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# Russell's viper venom affects regulation of small GTPases and causes nuclear damage

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ABSTRACT

Russell's viper with its five sub-species is found throughout the Indian subcontinent. Its venom is primarily hemotoxic. However, its envenomation causes damage to several physiological systems. The present work was aimed to study the dose and time dependent cytotoxic effects of Russell's viper venom (RVV) on human A549 cells grown *in vitro*. Time dependent changes have been observed in cellular morphology following exposure to RVV. Presence of stress granules, rounding-off of the cells, and formation of punctate structure and loss of cell–cell contact characterized the cellular effects. Fluorescence microscopic studies revealed that apoptotic cell population increased on exposure to RVV. Further to understand the mechanism of these effects, status of small GTPase (smGTPases) expression were studied by Western blot and RT-PCR; as smGTPases play pivotal roles in deciding the cellular morphology, polarity, cell movement and overall signaling cascade. It was shown for the first time that expression patterns of Rac, Rho and CDC42 genes are altered on exposure to RVV. Similarly, significant difference in the expression pattern of HSP70 and p53 at the mRNA levels were noted. Our results confirmed that RVV induces apoptosis in A549 cells; this was further confirmed by AO/EtBr staining as well as caspase-3 assay. All experiments were compared using RVV unexposed cells. We propose for the first time that RVV induces morphological changes in human A549 cells through modulation of smGTPase expression and affects the cellular-nuclear architecture which in turn interferes in proliferation and migration of these cells along with apoptosis.

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## 1. Introduction

Russell's viper is an old world snake falling under the elite viperine snake family. Its single species with five sub-species, namely, *russelli*, *pulchella* (sometimes considered synonymous with *russelli*), *formosensis Maki*, *limitis* and *nordicus* are distributed throughout south-East Asia. Envenomation by Russell's viper is known for its diverse patho-physiological consequences, e.g., myotoxicity, edema, pituitary insufficiency, 'dramatic hemorrhage' and renal failure apart from death. The amazing specificity of snake venom toxins to target physiological systems also made them potential molecules of drug development. A number of toxins with anti-cancer potentials have been purified in recent times from different snake venoms, including Russell's viper venom. The anti-cancer potential of Russell's viper venom lies in its cytotoxic effects. Most contemporary research in the development of anticancer therapeutics from venoms have focused on investigating the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells (Son et al., 2007; Yang et al., 2005).

It is well documented that Rho family of small GTPases play a vital role in the control of cell movement, morphology and adhesion by regulating the actin cytoskeleton (Bishop and Hall, 2000). Tumor invasion and metastasis involves various intracellular molecules, which includes formation of membrane protrusions like filopodia, lamellipodia, and pseudopodia (Abraham et al., 2001). In the recent past, studies have shown that Rho proteins are most likely involved in the cancer cell migration, tissue invasion and metastasis (Aznar and Lacal, 2001). Although, the cytotoxic effects Russell's viper venom has been observed, the true mechanism of

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the same is not known. We have studied the morphological changes in cultured lung adenocarcinoma cells following exposure to whole Russell's viper (*Vipera russelli russelli*) venom and tried to understand the mechanism. One of the major pathways for cellular morphological change is through the changes in cytoskeleton. Expression of Rac, Rho and CDC42 genes are known to be involved in cytoskeletal changes. In this study, we have attempted to understand the involvements of these systems in the RVV mediated cytotoxicity.

Since GTPase expression is important for cancer progression and metastasis, understanding regulation of GTPase is of clinical importance. In the present investigation we have addressed for the first time the cytotoxic effects of RVV on human *in vitro* cultured bronchoalveolar carcinoma-derived cell line (A549). A549 cells exhibit similarity to the type II alveolar cell phenotype and share many characteristics with the human primary epithelial cells (Fuchs et al., 2003). Therefore, this cell culture based work was considered as an appropriate *in vitro* model system to evaluate the cytotoxicity and the events involved in the cell death caused by RVV. In the present work, we analyzed the effect of RVV on A549 cell line in order to elucidate a putative direct cytotoxic action, cytotoxicity with respect to the expression and regulation pattern of small GTPases. We have also studied the involvement of caspase-3, Bcl2, Bax in relation to apopototic effects of RVV on A549 cells. We have studied for the first time the expression pattern of HSP70 at mRNA as well as in protein level. In this study, extent of nuclear damage and invasive property of A459 cells on exposure to RVV was noted.

## 2. Materials and methods

### 2.1. Chemicals

Dry pooled Russell's viper venom (RVV) was purchased from 'Calcutta Snake Park', Kolkata, India. All the general laboratory chemicals were purchased either from Sigma–Aldrich (USA), Bioline (India) or from Invitrogen (USA). Cell culture media, FBS and antibiotic antimycotic were bought from Cell One, USA or from Invitrogen USA. Human broncho-alveolar carcinoma derived (A549) cells was kindly provided by Dr. Samit Chattopadhyay National Centre for Cell Science (NCCS), Pune India. Primers were synthesized by IDT (USA/India). cDNA synthesis kit was purchased from Thermo Fisher, USA. Hsp70 mAB was purchased from Sigma–Aldrich (USA). Small GTPase (Rac1, RhoA and Cdc42) mAB were purchased from Cell Signaling Technology, Inc. USA. Hoechst 33342 was also purchased from Sigma–Aldrich (USA). For cell culture all the plastic wares were procured from Corning, USA. Phalloidin was purchased from Life Sciences Technology, USA. AO/EtBr was from Himedia, India. For invasion assay the inserts were purchased from Corning. Caspase 3 assay kit was procured from Invitrogen, USA.

### 2.2. In vitro culture of human A549 cells

A549 cells were cultured *in vitro* and maintained at 37 °C with 5% $CO_2$ in DMEM supplemented with heat inactivated 10% FBS, 1X antibiotic antimycotic solution and passaged every fourth day. For all experiments, cells were seeded at the density of $3–4 \times 10^4$ cells/ml either in a 6 well plate or in 25 $cm^2$ tissue culture flasks.

### 2.3. Preparation of RVV solutions

Dry RVV was reconstituted in 20 mM phosphate buffer (pH 7.4) over night at 4 °C; the solution was then centrifuged at 5000 rpm for 5 min at 4 °C to discard the cells debris and other particulate matters (Chakrabarty et al., 2000) [29]. This was further diluted with DMEM to obtain the desired concentration. RVV was prepared freshly every time from the original stock just before the treatment.

### 2.4. Methodology

#### 2.4.1. Changes in cell morphology

The effect of RVV induced morphological changes in A549 cell was assessed by simple microscopic method. After the cells were treated with different concentrations of RVV for 24 and 48 h, the cells from all the treated groups were photographed using inverted microscope using an attached digital camera (Nikon-Eclipse, TS 100F, USA) along with an unexposed sample as a control.

#### 2.4.2. Morphological assay for actin by FITC-phalloidin

A549 cells were seeded at a density of $1 \times 10^6$ cells/mL in six-well plates, and treated with different concentrations of RVV 2.5 μg/ml and 5 μg/ml for 24 and 48-h respectively. The actin filaments in A549 cells were visualized by staining with FITC-phalloidin. Cells in each group were fixed with 3.7% formaldehyde for 30 min at room temperature, rinsed three times with 1 × PBS, then treated with 0.1% Triton X-100 for 10 min at room temperature, and rinsed thrice with PBS. The cells were further incubated with (1:100) dilution FITC-phalloidin for 2 h in the dark at room temperature. To label the nuclei, Hoechst 33342 (10 μg/ml) was added for 15 min in the dark at room temperature. The cells were then washed in 1 × PBS to remove the unbound FITC-phalloidin and Hoechst 33342. Finally, the cytoskeletal and nuclear morphology was imaged by fluorescence microscope (Nikon).

#### 2.4.3. Cell viability assay

Exponentially growing cells were sub-cultured into T-flasks and allowed to attach before grouping and further treated with two different concentrations of RVV for 24 h and 48 h. Upon various time intervals drug treatments, cells were trypsinized and collected for staining with 0.5% of trypan blue with 1:1 ratio of the dye and cell suspension as described by Pratt and Willis (1985). 20 μl of mixed suspension taken onto haemocytometer and a minimum of two thousand cells including viable and dead cells were counted at 40X magnification using an inverted light microscope (Nikon Eclipse TS 100). Dead cells appeared blue in color, while viable cells appeared colorless. Three replicate counts were taken per sample. The percentage of cell viability was calculated as follows:

$$\left(\text{Viability } (\%) = \frac{\text{Number of non} - \text{stained cells}}{\text{Total cell number cells}} \times 100\right)$$

#### 2.4.4. Resazurin cytotoxicity assay

Cell viability through metabolic activity was assayed using Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one). Live and healthy cells reduce Resazurin to resorufin (blue to pink) and dihydroresorufin intracellularly (O'Brien et al., 2000). Cells were cultured to obtain a monolayer in a 24 well plate. RVV exposure at doses of 0 μg/ml (positive control), 1–10 μg/ml was applied. Positive difference in absorbance at $A_{600nm}$ and $A_{690nm}$ of each well culture against control was monitored and the percentage reduction was calculated and reported as a measure of toxicity as described by Anoopkumar-Dukie et al. (2005). The limiting value corresponding to 0% reduction was obtained by measuring absorbance at $A_{600nm}$ and $A_{690nm}$ of negative control (without cells or RVV).

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