รายงานวิจัยฉบับสมบูรณ์

โครงการ

การออกฤทธิ์ทางพยาธิสรีรวิทยาของพิษงูแมวเซา: บทบาทและกลไกขององค์ประกอบในพิษงู ที่เหนี่ยวนำให้เกิดภาวะไตวายเฉียบพลัน

Pathophysiological actions of Russell's viper venom: The role and mechanism of its fractional components induced acute renal failure.

โดย

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Pathophysiological actions of Russell's viper venom: The role and mechanism of its fractional components induced acute renal failure.

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Executive Summary

Project Title: Pathophysiological actions of Russell's viper venom: The role and mechanism of its fractional components induced acute renal failure.

Project Code: DBG5980012

Project Objective: Snakebite is an important public health problem in several countries in the tropics and Russell's viper (*Daboia siamensis*) is responsible of the events with a high morbidity and mortality. The most serious complication in the cases of Russell's viper bite is acute kidney injury (AKI), but its pathogenesis is uncertain. Russell's viper venom (RVV) consists of a mixture of several toxic enzymes, the effect of the whole RVV causing nephrotoxicity cannot formally exclude a potential interference of AKI with the function of other venom components. The present study, additional comparative studies of renal alterations caused by the whole RVV and its fractions were performed to confirm the precise role of each venom fractions in either intact rabbit kidney or isolated rabbit kidney. The overall objectives of the present study in terms of reference were to investigate effects of Russell's viper venom and its fractions on alterations of renal functions. The studies were carried out to clarify various pathophysiological changes on the mechanism responsible for induced AKI during envenomation whether involved renal function alterations of either intrarenal mechanisms or extrarenal mechanisms in experimental rabbits.

The experimental studies were carried out as follows;

1. The renal effects of Russell's viper venom and its fractions (phospholipase A_2, metalloprotease, L-amino acid oxidase, and phosphodiesterase) in isolated perfused rabbit kidney: A potential role for platelet activating factors.)
2. Proteomic comparison of venom proteins from juvenile, subadult and adult Russell's viper snakes
3. Comparative studies between the effects of juvenile, subadult and adult Russell's viper venoms on renal functions in *in vivo* studies in rabbits.

4. Pro- and anti-inflammatory cytokines release in urine and plasma from rabbits injected with Russell's viper venom and its fractions (phospholipase A_2, metalloproteinase, L-amino acid oxidase and phosphodiesterase) - *in vivo* and *in vitro* studies

5. The renal effects relating to oxidative status in plasma, and kidney tissue during envenomation with Russell's viper venom and its fraction (phospholipase A_2, metalloprotease, L-amino acid oxidase, and phosphodiesterase) - *in vivo* and *in vitro* studies.

6. Apoptosis relating to Bcl-2 family and transforming growth factor b1 signalling proteins in rabbit kidney injected with Russell's viper venom and its fractions

7. The renal effects of phospholipase A_2 purified from Russell's viper venom: the role of Ca^{++}, Na^+ and K^+ channel blockers

Materials and methods:

Adult male white New Zealand rabbits, weighing 2 to 3 kg, were used for experimental animals either *in vivo* or *ex vivo* studies in isolated perfused rabbit kidney (IPK). Russell's viper from easthern regions of Thailand were kept in captivity at the Snake Farm of the Queen Saovabha Memorial Institute (QSMI). The entire venom extracted by each snake were pooled into the glass vial specific for venom pool. The crude venom was lyophilized and stored at -20 °C until use. Effects of Russell's viper venom and its fraction including phospholipase A_2 (PLA_2), metalloprotease (MP), L-amino acid oxidase (LAAO) and phosphodiesterase (PDE) were prepared for studies on renal functions both *in vivo* and in isolated perfused rabbit kidney as *in vitro*, where appropriate experiments were divided into different Chapters and series of studies to cover both intrarenal mechanisms and extrarenal mechanisms.

Overview of results and conclusion:

The initial experiment was designed to study the effects of RVV and its fractions, including PLA_2, MP, LAAO and PDE on renal functions in IPK. Their effects on renal alterations whether being promoted by a platelet activating factor (PAF) was tested with a platelet activating factor receptor antagonist, WEB 2086, for a possible blockade of the renal effects in the presence of whole RVV and its fractions. The study can conclude that the renal effects induced by RVV and its fractions may be due to the direct effect on the kidney cells or indirectly causing the release of PAF as mediator that involve the renal alterations including the renal hemodynamics, glomerular and tubular functions. These renal effects would be due to the synergistic action of the different

components of the snake venom, instead of the action of a single component. The experiment in rabbit IPK is the first demonstration of the mechanism of the physio-pathological renal effects induced by RVV and its fractions (Chapter I).

Further studies for comparative purposes among venoms compositions from adults, subadults and juveniles of Russell's viper which were analyzed in two ways: first, by isolation and initial characterization of venom compositions and its venom enzymatic activities were performed for PLA_2 and MP as of dominant protein families and LAAO and PDE as minor protein families from adult, subadult and juvenile Russell's viper. Second, by characterization and quantification of the venom proteomes, the differential protein and its composition in the venoms by two dimensional electrophoresis (2DE) and venom protein profiles of individual adults, subadults and juveniles were compared using the mass spectrometric analysis (MS). Only proteins at 95% significance threshold were reported by the exponentially modified protein abundance index (emPAI) for label free quantification showed that emPAI for PLA_2 juvenile (816) nearly four time higher than adult Russell's viper. Other venom protein profiles for example, basic phospholipase A_2, vipoxin B chain (1.04), Kunitz-type serine protease inhibitor 3 (0.37), snake venom serine protease gussurobin (0.12) and group III snake venom metalloprotease (0.10) were apparent in the venom from juvenile snake only (Chapter II).

The effects of the mechanisms of venom action within the body to induce especially AKI during envenomation of varying size and ages of Russell's viper have been determined. This study indicate that the action of venom from juvenile Russell's viper has more effects on reduction of renal hemodynamics functions alterations including tubulo-interstitial nephritis and marked decrease in platelets count after envenomation as compared with those of subadult and adult Russell's viper venom (Chapter III).

Cytokines studies indicate that exposure to PLA2 and MP cause increase IFN-γ in both plasma and urine in in vivo but not for RVV, LAAO and PDE. In in vitro studied, IFN-γ increase in urine except LAAO and PDE. RVV may enhance venom fractions induced signaling cascades that elicit many pro- and anti-inflammatory pathways in a dose-dependent manner and may contribute to the mortality to secondary inflamation rather than the individual venom fraction (Chapter IV).

Studies on the renal effects relating to oxidative status in plasma and kidney tissue during envenomation with Russell's viper venom and its fraction. It can conclude the present study

reported that venom and its fraction is capable to cause marked alterations in some behavior and biochemical parameters by inducing an oxidative damage and inhibiting the antioxidant enzymes activities. The study recommended that attention should be paid to reduce the sources of envenomation to snake venom and using additional to conventional medicine for treatment of oxidative stress of kidney disorders (Chapter V).

The present study reported that RVV, PLA_2 and MP caused mild degree of proximal and distal tubulonephrosis in *in* vivo model while RVV and all of its fractions caused more damage in *in vitro* model by caused moderate to severe tubulonephrosis at proximal, distal tubule and collecting duct. The result of intrinsic pathway (BCL2) show lower level of BCL2 at all renal tubular cells in RVV and PLA_2 in both *in vivo* and *in vitro* model. While BAX value in *in vitro* model, MP and PLA_2 is lowere than control but LAAO and PDE has opposite result. All of these results indicate that, RVV and its fraction has cause tubular cells damage via apoptotic pathways in different pattern. In *in vitro* model has more damage than *in vivo* model which may be the result of extrarenal factors that reduce severity by compensatory mechanism.
(Chapter VI).

The further study was carried out to determine the renal effects of PLA_2 purified from RVV: the role of Ca++, Na+ and K+ channel blockers which the present results suggest that an administration of PLA_2 in the isolated rabbit kidney causes direct acute alterations of renal hemodynamics. Acute alterations of renal functions are probably affected by either the direct action of PLA_2 or indirect effect from ionic exchanges occurring in the renal vascular membrane of the treated kidney. The role of PLA_2 in causing renal vasoconstriction is depended on the presence of extracellular Ca^{2+}. PLA_2 activated to open L-type Ca^{2+} channel and Na^{+} channel in the renal vascular membrane with influx of Ca^{2+} and Na^{+} leading to vasoconstriction causing increases in RVR and PP levels.The role of PLA_2 in causing renal vasoconstriction is significantly attenuated by the effects of either Verapamil in inhibiting calcium influx or Amiloride in inhibiting sodium influx or Minoxidil in inhibiting potassium channel. (Chapter VII)

This report is based on the following publications, which will be referred to in the text by their roman numerals.

CHAPTER I

The Pathophysiological effects of Russell's viper (*Daboia siamensis*) venom and its fractions in isolated perfused rabbit kidney: A potential role for platelet activating factor

Publication in Toxicon-X, 7: 100046 (2020)

CHAPTER I

The Pathophysiological effects of Russell's viper (*Daboia siamensis*) venom and its fractions in isolated perfused rabbit kidney: A potential role for platelet activating factor

Abstract: The most serious complication in the cases of Russell's viper (*Daboia siamensis*) bite is acute kidney injury (AKI), but its pathogenesis is uncertain. The effects of Russell's viper venom (RVV) and its fractions, including phospholipase A_2 ($RvPLA_2$), metalloprotease (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) on renal functions were investigated in the isolated perfused rabbit kidney (IPK) from adult male white New Zealand rabbits. Moreover, their effects on renal alterations whether being promoted by a platelet activating factor (PAF) was tested with a platelet activating factor receptor antagonist, WEB 2086, for a possible blockade of the renal effects in the presence of whole RVV and its fractions. Whole RVV alone (1.0 mg/ml) caused significant initial decreases in perfusion pressure (PP) and renal vascular resistance (RVR) ($P < 0.05$) of the first 10 min., thereafter a gradual rise occurred throughout the perfusion time. These effects were abolished by WEB 2086 (200 μg/100 μl) prior to RVV administration showing a marked fall in the PP and RVR ($P < 0.05$) toward the basal level throughout the perfusion time. Administration alone either $RvPLA_2$ (280 μg/ml) or RvMP (280 μg/ml) caused significant increases in PP and RVR ($P < 0.05$) at 60 and 90 min. of the perfusion time. WEB 2086 completely abolished the effects induced by RvMP on PP and RVR ($P < 0.05$) but not for the $RvPLA_2$ effect throughout the perfusion time. Administration of 2 ml of RvLAAO (135 μg / ml) alone also caused increases in PP and RVR while administration of 2 ml of RvPDE (100 μg / ml) caused a reduction of both PP and RVR throughout the perfusion time and these effects were not prevented by pretreatment with WEB 2086. Whole RVV alone caused significant decreases in glomerular filtration rate (GFR), urinary flow (UF) including osmolar clearance (Cosm) ($P < 0.05$) with maximal effect at 90 min. These effects were not prevented by pretreatment with WEB 2086. Administration of each $RvPLA_2$, RvMP, RvLAAO and RvPDE alone caused increases in GFR, UF and Cosm throughout the perfusion time. WEB 2086 completely blocked the effects induced by either $RvPLA_2$ or RvMP on GFR, UF and Cosm ($P < 0.05$). These results were in contrast with those elicited by RvLAAO and RvPDE which WEB 2086 was unable to abolish their action on

GFR, UF and Cosm. The percent of fractional sodium excretion (%$FENa^+$) significantly increased at 90 min. after administration of whole RVV alone while the percent of fractional potassium excretion (%FEK^+) slightly increased. These effects were abolished by pretreatment with WEB 2086. Effects of $RvPLA_2$ and RvLAAO alone caused slight increases in %$FENa^+$ while %FEK^+ showed tendency to decline throughout the perfusion time and these effects were in similar manner alterations after pretreatment with WEB 2086. The effect of RvMP alone caused marked decrease in % FEK^+ ($P < 0.05$), while % $FENa^+$ slightly decreased throughout periods of study and these effects were prevented by pretreatment with WEB 2086. During RvPDE administration alone, both % $FENa^+$ and % FEK^+ were significantly increased ($P < 0.05$) with maximal effect at 30 min., this effect for % FEK^+ was prevented by WEB 2086 when it was injected previously to RvPDE. No alterations for the concentration of nitric oxide (NO) in urine were apparent during administrations of either whole RVV or its fractions. The histological evaluation profiled by the treated groups, in which kidneys were perfused with either whole RVV or its fractions, showed no remarkable lesions of glomerular part but mainly tubulonephrosis of the treated kidney. In conclusion, the renal effects induced by RVV and its fractions may be due to the direct effect on the kidney cells or indirectly causing the release of PAF as mediator that involve the renal alterations including the renal hemodynamics, glomerular and tubular functions. These renal effects would be due to the synergistic action of the different components of the snake venom, instead of the action of a single component. Our current experiment in rabbit IPK is the first demonstration of the mechanism of the physio-pathological renal effects induced by *Daboia siamensis* venom and its fractions.

Keywords: Russell's viper, *Daboia siamensis*, phospholipase A_2, metalloprotease, L-amino acid oxidase, phosphodiesterase, WEB 2086, isolated perfused kidney

1. Introduction

Snakebite is an important public health problem in several countries in the tropics and Russell's viper (*Daboia siamensis*) is responsible of the events with a high morbidity and mortality. The main and most serious complication during Russell's viper bite is acute kidney injury (AKI) (Kanjanabuch and Sitprija, 2008). The mechanisms acting within the body to induce AKI during envenomation are uncertain. The pathogenetic mechanisms underlying acute renal failure (ARF) may include renal vascular obstruction by fibrin micro-thrombi (DIC), ischemia or hypoperfusion due to the fall in blood pressure and hemolysis-induced pigment nephropathy due to some extent hemolytic (Sitprija et al., 1974). This manifestation can not exclude the presence of proteolytic enzymes and vasoactive substances which could promote, or even potentiate, the coagulant process in renal sites. *In vivo* studies in experimental animals have been shown that decreases in renal blood flow and glomerular filtration rate with an injection of the crude venom of Russell's viper (RVV) might be depended on changes of extrarenal factors via reductions of both systemic and renal circulations (Tungthanathanich et al., 1986; Thamaree et al., 1994). The etiology of RVV induced acute renal failure in humans and experimental animals is still not completely understood but probably involves a direct action of venom components on renal tubules and renal epithelial cells (Sitprija and Chaiyabutr, 1999). The possible existence of a direct nephrotoxic component was reported, which was not well described up to now. RVV is a complex mixture that contains both non-protein and protein components with different structures and specific biochemical activities. The major protein components of the RVV consist of a mixture of potentially toxic proteins, peptides and several enzymes (Sitprija and Sitprija, 2012). A possible effect of each venom component may dominate the clinical presentation causing local and systemic injury as well as haematological, cardiovascular and renal functions. The isolation and functional characterization of venom components will provide a basis for understanding the mechanisms and/or molecular models of venom action (Harvey et al, 1998). However, the potential role of each venom component of RVV has direct and/or indirect cytotoxic relating to AKI has not been studied in as much detail. Previously, studies done in experimental animals by Suwansrinon et al. (2007) demonstrated that administration of two different venom fractions of RVV either PLA_2 or MP showed alterations of the renal functions which changes in renal hemodynamics appeared to correlate better with MP than PLA_2. In addition, Mitrmoonpitak et al. (2013) have reported that injection of venom fraction either PLA_2 or MP would promote intense inflammatory mediators

with an elevation of plasma concentrations of pro-inflammatory cytokines for interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and PGE2. The plasma level of nitric oxide, a vasoactive mediator, was increased after PLA_2 fraction injection but not with MP fraction injection. It is not known what toxic fractions of RVV are the cause and what fractions play a key role in its effects for such responses. These *in vivo* models do not exclude the influence of higher order of extrarenal factors such as the nervous system, blood pressure, coagulation and other blood borne factors including corpuscles and hormones. However, few data are available to study the specific mechanisms in responsible for induction of AKI by RVV and its fractions, although a number of studies have been reported the direct effects of whole RVV on renal tubular and glomerular injury in the isolated perfused kidney (Ratcliffe et al., 1989; Willinger et al., 1995; Chaiyabutr et al., 2014).

Nevertheless, ARF can result from a variety of renal injuries and several processes that make a major contribution to the reduction in glomolular filtration rate (GFR) and renal blood flow (RBF) for characteristic of ARF. It has been reported that the kidney is capable of released inflammatory mediators in response to several stimuli (Pirotzky et al., 1984b). One of the research studies is the search for a factor(s) locally release into the kidney during envenomation that might be responsible for changes in renal vascular and glomerular functions associated with renal failure. The release of intrarenal factors in envenoming may be superimposed on renal effects of the venom action investigated. It has been demonstrated that platelet activating factor (PAF), acetylated alkyl phosphoglycerides constitute, a lipid mediator, can be released by several other cell types and organs, including the kidney. (Caramelo et al., 1984; Benveniste et al.,1972). PAF is a membrane-derived phospholipid with widely recognized pro-inflammatory activities. PAF may be one of the entities responsible for causing the hemodynamic changes in the ARF, because it can act as a vasodilator or vasoconstrictor, depending upon its concentration (Lo´pez-Novoa, 1999). The implication of PAF as an important pro-inflammatory mediator in the pathogenesis of ARF has been suggested in a variety of experimental and human studies (Caramelo et al., 1984; Ferreira and Fonteles, 1996). It has been reported that PAF also plays an essential role in signal transduction pathways for induction of nitric oxide (NO) production as a mediator exerting a vasodilator activity on afferent arterioles (Juncos et al., 1993). However, there are no studies evaluating the role of PAF during envenomation with Russell's viper venom, although PAF is an important mediator in the reductions of GFR and UF induced by *Bothrops* snakes venom which these effects being

blocked by WEB 2086 have been described in isolated perfused rat kidney (Monteiro and Fonteles, 1999; Havt et al., 2001). Considering those data, whether the pathogenesis of ARF induced by RVV is involved by a local generation of PAF in the kidney, which is not fully understood. We thus hypothesized that the action of venom either whole RVV or its venom fractions causes renal functional alterations involved the PAF pathway in the kidney. We decided to study whether the nephrotoxic effect of RVV and its fractions is somehow related to a concomitant generation of PAF and the specific PAF receptor antagonists (WEB 2086) is essential to evaluate for such studies.

Because RVV consists of a mixture of several toxic enzymes, the effect of whole RVV causing nephrotoxicity cannot formally exclude a potential interference of AKI with the function of other venom components. Thus, in the present study, additional comparative studies of renal alterations caused by whole RVV and its fractions, need to be performed in order to confirm the precise role of each venom fractions in the isolated kidney. Generation of PAF in kidney might involve the action of RVV and its fractions and since the study mechanisms of action of venom fractions for pathogenesis of ARF make the elucidation of their role's difficult *in vivo* studies; the studies in the isolated kidney model without the influence of extrarenal factors would help in this regard.

Therefore, two experimental protocols were performed. The study in protocol 1 was carried out to characterize the functional properties of PAF in the isolated rabbit kidney by using two different concentrations of exogenous PAF administrations and confirmation its function with pre-exposure to a specific platelet activating factor receptor antagonist, WEB 2086, as a blocker. Experiments in protocol 2 were designed to comparative studies the direct renal effects promoted by the whole RVV and isolated venom fractions from RVV ($RvPLA_2$, RvMP, RvLAAO and RvPDE). The role of local PAF generation during envenoming was tested by WEB 2086, as a blocker in the isolated perfused rabbit kidney. After treatments with either whole RVV or its venom fractions and WEB 2086, the renal histological examinations were verified. Defining the role of PAF in modulating the action of whole RVV and its venom fractions in the isolated perfused rabbit kidney without the influence of extrarenal factors will lead to a better understanding of these pathophysiological mechanisms involved in the features of AKI.

2. Materials and methods

2.1 Experimental animals

Adult male white New Zealand rabbits, weighing 2 to 3 kg, were obtained from the Animal house, Queen Saovabha Memorial Institute. The animals settled in stainless steel cages, fed a standard diet and water. Exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 26 ± 1^{o}C. The animals quarantined for 14 days before the experiments. Animals involved in this study were conducted with permission of the Ethic Committee of the Queen Saovabha Memorial Institute Animal Care and Use (approval number QSMI-ACUC-03-2016) in accordance with the guideline of the National Research Council of Thailand.

2.2. Isolated perfused kidney preparation

The preparation for isolated perfused rabbit kidney (rabbit IPK) was based on methods described previously (Chaiyabutr et al. 2014). Briefly, adult male white New Zealand rabbits were fasted for 24 h before the experiment with access to water ad libitum. The rabbit was anaesthetized with pentobarbital sodium (30 mg/kg, i.v). The animal was given 1,000 units heparin intravenously. After opening the abdomen, the left ureter was cannulated with polyvinyl catheter. The left renal artery was prepared for perfusion after careful exposure from its surrounding tissue, and was ligated above the renal artery of the left kidney. The 19-gauge stainless steel needle, approximately 1.0 inch in length with a smooth tip, served as the arterial cannula. The needle was inserted into the renal artery and flushed with heparinized saline (100 units/ml). The kidney was isolated with the renal vein and the ureter intact and immediately transferred to a thermostatically controlled tissue bath organ chamber (Radnoti, chamber for organ isolation procedures, catalog No. 166070, Grass Technologies, Monrovia, CA, USA). The fluid perfusing the kidney flowed from the cut end of the renal vein and the ureter. The isolated kidney was used to employ a recirculating perfusion design by means of a perfusion apparatus ex vivo. The working perfusate with an oxygenated modified Krebs–Henseleit solution at a temperature of 37°C and aerated with a gas mixture of O_2: CO_2 (95:5) was perfused through the renal artery by means of a recirculating a rotary pump (EYELA, Roller pump, RP-1000). The rabbit IPK was maintained at constant perfusion flow rate (40-60 ml/min) throughout the experiments.

The preparation of a modified Krebs Henseleit solution (MKHS) was described by Taft (2004). Total perfusate used per experiment was 100 ml consisting the composition (in mM) as following: 141 mM Na^{+}, 5.4 mM K^{+}, 1.9 mM Ca^{++}, 2.4 mM Mg^{++}, 126 mM Cl^{-}, 25 mM HCO_3^{-}, 2.44 mM SO_4^{2-}, 1.5 mM PO_4^{3-}, and 13 mM amino acids (consisted of alanine 1.470 mM, arginine 0.365 mM, asparagine 0.152 mM, aspartate 0.150 mM, cysteine 0.363 mM, glutamate 0.367 mM,

glutamine 1.470 mM, glycine 1.680 mM, histidine 0.177 mM, isoleucine 0.221 mM, leucine 0.298 mM, lysine 0.733 mM, methionine 0.242 mM, phenylalanine 0.236 mM, proline 0.235 mM, serine 0.733 mM, threonine 0.176 mM, tryptophane 0.054 mM, tyrosine 0.149 mM and valine 0.258 mM). Total perfusate also contained 100 mg D-glucose, 50 mg inulin including both 3 gm of bovine serum albumin (BSA fraction V, from Sigma Chemical Co. (St Louis. MO, USA) and 2 gm of dextran (Sigma Chemical Co.) as oncotic agent. The perfusion solution was adjusted at pH 7.4 and kept warm at 37°C by a pre-warming coil and oxygenation by the addition of a 1.2 μm filter in tissue bath organ chamber. Changes of perfusion pressure in the kidney were measured at the tip of the stainless-steel cannula with either a manometer or recorded on a Statham strain gauge pressure transducer, thereby allowing continuous record monitoring of perfusion pressure on the physiograph (Polygraph Model 79, Grass instruments Co.). The kidney was mounted in the perfusion system for 30 min. to allow the kidney approach to normal function as indicated by maintenance of urine flow and perfusion pressure, which was carefully kept at 100 mmHg. The perfusion pressure was measured at 5 min. intervals after equilibration. The first 30 min. of perfusion were considered to be internal control. The experimental period was divided into five intervals of 5, 10, 30, 60 and 90 min. of perfusion time. The experiments were conducted over 90 min. after either RVV or its fractions administration. In each interval period, samples of perfusate and urine were collected for 5 min. for determinations of sodium, potassium, inulin and osmolality.

2.3. Venom and chemical

A pool of venom obtained from 14 adult males and females Russell's viper (*Daboia siamensis*) snakes collected from the eastern region of Thailand, and maintained at Queen Saovabha Memorial Institute, The Thai Red Cross Society. Russell's viper venom (RVV) was milked, lyophilized and stored at -20º C. The crude RVV was isolated by fractionation methods for phospholipase A_2 ($RvPLA_2$), metalloprotease (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) for comparative purposes (Fig. 1). A platelet activating factor (PAF) (β-acetyl-γ-O-alkyl-L-α-phosphatidylcholine) (purchased from Sigma-Aldrich) was dissolved in solution containing 100 mM DMSO and 100 mM ethanol to give a stock solution of 200 μg/ml and stored at -20ºC. A platelet activating factor receptor antagonist, triazolobenzodiazepine substance (WEB 2086) was purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). The WEB 2086 was dissolved in solution containing 100 mM DMSO and 100 mM ethanol with gentle

warming to give a stock solution of 200 µg/100 µl and stored at -20º C. All chemicals were reagent grade.

2.3.1. The isolation of phospholipase A_2 from RVV ($RvPLA_2$)

Crude RVV was dissolved in buffer A (50 mM phosphate buffer pH 6.0). After centrifugation at 10,000 rpm for 5 min, the supernatant was applied to ion-exchange chromatography on HiTrap CMFF column (GE Healthcare, Sweden). The column was washed with 5 volumes of buffer A and elution was carried out with an increasing linear concentration gradient of NaCl from 0 – 1 M in buffer A at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and measured at absorbance 280 nm under an AKTA pure Fast Protein Liquid Chromatography system (FPLC, GE Healthcare, Sweden). Four peaks were observed and determined for PLA_2 activity. Fractions containing PLA_2 activity were pooled and further purified by size exclusion chromatography in a pre-equilibrated Superdex^{TM} 75 10/300GL column. (GE Healthcare, Sweden). Elution was carried out with 10 mM PBS pH 7.4 at room temperature. The flow rate was adjusted to 0.5 ml/min, and 1 ml fraction was collected in each tube. The proteins were determined by absorbance at 280 nm under a Unicorn 6.3 Software.

2.3.1.1. phospholipase A_2 activity

Phospholipase A_2 activity was conducted as described by Holzer and Mackessy (1996) with slight modifications. The sample (50 µl) was mixed with 3 mM 4-nitro-3-(octanoyloxy) benzoic acid 1:1 ratio (v/v) and incubated at 37 ºC for 20 min. Triton X-100 (2.5%) was added to the reaction mix and the absorbance was measured at 425 nm. A standard curve of absorbance as a function of chromophore (3-hydroxy-4-nitrobenzoic acid) concentration showed that a change in absorbance of 0.1 AU at 425 nm was equivalent to 25.8 nmoles of chromophore release.

2.3.2. The isolation of metalloproteinase from RVV (RvMP)

The supernatant of crude RVV was applied on Superdex^{TM} 75 10/300GL column equilibrated with 0.1 M sodium acetate buffer pH 6.7. The column was eluted at a flow rate of 0.4 ml/min, and fractions of 1 ml were collected under an AKTA pure FPLC system. Each fraction was assayed for enzymatic activity. The active fraction was collected, desalted, and concentrated by centrifugal ultrafiltration (Macrosep® 10K; Pall Corp, UK). The sample was then onto a Mono Q column

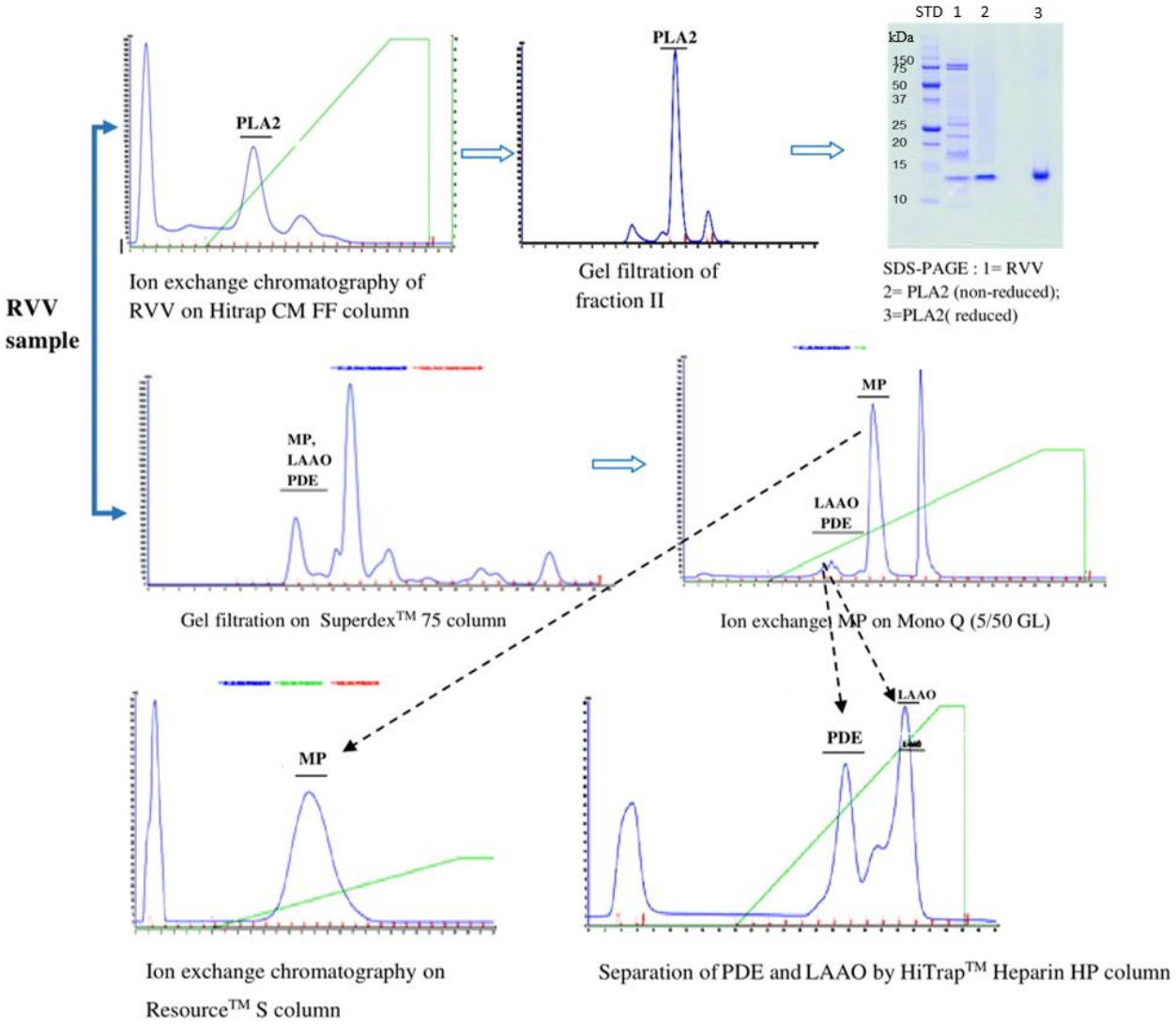
(5/15 GL; GE healthcare, Sweden) pre-equilibrated with buffer A (50 mM Tris-HCl buffer pH 8.0) and eluted with 60% linear gradient buffer B (1 M NaCl). Three peaks were separated, the active peak (2^{nd}) was further purified by Resource S column which was pre-equilibrated with 10 mM sodium phosphate buffer pH 6.7, and eluted with a linear gradient of 0 - 0.3 M NaCl.

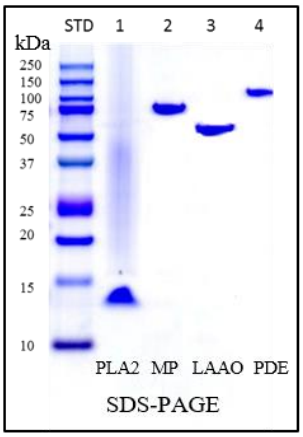
2.3.2.1. Metalloproteases activity

Metalloproteases activity was determined by estimation for the proteolytic activity by hydrolysis of heated casein as described by Anson (1938) with slight modifications. The reaction mixture consisting of 500 µl casein in 0.1 Tris-HCl pH 8.0 and 50 µl of venom was incubated for 2 hr at 37° C. The reaction was quenched by the addition of 500 µl of 5% trichloroacetic acid at room temperature. After centrifugation, the supernatant (400 µl) was mixed with 1 ml of 0.5 M Na_2CO_3 and 200 µl of diluted (1:5) Folin & Ciocalteau's phenol reagent. The mixture was then incubated at 37° C for 30 min. and absorbance was measured at 660 nm. One enzyme unit is defined as the amount of enzyme which hydrolyzes casein to produce color equivalent to 1.0 µmole of tyrosine per minute. The effect of protease inhibitor on the proteolytic activity of the sample was observed by pre-incubated the sample with EDTA (final concentration was 10 mM) at 37° C for 10 minutes. The mixture was then assayed the activities in the corresponding assay systems.

2.3.3. Isolations of phosphodiesterase from RVV(RvPDE) and L-amino acid oxidase from RVV (RvLAAO)

After the supernatant of crude RVV was applied on Superdex™ 75 10/300GL column. The active fraction was collected and the sample was then applied on a Mono Q column (5/15 GL; GE healthcare, Sweden). The first peak from Mono Q column expressed activity of phosphodiesterase and L-amino acid oxidase were purified by a HiTrap™ Heparin HP column (GE Healthcare, Sweden) pre-equilibrated with 50 mM Tris-HCl pH 8.0 and eluted with a linear gradient of 0-0.5 M NaCl (Fig. 1).





	Enzyme Specific Activity				Molecular weight (kDa)
	PLA2 (nM/mg)	Protease (uM//mg)	LAAO (U/mg)	PDE (U/mg)	
Crude venom	806	0.13	0.02	1.1	
PLA2	2099				13
Proteases		0.33			90
L-amino acid oxidases			40		60
Phosphodiesterase				14.0	110
Purification (fold)	2.6	2.5	200	12.7	

Fig.1. A schematic representation of the different work flows employed to fractionation of crude RVV

2.3.3.1. L-amino acid oxidase activity

The RvLAAO was carried out to determine activity according to Worthington Enzyme Manual (1977). A reaction mixture (510 µl) containing 0.1% L-leucine, 0.0065% o-dianisidine, and 0.007% horse radish peroxidase in 0.2 M Triethanolamine buffer pH 7.6 was incubated at 37 ºC. The reaction was started by adding fractions and monitored at 426 nm over 3 min.

2.3.3.2. phosphodiesterases activity

The RvPDE was carried out to determine activity as described by Lo *et al.,* (1966) with slight modifications using Ca-bis-p-nitrophenylphosphate as the substrate. The hydrolysis of the substrate was assessed by measuring the rate of increase in absorbance at 440 nm. One unit of enzyme activity was defined as the amount of enzyme that caused the increase of 0.001 absorbance unit per minute.

2.4. Experimental design

Two experimental protocols were carried out:

2.4.1. Experiment protocol 1

This study was assigned to demonstrate the functional properties of PAF whether the effectiveness of PAF were specific and receptor-mediated on renal functions in the isolated kidney. In vitro study in rabbit IPK was confirmed using a PAF receptor antagonist, WEB 2086 as a blocker in testing the action of PAF during exogenous PAF administration (IPK). Preparations of IPK were divided into six following groups (n = 4 each/group):

Group 1.1. The control group, following kidney excision and transfer to the recirculating perfusion system, control perfusions were conducted to establish viability of the preparation and to allow for the evaluation of any agents testing effects on kidney functions. The rabbit IPK was perfused with MKHS in the absence of any agent treatments in recirculating system for 120 min. (Control group).

Group 1.2. The triazolobenzodiazepine substance (WEB 2086), a receptor antagonist of platelet activating factor (200 µg/100 µl) was added only to the recirculating system into 100 ml of perfusate after the first 30-min equilibration period as internal control with a stabilization of basal

perfusion pressure. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 administration. (WEB 2086 group)

Group 1.3. After the 30-min equilibration of internal control period of perfusion, the rabbit IPK was treated only with 25 µl of exogenous PAF (200 µg/ml), which was added into 100 ml of perfusate of the recirculating system. This dose was arbitrarily chosen as a low dose PAF administration and a relevant total concentration of PAF in the perfusate was $9.5x10^{-8}$ M. The changes in renal functions were recorded thereafter for a 90 min. period after low dose PAF administration (PAF-L group).

Group 1.4. To test the effect of the PAF receptor antagonist (WEB 2086) whether could block the action of the low dose of exogenous PAF in IPK study. For interaction studies, 100 µl of WEB 2086 (200 µg/100 µl) was injected in the beginning of the perfusion, after the equilibration period, and 25 µl of PAF (200 µg/ml) was injected 30 min. later to the perfusate under the same protocol. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and a low dose PAF administration (WEB 2086 +PAF-L group).

Group 1.5. After the 30-min equilibration of internal control period of perfusion, the IPK was treated only with 75µl of exogenous PAF (200 µg/ml) which was added into 100 ml of perfusate of the recirculating system. This dose was arbitrarily chosen as a high dose PAF administration and a relevant total concentration of PAF in the perfusate was $27x10^{-8}$ M. The changes in renal functions were recorded thereafter for a 90 min. period after a high dose PAF administration (PAF-H group).

Group 1.6. To test the effect of the PAF receptor antagonist (WEB 2086) whether could block the action of the high dose of exogenous PAF in IPK study. For interaction studies, 100 µl of WEB 2086 (200 µg/100 µl) was injected in the beginning of the perfusion. After the equilibration period, and 75µl of PAF (200 µg/ml) was injected 30 min. later to the perfusate under the same protocol. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and a high dose PAF administration (WEB 2086 +PAF-H group).

2.4.2. Experiment protocol 2

The experiment in protocol 2 was assigned to study the effects of whole RVV and venom fractions ($RvPLA_2$, RvMP, RvLAAO and RvPDE) on renal functions in IPK study. Preparations

of rabbit IPK were divided into 10 experimental groups (n = 4 each/group). The experimental groups in protocols 2 were as follows:

Group 2.1. IPK was treated with 1 ml of the lyophilized RVV in normal saline (1 mg/ml) which was added into 100 ml of perfusate of the recirculating system after 30 min. of the equilibration period as internal control. This dose was arbitrarily chosen on the basis of an earlier investigation in experimental animals in either dogs or rabbits, where the dosage of LD_{50} of crude RVV by intravenously injection was 0.5 mg/kg body weight (Chaiyabutr et al., 2014; Tungthanathanich et al., 1986). This dosage converted to a concentration expressed in 10 μg/ml plasma by calculation with an estimation of the percentage of plasma volume in 5% of body weight. This concentration value was chosen for the dose of whole RVV used in IPK studies, which 1 mg of the RVV was added to 100 ml of perfusate to obtain the final concentration of 10 μg/ml. The changes in renal functions were recorded thereafter for a 90 min. period after whole RVV administration (RVV group).

Group 2.2. IPK was pretreated with 100 μl of WEB 2086 (200 μg/100 μl) after the 30-min. equilibration of perfusion and 1 ml of the whole RVV (1 mg/ml) was added into 100 ml of perfusate of the recirculating system later after 30 min. period of pretreatment with WEB 2086. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and whole RVV administrations (WEB 2086 +RVV group).

Group 2.3. IPK was treated only with 1 ml of the $RvPLA_2$ fraction (280 μg/ml) which was added into 100 ml of perfusate of the recirculating system after 30 min. of equilibration period as internal control. This dose was arbitrarily chosen and adjusted according to our previously described work in experimental animals (Mitrmoonpitak et al., 2013) using a dose of PLA_2 fraction at 140 μg/kg body weight by intravenously injection. This dosage converted to a concentration expressed in 140 μg/50 ml plasma by calculation with an estimation of the percentage of plasma volume in 5% of body weight. Therefore, the amount of $RvPLA_2$ using in the present study was adjusted by adding $RvPLA_2$ 280 μg into 100 ml of perfusate. The changes in renal functions were recorded thereafter for a 90 min. period after RvPLA2 administration (PLA_2 group).

Group 2.4. IPK was pretreated with 100 μl of WEB 2086 (200 μg/100 μl) after the 30 min. equilibration period of perfusion. Then 1ml of the $RvPLA_2$ (280 μg/ml) was added to the recirculating solution later after 30 min. period of pretreatment with WEB 2086. The changes in

renal functions were recorded thereafter for a 90 min. period after WEB 2086 and $RvPLA_2$ administrations (WEB 2086 +PLA_2 group).

Group 2.5. IPK was treated only with 1 ml of the RvMP fraction (280 μg/ ml) which was added into the perfusate of the recirculating system after 30 min. of equilibration period as internal control. This dose was arbitrarily chosen and adjusted according to our previously described work in experimental animals (Mitrmoonpitak et al., 2013) using a dose of MP fraction at 140 μg/kg body weight by intravenously injection. This dosage converted to a concentration expressed in 140 μg/50 ml plasma by calculation with an estimation of the percentage of plasma volume in 5% of body weight. Therefore, the amount of RvMP using in the present study was adjusted by adding RvMP 280 μg into 100 ml of perfusate. The changes in renal functions were recorded thereafter for a 90 min. period after RvMP administration (MP group).

Group 2.6. IPK was pretreated with 100 μl of WEB 2086 (200 μg/100 μl) after the 30 min. equilibration period of perfusion. Then 1ml of the RvMP (280 μg/ml) was added to the recirculating solution later after 30 min. period of pretreatment with WEB 2086. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and RvMP administrations (WEB 2086 + MP group).

Group 2.7. IPK was treated only with 2 ml of RvLAAO (135 μg/ml) which was added into 100 ml of perfusate of the recirculating system after 30 min. of equilibration period as internal control (LAAO group). This dose was arbitrarily chosen for comparative study with the other venom fractions. Therefore, an administration of RvLAAO was adjusted to 270 μg in 100 ml of perfusate. The changes in renal functions were recorded thereafter for a 90 min. period after RvLAAO administration (LAAO group).

Group 2.8. IPK was pretreated with 100 μl of WEB 2086 (200 μg/100 μl) after the 30 min of equilibration period of perfusion. Then 2 ml of the RvLAAO (135 μg/ml) was added to the recirculating solution later after 30 min. period of pretreatment with WEB 2086. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and RvLAAO administrations (WEB 2086 + LAAO group).

Group 2.9. IPK was treated only with 2 ml of RvPDE (100 μg/ml) which was added into the perfusate of the recirculating system after 30 min. of equilibration period as internal control of perfusion. This dose was arbitrarily chosen for comparative study with the other venom fractions. Therefore, an administration of RvPDE was adjusted to 200 μg in 100 ml of perfusate. The changes

in renal functions were recorded thereafter for a 90 min. period after RvPDE administration (PDE group).

Group 2.10. IPK was pretreated with 100 µl of WEB 2086 (200 µg/100 µl) after the 30 min equilibration of perfusion. Then 2 ml of RvPDE (100 µg/ml) was added to 100 ml of the recirculating solution later after 30 min. period of pretreatment with WEB 2086. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and RvPDE administrations (WEB 2086 + PDE group).

2.5. Samples collection and chemical analysis

Each experimental group was divided into six periods for renal function measurements after each specified treatment. Samples of urine and perfusate were collected at 5 min-intervals of each specified period (at 0, 5, 10, 30, 60 and 90 min. after treatment) for analysis of sodium, potassium, inulin, osmolality and urinary nitric oxide (NO) level. Sodium and potassium ion concentrations were determined by flame photometer (Flame Photometers, Laboratory Instrument, BWB Technologies UK Ltd) and osmolality was measured by osmometer (Fiske® Micro-osmometer Model210, Fiske® Associates, Norwood, Massachusetts, 02062, USA). The inulin concentration in both perfusate and urine was determined using the anthrone method (Young and Raisz, 1952). Urine samples stored at -40º C were determined for nitric oxide (NO) concentration. Nitrite and nitrate are the primary oxidation products of NO reacting with oxygen and, therefore, the nitrite/nitrate concentration in urine was used as an indicator of NO release. NO levels were quantified using an Arrowstraight™ Nitric Oxide measurement system (Lazar Research Laboratories, Los Angeles, CA, USA), which contained micro ion selective electrodes for independent measures of both nitrite and nitrate in 100 µl of urine sample. As a standard, the sodium nitrite and sodium nitrate were used (Sigma-Aldrich, Natick, MA, USA; for both). All procedures were conducted according to the manufacturer's protocol. Results were presented as a total NO (µM) calculated by summing the concentration values of nitrite $(NO_2)^-$ and nitrate $(NO_3)^-$

2.6. Histological studies of the isolated perfused kidney

The kidney was cut sagittally after 120 min. of perfusion period in either controlled kidney or treated kidney with whole RVV and its fractions. Kidney tissues were preserved in 10% neutral buffered formalin (v/v) for 24 hrs before tissue dehydration using graded alcohol and paraffin

embedding in paraffin blocks. The kidney tissues were then cut into 4 μm thickness serial sections and were stained with routine Hematoxylin and Eosin (H&E) and special staining with periodic acid-Schiff reaction (PAS) for morphologic study. Histological examinations were performed under a light microscope (Olympus, Tokyo, Japan) of the H&E and PAS -stained sections, which were used to assess changes in glomerulus, proximal and distal convoluted tubules, and collecting duct of the IPK. Each section was examined at least 50 glomeruli and 10 area of tubules from one section per kidney. Effects of whole RVV, $RvPLA_2$, RvMP, RvLAAO, RvPDE and WEB 2086 on changes in the glomeruli and renal tubules were assessed. Histopathological lesion was quantitated by a scoring system from 0 to 3+ (0, no lesion; 1+, mild lesion; 2+, moderate lesion; 3+, marked lesion). Glomerular congestion and crystal deposit in glomerulus, were graded from 0 to 3+ (0, no changes; 1+, mild changes; 2+, moderate changes; 3+, marked changes). Renal tubular changes (tubulonephosis and tubule-necrosis, in proximal tubule, distal tubule and collecting tubule) were graded from 0 to 3+ (0, no changes; 1+, mild changes; 2+, moderate changes; 3+, marked changes of sample).

2.7. Calculations for renal function

The perfusion pressure (PP), renal vascular resistance (RVR), urinary flow rate (UF), glomerular filtration rate (GFR), the fractional sodium (% FE_{Na^+}) and potassium (% FE_{K^+}) tubular excretions were determined as described in details by Chaiyabutr et al. (2014). The experimental results were compared with those of the internal control at 30 min. early in each group. The clearance (C) of inulin (Cin, GFR) and osmolality (Cosm) were calculated according to the standard formula (Smith, 1962). The flow rate of perfusate to the kidney and perfusion pressure of the system was used to calculate for the renal vascular resistance (RVR) using the standard formula (PP/perfusate flow rate). Fractional excretion of sodium (% $FENa^+$) or potassium (% FEK^+), was calculated by divided C_{Na^+} or C_{K^+} with GFR.

2.8. Statistical analysis

The data are presented as mean ± S.D. Significant differences between internal control and each specified time point of each experimental group were analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test, where appropriate, with a *P*-value< 0.05 being considered statistically significant

3. Results.

3.1. Effects of exogenous platelet activating factor (PAF) administration and platelet activating factor receptor antagonist, WEB 2086 on renal functions in IPK.

The study in experiment protocol 1, in cotrol conditions, perfused kidneys were preserved functionally, according to the data shown on Table 1, 2 and Fig. 2, 3, 4 and 5. The renal hemodynamics, glomerular filtration function, tubular sodium and potassium transport and osmolar clearance parameters were stable after perfusion with MKHS without any agents testing for over 120 min. (control group). In comparison with the control group, in the same method of rabbit IPK, when only WEB 2086 (200 μg/100 μL) was injected, the functional parameters remained stable throughout the perfusion time (WEB group) (Table 1, 2; Fig. 2, 3, 4 panels A, B; Fig 5 panel A).

In the rabbit IPK, increasing PAF availability by adding exogenouse PAF alone into the perfusate with either the low concentration (PAF-L = $9.5x10^{-8}$ M) or high concentration (PAF-H = $27x10^{-8}$ M) caused both increases in PP and RVR throughout the 90 min. of the perfusion time (Table 1; Fig. 2 panels C, D, E and F). However, these effects were abolished by WEB 2086 (200 μg/100 μl) when it was injected prior to administrations of PAF-L as shown in Fig. 2 panels C and D, whereas an increase in both PP and RVR was not completely prevented by WEB 2086 when it was injected prior to administrations of PAF-H. There were significant increases both PP and RVR at 60 min. ($p < 0.05$) and 90 min. ($p < 0.05$) period of the perfusion time as compared with the pretreated period (Table 1; Fig. 2 panels E, F).

Table1. Effects of platelet activating factor (PAF) administration either low dosage (PAF-L) or high dosage (PAF-H)) and pre-exposure of WEB 2086 on perfusion pressure (PP), renal vascular resistance (RVR), glomerular filtration rate (GFR) and urinary flow rate (UF) in the rabbit IPK.

Variables groups	Basal	5 min.	10 min.	30 min.	60 min.	90 min.
PP (mmHg):						
Control	103.8 ± 9.3	103.3 ± 8.8	102.3 ± 8.4	102.3 ± 7.9	102.8 ± 7.8	103.0 ± 7.8
WEB 2086	101.3 ± 2.2	100.5 ± 4.4	101.5 ± 2.8	103.3 ± 4.4	104.3 ± 5.3	105.3 ± 6.2
PAF-L	92.5 ± 7.6	99.8 ± 4.8	102.0 ± 9.7	106.8 ± 9.4	105.5 ± 8.9	106.3 ± 9.5
WEB 2086 +PAF-L	121.8 ± 12.5	98.5 ± 12.6	96.5 ± 12.2*	78.0 ± 11.7*	77.5 ± 11.1*	77.0 ± 10.1*
PAF-H	97.5 ± 8.9	111.8 ± 14.5*	111.8 ± 16.1	102.3 ± 6.7	103.0 ± 5.7	101.5 ± 10.1
WEB2086 + PAF-H	84.3 ± 4.9	87.5 ± 5.6	89.8 ± 7.6	93.0 ± 10.2	109.8 ± 11.8*	137.0 ± 22.2*
RVR (mmHg/ml/min):						
Control	1.86 ± 0.48	1.84 ± 0.47	1.83 ± 0.46	1.82 ± 0.45	1.83 ± 0.44	1.83 ± 0.44
WEB 2086	1.54 ± 0.04	1.53 ± 0.04	1.55 ± 0.06	1.57 ± 0.08	1.59 ± 0.09	1.60 ± 0.11
PAF-L	1.33 ± 0.46	1.51 ± 0.54	1.51 ± 0.56	1.52 ± 0.53	1.77 ± 0.79	1.76 ± 0.78
WEB2086 + PAF-L	2.3 ± 0.68	2.29 ± 0.38	2.27 ± 0.37	2.29 ± 0.37	2.25 ± 0.36	2.19 ± 0.35
PAF-H	1.74 ± 0.30	2.00 ± 0.42	2.00 ± 0.45	1.81 ± 0.26	1.82 ± 0.25	1.80 ± 0.27
WEB2086 + PAF-H	1.31 ± 0.14	1.35 ± 0.09	1.38 ± 0.08	1.42 ± 0.09	1.68 ± 0.16*	2.10 ± 0.34*
GFR (ml/min):						
Control	0.20 ± 0.03	0.17 ± 0.03	0.15 ± 0.04	0.17 ± 0.05	0.17 ± 0.05	0.16 ± 0.05
WEB2086	0.30 ± 0.19	0.31 ± 0.17	0.33 ± 0.18	0.31 ± 0.16	0.34 ± 0.19	0.31 ± 0.17
PAF-L	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	0.13 ± 0.02	0.12 ± 0.02
WEB2086 + PAF-L	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
PAF-H	0.10 ± 0.05	0.20 ± 0.17	0.15 ± 0.08	0.15 ± 0.11	0.14 ± 0.09	0.21 ± 0.14
WEB2086 + PAF-H	0.11 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.02
Urine Flow (ml/min):						
Control	0.13 ± 0.02	0.13 ± 0.02	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.02
WEB2086	0.28 ± 0.17	0.30 ± 0.17	0.30 ± 0.07	0.31 ± 017	0.31 ± 0.15	0.32 ± 0.18
PAF-L	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.13 ± 0.02	0.14 ± 0.02*	0.16 ± 0.03*
WEB2086 + PAF-L	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
PAF-H	0.12 ± 0.08	0.14 ± 0.11	0.22 ± 0.12	0.22 ± 0.14	0.23 ± 0.16	0.24 ± 0.16*
WEB2086 + PAF-H	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.01	0.13 ± 0.02

Data are presented as mean ± SD of four rabbit IPK from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): *$P < 0.05$, mean values of specified time period with respect to the basal period in each group. Basal is the internal control period prior to agent treatment of each experimental group. Control is the control group, perfusion with only MKHS. WEB 2086 is a platelet activating factor receptor antagonist, which was added 30 min. before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min. after the basal measurements of each experiment.

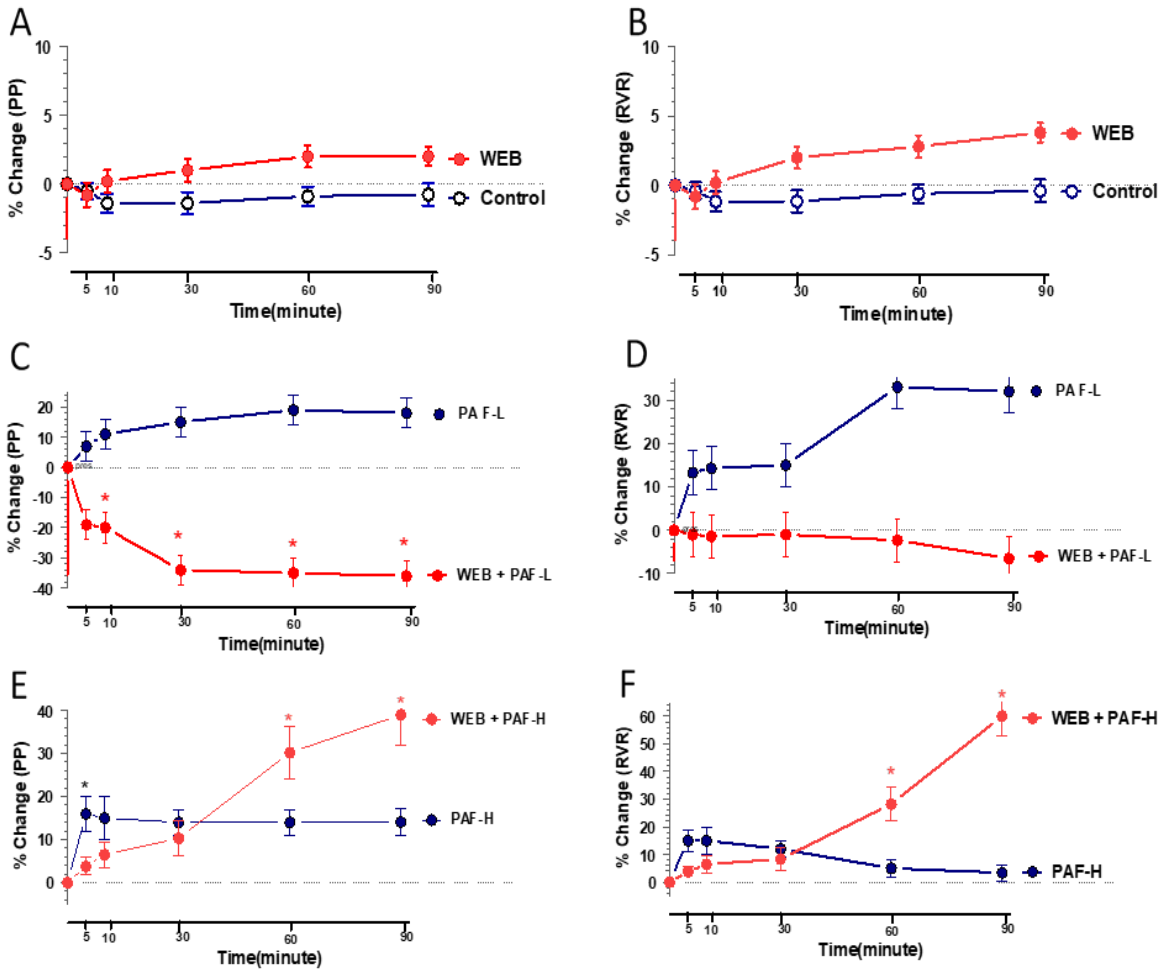


Fig. 2. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in perfusion pressure (PP) and renal vascular resistance (RVR) response to exogenous PAF administration by either low dosage (PAF-L) or high dosage (PAF-H)) which pretreated with WEB 2086 in the rabbit IPK. Panels A and B, comparative studies between the control group which were perfused by only MKHS (Control) and the group treated with only WEB 2086 (WEB). Panels C and D, comparative effects between the group treated with PAF-L alone (PAF-L) and the group treated PAF-L after pretreatment with WEB 2086 (WEB + PAF-L). Panels E and F, comparative effects between the group treated with PAF-H alone (PAF-H) and the group treated PAF-H after pretreatment with WEB 2086 (WEB + PAF-H) in 90 min of perfusion time. Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

The effect of administration of the PAF-L caused a slight reduction on GFR throughout the perfusion time while the UF was transient reduction at the first 10 min. followed by significant inclements at 60 min. ($p < 0.05$) and at 90 min. ($p < 0.05$) of the perfusion time as compared with the pretreated period. The high dose of PAF-H alone caused increases in GFR and UF throughout the perfusion time. These effects were abolished by WEB 2086 (200 μg/100 μl) when it was injected prior to administrations of either PAF-L or PAF-H (Table 1; Fig. 3 panels C, D, E, F).

The %$FENa^+$, %FEK^+ and Cosm in the administration of either PAF-L or PAF-H alone started to initially reduce at 5 min. period of the perfusion time, and thereafter there were progressively increases in the last period of the perfusion time. A significantly increase in %$FENa^+$ was apparent with maximal effect at 60 min. ($P < 0.05$) after administration of PAF-H alone. These effects were abolished by WEB 2086 (200 μg/100 μl) when it was injected prior to administrations of either PAF-L or PAF-H as shown in Table 2; Fig 4. panels C, D, E, F and Fig. 5 panels B, C.

Table 2. Effects of platelet activating factor (PAF) administration either low dosage (PAF-L) or high dosage (PAF- H) and pre-exposure of WEB 2086 on fractional excretion of sodium (%$FENa^+$), fractional excretion of potassium (%FEK^+) and osmolar clearance (Cosm) in the isolated perfused rabbit kidney.

Variables: groups	**Basal**	**5 min.**	**10 min.**	**30 min.**	**60 min.**	**90 min.**
FENa+ (%):						
Control	78.2 ± 9.1	92.3 ± 20.9	87.2 ± 12.3	90.7 ± 10.6	95.3 ± 11.1	78.8 ± 4.5
WEB 2086	93.8 ± 11.8	82.1 ± 12.2	71.95 ± 6.6	78.4 ± 5.3	92.4 ± 14.7	77.5 ± 5.8
PAF -L	84.3 ± 13.7	79.2 ± 14.8	83.0 ± 11.6	99.6 ± 25.4	90.8 ± 16.0	105.6 ± 25.8*
WEB2086 + PAF-L	98.4 ± 8.4	97.8 ± 6.7	87.9 ± 7.0	92.9 ± 7.6	102.6 ± 10.9	101.4 ± 11.0
PAF-H	100.9 ± 6.5	89.0 ± 9.5	115.3 ± 9.8*	122.4 ± 10.5*	129.8 ± 10.9*	98.4 ± 7.5
WEB2086 + PAF-H	93.8 ± 9.3	87.2 ± 4.7	93.6 ± 8.9	97.0 ± 6.1	92.6 ± 3.9	100.4 ± 4.1
FEK+ (%):						
Control	98.6 ± 5.1	98.2 ± 4.2	97.1 ± 4.5	98.8 ± 2.3	98.2 ± 9.4	81.5 ± 7.8
WEB 2086	105.5 ± 8.5	100.6 ± 10.6	92.8 ± 6.2	95.1 ± 3.1	93.8 ± 2.9	93.4 ± 2.5
PAF-L	120.7 ± 6.4	115.2 ± 8.6	119.7 ± 17.6	134.1 ± 22.3	123.3 ± 17.1	137.1 ± 19.7
WEB2086 + PAF-L	142.8 ± 31.8	161.7 ± 24.5	140.0 ± 35.1	144.8 ± 29.8	158.4 ± 26.5	165.5 ± 22.6
PAF-H	167.0 ± 31.8	142.9 ± 23.2	147.3 ± 34.8	173.6 ± 10.0	198.5 ± 37.2	166.7 ± 40.3
WEB2086 + PAF-H	185.7 ± 38.8	205.7 ± 34.9	179.9 ± 17.7	188.7 ± 31.1	175.9 ± 30.2	159.9 ± 21.9
Cosm(ml/min):						
Control	0.17 ± 0.07	0.13 ± 0.06	0.15 ± 0.07	0.11 ± 0.04	0.19 ± 0.09	0.20 ± 0.09
WEB 2086	0.24 ± 0.14	0.22 ± 0.14	0.26 ± 0.18	0.22 ± 0.13	0.17 ± 0.08	0.18 ± 0.08
PAF-L	0.12 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.13 ± 0.03	0.15 ± 0.03
WEB2086 + PAF-L	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
PAF -H	0.13 ± 0.07	0.13 ± 0.10	0.19 ± 0.12	0.17 ± 0.11	0.18 ± 0.13	0.21 ± 0.13
WEB2086 + PAF-H	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	0.10 ± 0.01

Data are presented as mean ± SD of four perfused kidneys from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period in each group. Basal is the internal control period prior to treatment of each experimental group. Control is the control group, perfusion with only MKSH. WEB 2086 is a platelet activating factor receptor antagonist, which was added 30 min. before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min. after the basal measurements of each experiment.

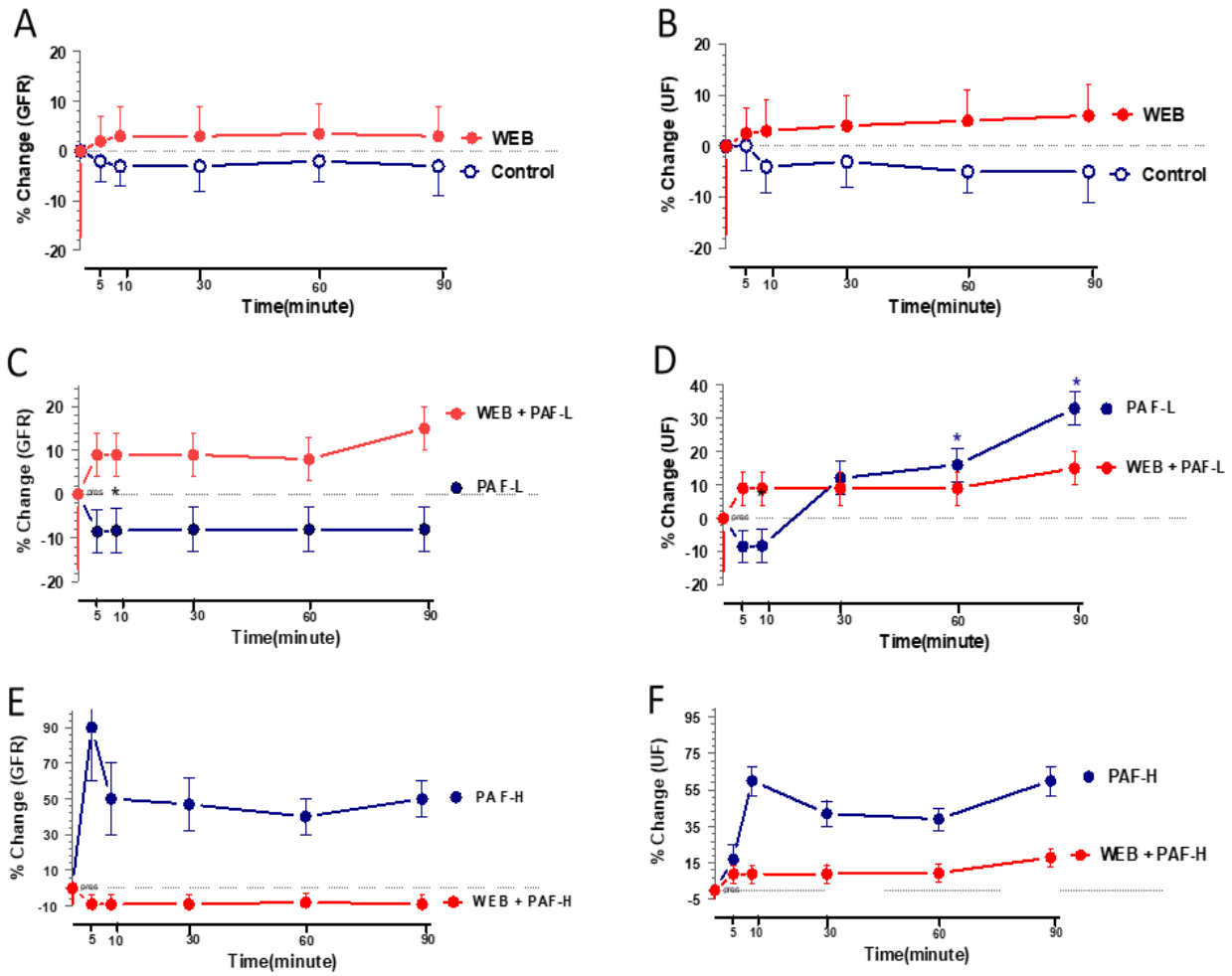


Fig. 3. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean glomerular filtration rate (GFR) and urine flow rate (UF) response to exogenous PAF administration by either low dosage (PAF-L) or high dosage (PAF-H) which pretreated with WEB 2086 on changes of in the rabbit IPK. Panels A and B, comparative studies between the control group which were perfused by only MKHS (Control) and the group treated with only WEB 2086 (WEB). Panels C and D, comparative effects between the group treated with PAF-L alone (PAF-L) and the group treated PAF-L after pretreatment with WEB 2086 (WEB + PAF-L). Panels E and F, comparative effects between the group treated with PAF-H alone (PAF-H) and the group treated PAF-H after pretreatment withWEB 2086 (WEB +PAF-H) in 90 min. of perfusion time. Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

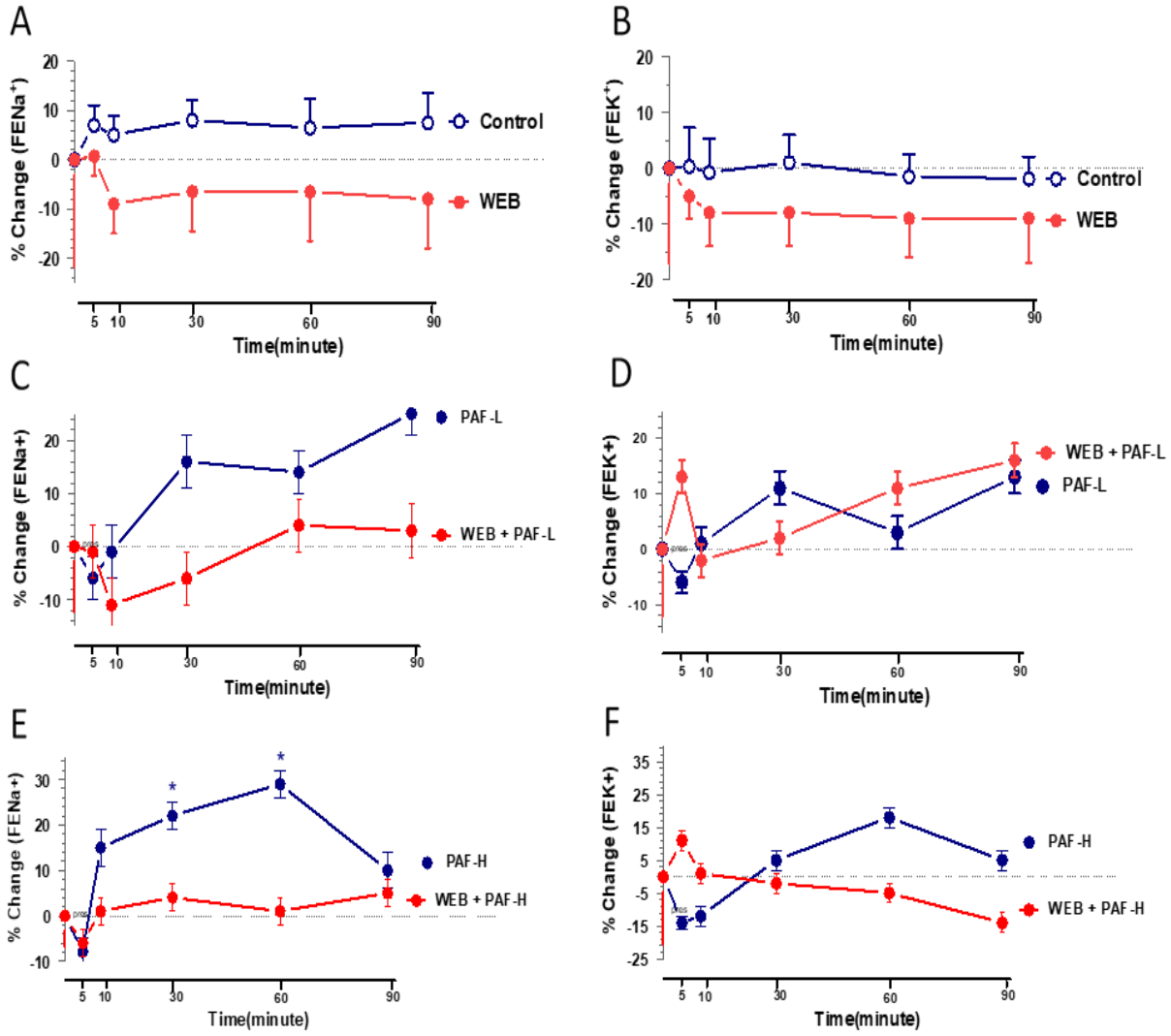


Fig. 4. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean fractional sodium excretion (%$FENa^+$) and fractional potassium excretion (%FEK^+) response to exogenous platelet activating factor (PAF) administration by either low dosage (PAF-L) or high dosage (PAF-H)) which pretreated with WEB 2086 in the isolated perfused rabbit kidneys (IPK). Panels A and B, comparative studies between the control group which were perfused by only MKHS (Control) and the group treated with only WEB 2086 (WEB). Panels C and D, comparative effects between the group treated with PAF-L alone (PAF-L) and the group treated PAF-L after pretreatment with WEB 2086 (WEB + PAF-L). Panels E and F, comparative effects between the group treated with PAF-H alone (PAF-H) and the group treated PAF-H after pretreatment with WEB 2086 (WEB + PAF-H) in 90 min of perfusion time. Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

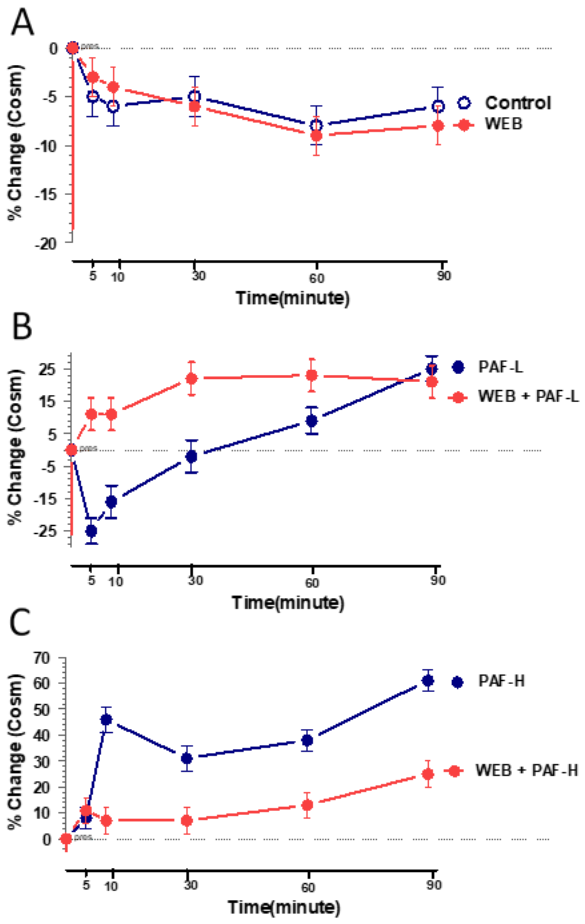


Fig. 5. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean osmolar clearance (Cosm) response to exogenous PAF administration by either low dosage (PAF-L) or high dosage (PAF-H)) which pretreated with WEB 2086 in the rabbit IPK. Panel A, comparative studies between control group which were perfused by only MKHS solution (Control) and the group treated with only WEB 2086 (WEB). Panel B, comparative effects between the group treated with PAF-L alone (PAF-L) and the group treated with PAF-L after pretreatment with WEB 2086 (WEB + PAF-L). Panel C, comparative effects between the group treated with PAF-H alone (PAF-H) and the group treated PAF-H after pretreatment with WEB 2086 (WEB + PAF-H) in 90 min. of perfusion time. Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

3.2. Effects of whole RVV, venom fractions (RvPLA$_2$, RvMP, RvLAAO, RvPDE) and WEB 2086 on renal hemodynamics

During administration of whole RVV alone (1mg/ml), the biphasic responses of renal hemodynamics were apparent. Significant initial decreases ($P < 0.05$) in PP and RVR occurred in the first phase as compared with those of the basal level of the internal control. This effect was transient and occurred at 5-10 min. after administration of whole RVV alone. A gradual rise in both PP and RVR occurred in 10 min. period of the perfusion time after the initial transient reduction, but the values remained below the pretreatment values throughout experimental period.

These effects were abolished by the specific PAF receptor antagonist, WEB 2086 when it was injected (200 μg/100 μl) prior to RVV, showing reversal with marked fall in PP and RVR (P < 0.05) throughout the perfusion time (Table 3; Fig. 6 panels A, B). The effects of administrations of venom fractions for $RvPLA_2$, RvMP, RvLAAO, and RvPDE on changes in renal hemodynamics show that administration of $RvPLA_2$ alone (280 μg/ ml) caused significant increases in PP and RVR (P< 0.05) with maximal effect at 60 min. and 90 min. period of the perfusion time. These effects were not prevented by WEB 2086 (200 μg/100 μl) when it was injected prior to administrations of either $RvPLA_2$. This combination of WEB 2086 and $RvPLA_2$ also caused significant increases in PP and RVR (P < 0.05) thoughout the perfusion time (Table 3; Fig. 6 panels C, D). Administration of RvMP alone (280 μg/ ml) caused significant increases in PP and RVR (P < 0.05) at 60 min. and 90 min. period of the perfusion time, but RvMP effects were lesser extent as compared to that of $RvPLA_2$ effect. These effects were abolished by pretreatment with WEB 2086 showing reversal of RvMP effects with the marked fall in PP and RVR (P < 0.05) throughout the perfusion time (Table 3; Fig. 6 panels E, F).

We have found that administration of RvLAAO alone (270 μg / 2ml) to the perfusate, increases in both PP and RVR levels were apparent as compared with the basal control levels. These effects were not prevented by pretreatment with WEB 2086 showing no reversal of RvLAAO effects with the marked increases in PP and RVR throughout the perfusion time (Table 3; Fig. 6 panels G, H). The administration of RvPDE alone (200 μg / 2 ml) to the perfusate caused decreases in both PP and RVR levels as compared with the basal control levels throughout the perfusion time. These effects were not prevented by WEB 2086 when it was injected prior to RvPDE (Table 3; Fig. 6 panels I, J).

Table 3. Effects of Russell's viper venom (RVV) and its fractions; phospholipase A_2 ($RvPLA_2$), metalloproteinase (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) and pre-exposure of WEB 2086 on perfusion pressure (PP) and renal vascular resistance (RVR) in the isolated perfused rabbit kidney.

Variables: groups	**Basal**	**5 min.**	**10 min.**	**30 min.**	**60 min.**	**90 min.**
PP (mmHg):						
RVV	102.0 ± 3.3	85.8 ± 8.8*	91.0 ± 4.2*	92.8 ± 5.6*	97.0 ± 3.8	98.3 ± 4.5
WEB 2086 + RVV	115.8 ± 8.5	95.0 ± 10.4	86.5 ± 11.0*	81.5 ± 10.5*	81.5 ± 10.7*	80.8 ±10.8*
RvPLA2	100.5 ± 10.1	105.4 ± 15.5	103.6 ± 11.6	114.7 ± 15.8	128.5 ± 22.8*	129.6 ±22.8*
WEB 2086 +Rv PLA2	119.3 ± 10.7	140.0 ± 5.5*	152.5 ± 8.7*	163.3 ± 16.2*	161.3 ± 1.4*	156.8 ±12.8*
RvMP	91.8 ± 4.4	95.0 ± 5.6	94.0 ± 4.8	93.5 ± 4.2	101.5 ± 9.1*	108.8 ±13 4*
WEB 2086+RvMP	104.5 ± 3.2	98.5 ± 2.7	96.5 ± 1.5	78.0 ± 5.7*	77.5 ± 6.3*	77.0 ± 5.9*
RvLAAO	105.3± 6.1	110.5 ± 3.6	114.5 ± 6.5.	116.0 ± 10.5	111.3 ± 12.5.	106.0 ±11.1
WEB2086 + RvLAAO	111.5± 3.9	120.8 ± 9.7	135.5 ± 20.0	142 ± 21.0	140.3 ± 19.3*	150.3 ±24.9*
RvPDE	94.0 ± 7.8	92.0 ± 7.6	92.3 ± 6.3	92.3 ± 5.8	90.7 ± 5.3	90.7 ± 5.7
WEB 2086 + RvPDE	82.0 ± 5.9	80.5 ± 5.63	79.3 ± 4.7	77.0 ± 3.1	74.5 ± 2.3	73.0 ± 2.5*
RVR (mmHg/ml/min):						
RVV	2.11 ± 0.22	1.79 ± 0.29*	1.89 ± 0.24	1.93 ± 0.25	2.01 ± 0.23	2.04 ± 0.26
WEB 2086 + RVV	2.03 ± 0.44	1.65 ± 0.28	1.51 ± 0.29	1.42 ± 0.27*	1.42 ± 0.28*	1.41 ± 0.28*
RvPLA2	1.65 ± 0.38	1.79 ± 0.49	1.73 ± 0.39	1.88 ± 0.33	2.07 ± 0.39*	2.08 ± 0.39*
WEB 2086 +Rv PLA2	2.03 ± 0.31	2.35 ± 0.24	2.53 ± 0.16*	2.68 ± 0.17*	2.67 ± 0.22*	2.60 ± 0.21*
RvMP	1.31 ± 0.09	1.36 ± 0.10	1.33 ± 0.12	1.34 ± 0.13	1.45 ± 0.15	1.55 ± 0.19*
WEB 2086+RvMP	1.58 ± 0.07	1.56 ± 0.05	1.52 ± 0.03	1.43 ± 0.08*	1.44 ± 0.08*	1.41 ± 0.08*
RvLAAO	1.48 ± 0.16	1.55 ± 0.25	1.59 ± 0.25	1.60 ± 0.21	1.54 ± 0.17	1.47 ± 0.12
WEB 2086+ RvLAAO	2.46 ± 0.38	2.7 ± 0.58	3.14 ± 0.59	3.28 ± 0.59	3.21 ± 0.88	3.45 ± 1.05
RvPDE	1.74 ± 0.46	1.7 ± 0.44	1.7 ± 0.42	1.69 ± 0.39	1.66 ± 0.38	1.66 ± 0.38
WEB 2086+ RvPDE	1.22 ± 0.15	1.19 ± 0.14	1.17 ± 0.13	1.13 ± 0.09	1.09 ± 0.07	1.08 ± 0.07

Data are presented as mean ± SD of four perfused kidneys from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): $*P < 0.05$, mean values of specified time period with respect to the basal period in each group. Basal is the internal control period prior to treatment of each experimental group. WEB 2086 is a platelet activating factor receptor antagonist, which was added 30 min. before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min after the basal measurements of each experiment.

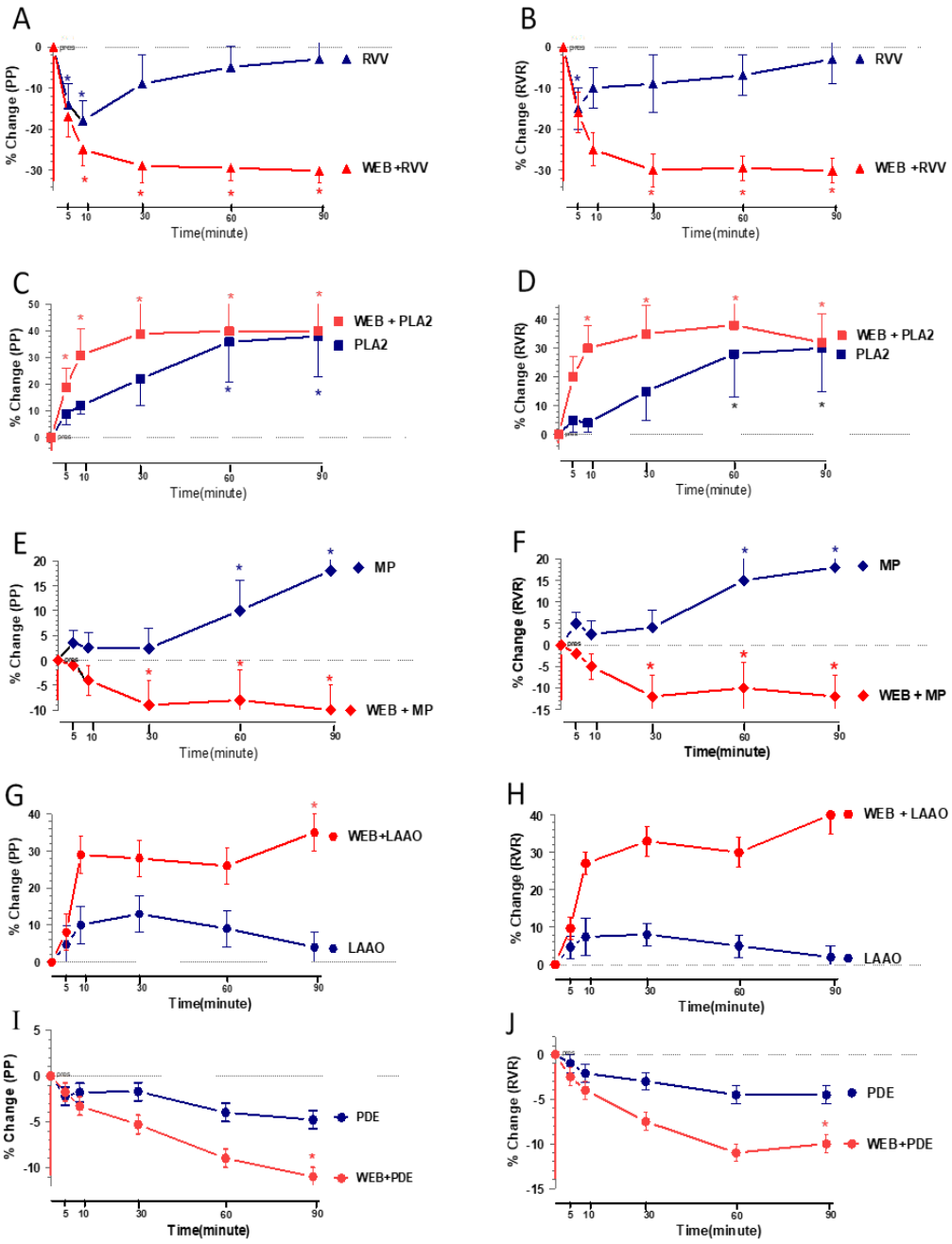


Fig. 6. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean renal perfusion pressure (PP) and renal vascular resistance (RVR) response to treatments of Russell viper venom (RVV) or pretreated with WEB 2086 in rabbit IPK. RVV: Panels (A, B); comparative effects between treating IPK with whole RVV alone (RVV) or pretreatment with WEB 2086 (WEB + RVV). Phospholipase A_2: Panels (C, D); comparative effects between treating IPK with RvPLA_2 alone (PLA_2) and pretreatment withWEB2086 (WEB + PLA_2). Metalloproteinase: Panels (E, F); comparative effects between treating IPK with venom RvMP alone (MP) and pretreatment withWEB 2086 (WEB + MP). L-amino acid oxidase: Panels (G, H); comparative effects between treating IPK with RvLAAO alone (LAAO) and pretreatment with WEB 2086 (WEB + LAAO). Phosphodiesterase: Panels (I, J); comparative effects between treating IPK with RvPDE alone (PDE) and pretreatment with WEB 2086 (WEB + PDE). Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

3.3. Effects of whole RVV, venom fractions (RvPLA$_2$, RvMP, RvLAAO, RvPDE) and WEB 2086 on glomerular filtration rate and urinary flow rate

The present study demonstrates that administration of whole RVV alone caused decreases in both GFR and UF with significant reductions ($P < 0.05$) observed at 60 min. and 90 min. period of the perfusion time. These effects were not prevented by WEB 2086 when it was injected prior to RVV showing the significant reduction in both GFR and UF ($P < 0.05$), which were still apparent thoughout the perfusion time although availability of WEB 2086 (Table 4; Fig. 7 panels A, B). It is interesting that administration alone of each of four venom fractions, namely RvPLA$_2$, RvMP, RvLAAO and RvPDE, caused increases in GFR and UF thoughout the perfusion time. These responses showed the opposite results in comparison to that of study using whole RVV alone. Either RvPLA$_2$ or RvMP alone caused significant increases both GFR and UF ($P<0.05$), which particularly observed at 60 min. and 90 min. of the perfusion time. These effects were abolished by WEB 2086 when it was injected prior to administration of either RvPLA$_2$ or RvMP. (Table 4; Fig. 7 panels C, D, E, F). In addition, administration of either RvLAAO or RvPDE alone caused significant increases in GFR and UF but these effects were not prevented by WEB 2086 when it was injected prior to administration of either RvLAAO or RvPDE, which increases in GFR and UF were still apparent thoughout the perfusion time although availability of WEB 2086. These effects indicate that WEB 2086, a platelet activating factor receptor antagonist did not induce blockade in either RvLAAO or RvPDE effects on glomerular functons in the perfused kidney (Table 4; Fig. 7 panels G, H, I, J).

Table 4. Effects of Russell's viper venom (RVV) and its fractions; phospholipase A_2 ($RvPLA_2$), metalloproteinase (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) and pre-exposure of WEB 2086 on glomerular filtration rate (GFR) and urinary flow rate (UF) in the isolated perfused rabbit kidney.

Variables	Basal	5 min.	10 min.	30 min.	60 min.	90 min.
GFR (ml/min):						
RVV	0.67 ± 0.08	0.67 ± 0.07	0.56 ± 0.15	0.48 ± 0.16	0.30 ± 0.08*	0.17 ± 0.04*
WEB 2086+RVV	0.59 ± 0.07	0.56 ± 0.11*	0.42 ± 0.10*	0.34 ± 0.08*	0.22 ± 0.05*	0.35 ± 0.11*
RvPLA2	0.33 ± 0.03	0.39 ± 0.04	0.47 ± 0.05	0.45 ± 0.07	0.66 ± 0.23*	1.00 ± 0.43*
WEB 2086+RvPLA2	0.69 ± 0.29	0.68 ± 0.26	0.74 ± 0.24	0.60 ± 0.17	0.52 ± 0.13	0.44 ± 0.12*
RvMP	0.24 ± 0.09	0.38 ± 0.16	0.53 ± 0.25	0.62 ± 0.26	0.70 ± 0.26*	0.76 ± 0.18*
WEB 2086+RvMP	0.20 ± 0.04	0.17 ± 0.02	0.16 ± 0.03	0.16 ± 0.03	0.19 ± 0.04	0.22 ± 0.08
RvLAAO	0.12 ± 0.03	0.14 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.16 ± 0.01*	0.14 ± 0.02
WEB 2086+RvLAAO	0.32 ± 0.03	0.32 ± 0.03	0.33 ± 0.03	0.35 ± 0.03	0.41 ± 0.11	0.56 ± 0.19*
RvPDE	0.15 ± 0.02	0.25 ± 0.04*	0.24 ± 0.06*	0.17 ± 0.01	0.21 ± 0.03	0.15 ± 0.03
WEB2086+RvPDE	0.10 ± 0.03	0.11 ± 0.02	0.11 ± 0.02	0.15 ± 0.03*	0.14 ± 0.02*	0.18 ± 0.03*
UF (ml/min):						
RVV	0.46 ± 0.10	0.45 ± 0.07	0.37 ± 0.09	0.32 ± 0.09	0.21 ± 0.05*	0.18 ± 0.05*
WEB 2086+RVV	0.66 ± 0.02	0.61 ± 0.09	0.48 ± 0.14	0.39 ± 0.14*	0.30 ± 0.11*	0.25 ± 0.09*
RvPLA2	0.18 ± 0.04	0.28 ± 0.04	0.30 ± 0.02	0.34 ± 0.04*	0.43 ± 0.10*	0.53 ± 0.19*
WEB 2086+RvPLA2	0.58 ± 0.22	0.60 ± 0.20	0.62 ± 0.16	0.59 ± 0.14	0.52 ± 0.11	0.44 ± 0.10
RvMP	0.20 ± 0.07	0.32 ± 0.10	0.38 ± 0.15	0.46 ± 0.21*	0.54 ± 0.20*	0.53 ± 0.18*
WEB 2086+RvMP	0.16 ± 0.01	0.14 ± 0.02	0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.04	0.13 ± 0.03
RvLAAO	0.11 ± 0.01	0.13 ± 0.02	0.15 ± 0.02*	0.16 ± 0.02*	0.17 ± 0.02*	0.16 ± 0.02*
WEB 2086+RvLAAO	0.15 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	0.26 ± 0.06*
RvPDE	0.10 ± 0.01	0.16 ± 0.01*	0.16 ± 0.01*	0.13 ± 0.01*	0.12 ± 0.02	0.11 ± 0.02
WEB 2086+RvPDE	0.13 ± 0.02	0.14 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.03	0.18 ± 0.03*

Data are presented as mean ± SD of four perfused kidneys from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): $*P < 0.05$, mean values of specified time period with respect to the basal period in each group. Basal is the internal control period prior to treatment of each experimental group. WEB 2086 was added 30 min before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min after the basal measurements of each experiment.

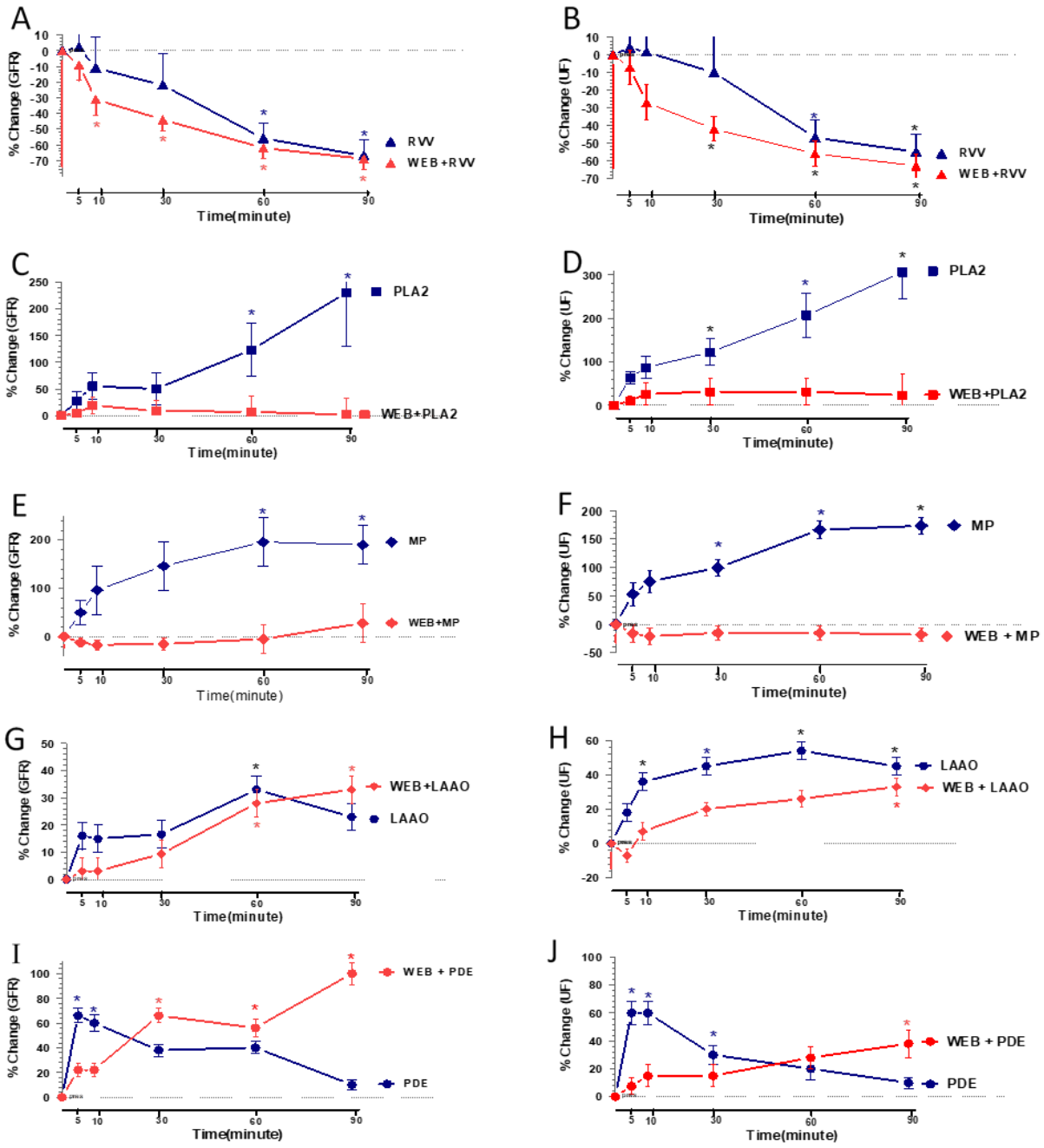


Fig. 7. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean mean glomerular filtration rate (GFR) and urine flow rate (UF) response to treatments of Russell viper venom (RVV) or pretreated with WEB 2086 in rabbit IPK. RVV: Panels (A, B); comparative effects between treating IPK with whole RVV alone (RVV) or pretreatment with WEB 2086 (WEB + RVV). Phospholipase A_2: Panels (C, D); comparative effects between treating IPK with RvPLA_2 alone (PLA_2) and pretreatment withWEB2086 (WEB + PLA_2). Metalloproteinase: Panels (E, F); comparative effects between treating IPK with venom RvMP alone (MP) and pretreatment withWEB 2086 (WEB + MP). L-amino acid oxidase: Panels (G, H); comparative effects between treating IPK with RvLAAO alone (LAAO) and pretreatment with WEB 2086 (WEB + LAAO). Phosphodiesterase: Panels (I, J); comparative effects between treating IPK with RvPDE alone (PDE) and pretreatment with WEB 2086 (WEB + PDE). Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

3.4. Effects of whole RVV, venom fractions ($RvPLA_2$, RvMP, RvLAAO, RvPDE) and WEB 2086 on renal fractional Na^+ and K^+ excretions

Fractional Na^+excretion (% $FENa^+$) increased after administration of whole RVV alone and the with maximal effect at 90 min. period of the perfusion time ($P<0.05$) while fractional K^+ excretion (% FEK^+) showed tendency to increase but was not statistically significant. These effects were abolished by WEB 2086 when it was injected prior to RVV (Table 5; Fig. 8 panels A, B).

In addition to the effect of administrations of venom fraction either $RvPLA_2$ or RvMP alone in IPK showed different responses on the renal tubular transport of Na^+ and K^+ in comparison to that of the study using whole RVV alone. Administration of $RvPLA_2$ alone showed slight increase in % $FENa^+$ while % FEK^+ showed tendency to decline at 60 and 90 min. period of the perfusion time. These effects were not prevented by WEB 2086 when it was injected prior to $RvPLA_2$. The marked increases in % $FENa^+$ ($P<0.05$) induced by $RvPLA_2$ after pretreatment with WEB 2086 but it was not apparent for % FEK^+ throughout the perfusion time (Table 5; Fig. 8 panels C, D). The effect of RvMP alone showed distinct differences in both % $FENa^+$ and % FEK^+ as compared to that of venom PLA_2. Administration of RvMP alone showed slight decrease in %$FENa^+$, while %FEK^+ significantly decreased ($P<0.05$) after addition and sustained reductions were apparent throughout the perfusion time. These effects were prevented by WEB 2086 when it was injected prior to RvMP, which the reversed effects to basal control levels of both %$FENa^+$ and %FEK^+ were apparent after pretreatment with WEB 2086 (Table 5; Fig. 8 panels E, F). The present studies demonstrate that administration of RvLAAO alone showed slight increased % $FENa^+$, while % FEK^+ showed further decreased throughout the perfusion time. These effects were not abolished by WEB 2086 which changes in %$FENa^+$ and %FEK^+ appeared in a similar manner in comparsion with that of injected RvLAAO alone (Table 5; Fig. 8 panels G, H). During Administration of RvPDE alone showed significant increases ($P < 0.05$) in both %$FENa^+$ and %FEK^+ with maximal effect at 30 min. period of the perfusion time. An increase in %FEK^+ was abolished by WEB 2086 when it was injected prior to RvPDE (Table 5; Fig. 8 panels I, J).

Table 5. Effects of Russell's viper venom (RVV) and its fractions; phospholipase A_2 ($RvPLA_2$), metalloproteinase (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) and pre-exposure of WEB 2086 on fractional sodium excretion (% $FENa^+$) and fractional potassium excretion (% FEK^+) in the isolated perfused rabbit kidney.

Variables: groups	**Basal**	**5 min.**	**10 min.**	**30 min.**	**60 min.**	**90 min.**
FENa+ (%):						
RVV	62.4 ± 13.1	64.6 ± 14.9	71.1 ± 14.5	70.6 ± 11.6	73.3 ± 11.1	92.1 ± 13.3*
WEB 2086 + RVV	97.3 ± 9.8	91.3 ± 10.2	90.7 ± 3.9	85.0 ± 8.9	95.4 ± 10.5	95.5 ± 15.1
RvPLA2	64.7 ± 4.3	65.7 ± 6.6	58.3 ± 5.1	69.4 ± 6.1	60.0 ± 10.6	58.5 ± 9.9
WEB 2086 + RvPLA2	76.6 ± 8.0	79.7 ± 4.7	89.1 ± 8.8	99.6 ± 9.6*	95.5 ± 7.3*	98.5 ± 2.7*
RvMP	83.8 ± 6.3	68.0 ± 4.2	71.6 ± 6.0	77.1 ± 8.9	80.0 ± 11.8	78.5 ± 16.5
WEB 2086 + RvMP	70.2 ± 8.0	75.8 ± 10.3	72.9 ± 8.8	70.4 ± 7.7	78.5 ± 6.7	77.2 ± 7.0
RvLAAO	90.9 ± 14.3	93.0 ± 16.9	111.7 ± 7.2*	103.5 ± 10.2	98.5 ± 8.0.2	105.5 ± 9.5
WEB 2086 + RvLAAO	83.2 ± 36.1	71.1 ± 30.1	81.6 ± 32.3	90.3 ± 24.8	92.1 ± 25,6	92.3 ± 25.5
RvPDE	60.0 ± 4.8	58.5 ± 5.4	63.7 ± 6.3	75.1 ± 4.2*	59.9 ± 14.2	71.7 ± 5.5
WEB 2086 + RvPDE	85.3 ± 6.0	96.9 ± 3.0	99.6 ± 10.5	90.3 ± 10.1	108.4 ± 13.1*	96.6 ± 4.8
FEK+ (%):						
RVV	84.7 ± 9.1	91.0 ± 7.4	94.3 ± 8.5	90.1 ± 8.6	92.0 ± 7.1	98.5 ± 9.1
WEB 2086 + RVV	104.6 ± 5.4	98.8 ± 6.3	91.1 ± 2.6	90.9 ± 3.2	98.5 ± 6.8	92.8 ± 5.4
RvPLA2	98.3 ± 8.6	109.8 ± 4.7	97.2 ± 3.0	108.2 ± 18.6	87.8 ± 19.3	87.6 ± 20.4
WEB 2086 + RvPLA2	111.3 ± 4.8	118.8 ± 5.3	123.6 ± 12.5	109.1 ± 11.5	110.0 ± 3.7	107.1 ± 6.7
RvMP	114.8 ± 7.3	117.1 ± 10.8	86.2 ± 11.6	77.2 ± 9.6*	74.9 ± 8.8*	79.8 ± 14.9*
WEB 2086 + RvMP	104.6 ± 5.8	123.6 ± 13.5	119.9 ± 10.8	119.8 ± 9.1	107.9 ± 13.1	114.2 ± 7.7
RvLAAO	169.5 ± 17.9	165.2 ± 18.7	198.0 ± 16.1	164.7 ± 19.8	157.6 ± 38.3	145.2 ± 33.5
WEB 2086 + RvLAAO	179.6 ± 33.3	180.4 ± 66.1	171.4 ± 55.5	167.6 ± 61.6	172.7 ± 58.1	158.6 ± 61.6
RvPDE	164.3 ± 15.7	164.7 ± 10.3	173.0 ± 13.5	232.8 ± 19.9*	182.7 ± 28.8	210.5 ± 14.5*
WEB 2086 + RvPDE	174.1 ± 25.7	198.1 ± 27.5	197.5 ± 23.1	160.7 ± 25.0	172.8 ± 31.7	163.2 ± 31.9

Data are preseted as mean ± SD of four perfused kidneys from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified tie period with respect to the basal period in each group. Basal is the internal control period prior to treatment of each experimental group. WEB 2086 was added 30 min before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min after the basal measurements of each experiment.

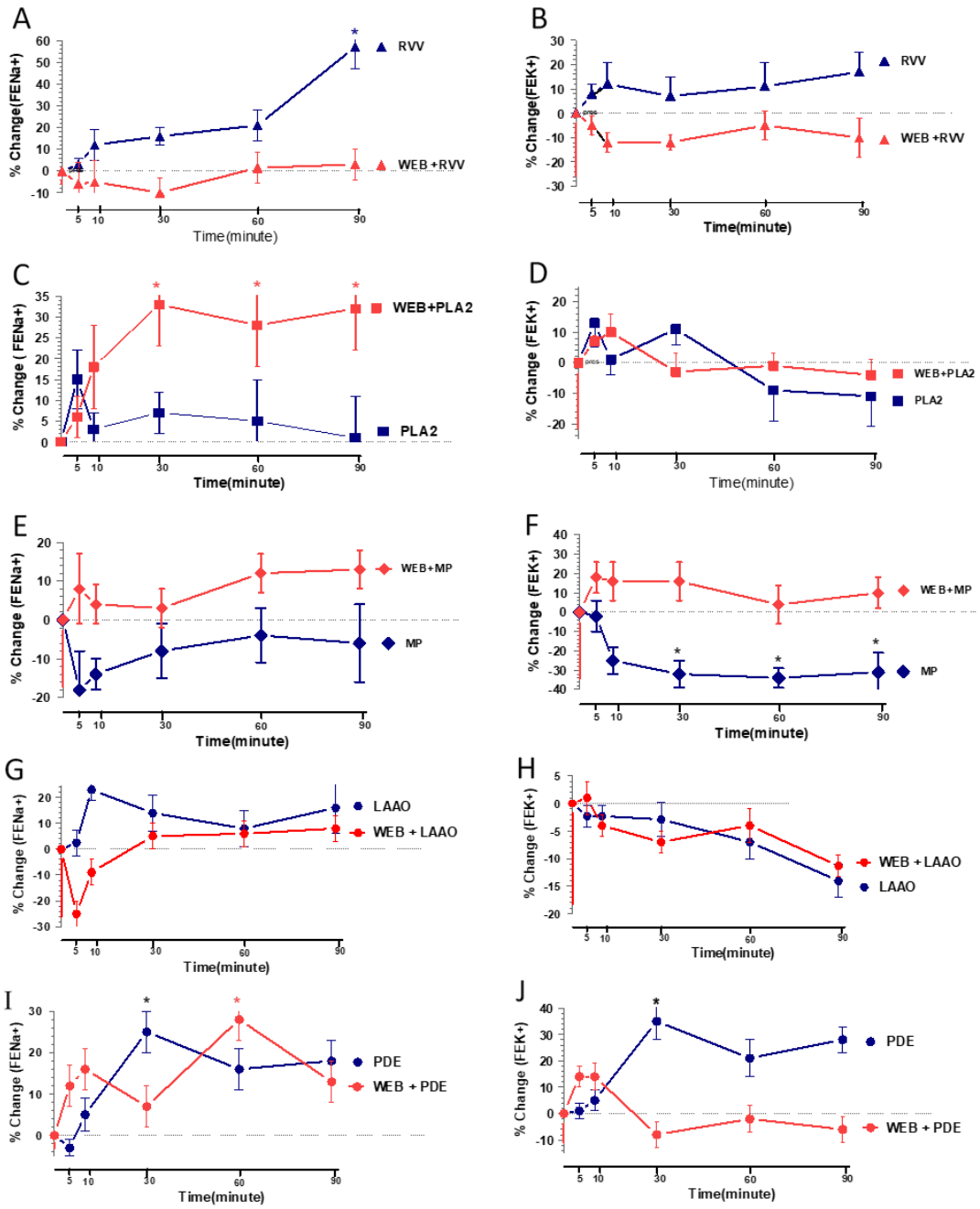


Fig. 8. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean fractional Na^+ excretion ($\%FENa^+$) and fractional K^+ excretion $^+$ ($\%FEK^+$) response totreatments of Russell viper venom (RVV) or pretreated with WEB 2086 in rabbit IPK. RVV: Panels (A, B); comparative effects between treating IPK with whole RVV alone (RVV) or pretreatment with WEB 2086 (WEB + RVV). Phospholipase A_2: Panels (C, D); comparative effects between treating IPK with $RvPLA_2$ alone (PLA_2) and pretreatment withWEB2086 (WEB + PLA_2). Metalloproteinase: Panels (E, F); comparative effects between treating IPK with venom RvMP alone (MP) and pretreatment withWEB 2086 (WEB + MP). L-amino acid oxidase: Panels (G, H); comparative effects between treating IPK with RvLAAO alone (LAAO) and pretreatment with WEB 2086 (WEB + LAAO). Phosphodiesterase: Panels (I, J); comparative effects between treating IPK with RvPDE alone (PDE) and pretreatment with WEB 2086 (WEB + PDE). Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test. *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

3.5. Effects of whole RVV, venom fractions (RvPLA$_2$, RvMP, RvLAAO, RvPDE) and WEB 2086 on renal osmolar clearance and urinary nitric oxide level

The present study demonstrates that whole RVV administration alone caused a significant reduction(P<0.05) of Cosm observed at 60 min and 90 min period of the perfusion time. These reductions could not be prevented by preatreatment of WEB 2086. (Table 6.; Fig. 9 panel A). Administration of venom fractions for RvPLA$_2$, RvMP, RvLAAO and RvPDE alone caused significant increases (P<0.05) in Cosm in IPK throughout the perfusion time. These effects were prevented by WEB 2086 when it was injected prior to administrations of venom fractions. (Table 6; Fig. 9 panels B, C, D, E). The present study determined NO formation in the IPK by measurement the urinary nitrate plus nitrite (NOx) concentrations taken at each experimental period. No significant alterations for the concentration of NO into urine were apparent during administrations alone either RVV or its venom fractions and urinary NO levels were not affected by WEB 2086 when it was injected prior to administrations of RVV and its fractions (Table 7).

Table 6. Effects of administrations of Russell' s viper venom (RVV) and its fractions; phospholipase A$_2$ (RvPLA$_2$), metalloproteinase (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) and pre-exposure of WEB 2086 on osmolar clearance (Cosm) in the isolated perfused rabbit kidney.

Variables: groups	**Basal**	**5 min.**	**10 min.**	**30 min.**	**60 min.**	**90 min.**
Cosm(ml/min):						
RVV	0.67 ± 0.36	0.67 ± 0.36	0.42 ± 0.04	0.32 ± 0.05	0.29 ± 0.07*	0.37 ± 0.19
WEB2086 + RVV	0.53 ± 0.05	0.52 ± 0.06	0.42 ± 0.13	0.41 ± 0.16	0.29 ± 0.11*	0.20 ± 0.07*
RvPLA2	0.18 ± 0.05	0.33 ± 0.06*	0.28 ± 0.03*	0.33 ± 0.04*	0.40 ± 0.08*	0.26 ± 0.13
WEB2086 + RvPLA2	0.49 ± 0.18	0.54 ± 0.20	0.60 ± 0.18	0.59 ± 0.14	0.49 ± 0.06	0.42 ± 0.10
RvMP	0.17 ± 0.07	0.30 ± 0.08	0.51 ± 0.09*	0.82 ± 0.15*	0.89 ± 0.09*	0.87 ± 0.09*
WEB 2086 +Rv MP	0.11 ± 0.03	0.10 ± 003	0.09 ± 0.03	0.10 ± 0.05	0.10 ± 0.05	0.10 ± 0.04
RvLAAO	0.10 ± 0.01	0.12 ± 0.03	0.13 ± 0.02	0.15 ± 0.02*	0.16 ± 0.02*	0.14 ± 0.02*
WEB 2086+RvLAAO	0.59 ± 0.12	0.48 ± 0.07	0.48 ± 0.06	0.62 ± 0.06	0.76 ± 0.20	1.08 ± 0.34*
RvPDE	0.09 ± 0.01	0.14 ± 0.01*	0.15 ± 0.01*	0.12 ± 0.01*	0.12 ± 0.02*	0.10 ± 0.02
WEB 2086 + RvPDE	0.12 ± 0.01	0.12 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.15 ± 0.03	0.16 ± 0.03

Data are presented as mean ± SD of four perfused kidneys from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): *P < 0.05, mean values of specified time period with respect to the basal period in each group. Basal is the internal control period prior to treatment of each experimental group. WEB 2086 is a platelet activating factor receptor antagonist, which was added 30 min before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min. after the basal measurements of each experiment.

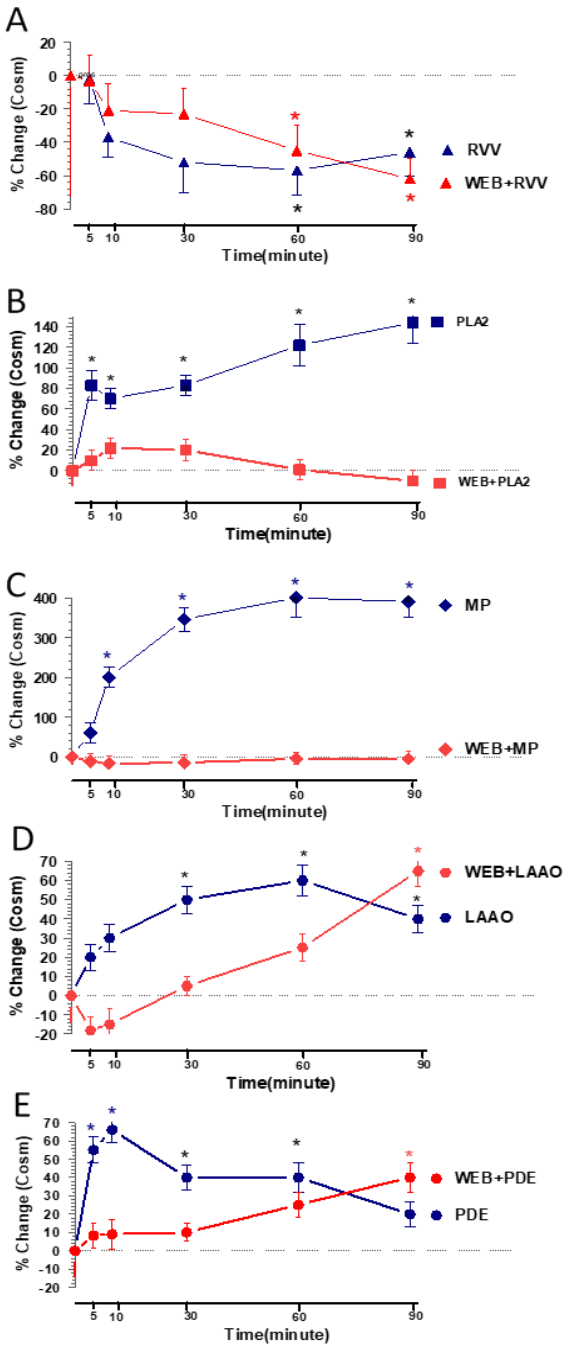


Fig. 9. Values of each parameter are presented as a percentage change relative to the baseline value at the start of the experiment. Time course of percent changes in mean osmolar clearance (C osm) response to treatments of Russell viper venom (RVV) or pretreated with WEB 2086 in rabbit IPK. RVV: Panels (A, B); comparative effects between treating rabbit IPK with whole RVV alone (RVV) or pretreatment with WEB 2086 (WEB + RVV). Phospholipase A_2: Panels (C, D); comparative effects between treating IPK with $RvPLA_2$ alone (PLA_2) and pretreatment withWEB2086 (WEB + PLA_2). Metalloproteinase: Panels (E, F); comparative effects between treating IPK with venom RvMP alone (MP) and pretreatment withWEB 2086 (WEB + MP). L-amino acid oxidase: Panels (G, H); comparative effects between treating IPK with RvLAAO alone (LAAO) and pretreatment with WEB 2086 (WEB + LAAO). Phosphodiesterase: Panels (I, J); comparative effects between treating IPK with RvPDE alone (PDE) and pretreatment with WEB 2086 (WEB + PDE). Each point represents mean ± SD of four experimental kidneys. *P*-values by one-way ANOVA with Bonferroni post hoc test: *Significant differences ($P < 0.05$) of specified time point with respect to the value of internal control in the same group.

Table 7. Effects of administrations of Russell' s viper venom (RVV) and its fractions; phospholipase A_2 ($RvPLA_2$), metalloproteinase (RvMP), L-amino acid oxidase (RvLAAO) and phosphodiesterase (RvPDE) and pre-exposure of WEB 2086 on urinary nitric oxide (NO) concentration in the isolated perfused rabbit kidney.

Variables: groups	Basal	5 min.	10 min.	30 min.	60 min.	90 min.
Urinary NO (mM):						
Control	0.75 ± 0.01	0.74 ± 0.02	0.75 ± 0.02	0.78 ± 0.03	0.80 ± 0.05	0.78 ± 0.01
WEB 2086	0.88 ± 0.08	0.77 ± 0.05	0.79 ± 0.05	0.79 ± 0.03	0.83 ± 0.06	0.80 ± 0.04
RVV	1.05 ± 0.03	0.98 ± 0.09	1.03 ± 0.08	0.99 ± 0.05	0.97 ± 0.03	1.08 ± 0.03
WEB 2086 + RVV	0.80 ± 0.04	0.82 ± 0.03	0.84 ± 0.07	0.86 ± 0.06	0.88 ± 0.06	0.88 ± 0.04
RvPLA2	1.26 ± 0.13	1.13 ± 0.21	1.15 ± 0.09	1.08 ± 0.15	1.20 ± 0.14	1.15 ± 0.12
WEB 2086 + RvPLA2	0.84 ± 0.13	0.89 ± 0.14	0.88 ± 0.17	0.85 ± 0.14	0.89 ± 0.06	0.91 ± 0.07
RvMP	0.93 ± 0.06	0.85 ± 0.21	0.88 ± 0.08	0.87 ± 0.06	0.90 ± 0.03	0.97 ± 0.08
WEB 2086 + RvMP	0.85 ± 0.04	1.02 ± 0.03	0.90 ± 0.07	0.91 ± 0.09	0.96 ± 0.05	0.87 ± 0.08
RvLAAO	0.95 ± 0.08	0.74 ± 0.09	0.94 ± 0.04	0.86 ± 0.05	0.89 ± 0.12	0.88 ± 0.12
WEB 2086 + RvLAAO	0.91 ± 0.08	0.96 ± 0.08	0.96 ± 0.02	0.87 ± 0.06	0.94 ± 0.09	1.11 ± 0.06
RvPDE	0.98 ± 0.13	0.97 ± 0.12	0.91 ± 0.07	0.94 ± 0.08	0.89 ± 0.06	0.90 ± 0.05
WEB 2086 + RvPDE	0.86 ± 0.09	0.93 ± 0.03	0.86 ± 0.09	0.94 ± 0.07	1.09 ± 0.04	0.82 ± 0.05

Data are presented as mean ± SD of four perfused kidneys from four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): $*P < 0.05$, mean values of specified time period with respect to the basal period in each group. Basal is the internal control period prior to treatment of each experimental group. WEB 2086 was added 30 min. before specified treatment of each experimental group. For calculations, samplings were made at 5, 10, 30, 60 and 90 min. after the basal measurements of each experiment. No significant changes in urinary NO concentrations (0.75-1.26 mM) at any point in all treated groups after 90 min. of administrations comparing with basal control period.

3.6. Effects of whole RVV, venom fractions ($RvPLA_2$, RvMP, RvLAAO, RvPDE) and WEB 2086 on kidney histological alterations.

Light microscopy of the rabbit IPK perfused with MKHS alone in the control group revealed a normal renal parenchyma of both glomerular and tubular part (Fig. 10 panels A, B, C, D). The rabbit IPK perfused with WEB 2086 alone showed no remarkable lesion both in glomerular and tubular parts except dilated lumen of proximal and distal tubule (Fig 10. panels E, F, G, H). The morphologic normal brush border was apparent in the proximal convoluted tubule with PAS staining in both control rabbit IPK and treated rabbit IPK with WEB 2086 alone (Fig. 10 panels C, D, G, H).

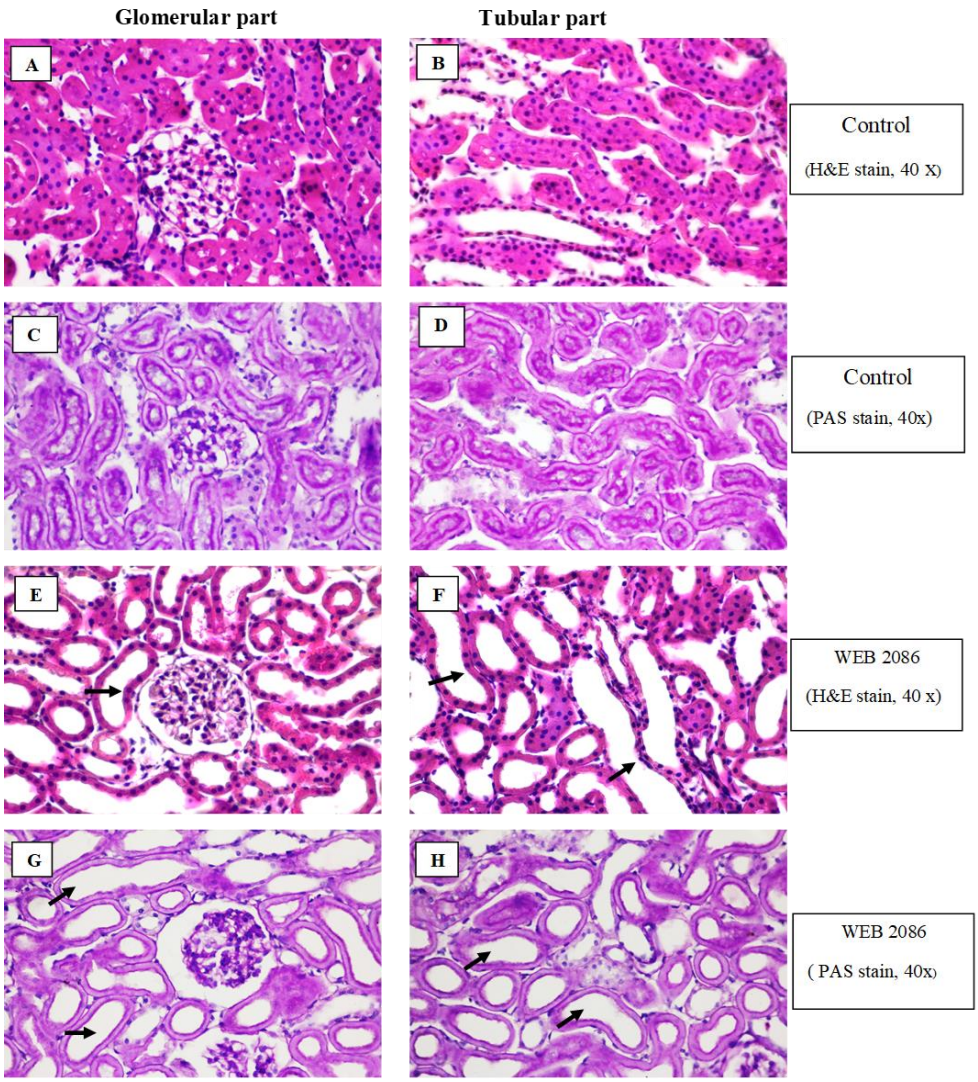


Fig. 10. Representative photomicrographs of kidney sections stained with H&E (panels A, B, E, F) and periodic acid-Schiff, PAS-stained sections (panels C, D, G, H) of glomerular and tubular parts of rabbit IPK (all slides: original magnification 40x). The kidney sections of the control kidneys taken 120 min after perfusion with MKHS alone (panels A, B, C, D) or taken 90 min. after administration of WEB 2086 alone (panels E, F, G, H). Note the morphologic normal brush border in the proximal convoluted tubule with PAS staining in both control IPK and treated IPK with WEB2086 alone (panels D, H). There was no remarkable lesion both in glomerular and tubular parts in both control IPK and treated IPK with WEB2086 alone except dilated lumen of proximal and distal convoluted tubules after treatment with WEB 2086 (indicating black arrows).

The sections from the rabbit IPK after 90 min. of administration with whole RVV alone produced no remarkable lesion in glomerular parts, while proximal and distal convoluted tubules revealed dilatation and moderate tubulonephrosis (+2) and collecting duct were severe tubulonephrosis (+3) (Fig. 11 panels A, B, C, D). Administration of whole RVV after pretreatment with WEB 2086 (WEB 2086 + RVV) showed no remarkable lesion in glomerular part, while proximal and distal convoluted tubules revealed dilatation and mild tubulonephrosis (+1) and collecting duct were mild tubulonephrosis (+1) (Fig. 11 panels E, F, G, H). However, no distinct brush border was

apparent in the proximal convoluted tubules in both IPK treated with whole RVV (Fig. 11 panel D) and the rabbit IPK treated with WEB 2086+RVV (Fig. 11 panel H).

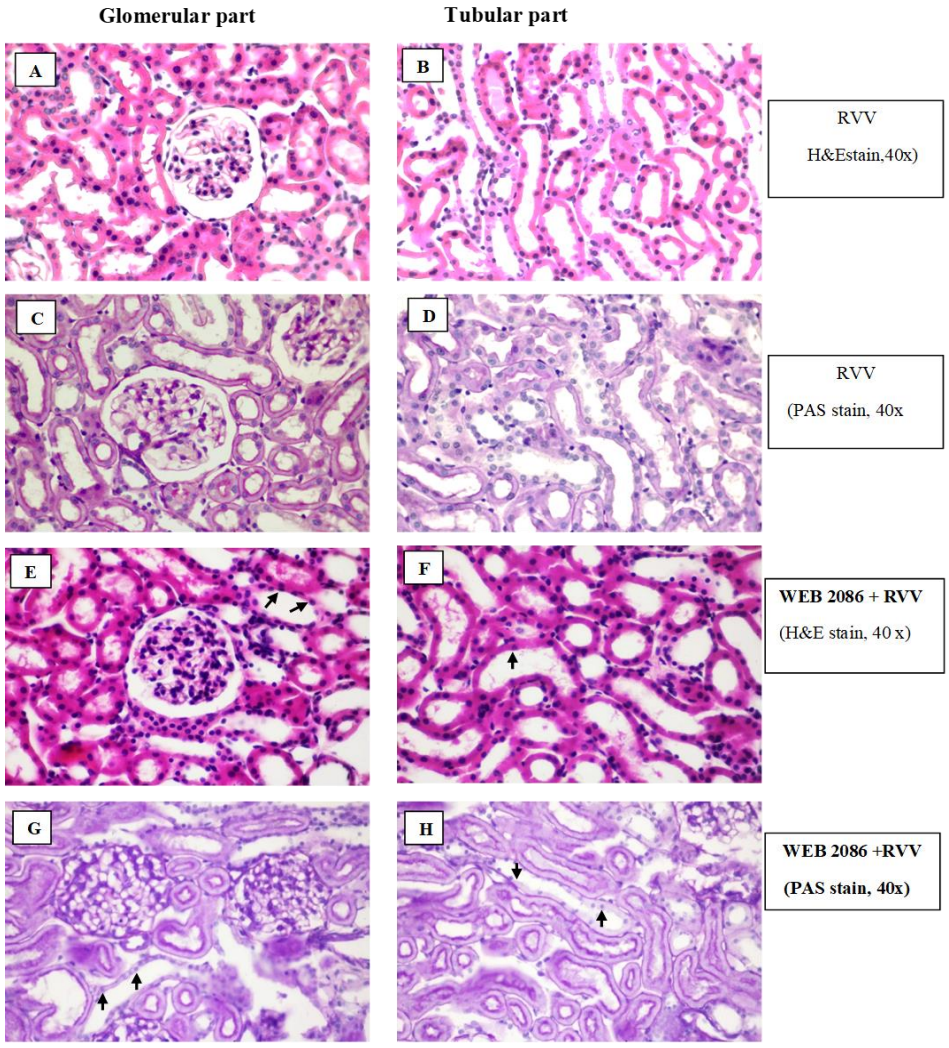


Fig.11. Representative photomicrographs of kidney sections stained with H&E (panels A, B, E, F) and periodic acid-Schiff, PAS-stained sections (panels C, D, G, H) of glomerular and tubular parts of rabbit IPK (all slides: original magnification 40x). The kidney sections of both glomerular and tubular parts are from the rabbit IPK taken 90 min. after administration of RVV alone (panels A, B, C, D) or taken 90 min. of administration of RVV after the pretreatment with WEB 2086 (WEB 2086+RVV) (panels E, F, G, H). Note the morphologic study with no distinct brush border were apparent in the proximal convoluted tubules in both IPK treated with RVV (panel D) and IPK treated with WEB2086+RVV (panel H). IPK treated with RVV alone showed no remarkable lesion in glomerular parts, while proximal and distal convoluted tubules revealed dilatation and moderate (+2) tubulonephrosis and collecting duct were severe (+3) tubulonephrosis (indicating black arrows). IPK treated with WEB 2086+RVV, there was no remarkable lesion in glomerular part, while proximal and distal convoluted tubules revealed dilatation and mild (+1) tubulonephrosis and collecting ducts were mild (+1) tubulonephrosis (indicating black arrows).

Administration of $RvPLA_2$ alone showed some glomeruli with severe unidentified crystals deposited in glomerular capillary lumen. Proximal and distal convoluted tubules revealed dilatation and moderate tubulonephrosis (+3) as well as collecting duct were moderate tubulonephrosis (+2) (Fig. 12 panels A, B, C, D). Administration of $RvPLA_2$ after pretreatment with WEB 2086 (WEB+$RvPLA_2$) showed no remarkable lesion in glomerular part but proximal

and distal convoluted tubules revealed dilatation and severe tubulonephrosis (+3) and collecting duct were severe tubulonephrosis (+3) (Fig. 12 panels E, F, G, H).

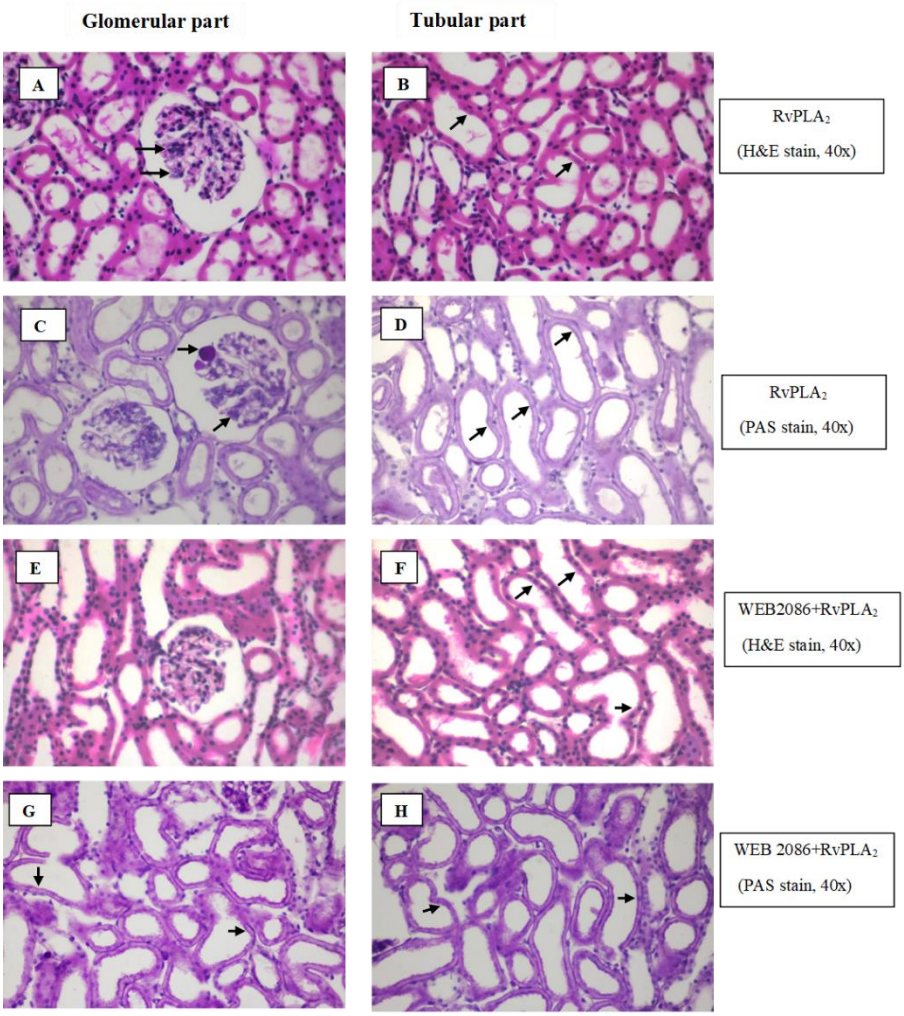


Fig. 12. Representative photomicrographs of kidney sections stained with H&E (panels A, B, E, F) and periodic acid-Schiff, PAS-stained sections (panels C, D, G, H) of glomerular and tubular parts of rabbit IPK (all slides: original magnification 40x). The kidney sections of both glomerular and tubular parts are from the rabbit IPK taken 90 min. after administration of $RvPLA_2$ alone (panels A, B, C, D) or taken 90 min. of administration of $RvPLA_2$ after the pretreatment with WEB 2086 (WEB 2086+$RvPLA_2$) (panels E, F, G, H). Note administration of $RvPLA_2$ alone showed some glomeruli with severe unidentified crystals deposited in glomerular capillary lumen (panels A, C, indicating black arrows) and proximal and distal convoluted tubules revealed dilatation and moderate (+3) tubulonephrosis and collecting ducts were moderate (+2) tubulonephrosis (panels B, D). IPK treated with WEB+PLA_2 showed no remarkable lesion in glomerular part but proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (indicating black arrows).

Administration of RvMP alone showed some glomeruli with severe unidentified crystals deposited in glomerular capillary lumen. and proximal and distal convoluted tubules revealed dilatation and severe tubulonephrosis (+3) and collecting duct were severe tubulonephrosis (+3) (Fig. 13 panels A, B, C, D). Administration of RvMP after pretreatment with WEB 2086 (WEB+RvMP) showed no remarkable lesion in glomerular part. Some glomeruli showed mild protein deposited in glomerular capillary lumen and proximal and distal convoluted tubules

revealed dilatation and severe tubulonephrosis (+3) and collecting duct were severe tubulonephrosis (+3) (Fig. 13 panels E, F, G, H).

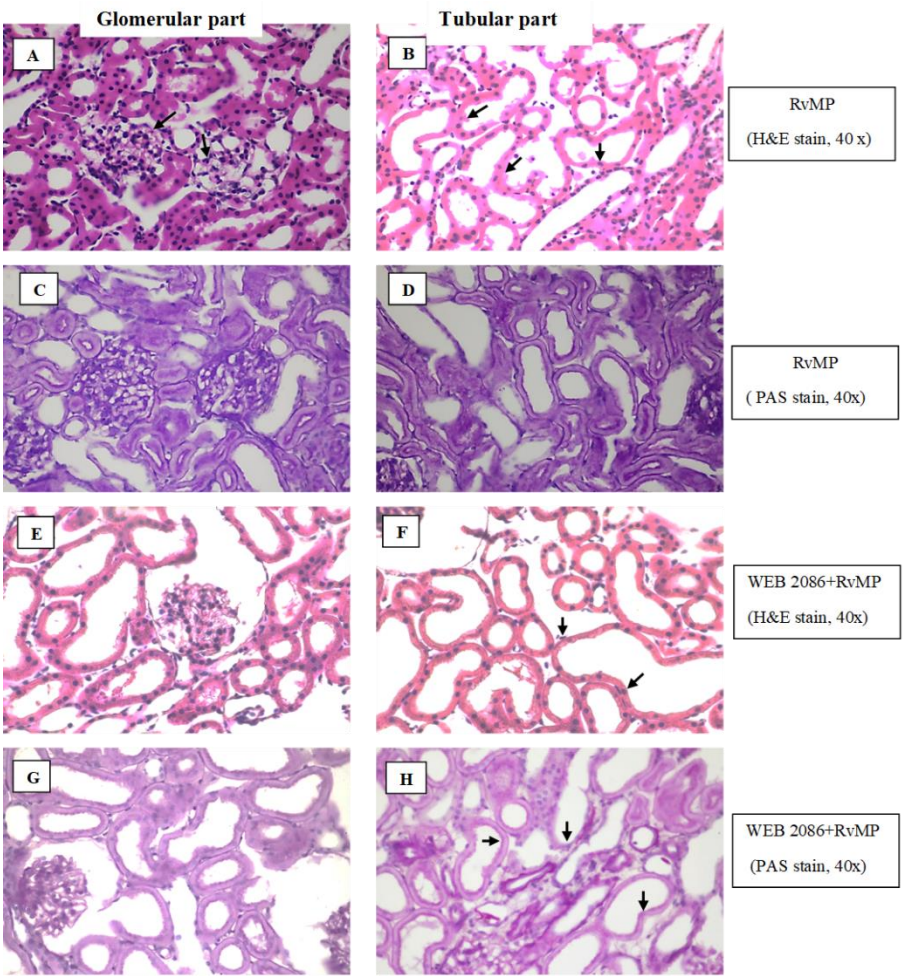


Fig. 13. Representative photomicrographs of kidney sections stained with H&E (panels A, B, E, F) and periodic acid-Schiff, PAS-stained sections (panels C, D, G, H) of glomerular and tubular parts of rabbit IPK (all slides: original magnification 40x). The kidney sections of both glomerular and tubular parts are from the rabbit IPK taken 90 min. after administration of RvMP alone (panels A, B, C, D) or taken 90 min. of administration of RvMP after pretreatment with WEB 2086 (WEB 2086+RvMP) (panels E, F, G, H). Note some glomeruli showed severe unidentified crystals deposited in glomerular capillary lumen after treatment with RvMP alone (panels A, C) and proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (panels B, D), indicating black arrows). IPK treated with WEB+MP showed no remarkable lesion in glomerular part. Some glomeruli showed mild protein deposited in glomerular capillary lumen and proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (panels F, H, indicating black arrows).

In addition to isolated kidney treated with RvLAAO alone, microscopic findings showed unidentified crystals which appeared to deposite in glomerular capillary lumen in some glomeruli (Fig.14, panels A, C). The proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (Fig 14 panels B, D). An administration of RvLAAO after pretreatment with WEB 2086 (WEB 2086+RvLAAO) showed no remarkable lesion in glomerular part and some glomeruli showed mild protein

deposited in glomerular capillary lumen. The proximal and distal convoluted tubules still appeared dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (Fig 14 panels E, F, G, H)

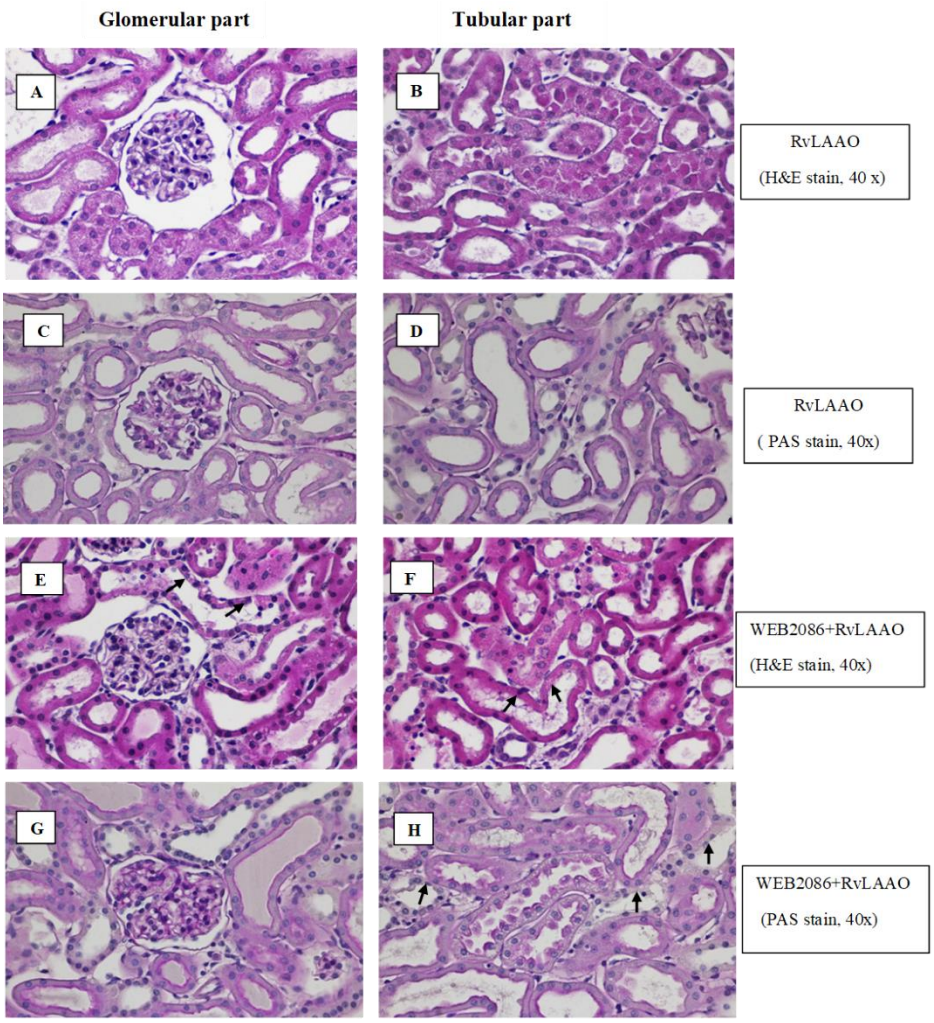


Fig. 14. Representative photomicrographs of kidney sections stained with H&E (panels A, B, E, F) and periodic acid-Schiff, PAS-stained sections (panels C, D, G, H) of glomerular and tubular parts of rabbit IPK (all slides: original magnification 40x). The kidney sections of both glomerular and tubular parts are from the rabbit IPK taken 90 min after administration of RvLAAO alone (panels A, B, C, D) or taken 90 min. of administration of RvLAAO after the pretreatment with WEB 2086 (WEB 2086+RvLAAO) (panels E, F, G, H). Note some glomeruli showed severe unidentified crystals deposited in glomerular capillary lumen after treatment with RvLAAO alone (panels A, C) and proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (panels B, D). IPK treated with WEB+ LAAO showed no remarkable lesion in glomerular part. Some glomeruli showed mild protein deposited in glomerular capillary lumen and proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (panels F, H, indicating black arrows).

The rabbit IPK treated with RvPDE alone showed severe unidentified crystals deposited glomerular capillary lumen in some glomeruli (Fig 15 panels A, C). Proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3)

tubulonephrosis (Fig. 15 panels B, D). Adminstraton of RvPDE after pretreatment with WEB 2086 (WEB+RvPDE) showed no remarkable lesion in glomerular part. Some glomeruli showed mild protein deposited in glomerular capillary lumen and proximal and distal convoluted tubules revealed dilatation and severe (+ 3) tubulonephrosis and collecting ducts were severe (+ 3) tubulonephrosis panels (Fig. 15 panels F, H).

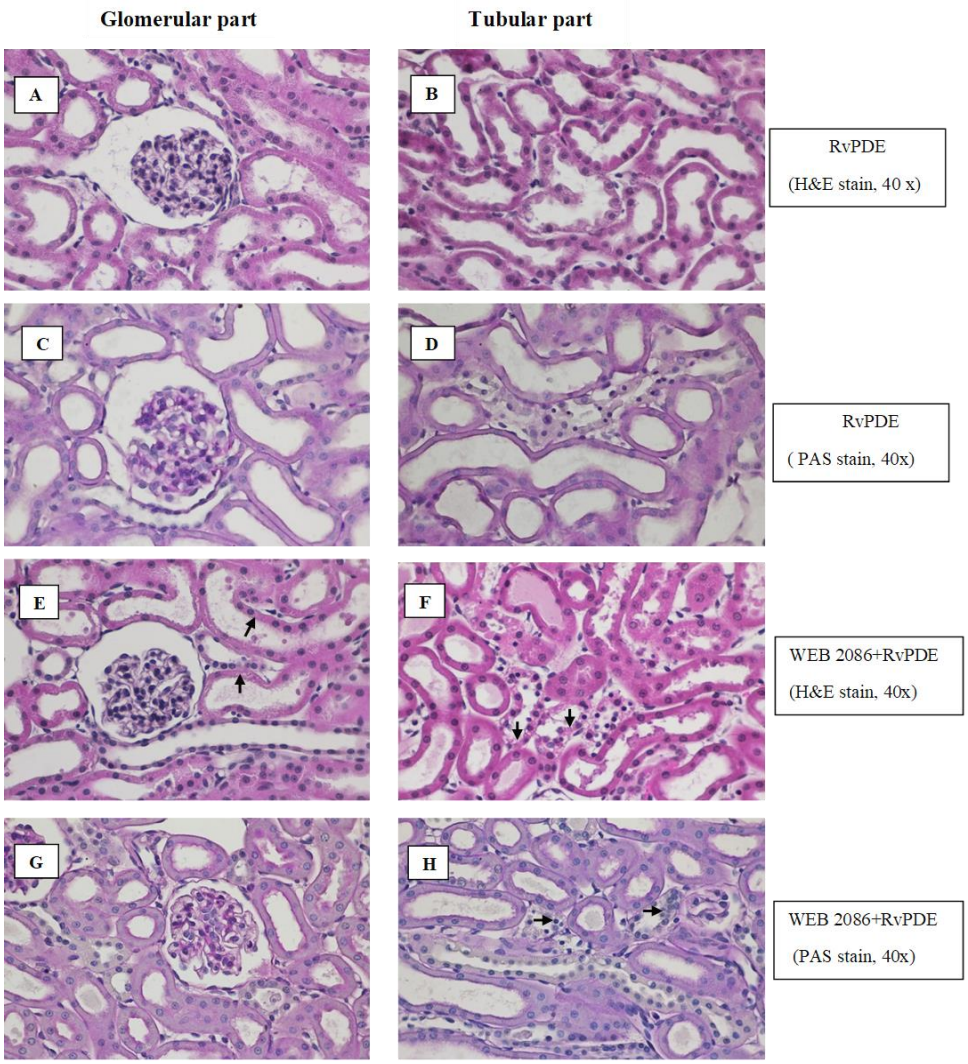


Fig.15. Representative photomicrographs of kidney sections stained with H&E (panels A, B, E, F) and periodic acid-Schiff, PAS-stained sections (panels C, D, G, H) of glomerular and tubular parts of rabbit IPK (all slides: original magnification 40x). The kidney sections of both glomerular and tubular parts are from the rabbit IPK taken 90 min after administration of RvPDE alone (panels A, B, C, D) or taken 90 min. of administration of RvPDE after the pretreatment with WEB 2086 (WEB 2086+RvPDE) (panels E, F, G, H).Note some glomeruli showed severe unidentified crystals deposited in glomerular capillary lumen after treatment with PDE alone (panels A, C) and proximal and distal convoluted tubules revealed dilatation and severe (+ 3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (B, D). IPK treated with WEB+PDE showed no remarkable lesion in glomerular part. Some glomeruli showed mild protein deposited in glomerular capillary lumen and proximal and distal convoluted tubules revealed dilatation and severe (+3) tubulonephrosis and collecting ducts were severe (+3) tubulonephrosis (panels (panels F, H, indicating black arrows).

4. Discussion

We have documented that the envenomation by whole RVV in the rabbit IPK shows changes in renal parameters causing acute kidney injury (AKI) (Chaiyabutr, et al., 2014). However, the physiopathological mechanisms of the whole RVV and its venom components-induced AKI have not been described by far. In the present study, renal alterations caused by whole RVV and its fractions ($RvPLA_2$, RvMP, RvLAAO and RvPDE) are comparatively studied to understand their effects on renal functions and whether their respective effects involve the PAF pathway that modulate the renal hemodynamics and glomerulo-tubular functions in the rabbit IPK model.

The present work in experiment protocol 1 was also designed to characterize the renal effects of PAF action by rabbit IPK method. We tested two different doses of exogenous PAF and observed changes in renal hemodynamics, glomerular functions and renal tubular transport in these two doses and to confirm whether a possible effect of exogenous PAF administration on renal functions could be inhibited by the effect of PAF receptor antagonist WEB 2086 in the rabbit IPK. Thus, an evaluating the PAF pathway involving in RVV and its fraction effect on renal functions was also performed by using a PAF receptor antagonist, WEB 2086 as a blocker. The experimental setup for the control group without any agent's treatments in the rabbit IPK showed that GFR, UF, PP, RVR, % $FENa^+$, % FEK^+ and Cosm were stable for a total of 120 min. period of the perfusion time. In the same method of the rabbit IPK, no significant effects induced by WEB 2086 on renal functions when only WEB 2086 (200 μg/100 μl) was injected, the functional parameters remained stable for over 90 min. period throughout the perfusion time. These results are agreed to the study of Monteiro et al. (1999) after administration of WEB 2086 only in isolated rat kidney.

The study in experiment protocol 1 shows that the effect of exogenous PAF administration alone either a low dose $9.5x10^{-8}$ M (PAF-L) or a high dose of $27x10^{-8}$ M (PAF-H) in the rabbit IPK increased both PP and RVR throughout periods of the perfusion time as compared with the pretreated period. These results demonstrate that the PAF action induced renal vasoconstriction in the rabbit IPK which appear contradictory to other previous reports *in vitro* studies in rats and hamsters, which have shown that the PAF action involves to dilate some vascular beds and constrict others (Kamata, et al., 1989; Dillon, et al., 1988). Whereas some studies show displaying a sustained renal vasodilation after the injection of PAF in isolated perfused rat kidneys (Schwertschlag, et al., 1987). The reason for this discrepancy is not clear, although the isolated

perfused rat kidney has become recognized as a suitable preparation for the study of renal functions (Maack 1980). We have found that in the same rabbit IPK preparation, the effect of PAF-L on increases in both PP and RVR were abolished by pretreatment with WEB 2086 (200 μg /100 μl), while blockade of PAF-H activity after pretreatment with WEB 2086 was not impressive when compared to the effect of PAF-L. This may be explained by the differences in dosage employed since the concentration of PAF-H ($27x10^{-8}$M) was higher than the concentration of PAF-L ($9.5x10^{-8}$ M) nearly three times, suggesting that the partial inhibition of WEB 2086 to the activity of PAF-H administration may be due to insufficient amount of WEB 2086 to abolish the direct renal vasoconstricting effects. In addition, a number of studies demonstrated that the concentrations of PAF in the renal circulation are between 5 x 10^{-10} and 2 x $l0^{-9}$ M (Badr et al., 1989; Tolins et al., 1989; Wang and Dunn,1987), and micropuncture studies on isolated rabbit afferent arterioles by Juncos et al. (1993) demonstrated that the direct action of PAF showed a receptor mediated biphasic effect by dilating them at low concentrations (4 x 10^{-10} M) while constricting them at higher concentrations. Therefore, exogenous PAF administration either PAF-L ($9x10^{-8}$M) or PAF-H ($27x10^{-8}$M) using in the present studies were 200 to 700-fold higher than those of other reports, which might contribute to cause renal vasoconstriction. However, it has been reported that PAF can induce renal vasoconstriction at least in part through stimulating production of thromboxane in the isolated perfused kidney (Camussi, 1986). A greater response in renal vasoconstriction by exogenous PAF-H administration may possibly involve more stimulating production of thromboxane although availability of WEB 2086.

Administration of exogenouse PAF-L caused slight reductions in GFR while UF including %FENa, %FEK and Cosm progressively increased throughout period of the perfusion time, whereas administration of PAF-H alone showed elevations of GFR including UF, %FENa, %FEK and Cosm. These results suggest that PAF modulates glomerular functions in a complex way that involves dosage employed. It has been reported that PAF exerts its effect on a specific PAF receptor within renal glomeruli (Honda et al., 2002) and increase in glomerular capillary permeability (Pirotzky et al., 1985). The possibility that PAF-H loading alone may presumably more binding to its specific receptors, activates glomerular capillary permeability leading to glomerular cytoskeleton alterations, enhancing filtrate traffic across the glomerular capillary wall resulting increases in GFR including UF, %FENa, %FEK and Cosm. However, these effects were

prevented by WEB 2086 when it was injected prior to either PAF-L or PAF-H administration in rabbit IPK.

Concerning the study in experiment protocol 2, further studies for the physiopathological mechanisms of the whole RVV and its fractions-induced AKI were investigated and their respective effects involved the PAF pathway modulating alteration of the renal functions were also performed in the rabbit IPK model. It is known that three possible mechanisms are responsible for the pathogenesis of snakebite relating to changes in hemodynamic, immunologic reactions and direct nephrotoxicity (Sitprija and Chaiyabutr, 1999). The renal damage with a direct tubulotoxic effect after Russell's viper bite has shown to associate with the disseminated intravascular coagulation (DIC) (Swe et al., 1997). Some researchers also showed the similarity of the importance of the systemic lesions caused by the coagulative process occurring in different types of snake bite in Viperidae family (Win-Aung et al., 1998). However, DIC has no relevance to the present *in vitro* studies in the rabbit IPK, since the perfusate using in the rabbit IPK is lack of fibrinogen and clotting factors.

In the present study, the experimental setup employed the dose concentration of whole RVV with 1 mg/100ml of perfusate used in IPK experiment, which was assumed to equalize that of $2LD_{50}$ of crude RVV used (0.5 mg/kg BW, i.v.) *in vivo* in the experimental dog (Tungthanathanich et al., 1986) and rabbit (Chaiyabutr et al., 2014). The dose of whole RVV used alone in this study in the rabbit IPK produce changes in the biphasic response of both PP and RVR. The pattern of these biphasic responses during envenomation with whole RVV are in agreement with the early report in isolated perfused rabbit kidney (Chaiyabutr et al., 2014). The biphasic responses of renal hemodynamics with initial transient decreased in PP in the first phase and a gradual rise in both PP and RVR occurred in 10 min. period of perfusion time, which indicate that the decrease in PP occurs through initial renal vasodilation and thereby decreasing RVR. Similar biphasic responses of changes in systemic blood pressure have been produced by RVV in previous study *in vivo* in experimental dogs (Tungthanathanich et al., 1986). Chaiyabutr et al. (1984, 1985a) demonstrated that the rise in systemic blood pressure following the initial transient decrease occurring in RVV envenomated animal is due to a compensatory mechanism, which involves releases of vasoconstrictor mediators either catecholamines or renin angiotensin system. It is known that the IPK has a cell-free medium without interference of systemic factors which is of paramount importance, since it eliminates the involvement of endogenous inflammatory mediators which are

not present in this system. However, a number of studies have been demonstrated that PAF as an inflammatory mediator can be released by kidney cells in response to stimuli of inflammatory cytokines and endotoxin e.g. glomerular cells (Morell et al., 1988; Whatley et al., 1989), endothelial cells (Whatley et al., 1989), both renal mesangial cells and renal medullary interstitial cells (Schlondorff and Neuwirth ,1986)), including in the isolated perfused kidneys (Pirotzky et al., 1984b). However, under basal conditions, no release of PAF product by resting kidney cells has been noted (Neuwirth et al., 1989). Nevertheless, Monteiro and Fonteles, (1999) demonstrated that PAF is released by kidney tissues during envenoming of *Bothrops jararaca* in the isolated perfused rat kidney, being involved as a mediator of the *B. jararaca* venom effects on renal functions. Thus, we speculate the treatment of whole RVV and its venom fractions in the rabbit IPK may promote the release of PAF synthesized by kidney cells. The present study strengthens the findings that PAF is indeed involved as a mediator of the RVV effect on vasoactive parameters PP and RVR. An elevation of both PP and RVR were completely abolished by WEB 2086 (200 μg/100 μl) when it was injected prior to whole RVV. As an explanation, the underlying mechanism of the changes in the biphasic response of renal hemodynamics (both PP and RVR) led to postulate that the liberation of PAF from kidney cells by stimulating of whole RVV alone would act as either a vasodilator or vasoconstrictor, depending upon its concentration (Lo'pez-Novoa, 1999). PAF would exert on receptors mediated biphasic effects on afferent arterioles, dilating them at low PAF concentrations during liberation in the initial period after whole RVV administration. However, the kidney cell in rabbit IPK was flooded with perfusate containing RVV in the recirculating system, and thus presumably maximally stimulating with a longer exposure to RVV, a further release of PAF products leading to an accumulation of the PAF concentrations within the renal tissue. Therefore, the constricting effect of renal arterioles would be apparent later and lasting until the end of the experiments. It is suggested that PAF locally released into the kidney plays a major role as a mediator in responsible for the effect of RVV on renal vascular contraction. A direct effect of PAF on contracting afferent arterioles in raising RVR has been noted (Juncos et al., 1993). However, the mechanism of PAF exerts a receptor-mediated biphasic effect on afferent arterioles, which do not relate to other vasoconstrictor mediators especially renin-angiotensin system (Schwertschlag et al., 1987) and sympathetic nervous system (Smith et al., 1982; Caillard et al., 1982) which can interact with formation of PAF. Since in the present study, there is neither

sympathetic innervations to the isolated kidney nor renin substrate in the preparation, an interaction of PAF with these systems would not be likely.

In addition, it has reported that the enhanced nitric oxide (NO) synthesis might be an important mechanism counteracting PAF-induced vasoconstriction (Juncos et al., 1993). If so, augmentation of PAF-induced vasoconstriction should be apparent when NO synthesis is decreased or nitric oxide inhibition. However, no significant changes in urinary NO concentrations at any point in all treated groups after 90 min. of administrations comparing with basal control period, indicating that PAF plays a role independent signal transduction pathways for NO production. A role of NO as a candidate for mediating the direct inhibitory effect of PAF during envenomation in IPK would be rule out.

The pathophysiological changes of renal functions in rabbit IPK treated with each venom fractions ($RvPLA_2$, RvMP, RvLAAO and RvPDE) were performed to compare with that of whole RVV. It is known that all snake species have different components in their venoms which PLA_2 and MP are dominant protein families and LAAO and PDE are secondary and minor protein families, repectively (Tasoulis and Isbister, 2017). PLA_2 in the venom is the principal component of snake venoms (Bon, 1997), and its pathophysiological effects have been extensively investigated (Kini, 2003). We found that each venom fractions for $RvPLA_2$, RvMP or RvLAAO injected alone into the rabbit IPK caused increases in PP and RVR throughout period of the perfusion time. An increase in both PP and RVR by administration of either $RvPLA_2$ or RvLAAO was not prevented by pretreatment with WEB 2086. These results were in contrast with those elicited by using RvMP including whole RVV, which both PP and RVR effects were abolished by pretreatment with the same amount of WEB 2086. This indicates that the action of either $RvPLA_2$ or RvLAAO not only induced generation of endogenous PAF to cause renal vasoconstriction, but also involved the disruption of PAF receptor-mediated signaling occurring in renal vasculature. The effect of $RvPLA_2$ on increases both PP and RVR may be explained by both specific and non-specific actions of PLA_2 on membrane trafficking system. The specific effect of $RvPLA_2$ may involve in the pathways of PAF synthesis (Snyder, 1985), depending on the activation of local calcium concentration (Lo´pez-Novoa, 1999). The specific action of PLA_2 is not only involved directly to PAF liberation, but it may exert its action in digests phospholipids of membranes of vascular smooth muscle cells, leading to cell damage. A major substrate for PAF synthesis is the phospholipid alkyl-acyl glycerophosphocholine, which gives rise lyso-PAF through the action of

PLA_2. It is therefore speculated that an increase in PAF may cause contraction of the renal vasculature and/or glomerular mesangial cells. Although the endothelial function was not demonstrated in the present studies, previous studies have suggested that an increase in renal PAF levels coexist with morphological evidence of endothelial damage, in which PAF's vasoconstrictor actions may be stronger (Lo´pez-Novoa, 1999). Another possible explanation for these results may reasonably be considered the content of PLA_2 has approximately 20-25%, by mass of the whole *Daboia* venom (Sharma et al., 2015; Tan et al., 2018). The $RvPLA_2$ using alone was analyzed for enzyme specific activity which was higher approximately 2.6 times than that of base of whole RVV used (Fig.1). Therefore, the high specific activity would be another possibility for non-specific effect of toxic $RvPLA_2$ on overt hydrolysis of the phospholipid of the biological membranes. However, our experiments in IPK are devoid of blood, we can assure that increases in PP and RVR were not promoted by pro-coagulant events. The $RvPLA_2$ fraction could induce lysis of endothelial cells and may induce not only the release of PAF but also other vasoconstrictor mediators, which superimpose increases in PP and RVR without abolishment by a selective platelet activating factor receptor antagonist, WEB 2086. However, further studies are required to investigate detailed mechanisms by which PAF activates $RvPLA_2$ activity.

According to administration of the RvLAAO fraction which showed similar results as $RvPLA_2$. An increase in both PP and RVR after pre-exposure of WEB 2086 (WEB+RvLAAO) failed to prevent vasoactive effects of RvLAAO fraction which may be explained by the mechanism of action of LAAO as a homodimeric flavoenzyme that catalyzes a redox reaction of different groups of amino acids, generating catabolic production of keto acids, ammonia and hydrogen peroxide (Moustafa et al., 2006, Tan and Fung, 2009). The hydrogen peroxide (H_2O_2) generated during the enzymatic reaction is a highly toxic oxygen reactive species that is capable of acting on intracellular components and cell membranes. Cytotoxicity caused by LAAO is mediated by H_2O_2 and triggers autophagy, apoptosis and necrosis in target cells (Zuliani, et al., 2009). This reactive oxygen species may act directly on cell membranes, leading to altering the permeability of the attacked area and induce endothelial injury (Du and Clemetson, 2002). Thus, it would suggest that the RvLAAO acts exclusively via a separate mechanism. The RvLAAO may induce lysis of endothelial cells not only the release of PAF but also other vasoconstrictor mediators, which could increase in PP and RVR herein without blocking of the WEB 2086. However, the present observation is not consistent with findings from another study identifying

the role of LAAO fraction isolated from *Bothrops marajoensis* venom by Dantas et al. (2015), which caused significant reductions of PP and RVR at 90 and 120 min. period of perfusion time in the isolated perfused rat kidney. It indicates that the action of venom LAAO fraction can be responsible for renal alterations by its respective venom. The differences in pathophysiological responses between RvLAAO and LAAO from *Bothrops* snake may be explained, at least in part, by the different doses of LAAO used in the studies. A dose of LAAO fraction used in rat IPK (10 μg/ ml of perfusate) by Dantas et al. (2015), was about 4 times higher than that of RvLAAO used in the present study in rabbit IPK (2.7 μg/ ml of perfusate). Therefore, the observed differences in renal hemodynamics response to LAAO from these two snake species may not be due to inherent difference of LAAO molecules, but may be resulted from the difference in levels of LAAO expression of proteolytic activity with amino acids as substrates resulting high production of H_2O_2 (Bregge-Silva et al., 2012), causing oxidative stress in the target cell. In addition, it is speculated for another explanation that the endothelial injurious stimuli of $RvPLA_2$ or RvLAAO fractions may cause the localized loss of cellular homeostasis of endothelial cell including cellular energy and membrane permeability to ions transport. Disruption in the integrity of the plasma membrane may lead to open sodium channels (ENaC) on the vascular smooth muscle cells as it does in epithelial cells (Garcia-Caballero et al., 2011; Jernigan and Drummond, 2005; Rossier and Stutts, 2009). Na^+ influx causes membrane depolarization and opening of calcium channel which results in Ca^{++} influx. Increased cytosolic Ca^{++} can cause vascular contraction. Both PP and RVR were therefore increased, which may exclude to PAF's potential role in pathophysiology, although availability of PAF receptors antagonists, WEB 2086.

An administration of the RvMP fraction alone also caused significant increases in PP and RVR at 60- and 90- min. period of the perfusion time as compared to that of $RvPLA_2$ effect which were abolished by the same amount of WEB. The pattern of changes was similar to that of the whole RVV effect after pretreatment with WEB 2086. This similarity suggests that the action of RvMP fraction may be explained, at least in part, by responsibility for renal hemodynamic of the whole RVV effect. According to the toxic MP constitute around 9% by mass of the venom reported in *Daboia siamensis* venom from China (Tan et al., 2018) and an analysis of the enzyme specific activity of RvMP in the present study was approximately 2.5 times higher than that of whole RVV (Fig.1), implying that RvMP fraction is a major component of RVV in regulation of renal hemodynamics. It is known that the hydrolytic enzymes activity of metalloproteinase from snake

venoms play multiple roles in the local and systemic effects (Gutierrez and Rucavado, 2000). Degradation of extracellular matrix proteins (ECM) by metalloproteinase has been noted (Baramova et al., 1989). It is possible that degradation of the ECM proteins by RvMP could lead to loss maintaining basement membrane structural and functional integrity of kidney cells especially vascular smooth muscle including renal mesangial cells and renal medullary interstitial cells. The generate PAF in response to the stimulus RvMP fraction would occur at those sites (Schlondorff and Neuwirth, 1986). It is possible that the disruption of PAF receptor-mediated signaling occurring in renal vasculature may not be affected by RvMP fraction. Therefore, it would speculate that enhanced PAF liberation induced by RvMP fraction alone may be a mediator for the renal vasoconstriction leading to increment of both PP and RVR.

In the present study, we also investigated the effects of RvPDE fraction on renal hemodynamics in the rabbit IPK. In contrast to other venom fractions, we found that administration of RvPDE fraction alone caused a progressive reduction on both PP and RVR throughout the perfusion time. These vasoactive effects were not prevented by pretreatment of PAF receptor antagonism, WEB 2086 (WEB+RvPDE) that the progressive declines in PP and RVR to the extent in similar manner as the effect of RvPDE alone. These results indicate that the response of reduction of renal hemodynamics to RvPDE was not direct mechanism of PAF action liberation as mediator. - Tan et al. (2018) reported that the PDE constitute around 0.25% by mass of the dried venom of *D. siamensis* from Guanxi, China. It may be possible that the catabolizing enzymes activity of PDE may be inadequate to promote endogenous PAF as a mediator of renal vasoconstriction in this rabbit IPK model. Another explanation for the reduction of renal hemodynamics might be due to hydrolytic activity of PDE associate with cell membranes and catabolize intracellular cAMP or cGMP, which play a role as a second messenger of intracellular signaling pathways (Beavo 1995). The action of PDE has shown to hydrolyse the 3′-phosphoester bond of cAMP to its biologically inactive 2′, 3′-cAMP-adenosine pathway (3′-AMP and 2′-AMP) and subsequently metabolize to adenosine and inosine (Jackson et al., 2009). If this is the case, one would expect that catalytic activity of exogenouse RvPDE causes a decrease in cAMP level in endothelial cell in the rabbit IPK model. A decrease in cellular cAMP levelcan not preserve its barrier function in protection of hypoxemia induced endothelial injury (Ogawa et al. 1992) leading to vascular relaxation. We predicted that the RvPDE fraction hydrolyse c-AMP within renal vascular cells which would

finally convert efficiently to increase adenosine and inosine levels. Since, adenosine is an important regulator of vascular tone. Thus, it may also be possible that adenosine induced relaxation of vascular smooth muscle by inhibiting calcium channel activity (Herlihy et al., 1976), leading to induce vasodilation excluding to PAF's potential role in vascular components (Dhananjaya and Souza, 2010). Actually, PDE with different isoforms have been identified to localize to the glomeruli, mesangial cells, cortical tubules, and inner medullary collecting duct which plays a role in the regulation of renal hemodynamics and kidney excretory function (Dousa,1999).

Changes in glomerular functions during envenomation with whole RVV relating to PAF pathway demonstrate that whole RVV administration alone caused significant progressive reductions of both GFR and UF. Pre-exposure of the rabbit IPK to WEB 2086 did not prevent the effects induced by whole RVV on the decreases in GFR and UF. It showed the opposite effect on both PP and RVR, suggesting the PAF effect induced by whole RVV mediate mostly on the vasoconstriction of renal vasculature, leading to efficient blockade by WEB 2086 without influencing the renal glomeruli. However, it has reported that the release of PAF occur both endothelial and renal mesangial cells by various stimuli (Snyder et al., 1996). The stimulus of *Bothrops jararacussu* venom in the isolated perfused rat kidney by Havt et al. (2001) demonstrated that PAF formation was released by the effect of venom using blockade of WEB 2086 as a marker. In the present study, it may presumably that the effect of whole RVV administration would give similar involvement of PAF liberation in the rabbit IPK. Thus, an effect of whole RVV alone on a progressive reduction in GFR and UF may be involed via PAF liberation in the regulation of glomerular function by acting directly through a contraction of mesangial cells (Schlondorff et al., 1986) with the consequent decrease in glomerular surface area. The question to consider is whether whole RVV stimulation might have promoted the release other substances synthesized by renal cells in IPK such as prostaglandins, cytokines, bradykinin (Barraviera et al., 1995; Faco et al., 2003). It is possible that the stimulation of whole RVV on the release of other mediators in renal tissure, especially the production of thromboxane B2 (TxB2), the stable metabolite of thromboxane A_2 (TxA_2), which may synergistic enhancement of mesangial cell contraction and thereby sustaining reduction of the glomerular filtration surface and ultrafiltration coefficient (Kf) (Mene et al.,1989). Our previous study on histological analysis of the rabbit IPK treated with RVV (10 μg/ml of perfusate) also showed no preservation of renal integrity and dilation of glomerular

capillary slits (Chaiyabutr et al., 2014). Destruction of the structure of the matrix in the glomerulus by the direct effect of whole RVV leads to loss of Kf coinciding with the release of other vasoconstrictor agents, resulting in decreased GFR and UF, which WEB 2086 could not prevent this contraction effect. Thus, the glomerular epithelial damage in this model is also an important cause in renal alterations induced by whole RVV which could be another mechanism for significant and sustained reduction in both GFR and UF (Fig.7 panels A, B).

It is interesting that administration of venom fractions alone for $RvPLA_2$, RvMP, RvLAAO and RvPDE increase in GFR and UF in the rabbit IPK, which showed the opposite results in using whole RVV alone. It indicates that different mechanisms of actions were apparent between whole RVV and its fractions in the regulation of glomerular functions. In addition to a rich in hydrolytic enzymes of both PLA_2 and MP in Viperid snake venom (Tan et al., 2018). The significant increases in GFR and UF during administration of either $RvPLA_2$ or RvMP alone could be explained, in part, by its hydrolytic enzyme properties that act on the cell membrane of renal glomeruli leading to increase the permeability of glomerular filtering membrane and disruption of glomerular basement membrane. These effects may lead to an increase in calculated inulin clearance in this model and thereby progressive increases in GFR and UF from the observation of each time point.

Normally, PAF exert its effects on a specific PAF receptor within renal glomeruli (Honda et al., 2002) and the role of PAF has been reported to be one of the mediators of increased glomerular capillary permeability (Pirotzky et al., 1985). The significant increases in both GFR and UF induced by $RvPLA_2$ or RvMP in this study were abolished by WEB 2086 injected prior to $RvPLA_2$ or RvMP. These findings indicate that endogenous PAF play a role as the mediators involving in either $RvPLA_2$ or RvMP effects in renal glomeruli, and partly contribute its effects coinciding with the hydrolytic enzyme's activity of PLA_2 or MP. However, the pretreatment of WEB 2086 in prevention the deleterious effects of glomerular functions promoted by $RvPLA_2$ or RvMP are likely to support the documented effect of PAF receptor antagonists on reduction histopathological lesions in models of nephrotoxic nephritis in rabbits (Livio et al., 1986) and in many kidney diseases with a vascular or glomerular etiology (Ortiz et al.,1991**;** Lo´pez-Novoa, 1999).

In contrast to the effects of $RvPLA_2$ and RvMP, progressive increases in GFR and UF by either RvLAAO or RvPDE effect was not prevented by pretreatment with WEB 2086. These findings indicate that the effect of RvLAAO or RvPDE on increases in GFR and UF do not involve the release of PAF receptor-mediated signaling in renal glomeruli. However, the present results for

the effect of RvLAAO on changes in GFR and UF did not agree to earlier report of Dantas et al (2015), who used LAAO from *Bothrops marajoensis* venom causing decreases in GFR and UF including PP and RVR in isolated rat kidney. These changes in glomerular function are related to a flavoenzymes activity of snake venom LAAO, which catalyze stereospecific oxidative deamination to give rise to alpha keto acids, ammonia and H_2O_2 (Bregge-Silva et al., 2012; Fox, 2013). The production of H_2O_2 may represent the prime cause enhancing glomerular membrane permeability, that could be responsible for increase in glomerular ultrafiltration. In the present circumstances RvLAAO alone caused a marked increase in UF, which suggest an estimate of glomerular filtrate delivered to renal tubules which may be a contributing factor by mechanism(s) not related to PAF action as mediator.

In addition, the effect of RvPDE alone increased in GFR and UF and sustained elevation throughout the perfusion time, which were not prevented by pretreatment with WEB 2086. It indicates that RvPDE may have renal glomeruli effect via mechanisms other than PAF receptor antagonism. Several evidences suggested that an increase in cyclic AMP levels in several tissues by either administration of vasopressin, parathormone, prostaglandins or infusion of dibutyryl cyclic AMP in micropuncture experiments (Baylis el al., 1976; Ichikawa and Brenner, 1977), decrease the glomerular ultrafiltration. The PDE plays an ancillary role in promoting catabolizing enzymes and hydrolyze the 3′-phosphoester bond of cAMP and cGMP (Dhananjaya and Souza, 2010). It is possible that an administration of exogenous RvPDE to the rabbit IPK, the hydrolytic activity of RvPDE might cause the reduction of cAMP/cGMP levels in renal glomeruli, leading to increase the glomerular ultrafiltration excluding to the role of PAF in renal glomeruli.

The studies on renal tubular functions of *whole* RVV and its venom fractions revealed that whole RVV administration alone showed increases in fractional Na^+ excretion (% $FENa^+$) with the significant effect at 90 min. period of the perfusion time, while fractional K^+ excretion (% FEK^+) showed tendency to increase but was not statistically significant. The effect of whole RVV on renal tubular sodium transport were consistent with results of previous studies in isolated perfused rat kidney (Ratcliffe et al., 1989). Although whole RVV caused increases in % $FENa^+$ and % FEK^+, it shows the opposite effect on GFR and UF, suggesting different mechanism in the regulation of urinary electrolytes excretion. Our previous studies demonstrated that the direct effects of whole RVV decrease absolute tubular Na^+ reabsorption of both proximal tubule and distal nephron in rabbit IPK using lithium as a marker for an evaluation (Chaiyabutr et al., 2014). Micropuncture

studies by measurement of the transmembrane potential in the proximal tubule of a Triturus kidney revealed that RVV causes depolarization in a dose-dependent manner (Chaiyabutr et al., 1985b), which might involve to an inhibition of Na^+-K^+-ATPase activity in renal tubules in both the renal cortex and medulla (Buranakarl et al., 1997). These findings may provide a mechanistic insight into the cellular events in modulation of ion channels activities (Sitprija and Sitprija, 2012), which whole RVV inhibits Na^+channel activity by which inactivation of Na^+-H^+ exchange (NHE3) in proximal tubules and Na^+ channels (ENaC) in the distal nephron. Subsequently, an elevation of increased cytosolic Na^+ would decrease Na^+ reabsorption through down regulation of NHE3 and ENaC at the apical border of renal tubules. Alternatively, RVV may directly inhibit NHE3 and ENaC activities. In addition to the reverse effects on %FENa^+ and % FEK^+induced by whole RVV were apparent after pretreatment with WEB 2086 throughout the perfusion time, indicating that there are PAF receptors present on the renal tubular epithelium to influence cellular processes. The effect of whole RVV on renal tubular transports of Na^+ and K^+ were mediated by the role of endogenous PAF influencing urinary electrolyte excretion independent of changes in GFR. The whole RVV in this study contained the protein mass from 10 -150 kDa which is similar range of 6-130 kDa of the Indian RVV (Kalita et al., 2018). This may be explained that the large molecular mass of venom proteins could not be filtered but to cause release of endogenous PAF by other glomerular cells that once filtered by kidney glomeruli could promote the renal tubular effects observed in this study. Thus, it is reasonable to postulate that PAF induced by whole RVV might not only act locally on a specific PAF receptor in glomerular and renal medullary interstitial cells (Asano et al., 1996), but also gain access to renal tubules through glomerular filtration, although the renal tubules do not produce PAF directly (Pirotzky et al., 1984b). The mechanism of whole RVV's action on urinary electrolytes excretion is probably mediated via PAF activity in regulation of ion channels activities. PAF action could inhibit the membrane Na-K-ATPase activity, which has been observed in a number of cell types (Catalan et al., 1994; Itadani et al., 1998). PAF inhibits solute reabsorption in the medullary thick ascending limb (mTAL) and collecting ducts via a cGMP-dependent pathway has also been noted (Bailly et al., 1992). The present data clearly demonstrate that WEB 2086 was able to prevent whole RVV effect on the renal tubular Na^+ and K^+ transports, which were mediated in part by PAF activity in inhibition of the membrane Na-K-ATPase activity. As described previously in this report, inhibition of membrane Na-K-ATPase activity by PAF can interfere the intracellular signaling cascade of ions channel in down regulation

of NHE3 and ENaC at the apical border of renal tubules. This is emphasized by the blockade caused by a selective platelet activating factor receptor antagonist, WEB 2086. However, the protein mass of whole RVV (10-150 kDa) may limit filtration to deliver to tubular fluid. It is probably that the relatively concentration of the whole RVV at least in the proximal part of the nephron may be occurred by secretory mchanisms in the tubular fluid via peritubular capillary. A further investigation is required to demonstrate patho-physiological significance of the cellular events in modulation of ion channels activities during envenomation. Our findings have provided more information by optical microscopy that direct acute effect of whole RVV on renal cells showed no distinct brush border in the proximal convoluted tubules, moderate degree of acute dilatation and severe tubulonephrosis (+3) (Fig.11 panels D, H). These histological alterations of renal cells by whole RVV are similar to that obtained previously by Chaiyabutr et al. (2014). The present data clearly demonstrate that pretreatment with WEB 2086 was able to prevent the deleterious effects promoted by whole RVV on % $FENa^+$ and % FEK^+. It is possible that the histological alterations affected by RVV may be reduced by WEB 2086, since it has been reported the role of PAF in nephrotoxic nephritis (NTN) that the PAF antagonists could reduce proteinuria and decrease the histopathological lesions (Livio, et al., 1986).

A number of studies on physiopathological alterations of renal tubular function induced by RVV fractions have not been described by far. In the present study, administration of RvMP fraction alone, the decreases in % $FENa^+$ and % FEK^+ throughout of the perfusion time showed different pattern of changes when compared to those effects by $RvPLA_2$, RvLAAO and RvPDE. The marked increase in GFR during administration of RvMP alone would account for the decreased fractional excretions of Na^+ and K^+. Moreover, it also showed the opposite effect on marked increases in UF and Cosm (Fig 7 panel E, F and Fig 9 panel C) and thus, these findings may provide a mechanistic insight into the ion renal tubular transport underlying RvMP property in the regulation of urinary electrolytes excretion. The hydrolytic property of either RvMP or $RvPLA_2$ may involve in direct acting on renal glomeruli and tubular cells leading to increase the cell membrane permeability resulting in the localized loss of ions homeostasis. Because a marked increase in GFR during administration of RvMP alone, increased amount of filtrate would be delivered to the tubular segment. Virtually all the Na^+ deliver out of the proximal nephon would be reabsorbed. It has been reported that the metalloprotease activity access renal tubular cells causing more degradation of plasma membrane and the cellular matrix proteins leading to loss

maintaining basement membrane structural integrity of kidney cells (Baramova et al., 1989). Optical microscopy demonstrates that direct acute effect of RvMP on renal cells showed dilatation and severe tubulonephrosis of both proximal and distal convoluted tubules including collecting ducts (Fig.13 panels B, D, F, H). Thus, changes in basement membrane structural integrity is probably cause leaky of tight junction which connect neighboring tubular cells and allows both Na^+ and K^+ pass directly between tubular fluid and extracellular fluid down their concentration gradients, thereby decreasing net urinary fractional Na^+ and K^+ excretion. Therefore, the permeability of the basal or apical membranes, or both, appears to differ considerably between $RvPLA_2$ and RvMP activity. The RvMP action on electrolytes transport can be explained by the paracellular pathway. It is known that transcellular Na^+ and K^+ movements across the renal tubular cells are regulated via ion channels activities of Na^+-H^+ exchange (NHE3) and Na^+ channels (ENaC) at apical membrane and Na^+-K^+-ATPase activity at basolateral membrane (Sitprija and Sitprija, 2012). The question arises as to whether these processes could obtain in IPK during administration among of $RvPLA_2$, RvMP, RvLAAO and RvPDE fractions. The possibility exists that the effects of administration either $RvPLA_2$ or RvLAAO alone adopted mainly transcellular routes for ion movements across the renal tubular epithelium which permit $\%FENa^+$ increase and $\%FEK^+$ decrease (Fig. 8 panels C, D, G, H). Therefore, it may be inferred that the basal pump activity during given either $RvPLA_2$ or RvLAAO fraction can cope with these changes. However, if the permeability of the tubular membrane is greater in the RvMP treatment, an increased rate of movement of both Na^+ and K^+ pass directly between tubular fluid and extracellular fluid down their concentration gradients might be expected and this might be greater than the transcellular active Na^+ and K^+transport. Thus, the effect of RvMP on ion transport can be interpreted by paracellular movements when changes in the reductions of both renal fractional excretions of Na^+ and K^+ occur under these conditions. After addition of either $RvPLA_2$ or RvMP, changes in basement membrane structural integrity was probably cause leaky of tight junction which Na^+ escaped into the urine might occur. It would create an osmotic diuretic effect resulting in an increase in osmolar clearance (Cosm) leading to increase in the rate of urine flow.

Reductions of both tubular fractional Na^+ and K^+ excretions by the effect of RvMP was prevented by WEB 2086 when it was given prior to RvMP administration. These results indicate that PAF played a role as mediator of RvMP-induced changes in the renal tubular Na^+ and K^+ transport in the rabbit IPK. Although, the kidney tubules do not produce PAF directly, but a

precursor has been identified and the activity of the enzyme acetyl-transferase has been shown in the glomeruli and in other kidney cells (Pirotzky et. al., 1984a). The size of $RvPLA_2$ and RvMP fractions have approximately 13 kDa and 90 kDa, respectively (Fig. 1) which are not filtered but to cause release of PAF by other glomerulular cells that once filtered by the glomeruli, could promote the tubular effects observed in this study.

The present studies were extended to demonstrate the tubular excretion of sodium and potassium during treatments of RvLAAO and RvPDE fractions. The treatment of RvLAAO alone showed slight increased % $FENa^+$ while % FEK^+ showed tendency to decline throughout period of the perfusion time. Administration of RvLAAO after pretreatment with WEB 2086 obtained renal fractional Na^+ and K^+ excretions in a similar manner as compared with that of administrations of RvLAAO alone. These findings suggest that PAF action did not involve on the renal tubular excretion of Na^+ and K^+ levels. These results differ from the study used LAAO from *Bothrops marajoensis* venom causing decreases in both Na^+ and K^+ tubular transport in isolated rat kidney (Dantas et al., 2015). It indicates that LAAO from different snake venom can be responsible for renal alterations caused by its respect venom. Since LAAO fraction from snake venom is flavoenzymes which catalyze stereospecific oxidative deamination of a L-amino acid to give rise to alpha keto acids, ammonia and hydrogen peroxide (Fox, 2013). The strong production of H_2O_2 via LAAO catalytic reaction (Bregge-Silva et al., 2012) may be a contributing factor enhancing membrane permeability of renal tubular cell that could be responsible for part of this effect to tubular Na^+ and K^+ transport by mechanism(s), which was not related to PAF mediator.

In addition, the maximal effects of administration of RvPDE alone caused increases in %$FENa^+$, % FEK^+ and osmolar clearance and sustained elevation throughout the perfusion time. These results were prevented by pretreatment with WEB 2086 resulting in reversal of % FEK^+ and osmolar clearance to basal control level, while progressive increase in % $FENa^+$ was still apparent. It indicates that RvPDE-induced generation of PAF as a mediator involvement of renal tubular transport of electrolytes, presumably as a result of inhibition of proximal tubular reabsorption. Although, PDE plays a role in promoting catabolizing enzymes and hydrolyze the 3′-phosphoester bond of cAMP and cGMP (Dhananjaya and Souza, 2010). Several studies demonstrated that cAMP and cGMP might play a role in the regulation electrolytes transport in the renal tubular cell. In micropuncture studies (Agus et al., 1971) and isolated perfused rat kidney (Monteiro et al., 1999), infusion of dibutyryl Db-cyclic AMP directly caused a significant decrease

in sodium transport at the proximal segment of the nephron. Thus, the hydrolytic property of RvPDE presumably cause reduction of cAMP/cGMP level in renal tubular cell leading to increase in the fractional tubular Na^+excretion. The RvPDE effect on %FENa^+ was not impressive when compared to those of % FEK^+ and osmolar clearance which were very efficiently blocked by WEB 2086, indicating that another pathway besides the G protein-coupled mechanism is also active in kidney tissues. However, evidence from an interaction taking place among the role of RvPDE on PAF and cAMP/cGMP in the controls the transport of ions across the tubular cell membrane is required to further study.

5. Conclusion

Identifying the mechanisms by which whole RVV and venom fractions of $RvPLA_2$, RvMP, RvLAAO and RvPDE induced renal alterations is a cause for AKI developing to ARF, somehow relating to a concomitant generation of PAF pathway and the specific PAF receptor antagonists (WEB 2086) is used to evaluate for such studies. Based on the fact that each venom fraction does not appear to act in a similar manner of changes in renal functions by which unlikely to account for the represent as whole RVV. The present study clearly indicates that changes in renal hemodynamics (i.e. PP and RVR) induced by either whole RVV or RvMP fraction could be mediated by a release of PAF, of which an increase in renal vasoconstriction in IPK perfused with RvMP presented the similar alterations being abolished by pretreatment of WEB 2086 as that of whole RVV. The changes in PP and RVR by the effects of $RvPLA_2$, RvLAAO and RvPDE could not be prevented by pretreatment with WEB 2086. Whole RVV caused significant decreases in GFR and UF, which were not prevented by pretreatment of WEB 2086, while $RvPLA_2$ and RvMP caused significant increases in GFR and UF and these effects were abolished by pretreatment with WEB 2086. Both RvLAAO and RvPDE showed its effects on increases in GRF and UF which these effects were not prevented by pretreatment of WEB 2086. Whole RVV caused increases in fractional Na^+excretion and fractional K^+ excretion by an action on renal tubules but had no apparent effect on Na^+ and K^+ transport after pretreatment with WEB 2086. The effects of $RvPLA_2$ and RvLAAO and RvPDE on increases in tubular Na^+ and K^+ transport would be due to its direct action on tubular membrane by mechanism(s) of transcellular rout which was not related to PAF mediator. The effect of RvMP on reductions of both tubular fractional Na^+ and K^+ excretions via a different mechanism being more emphasized by paracellular route, which were prevented by

pretreatment with WEB 2086. The present results, therefore, supported the hypothesis that RVV contain several different components that could act either individually or synergistically. Venom fractions isolated from RVV ($RvPLA_2$, RvMP, RvLAAO and RvPDE) were involved in the process of renal function alterations, and the renal effects observed would be due to the synergistic action of the different components of the venom, instead of the action of a single component.

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CHAPTER II

Proteomic comparison of venom proteins from juvenile, subadult and adult Russell's viper snakes

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Introduction

Envenomation by snakebites is a health problem and remains as a neglected tropical disease in many parts of the world by the World Health Organization (WHO). Russell's viper is considered as one of the most commonly encountered snakes and medically important species of venomous snakes. The phylogenetic analysis indicated that Russell's vipers constitute two distinct species, i.e. *Daboia russelii* in South Asia and *Daboia siamensis* in Southeast Asia. *Daboia siamensis* (*D. siamensis*) is one of the most important poisonous snakes which is distributed throughout Southeast Asian countries among many venomous snakes. Earlier studies from Myanmar were done by Myint-Lwin et al., (1985) reported that the clinical picture observed following bites by young and adult *D. siamensis* snakes varies, and over 50% of cases of *D. siamensis* bites are young snakes bite between two populations of *D. siamensis* (Tun-Pe et al., 1991). Further studies in biochemical and biological properties of *D. siamensis* venom by Tun-Pe et al., 1995 showed age-dependent variation. However, the variation in snake venom composition may be accompanied by different protein antigenicity that leads to the variation in clinical presentation. Although several studies in other snakes species have been reported on ontogenetic differences between venoms from adult and young snakes of the same species, variations about age-dependent, biological and biochemical features of venom extracts, differences in venom composition, toxicity, enzymatic activity, local effects and immunological aspects have been described (Minton, 1967, 1975; Fiero et al., 1972; Theakston and Reid, 1978; Meier and Freyvogel, 1980; Meier, 1986; Mackessy, 1988; Furtado et al., 1991; Tun-Pe et al., 1995; Mackessy et al., 2003; Mackessy et al., 2006; Arıkan et al., 2006; Guércio et al., 2006).

Regarding snake venom compositions contain an abundance of different toxic and nontoxic proteins, which many factors may cause of variation in venom compositions for examples taxonomic differences, age, geography, diet, seasonal variation, and sex-based variation (Chipaux et al., 1991; Chippaux, 2006; Mackessy, 2010). The detailed of the venom compositions profiled of *D. siamensis* venom has not yet been comprehensively determined, although a number of studies have described the difference in symptomatology after envenomation by *D. siamensis* from the

same species (Tun-Pe et al., 1995). Proteomic strategies have been applied to proteome profiling and comparative analysis in protein abundance, which show some extent among differences in terms of the protein type/family detected and the protein abundance quantitated, although it could also be partly confused. Knowledge of the venom compositions by the proteomic method would be very crucial to improve understanding of the venom complexity and variability. In addition, it has been reported in different types of snakes that the variation in snake venom composition may be accompanied by different protein antigenicity that leads to suboptimal immunoreactivity and weak neutralization by clinically used antivenoms (Oh et al., 2017; Wong et al., 2016). Moreover, the detailed characterization of individual and ontogenetic venom protein profile variations in juvenile, subadult and adult snakes, might throw some light on consideration of the necessity of using pooled venoms as a representative venom for antivenom production, the results may be of wider interest.

Based on this information whether venoms collected from *D. siamensis* in varying lengths and ages can show different action on both the functional and molecular compositional levels, further investigation is needed. Therefore, the aim to determine differences in the protein composition abundance among venoms from juvenile, subadult and adult of *D. siamensis*. Proteomic strategies were carried out to proteome profiling and comparative analysis in protein abundance. However, proteomic strategies have been mainly applied to proteome profiling and comparative analysis in protein abundance. Several regimens, including antivenom, have been tried to combat the severe effects of the toxin. The present study examined venom proteins on proteomic and biological characterization of *D. siamensis*. RVV specimens collected from Snake farm, QSMI, all crude lyophilized venoms obtained from varying ages and lengths of snakes from 3 different pooled venoms specimens. All venoms were analyzed with polyacrylamide disc gel electrophoresis, and their venom electrophoretic proteomic patterns were compared.

Materials and methods

Snakes and venom sample collections

Russell's viper (*Daboia siamensis*) snakes from eastern regions of Thailand were kept in captivity at the Snake Farm of the Queen Saovabha Memorial Institute (QSMI), Thailand. *Daboia siamensis* were maintained individually in plastic cages and were provided water ad libitum in the same animal care room in the Snake Farm. Once a month, the snakes are fed only with small

rodents in proportion to their weight (10–20% of the snake's body weight) All snakes were maintained under conditions of normal environmental temperature (average 27°C) and relative humidity (75%). Three groups of *D. siamensis* snakes both male and female in different lengths and ages in captivity were chosen for studies for juvenile (total length 22-27.5 cm), subadults (total length 53-74 cm) and adults (total length 76-127 cm). Snakes were weighed and measured body length at the time of each venom extraction in each group. Venom samples were extracted from 34 specimens, juvenile snakes after first shedding and first feeding of about 6 weeks old. Venom samples from subadult and adult snakes were obtained from 12 specimens and 85 specimens, respectively. The entire venom extracted by each snake was pooled into the glass vial specific for venom pool. The crude venom was lyophilized and stored at -20 °C until use. Lyophilized venoms were pooled from venoms of both sexes of *D. siamensis* by mixing amounts of samples from each specimen according to the length of the snakes.

Determination of venom enzymatic activities

The enzymatic activities of crude venoms from adult, subadult and juvenile of *D. siamensis* snakes were prepared based on methods described previously in detail (Chaiyabutr et al. 2020). Briefly, proteolytic activity for Metalloproteinase activity was determined using 2% casein in 0.5 M Tris-HCl pH 8.0 as substrate as described by Anson (1938). One unit of proteolytic activity was defined as the amount of enzyme hydrolyzing casein at an initial rate of Tyrosine 1.0 μM./min. Phospholipase A_2 activity was using 3 mM 4-nitro-3-(octanoyloxy) benzoic acid as a substrate. One unit of phospholipase A_2 activity is defined as the amount of enzyme caused a change of substrate in absorbance of 0.1 AU equivalent to 25.8 nmoles of chromophore release. (Holzer and Mackessy; 1996). The LAAO was carried out to determine activity according to Worthington Enzyme Manual (1977). One unit of L-amino acid oxidase activity was defined as the amount of venom caused the increment of 0.001 absorbance unit per min. The phosphodiesterase activity (PDE) was measured using bis (p-nitrophenyl) phosphate as substrate (Lo *et al.,* 1966). One unit of PDE activity was defined as the amount of enzyme that caused an increase of 0.001 absorbance unit per minute. The isolation of phospholipase A_2 from crude RVV by ion-exchange chromatography on HiTrap CMFF column (GE Healthcare, Sweden). Phospholipase A_2 activity was conducted as described by Holzer and Mackessy (1996). The isolation of metalloproteinase (MP). phosphodiesterase (PDE) and L-amino acid oxidase (LAAO) by gel filtration on Superdex^{TM} 75 10/300GL and column ion-exchange chromatography. Metalloproteinase activity

was conducted as described by Anson (1938). The LAAO was carried out to determine activity according to Worthington Enzyme Manual (1977). The PDE was carried out to determine activity as described by Lo *et al.*, (1966)

Venom and chemical analyses

Isolation and initial characterization of venom compositions

Venom compositions from adults, subadults and juveniles of *Daboia siamensis* were analyzed in two ways: first, by isolation and initial characterization of venom compositions and its venom enzymatic activities were performed for phospholipase A2 (PLA_2) and metalloproteinase (MP) as of dominant protein families and L-amino acid oxidase (LAAO) and phosphodiesterase (PDE) as minor protein families for comparative purposes among venoms from adult, subadult and juvenile *D. siamensis*. Second, by characterization and quantification of the venom proteomes, protein bands of interest (from Coomassie Brilliant Blue-stained TrisTricine 1DE) and protein spots (from 2-DE gels) were excised and submitted to in-gel reduction (10 mM dithiothreitol, 30 min at 65°C) and alkylation (50 mM iodacetamide, 2 h in the dark at room temperature), the differential protein and its composition in the venoms have been identified by two-dimensional electrophoresis (2DE). The *D.siamensis* venom proteins complexity was analyzed for an electrophoretic pattern by two dimensional (2D) SDS-PAGE on 12.5% polyacrylamide gels. The second part of venom analyses was performed on individual venom sample for protein extraction and quantification by proteomic analysis using mass spectrometry.

Two-dimensional polyacrylamide gel electrophoresis

The proteins of the venoms from juvenile, subadult and adult snakes were separated by two dimensional (2D) gels electrophoresis as previously described by Berkelman and Stenstedt, (1998**)**

First-dimensional isoelectric focusing (IEF). was performed using 150 µg of protein samples diluted in 125 µl of 60 mM DTT, 4% CHAPS and 0.5% of IPG buffer which were loaded into the 7 cm IPG gel strip containing a linear pH gradient from 3 to 10 (Immobilized pH Gradient (IPG), Amersham Bioscience Inc.) The 1st dimensional IEF in the solid phase with pH gradient was performed on Ettan IPGphor IEF System, (GE Healthcare Life Sciences, Buckinghamshire and UK). The running protocol was as recommended in the system instructions. The electrophoresis condition was:30 V for 12 h, 500V for 1 h,1000V for 1 h, 8000V for 8 h and 500 Vfor 4h.

Second dimensional SDS-PAGE: After IEF, the IPG gel strip was transferred to the 2nd dimensional SDS-PAGE (12.5% polyacrylamide gel). The electrophoresis was run under constant current 15mA/gel for 30 min and then 30 mA/gel until the bromophenol blue reached 0.5 cm from the bottom of the gel (Hofer SE 600, GE Amersham (GE Healthcare Life Sciences) Buckinghamshire and UK). Protein spots were visualized by Coomassie Blue R-250 staining and the following focusing conditions were used: 30 V for 6 h, 60 V for 6 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2 h. SDS-PAGE was done in a 16 cm 12% polyacrylamide gel. Coomassie blue was employed for protein staining.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

To analyze venom samples, two mg of crude, lyophilized venoms of juvenile, subadult and adult snakes were dissolved in lysis buffer (containing 1% Triton X-100 (Merck, Germany), 1%SDS, 1%NaCl. The venoms were measured for the protein concentration by Quick Start™ Bradford Protein Assay (Bio-Rad, Berkeley, CA, USA). The 30 µg of each venom were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, USA). The protein bands were visualized by Coomassie G-250 solution (Bio-Rad, USA). Whole gel lanes of snake venoms were cut into small pieces and kept at -80^{o}C until used.

Mass spectrometric analysis

Venom profiles of individual adults, subadults and juveniles were compared using the mass spectrometric analysis. Small pieces of whole gel lanes of snake venoms were destained by 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate was performed on gel pieces. Proteins were reduced by 4mM dithiothreitoland alkylated by 250 mMiodoacetamine (Sigma-aldrich, USA). The gel pieces were dehydrated by 100% ACN (Thermo Scientific, USA) and dried at room temperature. Tryptic digestion was performed by adding trypsin solution (Sigma-Aldrich, USA, T6567) at 1:100 ratio and incubated overnight at 37° C. The digested peptides were extracted by ACN and incubated for 15 minutes. Each supernatant was collected and dried using a centrifugal concentrator (TOMY, Japan). The samples were dissolved in 0.1% formic acid (Sigma-Aldrich, USA) and subjected to an Ultimate® 3000 Nano-LC systems (Thermo Scientific, USA) controlled by Chromeleon™ software version 7.2 (Thermo Scientific, USA). A microTOF-Q II (Bruker, Germany) was online coupled with the LC systems. All sample acquisitions were controlled by HyStar™ version 3.2 (Bruker, Germany). The data were processed and converted to mascot

generics files (.mgf) using Compass DataAnalysis™ software version 3.4 (Bruker, Germany) and performed database searches using Mascot Daemon software (Matrix Science, USA) against the NCBI Chordata database. Only proteins at 95% significance threshold were reported in this paper. The exponentially modified protein abundance index (emPAI) was used for semi-quantification (Ishihama et al. 2005). The proteins with more than two-fold difference in at least two biological replications were reported as differential proteins.

Results and discussion

The compositions of venom are compared the difference among venoms from adult, subadult and juvenile snakes of *D. siamensis*. Considering the characteristics of enzymatic activity of venoms from adult, subadult and juvenile snakes of *D. siamensis.* (Table 1, Figure 1). phospholipase A_2, protease, L-amino acid oxidase and phosphodiesterase for this analysis, the sample enzymatic activity showed slightly higher phospholipase A_2 activity in juvenile while L-amino acid oxidase and phosphodiesterase were lower as compared to others. Most of the venom protein from juvenile snakes had a large number of higher molecular weight protein bands (>10,000) than adult and subadult. The venom patterns of the adult were similar electrophoretically to that of subadult snakes (Figure 1)

Table1. A comparative profile of enzyme activities in a different age of *D.siamensis*

D.siamensis venom	Phospholipase A_2 (µM/mg)	Protease (U/mg)	L-Amino acid oxidase (U/mg)	Phosphodiesterase (U/mg)
Adult	4.47	0.0048	0.05	0.5
Subadult	4.71	0.0037	0.04	0.5
Juvenile	4.83	0.0046	0.02	0.2

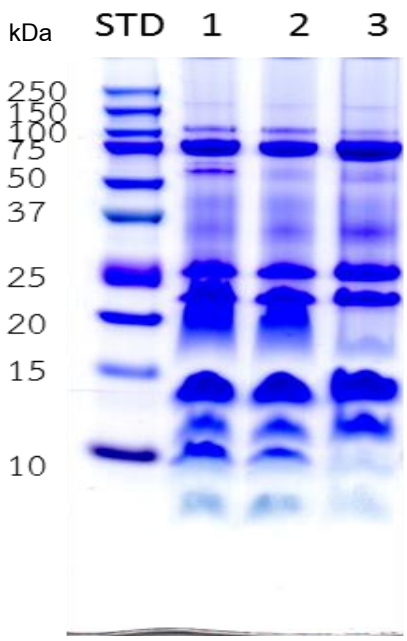

Figure 1. The SDS-PAGE patterns of all venoms were variable in terms of the total number of protein bands observed from adult (lane1), subadult (lane 2) and juvenile snakes (lane 3).

2-D gel electrophoresis of all *D.siamensis* venom and protein family compositions

The proteomic compositions of *D.siamensis* venom were investigated by 2-D electrophoresis and the separated protein bands were observed. The isolated toxins were assigned to the following protein families: serine protease (SVSP), metalloprotease, phospholipase A_2 L-amino acid oxidase and vascular endothelial growth factors (VEGFs) were identified. However, the present of low molecular mass less than 10 kDa, disintegrin cannot be excluded because we identified protein of more than 10 kDa (Figure 2). In all age have mostly the same compositions in the venom but differ in concentration which varied among age, especially some group of SVSP that was not apparent in adult venom.

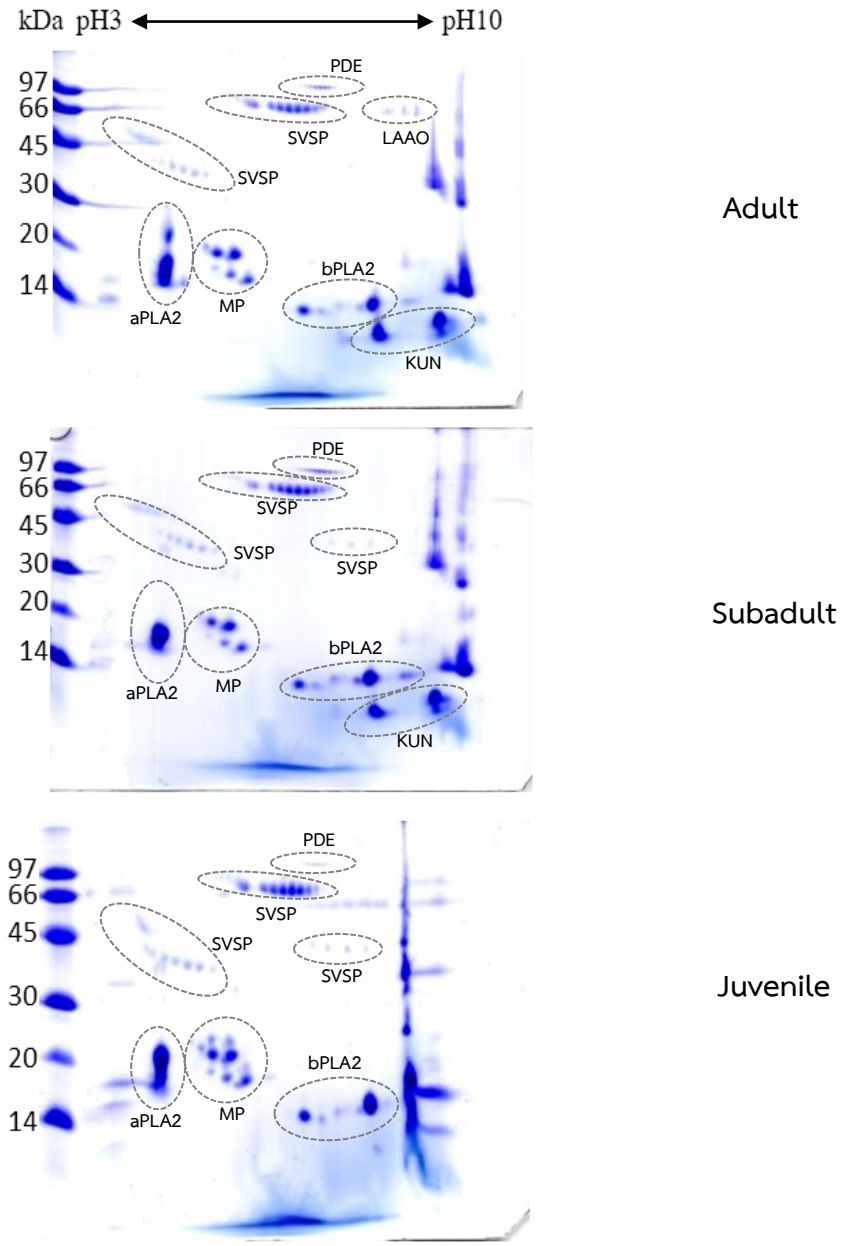

Figure 2. 2D-SDS-PAGE of venom proteins from adult, subadult and juvenile DS snakes. A total of 200 µg of total proteins from pooled venoms were isoelectrically focused (pI range 3-11) followed by separation by SDS-PAGE and Coomassie blue staining. Distinctly expressed protein spots among the three-length population are labelled. Molecular mass markers (in kDa) are indicated at the left of each gel.

Proteome alignment profiles

Proteome alignment profiles by Venn diagram analysis depicting the co-expressed (common region) and uniquely expressed proteins (green region, pink region and blue region)

discovered in the venoms of the adult, subadult and juvenile of *D. siamensis*. The present study, *D. siamensis* venom presented a total of 468 proteins across three groups of snakes. Of this total, 48 proteins were common in snake venoms of three ages as shown in Figure 3 and Supplementary table 2. Considering the proteins differentially or uniquely expressed in the groups of snakes (quantified and/or only identified), 130 proteins were observed in the juvenile group, 122 proteins in the adult group and 130 proteins were found in the subadult group. In relation to the common proteins, all groups of snakes presented 48 proteins equally distributed, 18 proteins were found between the juvenile and the adult snake, 15 proteins were common between the study of subadult and adult groups, and 5 proteins were present in the juvenile group and the subadult group (Figure 3).

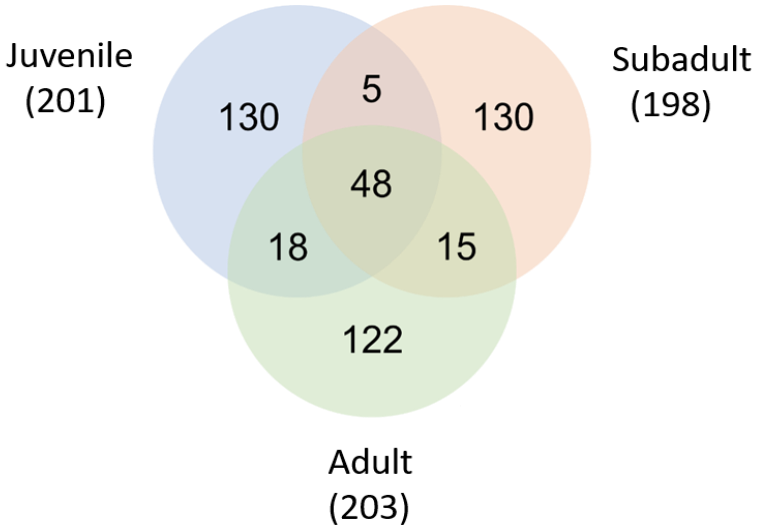

Figure 3. Venn diagram analysis of overlapping proteins showing the relation of exclusivity and interconnection of proteins found among venoms of juvenile, subadult and adult *D. siamensis* snakes.

The top-twenty most abundant proteins in venoms of juvenile, subadult and adult snakes are demonstrated in Figure 4-6 and Table 2. According to protein quantification, phospholipase A2 was extremely high in all snake venoms. The serine protease inhibitor was also high in venoms of all ages. Moreover, fibrinogenase and metalloproteinase were also found in a high proportion of snake venoms. Given that sequences of *D. siamensis* proteins are available in a sequence database, in different aging of the *D. siamensis* (Table 2). Since several snake venom proteins share high sequence similarity and analyzed by MS cover only a small fraction of their sequences, it was not possible to unequivocally determine the protein homologues from the sequenced

different aging of snakes. 2DE analysis of the spot with apparent MW of 10-20 kDa (Fig. 1, 2 and Table 1) that was present in similar quantities in adult, sub-adult and juvenile gels is presented here as an example. SDS-PAGE patterns of PLA2 the best matches corresponded to the adult and subadult(Fig.1). One of the determined sequences (LAAO) in the 2D-SDS-PAGE is present in both the adult and subadult but not in juvenile. (Fig 2). Comparison of the 2DE gels suggested that the concentration of LAAO increased during ontogenetic development.

However, the result of venom proteins only at 95% significance threshold were reported by the exponentially modified protein abundance index (emPAI) for label free quantification showed that emPAI for phospholipase A2 of juvenile (816) nearly four time higher than adult *D. siamensis*. Other venom protein profiles for example, basic Phospholipase A2 vipoxin B chain (1.04), Kunitz-type serine protease inhibitor 3 (0.37), snake venom serine protease gussurobin (0.12) and group III snake venom metalloproteinase (0.10) were apparent in the venom from juvenile snake only (Figure 4-6, Table 2).

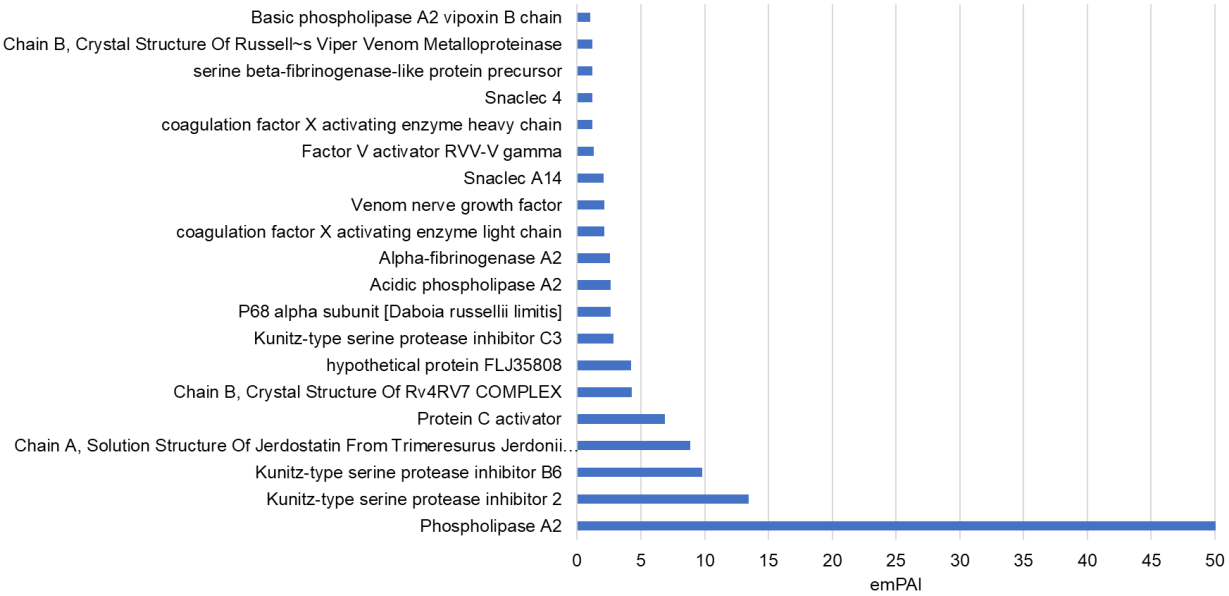

Figure 4. Top-twenty most abundant proteins in venom of juvenile *D. siamensis* snake

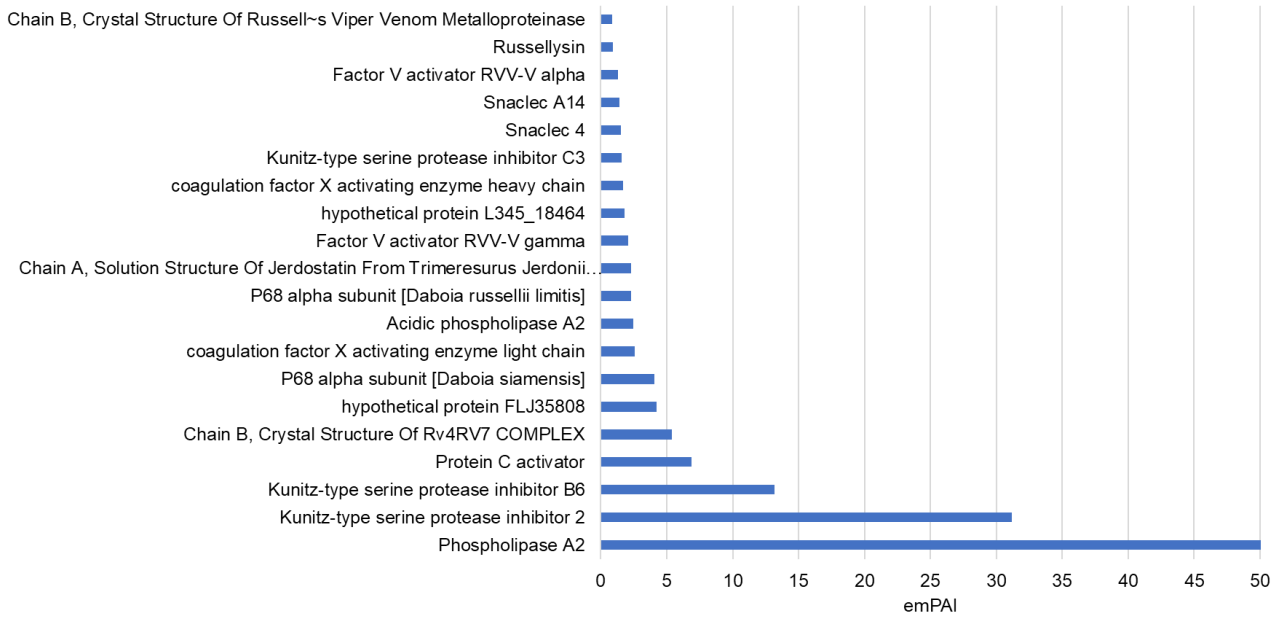

Figure 5. Top-twenty most abundant proteins in venom of subadult snake

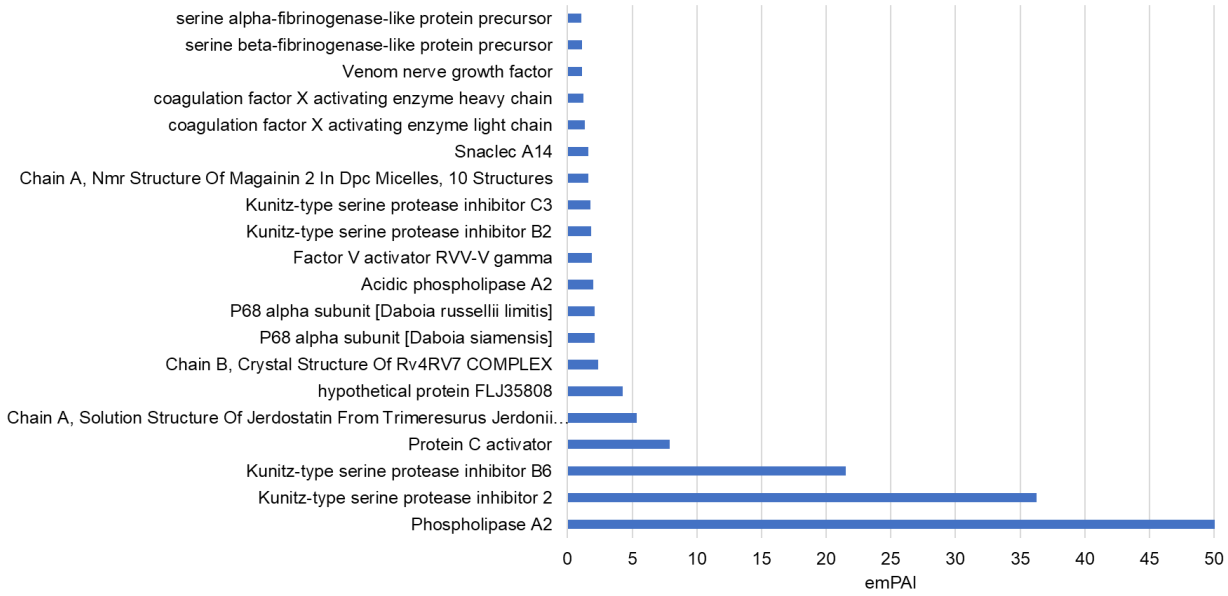

Figure 6. Top-twenty most abundant proteins in venom of adult snake

Table 2. Quantification of toxins in venoms from juvenile, sub adult and adult *D. siamensis* snakes

GI Number (Id)	Protein	emPAI		
		juvenile	sub adult	adult
gi|298351761	Phospholipase A2	816.07	816.07	266.23
gi|125041	venom basic protease inhibitor 2	13.45	31.19	36.25
gi|239977258	Trypsin inhibitor 6	9.83	13.16	21.5
gi|461511	Snake venom serine protease	6.9	6.9	7.91
gi|239977252	Trypsin inhibitor 3	2.84	1.57	1.79
gi|400714	Viperotoxin non-tox	2.61	2.49	1.97
gi|82116517	Alpha-fibrinogenase A2	2.57	N/A	N/A
gi|251205	metalloproteinase with disintegrin (platelet aggregation inhibitor)-like	2.13	2.57	1.33
gi|400499	venom nerve growth factor	2.13	0.58	1.12
gi|134130	Russell's viper venom FV activator gamma	1.31	2.1	1.87
gi|251204	metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and C-type lectin-like domains	1.22	1.71	1.24
gi|162329888	Chain B, Crystal Structure Of Russell's Viper venom Metalloproteinase	1.17	0.84	0.47
gi|129419	Basic Phospholipase A2 vipoxin B chain	1.04	N/A	N/A
gi|239977246	Trypsin inhibitor 1	0.9	N/A	0.92
gi|239977245	Trypsin inhibitor B1	0.41	0.37	0.37
gi|239977248	Kunitz-type serine protease inhibitor B2	0.41	N/A	1.82
gi|13959655	venom serine proteinase-like protein 2	0.39	0.12	0.13
gi|123913155	Kunitz-type serine protease inhibitor 3	0.37	N/A	N/A
gi|2851544	Vipoxin acidic component	0.27	0.59	0.56
gi|380875421	Beta-fibrinogenase	0.27	0.26	0.28
gi|425936533	Acidic Phospholipase A2 Cbl alpha	0.25	0.26	0.28
gi|380875417	Snake venom serine protease	0.25	0.12	0.13
gi|123915726	Basic Phospholipase A2 chain HDP-1P	0.24	0.23	N/A
gi|327478537	Snake venom vascular endothelial growth factor toxin	0.23	0.48	0.46
gi|73621852	Snake venom metalloproteinase	0.15	0.15	0.17
gi|134129	Russell's viper venom FV activator alpha	0.13	1.31	1.05
gi|82106261	Snake venom serine protease gussurobin	0.12	N/A	N/A
gi|387935408	Snake venom serine protease nikobin	0.12	N/A	0.13
gi|320579347	group III snake venom metalloproteinase	0.10	N/A	N/A
gi|82228619	Snake venom metalloproteinase	0.05	0.05	0.05
gi|215273878	Snake venom metalloproteinase	N/A	0.91	N/A
gi|58177210	Chain A, Crystal Structure Of Vr-1, A Vegf-F From A Snake venom	N/A	0.31	N/A
gi|381141431	Snake venom serine protease	N/A	0.12	N/A
gi|83523646	Group III snake venom metalloproteinase	N/A	0.05	N/A
gi|602696309	cytosolic Phospholipase A2 zeta	N/A	0.03	0.03

N/A: none of data

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CHAPTER III

Comparative studies between the effects of juvenile, subadult and adult Russell's viper venoms on renal functions in in vivo studies in rabbits.

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Introduction

Envenomation by snakebites is a health problem and remains as a neglected tropical disease in many parts of the world by the World Health Organization (WHO). Russell's viper is considered as one of the most commonly encountered snakes and medically important species of venomous snakes. The phylogenetic analysis indicated that Russell's vipers constitute two distinct species, i.e. *Daboia russelii* in South Asia and *Daboia siamensis* in Southeast Asia. *Daboia siamensis* (*D. siamensis*) is one of the most important venomous snakes which is distributed throughout Southeast Asian countries among many venomous snakes. It is one of the common biters capable of delivering lethal venom and responsible for a substantial number of deaths which the development of acute kidney injury (AKI) was an important clinical event and associated significantly with mortality (Alfred et al., 2019). The most common complication amongst lethal cases after *D. siamensis* bite is the process of acute renal failure (ARF), but its pathogenesis is not well understood. Earlier studies from Myanmar were done by Myint-Lwin et al., (1985) reported that the clinical picture observed following bites by young and adult *D. siamensis* snakes varies, and over 50% of cases of *D. siamensis* bites are young snakes bite between two populations of *D. siamensis* snakes (Tun-Pe et al., 1991). Further studies in biochemical and biological properties of *D. siamensis* venom by Tun-Pe et al., 1995 showed age-dependent variation. However, the variation in snake venom composition may be accompanied by different protein antigenicity that leads to the variation in clinical presentation. The degree of intraspecific venom effects from varying ages of *D. siamensis* snakes inducing AKI remains unclear. Even at the clinical picture observation following bites by *D.siamensis* snakes which the severity of symptoms after envenomation would expect to depend on both for the toxic components present in its venom and for the inoculated volume. The mechanisms of venom action within the body to induce especially AKI during envenomation of varying size and ages of *D. siamensis* have not been determined, although several studies in other snakes species have been reported on ontogenetic differences between venoms from adult and young snakes of the same species, variations about age-

dependent, biological and biochemical features of venom extracts, differences in venom composition, toxicity, enzymatic activity, local effects and immunological aspects have been described (Minton, 1967, 1975; Fiero et al., 1972; Theakston and Reid, 1978; Meier and Freyvogel, 1980; Meier, 1986; Mackessy, 1988; Furtado et al., 1991; Tun-Pe et al., 1995; Mackessy et al., 2003; Mackessy et al., 2006; Arıkan et al., 2006; Guércio et al., 2006).

Based on this information whether venoms collected from *D. siamensis* snakes in varying lengths and ages can show different action on both the functional and molecular compositional levels, further investigation is needed. Therefore, this study aimed to elucidate the underlying pathophysiological mechanisms of venoms from juvenile, subadult and adult *D. siamensis* snakes whether the action difference exists in the venoms affected to renal alterations in rabbit kidney in vivo.

Materials and Methods

Animals

Adult male white New Zealand rabbits, weighing 2 to 3 kg, were used for experimental animals *in vivo* studies. Animals were obtained from the Animal House, Queen Saovabha Memorial Institute (QSMI) and were settled in stainless steel cages, received water and fed a standard diet ad libitum, exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 26 ± 1 ^{o}C. The animals were quarantined for 14 d before experiments. *In vivo* experiments were performed in accordance with the permission of the Ethics Committee of the Queen Saovabha Memorial Institute Animal Care and Use (approval number QSMI-ACUC-03-2016) under the guideline of the National Research Council of Thailand.

Snakes and venom sample collections

Russell's viper (*Daboia siamensis*) snakes from eastern regions of Thailand were kept in captivity at the Snake Farm of the Queen Saovabha Memorial Institute (QSMI), Thailand. *Daboia siamensis* snakes were maintained individually in plastic cages and were provided water ad libitum in the same animal care room in the Snake Farm. Once a month, the snakes are fed only with small rodents in proportion to their weight (10–20% of the snake's body weight) All snakes were maintained under conditions of normal environmental temperature (average 27°C) and relative humidity (75%). Three groups of *D. siamensis* snakes both male and female in different lengths

and ages in captivity were chosen for studies for juvenile (total length 22-27.5 cm), subadults (total length 53-74 cm) and adults (total length 76-127 cm). Snakes were weighed and measured body length at the time of each venom extraction in each group. Venom samples were extracted from 34 specimens, juvenile snakes after first shedding and first feeding of about 6 weeks old. Venom samples from subadult and adult snakes were obtained from 12 specimens and 85 specimens, respectively. The entire venom extracted by each snake was pooled into the glass vial specific for venom pool. The crude venom was lyophilized and stored at -20 °C until use. Lyophilized venoms were pooled from venoms of both sexes of *D. siamensis* by mixing amounts of samples from each specimen according to the length of the snakes.

Animal Preparation

On the day prior to experimental study, adult male white New Zealand rabbits. were deprived of food but not of water for 12 hours. The animals were anaesthetized with sodium pentobarbital (50 mg/kg) by intravenous injection. The animal was tracheotomized for free the airway with an endotracheal tube. The jugular vein was cannulated with polyethylene tubes (PE 100) for infusion of the solution and renal clearance studies. The carotid artery was cannulated with a polyethylene tube (PE 100) for the recording of blood pressure and heart rate (Polygraph Model 79, Grass Instruments Co.) the left ureter was cannulated with a polyvinyl catheter (PV 120) via a retroperitoneal approach for urine collection.

Venom dose and administration protocol

A pool of Russell's viper (*Daboia siamensis*) venom was obtained from adult male and female *Daboia siamensis* snakes collected from the eastern region of Thailand and maintained at Queen Saovabha Memorial Institute. An earlier investigation in experimental animals in either dogs or rabbits, where the dosage of crude RVV in the lyophilized form that caused death in 50% of subjects (LD_{50}) by intravenous injection was 0.5 mg/kg body weight (Chaiyabutr et al., 2014; Tungthanathanich et al., 1986). However, a single venom dose of 0.1 mg/kg was arbitrarily chosen in the present work in rabbit based on preliminary experiments in which doses of 0.1 and 0.5 mg/kg were tested. Rabbits injected with 0.1mg/kg showed minimal systemic and renal function alterations, whereas those receiving 0.5 mg/kg generally died within a few minutes or hours after venom administration — this short survival time precluded adequate assessment of changes in renal functions. Therefore, the dose of 0.1 mg/kg provided the best combination of renal damage

(assessed histologically) concerning to survival time for3-4 h. Thus, a single venom dose of 0.1 mg/kg by intravenous injection was used in the present study.

Renal function

Three groups of male white New Zealand rabbits (n=4/group) were injected with lyophilized venom (0.1 mg/kg, i.v.) in 1 ml of 0.15 M NaCl in each group from those venoms of juvenile, subadult and adult of *D. siamensis*. In all groups, animals were anesthetized with pentobarbital sodium (50mg/kg i.v.) and after 30 min. equilibration time for renal function study. The control period for renal clearance studies, and circulatory measurements were begun before envenomation with venoms from juvenile, subadult and adult of *D. siamensis*. Urine collections along with arterial blood sampling at the midpoint of the urine collection were done. All changes were recorded at 5, 10, 30, 60, 90 and 120min. after envenomation. Systemic and renal hemodynamics including mean arterial blood pressure (MAP), heart rate (HR), packed cell volume (PCV), urine flow, inulin (In) clearance, para-aminohippuric acid (PAH) clearance, and urine electrolytes excretion and evaluation of haematological parameters were carried out. At the end of the experiment, animals were euthanasia with a high dose of pentobarbital sodium after which the kidneys were removed and immediately immersed in the appropriate fixative for further tissue processing.

Calculations for renal function

The study of both Inulin and PAH clearances was performed by the standard technique previously described (Chaiyabutr et al., 1984). Renal clearance (C) was calculated by a standard formula (C=UV/P) using the plasma and urine inulin and PAH levels for each period, where U = urinary concentration, V = urinary volume and P = plasma concentration. Inulin clearance was used to estimate the glomerular filtration rate and PAH clearance (CPAH) was used to estimate effective renal plasma flow. Fractional sodium ($FENa^+$) and potassium (FEK^+) excretions were calculated as CNa^+/Cin and CK^+/Cin, respectively.

Chemical analysis : The inulin concentration in both plasma and urine was determined by the anthrone method, which modified the method of Young and Raise (1952). The determination of PAH concentration in plasma and urine was carried out by the method of Bratton and Marshall as described by Smith (1962). The Na^+ and K^+ ion concentrations were determined by flame photometer (Flame Photometers, Laboratory Instrument, BWB Technologies UK Ltd), while the

osmolality was measured using an osmometer (Fiske® Micro-osmometer Model210, Fiske® Associates, Norwood, Massachusetts, 02062, USA). Estimation of the concentration of plasma urea was determined using a spectrophotometer (Coulombe and Fovreau, 1963). Plasma creatinine Creatinine was determined by the alkaline picrate method [Smith 1962]

Evaluation of haematological parameters

Haematological parameters were carried out for hemoglobin concentration (Hb) as well as, determination of red blood cells (RBCs), white blood cells (WBCs) and platelets count were performed by Auto-Hematology Analyzer Operation Manual (Mindray BC-5000 Vet, MindrayBio-medical Electronics Co. Ltd. Nanshan Shenzhen, China

Histological analysis.

At the protocol indicated above, the kidneys were removed and cut sagittally before fixation in buffered (pH 7.2) 10% formol solution for 48 h, followed by dehydration in a graded ethanol series and embedding in Histosec Kidney sections 5 μm thick were stained with H&E and periodic acid-Schiff, PAS.

Statistical Analysis

The results are expressed as mean ± one standard deviation (SD). One-way ANOVA with repeated measures were used with Bonferroni's post-hoc test to compare the number of changes among time points after the venom treatment within the same group. Comparisons between the two sets of data at each specified time point of the venom treated groups were performed by unpaired Student's t-test. Significance was accepted at the $P < 0.05$ level. All data were analyzed by GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Effects on general circulation (Table 1)

The present results show that mean arterial blood pressure (BP) markedly, decreased within 5 min. following the venom injection in rabbits. After a transitory decrease up to the 20 min, it gradually increased and, by 30 to 80 min. approached to the control level and maintained a lower level (Table 1)). Heart rate did not significantly change throughout the experimental period. The behavior of the mean arterial blood pressure responses showed differences among rabbits treated

with different types of venoms. The decrease in blood pressure of rabbits treated with juvenile venoms was apparent within 5 min. following the venom injection showed higher extent as compared with those of rabbits treated with either adult or subadult.. There was no significant change in packed cell volume (PCV), plasma osmolarity , osmolar clearance (Cosm) and free water clearance (CH_2O) after envenomation with all types of snake venoms.

Effects of snake venom on renal hemodynamics (Table 2)

Significant decreases in the glomerular filtration rate (GFR), the rate of urine flow (V) and effective renal plasma flow (ERPF) and effective renal blood flow (ERBF) were seen as compared to the control value especially at first 10 minutes, while renal vascular resistance (RVR) increased in stepwise fashion after envenomation in all groups (Table 2.). The filtration fraction (FF) showed no significant alterations throughout 120 minutes of experimental period after envenomation in all groups.

Effects of snake venom on the plasma electrolyte concentration, urinary electrolyte (Table3)

After administrations of either adult, subadult or juvenile *D.siamensis* venom, there were no differences in the plasma concentration of sodium (P_{Na}) after envenomation as compared to the control values, whereas the plasma concentration of potassium (P_K) increased stepwise with significance at 120 min after envenomation. The urinary excretion and fraction excretion of sodium ($U_{Na}V$, FE_{Na}) showed a stepwise decrease after envenomation with either adult, subadult or juvenile *D.siamensis* venom, whereas excretion and fraction excretion of potassium (U_KV, FE_K) showed a stepwise increase after envenomation as compared with control period.

Table 1 Changes in MAP, HR, PCV in experimental rabbits in response to different types of Russell's viper venoms administration.

	MAP (mmHg)	**HR (beats/min**	**PCV (%)**	**POsm (mEq/L)**	**Cosm (ml/min)**	**CH_2O (ml/min)**
Gr.I (Juvenile)						
Control	93.3±10.6	248±13	37.2±3.5	299.67±10.93	0.60±0.20	-0.24±0.14
5 min after RVV	53.3±14.4*	230±12	38.6±2.1	309.33±26.07	0.60±0.26	-0.30±0.09
10 min. after RVV	66.9±9.2*	231±11	39.2±2.7	305.00±19.62	0.19±0.20	-0.09±0.09
30 min. after RVV	84.2±16.4	230±8	37.9±1.7	296.00±7.80	0.30±0.18	-0.15±0.08
60 min. after RVV	91.8±12.6	225±12	36.1±1.1	322.00±45.07	0.32±0.13	-0.14±0.05
90 min. after RVV	94.3±13.5	228±11	37.8±3.2	306.33±14.9	0.36±0.14	-0.08±0.09
120 min. after RVV	102.2±11.7	221±16	36.8±4	312.67±24.01	0.42±0.17	-0.12±0.09
Gr.II (Subadult)						
Control	94.8±5.1	248±10.2	38.2±2.1	288.00±2.31	1.22±0.39	-0.37±0.24
5 min after RVV	45.2±7.8*	203±3.0	36.7±2.2	290.00±5.77	1.20±0.20	-0.47±0.05
10 min. after RVV	57.8±5.1*	228±4.9	36.7±2.2	290.00±5.77	0.18±0.07	-0.07±0.03
30 min. after RVV	62.7±10.1*	233±19.2	36.6±1.7	326.00±53.12	0.29±0.13	-0.14±0.06
60 min. after RVV	68.3±16.4	231±21.1	35.5±3.4	289.50±0.58	0.23±0.08	-0.11±0.09
90 min. after RVV	78.1±11.0	221±43.4	34.9±2.3	289.00±3.46	0.35±0.06	-0.16±0.03
120 min afterRVV	80.0±11.9	222±41.6	36.4±1.7	286.00±3.46	0.61±0.30	-0.22±0.05
Gr.III (Adult)						
Control	102.2±9.0	255±28	36.3±5.4	310.67±22.35	1.16±0.61	0.11±0.56
5 min after RVV	71.9±9.6*	244±36	36.2±3.1	306.00±10.32	0.56±0.40	-0.14±0.11
10 min. after RVV	81.7±6.5	246±44	35.4±3.9	301.67±5.24	0.40±0.19	-0.20±0.13
30 min. after RVV	82.8±13.2	245±35	34.4±3.9	300.00±9.34	0.56±0.19	-0.25±0.14
60 min. after RVV	80.8±11.8	206±30	32.5±5.3	303.67±16.06	0.27±0.20	-0.16±0.11
90 min. after RVV	85.8±11.6	218±27	32.9±4.9	299.00±7.10	0.21±0.09	-0.13±0.06
120 min. after RVV	90.0±11.0	211±55	31.6±6	319.00±31.84	0.24±0.07	-0.12±0.04

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Table 2. Glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), filtration fraction (FF) and urine flow rate (V) in experimental rabbits treated with different types of *D. siamensis* venoms**.**

	V (ml/min)	**GFR (ml/min)**	**ERPF (ml/min)**	**ERBF (ml/min)**	**RVR (mmHg/ ml/min)**	**FF (%)**
Gr.I (Juvenile)						
Control	0.32±0.16	5.78±1.02	35.4±23.57	56.83±39.65	2.07±0.83	20.14±8.71
5 min. after RVV	0.30±0.18	4.92±0.99	32.29±19.85	43.00±34.62	1.82±1.07	19.55±9.32
10 min. after RVV	0.10±0.11*	1.81±1.49*	12.73±16.64*	21.39±28.67*	8.98±6.74*	20.44±9.37
30 min. after RVV	0.15±0.11*	3.17±1.19*	21.69±16.45	35.04±27.35	3.65±2.44	19.14±9.73
60 min. after RVV	0.19±0.11*	4.42±1.26	31.20±31.7	49.22±50.81	2.75±1.15	20.44±9.74
90 min. after RVV	0.28±0.17	3.72±0.87	29.70±24.76	48.89±41.8	3.34±2.56	19.36±11.29
120 min. after RVV	0.30±0.21	4.05±0.93	30.09±26.96	49.95±47.38	3.72±2.16	22.79±13.92
Gr.II (Subadult)						
Control	0.85±0.33	6.90±1.39	31.16±8.94	50.14±12.75	1.96±0.39	22.74±4.72
5 min. after RVV	0.73±0.15	6.72±0.58	28.49±6.77	28.49±6.77*	1.71±0.68	24.34±4.40
10 min. after RVV	0.12±0.04*	1.09±0.33*	4.28±1.60*	6.70±2.26*	9.09±2.08*	26.15±4.12
30 min. after RVV	0.15±0.07*	4.02±0.54*	22.66±4.21	35.62±5.89	1.83±0.57	17.87±1.29
60 min. after RVV	0.12±0.01*	2.47±1.41*	8.97±4.96*	14.19±8.31*	6.31±3.77*	27.47±1.79
90 min. after RVV	0.19±0.04*	4.28±0.45	21.05±3.27	32.41±5.67	2.50±0.78	20.97±5.40
120 min. after RVV	0.40±0.27	5.22±0.80	20.43±6.37	32.06±9.63	2.75±1.17	26.58±4.65
Gr.III (Adult)						
Control	1.26±0.90	11.39±5.16	22.49±4.95	32.15±14.02	5.28±6.04	31.11±9.46
5 min. after RVV	0.42±0.39*	4.67±2.83*	14.48±3.69*	17.87±7.59*	6.85±7.65	30.63±13.71
10 min. after RVV	0.20±0.08*	7.10±8.96*	33.21±47.72	54.01±80.01	6.03±8.17	31.29±21.46
30 min. after RVV	0.32±0.13*	5.86±2.37*	24.25±21.17	38.16±35.62	3.48±1.76*	30.46±13.07
60 min. after RVV	0.11±0.10*	2.93±1.93*	11.61±6.64*	14.76±9.45*	7.76±5.58	23.45±6.39
90 min. after RVV	0.09±0.04*	2.64±1.05*	11.09±4.43*	13.90±5.25*	7.64±4.57	23.88±2.95
120 min. after RVV	0.12±0.05*	3.30±0.59*	13.32±2.47*	17.04±4.57*	5.60±1.80	24.88±1.74

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Table 3. Plasma levels (P), urinary excretions (UV) and fractional excretions (FE) of sodium ion and potassium ion in experimental rabbits treated with different types of Russell's viper venoms.

	P Na^+ (mEq/L)	UV Na^+ (μEq/min)	FE Na^+ (%)	P K^+ (mEq/L)	UV K^+ (μEq/min)	FE K^+ (%)
Gr.I (Juvenile)						
Control	137.23±3.13	65.90±29.92	8.61±3.87	1.9±0.1	3.9±0.9	38.03±13.47
5 min. after RVV	135.03±2.05	66.76±33.22	9.90±4.44	2.0±0.0	4.2±1.7	42.98±15.42
10 min. after RVV	138.67±7.04	18.28±24.79	8.68±6.72	2.0±0.1	3.2±1.5	33.79±22.57
30 min. after RVV	139.53±6.38	28.88±22.68	6.84±4.22	2.4±0.2	3.3±1.4	42.80±9.31
60 min. after RVV	139.60±6.37	27.22±16.73	4.80±3.26*	2.3±0.1	4.0±0.4	43.92±18.25
90 min. after RVV	139.10±5.68	31.76±19.14	6.51±3.63	2.5±0.2*	3.4±0.9	38.53±12.94
120 min. after RVV	142.73±8.59	34.56±17.54	6.21±2.91	2.5±0.1*	5.0±2.4	51.50±27.59
Gr.II (Subadult)						
Control	131.60±3.46	150.73±48.5	16.61±3.53	2.0±0.0	5.6±1.4	40.72±8.12
5 min. after RVV	134.65±4.45	151.56±33.6	16.60±2.23	1.8±0.1	5.3±0.7	44.41±6.81
10 min. after RVV	134.65±4.45	22.78±9.78	15.35±3.64	1.8±0.1	3.7±0.1	35.85±7.79
30 min. after RVV	128.30±2.77	31.85±15.82	5.94±2.11*	1.7±0.1	3.9±0.3	57.84±9.11
60 min. after RVV	132.75±3.06	23.71±5.56	8.98±4.08*	1.8±0.2	2.2±1.4*	46.03±6.67
90 min. after RVV	134.70±0.69	35.19±8.73	6.09±1.28*	2.1±0.4	3.6±0.7	42.55±13.69
120 min. after RVV	132.80±2.42	70.96±46.06	9.58±5.01*	2.0±0.3	3.7±0.6	36.66±6.49
Gr.III (Adult)						
Control	139.97±6.73	155.04±76.2	11.12±4.23	2.1±0.3	8.7±2.5	45.88±12.96
5 min. after RVV	141.10±7.39	65.96±56.56	9.21±3.74	2.2±0.4	3.3±2.2	34.69±11.43
10 min. after RVV	139.83±6.87	46.87±22.64	8.89±6.58	2.0±0.2	3.1±2.2	38.25±30.37
30 min. after RVV	137.10±9.84	68.95±26.31	8.68±1.70	2.1±0.2	5.5±4.0	43.05±9.02
60 min. after RVV	140.27±12.58	26.41±21.29	5.69±1.77*	2.3±0.3	3.0±2.4	44.94±11.14
90 min. after RVV	138.10±6.56	19.34±8.11	5.52±1.52*	2.2±0.1	2.3±1.2	39.30±12.16
120 min. after RVV	144.03±8.98	23.84±5.28	5.07±0.94*	2.5±0.3	2.8±1.2	34.06±12.62

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Table 4. Effects of RVV on haematological parameters of experimental rabbits at 10, 30, 60, 90 and 120 minutes after venom administration.

	RBC (10^6/µl)	Hb (g/dL)	MCV (fL)	Platelet (10^3/ µl)	Leukocyte (10^3/ µl)	Lymphocyt (%)	Monocyte (%)	Neutroph (%)
Gr.I (Juvenile)								
Control	5.49±0.49	11.50±0.9	66.40±0.7	303±61	1.98±0.32	58.57±18.6	6.90±1.17	25.93±20.67
5 min after RVV	5.34±0.37	11.22±0.5	70.83±1.9	255±87	1.62±0.51	58.15±15.2	6.25±2.12	28.05±16.62
10 min. after RVV	5.26±0.49	11.20±0.8	75.20±3.3	208±112	1.51±0.46	58.93±14.2	4.57±1.61	28.40±15.98
30 min. after RVV	5.20±0.49	10.97±0.8	73.83±5.1	139±29*	1.46±0.44	58.63±4.08	4.30±1.58	30.70±6.04
60 min. after RVV	5.10±0.37	10.83±0.8	71.57±3.5	141±56*	1.19±0.26	62.27±9.80	3.10±0.32*	29.20±9.24
90 min. after RVV	5.32±0.38	11.07±0.7	74.90±4.6	184±71	1.12±0.17	65.73±12.1	1.97±0.40*	27.37±11.58
120 min. after RVV	5.31±0.61	11.00±0.9	68.70±1.0	127±34*	1.22±0.29	65.27±14.7	2.63±0.29*	28.00±12.81
Gr.II (Subadult)								
Control	5.29±0.07	11.85±0.5	69.80±1.5	435±104	1.86±0.15	59.70±3.93	7.50±0.12	26.45±4.5
5 min after RVV	5.04±0.03	11.35±0.2	72.28±3.0	404±92	1.84±0.12	55.48±3.32	6.53±0.03	32.63±3.0
10 min. after RVV	4.79±0.02	10.85±0.0	74.75±4.6	372.5±79	1.83±0.09	51.25±2.71	5.55±0.17	38.80±1.6
30 min. after RVV	4.84±0.05	10.90±0.0	73.80±2.3	355.5±51	1.69±0.38	49.40±7.62	4.95±0.75	40.80±5.8
60 min. after RVV	4.60±0.03	10.60±0.2	74.85±4.6	316.5±27	1.12±0.14	60.00±0.00	4.40±0.46*	31.45±0.1
90 min. after RVV	4.63±0.10	10.60±0.0	71.95±2.8	301.0±12	1.35±0.13	48.65±1.91	4.85±0.98*	41.10±1.2
120 min. after RVV	4.92±0.18	11.00±0.1	72.00±2.4	315.5±19	1.83±0.03	31.90±0.12	3.80±1.62*	58.35±3.9
Gr.III (Adult)								
Control	5.18±0.92	11.20±1.2	69.30±3.7	538±346	2.27±0.14	60.50±16.5	2.82±2.09	28.53±20.
5 min after RVV	4.94±0.65	10.77±0.9	71.98±5.3	473±333	1.70±0.35	66.22±9.90	2.69±1.99	22.87±14.
10 min. after RVV	4.85±0.67	10.57±1.1	74.70±6.9	442±285	1.44±0.28	72.03±4.59	2.57±1.91	17.93±7.6
30 min. after RVV	4.77±0.85	10.33±1.3	71.97±4.9	430±243	1.59±0.69	67.57±9.95	3.09±2.84	23.80±10.
60 min. after RVV	4.51±1.05	9.70±1.79	72.43±4.4	374±185	1.32±0.34	69.93±3.54	2.92±2.22	20.80±3.8
90 min. after RVV	4.45±1.09	9.57±1.89	74.73±6.1	349±158	1.43±0.33	70.40±1.94	2.06±1.54	22.17±3.3
120 min. after RVV	4.47±1.10	9.63±1.88	69.97±3.2	319±115	1.59±0.63	67.87±10.2	1.72±1.34*	24.30±8.7

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Effects of snake venom on haematological parameters, blood urea nitrogen (BUN), and plasma creatinine levels (Table 4 and 5).

Concerning to the effects of *D.siamensis* venoms from adult, subadult and juvenile snakes on hematological parameters (Table 4) revealed that group I (Juvenile) showed a significant decrease in platelets count, monocyte, in comparison with group II (subadult) and III (adult). Meanwhile, a significant increase (P<0.05) in lymphocyte count was observed. While venom from subadult

and adult snake showed a non significant effect in the other parameters after 2 hours from venom injection of it when compared with pretreatment period as shown in Table 4.

The plasma creatinine concentration showed stepwise increases after envenomation in all groups, while The plasma urea concentration showed no significant changes in all groups after envenomation (Table 5)

Table 5. Effects of RVV on plasma urea and creatinine levels of experimental rabbits at 10, 30, 60, 90 and 120 minutes after venom administration

	Plasma Urea (mg%)	**Plasma Creatinine (mg%)**
Gr.I (Juvenile)		
Control	32.08±3.59	1.42±0.30
10 min. after RVV	36.23±7.25	1.58±0.46
30 min. after RVV	33.39±7.17	1.51±0.43
60 min. after RVV	34.81±8.32	1.65±0.56
90 min. after RVV	33.78±7.80	1.95±0.64
120 min. after RVV	35.73±8.75	2.00±0.59
Gr.II (Sub-adult)		
Control	24.08±3.15	1.49±0.07
10 min. after RVV	25.00±1.85	1.51±0.10
30 min. after RVV	24.50±4.61	1.51±0.03
60 min. after RVV	26.25±6.46	1.53±0.07
90 min. after RVV	24.72±4.45	1.60±0.03
120 min. after RVV	25.42±2.68	1.72±0.13
Gr.III (Adult)		
Control	32.38±4.42	1.90±0.53
10 min. after RVV	32.41±4.45	1.93±0.56
30 min. after RVV	33.97±5.72	1.95±0.33
60 min. after RVV	34.64±7.21	2.11±0.10
90 min. after RVV	33.44±6.70	2.21±0.16
120 min. after RVV	32.64±5.24	2.28±0.26

Effects of *D. siamensis* venom from adult. subadult and juvenile snakes on kidney histological alterations.

The sections from the rabbit kidney after 120 min. of administration with *D. siamensis* venom from the adult showed congestion of glomerulus (2/3 point). Renal tubules for Proximal convoluted showed diffuse acute tubulonephrosis (0/3 point). Distal convoluted tubules showed diffuse acute tubulonephrosis (0/3 point). Collecting ducts showed diffuse acute tubulonephrosis (0/3 point) (Fig.1.). Administration of *D. siamensis* venom from subadult snakes showed congestion of glomerulus part (2/3 point). Renal tubules for proximal convoluted tubules showed diffuse acute tubulonephrosis (0.3/3 point). Distal convoluted tubules showed diffuse acute tubulonephrosis (0/3 point). Collecting ducts showed diffuse acute tubulonephrosis (0/3 point) (Fig.2). Administration of *D. siamensis* venom from juvenile snakes showed no remarkable lesions of Glomerulus. Renal tubules showed diffuse acute tubulonephrosis (1.4/3 point) at proximal convoluted tubules. Distal convoluted tubules showed diffuse acute tubulonephrosis (0.8/3 point) and collecting ducts showed diffuse acute tubulonephrosis (1.0/3 point) (Fig.3).

Adult

H&E stain (40X) PAS stain (40X)

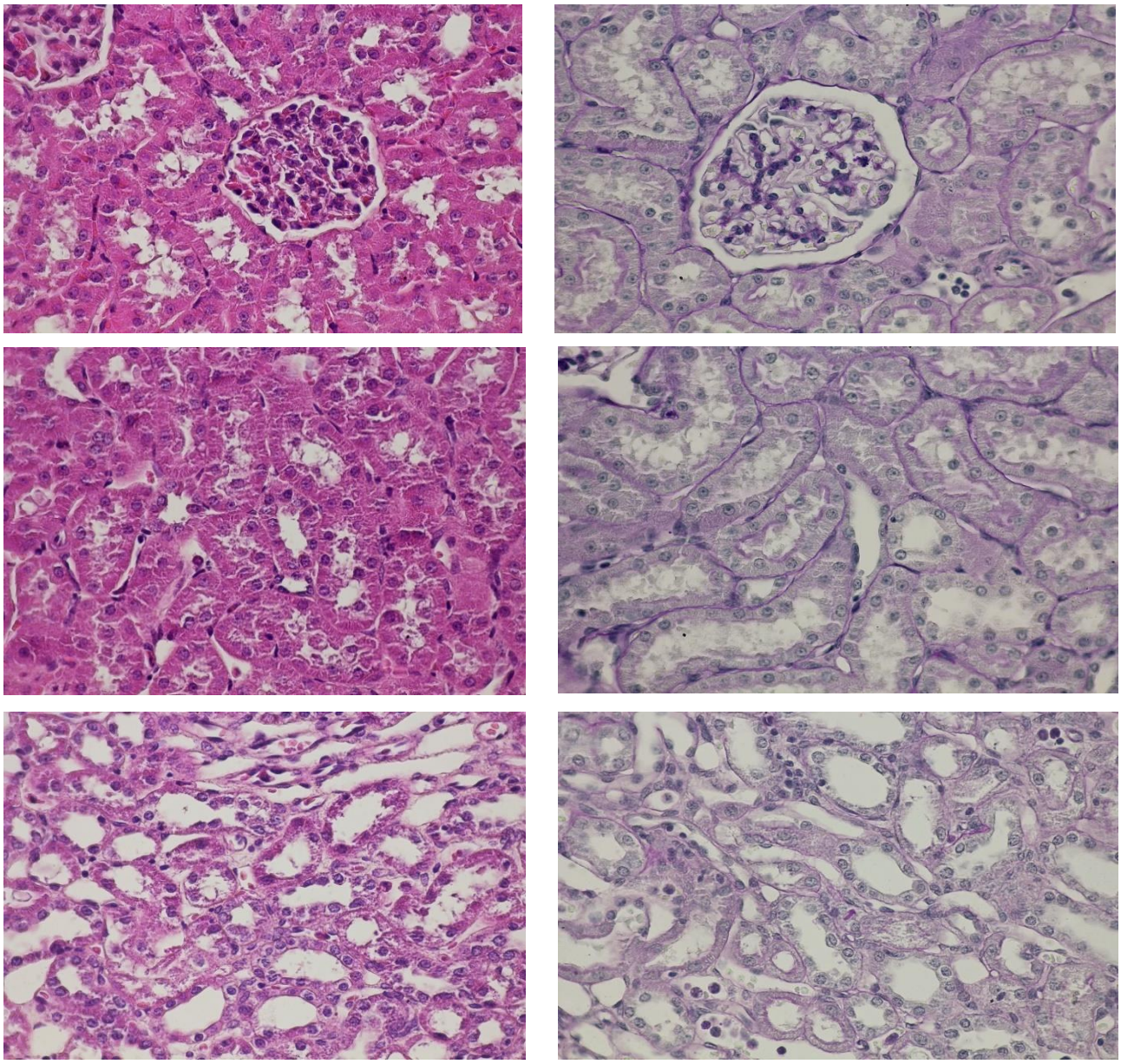

Figure 1
Representative photomicrographs of kidney sections stained with H&E and periodic acid-Schiff, PAS- stained sections of glomerular and tubular parts of rabbit kidneys after envenomation with adult *D.siamensis* venom

Subadult

H&E stain (40X) PAS stain (40X)

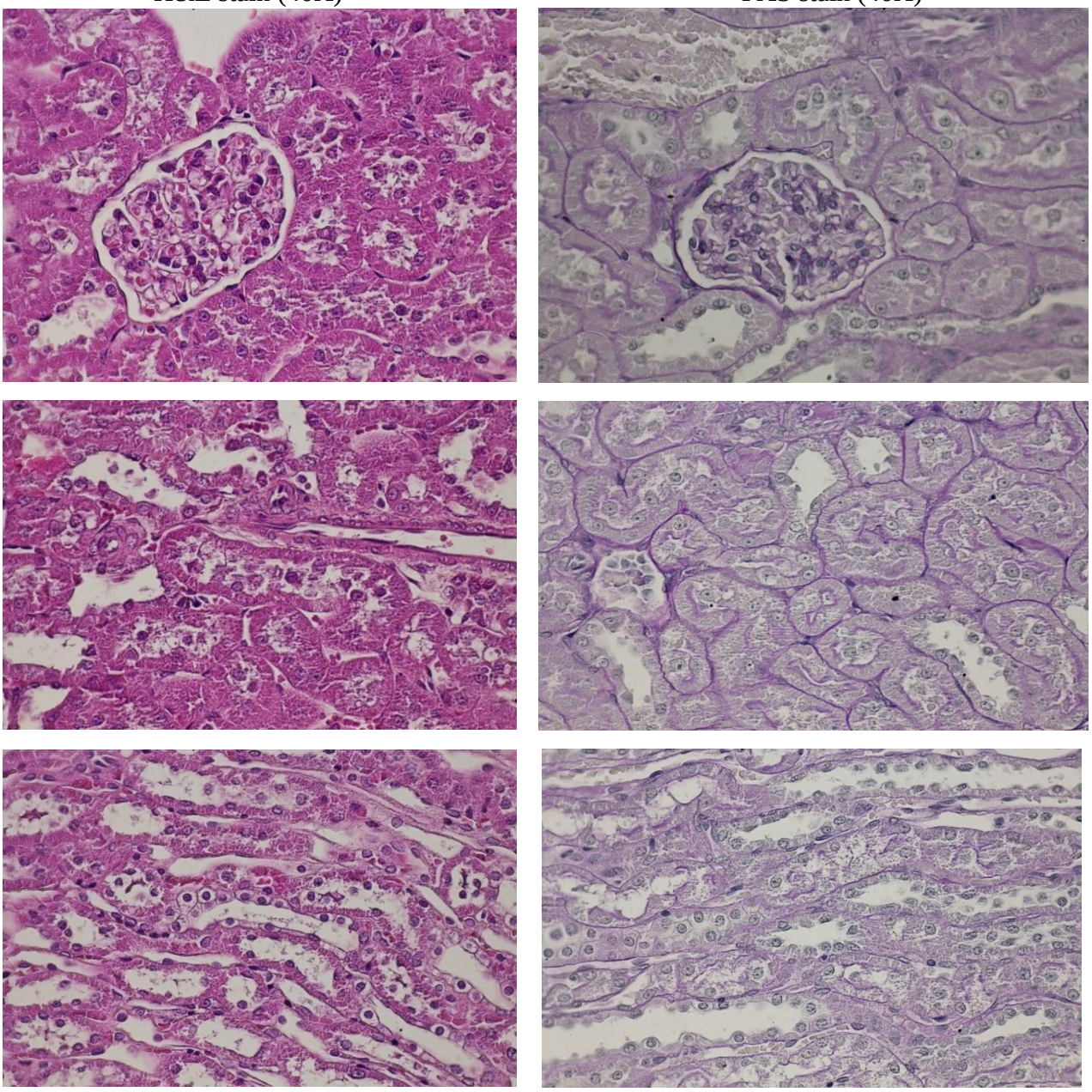

Figure 2
Representative photomicrographs of kidney sections stained with H&E and periodic acid-Schiff, PAS- stained sections of glomerular and tubular parts of rabbit kidneys after envenomation with subadult *D.siamensis in vivo*

Juvenile

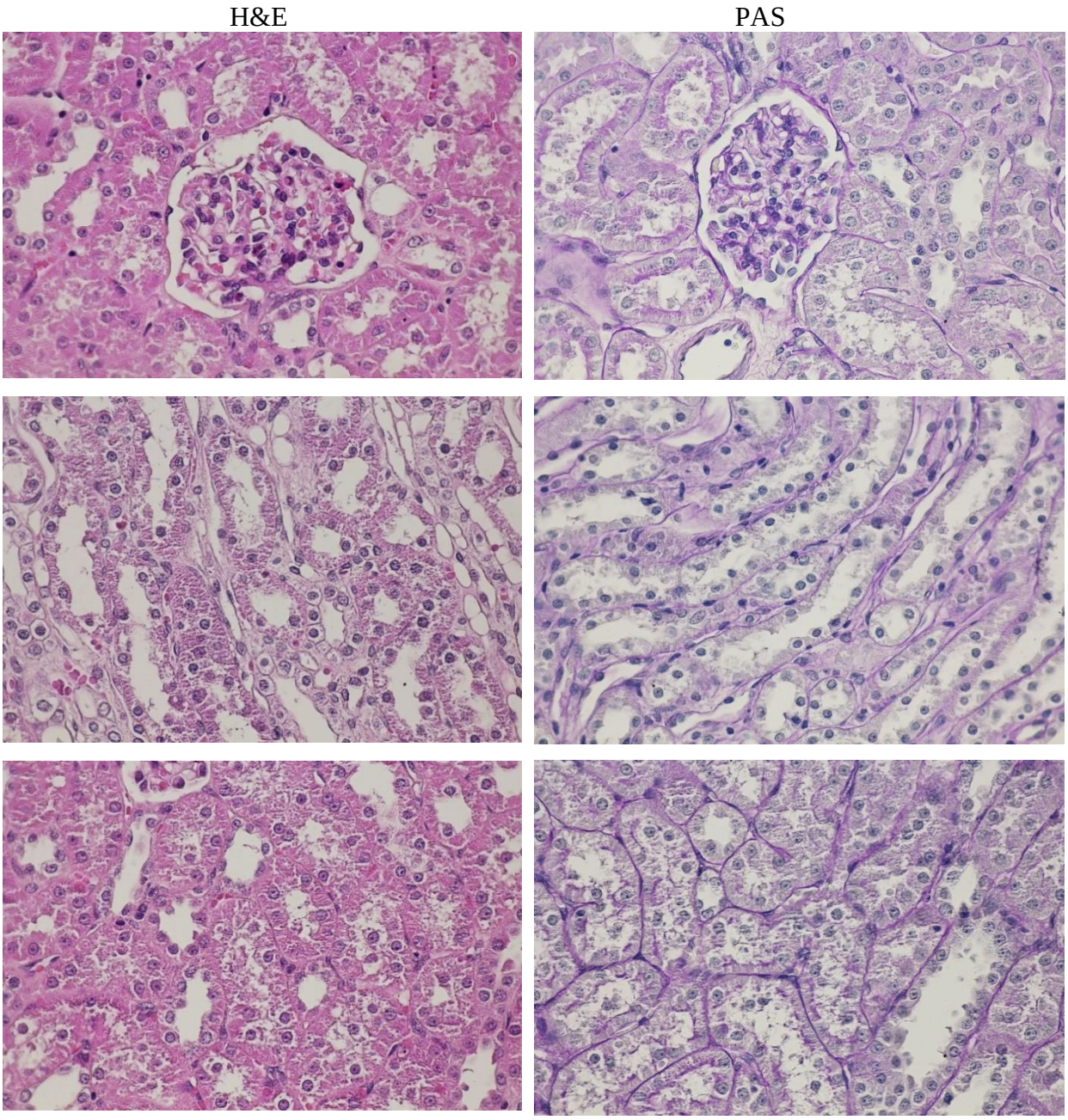

Figure 3, Representative photomicrographs of kidney sections stained with H&E and periodic acid-Schiff, PAS- stained sections of glomerular and tubular parts of rabbit kidneys after envenomation with juvenile *D. siamensis* venom

Discussion

The results of the present study show clearly that Russell's viper venom causes marked changes in renal hemodynamics and general circulation in the experimental rabbit. A decrease in arterial blood pressure within 5 min. following the venom injection. After a transitory decrease up to the 20 min, it gradually increased and, by 30 to 80 min. approached the control level and maintained a lower level. Renal blood flow and glomerular filtration rate decreased by approximately 22%, while renal vascular resistance increased. The pattern of these changes is similar to those of studies in dogs (Chaiyabutr et al. 1984, Tungthanathanich et al. 1984). The decrease in blood pressure during the first phase is due to the vasovagal effect which could be prevented by vagotomy (Lee and Lee. 1979)- The rise in blood pressure following the transient decrease has been explained by catecholamine release as a compensatory mechanism. Causing increased systemic vascular resistance (Tungthanathanich et al.,1984)- Previous studies in dogs indicated that an increase in renal vascular resistance with the decrease in total peripheral resistance over the subsequent 48 h of envenomation, resulted in a decrease in renal hemodynamics and electrolytes excretion. These findings have been interpreted as renal vasoconstriction induced by renin-angiotensin activity. (Tungthanathanich et al., 1986 Chaiyabutr et al 1985). However, at least in this model. the persistence of the low level of systemic blood pressure without compensatory mechanism after venom injection might relate the action of endogenous AII in response to hemodynamic alteration. This result could be the direct effect of a marked decrease in blood pressure which induced a resultant decrease in renal perfusion and glomerular filtration rate. In this situation, the decrease in urine flow appeared to accompany with a decrease in salt excretion. Creation of osmotic effect by electrolytes in the tubule could contribute to a decrease in urine flow. The electrolytes excretion after venom injection in rabbit coincided with the pattern of decreased renal blood flow and glomerular filtration rate. Thus, the reduction of sodium excretion could attribute to the decrease in filtered load, accompanying with a fall in glomerular filtration rate. A decrease in UNaV and %FENa after *D. siamensis* venom injection suggests that intrarenal regulates sodium excretion. Of interesting is the finding of the increase in UKV and %FEK. after venom injection, which might not be attributed to an increase in filtered load. The *D. siamensis* venom Is complex mixture that contains both non-protein and protein components with different structures of toxins especially phospholipase A2 which may involve this phenomenon. The possible explanation for these results may reasonably be considered the content of PLA_2 has

approximately 20-25% by mass of the whole *Daboia* venom (Sharma et al., 2015; Tan et al., 2018). Therefore, the high specific activity would be another possibility for the non-specific effect of toxic PLA_2 on overt hydrolysis of the phospholipid of the biological membranes, leading to cell damage. A tendency to increase the $\%FEK^+$ but this was not statistically significant. This is consistent with a previous study in the isolated rat kidney model (Ratcliffe et al., 1989). The venom of *D. siamensis* increased the $\%FEK^+$, it showed the opposite effect on the GFR and urine flow, suggesting a different mechanism in the regulation of urinary electrolytes excretion. A previous study in isolated rabbit kidney demonstrated that the direct effect of *D. siamensis* venom was to decrease the absolute tubular Na^+ reabsorption in both the proximal tubule and distal nephron in rabbit IPK when using lithium as a marker (Chaiyabutr et al., 2014). Micropuncture studies to measure the transmembrane potential in the proximal tubule of a Triturus kidney revealed that *D. siamensis* caused depolarization in a dose-dependent manner (Chaiyabutr et al., 1985), which might involve to the inhibition of Na^+-K^+-ATPase activity in the renal tubules of both the renal cortex and medulla (Buranakarl et al., 1997).

These findings may provide mechanistic insight into the cellular events in modulation of ion channels activities (Sitprija and Sitprija, 2012), where *D. siamensis* venom inhibits Na^+ channel activity by inactivation of Na^+-H^+ exchange (NHE3) in proximal tubules and Na^+ channels (ENaC) in the distal nephron. Subsequently, the elevated cytosolic Na^+ would decrease Na^+ reabsorption through down-regulation of NHE3 and ENaC at the apical border of the renal tubules. Alternatively, *D. siamensis* venom may directly inhibit NHE3 and ENaC activities. Additional information on the direct acute effect of *D. siamensis* venom from a juvenile snake on renal cells was obtained by optical microscopy, which revealed no distinct brush border in the proximal convoluted tubules, a moderate degree of acute dilatation, and severe tubulonephrosis (+3). These histological alterations of renal cells induced by *D. siamensis* venom are similar to those reported previously (Chaiyabutr et al., 2014). Comparative studies on the pathophysiological alterations of renal tubular function induced by different components of venom between adult and juvenile *D. siamensis* venoms are lacking. In the present study, administration of juvenile *D. siamensis* venom showed a different pattern of marked changes obtained by optical microscopy compared to those effects induced by venoms from both subadult and adult *D. siamensis* venoms.

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CHAPTER IV

Pro- and anti-inflammatory cytokines release in urine and plasma from rabbits injected with Russell's viper venom and its fractions (PLA2, MP, LAAO and PDE) - *in vivo* and *in vitro* studies

CHAPTER IV

Pro- and anti-inflammatory cytokines release in urine and plasma from rabbits injected with Russell's viper venom and its fractions (PLA_2, MP, LAAO and PDE) - *in vivo* and *in vitro* studies

Introduction

Snakebite is an important public health problem in several countries in the tropics and Russell's viper (*Daboia siamensis*) is responsible of the events with a high morbidity and mortality. The main and most serious complication during Russell's viper bite is acute kidney injury (AKI) (Kanjanabuch and Sitprija, 2008). The mechanisms acting within the body to induce AKI during envenomation are uncertain. The pathogenetic mechanisms underlying acute renal failure (ARF) may include renal vascular obstruction by fibrin micro-thrombi (DIC), ischemia or hypoperfusion due to the fall in blood pressure and hemolysis-induced pigment nephropathy due to some extent hemolytic (Sitprija et al., 1974). This manifestation cannot exclude the presence of proteolytic enzymes and vasoactive substances which could promote, or even potentiate, the coagulant process in renal sites. *In vivo* studies in experimental animals have been shown that decreases in renal blood flow and glomerular filtration rate with an injection of the crude venom of Russell's viper (RVV) might be depended on changes of extrarenal factors via reductions of both systemic and renal circulations (Tungthanathanich et al., 1986; Thamaree et al., 1994). The etiology of RVV induced acute renal failure in humans and experimental animals is still not completely understood but probably involves a direct action of venom components on renal tubules and renal epithelial cells (Sitprija and Chaiyabutr, 1999). The possible existence of a direct nephrotoxic component was reported, which was not well described up to now. RVV is a complex mixture that contains both non-protein and protein components with different structures and specific biochemical activities. The major protein components of the RVV consist of a mixture of potentially toxic proteins, peptides and several enzymes (Sitprija and Sitprija, 2012). A possible effect of each venom component may dominate the clinical presentation causing local and systemic injury as well as haematological, cardiovascular and renal functions. However, the potential role of each venom component of RVV has direct and/or indirect cytotoxic relating to AKI has not been studied in as much detail. Previously, studies done in experimental animals by Suwansrinon et al. (2007)

demonstrated that administration of two different venom fractions of RVV either PLA_2 or MP showed alterations of the renal functions which changes in renal hemodynamics appeared to correlate better with MP than PLA_2. Russell's viper is considered as one of the most commonly encountered snakes and medically important species of venomous snakes. The phylogenetic analysis indicated that Russell's vipers constitute two distinct species, i.e. *Daboia russelii* in South Asia and *Daboia siamensis* in Southeast Asia . *Daboia siamensis* (*D. siamensis*) is one of the most important poisonous snakes which is distributed throughout Southeast Asian countries among many venomous snakes. It is one of the common biters capable of delivering lethal venom and responsible for a substantial number of deaths which the development of acute kidney injury(AKI) was an important clinical event and associated significantly with mortality (Alfred et al., 2019). The most common complication amongst lethal cases after *D. siamensis* bite is the process of acute renal failure (ARF), but its pathogenesis is not well understood.

Venom toxin is characterized by robust induction of the systemic inflammatory response which can lead to cell damage, and potentially multiple organ failure. Venom toxin may stimulate the overproduction of pro-inflammatory mediators such as prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, and C-reactive protein (CRP), resulting in an acute inflammatory response. Via an inhibitory feedback effect, also induces anti-inflammatory cytokines such as IL-1 receptor antagonist, IL-4, IL-10, and IL-13. However, pre-exposure of innate immune cells such as macrophages to dose *D. siamensis* venom (0.5 mg/kg) to induce the robust expression of proinflammatory mediators to subsequent toxicity challenge. The mechanical feedback response leads to a shift away from a pro-inflammatory response, including production of TNF-α, IL-1β, IL-6, and PGE2 during envenomation with *D. Siamensis* venom toward a response with key anti-inflammatory features via IL-10 production have not been clarified. Knowledge on the envenomation with venom compositions relating to body cytokine system would be very crucial to improve understanding of the venom complexity and variability. Furthermore, when coupled with functional studies in the target organ i.e. kidney function, the knowledge will contribute toward elucidating the clinical pathophysiology of Russell's viper envenoming its fraction**.** Therefore, the aim of the present study was to investigate if and how envenomation with *D. Siamensis* venom and its fractions alter the inflammatory response to subsequent changes in renal mechanism via cytokine production

Materials and Methods

Animals

Adult male white New Zealand rabbits, weighing 2 to 3 kg, were used for experimental animals in *in vivo* studies. Animals were obtained from the Animal House, Queen Saovabha Memorial Institute (QSMI) and were settled in stainless steel cages, received water and fed a standard diet ad libitum, exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 26 ± 1 ^{o}C. The animals were quarantined for 14 d before experiments. *In vivo* experiments were performed in accordance with the permission of the Ethics Committee of the Queen Saovabha Memorial Institute Animal Care and Use (approval number QSMI-ACUC-03-2016) under the guideline of the National Research Council of Thailand.

Snakes and venom sample collections

Russell's viper (*Daboia siamensis*) snakes from eastern regions of Thailand were kept in captivity at the Snake Farm of the Queen Saovabha Memorial Institute (QSMI), Thailand. *Daboia siamensis* snakes were maintained individually in plastic cages and were provided water ad libitum in the same animal care room in the Snake Farm. Once a month, the snakes are fed only with small rodents in proportion to their weight (10–20% of the snake's body weight) All snakes were maintained under conditions of normal environmental temperature (average 27°C) and relative humidity (75%). Adult snake venoms were obtained from 85 specimens. The entire venom extracted by each snake were pooled into the glass vial specific for venom pool. The crude venom was lyophilized and stored at -20 °C until use. Lyophilized venoms were pooled from venoms of both sexes of *D. siamensis* by mixing amounts of samples from each specimen according to the length of the snakes.

Animal Preparation:

Experiments *in vivo* were carried out in adult male white New Zealand rabbits. The animals were deprived of food but not of water for 12 hours prior to the study. On the day of the experiment, the animals were anaesthetized with sodium pentobarbital (50 mg/kg) by intravenous injection. The animal was tracheotomized for free the airway with endotracheal tube. The jugular vein was cannulated with polyethylene tubes (PE 90) for infusion of the solution and renal clearance studies.

The carotid artery was cannulated with a polyethylene tube (PE 90) for the recording of blood pressure and heart rate (Polygraph Model 79, Grass Instruments Co.) The left ureter was cannulated with a polyvinyl catheter (PV 120)via a retroperitoneal approach for urine collection.

Venom dose and administration protocol

A pool of Russell's viper (*Daboia siamensis*) venom was obtained from adult male and female *Daboia siamensis* snakes collected from the eastern region of Thailand and maintained at Queen Saovabha Memorial Institute. An earlier investigation in experimental animals in either dogs or rabbits, where the dosage of crude RVV in a lyophilized form that caused death in 50% of subjects (LD_{50}) by intravenous injection was 0.5 mg/kg body weight (Chaiyabutr et al., 2014; Tungthanathanich et al., 1986). However, a single venom dose of 0.1 mg/kg was arbitrarily chosen in the present work in rabbit based on preliminary experiments in which doses of 0.1 and 0.5 mg/kg were tested. Rabbits injected with 0.1mg/kg showed minimal systemic and renal function alterations, whereas those receiving 0.5 mg/kg generally died within a few minutes or hours after venom administration — this short survival time precluded adequate assessment of changes in renal functions. Therefore, the dose of 0.1 mg/kg provided the best combination of renal damage (assessed cytokine study) in relation to survival time for3-4 h. Thus, a single venom dose of 0.1 mg/kg by intravenously injection was used in the present study. The RVV was isolated by fractionation methods for PLA_2, MP, LAAO and PDE for comparative purposes as described previously (Chaiyabutr et al., 2020).

Experimental design vivo

Two experimental protocols were carried out both *in vivo* and *ex vivo* in isolated rabbit kidneys:

Experiment protocol 1 for *in vivo* studies, five groups of male white New Zealand rabbits (n=4/group). Group I animal was injected with lyophilized venom (0.1 mg/kg, i.v.) in 1 ml of 0.15 M NaCl. The effects of intravenous administrations of venom fractions alone for PLA_2, (0.2mg/kg) in group II, MP(0.2 mg/kg) in group III, LAAO (0.15mg/kg) in group IV and PDE alone (0.1mg/kg) in group V on changes in cytokines levels in both plasma and urine.

Experiment protocol 2 for *in vitro* studies, the preparation for isolated perfused rabbit kidney (rabbit IPK) was based on methods described previously (Chaiyabutr et al. 2014). Five groups of IPK rabbits (n=4/group) were carried out. Group I , IPK was treated with the lyophilized RVV

in normal saline (1 mg/ml) which was added into 100 ml of perfusate of the recirculating system. Group II, PLA_2, (280 μg/ ml) was added into 100 ml of perfusate of the recirculating system. Group III, MP, (280 μg/ ml) was added into 100 ml of perfusate of the recirculating system. Group IV, LAAO (270 μg / 2ml) was added into 100 ml of perfusate of the recirculating system. Group V, PDE (200 μg / 2 ml) was added into 100 ml of perfusate of the recirculating system. The effects of administrations of venom and its fractions alone on changes in cytokines levels were determined in both perfusate and urine.

Samples collection and chemical analysis

Each experimental group was divided into six periods for cytokines measurements after each specified treatment. Samples of urine and plasma / perfusate were collected at intervals of each specified period (at 0, 10, 30, 60, 90 and 120 min after venom and its fractions treatment for cytokines analysis for pro-inflammatory cytokines (TNF-α, IL-1b และ IFN-γ) and anti-inflammatory cytokines: (IL-5, IL-4 และ IL-10

Cytokine measurement

Plasma and urine obtained from rabbit injected with crude venom, phospholipase A2, metalloprotease, phosphodiesterase or L-amino acid oxidase; and the perfusate of isolated perfused rabbit kidney tested with the same treatment as parallel experiments, these samples collected before and after venom administration at various times were used to measure anti- and pro-inflammatory cytokines. Quantitative measurement of the cytokine levels was performed by Enzyme-Linked Immunosorbant Assay (ELISA) kits using specific antibodies, cytokine standards and protocols according to the manufacturer's instructions (Nori® Rabbit cytokine ELISA Kit, Genorise Scientific, Inc., USA). Briefly, the cytokine standards and tested samples were duplicated added to 96-well microplate pre-coated with specific antibody against each cytokine. After 1 h incubation at room temperature and 3 times washing, the detection antibody was added and incubated for 1 h at room temperature. Then the plate was 3 times washed and incubated for 20 min with biotin-streptavidin HRP conjugate. After a final wash, the reaction was developed by the addition of substrate solution. Within 20 min, the reaction was stopped and the optical densities were measured at 450 nm in a microplate reader (Sunrise, TECAN, Austria). For each parameter, the levels of ant-inflammatory cytokines; IL-4, IL-5 and IL-10, and pro-inflammatory cytokines;

IL-1β, IFN-γ and TNF-α, were estimated from a standard curve and results were expressed as pg/ml.

Statistical Analysis

The results are expressed as mean ± one standard deviation (SD). One-way ANOVA with repeated measures were used with Bonferroni's post-hoc test to compare the number of changes among time points after the venom and venom fractions treatment within the same group.

Table 1: Concentrations of cytokines in plasma and urine during administration of *D. siamensis* venom and its fraction (PLA_2, metalloprotease) in rabbit

		IL-1 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	IFNγ (pg/ml)
Gr.I (RVV): In vivo							
Plasma	Control	565.36±402.82	71.48±35.47	58.70±9.63	7.83±3.40	73.98±14.70	13.60±10.95
	10 min.	-	90.73±21.41	-	-	87.86±12.16	14.72±10.06
	30 min.	524.62±322.92	109.97±7.35	64.59±10.95	8.87±5.01	101.75±9.62*	15.84±9.17
	60 min.	508.08±303.69	127.62±10.02	70.74±18.39	7.94±3.27	70.27±11.56	13.52±6.08
	90 min.	-	97.14±6.80	-	-	61.02±22.67	13.72±7.19
	120 min.	490.33±275.00	108.37±16.90	71.54±9.11	7.80±4.99	51.76±33.79	13.91±8.29
Urine	Control	385.86±9.86	140.45±16.67	45.33±11.39	7.50±0.50	88.79±21.03	9.75±3.54
	10 min.	-	136.44±28.37	-	-	75.83±27.41	9.17±2.81
	30 min.	373.36±23.66	132.43±40.07	56.03±14.91	7.76±0.29	62.87±33.79	8.59±2.07
	60 min.	362.87±12.24	109.97±27.78	50.94±19.27	7.65±0.95	64.72±39.01	6.82±0.93
	90 min.	-	126.01±26.79	-	-	72.13±33.39	7.01±1.36
	120 min.	381.42±17.91	132.43±40.07	39.71±10.16	6.50±0.52	79.53±27.77	7.20±1.80
Gr.II (PLA2): In vivo							
Plasma	Control	411.68±80.10	172.52±81.14	80.77±15.32	4.33±1.72	246.66±69.00	4.27±1.85
	10 min.	-	137.24±53.96	-	-	239.38±6.38	5.49±1.68
	30 min.	409.66±66.37	101.95±26.79	86.79±2.27	5.16±1.17	232.09±103.76	6.70±1.50
	60 min.	404.82±75.17	122.80±31.31	83.58±1.13	5.38±2.11	210.24±98.74	5.89±2.08
	90 min.	-	87.52±20.41	-	-	206.60±75.17	5.95±2.02
	120 min.	362.47±36.75	153.28±60.16	94.01±37.45	4.05±1.17	202.96±51.61	6.01±1.97
Urine	Control	393.12±23.78	109.97±7.35	41.05±12.92	6.32±1.72	296.19±94.48	7.90±2.28
	10 min.	-	120.40±20.51	-	-	256.13±108.73	7.93±1.65
	30 min.	385.05±24.17	130.82±33.68	48.54±5.46	7.54±0.86	216.07±122.99	7.97±1.01
	60 min.	382.23±35.33	127.62±29.40	50.41±7.92	7.83±1.49	217.53±166.66	7.90±0.96
	90 min.	-	140.45±9.62	-	-	216.07±139.70	9.40±1.38
	120 min.	396.75±25.39	117.99±33.80	53.35±2.82	9.57±4.96	214.61±112.74	10.90±1.80*
Gr.III (Metalloprotease): In vivo							
Plasma	Control	2,118.32±857.36	161.38±95.51	446.72±521.5	5.21±1.52	336.88±147.93	5.31±1.97
	10 min.	-	179.82±74.87	-	-	291.52±98.65	5.37±1.91
	30 min.	719.45±343.07	198.27±54.24	69.13±10.21	7.32±0.47	246.16±49.37	5.43±1.85
	60 min.	727.11±333.90	156.73±33.93	70.34±2.84	8.48±2.58	246.16±158.95	6.82±2.31
	90 min.	-	164.88±35.86	-	-	214.69±157.35	7.22±2.26
	120 min.	729.93±327.40	173.02±37.78	67.93±13.05	8.70±3.21	183.21±155.75	7.63±2.20*
Urine	Control	402.00±31.30	191.10±59.03	46.13±9.64	6.02±0.74	55.46±25.05	8.90±2.81
	10 min.	-	177.16±36.10	-	-	65.65±28.31	10.36±2.45
	30 min.	450.00±24.99	163.23±13.16	57.90±5.01	8.46±0.28	75.83±31.58	11.83±2.09
	60 min.	420.95±29.15	160.70±35.10	57.63±0.46	8.24±0.52	70.27±3.21	11.37±1.67
	90 min.	-	168.30±28.52	-	-	79.53±20.09	11.64±1.74
	120 min.	428.22±12.94	175.90±21.94	53.62±2.45	7.46±1.72	88.79±36.98	11.90±1.81*

Table 2: Concentrations of cytokines in plasma and urine during administration of *D. siamensis* venom fractions (L-amino acid oxidase and phosphodiesterase) in rabbit

		IL-1 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	IFNγ (pg/ml)
Gr.IV (L-amino acid oxidase): In vivo							
Plasma	Control	654.71±47.56	515.65±158.13	48.91±7.08	214.41±99.78	135.21±22.47	49.91±9.12
	10 min.	707.78±73.57	533.42±175.52	45.71±14.88	227.86±117.03	133.91±73.59	52.74±9.12
	30 min.	733.53±38.64	504.76±186.77	49.75±7.07	216.38±111.83	139.11±25.25	48.85±5.87
	60 min.	724.60±28.98	502.11±197.54	50.25±11.19	226.64±134.28	161.21±51.48	50.97±9.24
	90 min.	735.10±85.46	496.61±201.81	49.58±10.86	218.82±128.04	140.41±67.60	47.57±9.56
	120 min.	712.51±101.06	480.52±199.96	48.07±10.48	229.70±136.04	141.71±130	53.40±18.68
Urine	Control	648.14±21.92	128.37±2.19	21.74±5.64	61.71±7.54	83.20±61.09	15.16±0.80
	10 min.	662.33±14.77	131.25±4.94	23.75±2.36	60.71±9.10	89.71±65.56	12.90±0.67
	30 min.	663.91±49.76	134.65±12.58	25.77±3.70	57.99±7.85	153.41±61.93*	13.07±0.46
	60 min.	653.40±23.46	129.94±13.03	31.6±6.62	61.43±6.13	94.91±82.37	13.69±2.14
	90 min.	671.53±34.85	130.21±13.10	21.29±6.25	57.17±20	48.54±61.6	13.69±0.79
	120 min.	704.37±15.97	124.71±12.38	22.63±4.37	60.71±3.03	116.14±28.64*	14.90±1.24
Gr.V (Phosphodiesterase): In vivo							
Plasma	Control	734.58±206.77	519.87±82.24	37.65±11.81	198.16±25.76	119.61±84.28	41.89±20.56
	10 min.	751.39±183.38	519.19±74.04	37.81±11.66	225.48±4.04	157.31±41.25	43.33±22.27
	30 min.	742.72±200.41	547.85±80.49	35.29±8.11	221.13±9.42	131.74±30.47	44.12±21.8
	60 min.	747.19±183.86	514.38±108.18	36.64±7.46	226.98±22.30	146.91±94.41	39.75±18.71
	90 min.	733.00±174.94	515.36±91.34	40.50±8.04	227.11±21.34	161.21±77.95	40.46±17.73
	120 min.	719.87±176.08	511.24±128.01	35.46±5.55	215.15±31.33	109.21±46.22	38.45±15.05
Urine	Control	642.10±25.38	113.98±2.76	23.75±1.69	65.42±13.26	88.41±18.75	11.66±1.51
	10 min.	632.12±21.44	112.15±3.15	27.79±3.18	62.07±10.09	109.21±27.52	11.64±0.74
	30 min.	675.99±19.93	117.64±3.97	26.67±3.94	60.26±6.82	119.61±29.72	11.6±1.10
	60 min.	617.40±25.78	113.46±6.43	31.82±1.69	55.63±2.28	104.01±38.45	12.59±1.23
	90 min.	626.60±23.00	121.31±10.21	25.32±1.94	51.92±1.65	95.34±25.65	10.67±0.74
	120 min.	645.25±43.27	114.37±7.68	25.77±0.39	52.19±3.57	105.74±21.02	10.42±1.54

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Table 3: Concentrations of cytokines in plasma and urine during administration of *D. siamensis* venom and its fraction (PLA_2, metalloprotease) in isolated perfused rabbit kidney

		IL-1β (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	IFNγ (pg/ml)	NO (mmol)
Gr.I (RVV): In vitro								
Perfusate	Control	285.83±28.30	172.52±22.74	66.19±7.63	6.39±0.83	77.68±66.26	6.43±4.57	-
	10 min.	309.63±17.96	235.08±53.65	73.15±3.21	6.76±0.33	88.79±27.96	8.05±3.76	-
	30 min.	308.82±16.64	244.70±60.16	73.95±0.00	7.13±1.66	92.49±43.14	10.75±2.50	-
	60 min.	305.19±36.68	233.47±112.0	77.69±8.80	6.17±0.72	159.14±57.81*	13.60±2.79*	-
Urine	Control	368.92±16.11	393.83±197.6	64.85±20.87	8.51±2.11	229.50±116.78	8.05±5.69	1.05±0.05
	10 min.	376.58±11.54	282.37±72.70	88.39±18.97	12.63±3.59	194.78±121.30	21.77±17.50*	1.03±0.16
	30 min.	384.25±11.94	478.88±183.1	106.05±2.89	10.72±2.10	258.66±213.26	19.46±8.92*	0.99±0.08
	60 min.	372.95±16.64	382.99±150.6	115.15±51.27	13.38±3.61	276.71±162.16	27.25±19.37*	1.00±0.00
Gr.II (PLA2): In vitro								
Perfusate	Control	312.85±31.66	300.04±146.7	79.83±9.71	6.50±2.05	240.83±22.22	6.12±3.21	-
	10 min.	340.68±26.08	383.64±92.15	88.93±15.75	6.80±1.67	169.45±134.0	8.51±0.93	-
	30 min.	354.80±19.47	325.37±114.3	81.71±9.30	7.68±1.85	109.72±22.22	9.90±1.41	-
	60 min.	356.01±12.28	530.58±195.4	77.16±12.04	7.06±1.80	165.08±51.61	12.21±1.86*	-
Urine	Control	383.84±20.21	292.44±79.35	63.78±12.90	8.20±3.30	335.03±182.0	7.43±5.55	3.40±0.30
	10 min.	373.36±23.16	370.97±133.8	99.09±27.57	9.81±2.46	243.38±58.34	12.52±9.79	3.18±0.09
	30 min.	377.39±7.87	353.24±199.3	91.07±18.60	9.53±2.10	282.26±168.3	13.29±5.82	3.08±0.20
	60 min.	375.78±29.65	406.44±77.13	95.88±28.89	9.45±1.02	298.93±131.3	18.00±5.71*	3.08±0.13
Gr.III (Metalloprotease): In vitro								
Perfusate	Control	313.26±5.97	186.03±126.4	62.45±20.15	6.61±2.01	212.84±24.21	4.27±4.94	-
	10 min.	290.26±11.24	213.90±66.40	64.05±9.64	7.61±0.50	218.39±77.76	5.43±4.50	-
	30 min.	287.44±9.86	239.23±107.2	66.73±5.79	7.80±0.36	322.07±71.42	5.97±4.95	-
	60 min.	302.36±32.23	282.30±183.2	77.16±3.68	6.36±1.22	255.42±114.0	7.66±4.55	-
Urine	Control	381.83±45.54	212.57±57.41	102.57±26.45	7.06±2.78	111.01±17.85	9.21±3.70	0.94±0.11
	10 min.	403.21±43.02	595.24±212.68	107.12±19.73	9.64±0.86	286.89±150.3	9.75±1.77	0.88±0.16
	30 min.	408.85±48.26	352.97±189.35	92.14±8.58	9.60±0.65	140.63±110.6	11.98±1.97	0.85±0.08
	60 min.	447.58±27.87	289.06±78.55	83.85±20.53	10.12±0.99	99.90±11.56	15.76±1.19*	0.91±0.07

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Table 4: Concentrations of cytokines in plasma and urine during administration of *D. siamensis* venom fractions (L-amino acid oxidase and phosphodiesterase) in isolated perfused rabbit kidney

		IL-1β (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	IFNγ (pg/ml)	NO (mmol)
Gr.IV (L-amino acid oxidase): In vitro								
Perfusate	Control	485.25±72.11	129.68±31.24	26.72±7.39	52.37±5.10	175.51±80.38	13.37±0.98	-
	10 min.	643.68±13.31	121.83±4.38	25.77±9.13	59.17±10.2	100.11±38.30	13.69±2.40	-
	30 min.	639.47±40.67	115.81±5.66	19.33±5.39	57.90±5.62	204.55±153.5	14.19±1.59	-
	60 min.	640.52±40.62	131.78±15.51	28.40±4.60	58.72±10.4	144.31±173.0	12.72±0.92	-
Urine	Control	516.25±50.65	139.76±19.39	37.20±2.05	57.63±7.15	137.81±60.42	14.99±0.65	3.40±0.30
	10 min.	620.29±16.65	127.85±5.23	38.77±5.43	60.44±5.59	223.61±127.3	14.81±1.50	3.18±0.09
	30 min.	639.21±14.06	126.41±3.42	33.84±7.44	62.25±6.59	193.71±133.0	15.31±0.13	3.08±0.20
	60 min.	625.29±30.24	144.86±9.35	33.39±4.58	64.33±6.95	131.31±29.84	15.75±0.66	3.08±0.13
Gr.V (Phosphodiesterase): In vitro								
Perfusate	Control	511.53±44.55	105.73±3.15	19.50±4.41	42.95±2.84	107.47±51.04	9.86±0.13	-
	10 min.	535.96±15.11	104.16±9.30	21.06±1.03	41.77±5.40	227.51±105.7*	10.16±0.72	-
	30 min.	544.10±25.04	106.91±6.31	22.63±2.36	42.95±6.54	144.31±17.18	10.75±1.19	-
	60 min.	539.90±40.58	109.01±10.66	19.72±5.22	41.77±5.32	157.31±59.95	10.36±0.79	-
Urine	Control	568.54±19.43	117.90±2.16	30.70±6.10	53.73±5.81	109.21±25.48	12.81±2.57	0.94±0.11
	10 min.	594.55±20.24	99.19±20.21	26.44±1.69	56.09±3.22	115.71±51.37	12.60±1.71	0.88±0.16
	30 min.	606.11±30.51	110.71±5.93	27.11±3.32	56.09±5.57	148.21±92.88	12.63±1.76	0.85±0.08
	60 min.	581.41±17.00	118.04±5.95	25.55±7.49	54.00±4.96	53.30±55.26	13.10±1.82	0.91±0.07

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values of specified time period with respect to the basal period (control) in each group.

Results and discussion

Envenomation with RVV, the kidney is one of the first organ systems affected by venom toxin (Shastry et al., 1977). Early reports indicate that *D.siamensis* venom and its fractions induced renal hypoperfusion contributes to an ischemia-reperfusion insult that potentially leads to the activation of renal inflammation. An elevation of renal blood flow has always been recognized as strategy for AKI after envenomation . After venom injection, the renal fraction (% cardiac output) has been shown to reduce while the ratio of renal vascular resistance and total peripheral resistance was markedly increased. These findings suggest that an intrarenal mechanism seem to be responsible for the reduction of renal blood flow and filtration rate (Chaiyabutr et al., 1984). Therefore, our purpose here was to investigate the effects of *D. siamensis* and its fractions with changes in inflammatory cytokine system . To this end, we conducted assessments of pathology images of kidney relating to the levels of anti inflammatory cytokines; IL-4, IL-5 and IL-10, and

pro-inflammatory cytokines; IL-1β, IFN-γ and TNF-α, were estimated after envenomation. Our results showed that, as compared with control period, fraction of PLA_2 and MP caused increases in IFN-γ in plasma and urine which could ameliorate the severity or postpone the development of renal tubular injury in the rabbit model of *D.siamensis* envenomation. Meanwhile, RVV, PLA_2, MP, LAAO and PDE showed no effects on cytokine release. Venom toxin may stimulate the overproduction of pro-inflammatory mediators such as prostaglandin E2 (PGE2), TNF-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, and C-reactive protein (CRP), resulting in an acute inflammatory response. Via an inhibitory feedback effect. In addition to venom toxins, cytokine releases (e.g., TNF-α, IFN-γ, and IL) has been recognized as a biological mediator that plays an important role in the damage process. It can conclude that IL-4 was more effective in improving renal function after envenomation. This was associated with decreases in renal inflammatory response and oxidative stress. In the future study, we will replenish the detected parameters and added local renal oxidative and inflammatory response to make the investigation more integrated.

These results indicate that exposure to PLA_2 and MP cause increase IFN-γ in both plasma and urine in *in vivo* but not for RVV, LAAO and PDE. In *in vitro* studied, IFN-γ increase in urine except LAAO and PDE. RVV may enhance venom fractions induced signaling cascades that elicit many pro- and anti-inflammatory pathways in a dose-dependent manner. However, in the presence of RVV and PLA_2 significantly enhanced the RVV-induced production of TNF-α, IL-6, and IL-10 but not IL-1β in venom fractions compared to the control. These data may support recent evidence showing that pretreatment of RVV can induce a hyporesponsive state to subsequent secondary challenge with high-dose RVV in innate immune cells, while PLA_2 fraction results in more robust expression of pro-inflammatory cytokines. Interestingly, our results demonstrated that the RVV and stimulated production of IFN-γ but not IL-10 by IL4 which strongly downregulated in the RVV and PLA_2S groups compared to the other. In conclusion, these findings suggest that envenomation with RVV may contribute to the mortality to secondary inflammation rather than the individual venom fraction.

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CHAPTER V

The renal effects relating to oxidative status in plasma, and kidney tissue during envenomation with Russell's viper venom and its fraction (PLA_2, metalloprotease, L-amino acid oxidase, and phosphodiesterase) - *in vivo* and *in vitro* studies.

CHAPTER V

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Introduction

Russell's viper is considered as one of the most commonly encountered snakes and medically important species of venomous snakes. The phylogenetic analysis indicated that Russell's vipers constitute two distinct species, i.e. *Daboia russelii* in South Asia and *Daboia siamensis* in Southeast Asia . *Daboia siamensis* (*D. siamensis*) is one of the most important poisonous snakes which is distributed throughout Southeast Asian countries among many venomous snakes. It is one of the common biters capable of delivering lethal venom and responsible for a substantial number of deaths which the development of acute kidney injury(AKI) was an important clinical event and associated significantly with mortality (Alfred et al., 2019). Despite the effects of Russell's viper bite has the significant impact on the health disorders which are attributed to renal failure, mechanisms acting within the body to induce acute renal failure remains unknown about the different toxic proteins and peptides that are the major components of Russell's viper venom (RVV) (Gutiérrez, et al 2017). Interpretation of clinical data of snake bite is difficult, since multiple factors are involved and close observation cannot be made immediately after the bite. The cause effect relationship can better be defined in an experimental model. During a study of envenomation with crude RVV in experimental animals (Tungthanathanich et al., 1986) it was noted that marked initial hypotension with increased renal vascular resistance and slight increase in total systemic vascular resistance, later followed by decreased systemic vascular resistance, increased renal vascular resistance, decreased renal blood flow and glomerular filtration rate. Renal fraction decreased while cardiac output and blood volume remained unchanged throughout the 48 hr after envenomation . However, the action of crude RVV is the combined effect of all components of enzymatic and nonenzymatic protein and peptide toxins present in the

venom on a variety of physiological targets. Accordingly, most toxins have evolved to the clinical complications of severe systemic and local pathology particularly kidney precisely target (Girish and Kemparaju 2011; Ushanandini et al. 2006).

A possible effect of each venom component (e.g. phospholipase A_2 (PLA2), metalloprotease (MP), L-amino acids oxidase (LAOOs) and phosphodiesterase (PDE) may dominate the clinical presentation causing local and systemic injury as well as haematological, cardiovascular and renal functions. However, the potential role of each venom component of RVV relating to AKI has not been studied in as much detail. Toxicity by reactive oxygen species (ROS) has been well known as a major cause of cellular injury in various organ include kidney. It has been demonstrated that the oxidative stress is a secondary effect of certain snake bite that oxidative stress may play a central role in renal toxicity of snake envenomation. Snake envenoming will exacerbate with subsequent generation of a variety ROS involving in the inflammatory reactions that contribute to oxidative damage and further affecting the cellular physiology and play a significant role in the pathological conditions (Carroll *et al*., 2007).A state of imbalance between pro-oxidative and antioxidative processes has been established, and defined as oxidative stress with increased amounts of ROS present (Wardle, 2005). ROS are involved in cell signaling and cause activation of proinflammatory and mitogenic cellular pathways, which enhance the progression of renal toxicity and cause a progressive decline in renal function.5–7 Glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and nitric oxide synthase are the most important antioxidant enzymes that detoxify ROS molecules in the kidneys.(Galle, J., 2001). Excess ROS and an imbalance of cellular antioxidant capacity has shown to play a causal role in kidney dysfunction in animal models (Sebastin Santhosh et al., 2013).

The present study explores the action of different venom fractions that are the major components of RVV on kidney functions, and synthesize experimental data to illustrate - whether increased oxidative stress can lead to dysfunction of this organ. For this purpose a profile consisting of four antioxidant biomarkers, SOD, catalase , GSH and MDA were measured in plasma, urine and the kidney of rabbit animal model envenomated with crude RVV and its fractions,(PLA2, MP, LAAOs and PDE). Because kidney is the target for RVV, it is important to screen for the potential effects of the venom and its fractions on induced oxidative damage in the kidney of experimental animals. Studies were conducted in in vivo experiments in rabbits , with follow-up the IPK model. The results may be of wider interest.

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Materials and Methods

Animals

Adult male white New Zealand rabbits, weighing 2 to 3 kg, were used for experimental animals in *in vivo* studies. Animals were obtained from the Animal House, Queen Saovabha Memorial Institute (QSMI) and were settled in stainless steel cages, received water and fed a standard diet ad libitum, exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 26 ± 1 ºC. The animals were quarantined for 14 d before experiments. Experiments were performed in accordance with the permission of the Ethics Committee of the Queen Saovabha Memorial Institute Animal Care and Use (approval number QSMI-ACUC-03-2016) under the guideline of the National Research Council of Thailand.

Snakes and venom sample collections

Russell's viper (*Daboia siamensis*) snakes from easthern regions of Thailand were kept in captivity at the Snake Farm of the Queen Saovabha Memorial Institute (QSMI), Thailand. *Daboia siamensis* snakes were maintained individually in plastic cages and were provided water ad libitum in the same animal care room in the Snake Farm. Once a month, the snakes are fed only with small rodents in proportion to their weight (10–20% of the snake's body weight) All snakes were maintained under conditions of normal enveronmental temperature (average 27°C) and relative humidity (75%). Adult snake venons were obtained from 85 specimens. The entire venom extracted by each snake were pooled into the glass vial specific for venom pool. The crude venom was lyophilized and stored at -20 °C until use. Lyophilized venoms were pooled from venoms of both sexes of *D. siamensis* by mixing amounts of samples from each specimen according to the length of the snakes.

Animal Preparation*:*

Experiments *in vivo* were carried out in adult male white New Zealand rabbits. The animals were deprived of food but not of water for 12 hours prior to the study. On the day of the experiment, the animals were anaesthetized with sodium pentobarbital (50 mg/kg) by intravenous injection. The animal was tracheotomized for free the airway with endotracheal tube. The jugular vein was

cannulated with polyethylene tubes (PE 90) for infusion of the solution and renal clearance studies. The carotid artery was cannulated with a polyethylene tube (PE 90) for the recording of blood pressure and heart rate (Polygraph Model 79, Grass Instruments Co.) The left ureter was cannulated with a polyvinyl catheter (PV 120)via a retroperitoneal approach for urine collection.

Venom dose and administration protocol

A pool of Russell's viper (*Daboia siamensis*) venom was obtained from adult male and female *Daboia siamensis* snakes collected from the eastern region of Thailand and maintained at Queen Saovabha Memorial Institute. An earlier investigation in experimental animals in either dogs or rabbits, where the dosage of crude RVV in a lyophilized form that caused death in 50% of subjects (LD_{50}) by intravenous injection was 0.5 mg/kg body weight (Chaiyabutr et al., 2014; Tungthanathanich et al., 1986). However, a single venom dose of 0.1 mg/kg was arbitrarily chosen in the present work in rabbit based on preliminary experiments in which doses of 0.1 and 0.5 mg/kg were tested. Rabbits injected with 0.1mg/kg showed minimal systemic and renal function alterations, whereas those receiving 0.5 mg/kg generally died within a few minutes or hours after venom administration — this short survival time precluded adequate assessment of changes in renal functions. Therefore, the dose of 0.1 mg/kg provided the best combination of renal damage (assessed cytokine study) in relation to survival time for3-4 h. Thus, a single venom dose of 0.1 mg/kg by intravenously injection was used in the present study. The crude RVV was isolated by fractionation methods for phospholipase A_2 (PLA_2), metalloprotease (MP), L-amino acid oxidase (LAAO) and phosphodiesterase (PDE) for comparative purposes as described previously(Chaiyabutr et al., 2020).

Experimental design

Two experimental protocols were carried out both *in vivo* and in isolated rabbit kidneys:

Experiment protocol 1 for in vivo studies, five groups of male white New Zealand rabbits (n=4/group). Group I : animal was injected with lyophilized venom (0.1 mg/kg, i.v.) in 1 ml of 0.15 M NaCl. The crude RVV was isolated by fractionation methods for phospholipase A_2 (PLA_2), metalloprotease (MP), L-amino acid oxidase (LAAO) and phosphodiesterase (PDE) for comparative purposes as described previously (Chaiyabutr et al., 2020). The effects of intravenous administrations of venom fractions alone for PLA_2, (0.2 mg/kg) in group II, MP (0.2mg/ kg) in

group III, LAAO (0.15mg/kg) in group IV and PDE alone (0.1mg/kg) in group V on changes in antioxidant biomarkers in both plasma and urine.

At the end of the in vivo study, all rabbit were sacrificed using intravenous injection of an overdose (60 mg/kg BW) of thiopental sodium. The abdominal walls are opened. Some pieces of the kidney were collected, frozen in liquid nitrogen, and stored at -70ºC for analysis the activities of SOD, CAT and concentrations of GSH and MDA in the kidney. Both plasma and urine were kept at-20ºC for determination of concentrations of GSH and MDA. The kidney was collected and divided into two portions, and then stored in 4% paraformaldehyde and liquid nitrogen, respectively.

Experiment protocol 2 for in vitro studies, the preparation for isolated perfused rabbit kidney (rabbit IPK) was based on methods described previously (Chaiyabutr et al., 2014). Five groups of IPK rabbits (n=4/group) were carried out. Group I , IPK was treated with 10 μg/ml of the lyophilized RVV in normal saline which was added into 100 ml of perfusate of the recirculating system. Group II, PLA_2, (300 μg/ ml) was added into 100 ml of perfusate of the recirculating system. Group III, MP, (250 μg/ ml) was added into 100 ml of perfusate of the recirculating system. Group IV, LAAO (300 μg / 2ml) (280 μg/ ml) was added into 100 ml of perfusate of the recirculating system. Group V, PDE (200 μg / 2 ml) was added into 100 ml of perfusate of the recirculating system. The effects of administrations of venom and its fractions alone on changes in antioxidant biomarkers were determined in both perfusate and urine.

Samples collection and chemical analysis

Each experimental group was divided into six periods for cytokines measurements after each specified treatment. Samples of urine and plasma / perfusate were collected at intervals of each specified period (at 0, 10, 30, 60, 90 and 120 min after venom and its fractions treatment for cytokines analysis for catalase activity in kidney ,Total antioxidant status (TAS) , Reduced glutathione (GSH), Superoxide dismutase (SOD), Lipid peroxidation (MDA)

At the end of experiment, animals were euthanasia a with high dose of pentobarbital sodium after which the kidneys were removed and immediately immersed in the liquid nitrogen for further tissue processing .

Biochemical investigation:

In the kidney samples, urine, plasma and perfusate, the following parameters were investigated: Catalase (CAT), Total antioxidant status (TAS), Glutathione reductase activity (GSH)SOD, Superoxide dismutase (SOD) and Lipid peroxidase assay (MDA).

Catalase activity (CAT): Catalase was determined by means of the UV spectrophotometric method described by Luck (1971). H_2O_2 was used as substrate. The UV absorption of the H_2O_2 solution was recorded at 240 nm after reaction of H_2O_2 with catalase. From the decrease in optical density, the enzyme activity of homogenated kidney cortex at 1% Triton X-100, was calculated. The amount of H_2O_2 decomposed was calculated on the basis of the molar extinction coefficient of H_2O_2 (0.71 L mol^{-1} cm^{-1}) and the results were expressed as mmol of H_2O_2 decomposed min^{-1} mg^{-1} protein.

Total antioxidant status (TAS): For TAS quantitation, a commercial kit (catalog no. NX2332; Randox, Ltd., Crumlin, United Kingdom) was used. Briefly, ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) was incubated with metmyoglobin and hydrogen peroxide to produce the radical cation ABTS•+. This has a relatively stable blue-green color that can be measured at 600 nm. Based on their concentration, antioxidants will cause a suppression of the color production.

Glutathione reductase activity (GSH): Glutathione reductase is also called GSH; it is an enzyme that reduces glutathione disulfide (GSSG) to sulfhydryl form GSH, which is an The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm and is directly proportional to the GR activity in the sample. The Glutathione Reductase Assay Kit can be used to measure GR activity in plasma, perfusate, urine and tissue homogenates .

Determination of superoxide dismutase (SOD) activity: Superoxide dismutase (SOD) activities (total SOD) was determined using the pyrogallol assay following the procedure described by Marklund and Marklund(1974), based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD. Briefly, 100 µL of 20 mM pyrogallol solution (in HCl 0.01 M) was added to 2.8 µL of tris buffer (containing 50 mM of tris buffer and 1 mM of ethylene diamine tetra acetic) and 100 µL of tissues supernatants and then mixed.

Measurement was taken at 420 nm after 1 min 30 sec and 3 min 30 sec using a visible spectrophotometer (DU 720 UV, Beckman Coulter, Palo Alto, CA). The percentage of inhibition of pyrogallol autoxidation was calculated and 1 U of **enzymatic activity** was defined as the quantity of enzyme necessary to achieve a 50% inhibition of autoxidation at 25°C mg^{-1} of protein(unit SOD/ml/mg protein).

Lipid peroxide assay (MDA): Lipid peroxide formation was assayed by the method of Ohkawa *et al*.(1979) for plasma, perfusate, urine and kidney tissue homogenates. The results were expressed as nmol of malondialdehyde (MDA) mg^{-1} of protein. The MDA is a degradation product of peroxidized lipids. The pink color of the TBA-MDA chromophore has been taken as an index of LPO (absorption maximum at 532 nm).

Results

Table 1: Changes of oxidative stress markers after envenomation by *D.siamensis* venom and its fractions in intact rabbit kidney (In vivo) and in isolated perfused rabbit kidney (In vitro)

	Catalase (unit/mg protein)	**SOD (Unit/ml/mg protein)**	**GSH (µmol/mg kidney protein)**	**MDA (nmol/mg kidney protein)**
In vivo (Intact kidney)				
Control	187.36±62.95	38.71±20.45	0.26±0.06	0.06±0.02
Gr.1: RVV	274.27±91.31	103.02±8.57*	1.10±0.28*	0.17±0.03
Gr.2: PLA2	272.55±24.38	94.29±31.53	0.69±0.09	0.09±0.03
Gr.3: Metalloprotease	417.54±148.70*	114.26±34.70*	0.58±0.10	0.07±0.03
Gr.4: L-amino acid oxidase	447.81±102.69*	200.31±47.66*	0.99±0.22	0.17±0.03
Gr.5: Phosphodiesterase	234.03±36.40	91.74±42.92	0.63±0.20	0.11±0.02
In vitro (IPK)				
Control	142.52±76.18	57.64±14.64	0.30±0.14	0.06±0.01
Gr.1: RVV	308.54±43.40*	50.20±18.25	0.79±0.07	0.13±0.01
Gr.2: PLA2	469.69±99.85*	92.89±63.41	0.85±0.28	0.09±0.02
Gr.3: Metalloprotease	421.55±70.39*	114.51±43.21*	0.63±0.06	0.05±0.03
Gr.4: L-amino acid oxidase	330.39±63.07*	108.73±22.62	0.62±0.12	0.11±0.02
Gr.5: Phosphodiesterase	146.13±35.00	97.39±18.98	0.44±0.10	0.08±0.01

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values respect to the basal period (control) in each group.

Table 2: Changes of TAS levels in urine, plasma and perfusate after envenomation by *D.siamensis* venom and its fractions in intact rabbit kidney (In vivo) and in isolated perfused rabbit kidney (In vitro)

In vivo:		**TAS (% inhibition)**				
Time after envenomation (min)		**0**	**10**	**30**	**60**	**90**
Gr.1: RVV	Plasma	5.27±4.61	13.69±4.7	18.54±6.5	25.04±3.3	26.13±2.7
	Urine	18.22±8.5	20.88±6.6	35.23±6.7	35.79±1.3	38.71±5.5
Gr.2: PLA2	Plasma	2.33±1.56	6.22±1.39	13.34±9.1	19.76±5.6	17.75±3.6
	Urine	29.50±7.7	33.76±5.1	29.69±2.2	30.09±11.53	32.14±5.4
Gr.3: Metalloprotease	Plasma	4.11±0.77	8.57±0.51	16.80±1.87	20.52±1.95	21.12±1.56
	Urine	30.47±10.62	27.71±14.47	22.41±13.39	27.96±16.33	32.38±20.46
Gr.4: L-amino acid oxidase	Plasma			----		
	Urine					
Gr.5: Phosphodiesterase	Plasma			----		
	Urine					
In vitro:		**TAS (% inhibition)**				
Time after envenomation (min)		**0**	**10**	**30**	**60**	**90**
Gr.1: RVV	Perfusate	2.39±0.41	3.21±1.44	1.92±1.08	0.70±0.44	1.55±1.39
	Urine	3.98±2.27	2.12±1.04	1.66±0.72	1.76±1.65	2.16±1.17
Gr.2: PLA2	Perfusate	3.03±0.80	3.78±1.04	3.63±0.46	3.33±0.66	3.57±0.36
	Urine	-11.68±19.15	-9.97±12.88	-14.09±16.06	-14.18±17.41	-14.18±17.41
Gr.3: Metalloprotease	Perfusate	5.64±1.05	5.87±1.46	5.68±2.05	5.52±1.17	5.87±1.52
	Urine	6.37±4.45	8.90±1.91	8.51±1.48	8.01±1.54	7.93±1.44
Gr.4: L-amino acid oxidase	Perfusate			---		
	Urine					
Gr.5: Phosphodiesterase	Perfusate			---		
	Urine					

Table 3: Changes of MDA levels in urine, plasma and perfusate after envenomation by *D.siamensis* venom and its fractions in intact rabbit kidney (In vivo) and in isolated perfused rabbit kidney (In vitro)

In vivo:		**MDA (nmol/ml)**				
Time after envenomation (min)		**0**	**10**	**30**	**60**	**90**
Gr.1: RVV	Plasma	4.22±0.68	6.26±4.11	8.83±3.20*	7.05±4.48*	5.62±1.82
	Urine	5.66±4.80	5.27±3.62	10.41±4.07	16.33±11.94*	15.15±4.10*
Gr.2: PLA2	Plasma	5.18±1.15	4.49±0.10	5.58±2.89	5.27±1.77	5.98±1.39
	Urine	1.67±0.33	3.77±0.50	5.74±1.36*	9.15±5.19*	6.78±2.64*
Gr.3: Metalloprotease	Plasma	3.11±0.32	4.95±1.48	4.71±0.66	4.16±3.61	5.06±1.44
	Urine	4.18±1.34	2.73±1.45	1.34±0.93*	3.65±0.65	3.55±0.57
Gr.4: L-amino acid oxidase	Plasma	7.06±0.12	9.62±0.06	15.18±1.22*	14.66±3.37*	9.79±1.41
	Urine	6.45±1.26	8.47±1.83	11.65±1.40*	10.79±0.39*	8.50±0.86
Gr.5: Phosphodiesterase	Plasma	3.60±0.78	4.19±0.28	7.11±0.56*	6.19±0.20	4.22±0.64
	Urine	4.85±1.77	9.56±2.65*	9.60±1.38*	5.93±2.86	6.04±1.50
In vitro:		**MDA (nmol/ml)**				
Time after envenomation (min)		**0**	**10**	**30**	**60**	**90**
Gr.1: RVV	Perfusate	0.90±0.08	2.61±1.43	0.96±0.15	3.01±2.04	2.84±1.51
	Urine	11.42±6.35	18.00±9.02	19.85±7.66*	24.06±4.88*	36.95±13.33*
Gr.2: PLA2	Perfusate	0.76±0.57	0.75±0.58	1.25±0.89*	1.14±0.33*	0.89±0.38
	Urine	6.68±4.37	5.58±2.82	5.57±3.70	6.15±3.70	6.83±4.07
Gr.3: Metalloprotease	Perfusate	0.43±0.59	0.41±0.21	0.65±0.40	0.46±0.30	0.35±0.25
	Urine	1.31±0.76	3.13±1.84*	3.07±1.46*	1.74±0.68	2.00±1.34
Gr.4: L-amino acid oxidase	Perfusate	6.29±1.01	7.69±1.77	9.99±1.40	9.25±0.57	6.44±0.90
	Urine	4.54±0.85	7.10±0.58	9.75±1.31*	7.60±1.62	3.86±1.15
Gr.5: Phosphodiesterase	Perfusate	3.58±0.35	6.21±1.51	6.30±0.64	5.45±0.92	3.82±0.34
	Urine	4.95±1.30	6.17±0.96	7.04±2.31	7.94±1.43	5.27±2.19

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values respect to the basal period (control) in each group.

Table 4: Changes of GSH levels in urine, plasma and perfusate after envenomation by *D.siamensis* venom and its fractions in intact rabbit kidney (In vivo) and in isolated perfused rabbit kidney (In vitro)

In vivo:		**GSH (mmol/l)**				
Time after envenomation (min)		**0**	**10**	**30**	**60**	**90**
Gr.1: RVV	Plasma	4.69±0.76	6.67±1.77	13.02±1.20*	16.40±3.50*	38.83±6.35*
	Urine	20.64±3.78	26.36±13.80	50.64±11.89	100.64±22.21*	109.93±20.86*
Gr.2: PLA2	Plasma	5.68±1.57	9.57±1.52	17.79±1.01*	16.00±0.51*	28.04±1.77*
	Urine	21.00±2.64	34.43±11.52	26.18±12.43	51.54±10.85*	71.54±14.51*
Gr.3: Metalloprotease	Plasma	1.71±0.30	4.39±3.59	10.14±5.66*	14.07±3.54*	11.68±4.39*
	Urine	7.55±2.05	10.86±4.19	15.13±9.34	25.45±13.58*	19.00±12.59*
Gr.4: L-amino acid oxidase	Plasma	34.31±6.53	36.23±12.51	48.92±7.61	29.69±7.61	19.97±10.48
	Urine	56.62±11.0	94.50±19.58	72.96±23.00	68.73±16.62	66.42±8.34
Gr.5: phosphodiesterase	Plasma	8.92±3.26	13.39±1.29	30.46±3.26	25.08±1.09	13.92±3.81
	Urine	31.04±5.90	68.54±24.60	65.46±18.48	41.81±19.45	33.64±18.64
In vitro:		**GSH (mmol/l)**				
Time after envenomation (min)		**0**	**10**	**30**	**60**	**90**
Gr.1: RVV	Perfusate	2.39±0.62	2.13±0.58	2.57±0.87	7.68±1.89*	13.11±3.16*
	Urine	4.30±2.18	5.61±0.95	12.75±1.90*	18.21±7.42*	11.32±5.27*
Gr.2: PLA2	Perfusate	3.64±1.52	4.56±2.07	4.34±0.84	14.96±3.87*	15.11±1.78*
	Urine	3.38±1.9	7.02±1.41	15.98±3.00*	11.82±3.53*	8.93±4.27
Gr.3: Metalloprotease	Perfusate	1.93±0.35	1.95±0.40	2.76±0.90	4.73±1.30*	4.68±1.37*
	Urine	2.34±0.58	4.88±0.86	4.04±0.83	10.29±2.16*	6.52±2.04*
Gr.4: L-amino acid oxidase	Perfusate	26.04±5.97	32.38±12.82	47.58±8.32	38.73±8.80	26.62±5.37
	Urine	18.92±4.07	31.38±2.67	27.77±6.95	19.81±7.13	9.69±4.12
Gr.5: Phosphodiesterase	Perfusate	10.08±2.04	25.08±8.99*	25.85±3.50*	19.12±3.35	11.42±2.12
	Urine	22.77±10.5	40.65±16.51	66.04±14.22*	60.27±22.45*	37.03±8.60

Data are preseted as mean ± SD of four different animals in each group. *P*-values analyzed by ANOVA (Bonferoni test): **P* < 0.05, mean values respect to the basal period (control) in each group.

Results and discussion

The present study evaluated the effect of *D. siamensis* and its fractions on the development of different behaviors, the antioxidant status in kidney functions and revealed the ability of bodily response to protect kidney against the deleterious effect of venom. The pathogenic role of reactive oxygen species in kidney injury is well established (Thadhani et al., 1996; Zager et al., 1999; Sapirstein et al., 2003) The cortex of kidney is region known to be particularly susceptible in toxin and infection disease and have an important role in Several studies suggest a general decline in renal functions affected by snake venom toxin. Because kidney is the target for RVV, the present study assessed whether it is important to screen for the potential effects of the venom and its fractions on induced oxidative damage in the kidney of experimental animals.

In the present experiment, there was a significant increase in lipid peroxidation after envenomation with venom and its fractions, measured in terms of TBARS levels in both in vivo and IPK model. Venom and its fractions causes the peroxidation of membrane lipids and thus causes membrane damage. These results are in agreement with studies of Isac de Castroa (2004) showed that venom exposure enhanced the kidney lipid peroxidative damage with concomitant alterations in the enzymatic defense system. Tubular toxicity is independent of extracellular calcium and mediated in part by lipid peroxidation.

The presence of either venom or its fractions particular PLA2 might affect the lipid membrane, in increased lipid peroxidation**.** The result is a substantial increase in the rate of phospholipid peroxidation in kidney cells, leading to membrane damage and cell death. The increased lipid peroxidation is, at least in part, due to an inhibition of SOD in the kidney tissue. The SOD presents the first line of defense against superoxide, as it dismutases the superoxide anion to H_2O_2 and O2(Fridovich I., 1983). Because the SOD enzyme generates H_2O_2, it works in collaboration with H2O2 removing enzymes. Catalase converts H_2O_2 to water and oxygen. Catalase is present in the peroxisomes of mammalian cells and probably serves to destroy H_2O_2 generated by oxidase enzymes located within these subcellular organelles (Fridovich, I., 1983).

However, the kidney is an organ that is especially susceptible to peroxide damage because of several factors, such as, high oxygen turnover, low mitotic exposure affects the kidney more than any other organ.

Glutathione peroxidase (GP_X) is an enzyme family with peroxides activity whose main biological role is to protect the organism from oxidative damage and help to prevent lipid peroxidation of cellular membrane by removing the free peroxides in the cell(Kankofer, M., 2002). Oxidative stress has been implicated in the pathogenesis of a number of disorders and its extent of injury is generally related to an increase or decrease of one or more free radical scavenging enzymes of which GPX is one. In the present study, venom and its fractions causes produced significant increase of glutathione (GSH) and glutathione reductase activities would be decreased.

It can conclude the present study reported that venom and its fraction is capable to cause marked alterations in some behavior and biochemical parameters by inducing an oxidative damage and inhibiting the antioxidant enzymes activities. The study recommended that attention should be paid to reduce the sources of envenomation to snake venom and using additional to conventional medicine for treatment of oxidative stress of kidney disorders.

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CHAPTER VI

Apoptosis relating to Bcl-2 family and transforming growth factor b1 signaling proteins in rabbit kidney injected with Russell's viper venom and its fractions

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Apoptosis relating to Bcl-2 family and transforming growth factor b1 signaling proteins in rabbit kidney injected with Russell's viper venom and its fractions

Introduction

Apoptosis or programmed cell death is a process of cell death morphologically characterized by cell volume reduction, chromatin condensation, DNA and nuclear fragmentation, and apoptotic body formation. Apoptosis is a gene-regulated occurrence characterized by special morphological features, including condensation of chromatin, shrinkage of cell and nucleus, membrane blebbing, and DNA fragmentation. It is known that apoptosis implicates in several cellular degenerative disorders from different etiologies. Apoptosis is mediated by two distinct pathways: the extrinsic or caspase pathway and the intrinsic or Bcl-2 family pathway. The extrinsic pathway causes the cleavage of effector caspases resulting in irreversible cell death. Caspase enzymes are working as a cascade and caspase 3 is the most important member of this family which may plays an effective role in apoptosis of kidney cell. The intrinsic pathway of apoptosis is regulated by the Bcl-2 gene family. Bcl-2 family is a set of cytoplasmic proteins that regulate apoptosis. There are 2 types of proteins in the Bcl-2 family that have either pro-apoptotic (e.g. Bax, Bad) or anti-apoptotic (e.g. Bcl-2, Bcl-XL) effects. During envenomation, an imbalance between Bcl-2 and Bax can cause either an apoptotic or an anti-apoptotic effect on cells. Apoptosis can be proposed as a possible mechanism for venom-induced kidney cell death. As current evidence does not support proliferation as a major mechanism of increased cell density in acute kidney injury after envenomation. This pathogenesis is not well understood. Whether this process can happen even after specific antivenom treatment.

The aims of this study are to evaluate signal transduction pathways involved apoptosis in renal failure induced by fractionated components from Russell's viper venom, by which (1) to determine the percent of cells undergoing apoptosis; (2) to evaluate the abundance and spatial distribution of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins; and (3) to evaluate the Caspase-3 activity.

Materials and Methods

Animals*:* Adult male New Zealand white rabbits weighing between 2-3 kg will be used for the study.

In *in vivo* study, animals will be divided into five groups of 4 animals each. Animals will be fasted for 24 h with free access to water and will be anesthetized with pentobarbital sodium *(*25 mg/kg*)* for further experiments.

In *in vitro* study, isolated perfused rabbit kidney will be prepared for experimental studies. The procedure for organ preparation for isolated perfused kidney will be described as in Experiment 1.

Protocol of study

Either in vivo or in vitro experiments will be conducted over 120 min after venom administration. Russell's viper *(Daboia siamensis)* venom will be milked and lyophilized at Queen Saovabha Memorial Institute, The Thai Red Cross Society. The lyophilized of Russell's viper venom will be fractionated to obtain metalloprotease, PLA_2, L- amino acid oxidase and phosphodiesterase of high purity in normal saline. These fractionated venoms will be used for studies both in vivo and in vitro studies. Two series of studies will be performed as following;

Series I*:* In vivo studies will be performed in five groups of experimental rabbits as following;

Group 1. After animal preparation for study of renal functions, the first 30 min of the experiment will be used as internal control and crude Russell's viper venom is intravenously injected to 0.5 mg/kg to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 2. After animal preparation for study of renal functions, the first 30 min of the experiment will be used as internal control and venom fraction of PLA_2 *(*0.2 mg/kg*)* is intravenously injected to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 3. After animal preparation for study of renal functions, the first 30 min of the experiment will be used as internal control and venom fraction of metalloprotease *(*0.2 mg/kg*)* is intravenously injected to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 4. After animal preparation for study of renal functions, the first 30 min of the experiment will be used as internal control and venom fraction of L-amino acid oxidase *(*0.25 mg/kg*)* is intravenously injected to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 5. After animal preparation for study of renal functions, the first 30 min of the experiment will be used as internal control and venom fraction of phosphodiesterase *(*0.25 mg*/*kg*)* is intravenously injected to study apoptosis pathway in kidney cell. *(*n=4*)*

Series II*:* In vitro studies, five groups of studies **in isolated perfused rabbit kidneys** will be performed as following;

Group 1. After the preparation of isolated perfused rabbit kidneys, the first 30 min of the experiment will be used as internal control and crude venom is added to the perfusion system to obtain the final concentration of crude Russell's viper venom *(*10 µg*/*ml*)* to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 2. After the preparation of isolated perfused rabbit kidneys, the first 30 min of the experiment will be used as internal control and venom fraction of PLA_2 *(*400µg*/*100ml*)* is added to the perfusion system to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 3. After the preparation of isolated perfused rabbit kidneys, the first 30 min of the experiment will be used as internal control and venom fraction of metalloprotease *(*400µg*/*100ml*)* is added to the perfusion system to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 4. After the preparation of isolated perfused rabbit kidneys, the first 30 min of the experiment will be used as internal control and venom fraction of L-amino acid oxidase *(*5µg*/*ml*)* is added to the perfusion system to study apoptosis pathway in kidney cell. *(*n=4*)*

Group 5. After the preparation of isolated perfused rabbit kidneys, the first 30 min of the experiment will be used as internal control and venom fraction of phosphodiesterase *(*5µg*/*ml*)* is added to the perfusion system to study apoptosis pathway in kidney cell. *(*n=4*)*

At the end of the 120 minute after envenomation, Kidney tissues *(*medulla and cortex*)* will be removed and placed on an ice-cooled cutting board. Kidney tissues will be dissected and divided to two portions. One portion with snap frozen in liquid nitrogen and stored at −70◦C for extraction of DNA, RNA, and protein and other portion of tissue will be kept in 10*%* formaldehyde solution for other analysis.

1. Isolation of total RNA*:* Total RNA will be isolated from processed kidney tissue of rabbits using Trizol Isolation Reagent *(*Molecular Research Center, USA*)* according to the manufacturer instructions.

2. Synthesis of cDNA: Total RNA will be reverse transcribed (RT) into cDNA with Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following the kit protocol. A quantitative polymerase chain reaction assay (Real-time RT-PCR) will be carried out using RBC ThermOne Real-Time Premix (SYBR Green), (RBC Bioscience, Taiwan) to determine the levels of Bcl-2, Bcl-xL, and Bax mRNA expressions. A house keeping gene such as GAPDH gene (Glyceraldehydes-3-phosphate dehydrogenase) will be amplified under the same condition as an internal control for normalization of gene expression levels.

3. Western blot analysis:

Kidney tissues collected will be homogenized with ice-cold homogenizing buffer with protease inhibitor and proteins will be measured. Kidney tissue protein lysates (per well) will be separated by SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Blots are blocked with blocking buffer (5% not-fat dried milk in PBS). After blocking, blots will be incubated with anti-Bcl-2 polyclonal antibody (1/500, v/v), anti-Bcl-xL polyclonal antibody (1/1000, v/v), and anti- Bax polyclonal antibody (1/250, v/v) for 20 hours at 4◦C. Blots will be washed with 0.1% tween 20 in PBS and incubated with HRP conjugated secondary antibody (1/5000, v/v) at room temperature. The Bcl-2, Bcl-xL and Bax protein bands were visualized using enhanced chemiluminescnces method.

4. Detection of Caspase-3 activity: Caspase-3 activity will be determined using the Caspase-3/CPP32 Fluorometric Assay Kit. For each assay, tissue cell lysate will be used. Samples will be read in a fluorimeter. Caspase-3 activity will be determined by comparing fluorescence of 7-amino-4-trifluoromethyl coumarin in control and treated kidney tissues. Determination of the apoptotic cells within kidney tissue by assessing the immunoreactivity of cleaved caspase-3 and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay using tissue microarray technique will also be carried out.

Determination for the percent of cells undergoing apoptosis pathways:

The apoptosis pathways were investigated by determination of parameters as followed: spatial distribution of proapoptotic (Bax) proteins and anti-apoptotic (Bcl-2) proteins using tissue microarray technique.

Statistical analysis*:* All results are evaluated using the mean ± S.D. The results will be analyzed with t-tests.

Histopathology

Tissue collection and preparations

The kidneys were obtained from necropsy cases in each group. Each kidney was divided into2 parts: one was preserved in 10% buffered formalin for histopathological examination, and another one was immediately collected and snapped frozen in liquid nitrogen before stored at -80°C for extraction of mRNA.

Tissue samples were preserved in 10%buffered formalin for 24 hours, routinely histologic processed and embedded in paraffin wax. Four-micrometer-thickness serial sections were stained with routine Hematoxylin and Eosin (H&E) staining for histopathological study under light microscope and special staining with Periodic Acid-Schiff reaction (PAS) in order to glomerular and tubular lesion study.

Histopathological lesions in the cortex and medulla of renal tissues were examined and scored under a light microscope by a veterinary pathologist. Microscopic lesions of glomerular and tubular compartments including tubular dilatation, tubular cell flattening, tubular cell vacuolization and deposition of tubular cast were noted.

Results

Table 1**:** Histopathological lesions in rabbit intact kidneys after administration of snake venom and its fractions.

In vivo

Histopathology

Lesion / Group	Control	RVV	PLA2	MET	LAAO	PDE
Glomerular congestion	2.93±0.10	2.80±0.40	3.00±0.00	3.00±0.00	0	0
Crystal deposit in glomerulus	0	0	0	0	0	0
Tubulonephrosis, proximal	0	1.25±0.17	1.40±0.37	1.50±0.71	0	0
Tubulo-necrosis, proximal	0	0	0	0	0	0
Tubulonephrosis, distal	0	1.10±0.08	1.15±0.13	1.10±0.42	0	0
Tubulo-necrosis, distal	0	0	0	0	0	0
Tubulonephrosis, collecting	0	2	2	2	0	0
Tubulo-necrosis, collecting	0	0	0	0	0	0

BCL-2

Lesion / Group	Control	RVV	PLA2	MET	LAAO	PDE
Proximal convoluted tubules	0.55±0.69	0.03±0.16	0.20±0.40	0.53±0.60	0.50±0.49	0.08±0.13
Distal Convoluted tubules	0.95±0.89	0.20±0.41	0.40±0.47	0.73±0.55	0.50±0.49	0.08±0.13
Collecting ducts	0.60±0.50	0.05±0.22	0.05±0.22	0.13±0.34	0.58±0.33	0.03±0.04
Total	0.70±0.72	0.09±0.29	0.22±0.41	0.46±0.56	0.53±0.43	0.06±0.10

BAX

Lesion / Group	Control	RVV	PLA2	MET	LAAO	PDE
Proximal convoluted tubules	0.60±0.60	1.00±0.56	1.43±0.81	1.40±0.67	0.45±0.47	0.23±0.23
Distal Convoluted tubules	0.05±0.22	0.00±0.00	0.13±0.34	0.03±0.16	0.45±0.47	0.23±0.23
Collecting ducts	0.90±0.72	0.40±0.60	0.73±0.63	0.90±0.55	1.25±0.42	0.28±0.18
Total	0.52±0.65	0.47±0.62	0.72±0.82	0.78±0.76	0.72±0.25	0.24±0.18

Remark: Lesion score showed in the table is semi-quatitative analysis: 0 = No remarkable lesion **(**NRL**)**; 1 = mild degree change; 2 = moderate degree change; 3 = severe degree change

Table 2**:** Histopathological lesions in isolated rabbit kidneys after administration of snake veom and its fractions.

In vitro

Histopathology

Lesion / Group	Control	RVV	PLA2	MET	LAAO	PDE
Glomerular congestion	0.05±0.09	0.05±0.05	0.24±0.48	0	0	0
Crystal deposit in glomerulus	0	2.23±0.16	1.77±1.20	1.05±0.94	0	0
Tubulonephrosis, proximal	0	2.03±0.21	2.63±0.62	2.70±0.54	2.32±0.67	1.80±0.12
Tubulo-necrosis, proximal	0	0.05±0.10	0.15±0.30	0.10±0.14	0	0
Tubulonephrosis, distal	0	1.68±0.56	2.45±0.84	2.83±0.24	2.36±0.74	1.75±0.15
Tubulo-necrosis, distal	0	0.03±0.05	0.35±0.26	0.15±0.24	0	0
Tubulonephrosis, collecting	0	2.43±0.26	2.95±0.10	2.75±0.50	2.54±0.56	1.83±0.24
Tubulo-necrosis, collecting	0	0.18±0.29	0.13±0.25	0.18±0.22	0	0

BCL-2

Lesion / Group	Control	RVV	PLA2	MET	LAAO	PDE
Proximal convoluted tubules	2.18±0.59	1.11±0.63	1.03±0.90	2.33±0.08	2.40±0.22	2.47±0.44
Distal Convoluted tubules	2.53±0.64	0.95±0.71	1.30±0.96	2.50±0.64	2.40±0.22	2.47±0.44
Collecting ducts	2.50±0.56	0.93±0.86	0.67±0.51	1.45±0.71	2.62±0.37	2.75±0.23
Total	2.44±0.61	0.99±0.74	1.00±0.85	2.09±0.81	2.47±0.17	2.56±0.28

BAX

Lesion / Group	Control	RVV	PLA2	MET	LAAO	PDE
Proximal convoluted tubules	1.16±0.74	2.31±0.65	1.83±0.79	1.93±0.66	2.52±0.40	2.10±0.59
Distal Convoluted tubules	1.00±0.74	0.49±0.69	0.52±0.73	0.30±0.56	2.52±0.40	2.10±0.59
Collecting ducts	0.47±0.70	1.46±0.98	0.68±0.83	0.58±0.50	2.60±0.26	2.43±0.43
Total	0.83±0.78	1.42±1.08	1.01±0.98	0.93±0.91	2.55±0.33	2.21±0.51

Remark: Lesion score showed in the table is semi**-**quatitative analysis: 0 = No remarkable lesion **(**NRL**)**; 1 = mild degree change; 2 = moderate degree change; 3 = severe degree change

Histopathological result

Main lesion of the kidneys was located in cortex which is composed of glomerulus, proximal convoluted tubules and distal convoluted tubules**.** Glomerulus of the group IPK**-**PLA_2 and IPK**-**Metallo showed diffuse unidentified crystals; fragile, translucent and spherical**-**shaped crystals, deposited in glomerular capillary lumen without inflammatory response**.** Most of affected tubules were proximal and distal convoluted tubules showed various degrees of tubulonephrosis**.** The tubules were hydropic degeneration; swelling, cloudy swelling of cytoplasm and some contained homogeneous eosinophilic hyaline droplets in their cytoplasm**.** There was no evidence of necrosis and infiltration of acute inflammatory cell infiltration **(**neutrophil and macrophage**)** in affected tubules in all groups**.** Other old lesions of multifocal chronic interstitial nephritis; tubular degeneration and necrosis, infiltration of chronic inflammation cells **(**lymphocytes and plasma cells**)** and increased fibrotic tissue in interstitium was noted in each group, but this lesion was not interfere with experimental observed lesion

*Protein expressions of Bcl**-**2 family protein **(**Bax and Bcl–2**)** by using immunohistochemistry method*

Bax and Bcl**-**2 protein expression of positive control; canine lymphoma and normal rabbit kidney, lymph node and spleen were performed by immunohistochemistry as previously described**.**

Results and discussion

Acute renal failure (ARF) is the most serious complication of Russell's viper (*Daboia siamensis*) envenomation. Acute tubular necrosis (ATN) is the most common cause of ARF and pathogenesis of ATN in snakebite envenomation may be related to many factors such as hemodynamic disturbances, immunologic reactions and direct nephrotoxicity, although evidence for the latter factor is still lacking (Sitprija and Chaiyabutr, 1999). The pathogenesis mechanisms underlying acute renal failure (ARF) may include renal vascular obstruction by fibrin micro-thrombi (DIC), ischemia or hypoperfusion due to the fall in blood pressure and hemolysis-induced pigment nephropathy due to some extent hemolytic (Sitprija et al., 1974). This manifestation cannot exclude the presence of proteolytic enzymes and vasoactive substances which could promote, or even potentiate, the coagulant process in renal sites. The etiology of RVV induced

acute renal failure in humans and experimental animals is still not completely understood but probably involves a direct action of venom components on renal tubules and renal epithelial cells via the process of apoptosis

The present study was designed to investigate the effect of *D. siamensis* venom and its fractions on kidney function relating to apoptosis pathways; the intrinsic and extrinsic pathways. The intrinsic pathway is regulated by BCL2 family members and effector function is mediated through release of proteins such as cytochrome c through the process of mitochondrial outer membrane permeability (Youle and Strasser, 2008). Apototic pathway is controlled by apoptotic inhibitor (BCL2) and apoptotic promoters (BAX).

It can conclude the present study reported that RVV, PLA_2 and MP caused mild degree of proximal and distal tubulonephrosis in *in* vivo model while RVV and all of its fractions caused more damage in *in vitro* model by caused moderate to severe tubulonephrosis at proximal, distal tubule and collecting duct. The result of intrinsic pathway (BCL2) show lower level of BCL2 at all renal tubular cells in RVV and PLA_2 in both *in vivo* and *in vitro* model. While BAX value in *in vitro* model, MP and PLA_2 is lower than control but LAAO and PDE has opposite result. All of these results indicate that, RVV and its fraction has cause tubular cells damage via apoptotic pathways in different pattern. In *in vitro* model has more damage than *in vivo* model which may be the result of extrarenal factors that reduce severity by compensatory mechanism.

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CHAPTER VII

The renal effects of phospholipase A_2, metalloprotease, L-amino acid oxidase, and phosphodiesterase purified from Russell's viper venom: the role of Ca^{++}, Na^{+} and K^{+} channel blockers

CHAPTER VII

The renal effects of phospholipase A_2, purified from Russell's viper venom: the role of Ca^{++}, Na^{+} and K^{+} channel blockers

Introduction

Acute renal failure (ARF) is the most serious complication of Russell's viper (*Daboie siamensis*) envenomation. Acute tubular necrosis (ATN) is the most common cause of ARF and pathogenesis of ATN in snakebite envenomation may be related to many factors such as hemodynamic disturbances, immunologic reactions and direct nephrotoxicity, although evidence for the latter factor is still lacking (Sitprija and Chaiyabutr, 1999). The pathogenesis mechanisms underlying acute renal failure (ARF) may include renal vascular obstruction by fibrin micro-thrombi (DIC), ischemia or hypoperfusion due to the fall in blood pressure and hemolysis-induced pigment nephropathy due to some extent hemolytic (Sitprija et al., 1974). This manifestation cannot exclude the presence of proteolytic enzymes and vasoactive substances which could promote, or even potentiate, the coagulant process in renal sites. The etiology of RVV induced acute renal failure in humans and experimental animals is still not completely understood but probably involves a direct action of venom components on renal tubules and renal epithelial cells (Sitprija and Chaiyabutr, 1999) and most serious complication during Russell's viper bite is acute kidney injury (AKI) (Kanjanabuch and Sitprija, 2008) Systemic manifestations such as bleeding and hemodynamic instability may also occur which may evolve to disseminate intravascular coagulation. Several factors have been implicated in the pathogenesis of *Daboia siamensis* venom induced-ARF such as renal vasoconstriction and consequent renal ischemia, hemolysis, glomeruli fibrin deposition and vascular injury (Sitprija et al., 1974). Renal tubular injury may result either from renal ischemia, hemoglobinuria or coagulation disturbances. It may also result from a direct nephrotoxic effect and its renal mechanism is scarce (Sitprija and Chaiyabutr, 1999). An experimental model using *D. siamensis* infused intravenously in dogs was described, which Tungthanathanich et al (1986) demonstrated that intravenous *D. siamensis* venom caused impairment of glomerular filtration and renal blood flow. In addition, perfusion of isolated rabbit kidneys with *D. siamensis* venom reduced perfusion pressure and increased vascular resistance and appears to have a direct cytotoxic effect on renal tubular epithelial cells (Chaiyabutr et al.,

2014, 2020). However, clear evidences of mechanism of direct effect of *D. siamensis* venom on renal tubules are still lacking. It is unknown whether venom fraction like phospholipase A2, a major venom component affect a possible direct venom tubular toxicity on ions channels at tubular cell membrane. Use of isolated kidney allows the investigation of venom direct effect without interference of venom systemic and hemodynamic effects. On this background, the present study was designed to evaluate a direct effect of phospholipase A2 fraction from *D. siamensis* venom on isolated perfused rabbit kidney using channel blockers on venom tubular toxicity.

Materials and Methods

Experimental animals

Adult male white New Zealand rabbits, weighing 2 to 3 kg, were obtained from the Animal house, Queen Saovabha Memorial Institute. The animals settled in stainless steel cages, fed a standard diet and water. Exposed to a 12 h light/dark cycle, and maintained at a laboratory temperature of 26 ± 1°C. The animals quarantined for 14 days before the experiments. Animals involved in this study were conducted with permission of the Ethic Committee of the Queen Saovabha Memorial Institute Animal Care and Use (approval number QSMI-ACUC-03-2016) in accordance with the guideline of the National Research Council of Thailand.

Isolated perfused kidney preparation

The preparation for isolated perfused rabbit kidney (rabbit IPK) was based on methods described previously (Chaiyabutr et al. 2014). Briefly, adult male white New Zealand rabbits were fasted for 24 h before the experiment with access to water ad libitum. The rabbit was anaesthetized with pentobarbital sodium (30 mg/kg, i.v). The animal was given 1,000 units heparin intravenously. After opening the abdomen, the left ureter was cannulated with polyvinyl catheter. The left renal artery was prepared for perfusion after careful exposure from its surrounding tissue, and was ligated above the renal artery of the left kidney. The 19-gauge stainless steel needle, approximately 1.0 inch in length with a smooth tip, served as the arterial cannula. The needle was inserted into the renal artery and flushed with heparinized saline (100 units/ml). The kidney was isolated with the renal vein and the ureter intact and immediately transferred to a thermostatically controlled tissue bath organ chamber (Radnoti, chamber for organ isolation procedures, catalog No. 166070, Grass Technologies, Monrovia, CA, USA). The fluid perfusing the kidney flowed

from the cut end of the renal vein and the ureter. The isolated kidney was used to employ a recirculating perfusion design by means of a perfusion apparatus ex vivo. The working perfusate with an oxygenated modified Krebs–Henseleit solution at a temperature of 37°C and aerated with a gas mixture of O_2: CO_2 (95:5) was perfused through the renal artery by means of a recirculating a rotary pump (EYELA, Roller pump, RP-1000). The rabbit IPK was maintained at constant perfusion flow rate (40-60 ml/min) throughout the experiments.

The preparation of a modified Krebs Henseleit solution (MKHS) was described by Taft (2004). Total perfusate used per experiment was 100 ml consisting the composition (in mM) as following: 141 mM Na^+, 5.4 mM K^+, 1.9 mM Ca^{++}, 2.4 mM Mg^{++}, 126 mM Cl^-, 25 mM HCO_3^-, 2.44 mM SO_4^{2-}, 1.5 mM PO_4^{3-}, and 13 mM amino acids (consisted of alanine 1.470 mM, arginine 0.365 mM, asparagine 0.152 mM, aspartate 0.150 mM, cysteine 0.363 mM, glutamate 0.367 mM, glutamine 1.470 mM, glycine 1.680 mM, histidine 0.177 mM, isoleucine 0.221 mM, leucine 0.298 mM, lysine 0.733 mM, methionine 0.242 mM, phenylalanine 0.236 mM, proline 0.235 mM, serine 0.733 mM, threonine 0.176 mM, tryptophane 0.054 mM, tyrosine 0.149 mM and valine 0.258 mM). Total perfusate also contained 100 mg D-glucose, 50 mg inulin including both 3 gm of bovine serum albumin (BSA fraction V, from Sigma Chemical Co. (St Louis. MO, USA) and 2 gm of dextran (Sigma Chemical Co.) as oncotic agent. The perfusion solution was adjusted at pH 7.4 and kept warm at 37°C by a pre-warming coil and oxygenation by the addition of a 1.2 μm filter in tissue bath organ chamber. Changes of perfusion pressure in the kidney were measured at the tip of the stainless-steel cannula with either a manometer or recorded on a Statham strain gauge pressure transducer, thereby allowing continuous record monitoring of perfusion pressure on the physiograph (Polygraph Model 79, Grass instruments Co.). The kidney was mounted in the perfusion system for 30 min. to allow the kidney approach to normal function as indicated by maintenance of urine flow and perfusion pressure, which was carefully kept at 100 mmHg. The perfusion pressure was measured at 5 min. intervals after equilibration. The first 30 min. of perfusion were considered to be internal control. The experimental period was divided into five intervals of 5, 10, 30, 60 and 90 min. of perfusion time. The experiments were conducted over 90 min. after either RVV or its fractions administration. In each interval period, samples of perfusate and urine were collected for 5 min. for determinations of sodium, potassium, inulin and osmolality.

Venom and chemical

A pool of venom obtained from 14 adult males and females Russell's viper (*Daboia siamensis*) snakes collected from the eastern region of Thailand, and maintained at Queen Saovabha Memorial Institute, The Thai Red Cross Society. Russell's viper venom (RVV) was milked, lyophilized and stored at -20º C. The crude RVV was isolated by fractionation methods for phospholipase A_2 (PLA_2).

The isolation of phospholipase A_2 from RVV (PLA_2)

Crude RVV was dissolved in buffer A (50 mM phosphate buffer pH 6.0). After centrifugation at 10,000 rpm for 5 min, the supernatant was applied to ion-exchange chromatography on HiTrap CMFF column (GE Healthcare, Sweden). The column was washed with 5 volumes of buffer A and elution was carried out with an increasing linear concentration gradient of NaCl from 0 – 1 M in buffer A at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and measured at absorbance 280 nm under an AKTA pure Fast Protein Liquid Chromatography system (FPLC, GE Healthcare, Sweden). Four peaks were observed and determined for PLA_2 activity. Fractions containing PLA_2 activity were pooled and further purified by size exclusion chromatography in a pre-equilibrated Superdex^{TM} 75 10/300GL column. (GE Healthcare, Sweden). Elution was carried out with 10 mM PBS pH 7.4 at room temperature. The flow rate was adjusted to 0.5 ml/min, and 1 ml fraction was collected in each tube. The proteins were determined by absorbance at 280 nm under a Unicorn 6.3 Software.

Experiment protocol

The experiment in protocol was assigned to study the effects of whole RVV and venom fractions (PLA_2,) on renal functions in IPK study. Preparations of rabbit IPK were divided into 10 experimental groups (n = 4 each/group). The experimental groups in protocols 2 were as follows:

Group II: (PLA_2)

IPK was treated with 1 ml of the lyophilized RVV in normal saline (1 mg/ml) which was added into 100 ml of perfusate of the recirculating system after 30 min. of the equilibration period as internal control. This dose was arbitrarily chosen on the basis of an earlier investigation in experimental animals in either dogs or rabbits, where the dosage of LD_{50} of crude RVV by

intravenously injection was 0.5 mg/kg body weight (Chaiyabutr et al., 2014; Tungthanathanich et al., 1986). This dosage converted to a concentration expressed in 10 μg/ml plasma by calculation with an estimation of the percentage of plasma volume in 5% of body weight. This concentration value was chosen for the dose of whole RVV used in IPK studies, which 1 mg of the RVV was added to 100 ml of perfusate to obtain the final concentration of 10 μg/ml. The changes in renal functions were recorded thereafter for a 90 min. period after whole RVV administration (RVV group).

Group II (PLA_2 + Free Ca^{2+} perfusate)

IPK was pretreated with 100 μl of WEB 2086 (200 μg/100 μl) after the 30-min. equilibration of perfusion and 1 ml of the whole RVV (1 mg/ml) was added into 100 ml of perfusate of the recirculating system later after 30 min. period of pretreatment with WEB 2086. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and whole RVV administrations (WEB 2086 +RVV group).

Group III (Verapamil + PLA_2)

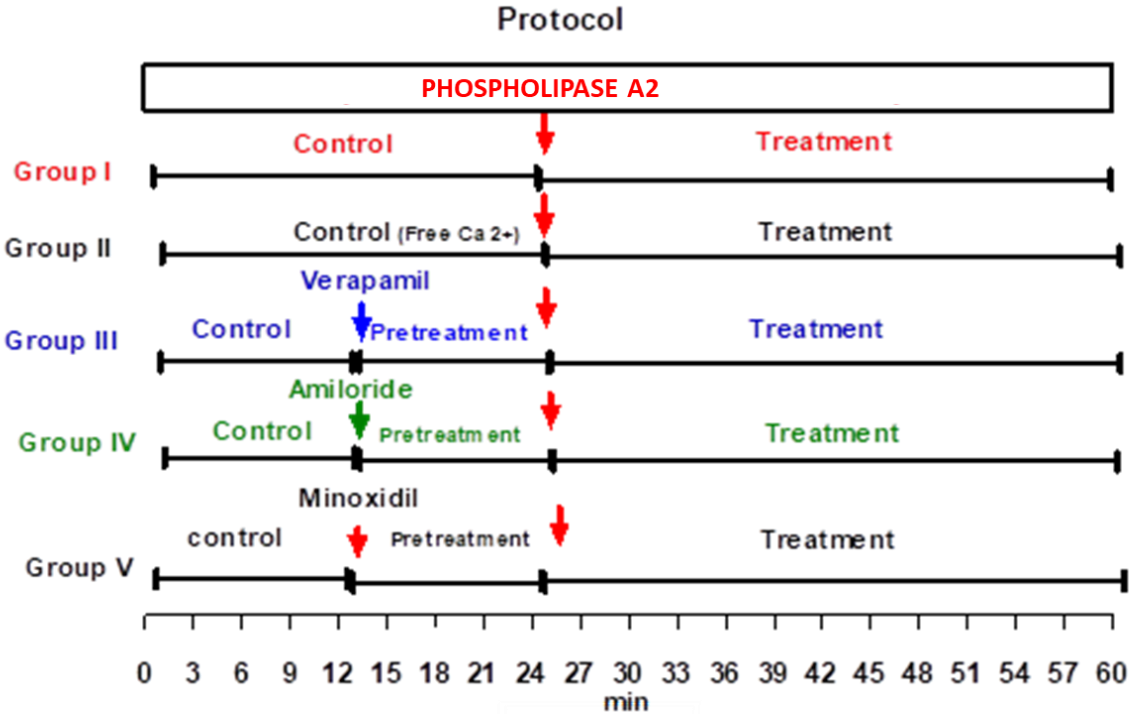
was treated only with 1 ml of the $RvPLA_2$ fraction (280 μg/ml) which was added into 100 ml of perfusate of the recirculating system after 30 min. of equilibration period as internal control. This dose was arbitrarily chosen and adjusted according to our previously described work in experimental animals (Mitrmoonpitak et al., 2013) using a dose of PLA_2 fraction at 140 μg/kg body weight by intravenously injection. This dosage converted to a concentration expressed in 140 μg/50 ml plasma by calculation with an estimation of the percentage of plasma volume in 5% of body weight. Therefore, the amount of $RvPLA_2$ using in the present study was adjusted by adding $RvPLA_2$ 280 μg into 100 ml of perfusate. The changes in renal functions were recorded thereafter for a 90 min. period after RvPLA2 administration (PLA_2 group).

Group IV (Amiloride + PLA_2)

IPK was pretreated with 100 μl of WEB 2086 (200 μg/100 μl) after the 30 min. equilibration period of perfusion. Then 1ml of the $RvPLA_2$ (280 μg/ml) was added to the recirculating solution later after 30 min. period of pretreatment with WEB 2086. The changes in renal functions were recorded thereafter for a 90 min. period after WEB 2086 and $RvPLA_2$ administrations (WEB 2086 +PLA_2 group).

Group V (Minoxidil + PLA_2)

IPK was treated only with 1 ml of the RvMP fraction (280 µg/ ml) which was added into the perfusate of the recirculating system after 30 min. of equilibration period as internal control. This dose was arbitrarily chosen and adjusted according to our previously described work in experimental animals (Mitrmoonpitak et al., 2013) using a dose of MP fraction at 140 µg/kg body weight by intravenously injection. This dosage converted to a concentration expressed in 140 µg/50 ml plasma by calculation with an estimation of the percentage of plasma volume in 5% of body weight. Therefore, the amount of RvMP using in the present study was adjusted by adding RvMP 280 µg into 100 ml of perfusate. The changes in renal functions were recorded thereafter for a 90 min. period after RvMP administration (MP group).



Results

	UF (ml/min)	GFR (ml/min)	PP (mmHg)	RVR mmHg/ml/min	%TNa^+ (%)	%TK^+ (%)	Cosm (ml/min)
Gr.I (PLA_2)							
Control period							
3 min. Pretreatment	0.15±0.06	0.31±0.14	95.25±17.08	2.71±0.77	56.94±27.62	91.83±9.32	0.38±0.22
6 min. Pretreatment	0.18±0.10	0.37±0.21	96.75±15.31	2.75±0.81	56.03±26.93	92.85±10.23	0.41±0.28
9 min. Pretreatment	0.21±0.11	0.38±0.18	95.75±15.88	2.79±0.80	60.03±26.59	99.46±10.78	0.52±0.44
12 min. Pretreatment	0.22±0.12	0.46±0.32	96.25±14.89	2.79±0.80	56.68±27.48	91.80±18.46	0.52±0.39
15 min. Pretreatment	0.25±0.16	0.50±0.34	96.25±14.89	2.86±0.82	58.74±28.14	97.00±18.45	0.63±0.53
Treated PLA_2 period							
3 min. after PLA_2	0.27±0.20	0.63±0.52	106.25±13.07	2.98±0.80	47.52±20.42	75.33±26.65	0.83±0.86
6 min. after PLA_2	0.26±0.17	0.49±0.28	110.25±14.52	3.03±0.83	57.23±25.65	87.11±26.30	0.72±0.57
9 min. after PLA_2	0.23±0.14	0.43±0.21	112.50±13.18	3.00±0.76	54.68±23.34	87.42±27.64	0.62±0.51
12 min. after PLA_2	0.23±0.11	0.42±0.16	113.50±9.26	3.02±0.74	59.04±28.78	88.54±33.52	0.52±0.34
15 min. after PLA_2	0.25±0.10	0.40±0.15	115.75±9.54	3.04±0.72	69.87±26.53	93.40±13.48	0.54±0.27
18 min. after PLA_2	0.26±0.12	0.39±0.18	117.50±6.86	3.04±0.72	73.01±27.90	97.20±9.66	0.54±0.15
Gr.II (PLA2+Free Ca^{2+} perfusate)							
Control period							
3 min. Pretreatment	0.48±0.27	0.64±0.36	75.25±16.88	1.64±0.85	75.75±25.27	76.89±14.75	0.38±0.20
6 min. Pretreatment	0.47±0.30	0.56±0.32	76.75±17.39	1.68±0.88	82.91±34.07	83.28±13.34	0.42±0.27
9 min. Pretreatment	0.44±0.30	0.54±0.34	78.25±17.48	1.71±0.89	79.06±23.64	80.91±17.92	0.34±0.20
12 min. Pretreatment	0.45±0.30	0.49±0.32	79.00±16.69	1.72±0.88	91.25±42.06	89.20±14.75	0.35±0.22
15 min. Pretreatment	0.44±0.30	0.48±0.32	79.50±16.22	1.73±0.87	91.35±42.99	89.30±18.37	0.34±0.20
Treated PLA2 period							
3 min. after PLA_2	0.50±0.39	0.52±0.34	79.25±15.00	1.72±0.84	93.41±38.51	90.64±20.94	0.38±0.28
6 min. after PLA_2	0.44±0.35	0.43±0.27	78.75±14.08	1.70±0.81	97.78±49.67	89.97±23.48	0.35±0.24
9 min. after PLA_2	0.41±0.31	0.39±0.24	78.75±14.08	1.70±0.81	104.05±59.69	93.92±22.06	0.30±0.21
12 min. after PLA_2	0.40±0.29	0.43±0.26	79.25±14.17	1.71±0.81	89.21±41.57	83.35±30.57	0.33±0.22
15 min. after PLA_2	0.40±0.29	0.45±0.26	79.00±14.12	1.71±0.81	88.79±45.13	81.70±30.96	0.31±0.19
18 min. after PLA_2	0.42±0.29	0.49±0.30	79.00±14.12	1.71±0.81	88.26±46.60	80.67±36.15	0.31±0.20
Gr.III (Verapamil+PLA_2)							
Control period							
3 min. after control	0.11±0.03	0.26±0.07	101.00±2.83	1.51±0.16	39.81±8.02	93.67±7.28	0.10±0.01
6 min. after control	0.12±0.03	0.30±0.09	100.25±4.99	1.52±0.15	42.15±10.30	98.33±7.35	0.10±0.00
9 min. after control	0.13±0.04	0.29±0.09	100.50±4.93	1.52±0.16	44.74±14.13	99.24±14.72	0.11±0.01
12 min. after control	0.15±0.04	0.31±0.10	100.75±5.91	1.52±0.17	39.72±8.11	101.97±17.37	0.13±0.04
Pretreated Verapamil							
3 min. after Verapamil	0.17±0.08	0.42±0.24	104.25±7.14	1.58±0.13	42.86±11.21	85.56±8.04	0.19±0.07
6 min. after Verapamil	0.18±0.10	0.43±0.27	105.50±6.66	1.58±0.14	45.86±10.32	90.96±6.04	0.22±0.09
9 min. after Verapamil	0.21±0.13	0.45±0.30	106.25±7.09	1.59±0.07	48.20±7.98	95.53±8.49	0.25±0.10

12 min. after Verapamil	0.21±0.12	0.48±0.32	107.00±6.68	1.61±0.09	48.15±11.59	92.74±7.41	0.26±0.14
15 min. after Verapamil	0.28±0.19	0.56±0.34	106.50±7.23	1.59±0.06	50.54±9.28	97.01±10.30	0.28±0.09
Treatment Verapamil+PLA_2							
3 min. after Verapamil+PLA_2	0.25±0.17	0.54±0.41	108.25±6.90	1.60±0.08	50.09±6.87	89.15±12.88	0.30±0.17
6 min. after Verapamil+PLA_2	0.26±0.16	0.55±0.40	110.50±6.14	1.65±0.12	50.33±7.47	89.76±12.03	0.29±0.19
9 min. after Verapamil+PLA_2	0.25±0.14	0.53±0.36	110.50±7.72	1.63±0.09	51.48±8.20	89.24±7.92	0.31±0.17
12 min. after Verapamil+PLA_2	0.23±0.13	0.46±0.33	111.25±5.56	1.65±0.12	54.13±7.46	91.84±12.00	0.23±0.17
15 min. after Verapamil+PLA_2	0.22±0.14	0.42±0.31	115.00±5.23	1.68±0.17	55.69±5.68	92.84±12.09	0.22±0.21
18 min. after Verapamil+PLA_2	0.22±0.13	0.42±0.29	118.75±9.32	1.70±0.19	57.36±6.42	90.20±11.38	0.22±0.22

	UF (**ml/min**)	**GFR** (**ml/min**)	**PP** (**mmHg**)	**RVR** **mmHg/ml/min**	**%TNa^+** **(%)**	**%TK^+** **(%)**	**Cosm** (**ml/min**)
Gr.IV (Amiloride+PLA2)							
Control period							
3 min. after control	0.18±0.11	0.25±0.13	92.00±9.27	1.34±0.55	73.11±17.88	101.56±7.70	0.16±0.08
6 min. after control	0.19±0.12	0.26±0.15	92.00±9.97	1.35±0.56	71.46±17.13	99.28±4.61	0.17±0.09
9 min. after control	0.20±0.11	0.26±0.11	92.00±10.80	1.34±0.54	74.48±21.80	102.83±12.49	0.18±0.11
12 min. after control	0.21±0.11	0.27±0.10	92.50±10.97	1.35±0.54	75.25±22.45	104.20±15.51	0.19±0.09
Pretreated Amirolide							
3 min. after Amirolide	0.20±0.09	0.26±0.09	91.25±11.53	1.33±0.52	75.74±24.85	99.85±16.84	0.18±0.06
6 min. after Amirolide	0.21±0.08	0.27±0.07	90.75±11.03	1.33±0.52	75.40±22.77	93.18±15.35	0.18±0.05
9 min. after Amirolide	0.20±0.05	0.27±0.06	91.25±10.53	1.34±0.52	73.56±22.24	88.02±16.13	0.19±0.03
12 min. after Amirolide	0.22±0.04	0.30±0.06	92.00±10.92	1.36±0.51	73.11±21.18	85.51±16.19	0.20±0.01
15 min. after Amirolide	0.22±0.02	0.30±0.07	92.50±12.29	1.41±0.50	74.92±22.83	86.61±18.30	0.23±0.04
Treatment Amirolide+PLA_2							
3 min. after Amirolide+PLA_2	0.34±0.21	0.41±0.17	98.00±21.02	1.49±0.47	77.97±15.46	89.35±16.96	0.28±0.10
6 min. after Amirolide+PLA_2	0.36±0.21	0.43±0.16	99.75±22.57	1.49±0.47	77.65±15.68	87.59±19.14	0.29±0.11
9 min. after Amirolide+PLA_2	0.38±0.24	0.45±0.18	99.75±22.28	1.51±0.47	78.42±14.96	88.05±18.74	0.33±0.19
12 min. after Amirolide+PLA_2	0.40±0.25	0.46±0.17	101.25±23.50	1.52±0.48	80.17±17.33	90.70±22.00	0.36±0.18
15 min. after Amirolide+PLA_2	0.41±0.24	0.48±0.15	101.50±23.56	1.51±0.47	80.64±18.92	89.61±22.18	0.35±0.14
18 min. after Amirolide+PLA_2	0.42±0.21	0.49±0.12	101.25±23.50	1.51±0.47	81.78±21.14	90.08±24.05	0.31±0.05
Gr.V (Minoxidil+PLA2)							
Control period							
3 min. after control	0.23±0.20	0.32±0.23	63.25±9.84	0.87±0.38	74.77±22.72	194.94±56.69	0.18±0.14
6 min. after control	0.25±0.23	0.37±0.31	64.25±10.72	0.88±0.38	69.40±16.13	182.80±41.67	0.21±0.16
9 min. after control	0.26±0.25	0.33±0.21	64.00±10.80	0.88±0.37	74.41±23.65	198.52±78.33	0.20±0.18

12 min. after control	0.26±0.24	0.28±0.16	64.25±10.21	0.87±0.37	98.24±61.23	253.09±183.12	0.24±0.19
Pretreated Minoxidil							
3 min. after Minoxidil	0.29±0.27	0.34±0.24	63.75±10.90	0.85±0.36	76.44±26.55	197.85±92.46	0.23±0.20
6 min. after Minoxidil	0.29±0.28	0.33±0.23	63.25±10.50	0.88±0.37	78.77±27.31	206.15±99.52	0.24±0.18
9 min. after Minoxidil	0.29±0.27	0.34±0.24	64.25±11.30	0.86±0.37	78.20±22.20	199.94±99.12	0.19±0.16
12 min. after Minoxidil	0.30±0.28	0.51±0.53	64.00±10.86	0.88±0.37	66.06±15.81	164.59±91.43	0.26±0.21
15 min. after Minoxidil l	0.32±0.30	0.40±0.31	64.75±11.24	0.89±0.37	73.23±16.51	179.01±89.98	0.23±0.19
Treatment Minoxidil+PLA_2							
3 min. after Minoxidil+PLA_2	0.34±0.30	0.68±0.79	65.75±11.79	0.94±0.42	63.52±21.80	177.01±111.54	0.26±0.20
6 min. after Minoxidil+PLA_2	0.32±0.28	0.43±0.35	66.00±11.92	1.00±0.48	72.09±8.52	173.65±88.92	0.27±0.21
9 min. after Minoxidil+PLA_2	0.34±0.27	0.40±0.27	69.25±10.69	1.03±0.49	78.42±17.26	184.29±98.61	0.28±0.21
12 min. after Minoxidil+PLA_2	0.34±0.26	0.35±0.22	73.25±11.56	1.00±0.48	89.55±33.19	206.11±113.26	0.33±0.26
15 min. after Minoxidil+PLA_2	0.34±0.24	0.37±0.22	74.00±13.78	1.01±0.48	86.97±29.05	187.68±87.84	0.27±0.18
18 min. after Minoxidil+PLA_2	0.35±0.22	0.36±0.20	73.00±11.34	1.00±0.49	94.47±43.03	194.45±92.13	0.30±0.18

Results and discussion

A marked increases in both PP and RVR levels were apparent during perfusion of PLA2 alone (gr.I). The role of PLA2 on PP and RVR indicating renal vasoconstriction were significantly attenuated by pretreatment of either Verapamil in inhibiting calcium influx (gr.III) or Amiloride in inhibiting sodium influx (gr.IV). In contrast, perfusion of free Ca^{++} in perfusate (gr.II), PLA2 did not affect to PP and RVR levels. An acute effect of PLA2 alone (gr.I) and in pre-treated isolated kidney with Verapamil(gr.III) showed biphasic responses with an initial increase in GFR (~15 min) and followed by a gradual decrease after given PLA2, which differed from the pre-treated isolated kidney with Amiloride (gr.IV) causing stepwise increases in GFR despite continued PLA2 infusion. An infusion of free Ca^{++}in perfusate (gr.II), PLA2 did not affect to the GFR level. An acute effect of PLA2 alone(gr.I) and in pre-treated isolated kidney with Verapamil (gr.III) showed slight increases in UF, which differed from the pre-treated isolated kidney with Amiloride (gr.IV) causing stepwise increases in UF despite continued PLA2 infusion. In (gr.II), perfusion of free Ca^{++} in perfusate, PLA2 did not affect to the UF level. Minoxidil (gr.V) slightly inhibit the effect of PLA_2 on PP and RVR. An acute effect of PLA2 alone (gr.I) and in pre-treated isolated kidney with either Verapamil (gr.III) or Amiloride (gr.IV) showed increases in fractional Na^+ tubular transport during continued PLA2 infusion. In (gr.II), perfusion of free Ca^{++} in

perfusate, PLA2 did not affect to the fractional Na+ excretion. An acute effect of PLA2 alone (gr.I and in pre-treated isolated kidney with Verapamil (gr.III) and Minoxidil (gr.V) showed unalteration of fractional K^+ tubular transport during continued PLA2 infusion, which differed from the pre-treated isolated kidney with Amiloride (gr.IV) causing marked decreases in fractional K^+ tubular transport despite continued PLA2 infusion. In (gr.II), perfusion of free Ca++ in perfusate, PLA2 did not affect to the fractional K^+ excretion.

Conclusion

The present results suggest that an administration of PLA2 in the isolated rabbit kidney causes direct acute alterations of renal hemodynamics. Acute alterations of renal functions are probably affected by either the direct action of PLA2 or indirect effect from ionic exchanges occurring in the renal vascular membrane of the treated kidney. The role of PLA2 in causing renal vasoconstriction is depended on the presence of extracellular Ca^{2+}. PLA2 activated to open L-type Ca^{2+} channel and Na^+ channel in the renal vascular membrane with influx of Ca^{2+} and Na^+ leading to vasoconstriction causing increases in RVR and PP levels. The role of PLA2 in causing renal vasoconstriction is significantly attenuated by the effects of either Verapamil in inhibiting calcium influx or Amiloride in inhibiting sodium influx or Minoxidil in inhibiting potassium channel.

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