Systemic pathological effects induced by cobra (*Naja naja*) venom from geographically distinct origins of Indian peninsula

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S U M M A R Y

Indian cobra (*Naja naja*) venom from different geographical locations varied in its composition and biochemical, pharmacological and immunological properties. Recently it has been shown that the variation in composition of venom from different geographical origin of Indian peninsula is due to the quantitative difference in the same components and also the presence of different biochemical entities with respect to their origin. This disparity in venom composition may be due to several environmental factors. However, very little is known about the systemic effects on vital organs caused by the venom due to regional variation. In the present investigation, the venom samples procured from eastern, western and southern regions were compared for histopathological effects on skeletal muscle and some vital organs (heart, lungs, liver and kidney) in the mouse model. All the three venom samples damaged vital organs such as cardiac muscle, gastrocnemius muscle, liver, lungs and kidneys; however, the extent of damage varied greatly. Eastern venom predominantly damaged cardiac muscle and kidney, western venom injured the liver and the southern venom affected the lung. In addition, the eastern venom caused the recruitment of a flux of inflammatory cells in the skeletal muscle unlike southern and western venom samples. These results suggest the diversity of target-specific toxins in all the three regional venoms. Thus, the study explores the possible variations in the pathological effects of cobra (*Naja naja*) venom samples on vital organs due to geographical distribution in the Indian subcontinent. It also emphasizes the importance of intra-specific variation of venom samples for the production of efficacious and region-specific therapeutic antivenom.

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Introduction

Despite snake venoms being a depot of target-specific toxins, some of them may serve as drugs or prototypes for drug design, but the management and neutralization of fatal snakebite are of priority. Antivenom is the preferred and worldwide choice of treatment for snakebites. Therefore, polyvalent (prepared against the venoms of few snakes), bivalent (prepared against the venoms of two snakes) and monovalent (prepared against the venom of one snake) antivenoms are currently available for therapeutic use. Although antivenom therapy acknowledges a high rate of success, several factors restrict its efficacy, such as lack of information of the bitten species (in most cases), antivenom dosage to be administered and its stability. Over and above, the therapy is much more complicated due to the factor of venom variability. The variation in venom composition and pharmacology has been addressed for different snake venoms at several levels (Chipppaux et al., 1991). Studies on understanding the intra-specific variability of venoms are gaining much attention with the intention of production of efficacious therapeutic antivenom and have been studied extensively (Goncalves, 1956; Glenn et al., 1983; Silveira et al., 1990; Yang et al., 1991; Lomonte and Carmona, 1992; Oguiura et al., 2000; Ferquel et al., 2007).

In India, cobra (*Naja naja*), Russell's viper (*Vipera/Daboia russellii*), krait (*Bungarus cereulus*) and saw-scaled viper (*Echis carinatus*) are considered to be the major poisonous snakes and are endemic in the subcontinent (Jadhav and Kapre, 1991). Indian cobra (*N. Naja*) is an identified and well-characterized snake of India and it is classified as a separate species after taxonomic classification of *Naja naja* species (Wuster, 1996); therefore, studies on Indian cobra (*N. naja*) venom composition variation due to geographical distribution are not to be confused with the presence of subspecies under the same genus. In the recent past, attention has been paid to understand the intra-specific variability of *N. naja* venom due to distant geographical distribution within the subcontinent with the intention of developing a more effective therapeutic antivenom. Though different regional *N. naja* venom shared similar biochemical composition, but varied greatly

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in their abundance of target-specific toxins. Studies from our laboratory and others have discovered marked variability among the different regional N. naja venom in it's immunological propetly (Mukerjee and Maity, 1998, 2002; Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2006, 2007). More recently, we have reported the presence of PLA$_2$ in eastern regional N. naja venom, which is absent in both southern and western regional venoms (Shashidharamurthy and Kemparaju, 2006). These studies have concluded that the antivenom prepared against one regional venom may not be effective against the other regional venoms. Extending the study further, in the present investigation, we report the differential histopathological effects of three geographically distant N. naja venom samples on vital organs such as heart, liver, lung, kidney and skeletal muscle in a mouse model.

Materials and methods

Indian cobra (Naja naja) venom samples (pooled and lyophilized from 4 to 6 adult snakes of both the sexes) were purchased in three batches from Hindustan park (Kolkata, West Bengal), Haffkine Institute (Mumbai, Maharashtra) and Irulla Snake Catchers (Chennai, Tamil Nadu). Creatine kinase-MB (CK-MB) diagnostic kit is from Crest Biosystems (Mysore, India). SGOT and SGPT assay kit was from Agappe (Mysore, India). Male Swiss Wistar mice weighing 20–22 g used for the histopathological studies were from the animal house facility, Department of Zoology, University of Mysore, Mysore, India. All the animal experiments were carried out according to the Animal Ethical Committee Protocol. All other reagents and chemicals used were of analytical grade. The LD$_{50}$ (mg/kg body weight of mice) were found to be 0.7 mg, 2.2 and 1.2 mg for eastern, southern and western regional venoms (Shashidharamurthy et al., 2002), respectively; these values were used for all the in vivo studies unless otherwise indicated.

Histopathological studies of vital organs

The histopathological studies were carried out as described earlier (Peichoto et al., 2006). Three groups of mice (N = 9 in each group) were injected (i.p.) independently with half the LD$_{50}$ dose, which was previously established in our laboratory (Shashidharamurthy et al., 2001) of eastern, western and southern regional venom samples in a volume of 0.3 ml saline, respectively. The fourth group (n = 5) injected with saline alone served as specificity control. After 5 h, each mouse was anaesthetized and blood was drawn from abdominal vena cava and serum samples were used for analysis of the tissue marker enzymes and creatinine estimation. Later autopsy was done and tissue samples from liver, kidney, lungs and heart were stored immediately in Bouin's solutions. For myonecrosis, studies were carried out as described (Prasad et al., 1999). Briefly, the venom samples (50 μg in 50 μl saline) from all the three regions were injected (i.m.) independently into three groups of mice (n = 5 for each group of mice) into the right thigh muscle/gastrocnemius muscle and the left thigh muscle was injected with saline, which served as specificity controls. The animals were sacrificed after 3 h. The muscle tissue around the site of injection was removed and fixed immediately in Bouin's solution. After 2 days all the tissues samples were removed, washed with distilled water and then dehydrated by processing through different grades of alcohol, and chloroform:alcohol mixtures and embedded in paraffin. The embedded tissues were cut into 5-μm-thick sections using Spencer '800' microtome and stained with hematoxylin and eosin. The stained tissue sections were observed under a Leitz

Wetzlar Germany type-307-148.002 microscope and photographs were taken using a photometrics colorsnap CF camera (Roper Scientific Photometrics) attached to the microscope.

Creatine kinase activity

Heart-specific isoform, creatine kinase-MB (CK-MB) activity was assayed according to the method of IFCC (1989) as described in the reagent/substrate start scheme of manufacture's protocol using Crest Biosystems diagnostic kit. Briefly, reagent-L1 (0.8 ml) and 50 μl of serum sample were incubated for 5 min at 37 °C. Reaction was initiated by adding 0.2 ml of reagent-L2 and optical density was read at 340 nm for 3 min and recorded at an interval of 1 min. Activity is expressed as units/l.

Serum glutamate-oxaloacetate transaminase and Serum glutamate-pyruvate transaminase (SGOT and SGPT) activities

SGOT and SGPT activities were assayed according to the method of Thefeld et al. (1974) as described in the reagent start scheme of manufacture's protocol using an Agappe diagnostic kit. Briefly, reagent-1 (0.8 ml) and reagent-2 (0.2 ml) were added with 0.1 ml of mouse serum sample and incubated for 1 min at 37 °C. Optical density was read at 340 nm for 3 min and recorded at an interval of 1 min. Activity is expressed as units/l.

Estimation of creatinine

Serum creatinine content was assayed according to the method of Fabiny and Ertinghausen (1971) as described in the reagent start scheme of manufacture's protocol using a CPC diagnostic kit. Briefly, reagent-1 (0.5 ml) and reagent-2 (0.5 ml) were added with (0.1 ml) of serum sample and immediately optical density was read at 500 nm. Activity is expressed as units/l.

Results

Map of the Indian peninsula showing the state and location of cobra (N. naja) venom samples purchased

Based on the geographical origin, the N. naja venom samples procured from Hindustan Park (Kolkata), Haffkine Institute (Mumbai) and Irulla Snake Catchers (Chennai) were referred to as the venom of eastern, western and southern regions of Indian Peninsula, respectively (Fig. 1).

The cardiotoxicity of the Indian Cobra (N. naja) venom varies from region to region

The cobra venom is found to be highly cardiotoxic; therefore we studied the effect of venom samples from different geographical origins on heart muscle. Noticeably, the eastern venom caused the accumulation of erythrocytes in the lumen of the blood vessel (Fig. 2a, Section B). The clumping of erythrocytes may be due to the clot formation induced by the venom. Interestingly, erythrocyte clumping was not observed in the tissue sections from mice injected with either southern (Fig. 2a, Section C) or western (Fig. 2a, Section D) venoms. Despite the necrosis of venom-treated tissues sections, they are not readily distinguishable from one another. Therefore we determined the creatine kinase isoenzyme (CK-MB) activity in the serum from venom-treated mice (CK-MB serves as a marker for myocardial infraction) was determined. The CK-MB assay revealed varied levels of activity. The serum sample

from eastern venom-injected mice showed the highest activity followed by southern and western venom with least activity among the three venoms studied (Fig. 2b). Further, serum glutamate-oxaloacetate transaminase (SGOT) activity was determined. Since its level increases with tissue damage especially associated with the cardiac muscle, liver tissue, skeletal muscle and kidneys. The SGOT activity varied as

eastern > western > southern venom samples (Fig. 2c). These results suggest that the cardiotoxicity of the *N. naja* venoms varied as eastern > southern > western venoms.

Fig. 3. Photomicrographs of gastrocnemius muscle of mouse. Light micrographs of gastrocnemius muscle (40 ×) taken from a mouse after the i.m. Injection of tested dose (10 μg) of *Naja naja* venom from eastern (B), southern (C) and western (D) regions of India. Control was obtained from saline-injected mice (A). Note the presence of necrotic cells (N), inflammatory cells (I) and more interstitial space (S). Arrows indicate region of necrosis.

Fig. 2. (a) Photomicrographs of cardiac muscle of mouse. Light micrographs of cardiac muscle (40 ×) taken from a mouse after the i.m. injection of tested dose (LD$_{25}$) of *Naja naja* venom from eastern (B), southern (C) and western (D) regions of India. Control was obtained from saline-injected mice (A). Note the presence of necrotic cells (N) and inflammatory cells (I). Arrows indicate region of necrosis. (b) Cardiac-specific serum creatine kinase (CK-MB) activity. Heart-specific isoform, creatine kinase-MB (CK-MB) activity was assayed using a Crest Biosystems diagnostic kit. Reagent-L1 and serum sample were incubated for 5 min at 37 °C. Reaction was initiated by adding reagent-L2 and optical density was read at 340 nm for 3 min and recorded at an interval of 1 min. Activity was expressed as units/l. Values represent mean ± SEM of nine experiments. (c) Serum glutamate-oxaloacetate transaminase (SGOT) activity. SGOT activity was assayed using an Agappe diagnostic kit. Reagent-1 and reagent-2 were added with serum sample and incubated for 1 min at 37 °C. Optical density was read at 340 nm for 3 min and recorded at an interval of 1 min. Activity was expressed as units/l. Values represent mean ± SEM of nine experiments.

Fig. 1. Schematic representation of the state and locations of Indian cobra venom samples purchased from eastern (Kolkata, West Bengal), western (Mumbai, Maharastra) and southern (Chennai, Tamil Nadu) regions of Indian peninsula.
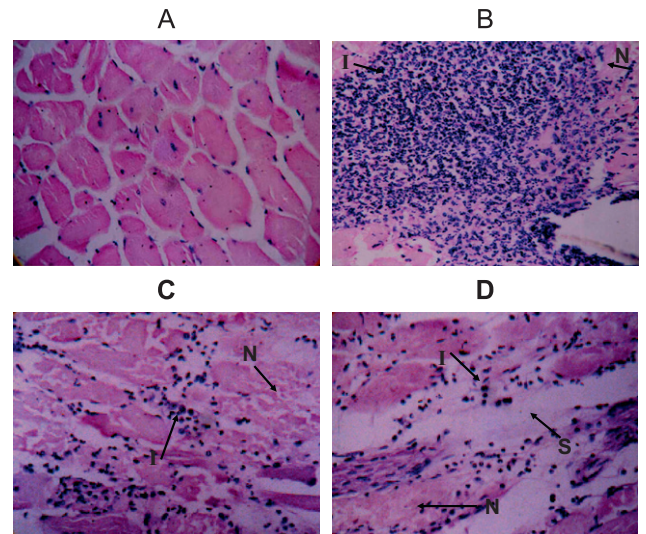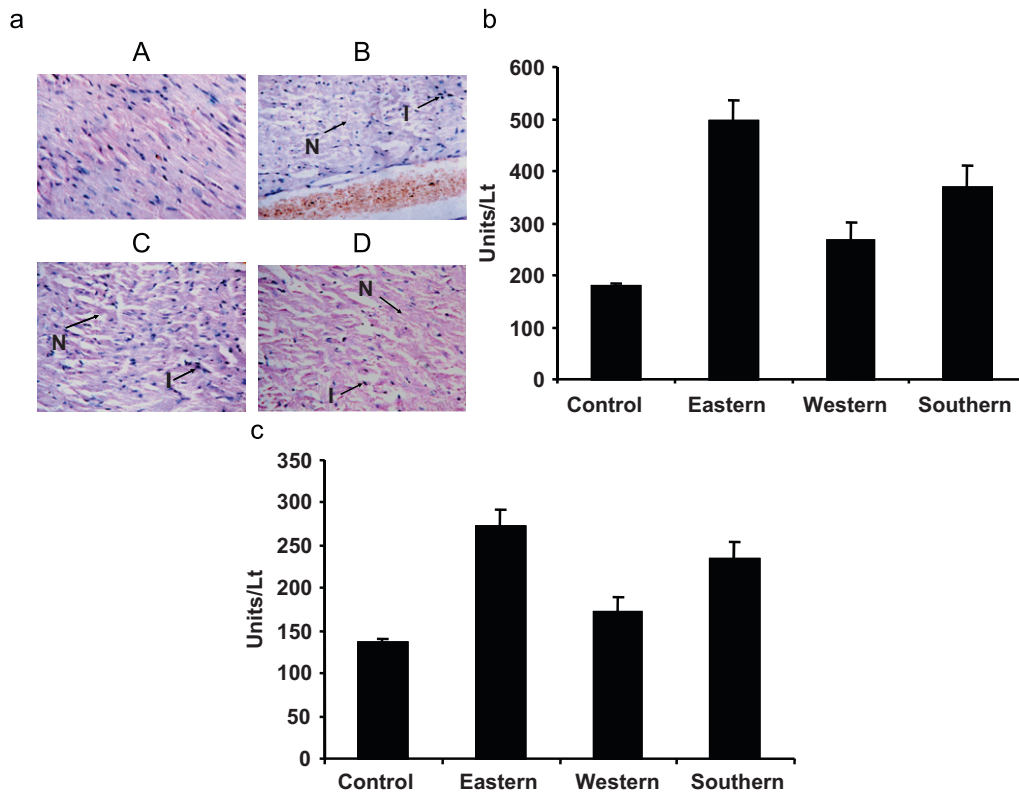
Effect of three regional N. naja venom samples on skeletal muscle

Damage of muscle tissue was observed with the appearance of necrotized myocytes in all the three sections. Fig. 3 shows the varied effect of the three regional venoms on the skeletal muscle of mice. Widening of the interstitial space is conspicuous in western venom-injected tissue section (Fig. 3D). The saline-injected control mice (Fig. 3A) revealed intact myocytes with no infiltration of inflammatory cells. There has been a burst of inflammatory response in case of eastern venom-injected mice tissue section, in which a dense herd of inflammatory cells was seen at the site of injection (Fig. 3B), while marginal infiltration of inflammatory cells was seen in tissue sections of mice injected with the southern (Fig. 3C) and western (Fig. 3D) venoms. The massive infiltration of inflammatory cells was observed only in eastern venom-injected tissue section. These results suggest the presence of variation in the myotoxicity of these venoms on skeletal muscle.

Effect of three regional N. naja venom samples on liver

The tissue sections from liver did not show marked differences among the three regional venoms as no conspicuous signs of dystrophy was observed when compared to saline-injected control tissue section (Fig. 4a, Section A). Nevertheless, eastern venom (Fig. 4a, Section B) appears to cause the clumping of erythrocytes in blood vessel, revealing the accumulation of erythrocytes in the lumen of blood vessel as seen in cardiac muscle section (Fig. 2a). In contrast, liver tissue sections injected with the southern (Fig. 4a, Section C) and western venoms (Fig. 4a, Section D) are clear but appear dilated. However, when the level of serum SGPT activity was measured, noticeable difference was observed. Mice injected with eastern, southern and western venoms showed about 1.3-, 2.4- and 1.7-fold elevated enzyme response compared to the saline-injected control sample (Fig. 4b). SGPT is found predominantly in liver and serve as a marker of liver tissue destruction. These data suggest that the eastern venom is least hepatotoxic, while western venom has the highest hepatotoxicity among the three venoms studied.

Effect of three regional N. naja venom samples on lung and kidney

The lung tissue sections from mice injected with southern (Fig. 5C) and western (Fig. 5D) venoms showed the rupture and congestion of blood vessels. The congestion was more prominent in western venom, while eastern venom (Fig. 5B) did not, but revealed irregular and protruded vessel wall morphology. However, all the three tissue sections that were treated with respective regional venoms revealed infiltration of inflammatory cells. On the other hand, the enhanced response was observed in southern venom as the lumen of the vessel was crowded with infiltered leukocytes. The tissue section from saline-treated control mice revealed normal blood vessel morphology with spherical vessel wall and minimal inflammatory cells (Fig. 5A). In contrast, though kidney sections from mice injected with eastern (Fig. 6B), southern (Fig. 6C) and western (Fig. 6D) venoms revealed altered histology compared to the saline-injected control mice (Fig. 6A), we did not observe any noticeable difference among the three regional venoms injected. However, the maximum damage appears to be associated with the eastern venom-injected tissue section (Fig. 6B). The level of serum creatinine, a metabolic marker for kidneys dysfunction, did not record significant differences in its content; in all the three
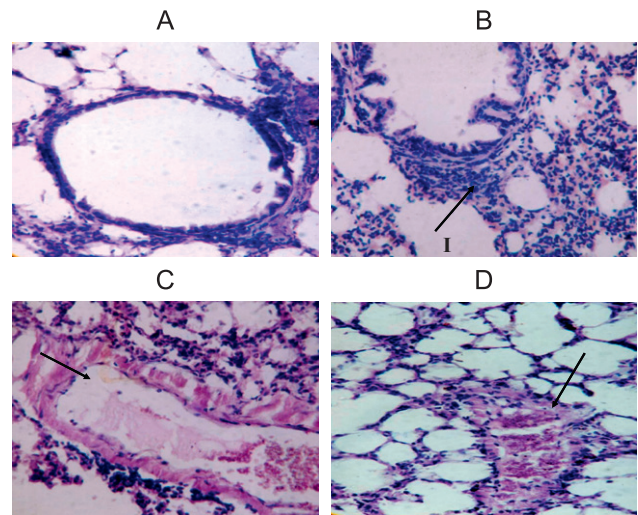

Fig. 5. Photomicrographs of lung tissue of mouse. Light micrographs of lung tissue (40 ×) taken from a mouse after the i.p. injection of tested dose (LD$_{25}$) of *Naja naja* venom from eastern (B), southern (C) and western (D) regions of India. Control was obtained from saline-injected mice (A). Note the presence of inflammatory cells (I). Arrows indicate blood vessel congestion.
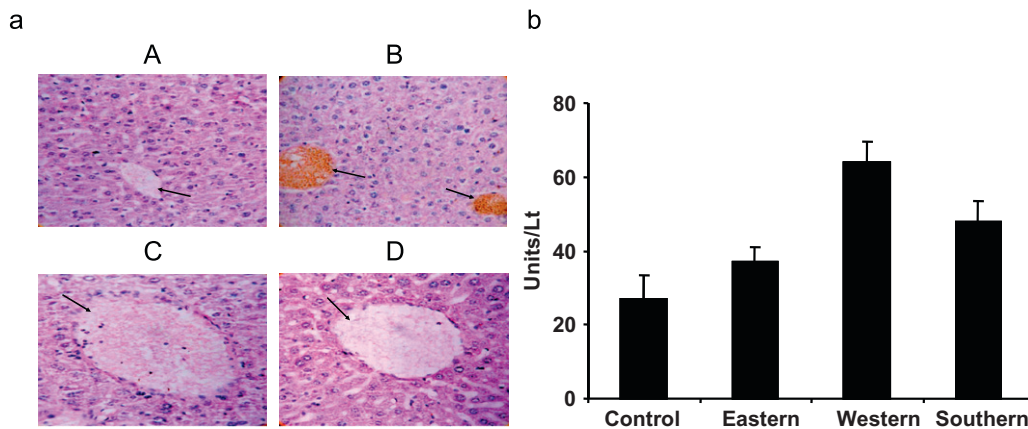

Fig. 4. (a) Photomicrographs of liver tissue of mouse. Light micrographs of liver tissue (40 ×) taken from a mouse after the i.p. injection of tested dose (LD$_{25}$) of *Naja naja* venom from eastern (B), southern (C) and western (D) regions of India. Control was obtained from saline-injected mice (A). Arrows indicate blood vessel congestion and dialation. (b) Serum glutamate-pyruvate transaminase (SGPT) activity. SGPT activity was assayed using an Agappe diagnostic kit. Reagent-1 and reagent-2 were added with serum sample and incubated for 1 min at 37 °C. Optical density was read at 340 nm for 3 min and recorded at an interval of 1 min. Activity was expressed as units/l. Values represent mean ± SEM of nine experiments.
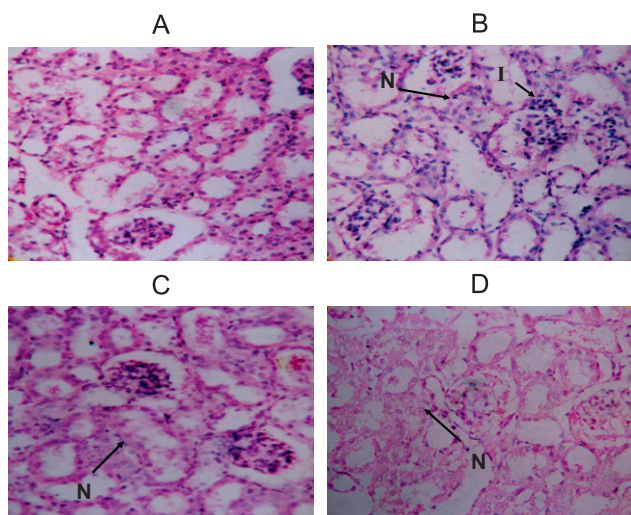
Fig. 6. Photomicrographs of kidney tissue of mouse. Light micrographs of kidney tissue ($40\times$) taken from a mouse after the i.p. injection of tested dose (LD_{25}) of *Naja naja* venom from eastern (B), southern (C) and western (D) regions of India. Control was obtained from saline-injected mice (A). Note the presence of tubular necrosis (N), inflammatory cells (I). Arrows indicate region of tubular necrosis.

cases, the values were very close to the saline-injected control values (data not shown).

Discussion

The intra-species variation in snake venom composition is a serious medical concern for researchers, as it would help in the production of efficacious antivenom. Earlier reports describe the variation in biochemical and pharmacological properties of *N. naja* venom from geographically distant regions in the Indian sub-continent (Mukerjee and Maity, 1998, 2002; Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2006). In the present investigation, we explored the systemic pathological changes induced by the *N. naja* venom on vital organs due to distinct geographical origin. The three regional venom samples vary greatly in their histopathological properties. For instance, all the three regional venoms showed varied effect on the cardiac muscle. The respective tissue sections from the three regional venom-injected mice revealed necrotized and distorted musculature unlike saline-injected control tissue section; however, the venom-induced necrosis of myocardium varies as eastern > southern > western venom samples. To biochemically distinguish the variation in necrosis of heart muscle, the CK-MB and SGOT enzyme activity was determined in the serum from venom-injected mice. CK-MB is one of the most important myocardial markers, with well-established role in confirming acute myocardial infarction (Apple and Preese, 1994). Creatine kinase (CK) is a dimer of M and B subunits, found primarily in muscle, myocardium, brain and lung tissue, which exists as three dimeric isoenzymes: CK-MM (CK-3), CK-MB (CK-2) and CK-BB (CK-1). The CK-MB accounts for 5% to 50% of total CK activity in myocardium. It is important to note that the serum level of CK-MB and SGOT enzymes (SGOT activity increases when the tissue destruction is associated with the cardiac muscle, liver tissue, skeletal muscle and kidneys) revealed a similar pattern of response. These results suggest that the cardiotoxins in these venom samples vary quantitatively depending on their geographical distribution. Thus the cardiotoxicity of the *N. naja* venoms varied as eastern > southern > western. Another most striking difference is the

recruitment, infiltration and confluence of inflammatory cells in the respective venom-injected skeletal muscle. The myotoxicity is varied as eastern ≥ southern > western. In addition, our earlier study reported that Indian cobra venom samples from different geographical origins was found to vary in phospholipase A_2 content and eastern venom exhibited a distinct PLA_2 enzyme while it was absent in southern and western venom samples (Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2006). Therefore the generation of inflammatory cells recruiting agents by the eastern venom is more likely where phospholipase and proteolytic degradation products have contributed to leukocytes' recruiting property. Therefore, it appears that the eastern venom is more inflammatory than the other two venoms studied. Accordingly, the edema-forming potency of eastern venom was comparatively more than that of the other two venoms studied (data not shown). Edema formation is categorized as an inflammatory response and characterized by swelling of local area at the envenomed region. Similar studies on variation in myotoxic property of *Brothrops* venom were established (Zamuner, et al., 2004). Further, the liver tissue section and SGPT enzyme activity in serum suggest that the eastern venom is least hepatotoxic, while western venom has the highest hepatotoxicity among the three venoms studied. However, the lung and kidney tissue sections from mice injected with eastern, southern and western venom revealed altered histology compared to the saline-injected mice, but no noticeable difference among the three regional venoms injected was observed. These results suggest the diversity of target-specific toxins in all the three regional venoms studied. The target-specific PLA_2 isoenzymes, which cause hemorrhage in lung, liver, pituitary, thyroid and kidney, have been isolated and characterized from *Vipera russelli* venom (Vishwanath., (1986); Kasturi and Gowda, 1989). Considering the above-discussed biochemical, pharmacological and immunological variations in snake venom, the therapy for snake venom envenomation becomes less effective and more complicated. Recently, it has been shown that antivenom prepared against particular regional venom is reported to be ineffective or partially effective against the toxicity of other regional venoms due to the varied composition of venom components within the same species (Phillips et al., 1988; Prasad et al., 1999, Mukerjee and Maity, 2002; Shashidharamurthy and Kemparaju, 2007). These observations provide direct evidence for variability in specificity and neutralizing efficacy of the antivenom, which in turn suggests possible antigenic variability among the different regional venoms.

In conclusion, the histopathological and biochemical studies revealed the diverse toxic effects of the three regional venoms on vital organs such as cardiac muscle, lung, liver, kidney and gastrocnemius muscle. Thus, the data suggest all three regional venom vary quantitatively and functionally in target-specific toxins depending on the distinct geographical origin. Further, this study not only contribute towards understanding the intra-species venom variability but also emphasises the importance in production of efficacious and region-specific therapeutic anti-venom.

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