohydride were added into the mixtures and dialyzed in PBS. The mixtures were separated by Gel Filtration Chromatography.

Detection Russell's viper snake venom by ELISA method

Plates were coated with 50 µl/well of 0.05 M carbonate buffer pH 9.6 containing horse monovalent anti-venom (1:2000) and incubated 3 hr at 37oC. After washing three times with PBS pH 7.0, plate was blocked with 1% skim milk in PBS for 1 h. and washed with PBS. A sample or serial dilution of snake venom was added into plate. After washing, peroxidase-conjugated specific horse IgG (1:20) was added for 1 h. and washed with PBS. Finally, substrate (OPD) was added into plate and incubated for 30 min, 0.5 M sulfuric acid was used as stopping solution. The absorbance was measured at 492 nm using ELISA plate reader. Concentration of samples was calculated from standard plotted using a four parameter logistic regression from TECAN Instrument

Blood collection for determination of creatinine and blood urea nitrogen (BUN)

At various time points during the animal experiments (0, 3, 6, 9, and 12 h post-injection of venom or 0.9% NaCl), approximately 0.5 mL of blood was taken via the carotid artery and collected in to 1.5 mL Eppendorf tubes. After collection, the samples were centrifuged at 5,500 rpm for 10 min. The supernatant was stored at –20 °C for no longer than 12 h, before determination of creatinine and BUN levels. Creatinine and BUN levels were measured at 37 ºC via an automated process using Flex® reagent cartridges and a Dimension® clinical chemistry system supplied by Siemens Healthineers (Germany). Plasma BUN values were measuring using 340 and 383 nm wavelengths by bichromatic rate, whereas plasma creatinine level was measured using 540 and 700 nm wavelengths using bichromatic rate.

Histopathological Studies

The right gastrocnemius muscle and both kidneys were removed and preserved in 10% formaldehyde before being embedded in paraffin. Embedded samples were cut and stained with hematoxylin-eosin (H&E) and/or periodic acid Schiff (PAS). Tissue examination was performed under a light microscope (Olympus BH-2, Olympus Optical Co., Japan). Areas in the slide with pathological changes due to typical myotoxicity and nephrotoxicity were photographed using an Olympus C-35AD camera (Olympus Optical Co., Japan).

Data Analysis and Statistics

Increases in plasma BUN and creatinine were calculated by subtracting the values of the control group from the treatment group, and then presented as mean ± standard error of the mean (SEM). The 95% confidence interval (95% CI) was also calculated. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., USA). Multiple comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Statistical significance was indicated where $P < 0.05$.

Scope of research

Materials

- Freeze-dried venom of *Daboia siamensis* will be used entire the study and obtained from snake farm of Queen Saovabha Memorial Institute of Thai Red Cross, Bangkok, Thailand

Examination of *Daboia siamensis* venom Pharmacokinetic using the anaesthetized rats

- To determination of dose-dependent myotoxic effects of *Daboia siamensis* venom on Sprague-dawley rats by measuring serum CK level
- Blood samples will be collected before and after venom administration at the maximum time point of 9 h for the determination of CK and LDH level.
- Plasma venom concentration will be monitored at the maximum of 6 h
- The relationship between time and severity of nephrotoxicity following Russell's viper envenomation will be investigated under the examination of serum CK level and pathological study.
- Different routes of venom administration (i.v., i.m. and s.c.) will be performed for the examination of *Daboia siamensis* venom pharmacokinetic variations.

Pathological study

- To determine morphological changes of rat's kidney following snake venom administration under light-microscope. All tissues will be stained using H & E.

Results

Serum venom concentrations

The time course of serum concentrations after i.v. administration of *D. siamensis* venom (100 µg/kg, *n* = 4) is shown in Figure 5. The highest concentration of approximately 290 ng/mL was seen with the first sample at 5 min, after which there was an exponential decline over the next 1 h.

Venom injection via the s.c. route caused a slight increase in venom concentrations (absorption) to a peak value of approximately 30 ng/mL at 6 h. Venom injection via the i.m. route only reached a peak concentration of approximately 40 ng/mL at 15 min. For i.m. venom administration, a plateau in the venom concentration was observed at approximately 20 ng/mL from 120 min to the last sampled time point of 360 min.

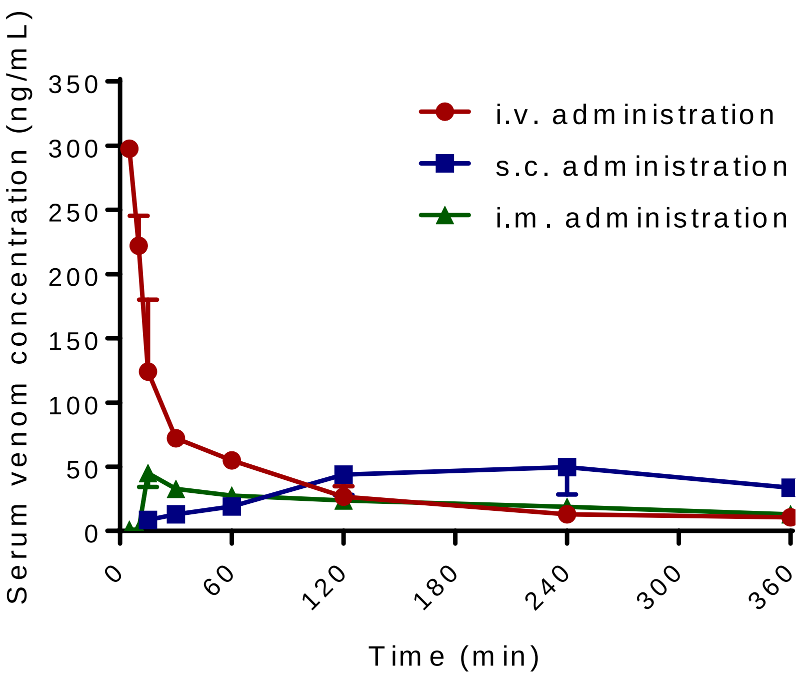


Figure 5. Plots of the serum venom concentrations versus time in rats administered D. siamensis venom (100 µg/kg) vis three different routes of administration, i.v. (red), s.c. (blue) or i.m. (green). The points are the mean ± SEM.

In vitro myotoxic effects in anaesthetized rats

The plasma CK concentrations of rats injected with *D. siamensis* venom 250 and 750 µg/kg (i.m.) were not different to control rats and were within the normal rang until 12 h of the venom injection (*n* = 3-4).

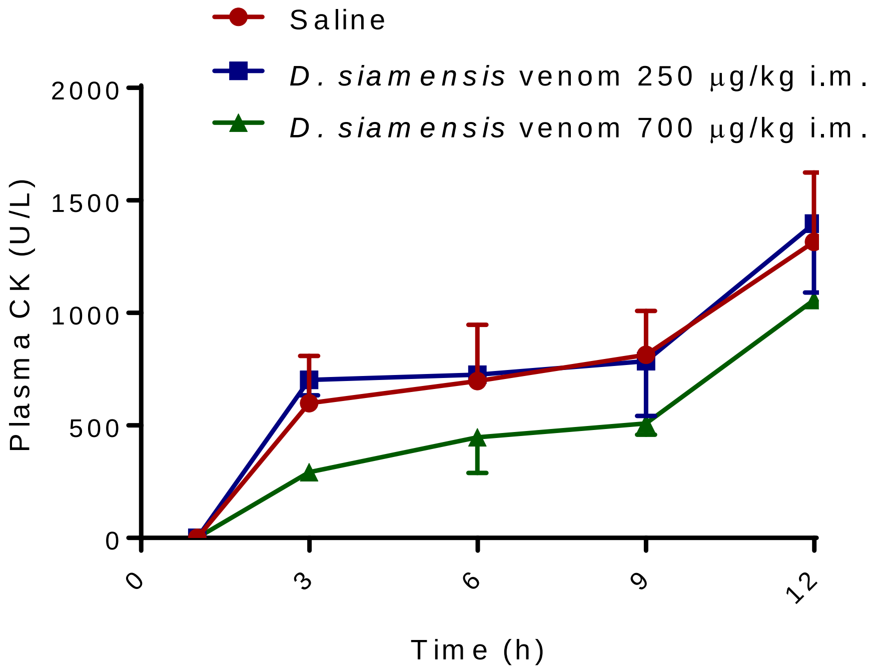


Figure 6. Plasma CK concentrations in aneasthetised rats (*n* = 4) intramuscularly injected with either 250 or 700 µg/kg D. siamensis venom compared to the saline injected rats (*n* = 3)

The effectiveness of DSAV on Russell's viper-induced nephrotoxicity

A significant increase in plasma BUN levels were observed following the administration of *D. siamensis* venom (700 µg/kg) via the intramuscular (i.m.) route into the anaesthetised rat, when compared to the control group (Supporting information, S1). Time course sampling (every three hours) revealed that BUN increased at each time point up to the end of the experiment (12 hrs, Figure 6A). The intravenous administration of DSAV (i.v.) at 3× the scaled recommended therapeutic dose (*i.e.*, 1 mL per 0.6 mg of *D. siamensis* venom) prior to the injection of venom resulted in a significant reduction in plasma BUN levels compared to the venom only controls (n=4-5, $P < 0.05$) (Figure 6A). However, no significant reduction in BUN levels was observed with a reduced therapeutic dose of 2x that recommended. The administration of antivenom 1 h after the i.m. administration of venom also did not significantly decrease plasma BUN levels compared to the administration of venom alone (n = 4-5, $P < 0.05$, one-way ANOVA, followed by Bonferroni's *t*-test, Figure 6B).

In addition to BUN, the intramuscular administration of *D. siamensis* venom (700 µg/kg) also resulted in significant increases in plasma creatinine levels compared to the control group (Figure 7A and B). Creatinine levels also increased over time and were significantly reduced when DSAV at 3× the recommended therapeutic dose (n = 4-5, $P < 0.05$) was intravenously administration prior to the injection of venom, but no significant effect was observed when 2× the recommended dose was administered (Figure 7A). However, in contrast with BUN, the administration of antivenom (i.v., infusion; 3× recommended titre) 1 h after the i.m. administration of venom caused a significant decrease in plasma creatinine compared to the administration of venom alone (n = 4-5, $P < 0.05$, one-way ANOVA, followed by Bonferroni's *t*-test, Figure 7B).

Figure 7. High doses of *D. siamensis* monovalent (DSAV) antivenom are required to abrogate increased plasma BUN levels caused by the administration of Thai *D. siamensis* venom. (A) The graphs show increases in the blood urea nitrogen (BUN) concentrations of rats administered with (i) *D. siamensis* venom (700 µg/kg, i.m.), and (ii) venom alongside the pre-administration of DSAV at two times the recommended therapeutic dose and (iii) venom alongside the pre-administration of DSAV at three times the recommended therapeutic dose. (B) Prior administration of DSAV at three times the recommended therapeutic dose significantly prevented the increase plasma BUN compared with antivenom given 1 h after venom. Data is displayed for BUN of rats administered with (i) *D. siamensis* venom (700 µg/kg, i.m.), (ii) venom alongside the pre-administration of DSAV at three times the recommended therapeutic dose, and (iii) venom and antivenom (3x recommended dose) 1 hr after venom administration. The data displayed is presented as increased levels compared to the control (normal saline, *n*=4-5) and represent mean measurements (*n*=4-5), with error bars representing SEM. * $P < 0.05$, compared to *D. siamensis* venom alone (one-way ANOVA, followed by Bonferroni *t*-test).

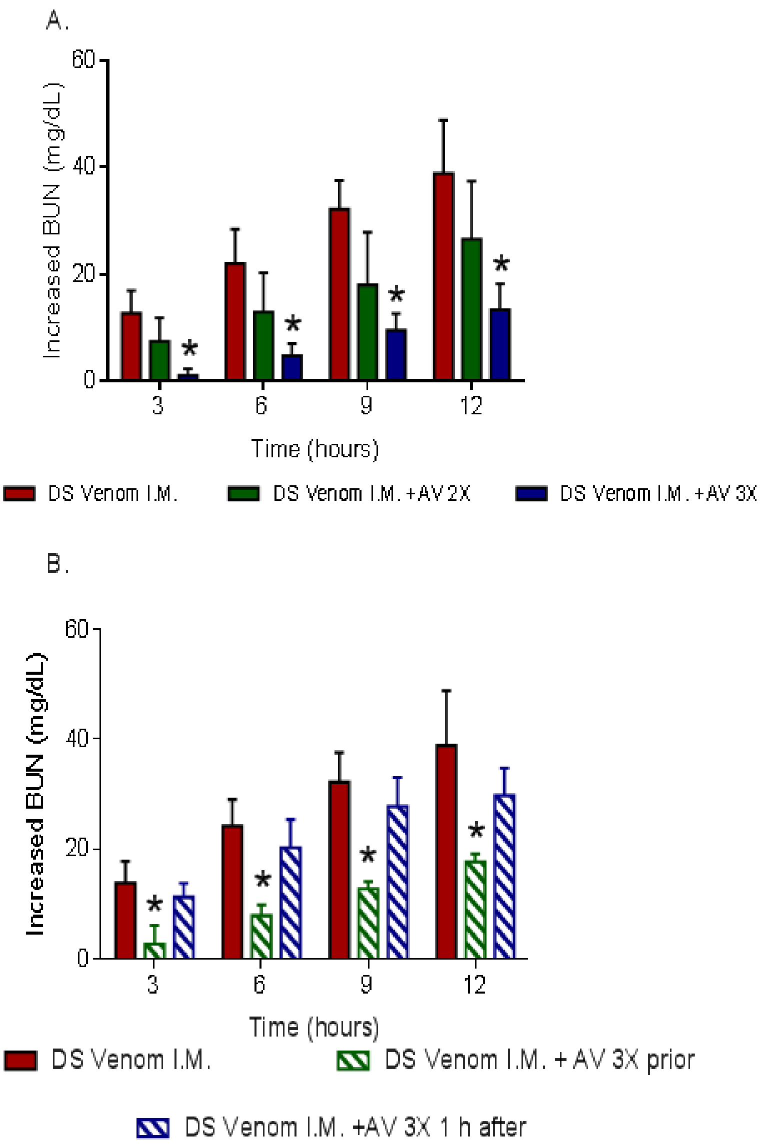
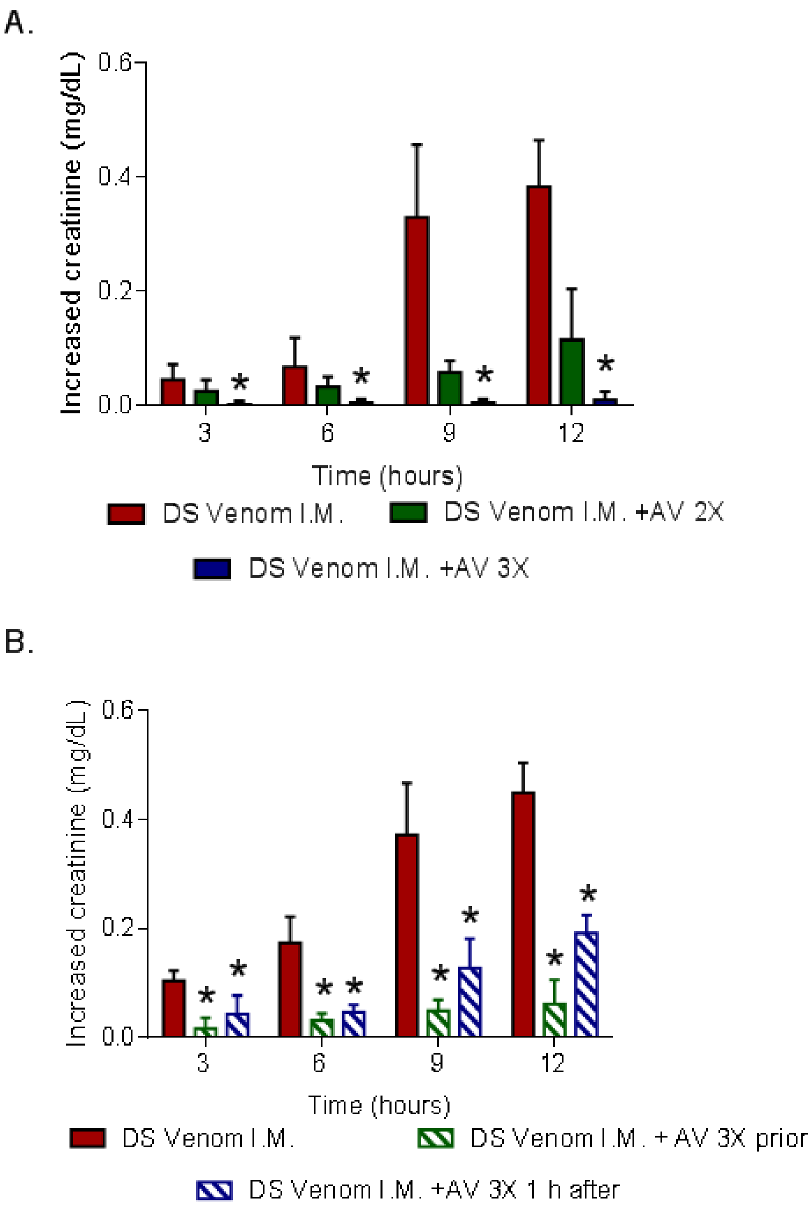


Figure 8. High doses of *D. siamensis* monovalent (DSAV) antivenom are required to abrogate increased plasma creatinine levels caused by the administration of Thai *D. siamensis* venom. (A) Plasma creatinine concentrations of rats administered with (i) *D. siamensis* venom (700 µg/kg, i.m.), and (ii) venom alongside the pre-administration of DSAV at two times the recommended therapeutic dose and (iii) venom alongside the pre-administration of DSAV at three times the recommended therapeutic dose. (B) Delayed administration of DSAV still results in significantly reduced plasma creatinine levels induced by *D. siamensis* venom *in vivo*. The graphs show plasma creatinine concentrations of rats administered with (i) *D. siamensis* venom (700 µg/kg, i.m.), (ii) venom alongside the pre-administration of DSAV at three times the recommended therapeutic dose, and (iii) venom and antivenom (3x recommended dose) 1 hr after venom administration. The data displayed is presented as increased levels compared to the control (normal saline, n=4-5) and represents mean measurements (n=4-5), with error bars representing SEM. * $P < 0.05$, compared to *D. siamensis* venom alone (one-way ANOVA, followed by Bonferroni t-test).



Histopathological Studies

Skeletal muscle

Skeletal muscle displayed a minor degree of myofiber disintegration and neutrophilic infiltration 12 h following administration of *D. siamensis* venoms.

Figure 9. Morphological changes (H&E stain, 400× magnification) of rat gastrocnemius muscle following intramuscular (i.m.) administration of (**a**) vehicle control (normal saline 50 µL), *D. siamensis* (700 µg/kg) venom for (**b**) 12 h. Diamond shapes indicate neutrophilic infiltrate, triangles indicate disintegrating myofibers. Scale = 50 µm.

Rat kidneys exhibited mild to moderate morphological changes at the 12 h time points following *D. siamensis* venom administration (700 µg/kg; i.m.). These changes were characterized by the presence of hyaline cast, dilatation of renal capillary, diffuse or focal glomeruli and/or interstitial vessels congestion (Figure.10) and tubular injury with loss of brush border. Pre-administration of *D. siamensis* antivenom (3 x recommended triter) prevented morphological changes of kidney. Administration of antivenom following *D. siamensis* venom administration following venom injection at 1 h partially prevented *D. siamensis* venom-induced renal injury (Figure 10D).

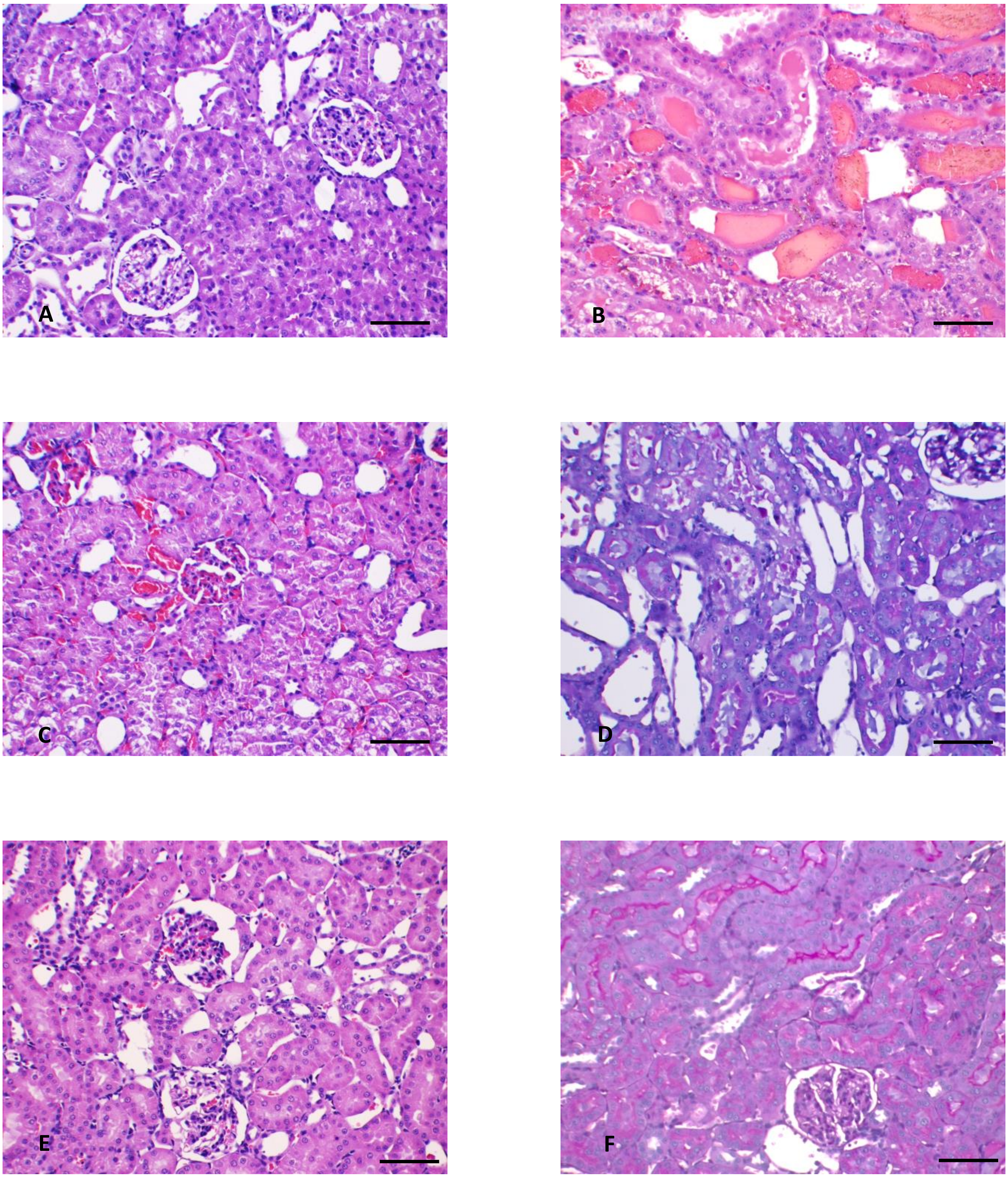


Figure 10. Morphological changes (H&E stain; 400× magnification) of rat kidneys following intramuscular administration of (**A**) vehicle control, (**B**) *D. siamensis* venom, (**C**) pre-administration of antivenom prior to venom administration and (**E**) administration of antivenom following venom injection. Morphological changes (PAS stain; 400× magnification) of rat kidneys following intramuscular (i.m.) administration of *D. siamensis* venom following Pre-administration of antivenom for (**D**) and (**F**) administration of antivenom following venom injection.

Scale = 50 µm.

Discussion

This study reports the changes in serum venom concentrations over 6 h, and the changes in CK, BUN and creatinine over 12, following the administration of *D. siamensis* venom. There were significant increases in BUN and creatinine concentrations 3 h after administration of 700 µg/kg. These data indicated the evidence of nephrotoxicity of *D. siamensis* venom.

After i.m. venom administration, we observed relative rapid absorption into the central compartment until approximately 30 min post-administration of venom. After this point, the venom concentrations slightly decrease to a plateau. Whereas, a slight increase in venom concentration following s.c. administration was observed until it reached to maximum at 12 h time point suggesting that there may be other processes in addition to absorption and distribution, such as binding to something in central compartment [8].

Systemic myotoxicity is observed following envenoming by sea snake, some viperids [62, 63] and elapids [12]. Clinical outcomes following systemic venom-induced myotoxicity include widespread muscle injury with associated myalgia, elevation of plasma CK level, myoglobinuria [42] and hyperkalaemia due to extensive muscle cell damage [64]. Previous work has shown that systemic envenoming by *D. russelii* could lead to neurotoxic envenoming [63], myoglobinuria and acute renal failure. Although severe generalized myalgia [65] and neurotoxicity following envenoming by *D. siamensis* have been anecdotally reported in Thailand, studies regarding myotoxicity have yet to be elucidated.

The determinations of CK, creatinine and BUN are important to assist in the diagnosis of muscular damage and nephrotoxicity. Our data showed that *D. siamensis* venom caused significant increases in serum creatinine and BUN levels following venom injection. This result is consistent with a case report that showed a rise in renal enzymes in victims after systemic envenoming [66]. In addition, elevation of serum BUN and creatinie levels after 3 h envenoming is indicating acute kidney injury in envenomed animals.

In the present study, significant increase in CK elevation did not observe within 12 h as seen for viperid myotoxins [42] or coral snake venoms [46]. This result is in agreement with a recent study indicating no significant elevation of CK level following Sri Lankan Russell's viper envenoming at the 6 h time point [63]. Our data indicate that envenoming by D. siamensis do not associate to myotoxicity. The variation in CK levels following venom administration could be attributed to differences in either the pharmacokinetics of venom distribution or method of administration. Indeed, administrations of venom via i.m. or s.c. (subcutaneous) may cause a slower rise in venom concentration, resulting in a delayed increase in CK levels [8]. In contrast, i.v. administration induced a rapid elevation in CK levels due to the venom having 100% bioavailability compared to i.m. or s.c. administration [8].

Snake venom-induced skeletal muscle damage is characterized by hypercontraction of myofilaments, disruption of the plasma membrane, and tissue necrosis including release of CK [38]. In fact, renal damage can be induced by the myotoxic effect of toxins that cause nephrons to be overloaded by proteins e.g. myoglobin from decayed tissue tubules and result in secondary acute kidney injury [43, 67, 68]. Moreover, direct cytotoxic/myotoxic effects of venom components are also responsible for these kidney injuries [69, 70].

In the present study, we found that *D. siamensis* venom induced time-dependent nephrotoxic activities but not for skeletal muscle damage. A lower degree of muscle necrosis was detected as early as 12 h after venom administration. In the kidneys, the presence of hyaline cast was seen in glomerular and interstitial tissues after venom administration. Twelve hours after venom injection, extensive tubular injury which was characterized by diffused tubular necrosis, completely obstructed lumen by casts and loss of brush border were detected. These renal morphologic changes have also been found in tissues exposed to the venoms of some Russell's vipers [71], *Micrurus* species [46] and other terrestrial elapids [37, 72].

In this study, we demonstrated that *D. siamensis* venom from Thailand displayed significant nephrotoxicity in an animal model and with histological evaluation of tissues. Clinicians need to be aware that systemic envenoming by *D. siamensis* may cause nephrotoxicity and other associated outcomes.

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Research outputs

Policy:

The results of this study display significant benefit for improving the treatment strategy(e.g. establishment of National guideline or encouraging an appropriate antivenom use) of severe snake envenomingin Thailand and other Southeast Asian countries. These findings will therefore have a substantial impact on the low-income, rural populations of Southeast Asia who suffer the greatest burden of snakebite.

Economy:

Cross-neutralisation occurs between Russell's viper venoms from different locations of Asia (e.g. Indonesia, Sri Lanka and Thailand) and their respective antivenoms e.g. manufactured antivenoms from Thai red cross and oversea manufacturer's.

Academic:

To study the cross-neutralisation of the Asian piver antivenom is important because (i) identifying the snake responsible for a bite, and therefore the treatment to deliver, is difficult and (ii) the availability and affordability of antivenoms to rural populations is sporadic.

To optimize outcomes, it is essential that research into the pharmacological effects of venoms and possible treatment strategies is influenced by clinicians. For a mechanistic understanding of venoms to be useful it must reflect the clinical envenoming syndromes. Venoms contain large numbers of toxic components, but only a few appear to be important in human envenoming. Thus, focusing on the effects of the clinically important components will allow a better understanding of human envenoming, testing currently available treatments such as antivenom, and the development of potential new therapies. Therefore, our stakeholders will be those who working in ministry of health (esp. People who responsible for establishing and updating national guideline for snakebite), clinicians, pharmaceutical companies including biomedical scientists. We have a strong connection with the people who are responsible for

National Health Policy and have an important role to establish the therapeutic guild line such as;

- The Deputy Director in Academic Affair of Thai Red Cross Society (Prof. Narongsak Chaiyabutr).
- Head of Division of Toxicology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok (Assist. Prof. Suchai Suteparuk).

<u>Public:</u>

To maximize potential outcomes following the visit, we need to make effective communication between our research group and people who have the major role in making the health policy. We may organize the conferences, meeting and training with the officers who working in the ministry of health or clinicians. Face-to-face meetings will be held with the individuals identified in the stakeholders section, for example. We will also spread our knowledge to local communities in order to facilitate understanding of venomous snakes and provide an overview of the basic treatment of snakebite victims. In addition, the outomces of this study will be published in open access scientific journals to facilitate the communication of our results to other researchers in diverse scientific fields

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

- ข้อมูลส่วนหนึ่งของงานวิจัยนี้ได้ส่งตีพิมพ์ในวารสาร Plos Neglected Tropical Diseases ซึ่งอยู่ระหว่างการตีพิมพ์ (รายละเอียดตามภาคผนวก)

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)

- ส่งเสริมและเผยแพร่ประสิทธิภาพของเซรุ่มต้านพิษงูจากสถานเสาวภา สภากาชาดไทย ในการรักษาผู้ป่วยที่ผู้งูกัดในประเทศต่าง ๆ ในภูมิภาคเอเชีย

2.2 เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

- นำองค์ความรู้ที่ได้ไปสร้างนโยบายสาธารณะสุขในการรักษาผู้ป่วยที่ถูกงูกัด

2.3 เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

- เกิดความร่วมมือจากหน่วยงานการศึกษาและหน่วยงานทางสาธารณะสุขในการความร่วมมือเพื่อพัฒนาการดูแลและรักษาผู้ป่วยที่ถูกงูกัด

2.4 เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

-นำองค์ความรู้ที่ได้ไปประยุกต์ใช้ในการเรียนการสอนให้กับผู้เรียนตั้งแต่ระดับประถมศึกษาถึงระดับบัณฑิตศึกษา