

Scorpion (*Odontobuthus doriae*) venom induces apoptosis and inhibits DNA synthesis in human neuroblastoma cells

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Abstract Scorpion and its organs have been used to cure epilepsy, rheumatism, and male impotency since medieval times. Scorpion venom which contains different compounds like enzyme and non-enzyme proteins, ions, free amino acids, and other organic inorganic substances have been reported to possess antiproliferative, cytotoxic, apoptogenic, and immunosuppressive properties. We for the first time report the apoptotic and antiproliferative effects of scorpion venom (*Odontobuthus doriae*) in human neuroblastoma cells. After exposure of cells to medium containing varying concentrations of venom (10, 25, 50, 100, and 200 µg/ml), cell viability decreased to 90.75, 75.53, 55.52, 37.85, and 14.30%, respectively, after 24 h. Cells expressed morphological changes like swelling, inhibition of neurite outgrowth, irregular shape, aggregation, rupture of membrane, and release of cytosolic contents after treatment with venom. Lactate dehydrogenase (LDH) level increased in 50 and 100 µg/ml as compared to control, but there was no significant increase in LDH level at a dose of 10 and 20 µg/ml. Two concentrations viz. 50 and 100 µg/ml were selected because of the profound effect of these concentrations on the cellular health and population. Treatment with these two concentrations induced reactive nitrogen intermediates and depolarization in mitochondria. While caspase-3 activity increased in a concentration-dependent manner, only 50 µg/ml was able to fragment

DNA. It was interesting to note that at higher dose, i.e., 100 µg/ml, the cells were killed, supposedly by acute necrosis. DNA synthesis evidenced by bromodeoxyuridine (BrdU) incorporation was inhibited in a concentration-dependent manner. The cells without treatment incorporated BrdU with high affinity confirming their cancerous nature whereas very less incorporation was noticed in treated cells. Our results show apoptotic and antiproliferative potential of scorpion venom (*O. doriae*) in human neuroblastoma cells. These properties make scorpion venom a valuable therapeutic agent in cancer research.

Keywords Scorpion venom · *Odontobuthus doriae* · Mitochondrial membrane potential · Caspase-3 · BrdU

Introduction

Use of animals and products obtained from their organs have been part of medicinal substances since medieval times [1]. Traditional healers used scorpions and other poisonous animals to treat different types of diseases. The use of animal toxins to determine their mechanism of action on different systems of body could prove to be starting point for designing new treatments of various diseases. Venom's characteristics to stun the prey by its poisonous effect could be used as initial guide to develop a chemical with gentler and therapeutic properties and attention of scientists are now focused to explore the potential of venom for developing medicine for incurable diseases. Research on effects of scorpion venom as a treatment for brain cancer is being carried out by many groups of scientists [2].

Scorpion venom contains different group of compounds such as proteins (including several enzymes like hyaluronidases,

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phospholipases, sphingomyelinases [3], acetylcholinesterase, alkaline phosphatase, and proteolytic enzymes [4], some peptide fractions with molecular masses ≤ 10 kDa, ions, free amino acids, and neurotransmitters [5]. Some of these low molecular-mass, basic, and cysteine-rich peptides can affect different ion channels, in particular those for Na^+ , K^+ , Ca^{2+} , and Cl^- in excitable membranes [6] and can be a driving force for cytotoxicity. Blockers of cell membrane channels play an important function in cellular mitogenesis [7] and also are identified to control signal transduction in the metastatic cascades [8]. Scorpion venom has been previously used as source of drugs that have been used to treat different diseases like epilepsy, acute and chronic convulsion, tetanus, and subcutaneous nodules [9]. Moreover, scorpion venom is reported to decrease the growth of cancer cells [10, 11] and can also inhibit primary tumors in vivo [2, 12]. Scorpion venom (*Alumnus gravimanus*) has been reported to be hypotensive in cats [13]. Moreover, Scorpion toxin II is potent in inducing contracture and spontaneous contractions of the chick biventer cervicis muscle [14] apart from contractile effects on guinea pig ileum [15]. Some scorpion (*Herders gertschi*) venom peptides have been demonstrated to have selective activity on ryanodine receptors [16]. Keeping in view these properties of scorpion venoms, we investigated the cytotoxic effect of venom from *Odontobuthus doriae* (Thorell, 1876) in the human neuroblastoma cells. This study is first of its kind that highlights the cytotoxic properties of *O. doriae* venom and explains a possible mechanism of apoptosis.

Materials and methods

Chemicals

DMEM-F12 (Gibco, USA), Fetal bovine serum (Gibco, USA), Trypsin–EDTA (Sigma-Aldrich, USA), Phenol red (Sigma-Aldrich, USA), Trypan blue (Sigma-Aldrich, USA), PBS (Gibco, USA), Penicillin–streptomycin solution (Sigma-Aldrich, USA), Antibiotic–antimycotic (Invitrogen, USA), DMSO [Dimethylsulfoxide] (Sigma-Aldrich, USA), In vitro toxicology assay kit: lactic dehydrogenase based (Sigma-Aldrich, USA), JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide] (Sigma-Aldrich, USA), Griess reagent (Sigma-Aldrich, USA), Triton X-100 (Sigma-Aldrich, USA), Agarose (Sigma-Aldrich, USA), Fixative (Sigma-Aldrich, USA), BrdU [5-Bromo-2'-Deoxyuridine] (Sigma-Aldrich, USA), DAB [Diaminobenzidine] (Sigma-Aldrich, USA), primary monoclonal anti-bromodeoxyuridine (BrdU) antibody (Sigma-Aldrich, USA), Anti-mouse IgG (Fab specific)-peroxidase antibody produced in goat (Sigma-Aldrich, USA), NGS [Normal goat

serum] (Gibco, USA), U Bottom 24-wells plate (Sigma-Aldrich, USA), and U Bottom 96-wells plate (Sigma-Aldrich, USA). All the other chemicals and reagents were of analytical grade and purchased locally.

Scorpion venom

Odontobuthus doriae is an excavator scorpion and usually rests underground. For this study, scorpions were collected from Baghershahr in Tehran province of Iran. Scorpions were housed in ventilated cages for more than 1 year and were fed with live housefly larva, flour beetle larva, and locust. Venom was obtained monthly by mild electrical stimulation (20 V, 500 mA) and was solubilized in sterile double distilled water. After centrifugation at $8000\times g$ for 15 min at 4°C , supernatant was immediately lyophilized and stored at -20°C until use [17]. The crude venom was dissolved in DMEM-F12 and protein content was determined by Bradford method [18]. Venom was disinfected with 1.5% antibiotic–antimycotic solution.

Cell culture

SH-SY5Y cells were procured from National Centre for Cell Science (NCCS) Pune, India. Cells were grown in 75-ml plastic flasks in DMEM-F12 medium supplemented with 10% heat inactivated fetal bovine serum, 10 $\mu\text{l}/\text{ml}$ Penicillin–streptomycin solution or 1% antibiotic–antimycotic solution and incubated in CO_2 incubator (37°C , 5% CO_2 , humidified atmosphere). Medium was replaced three times a week. A 100 μl of cell suspension was stained with trypan blue (0.2%) and viable cells were counted using a hemocytometer.

Cell treatments

For treatment with different concentrations of venom, cells were seeded in sterile 24- or 96-well plate with utmost care taken to keep the cell number equal in all the wells. Following overnight incubation, medium was aspirated and medium with different concentrations of venom was added. The seeding density was different in different tests as per the procedure followed.

Determination of cell viability (MTT reduction)

Cells were seeded in 96-well plate at a density of 1×10^4 cells per well and incubated overnight. Medium was removed and cells were exposed to medium containing varying concentrations of venom (10, 25, 50, 100, and 200 $\mu\text{g}/\text{ml}$) for 24 h. 20 μl of MTT stock solution (5 mg/ml) was added to each well. This was followed by incubation for 1 h. When purple-colored precipitates were visible under the

microscope, medium was carefully discarded. For solubilization of formazan crystals (MTT formazon) [19], 200 μ l of DMSO was added to each well and cells were incubated in dark at room temperature for 2 h. The color (purple) developed was measured at 570 nm by a microplate reader (Bio-Rad, USA).

Lactate dehydrogenase (LDH) assay

Cells were seeded in 96-well plate at a density of 2×10^4 cells/well in culture medium. After overnight incubation, medium was replaced and cells were exposed to varying concentrations of venom (10, 20, 50, and 100 μ g/ml). Cells were incubated for 24 h and LDH activity was measured in the cell lysate and supernatants using in vitro toxicology assay kit (Sigma) in accordance with the manufacturer's instructions [20]. The absorbance was determined at 490 nm using plate reader.

Reactive nitrogen species assay

Nitrite production was determined in the supernatants of cultured cells [21]. The cells were seeded in 96-well plate at density of 1×10^4 cells/well. Cells were incubated overnight. Thereafter medium was discarded and cells were exposed to medium containing venom (50, 100 μ g/ml). After 24 h, medium from each well was transferred to fresh tube. After centrifugation at $500 \times g$ for 5 min at 4°C , 100 μ l of the supernatant was transferred to fresh 96-well plate, mixed with an equal volume of Griess reagent (0.04 g/ml PBS, pH 7.4), and incubated at room temperature for 10 min. The absorbance was measured within 30 min at 540 nm by a microplate reader (Bio-Rad, USA). Nitrite concentration in control and treated cells were calculated using sodium nitrite standard reference curve and expressed as $\mu\text{M/ml}$.

Mitochondrial membrane potential (ψ /m) determination

Mitochondrial membrane potential was measured with specific cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) [22]. Briefly, cells were seeded in 24-well plate at a density of 1×10^6 cells/well and incubated overnight. Medium was replaced and cells were exposed to 50 and 100 μ g/ml of venom and incubated for 24 h. The harvested cells were incubated in 0.5 ml JC-1 (10 μM) for 8 min at room temperature in dark. After centrifugation for 5 min at $500 \times g$ and washing twice by PBS (pH 7.4) to remove unincorporated dye, pellets were resuspended in 2 ml PBS (pH 7.4). Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm) were measured using a spectrofluorimeter (spectrometer LS50B,

Perkin Elmer). The mitochondrial membrane potential was estimated as the ratio of green to red fluorescence.

Measurement of caspase-3 activity

Caspase-3 activity was evaluated using a commercially available Caspase-3/ CPP32 Colorimetric assay kit (Biovision) according to the manufacturer's instructions. Briefly, cells were seeded in 24-well plate at a density of 1×10^6 cells/well overnight. Medium was discarded and cells were exposed to different concentrations of venom (50 and 100 μ g/ml) for 24 h. After harvesting, the cells were washed with PBS (pH 7.4) and pelleted by centrifugation at $5000 \times g$ for 5 min at 4°C . The cells were resuspended in 100 μ l chilled cell lysis buffer [10 mM Tris, 1 mM EDTA, and 1% Triton X-100; pH 7.4] and incubated on ice for 20 min. Aliquots were analyzed for protein [18], and lysate volume equivalent to 50 μ g of protein was brought to 100 μ l with lysis buffer in 96-well plate and mixed with reaction buffer (containing 10 mM DTT). All the samples were incubated for 1 h at 37°C after adding 5 μ l of 4 mM DEVD-pNA (200 μM). The absorbance was read at 405 nm in microplate reader (Bio-Rad, USA).

DNA fragmentation analysis

Cells were seeded in 24-well plate at a density of 4×10^6 cells/well and incubated overnight. Thereafter medium was carefully discarded and cells were exposed to 500 μ l medium containing 50 and 100 μ g/ml of venom for 24 h. The cells were harvested and centrifuged at $500 \times g$ for 5 min at 4°C . Pellet was then washed twice with 0.5 ml ice-cold TE buffer (Tris-EDTA) and collected by centrifugation at $500 \times g$ for 5 min at 4°C . Cells were lysed in 50 μ l of chilled DNA lysis buffer (0.5% Triton X-100, 20 mM EDTA and 5 mM Tris-HCl, pH 8.0) for 30 min on ice. Extraction of DNA was carried out by adding 50 μ l of 0.1 mg/ml proteinase K, 150 mM NaCl, and 0.2% (w/v) SDS and incubated at 50°C for 3 h. Nucleic acid was extracted by adding equal volume of solution containing phenol/chloroform/isoamyl alcohol (25:24:1) [23]. After centrifugation at $1000 \times g$ for 5 min at 10°C , supernatant was transferred to a fresh tube. DNA was precipitated by two volumes of cold absolute ethanol and pelleted by centrifugation ($20000 \times g$ for 30 min at 4°C). Supernatant was carefully discarded by rapidly inverting tubes and DNA was washed twice by ice-cold 70% ethanol. It was then air dried for 5–10 min and mixed in 50 μ l TE buffer containing RNase (0.2 mg/ml). After incubation at 55°C for 1 h. DNA was stored at -20°C until use. The extracted DNA was analyzed by loading 10–20 μ g into 1.5% agarose gel containing ethidium bromide (1 μ g/ml). The gel was visualized with ethidium bromide by UV transilluminator

(Nugen Scientific, India with UV Photo MW version 11.01 for windows).

BrdU incorporation (immunocytochemical study)

Cells were seeded in 96-well plate at density 4×10^4 cells/well and incubated overnight. Thereafter cells were exposed to 50 and 100 $\mu\text{g/ml}$ of venom for 22 h and then pulsed with 10 μM BrdU (5-bromo-2'-deoxyuridine) for 2 h [24]. Medium was carefully discarded and the cells were subsequently washed twice with PBS (pH 7.4) and dehydrated using ethanol. Optimal fixation of cells was achieved by 4% paraformaldehyde for 30 min and after washing twice with PBS (pH 7.4) containing 1 N HCl on ice for 10 min and 2 N HCl for 10 min at room temperature. After brief rinsing in PBS (pH 6.0), cells were incubated in methanol containing 0.3% H_2O_2 for 15 min followed by incubation in blocking buffer (1.5% NGS, 0.5% BSA, and 0.1% Triton X-100) for 30 min. Finally cells were incubated with monoclonal anti-BrdU antibody (1:100) in 10% NGS at 4°C overnight. Cells were washed twice with PBS (pH 7.4) and immediately incubated with anti-mouse secondary antibody (1:200) for 1 h at room temperature. Cells were washed twice with PBS (pH 7.4) and peroxide complex was visualized with DAB (3,3'-Diaminobenzidine tetrahydrochloride) under microscope. After dehydration with ethanol (70, 80, 90, and 95%), BrdU-positive (brown) cells were photographed with an inverted microscope (Nikon, Japan).

Statistical analysis

All the data presented are mean \pm SEM. Analysis between the groups was carried out with one-way ANOVA (analysis of variance) with post hoc analysis by Tukey–Kramer multiple comparison method. Any variation with $P < 0.05$ was considered to be significant.

Results

Cell morphology

The cells were monitored for 24 h after exposure with 50 and 100 $\mu\text{g/ml}$ of *O. doriae* venom. The control, round-shaped cells switched to polygonal form after overnight incubation. These cells after exposure by 50 $\mu\text{g/ml}$ of venom were characterized by swelling, inhibition of neurite outgrowth, irregular shape, rupture of membrane, and release of cytosolic contents. In other experiment, the round-shaped cells without overnight incubation were exposed to similar concentration of venom. In this case, cells exhibited aggregation, swelling, absence of neurites,

rupture of membrane, and release of cytosolic contents. The cells expressed similar acute changes by treatment with 100 $\mu\text{g/ml}$ of venom. Both the sets of experiments demonstrated strong dose and effect relationship with respect to morphological changes (Fig. 1).

Cell viability (MTT reduction)

Venom decreased cell viability in a dose-dependent manner (Fig. 2). At a dose of 20 $\mu\text{g/ml}$ and above, cell viability decreased significantly. While treatment with 10 μg did not affect the cell viability (90.75%) but at 20, 50, 100, and 200 $\mu\text{g/ml}$, viability decreased to 75.53% ($P < 0.01$), 55.52% ($P < 0.001$), 37.85% ($P < 0.001$), and 14.30% ($P < 0.001$), respectively.

LDH release

After determining cell viability, LDH release was evaluated 24 h later to confirm cell death. No significant increase in LDH was observed at 10 and 20 $\mu\text{g/ml}$ as compared to control but at higher dose of 50 and 100 $\mu\text{g/ml}$, LDH increased significantly ($P < 0.001$) (Fig. 3). Therefore, these two doses were considered as optimal dose to carry out all other analysis.

Reactive nitrogen intermediates (RNI)

Concentration of nitrite (NO production) in the supernatant increased in a concentration-dependent manner. Treatment with 50 and 100 $\mu\text{g/ml}$ of venom increased nitrite content to a significant ($P < 0.001$) level as compared to the control cells (Fig. 4).

Mitochondrial membrane potential (ψ/m)

Change of mitochondrial membrane potential can affect the cellular health. Several cellular transduction molecules affect the mitochondrial membranes and can initiate apoptosis. We investigated whether scorpion venom can change the membrane potential (ψ/m) as a function of green/red fluorescence intensity of JC-1 and compared it among different treatment groups (Fig. 5). Mitochondrial membrane potential decreased significantly at the two concentrations of venom viz. 50 $\mu\text{g/ml}$ ($P < 0.01$) and 100 $\mu\text{g/ml}$ ($P < 0.001$).

Caspase-3 activity

Caspase-3 is the major executor of cell death. It can lead to cellular death either by DNA breakage or lysis of cytoskeletal proteins. The elevation of caspase-3 following venom treatment was checked by colorimetric method in cell

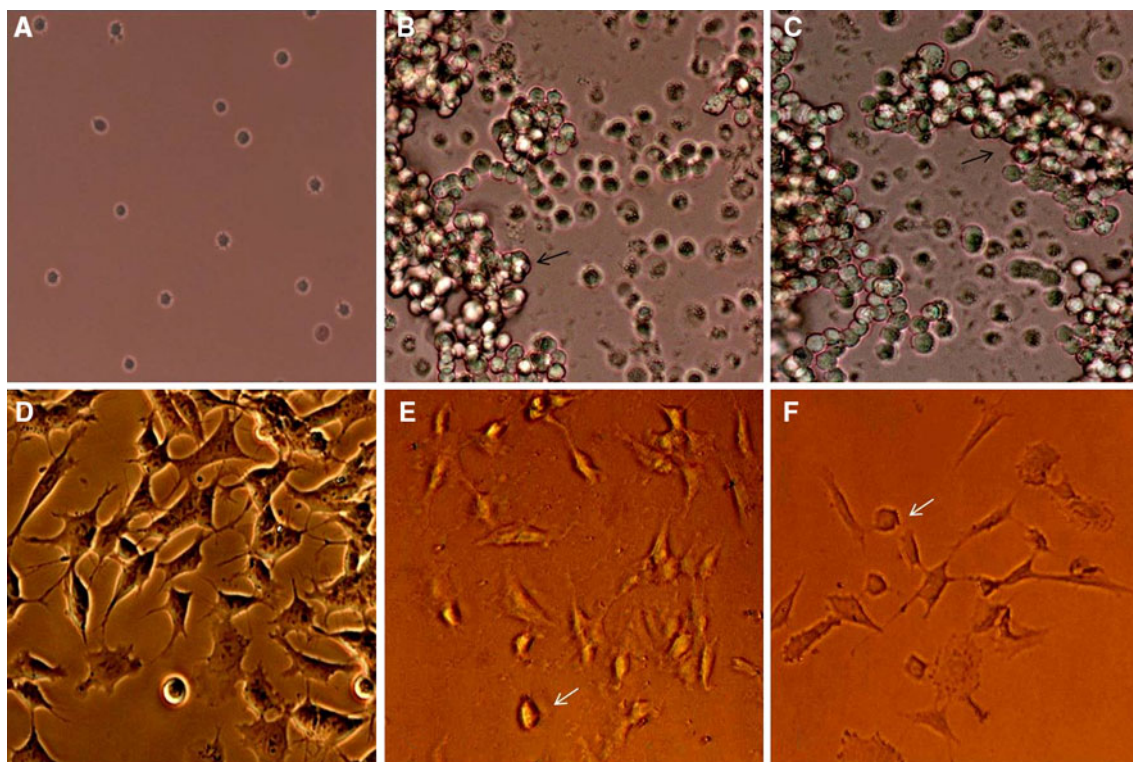


Fig. 1 Microscopic characteristics ($\times 200$) of cultured human neuroblastoma cells 24 h after exposure with 50 and 100 $\mu\text{g/ml}$ of *O. doriae* venom. **a** Round-shaped cells after seeding in 96-wells plate. **b** Incubated round cells with 50 $\mu\text{g/ml}$ of venom show loss of neurite out growth, aggregation (*black arrows*) and swelling. **c** Incubated round cells with 100 $\mu\text{g/ml}$ of venom expressed similar intense changes. **d** The round-shaped cells changed to polygonal form

with granulated contents, homogeneous and distinct edges after overnight incubation. **e** Polygonal cells after incubation with 50 $\mu\text{g/ml}$ of venom inhibited neurite out growth, irregular shape, rupturing of cell membrane (*white arrows*), releasing of cellular contents and swelling (*white arrows*). **f** Incubated polygonal cells exposed to 100 $\mu\text{g/ml}$ of venom exhibited acute similar changes

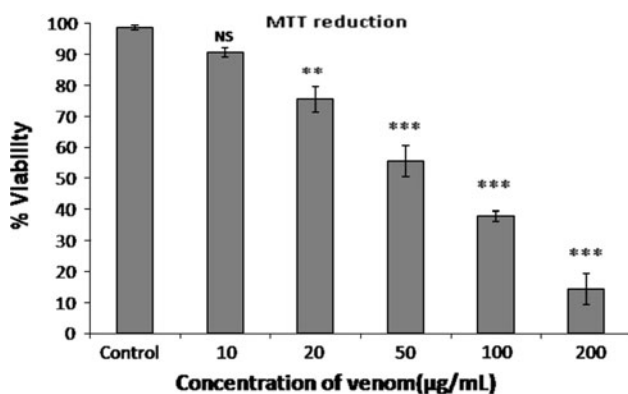


Fig. 2 MTT reduction in SH-SY5Y cells after 24 h exposure with varying concentrations of *O. doriae* venom. Cell viability was expressed as the proportion of absorbance values normalized to the control group after subtracting the blank absorbance from samples and the control. The data are expressed as the mean \pm SEM of three independent experiments. Significances indicated are shown in comparison to control. *NS* non-significant. ** $P < 0.01$. *** $P < 0.001$

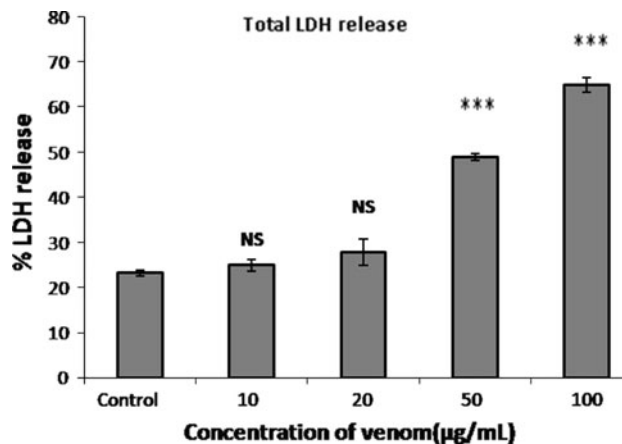


Fig. 3 Lactate dehydrogenase release in SH-SY5Y cells after 24 h exposure with varying concentrations of *O. doriae* venom (10, 20, 50, and 100 $\mu\text{g/ml}$). The % LDH release from the cells was determined by dividing absorbance of the culture supernatants to absorbance of supernatant plus cells lysate. The data were expressed as the mean \pm SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. *NS* non-significant. *** $P < 0.001$

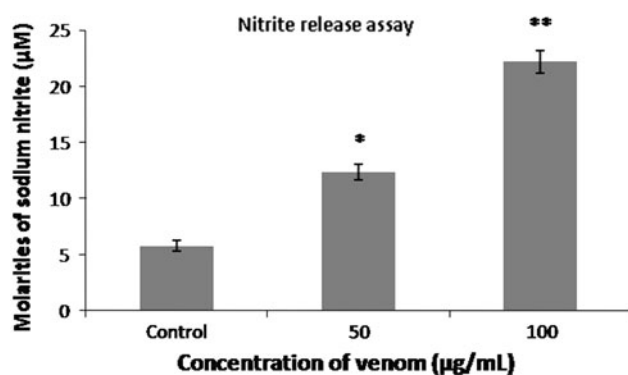


Fig. 4 Nitric oxide production was determined by measuring nitrite in culture medium of control and treated cells with 50 and 100 µg/ml of scorpion venom. Concentration of NO was determined by comparing with the absorbance value of nitrite standard curve and expressed as µM/ml. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. * $P < 0.05$. ** $P < 0.01$

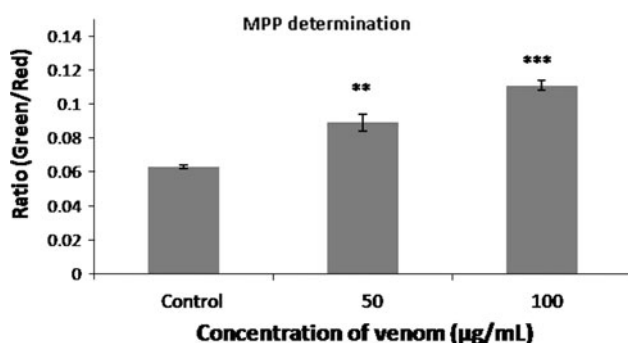


Fig. 5 Mitochondrial membrane potential was measured by using specific cationic dye JC-1 in control and treated cells. Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm) were measured. The mitochondrial membrane potential was estimated as the ratio of green to red fluorescence. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are in comparison to control. ** $P < 0.01$. *** $P < 0.001$

lysates. Caspase-3 activity increased in a dose-dependent manner (Fig. 6). 50 µg/ml of venom was able to elevate the activity to 0.956 µM pNA liberated/hr/ml in comparison to 0.671 µM pNA liberated/hr/ml in the non-treated control ($P < 0.05$) whereas 100 µg/ml elevated the activity to 1.71 µM pNA liberated/hr/ml ($P < 0.001$).

DNA fragmentation analysis

DNA was extracted from the cells after venom treatment and resolved on 1.5% agarose. DNA fragmentation (200–300 bp) was observed at a dose of 50 µg/ml but at 100 µg/ml, venom did not induce DNA breakage (Fig. 7).

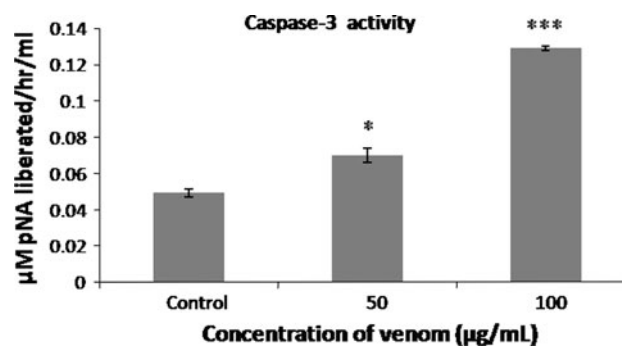


Fig. 6 Caspase-3 activity was determined by measuring DEVD-pNA hydrolysis and was performed in lysates of control and samples (treated with 50 and 100 µg/ml for 24 h). The absorbance of cell lysate and buffer was subtracted to calculate the caspase-3 activity. Data represent mean ± SEM in triplicate. Significances indicated are shown in comparison to control. * $P < 0.05$. *** $P < 0.001$

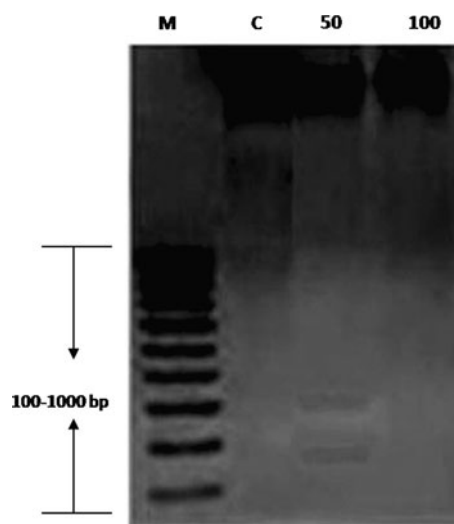


Fig. 7 DNA fragmentation analysis following treatment with the venom of *O. doriae* in human neuroblastoma cell. Note that at 50 µg/ml, venom produced two fragments (200 and 300 bp), but at higher dose level, failed to induce fragmentation. *C* control cells. *M* DNA marker. *50* treated cells with 50 µg/ml of venom. *100* treated cells with 100 µg/ml of venom

BrdU incorporation

Immunocytochemistry was carried out to check whether *O. doriae* venom can affect the synthesis of DNA in cells using a thymine analog BrdU which gets incorporated during DNA synthesis. The scorpion venom decreased the number of nuclei undergoing DNA synthesis. At 100 µg/ml, most of the cells synthesizing DNA were eliminated, while 50 µg/ml of venom inhibited the synthesis remarkably as compared to control (Fig. 8).

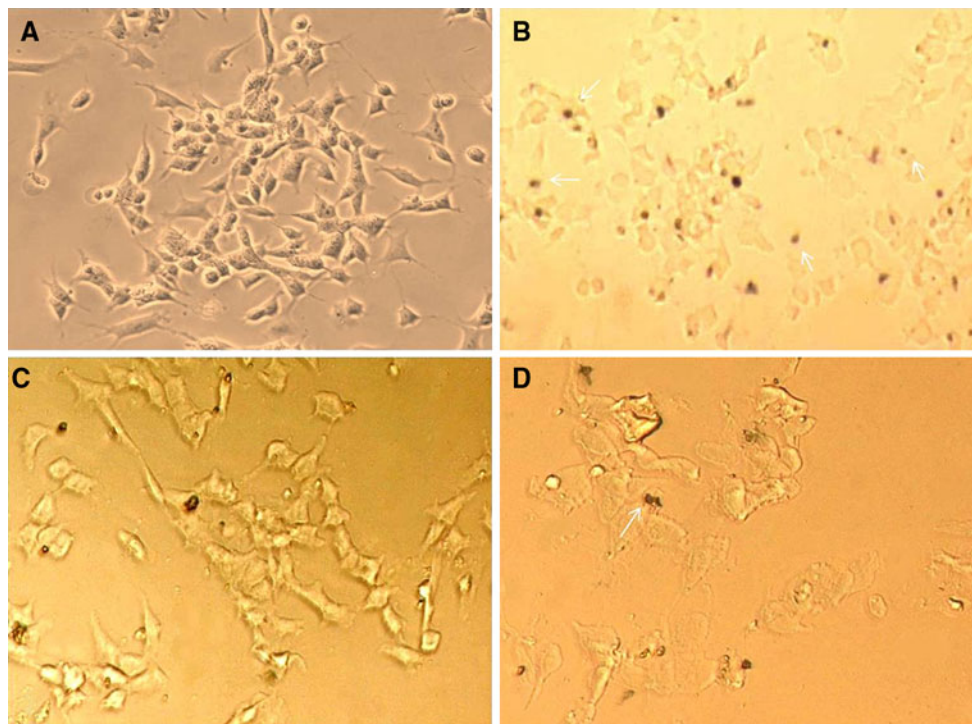


Fig. 8 Effect of scorpion venom on the synthesis of DNA in human neuroblastoma cells evidenced by immunocytochemical detection of thymidine analog BrdU using monoclonal antibody. **a** Cells without the BrdU incorporation. **b** Control cells pulsed with BrdU (*white*

arrow shows BrdU positive cells). **c** Inhibition of the DNA synthesis following venom (50 µg/ml) treatment. **d** Inhibition of DNA synthesis by dose of 100 µg/ml of venom

Discussion

Exposure with venom was characterized by a clear change in cell morphology and the results demonstrated strong correlation between dose and effect (Fig. 1).

Neuroblastoma cells after exposure with venom of *O. doriae* exhibited aggregation, swelling, inhibition of neurite outgrowth, irregular shape, rupture of membrane, and release of cytosolic contents (Fig. 1). Since scorpion venoms are known to increase platelet aggregation [25] and also contain phospholipases [3] and proteolytic enzymes [4], it is possible that they are responsible for inducing of aggregation [26, 27] and swelling [4] in cells after exposure with venom.

The decrease in cell viability was concentration-dependent (Fig. 2). MTT reduction was detected at a dose of 20 µg/ml and above ($P < 0.001$), while 200 µg/ml killed most of the cells. LDH level increased in cultures (Fig. 3) but low doses of venom (10 and 20 µg) could not induce cell death whereas high doses (50, 100 µg) increased LDH significantly ($P < 0.001$). The inverse relation of cell viability and LDH confirmed the cytotoxicity of venom in human neuroblastoma cells. In order to further differentiate between apoptotic and necrotic pathways, several parameters were addressed.

Since, uncontrolled proliferation is the hallmark of cancerous cells, the stoppage of DNA replication and induction of cell death are the two targets of developing molecules with therapeutic potential. Apoptosis is a networked phenomenon with intricate mechanisms. Several biomolecules present in the cells can initiate apoptosis. Nitric oxide (NO) is a molecule with several important functions, but its over expression can affect cellular health through multidimensional modes [28]. NO is reported to affect mitochondrial function by degrading the endomembrane system [29] and alter the structure of several ion channels [30]. Furthermore oxidative stress produced by NO can induce extensive protein oxidation and degradation and significant increase in cell blebbing, rounding up, and over all size [31]. Oxidative stress can also cause extreme results including senescence and even cell death by either apoptosis or necrosis [31]. In this study, we assayed nitrite content in cells following venom exposure. There was a twofold and fourfold increase ($P < 0.001$) in nitrite level when exposed to a dose of 50 and 100 µg/ml, respectively (Fig. 4).

Elevated nitrite can damage the biomembranes including mitochondrial membrane and can lead to the formation of permeability transition pore (PTP) [32] and leakage of several proapoptotic factors such as cytochrome-c in the

cytoplasm [33]. In order to evaluate the mitochondrial involvement, a cationic dye JC-1 with dual fluorescence properties was used. Venom induced depolarization (Fig. 5) at 50 $\mu\text{g/ml}$ ($P < 0.01$) as well as 100 $\mu\text{g/ml}$ significantly ($P < 0.001$). Change in the depolarization between two concentrations was significant too ($P < 0.01$). Loss of MMP and formation of PTP can lead to caspase-3 activation as suggested earlier [34]. Caspase-3 activity increased in the cell lysates following venom exposure in a concentration-dependent manner. 50 $\mu\text{g/ml}$ of dose enhanced the activity significantly ($P < 0.05$) as compared to the control but 100 $\mu\text{g/ml}$ induced a higher increment ($P < 0.001$) (Fig. 6). The increment within the treatments was also significant ($P < 0.01$).

Scorpion venom can induce DNA fragmentation as demonstrated earlier [9]. In this study DNA fragmentation was noticed in 50 $\mu\text{g/ml}$ treated cells with fragments corresponding to 200 and 300 bp, whereas in 100 $\mu\text{g/ml}$ treated cells, no DNA fragmentation could be observed. (Fig. 7). The most possible reason behind this could be the acute necrosis which may elevate caspase-3 sharply [35] but eventually fail to fragment DNA. This was also confirmed by the morphological analysis which revealed severely swollen cells with lesser apoptotic bodies following 100 $\mu\text{g/ml}$ venom treatments (Fig. 1).

Cancer cells divide in an uncontrolled manner which may be ascribed to the mutation of one or more tumor suppressor genes and faster replication of DNA. Recent research has been focused on the agents that can suppress the replication of DNA either by genetic or epigenetic mechanisms. In earlier studies, scorpion venom has been demonstrated to hamper proliferation of prostate cancer cells and human leukemia cells [9, 36]. In order to confirm whether venom (*O. doriae*) can inhibit DNA synthesis in proliferating neuroblastoma cells, BrdU incorporation was checked with immunocytochemistry. The cells without treatment incorporated BrdU with high affinity confirming their cancerous nature. Treatment with venom decreased DNA synthesis in a concentration-dependent manner (Fig. 8). Taken together, the results reported in this study establish the apoptotic and antiproliferative potential of scorpion (*O. doriae*) venom. Further studies based on better molecular methods may be employed to establish the mechanism of signal transduction after venom exposure to cells.

Conclusion

Investigations in this study have highlighted proapoptotic effect of venom at a lower concentration (50 $\mu\text{g/ml}$) and acute necrosis at higher concentration (100 $\mu\text{g/ml}$). This study assumes significance in view of the fact that scorpion

venom stops the synthesis of DNA in cancerous cells. All these results make scorpion (*O. doriae*) venom a favorable candidate in the neuroblastoma research pending further studies to establish the fact.

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Conflict of interest The authors declare that there are no conflicts of interest.

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