Determination of Nephrotoxicity, Hepatotoxicity and Cardiovascular Disturbances following Malayan Pit Viper (Calloselasma rhodostoma) Envenoming: Histopathological study and The Protective Effect of Hemato Polyvalent Snake Antivenom from Thailand

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Research Article

Keywords: venom, snake, antivenom, Malayan pit viper, nephrotoxicity, kidney

Posted Date: March 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1410806/v1

Abstract

Calloselasma rhodostoma (Malayan pit viper) is a medically important snake species found widely distributed across Southeast Asia. Systemic coagulopathy causing severe hemorrhage, and local tissue injury, are commonly found following *C. rhodostoma* envenoming. However, the unusual nephrotoxicity and congestive heart failure have been reported in a patient who had a long length of hospital stay. In this study, we determined the effect of *C. rhodostoma* envenoming on cardiovascular disturbances and morphological changes of liver, heart and kidney using animal models. We also evaluated the protective effect of Hemato polyvalent antivenom (HPAV; The Queen Saovabha Memorial Institute (QSMI) of the Thai Red Cross Society, Thailand) to neutralise histopathological effect on *C. rhodostoma* envenomed tissues. Intravenous administration of *C. rhodostoma* venom (1000 µg/kg, i.v.) caused rapid decrease in mean arterial pressure (MAP) followed by complete cardiac collapse in anaesthetized rats. Moreover, intraperitoneal (i.p.) administration of *C. rhodostoma* venom (11.1 mg/kg; 3xLD_{50}) for 24 h caused cellular lesions in liver and heart tissues. *C. rhodostoma* venom also induced nephrotoxicity indicated by the presence of tubular injury, interstitial vascular congestion and inflammatory infiltration in the whole area of kidney. Administration of HPAV at recommended doses by manufacturer 15 min prior to or after experimentally envenoming of *C. rhodostoma* venom significantly reduced morphological changes of liver, heart and kidneys. This study found that *C. rhodostoma* envenoming can induce cardiovascular disturbances, hepatotoxicity and nephrotoxicity. We also highlighted the potential broad utility of the HPAV for treating *C. rhodostoma* envenomings to neutralize histopathological effect of *C. rhodostoma* venom. However, the early delivery of antivenom in recommended doses by manufacturer appear to capable of preventing envenoming outcomes.

Introduction

Snakebite envenoming is a major contributor to morbidity and mortality in rural communities of sub-Saharan Africa, South Asia and Southeast Asia[1,2]. Annually, it is estimated that snake envenoming results in 81,000-138,000 deaths worldwide and is also responsible for permanent physical or psychological disabilities, including blindness, amputation, and post-traumatic stress disorders[2]. In Thailand, the National Health Security Office (NHSO) reported 7.9 snakebite cases per 100,000 people in 2017. Five species of venomous snakes i.e. monocled cobra (*Naja kaouthia*), Malayan krait (*Bungarus candidus*), Russell's viper (*Daboia siamensis*), Green Pit Viper (*Trimeresurus* spp.) and Malayan Pit Viper (*Calloselasma rhodostoma*) have been classified as venomous snakes of category 1 causing high mortality and morbidity rates in the Public Health System of Thailand[3].

The Malayan Pit Viper (*Calloselasma rhodostoma*: subfamily Crotalinae, formerly known as *Angkistrodon rhodostoma*) is the species responsible for the most cases of envenoming in Thailand, being responsible for 38% of bites[3]. *C. rhodostoma* has also been recognised as a species that causes high levels of morbidity, disability or mortality in Cambodia, the Indonesian Islands of Java and Madura, the Peninsular Malaysia, Myanmar and Vietnam[2–4]. A number of studies have reported the basic proteomic profile of *C. rhodostoma* venom indicates the presence of phospholipase A_2 (PLA_2), snake venom metalloproteinases

(SVMP), flavin monoamine oxidase and serine protease toxin families[5–7]. In addition, aminopeptidase, glutaminyl-peptide cyclotransferase and ankyrin repeats were recently identified from the Malaysian *C. rhodostoma* venom[8].

The most significant clinical manifestations following *C. rhodostoma* envenoming are coagulopathy resulting petechiae, epistaxis, hematuria, hemoptysis including uterine, gastrointestinal and central nervous system haemorrhage, as well as disseminated intravascular coagulopathy (DIC), and shock. Local painful swelling and tissue necrosis at the bite-site are also commonly observed. The local tissue damage is likely to be due to the effects of myotoxic PLA_2 in the venom. Snake venom PLA_2s cause disruption of plasma membrane integrity and sarcolemma damage resulting in Ca^{2+} influx into the cytoplasm[9]. Moreover, the increase in intracellular Ca^{2+} also induces myofilament hypercontraction and mitochondrial dysfunction, leading to irreversible muscle damage and tissue necrosis[10,11].

In addition to hemodynamic disturbances, envenomings by medically important Asian vipers *e.g.* Russell's viper (*Daboia* spp.), Green pit viper (*Trimersurus* spp.), Hump-nosed pit viper (*Hypnale* spp.) or Saw-scaled viper (*Echis* spp.) can induce nephrotoxicity, which is characterized by haematuria, tubular necrosis and acute renal failure[12,13]. Interestingly, a ten year retrospective study of *C. rhodostoma* envenoming in southern Thailand indicated that acute kidney injury (AKI) and congestive heart failure occurred in some patients envenomed by this species[14,15]. Administration of antivenom is the primary treatment strategy for victims of snake envenoming. In Thailand, The Queen Saovabha Memorial Institute (QSMI: Thai Red Cross Society, Bangkok, Thailand) produces two antivenoms which can be used for treatment of *C. rhodostoma* envenoming. These are a monovalent antivenom which only contains polyclonal antibodies derived from equine plasma hyperimmunized with *C. rhodostoma* venom, and a polyvalent antivenom, which comprises of antibodies sourced from animals immunized with *C. rhodostoma* venom and venoms from other medically important hematotoxic snake species.

Although previous studies have proven the effectiveness of snake antivenom from QSMI to neutralize circulating venom and reverse systemic symptoms[16,17], there are still controversies regarding the risk of hypersensitivity and the high amounts of antivenom required to reverse nephrotoxicity following *C. rhodostoma* envenoming[14,18]. Moreover, administration of monovalent antivenom at the three times higher than the recommended therapeutic concentration was required to prevent nephrotoxicity following envenoming by Asian viper [19]. In the present study, we examined the effect of experimental *C. rhodostoma* envenoming in mice on the kidney, liver and heart, in the absence and presence of Hemato polyvalent antivenom (HPAV) using light microscopy and transmission electron microscopy (TEM). Evaluation of cardiovascular activity, i.e. effect on mean arterial pressure (MAP) and heart rate, was also examined in the anaesthetized rat.

Material And Methods

Snake venoms

Malayan pit viper venom (*C. rhodostoma* venom, batch number: 607.002), pooled and lyophilized from 9 Indonesian specimens and 62 Malaysian specimens, was purchased from Latoxan (Valence, France). Freeze-dried venom samples were stored at 4 °C, prior to use. When required, venom was weighed, reconstituted in phosphate-buffered saline (PBS) and protein concentration measured using BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

Antivenoms

Hemato polyvalent Snake antivenom (HPAV; Lot NO: HP00216, expiry date 08/03/2021) was purchased from QSMI of Thai Red Cross Society, Bangkok, Thailand. The freeze-dried antivenoms were dissolved with pharmaceutical grade water supplied by the manufacturer. The dissolved antivenoms were then stored at 4 ºC prior to use.

Animal ethics and care

Male Sprague-Dawley rats and Jcl-ICR mice were purchased from Nomura-Siam International Co. Ltd., Bangkok, Thailand. Animals were housed in stainless steel containers with access to food and drinking water *ad libitum*. Approvals for all experimental procedures were obtained from the Subcommittee for Multidisciplinary Laboratory and Animal Usage of Phramongkutklao College of Medicine and the Institutional Review Board, Royal Thai Army Department, Bangkok, Thailand (Documentary Proof of Ethical Clearance Number: IRBRTA S055b/64_Xmp and IRBRTA S054b/64_Xmp) in accordance with the U.K. Animal (Scientific Procedure) Act, 1986 and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). **All methods were performed in accordance with the relevant guidelines and regulations**. **The study is reported in accordance with ARRIVE guidelines** (https://arriveguidelines.org).

Anaesthetized rat preparation

Male Sprague-Dawley rats weighting 280-350 g were anaesthetized using separate injections of Zoletil (20 mg/kg, i.p.) and Xylazine (5 mg/kg, i.p.). Additional anesthetic was administered throughout the experiment as required. A midline incision was made in the cervical region, and cannulae inserted into the trachea, jugular vein and carotid artery, for artificial respiration (if required), administration of drugs/venom and measurement of blood pressure, respectively. Arterial blood pressure was recorded using a Gould Statham P23 pressure transducer filled with heparinized saline (25 U/ml). Systemic blood pressure was monitored on a MacLab system (ADInstruments). Pulse pressure was defined as the difference between systolic and diastolic blood pressures. Mean arterial pressure (MAP) was defined as diastolic blood pressure plus one-third of pulse pressure. The rats were kept under a heat lamp for the entire experiment to maintain body temperature. At the conclusion of the experiment, animals were killed by an overdose of pentobarbitone (i.v.). Liver, heart and kidneys were removed for further histopathological studies.

In vivo venom lethality

As an essential prerequisite to assessing antivenom efficacy, we first determined the median murine lethal dose (LD_{50}) for *C. rhodostoma* venom using WHO-recommended protocols[20]. Briefly, groups of 4-5 male Jcl:ICR mice (18-22 g) received an intraperitoneal injection of varying doses of venom in 100 µl PBS and, 24 h later, the number of surviving mice in each group was recorded. The venom LD_{50} (i.e. the amount of venom that causes lethality in 50% of a population of injected mice) and corresponding 95% confidence limits of each venom concentration were calculated using probit analysis[19,21].

To determine the effectiveness of HPAV, a challenge ($3xLD_{50}$) dose of *C. rhodostoma* venom was intraperitoneally administered to animals. The neutralizing effect of HPAV was examined when administered 15 min prior to, or 15 min after, *C. rhodostoma* venom.

Histopathological studies

Histological preparation for Haematoxylin and Eosin (H&E) staining

Histopathological examination of heart, liver and kidney of envenomed animals was determined following previously described methods[22]. At the conclusion of the in vivo venom lethality test (i.e. 24 h), all animals were sacrificed, and the liver, heart and both kidneys removed and preserved in 10% formalin. All tissues were dehydrated in graded series of ethanol through 70, 80, 90, 95 and 100% with two changes for 1 h each. Three washings of xylene, for 30 min each, were then completed before being embedding the tissues in paraffin. Embedded samples were cross-sectionally cut and stained with H&E. Tissues were examined and photographed under an Olympus light microscope (BX-50, Olympus, Japan). Degrees of severity in morphological changes of liver, heart and kidneys were evaluated as previously described (Table 1, 2 and 3).

Histological preparation for Transmission electron microscopy method

Pieces of liver, heart and kidney tissues (~1 mm^3) were immediately fixed in 2.5% buffered glutaraldehyde. The specimens were post-fixed in 1% osmium tetroxide, dehydrated, infiltrated with propylene oxide and embedded in resin. Semi-thin sections about 0.5 µm or 1.0 µm were stained with Toluidine blue, used as a guideline to the area of interest and further trimmed. Ultrathin sections about 60 nm were cut on an ultramicrotome. They were stained with uranyl acetate and lead citrate. Ultrathin sections are spread mostly on 200 or 300 mesh copper grids and stained with uranyl acetate and lead citrate solutions. The sections were examined and photographed by Transmission electron microscope (TEM-JEM2010, JEOL, Japan)

Table 1

Pathological characteristic and evaluation criteria for degree of morphological changes in liver[23]

Tissue change	Description	Score
Congestion	• No congestion • In few sinusoids and vessels • In about half of sinusoids and vessels • In almost all sinusoids and vessels	0 + ++ +++
Inflammatory infiltration	• No inflammatory infiltrate • 1-3 inflammatory foci/ section • 4-6 inflammatory foci/ section • >6 inflammatory foci/ section	0 + ++ +++
Necrosis	• No necrosis • Focal necrosis • Zonal necrosis • Confluent necrosis	0 + ++ +++

Table 2

Pathological characteristic and evaluation criteria for degree of morphological changes in kidneys[23]

Tissue change	Description	Score
Congestion	• No congestion • Focal glomeruli and interstitial vessels • Diffuse glomeruli and interstitial vessels • In almost all glomeruli and interstitial vessels or hemorrhage	0 + ++ +++
Inflammatory infiltration	• No inflammatory infiltrate • 1-3 inflammatory foci/ section • 4-6 inflammatory foci/ section • >6 inflammatory foci/ section	0 + ++ +++
Tubular injury	• No evidence of tubular injury • Less than 50% loss of brush border • More than 50% loss of brush border with cell sloughing off • Tubular necrosis is found in most area	0 + ++ +++

Table 3

Pathological characteristic and evaluation criteria for degree of morphological changes in heart [24]

Tissue change	Description	Score
Myocardial Damage	• No lesions	0
	• Slight derangement of muscle fibers, few inflammatory cells and vacuoles	0.5
	• Focal lesions of the subendocardial portion of the apex and mid-ventricle, inflammatory cells, interstitial edema, vacuolization of myocytes	1
	• Focal lesions of the subendocardium of the apical and mid ventricular region with right ventricular involvement	1.5
	• Focal lesions extending over a wider area of both ventricles	2
	• Focal lesions extending over a wider area of both ventricles, extensive inflammatory cell infiltration, interstitial edema, rupture of myofibers	2.5
	• Confluent lesions of the apex, mid-left ventricle and right ventricle, extensive inflammatory cell infiltration, profuse edema	3
	• Confluent lesions throughout the heart	4

Data analysis and Statistics

For the anaesthetized rat experiments, sample sizes are based on the number of animals required to provide >85% power to detect an effect size of 35% with a confidence level (α) of 5% for the *in vivo* endpoint measure of blood pressure (standard deviation (SD) <15%). This ensured that experimental design was sufficiently power. Statistical analysis was performed using Prism 5.0 software (GraphPad Software, San

Diego, CA, USA). Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Data were expressed as mean ± SEM.

Results

Effect of C. rhodostoma venom on blood pressure and heart rate

Intravenous administration of saline (200 µl) did not affect rat blood pressure and heart rate (2A). *C. rhodostoma* venom (500-1000 µg/kg) displayed hypotensive effects when administered to anaesthetized rats. *C. rhodostoma* venom (500 µg/kg, i.v., Fig. 1A, 2B) decreased MAP to 45 ± 4 mmHg (Fig. 1A) at 20 s after administration. While the higher dose of venom (i.e. 1000 µg/kg, i.v.) caused a similar reduction in MAP (43 ± 1 mmHg, Fig. 1A) at 20 s but caused cardiovascular collapse (i.e. undetectable blood pressure) within 3 min (Fig. 2C).

Intravenous administration of *C. rhodostoma* venom (500-1000 µg/kg) also caused a significant reduction in heart rate at 20 s (Fig. 1B). With abolition of heart rate observed for the higher dose of venom after 3 min (Fig. 1B).

Neutralization of venom lethality in vivo by Hemato polyvalent antivenom

The lethal effect (expressed as murine LD_{50}) of *C. rhodostoma* venom was 3.706 mg/kg (i.p.). To determine the neutralizing effect of HPAV on *C. rhodostoma* venom lethality, we challenged groups of mice with three times the respective LD_{50} dose of venom (i.e. 11.1 mg/kg, ip.) and determined the effectiveness of the administration of HPAV 15 min prior to, and 15 min after, intraperitoneal administration of *C. rhodostoma* venom. The prior administration (i.e. 15 min) of HPAV at the recommended titre neutralized the toxicity of *C. rhodostoma* venom, whereas the administration of HPAV (i.p., recommended titre) 15 min after venom administration still resulted in the deaths of 25% of animals tested.

Histopathological studies

Effect of C. rhodostoma venom on morphological changes of liver and effect of HPAV on histopathology of liver

Administration of *C. rhodostoma* venom ($3xLD_{50}$; i.p.) caused congestion of sinusoid (Fig. 3A), diffused hepatic necrosis, amyloidosis, edema of hepatocyte cell (Fig. 3B) and congestion of central vein (Fig. 3E). Inflammatory cells with lymphocytes and Kupffer cells were detected throughout liver tissues from envenomed mice including portal triad (Fig. 3C and 3D).

Under TEM determination, diffusion of necrotic hepatocytes and mitochondrial swelling (Fig. 4A) were detected together with damage of cytoplasm. These were indicated by the presence of dilated blood sinusoids around the boundary of hepatocytes. Expanded sinusoid with cell debris were also observed in the liver from envenomed mice (Fig. 4B).

The absence in amyloidosis and the decreases in vascular congestion of central vein of the liver were detected (Fig. 3F) in mice which received the recommended doses of HPAV either before or after the administration of *C. rhodostoma* venom (Table 4). Moreover, administration of HPAV also minimized the level of lymphocytes including pyknotic nuclei and eosinophilic cytoplasm from necrotic hepatocyte induced by *C. rhodostoma* venom (Fig. 4C, D, E and F).

Table 4

Pathological score of liver tissues following the intraperitoneal administration of saline (negative control), *C. rhodostoma* venom, hemato-polyvalent antivenom 15 min prior or after administration of *C. rhodostoma* venom (0 is indicated undetectable lesion, + is indicated the presence of pathological lesion).

Group	Liver (scores)		
	Congestion	Inflammatory infiltration	Necrosis
Saline	0	0	0
C. rhodostoma venom (CRV, IP)	+++	++	+++
HPAV (IP)	0	0	0
CRV followed by HPAV 15 min later	+	+	+
HPAV followed by CRV 15 min later	+	+	+

Effect of C. rhodostoma venom on morphological changes of heart tissues

Heart tissue from mice 24 h after administration of *C. rhodostoma* venom indicated derangement of cardiac muscle fibers and few inflammatory cells (Fig. 5A). Cardiac muscle fiber hypertrophy and mitochondrial swelling were found under TEM (Fig. 5C.). Cardiac vessels also showed endothelial cell swelling and macrophage infiltration (Fig. 5E).

Administration of HPAV, prior to or after the administration of *C. rhodostoma* venom, significantly prevented endothelial cell swelling (Fig. 5B) and pathological changes of cardiac muscle fiber (Fig. 5D and 5F and Table 5).

Table 5

Pathological changes of heart tissue following the intraperitoneal administration of saline (negative control), *C. rhodostoma* venom, hematopolyvalent antivenom 15 min prior to or 15 min after administration of *C. rhodostoma* venom (0 is indicated undetectable lesion, + is indicated the presence of pathological lesion).

Group	Heart (scores)
	Myocardial Damage
Saline	0
C. rhodostoma venom (CRV, IP)	2.5
HPAV	0.5
CRV followed by HPAV	1.5
HPAV followed by CRV	1.5

Effect of C. rhodostoma venom on morphological changes of kidney and the protective effect of HPAV on pathohistology of kidney

Histopathological examination of kidneys dissected at the 24 h time point following *C. rhodostoma* venom administration (3xLD_{50}; i.p.) indicated the presence of hyaline cast, dilatation of renal capillary, diffuse or focal glomerular atrophy (Fig. 6B and 6C) and/or congestion of interstitial vessels (Fig. 6E) and tubular injury (Fig. 6A) with loss of brush border. Disarrangement of vascular smooth muscle was also detected in envenomed tissues (Fig. 6D).

Histopathological study of kidney under TEM revealed disarrangement of pedicel and ruptured of glomerular (Fig. 7A.). Tubular injury and lysis of tubular nucleus were detected in envenomed kidneys (Fig. 7B).

Dilated glomerular capillaries (Fig. 7C) and epithelial cell swelling of renal tubule (Fig. 7D) were rarely detected in mice which received HPAV 15 min after the administration of *C. rhodostoma* venom. The decrease in tubular castminor degree of interstitial vessel congestion (Fig. 6F), and vacuolar degeneration (Fig. 7E and 7F) were still observed throughout the entire the tissue of animals which received HPAV prior to or after to venom administration (Table 6).

Table 6

Pathological changes of kidney tissues following the intraperitoneal administration of saline (negative control), *C. rhodostoma* venom, hematopolyvalent antivenom 15 min prior to or 15 min after administration of *C. rhodostoma* venom (0 is indicated undetectable lesion, + is indicated the presence of pathological lesion).

Group	kidney (scores)		
	Congestion	Inflammatory infiltration	Tubular injury
Saline	0	0	0
C. rhodostoma venom (CRV, IP)	+++	++	+++
HPAV (IP)	0	0	0
CRV followed by HPAV	+	+	+
HPAV followed by CRV	+	+	+

Discussion

Calloselasma rhodostoma is an endemic pit viper species in South East Asia. Severe local effects including swelling, blistering, compartment syndrome and tissue necrosis are commonly observed following envenoming by *C. rhodostoma* in Thailand[15]. In addition, coagulopathy resulting in hemorrhage is a major systemic outcome of *C. rhodostoma* envenomed patients[14]. *C. rhodostoma* envenoming-induced acute kidney injury and cardiovascular events i.e. congestive heart failure were also reported in a patient with long length of hospital in Southern Thailand[14]. These have raised our interest to investigate the pathological effects behind nephrotoxicity and cardiovascular disturbances using histopathological analysis. In this study the effectiveness of HPAV to inhibit morphological changes following *C. rhodostoma* envenoming was also investigated.

Administration of monospecific antivenom remains an effective treatment for viper envenomings. However, the availability of, and access to, geographically-appropriate antivenom, and correct identification of the biting species, remains problematic in many rural areas. Administration of polyvalent antivenom is a valid option for snakebite patients in order to minimize the occurrence of incorrect antivenom application due to diagnostic error. Previously, a number of studies exhibited the effectiveness of hemato polyvalent antivenom (HPAV) from Thailand to inhibit toxicity from various Asian hematotoxic and nephrotoxic snakes i.e. *Daboia* spp., *Trimeresurus* spp., *Challoselasma* spp., including *Hypnale* spp.[16,17,19]. HPAV was demonstrated to effectively neutralise procoagulant and hemorrhagic activities of all venoms tested. Moreover, HPAV also displayed a preventive effect on the occurrence of *Daboia siamensis* venom-induced hematuria and proteinuria in envenomed animals[17]. Interestingly, the potency of HPAV was shown to be generally higher than that of *C. rhodostoma* monovalent antivenom in neutralization of lethality, coagulation, hemorrhage and necrosis of challenge dose ($5xLD_{50}$) of *C. rhodostoma* venom suggesting the presence of higher antibodies and synergistic cross-neutralizing component of HPAV[17]. Similarly, a previous study also indicated that early administration of higher concentrations of *Daboia siamensis* monovalent antivenom than the recommended titre (i.e. 1 ml of antvenom for 0.6 mg *Daboia siamensis* venom) was required to prevent nephrotoxicity following Russell's viper envenoming[19]. In the present study, administration of HPAV at the recommended concentration (1 ml of antivenom to neutralise 1.6 mg

Malayan pit viper venom) displayed neutralizing activity on histopathological changes of heart, liver and kidney tissues either 15 min prior to or 15 min after administration of *C. rhodostoma* venom ($3xLD_{50}$) in mice. In a preliminary study, we found that intraperitoneal administration of *C. rhodostoma* venom ($3xLD_{50}$) was lethal in animals tested within 1 h. Therefore, administration of HPAV 15 min after *C. rhodostoma* envenoming was chosen as a suitable time point to examine the effectiveness of HPAV after envenoming. There was no remarkable different in the protective effects of HPAV on envenomed mice when the antivenom was administered either 15 min prior to or after envenoming.

C. rhodostoma venom is a rich source of biological proteins such as snake venom metalloproteinase (SVMPs), phosphodiesterase (PDEs), phospholipase A_2 (PLA_2s), and snake venom serine protease (SVSPs). Recently, aminopeptidase, glutaminyl-peptide cyclotransferase along with ankyrin repeat were identified in Malaysian *C. rhodostoma* venom[8]. These toxic proteins were shown to be responsible for a number of hematologic outcomes (e.g. hemorrhage, hypotension and inflammation)[25,26] and cellular necrosis[27,28], which may involve nephrotoxicity, hepatotoxicity and cardiovasrcular disturbances observed following *C. rhodostoma* envenoming.

Cardiovascular effects observed following snakebite envenoming have been reported in envenomed victims of snakes from the family Elapidae (i.e. *Pseudonaja textilis*, *Oxyuranus scutellatus* and *Bungarus candidus*) and Viperidae (i.e. *Echis ocellatus*). In this study, intravenous administration of 500 µg/kg of *C. rhodostoma* venom caused a rapid, but transient, decrease in blood pressure and heart rate follow by a more prolonged hypotensive effect for a few minutes. In contrast, while administration of 1000 µg/kg of *C. rhodostoma* venom also lowered mean arterial pressure and heart rate, this was followed by cardiovascular collapse. The mechanism behind cardiovascular collapse following snakebite envenoming has been demonstrated to involve vascular mediators (e.g. nitric oxide and prostacyclin) and autonomic adaptation[30,31]. Previously we reported, OSC3, an isolated PLA_2 from *Oxyuranus scutellatus* (Taipan) venom, induced a transient decrease in MAP in anaesthetized rats and caused vascular relaxation in mesenteric arteries, which was due to a combination of release of dilator autacoids and direct relaxation of vascular smooth muscle involving the cAMP/protein kinase A cascade[31,32]. Previously, we have shown that prior administration of hexamethonium or atropine significantly attenuated cardiac toxicity of Malayan krait (*B. candidus*) venom [30] suggesting the involvement of ganglionic nicotinic receptors and muscarinic acetylcholine receptors. Moreover, administration of a prothrombin activator from *P. textilis* venom induced cardiovascular collapse of anaesthetized rats suggesting that prothrombin activator-like toxin may be a contributor to snake venom-induced rapid cardiovascular collapse[33]. Indeed, acute coronary syndrome was reported in a *C. rhodostoma* envenomed patient, which was relieved by administration of antithrombotic agents for 5 days[15]. In our current study, *C. rhodostoma* venom caused swelling in the vasculature of cardiac muscle and the presence of macrophages in cardiac vessels indicating a direct effect of venom on tissues. The histopathological examination of heart tissue indicated that *C. rhodostoma* venom caused extensive hypertrophy of cardiac myofibers and mitochondrial swelling within 24 h after envenoming. These morphological changes in cardiac tissues can be attributed to the presence of cellular cytotoxic components of venom.

In this work, the effect of *C. rhodostoma* venom on histopathology of liver tissue was also investigated. Hepatocyte vacuolation, prominent van Kupffer cells and congestion in central vein were detected in the liver. We also detected the presence of lymphocytes pyknotic nuclei and eosinophilic cytoplasm causing amyloidosis in some areas. This indicates the presence of inflammatory effect on hepatic tissue. In fact, these hepatic injuries were also observed following *Naja haje*[34] and *Crotalus durissus terrificus*[35] envenoming. These hepatotoxic effects included an elevation in bilirubin, increases in serum alanine, aminotransferase, aspartate aminotransferase, γ-glutamyl transferase and alkaline phosphatase. The mechanism behind snake venom induced-hepatotoxicity was demonstrated to be associated with liver apoptosis indicated by the rise in lipid peroxidation and nitric oxide production[34]. In fact, snake venom L-amino acid oxidase (LAAOs) becomes an important cytotoxic agent causing cell death in several organisms via the release of reactive oxygen species, hydrogen peroxide (H_2O_2)[36].

There are 5 groups of Asian snakes which have been reported to cause nephrotoxicity i.e. Russell's vipers, green pit vipers, saw-scaled viper, hump-nosed pit viper and sea-snake. In the present work, we demonstrated the nephrotoxicity induced by *C. rhodostoma* venom. In Thailand, acute kidney injury and rhabdomyolysis (2 patients) were clinically reported following *C. rhodostoma* envenoming. Of these patients. one patient died from rhabdomyolysis following recovery from systemic bleeding[15]. Nephrotoxicity is commonly induced by snakes with hemotoxic and myotoxic effects e.g. vipers, Australian elapids and sea snakes. Clinical manifestations of renal involvement include proteinuria, hematuria, pigmenturia and acute kidney injury[37]. We previously demonstrated that Asian Russell's viper (*Daboia* spp.) venoms contain nephrotoxic substances e.g. $SVPLA_2$ and SVMP causing glomerulonephritis, interstitial congestion, tubular necrosis and cortical necrosis in envenomed tissues[38]. The present study showed that *C. rhodostoma* venom caused tubular necrosis with cytoplasmic eosinophilia and pyknotic nuclei, indicating the presence of inflammation of the renal tubule, similar to nephrotoxic lesions induced by other species. To treat and prevent nephrotoxicity, apart from administration of snake antivenom, early plasmapheresis and blood exchange have been applied when snake antivenom was unavailable. Moreover, plasmapheresis, blood exchange, peritoneal or hemodialysis were addressed to perform as early as possible for prevention of AKI. In addition, urine alkalization by sodium bicarbonate also help to prevent AKI in patient who has myoglobinuria or hemoglobinuria[37].

In this study, we have demonstrated that *C. rhodostoma* envenoming causes profound histopathological changes in heart, liver and kidney tissues. Further pharmacological and physiological determinations may enable a better understanding and management of Malayan pit viper envenoming. These data also indicate that the morphological anomalies observed in envenomed tissues may contribute to cardiovascular disturbances and nephrotoxicity in envenomed victims. Early monitoring of cardiovascular and renal function together with appropriate snake antivenom administration are required to prevent the life-threatening outcomes.

Declarations

Acknowledgments:

The authors wish to acknowledge the Office of Research Development, Phramongkutklao College of Medicine & Phramongkutklao Hospital (ORD, PCM & PMK, Bangkok, Thailand). W.C.H was funded by an Australian National Health and Medical Research Council (NHMRC) Centres for Research Excellence Grant ID: 1110343.

Data availability:

The datasets used and/or analysed during the current study available from the corresponding author (J.C.) on reasonable request.

Conflicts of Interest:

The authors declare no conflict of interest.

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Figures

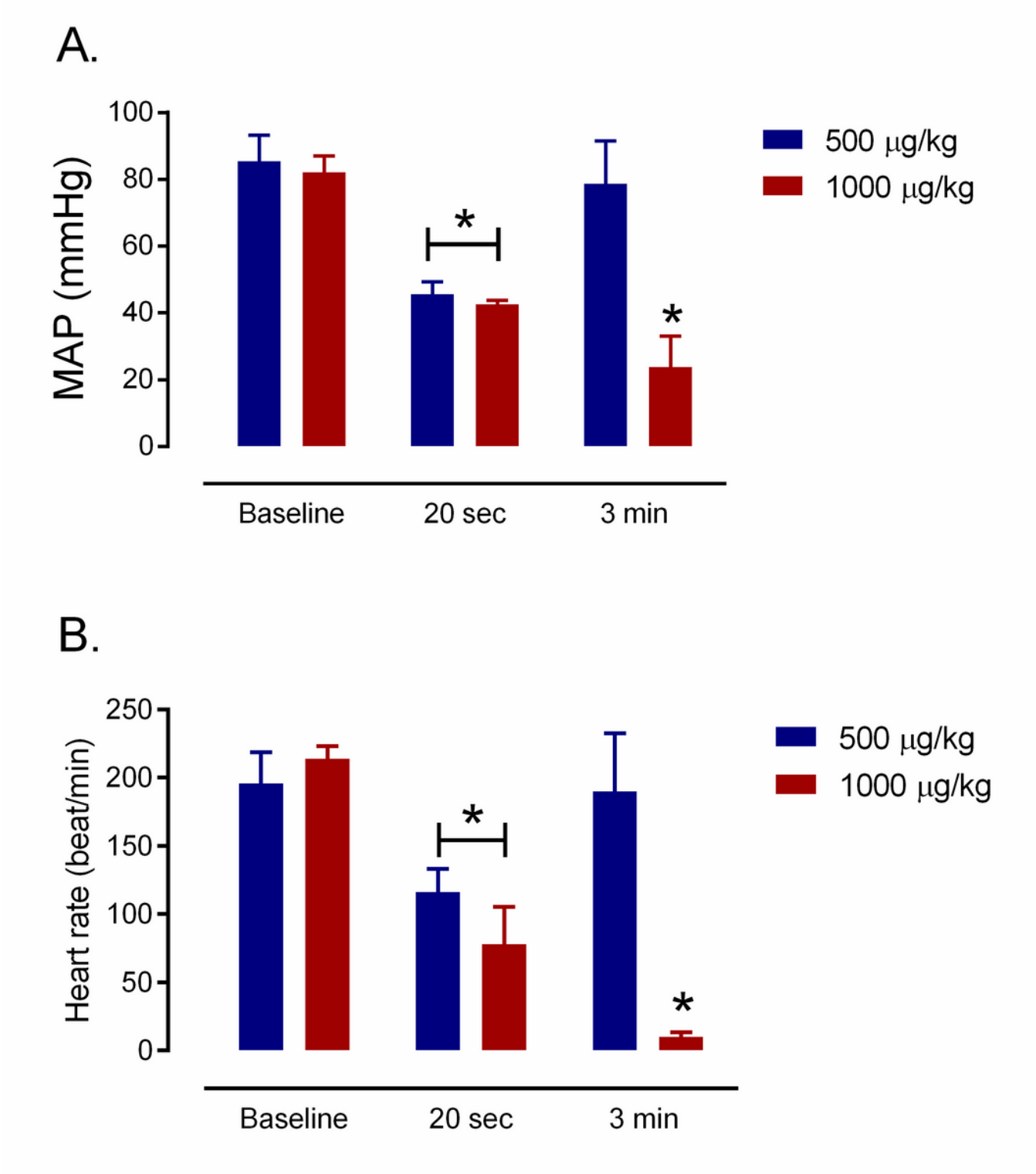

Figure 1

(A) Mean arterial pressure (MAP) before and following the administration of *C. rhodostoma* venom (500-1000 µg/kg, i.v., *n*=5) at 20 s and 3 min. (B) Effect of intravenous administration of *C. rhodostoma* venom (500-1000 µg/kg) on rat heart rate at 20 s and 3 min (*n* = 5).

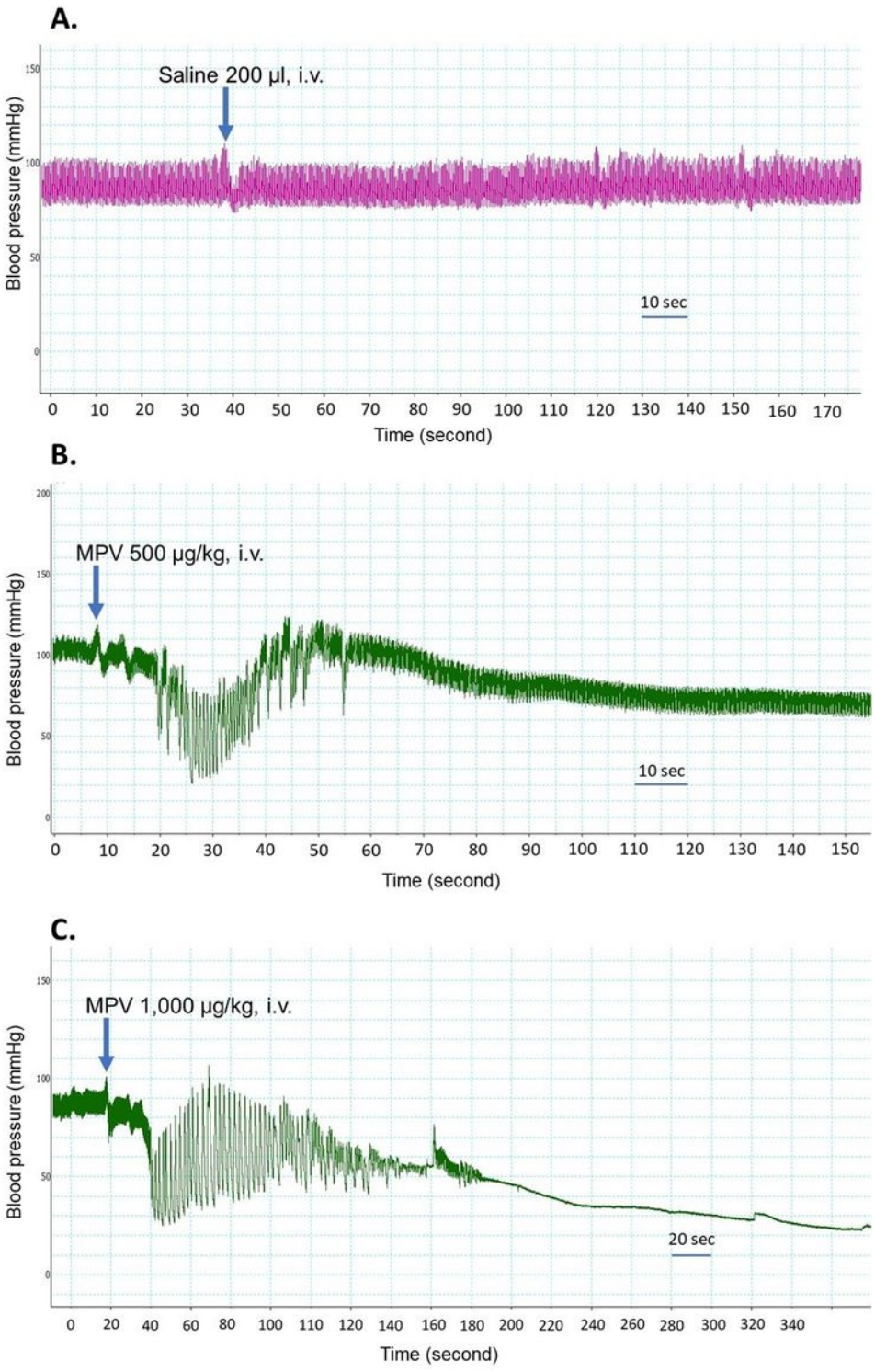

Figure 2

Traces showing the effect of intravenous administration of (A) saline 200 µl, (B) *C. rhodostoma* venom (MPV) 500 µg/kg or (C) *C. rhodostoma* venom (MPV) 1000 µg/kg on MAP of anaesthetized rats.

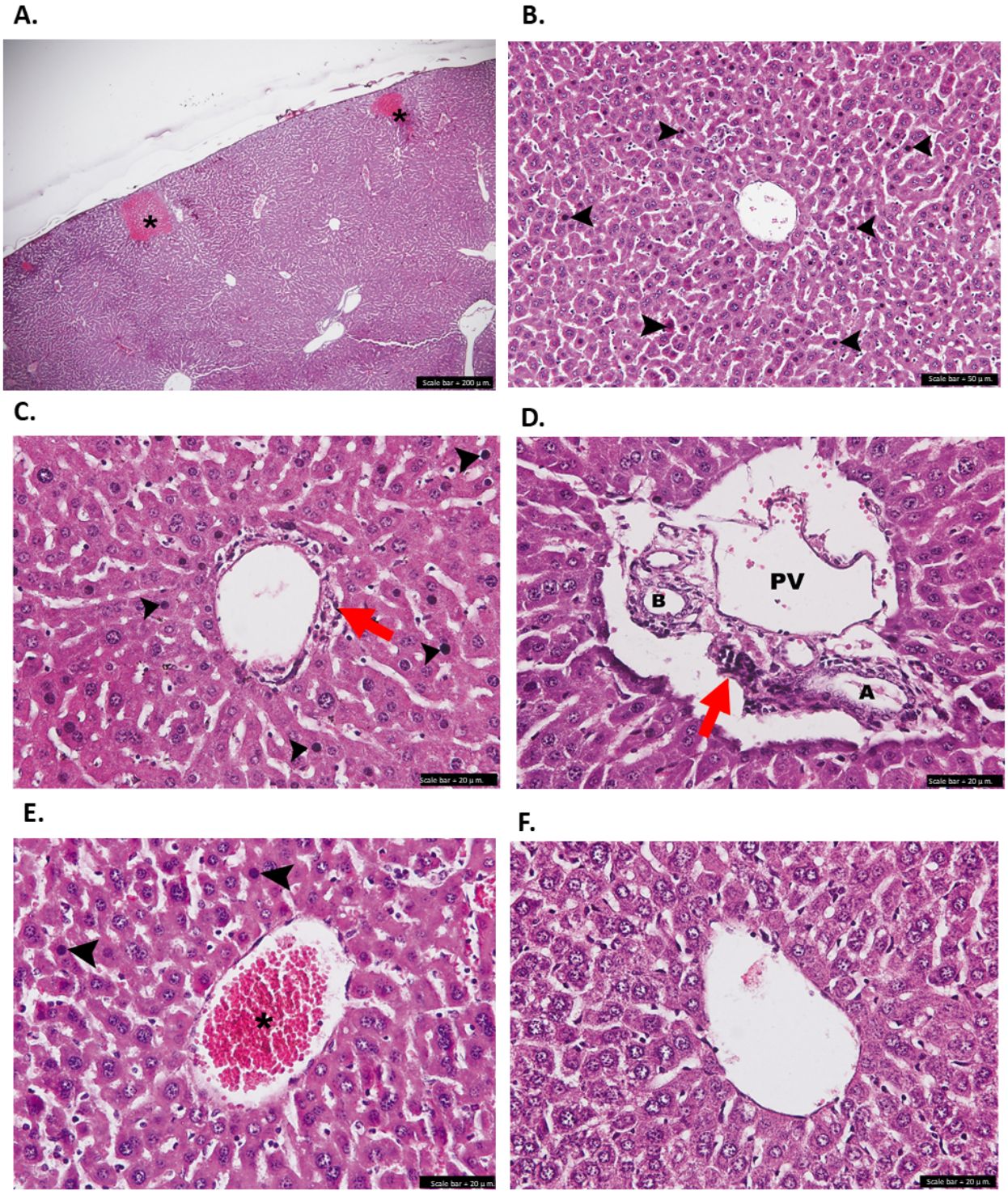

Figure 3

Histopathological changes in the livers (H & E stain) following 24-h intraperitoneal administration of *C. rhodostoma* venom (3xLD50), in mice, indicated (A.) congestion and amyloidosis (asterisks; scale bar = 200 µm), (B.) hepatic necrosis with pyknotic nuclei and eosinophilic cytoplasm (scale bar = 50 µm), (C.) inflammatory cells of hepatocyte (scale bar = 20 µm) and (D.) portal triad (scale bar = 20 µm; letter-B = bile duct, letter-PV = Portal vein, letter-A = hepatic artery), including (E.) congestion of central vein (scale bar = 20 µm). (F.) The protective effect of prior administration (15 min) of HPAV on *C. rhodostoma* venom induced hepatotoxicity (scale bar = 20 µm). indicates pyknotic nuclei and eosinophilic cytoplasm. Red arrows indicate inflammatory cells of hepatocyte.

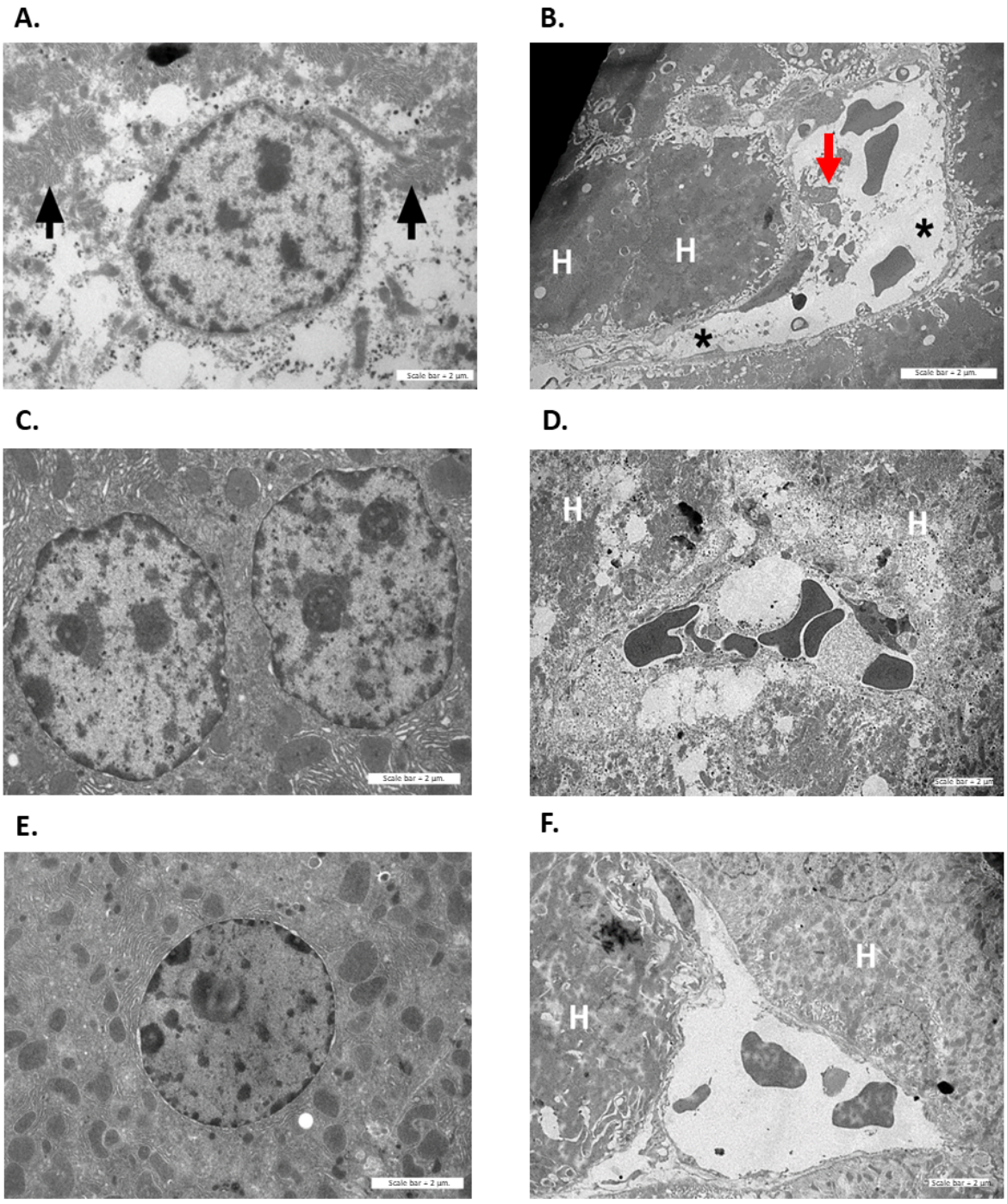

Figure 4

The histopathological examination of the liver under Transmission electron microscopy indicates that administration of *C. rhodostoma* venom (3xLD_{50}, i.p.), in mice, causes (A.) disappearance of normal cell organization swelling mitochondrial structures (black arrows), (B.) necrotic hepatocytes and the presence of cell debris in liver sinusoid (red arrow). The effect of 15 min prior administration of HPAV on *C. rhodostoma* venom induced hepatotoxicity of (C.) hepatocytes and (D.) sinusoid. The effect of HPAV

administration (i.p.) 15 mins following venom administration on morphological changes of (E.) hepatocyte and (F.) sinusoid. Scale bar = 2 µm. H indicates necrotic hepatocytes. Asterisks indicate dilated blood sinusoid of hepatocytes.

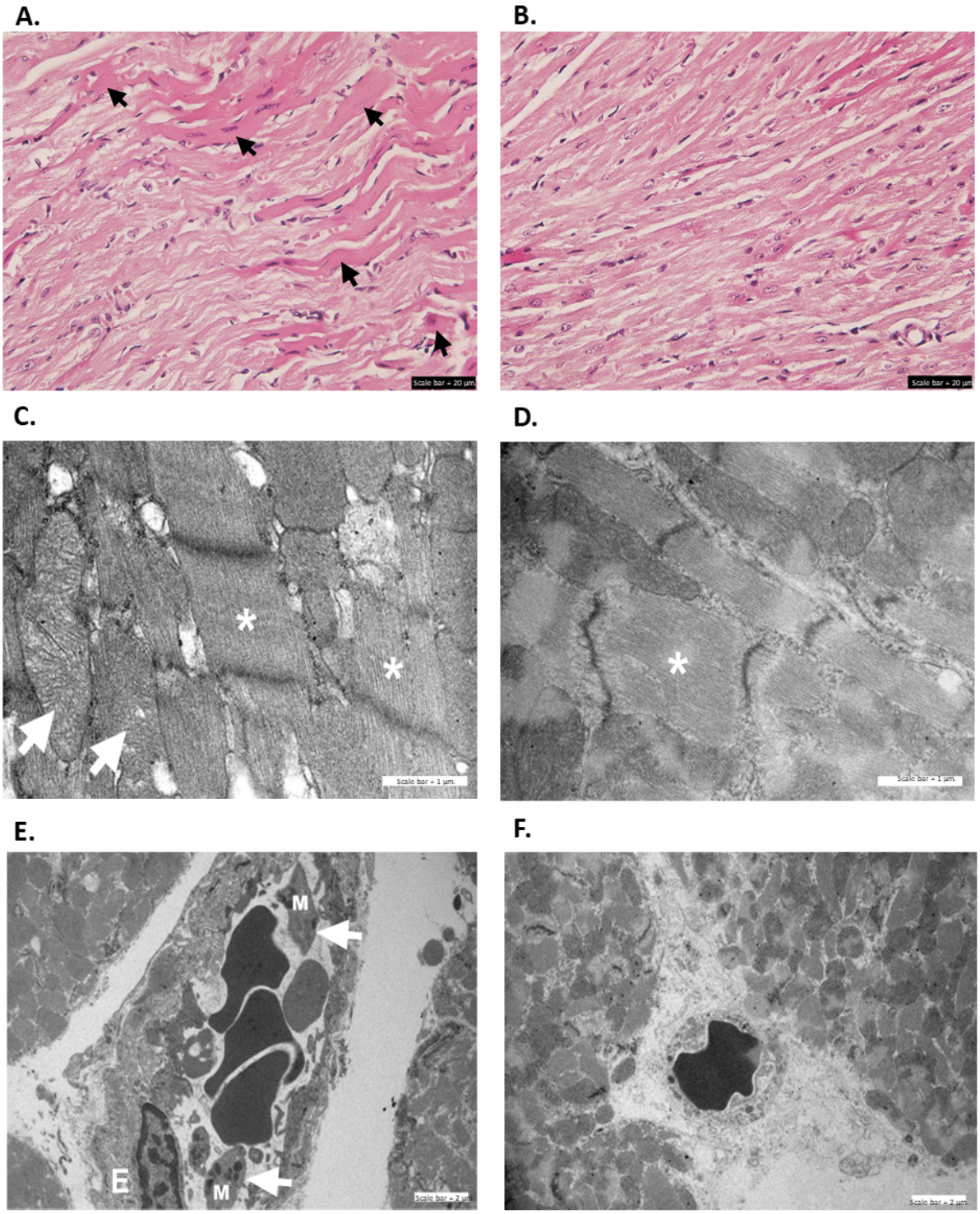

Figure 5

Histopathological examination of heart tissue (H & E staining) following 24-h intraperitoneal administration of *C. rhodostoma* venom ($3xLD_{50}$), in mice, indicated (A.) hypertrophy of cardiac tissues (black arrows; scale bar = 20 μm) and (B.) the protective effect of prior administration of HPAV on *C. rhodostoma* venom-induced cardiac tissue damage (scale bar = 20 μm). The histopathological determination of heart tissue under TEM indicates that administration of *C. rhodostoma* venom ($3xLD_{50}$, i.p.) causes (C.) cardiac muscle fiber hypertrophy and mitochondrial swelling (white arrows; scale bar = 1 μm) including (E.) endothelial cell swelling (letter-E; scale bar = 2 μm) and the presence of macrophage (white arrows). The protective effect of prior administration (15 min) of HPAV on *C. rhodostoma* venom-induced cardiac tissue damage (D. and F.). White asterisk indicates cardiac muscle fiber hypertrophy. Letter-M indicates macrophage infiltration.

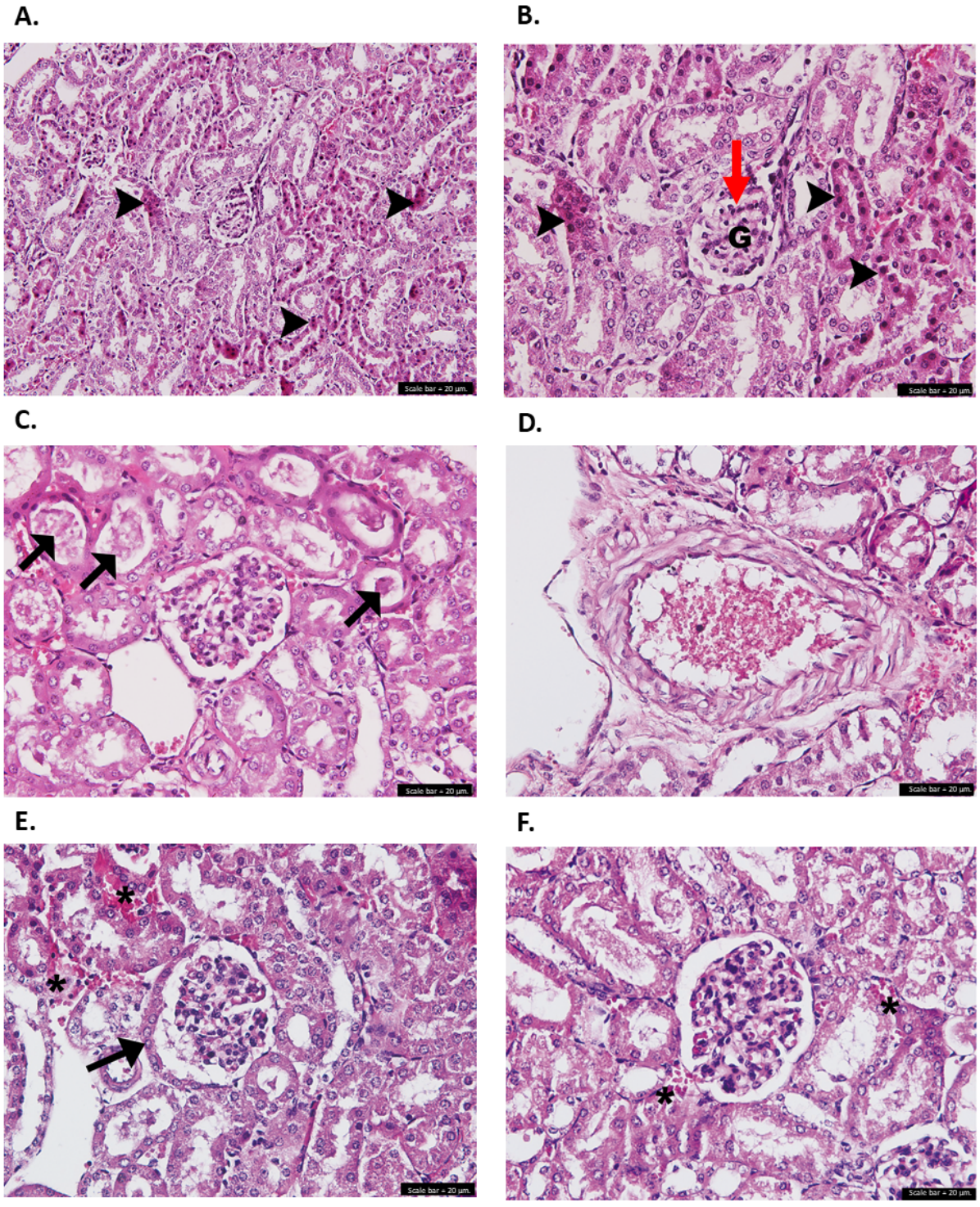

Figure 6

Histopathological changes of kidney tissues (H & E staining; scale bar = 20 μm) following 24-h intraperitoneal administration of *C. rhodostoma* venom ($3xLD_{50}$), in mice, indicating (A.) tubular necrosis, (B.) glomerular atrophy (red arrow), (C.) tubular cast(black arrow), (D.) disintegration of arterial wall in tunica intima, and (E.) interstitial vessel congestion of glomerulus (asterisks) and hypertrophy of parietal

cell (black arrow). (F.) The effect of prior administration of HPAV on prevention of venom-induced nephrotoxicity. * indicates interstitial vessel congestion. indicates tubular necrosis.

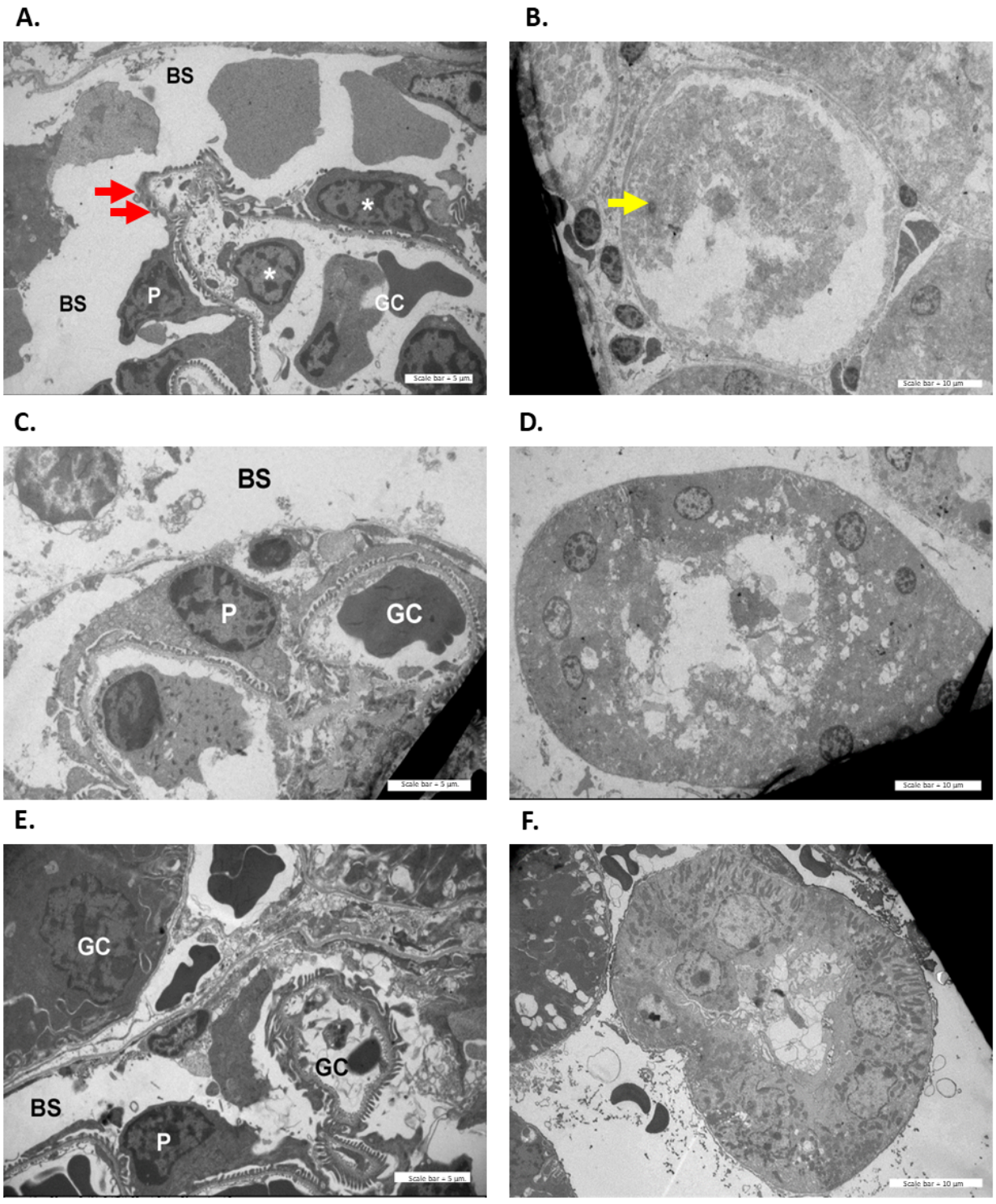

Figure 7

The histopathological determination of kidney tissues under TEM indicates that administration of *C. rhodostoma* venom (3xLD_{50}, i.p.), in mice, causes (A.) tubular injury of glomerulus, disarrangement of pedicel (red arrow), dilated glomerular capillaries (white asterisks), and (B.) a loss of cellular organelles in cytoplasm of renal tubule (yellow arrow). Histopathological study showing (C.) dilated glomerular capillaries and (D.) epithelial cell swelling of renal tubule of mice which received HPAV 15 min after the administration of *C. rhodostoma* venom. The protective effect of prior administration of HPAV on venom-induced morphological changes of (E.) glomerulus and (F.) renal tubule. BS indicates Bowman.s space; GC indicates glomerular capillaries; P indicates podocyte; M indicates mesangium cell. Scale bars of A., C. and E. = 5 μm; Scale bars of B., D. and F. = 10 μm.