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

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SUMMARY

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.

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 (Chippaux 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 propetry (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