Scorpion venom (Odontobuthus doriae) induces apoptosis by depolarization of mitochondria and reduces S-phase population in human breast cancer cells (MCF-7)

Jamil Zargar a, Sadiq Umar a, Mir Sajad a, M. Naime b, Shakir Ali b, Haider A. Khan a,⇑

a Clinical Toxicology Laboratory, Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), New Delhi 110 062, India
b Department of Biochemistry, Jamia Hamdard (Hamdard University), New Delhi 110 062, India

Abstract

Venom of some species of scorpions induces apoptosis and arrests proliferation in cancer cells. This is an important property that can be harnessed and can lead to isolation of compounds of therapeutic importance in cancer research. Cytotoxicity was investigated using MTT reduction and confirmed with lactate dehydrogenase release following venom exposure. Apoptosis was evaluated with determination of mitochondrial membrane potential, reactive nitrogen species assay, measurement of Caspase-3 activity and DNA fragmentation analysis. To confirm that venom can inhibit DNA synthesis in proliferating breast cancer cells, immunocytochemical detection of BrdU incorporation was done. Our results demonstrated that venom of Odontobuthus doriae not only induced apoptosis but lead to the inhibition of DNA synthesis in human breast cancer cells (MCF-7). Cell viability decreased with parallel increment of LDH release in dose dependent manner after treatment with varying concentrations of venom. Moreover, venom depleted cellular antioxidants evidenced by depression of GSH and Catalases and concomitantly increased reactive nitrogen intermediates (RNI). These events were related to the depolarization of mitochondria and associated Caspase-3 activation following venom treatment in a concentration dependent manner. Finally, fragmentation of nuclear DNA following venom treatment confirmed the apoptotic property of the said venom. These results suggest that venom of O. doriae can be potential source for the isolation of effective anti-proliferative and apoptotic molecules.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Recent investigations in vitro as well as in vivo have demonstrated that scorpion venom has ability to induce apoptosis and inhibition of DNA synthesis in variety of cells (Soroceanu et al., 1998; Omran, 2003; Wang and Ji, 2005; Das Gupta et al., 2007) with unclear mechanism. Venom of Heterometrus bengalensis induced apoptosis and antiproliferative effect in human leukemic cell lines (Das Gupta et al., 2007) and Buthus martensiis Karsch (Wang and Ji, 2005) and Leirus quinquestriatus (Omran, 2003) venoms inhibit growth of primary brain and glioma tumors, respectively. Results of these findings strongly indicate the presence of compounds in scorpion’s venom that can bring about a breakthrough in pursuit of proapoptotic components. It is clear that the induction of apoptosis and inhibition of proliferation in tumor cells are widely recognized as common factors in the biological response of cancer and key mechanisms to a variety of therapeutic manoeuvres (Dowsett et al., 1999). Several studies have been attempted to find the anti apoptotic and antiproliferative properties of scorpion’s venoms (Petricevich, 2010).

Previous studies have demonstrated that scorpion venom contains a rich source of polypeptides and enzymes with a variety of biological functions (Caliskan et al., 2009). Different species of scorpions have been reported to have around 70 peptides (Possani et al., 1999). It was reported that biological effects of scorpion venom are mainly due to the presence of low-molecular-weight proteins that exert powerful effects on excitable cells (Jalali et al., 2005) through interaction with different cellular ion channels such as Na+ (Ji et al., 2002), K+ (Ji et al., 2003) and Cl− (De Bin et al., 1993) without mechanistic characterization. Advanced methods of fractionation, chromatography and peptide sequencing have made it possible to characterize the components of scorpion venoms (Petricich, in press).

Odontobuthus doriae (Thorell, 1876) is one of the medically important Buthide scorpions of Middle East (Mirakabadi et al., 2006). Envenomation after scorpion sting can cause various effects ranging from local pain, inflammation and necrosis to muscle paralysis, and it can be deadly for children (Jalali et al., 2007).

Two of the protein toxins including OD1 (Jalali et al., 2005) and OdK1 (Abdel-Mottaleb et al., 2006) have been identified from venom of O. doriae that target voltage-gated Na+ and K+ ion channels, respectively.
In this study we highlight the cytotoxic effects of *O. doriae* venom and a possible mechanism of apoptosis and antiproliferation on the human breast cancer cells (MCF-7).

2. Materials and methods

2.1. Antibodies and reagents

DMEM (Sigma–Aldrich, USA), Fetal bovine serum (Gibco, USA), Trypsin–EDTA (Sigma–Aldrich, USA), Trypan blue (Sigma–Aldrich, USA), PBS (Gibco, USA), Penicillin–streptomycin solution (Sigma–Aldrich, USA), Antibiotic–antimycotic (Invitrogen, USA), Phenol red (Sigma–Aldrich, USA), in vitro Toxicology Assay kit: Lactic dehydrogenase based (Sigma–Aldrich, USA), Griess reagent (Sigma–Aldrich, USA), JC-1 [5,5′,6′-tetrachloro-1,1′,3′,3′-tetracyanobenzimidazolyl-carbocyanine iodide] (Sigma–Aldrich, USA), DMSO [Dimethylsulfoxide] (Sigma–Aldrich, USA), Triton X-100 (Sigma–Aldrich, USA), Agarose (Sigma–Aldrich, USA), Fixative (Sigma–Aldrich, USA), BrDU [5-Bromo-2′-deoxyuridine] (Brdu) antibody (Sigma–Aldrich, USA), DAB [Diaminobenzidine] (Sigma–Aldrich, USA), Primary monoclonal anti bromodeoxyuridine (Brdu) antibody (Sigma–Aldrich, USA), NGS [Normal goat serum] (Gibco, USA), Anti-mouse IgG (Fab specific)-peroxidase antibody produced in goat (Sigma–Aldrich, USA), U Bottom 12-wells plate (Sigma–Aldrich, USA), U Bottom 96-wells plate (Sigma–Aldrich, USA). All the other chemicals and reagents were of analytical grade and purchased locally.

2.2. Scorpion venom

Scorpions were collected from southern regions of Tehran province in Iran. Crude venom was obtained monthly by mild electrical simulation and was solubilized in sterile double distilled water. After centrifugation at 8000 × g for 15 min at 4 °C, supernatant was immediately freeze dried and stored at −20 °C until further use (Borges et al., 2006). Venom was reconstituted in serum free DMEM without phenol red and protein content was estimated by Bradford method (Bradford, 1976). Venom was disinfected with 10% heat inactivated fetal bovine serum, 10 µl/ml penicillin–streptomycin solution or 1% antibiotic–antimycotic and incubated in CO2 incubator (37 °C, 5% CO2 and humidified atmosphere). The medium was replaced three times a week. For enumeration, 100 µl of cells concentration were stained with trypan blue (0.2%) and cells were counted using a haemocytometer.

2.3. Cell culture

MCF-7 cells were procured from national facility for animal tissue and cell culture (NCCS), Pune, India. Cells were grown in 75 ml plastic flasks in DMEM medium supplemented with 10% heat inactivated fetal bovine serum, 10 µl/ml penicillin–streptomycin solution or 1% antibiotic–antimycotic and incubated in CO2 incubator (37 °C, 5% CO2 and humidified atmosphere). The medium was replaced three times a week. For enumeration, 100 µl of cells concentration were stained with trypan blue (0.2%) and cells were counted using a haemocytometer.

2.4. Cell treatments

For treatment with venom, cells were seeded in sterile 12 or 96-well plate with care taken to keep the cell number almost equal in all the wells. Following overnight incubation (37 °C and 5% CO2), medium was aspirated and medium with different concentration of venom was added. The seeding density was different in different tests as indicated therein.

2.5. Determination of cell viability (MTT reduction)

Cytotoxicity of scorpion venom was carried out by MTT reduction assay (Mosmann, 1983). Cells were seeded in 96-well plate at a density of 2 × 10^4 cells per well and incubated overnight. Media was removed and cells exposed to medium containing varying concentrations of venom (10, 25, 50, 100 and 200 µg/ml) for 24 h at 20 °C 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, media was carefully discarded. For solubilization of formazan crystals (MTT formazan), 200 µl of DMSO (dimethylsulfoxide) was added to each well and cells were incubated in dark at room temperature for 2 h. The color (purple) development was measured at 570 nm by a microplate reader (Bio-Rad, USA).

2.6. Lactate dehydrogenase assay (LDH)

Release of lactate dehydrogenase (LDH) was analyzed as a marker for cell membrane integrity to determine cell viability (Decker and Lohmann-Matthes, 1988) after exposure with venom. 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 µg/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. The absorbance was determined at 490 nm using plate reader.

2.7. Reactive nitrogen species assay

Nitrite production was determined in the supernatants of cultured cells (Ding et al., 1988). The cells were seeded in 96-well plate at density of 2 × 10^4 cells/well. Cells were incubated overnight. Thereafter media was discarded and cells exposed to medium containing venom (50, 100 µg/ml). After 24 h media from each well was transferred to fresh tube. After centrifugation at 500 × g for 5 min at 4 °C, 100 µl of the supernatant were transferred to fresh 96-well plate, mixed with an equal volume of Griess reagent (0.04 g/ml in 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 µM/ml.

2.8. Reduced glutathione (GSH)

Total reduced glutathione (GSH) was measured following the method of Sedlak and Linsay (1968). Cells were seeded in 12-well plate at a density of 5 × 10^5 cells/well in culture medium. After overnight incubation, culture medium was replaced with medium containing 50 and 100 µg/ml of venom for 24 h. After harvesting, the cells were washed with PBS (pH 7.4) and pelleted by centrifugation at 5000 × g for 5 min at 4 °C. The cells were freeze fractured by incubation in −20 °C for 30 min and were resuspended in 200 µl chilled cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% Triton X-100 for 30 min at room temperature. Thereafter, cell lysates were sonicated for 10–15 min and centrifuged at 2000 × g for 10 min and supernatant was collected. After estimation of protein by Bradford assay (Bradford, 1976), an aliquot of supernatant was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4 °C for 2 h. The contents were centrifuged at 500 × g for 15 min and supernatant was collected. 20 µl sample was mixed with 75 µl lysis buffer, 55 µl Tris buffer (pH 8.5) containing 0.02 M EDTA and 25 µl DTNB [5,5′-dithio bis (2-N-azonoic acid)]. Absorbance of the chromogen (yellow) was read at 412 nm by a microplate reader (Sajad et al., 2010). The result was expressed as µg GSH/mg protein using molar extinction coefficient of 13600.
2.9. Catalase activity

Catalase activity in the cell lysates was assayed as per Sinha (1972). Cells were seeded in 12-well plate at a density of 5 x 10^4 cells/well in culture medium. After overnight incubation, medium was replaced with another medium containing 50 and 100 μg/ml of venom for 24 h. After harvesting, the cells were pelleted by centrifugation at 5000 x g for 5 min at 4 °C. Thereafter, cells were freeze fractioned by incubation in -20 °C for 30 min following incubation with 200 μl lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% Triton X-100] for 30 min at room temperature. Lysates were sonicated for 10–15 min, centrifuged at 2000 x g for 10 min and supernatant was collected. After estimation of protein by Bradford assay (Bradford, 1976), in an eppendorf tube 5 μl of sample/well was mixed with 50 μl of lysis buffer, 20 μl of distilled water and 25 μl of hydrogen peroxide (15%). After shaking, the samples were incubated for 2 min at 37 °C and mixed with 100 μl of dichromate acid reagent (0.1 M potassium dichromate in glacial acetic acid) following heating in a water bath at 100 °C for 10–15 min until the color of the sample was changed to greenish/faint greenish. 200 μl of sample was transferred into flat bottomed 96-well plates and absorbance was read at 570 nm in a plate reader (Sajad et al., 2010). The results were converted into activity using molar extinction of Catalases as 43.6. and express as micro moles of hydrogen peroxide consumed/min/mg protein.

2.10. Mitochondrial membrane potential (ψm) determination

Mitochondrial membrane potential was measured with specific cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Smiley et al., 1991). Briefly, cells were seeded in 12-well plate at a density of 1 x 10^5 cells/well and incubated overnight. Medium was replaced and cells were exposed to 50 and 100 μg/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 x 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.

2.11. 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 12-well plate at a density of 1 x 10^4 cells/well overnight. Medium was discarded and cells were exposed to different concentrations of venom (50 and 100 μg/ml) for 24 h. After harvesting, the cells were washed with PBS (pH 7.4) and pelleted by centrifugation at 5000 x g for 5 min at 4 °C. The cells were resuspended in 100 μl 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 (Bradford, 1976), and lystate volume equivalent to 50 μg of protein was brought to 100 μl 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 μl of 4 mM DEVD-pNA (200 μM). The absorbance was read at 405 nm in microplate reader (Bio-Rad, USA).

2.12. DNA fragmentation analysis

Cells were seeded at a density of 5 x 10^5 cells/well in 12-well plate and incubated overnight. Thereafter media was carefully aspirated and cells were exposed to 500 μl medium containing 50 and 100 μg/ml of venom and incubated for 24 h. The cells were harvested and centrifuged at 500 x g for 5 min at 4 °C. Pellet was then washed twice with 0.5 ml TE buffer (Tris–EDTA). Cells were lysed in 50 μl of chilled DNA lysis buffer (0.5% Triton X-100, 25 mM EDTA and 25 mM Tris–HCl, pH 7.4) for 30 min on ice. Extraction of DNA was carried out by adding 50 μl 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) (Kweon et al., 2004). After centrifugation at 1000 x 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 x g for 30 min at 4 °C). Supernatant was carefully discarded by rapidly inverting tubes and DNA was washed twice with ice-cold 70% ethanol. It was then air dried for 5–10 min and mixed in 50 μl 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 μg into 1.5% agarose gel containing ethidium bromide (1 μg/ml). The gel was visualized with ethidium bromide by UV transilluminator (Nugen Scientific, India with UV Photo MW version 11.01 for windows).

2.13. Immunocytochemical study (BrdU incorporation)

Cells were seeded in 96-well plate at density 2 x 10^4 cells/well and incubated overnight. Cells were exposed to 50 and 100 μg/ml of venom and incubated for 22 h and pulsed with 10 μM (Dover and Patel, 1994) 5-bromo-2’-deoxyuridine for 2 h. 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 followed by 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% H2O2 for 15 min and 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 1:200 anti-mouse secondary antibody for 1 h with constant shaking at room temperature. Cells were washed two times with PBS (pH 7.4) and peroxide complex was visualized with DAB (3,3’-diaminobenzidine tetrahydrochloride). Cells were briefly counterstained using Basic Fuschin (0.1%) in ethanol. After dehydration in ethanol (70%, 80%, 90% and 95%), brown positive stained cells were photographed with an inverted microscope (Nikon, Japan). Analysis of BrdU-immunoreactive cells, a person blind to the experimental protocol was employed for counting. Cells were counted using Image J software with cell counting jar (National Institutes of Health, USA). The numbers of BrdU-labeled cells were averaged from seven different photomicrographs in three replicate wells. For data analysis, numbers were expressed as percentage of BrdU (+) cells per photograph.

2.14. Statistical analysis

All the data presented are mean ± 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.
3. Results

3.1. Analysis of cell morphology

Changes in the cell morphology were monitored for 24 h after exposure with 50 and 100 μg/ml of venom. The cells changed from round shape to polygonal with slightly granulated contents and distinct edges after overnight incubation at 37 °C and 5% CO₂.

Under similar conditions, exposure with 50 μg/ml of venom exhibited increasing cellular contents, shrinkage, swelling, rupture of membrane (black arrows) and release of cytosolic contents after 24 h. Venom (100 μg/ml) of presented similar changes but with marked acute necrosis (Fig. 1). In other experiments, the round shaped cells without overnight incubation were exposed to similar concentration of venom. In this case, cells did not switch to polygonal form after overnight incubation. These cells, 24 h after exposure with 50 μg/ml of venom, exhibited aggregation, swelling, rupture of membrane and release of cytosolic contents. A 100 μg/ml dose resulted in loss of cells with acute necrosis with increased cellular adhesiveness (Fig. 1).

3.2. Cell viability (MTT assay)

Venom decreased cell viability in a dose dependant manner (Fig. 2). At a dose of 10, 20, 50, 100 and 200 μg/ml of venom; cell viability decreased to 86.8 ± 2.38, 80.6 ± 0.37, 67.7 ± 3.3, 62.2 ± 0.76 and 39.9 ± 1.2%, respectively. Venom (10 μg/ml) affected cell viability significantly (p < 0.01), but a dose of 20 μg or more, decreased the cell viability to a greater significant level (p < 0.001).

3.3. Effect on lactate dehydrogenase (LDH)

Lactate dehydrogenase release was evaluated to confirm results of MTT assay. Release of LDH increased in dose dependent manner (Fig. 3). At a dose of 10, 20, 50 and 100 μg/ml of venom; release of LDH increased to 19.73 ± 0.66 (p < 0.05), 21.51 ± 0.74 (p < 0.001), 29.89 ± 0.51 (p < 0.001) and 35.08 ± 0.98% (p < 0.001), respectively. No significant increase in LDH was observed at 20 μg/ml as compared to 10 μg/ml but at two higher doses (50 and 100 μg/ml), LDH increased significantly (p < 0.01). Therefore these two doses were considered as optimal dose to carry out all other analysis.

3.4. Reactive nitrogen intermediates (RNI)

NO production increased in the cells after exposure with venom in a concentration dependant manner. The selected optimal doses of venom increased concentration of nitrite (NO production) in the supernatant significantly as compared to control (Fig. 4). Dose 100 μg/ml of venom increased the nitrite content from 6.34 ± 0.14 μM/ml in controls to 9.38 ± 0.46 μM/ml (p < 0.001)
where as at 50 μg/ml, 8.32 ± 0.07 μM/ml (p < 0.01) concentration was observed.

3.5. Reduced glutathione (GSH)

Treatment of cells with the two selected doses of venom resulted in decrease in glutathione level in a dose dependent manner (Fig. 5). GSH in control cells was 0.245 ± 0.01 μgm GSH/mg protein whereas in treated cell with 50 and 100 μg/ml, decrement was 0.035 ± 0.005 (p < 0.001) and 0.031 ± 0.007 (p < 0.001) μgm GSH/mg protein, respectively (Fig. 5). Depletion in glutathione level was not significant between the two doses as compared to each other.

3.6. Catalase activity

The two concentration of venom viz. 50 μg/ml and 100 μg/ml significantly decreased the catalase activity as compared to control. At 50 and 100 μg/ml of venom, the activity reduced to 2.75 ± 0.22 (p < 0.001) and 2.44 ± 0.40 (p < 0.001), respectively, in comparison to 16.82 ± 0.52 μmol of hydrogen peroxide consumed/min/mg protein in control (Fig. 6). Again the decrement in catalase activity between the two doses was not significant.

3.7. Change in mitochondrial membrane potential (ψ/m)

The ratio of green/red fluorescence intensity of JC-1 in control cells was 0.17 ± 0.006 and in treated cell with 50 and 100 μg/ml, increment was 0.21 ± 0.03 and 0.62 ± 0.014, respectively (Fig. 7). Therefore mitochondrial membrane potential as a function of green/red fluorescence decreased significantly at the two concentrations of venom viz. 50 μg/ml (p < 0.05) and 100 μg/ml (p < 0.001).

3.8. Caspase-3 activity

The elevation of Caspase-3 following venom treatment is indicative of cellular apoptosis either by breakage of DNA or lysis of cytoskeletal protein. Upregulation of Caspase-3 expression was checked by colorimetric method dependent on release of pNA (p-nitroanilide) in cell lysates of MCF-7 cells. Caspase-3 activity increased in a dose dependent manner (Fig. 8). At 50 and 100 μg/ml of venom, the activity increased to 0.033 ± 0.001 (p < 0.05) and 0.0587 ± 0.005 (p < 0.001) μL pNA liberated/h/ml, respectively, in comparison to 0.021 ± 0.001 μL pNA liberated/h/ml in control. Also the increment in caspase activity was in close agreement with change in mitochondrial potential.

3.9. DNA fragmentation analysis

DNA was extracted from the cells after venom treatment and resolved on 1.5% agarose. Both the concentrations viz. 50 μg/ml...
was performed in lysates of control and samples (treated with 50 and 100 μg/ml for 24 h). The absorbance of cell lysates and buffers were subtracted to calculate the caspase-3 activity. Comparison of the absorbance of pNA of a treated sample with an untreated control allows determination of the fold increase in Caspase-3 activity. Data represent mean ± SEM in triplicate. Significances indicated are in comparison to control. *p < 0.05, ***p < 0.001.

Fig. 7. Mitochondrial membrane potential was estimated by using JC-1 (10 μM) probe in control and sample cells (treated with 50 and 100 μg/ml) for 24 h. Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm) were measured. The ratio of green/red fluorescence as a measure of mitochondrial membrane potential was calculated. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are in comparison to control.

3.10. 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 (Fig. 10). At 50 μg/ml, numbers of cells incorporating BrdU was reduced to 12.51 ± 0.96% (P < 0.01), while 100 μg/ml of venom inhibited incorporation to 5.39 ± 1.05% (P < 0.001) as compared to control (24.3 ± 1.6).

Fig. 8. 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 lysates and buffers were subtracted to calculate the caspase-3 activity. Comparison of the absorbance of pNA of a treated sample with an untreated control allows determination of the fold increase in Caspase-3 activity. Data represent mean ± SEM in triplicate. Significances indicated are shown in comparison to control. *p < 0.05, ***p < 0.001.

Fig. 9. Genomic DNA fragmentation analysis following, treatment of scorpion venom (O. doriae) in MCF-cells. DNA was extracted, and resolved on a 1.5% agarose gel and visualized with ethidium bromide. C, control cells. M, DNA Marker. 50, treated cells with 50 μg/ml of venom. 100, treated cells with 100 μg/ml of venom.

and 100 μg/ml induced fragmentation of DNA in 200–400 bp regions as compared to the control cells (Fig. 9).

3.10. 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 (Fig. 10). At 50 μg/ml, numbers of cells incorporating BrdU was reduced to 12.51 ± 0.96% (P < 0.01), while 100 μg/ml of venom inhibited incorporation to 5.39 ± 1.05% (P < 0.001) as compared to control (24.3 ± 1.6).

4. Discussion

Scorpion venom can induce apoptosis in different types of cells including cancer cells (Gomes et al., 2010). This is an important property which can lead to isolation of compounds of therapeutic importance in cancer research. In this study, we analyzed apoptosis and antiproliferative effects of scorpion venom (O. doriae) to elucidate this probable mechanism of action in breast cancer cells. Scorpion venoms contain enzymes, such as acetylcholinesterase, phospholipase A2, alkaline phosphatase and proteolytic enzymes with gelatinolytic activity (Incesu et al., 2005). Cells after venom treatment exhibited increasing necrosis in a dose dependent manner. It suggests that proteolytic enzymes are responsible for necrotic activity (Incesu et al., 2005).

Morphological findings suggested reduction in cell viability. Our investigation showed reduced MTT and increased LDH level in the treated cells. These parameters are used as markers for cell membrane integrity and index for cytotoxicity (Decker and Lohmann-Matthes, 1988). These results lend support to the finding where cell viability of MCF-7 decreased after treatment with scorpion venom in a dose dependent manner.

Since venom is a complex mixture of several organic and inorganic compounds, we investigated venom induced oxidative stress on the cells. Venom was found to reduce the cellular antioxidant level which may be due to induction of reactive oxygen species.

Nitric oxide has been previously demonstrated to affect mitochondrial functioning by degrading the endomembrane system (Finocchietto et al., 2009) and altering the structure of several ion channels (Leonelli et al., 2009). More over, nitrite is a potent reactive intermediate and in presence of superoxide, can form peroxynitrite (Liaudet et al., 2009). All these probable mechanisms can lead to deleterious consequences on the cellular health. The primary product of nitric oxide metabolism, nitrite was assayed in MCF-7 cells following venom treatment. Scorpion venom of O. doriae elevated NO production which was clearly observed after 24 h of treatment. Earlier studies demonstrated that induction of inducible nitric oxide synthase is an essential part of tumor necrosis factor-α-induced apoptosis in MCF-7 (Binder et al., 1999; Mooney et al., 2002). Moreover other studies have also suggested role of nitric oxide in induction of apoptosis in mammalian cells like human neuroblastoma cells (Zargan et al., 2011) and rat hippocampal neuronal cells (Sajad et al., 2009). Elevated nitrite can damage the biomembranes including mitochondrial membranes and can aid in the formation of permeability transition pore (Vieira and Kroemer, 2003) and leakage of pro-apoptotic fac-

tors including cytochrome-c in the cytoplasm (Caroppi et al., 2009).

In order to evaluate the mitochondrial involvement in the venom induced rise in nitrite level, a cationic dye JC-1 with dual fluorescence properties was used. Scorpion venom was found to induce depolarization in mitochondria in breast cancer cells.

Caspase-3 is a key protease required for the execution of apoptosis (Wimmer et al., 2004). We investigated its activity to find out whether scorpion venom can lead to apoptosis by elevation of Caspase-3 activity. It was observed that Caspase-3 activities increased in the cell lysates of MCF-7 cells following treatment with venom of *O. doriae*. These results were in close agreement with enhanced depolarization of mitochondrial membrane and production of nitric oxide in MCF-7. Recent investigations in spider and *O. doriae* venom have demonstrated their ability to induce apoptosis in HeLa and SH-SY5Y cells, respectively, resulting from an increase in Caspase-3 activity (Li et al., 2005; Zargan et al., 2011).

Fig. 10. Effect of scorpion venom on the synthesis of DNA in MCF-7 cells visualized by immunocytochemical analysis of thymidine analog BrdU (pulsed for 1 h) using monoclonal antibody. (A) Fixed cells without the BrdU incorporation. (B) Normal cells pulsed with BrdU (arrow). (C) inhibition of the DNA synthesis following treatment with 50 μg/ml of venom (arrow). (D) 100 μg/ml of venom shows inhibition of DNA synthesis to a greater extent as compared to the lower dose. (E) Frequency of BrdU positive cells. 50, treated cells with 50 μg/ml of venom. 100, treated cells with 100 μg/ml of venom. Data represent mean ± SEM and significances indicated are shown in comparison to control. ***(p < 0.01), ***(p < 0.001).
Fig. 11. Venom of O. doriae induces reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) in MCF-7 cells. Nitric oxide depolarizes mitochondria. Furthermore nitric oxide can lead to formation of permeability transition pore and leakage of pro-apoptotic factors leading the elevation of caspases in the cytoplasm. Together ROS and RNI can lead to the fragmentation of nuclear DNA and trigger apoptosis thereof.

These studies highlight the apotogenic and growth arresting properties of venom of O. doriae and probable mechanism of cell death in MCF-7 (Fig. 11). The most practical importance of this study lies in the investigation of anti-proliferative and apoptosis-inducing properties of O. doriae venom fraction constituents for developing new drugs for cancer and other incurable diseases.

5. Conflict of interest statement

None declared.

Acknowledgments

Authors acknowledge and thank NCCS, Pune for timely shipment of the cell lines. Biochemistry Department of Hamdard University also acknowledges the support of DST to establish infrastructure for animal tissue culture facility under FIST program.

References


