



Cytotoxic Effect of Snake (*Echis Carinatus*) Venom on Human Embryonic Kidney Cells (HEK 293)

Mahboobeh Balali Bahadorani¹ and Abbas Zare Mirakabadi^{2*}

¹Department of Biology, Karaj Branch, Islamic Azad University, Iran

²Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Iran

*Corresponding author: Abbas Zare Mirakabadi, Professor, Biochemistry, Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Karaj, Iran, Tel: 02634552006, E-mail: zareabbas83@gmail.com

Abstract

Background: The venom of snake (*Echis carinatus*) induces hemorrhage and necrosis locally at the bite site as well as acute renal failure (ARF) as a consequence of morphological and functional alterations in glomerular and tubular cells.

Objectives: It is not clear that ARF results from a direct cytotoxic effect on renal epithelia or from a renal ischemia due to systemic hemodynamic disturbances. This work investigated the *in vitro* effect of *Echis Carinatus* crude venom, using cultured Human embryonic kidney (HEK 293) mono layers as a model to see the cytotoxic effect of *Echis carinatus* venom.

Materials and methods: The effect of *Echis Carinatus* snake venom on HEK 293 cells viability was determined by MTT assay and neutral red uptake assay. The integrity of cell membrane through LDH release was measured with the LDH Kit. Morphological changes of endothelial cells were also evaluated using a phase contrast microscope.

Results: In MTT assay, crude venom induced dose dependent cytotoxic effects on HEK 293 cells which were confirmed by neutral red assay. Crude venom caused changes in the integrity of cell membrane determined by rise in LDH release too.

Conclusions: Based on the results obtained in the present study it may be concluded that the damage induced by *E. carinatus* venom on Kidney is probably related to the direct effect as well as indirect effect including hypotension, hemolysis, hemoglobinuria, rhabdomyolysis, and myoglobinuria of this venom on kidney which may lead to Acute renal failure (ARF).

Keywords

Snake venom, Cytotoxicity effect, HEK 293, Acute renal failure, *Echis carinatus*

Introduction

The morbidity and mortality associated with snake bite are serious public health problems in many regions of the world [1]. It is estimated that the true incidence of snake envenomation could exceed 5 million per year [2,3]. Viper bites are more common than other poisonous snake bites in human beings. *Echis carinatus* snakes due to presence of specific enzymes like metalloproteases

(SVMPs), hyaluronidases and phospholipases A2 (PLA2s), which often complement each other's functions, make progressive tissue necrosis and permanent physical deformities [4,5]. It seems that metalloproteases can cause lysis of structural proteins including basal lamina [6]. It is well established fact that some snake venoms including *E. carinaus* can cause local tissue damage which brings about pain and edema leading to performance tissue loss. On the other hand the venom can cause systemic effects including, anemia, hypotension hemorrhage as well as acute renal failure [7,8]. Acute renal failure (ARF) is mainly observed following bites by the snakes which belong to the viperidae group [9,10]. The ARF which happen after snake bite is usually reversible, but if acute cortical necrosis occurs, it may lead to an incomplete recovery [11]. Acute Kidney Injury is diagnosed by biochemical monitoring which presents a late indication of a functional change in glomerular function rate [12]. It is thought that *Echis carinatus* snake venom can induces ARF as a consequence of morphological and functional alterations in glomerular and tubular cells [13]. A few reports indicate the cytotoxicity of *Echis* venom [14,15]. Hoda Khalid (2015) recently investigated the cytotoxic effect of crude venom of *Echis ocellatus* [14]. Moreover the Rebecca D Pierce (2011) used polyethyleneimine (PEI) to enhance cellular adherence, and to determine whether the substrate attachment influenced the survival of cells treated with crude *E. carinatus* venom. It is still unclear whether the ARF results from a direct cytotoxic effect on renal epithelia or from a renal ischemia due to systemic hemodynamic disturbances [15]. In order to elucidate a putative direct cytotoxic action of *Echis carinatus* venom we used an *in vitro* system, using cultured Human embryonic kidney (HEK 293) cells monolayers as a model.

Materials and Methods

Venom preparation

Ten milligrams of lyophilized *Echis carinatus* crude venom was obtained from the Venomous Animals and Antivenom Production Department of Razi Vaccine and Serum Research Institute, Iran. The venom was dissolved in 10ml of DMEM culture media and stored at -20°C until use.

Reagents

Dulbecco's Modified Eagle's medium, high glucose (DMEM),

Fetal Bovine Serum (FBS), Penicillin-Streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland), MTT(3-(4,5-dimethylthiazolyl)-2), 5-diphenyltetrazolium bromide), neutral red dye (NR), DMSO (dimethyl sulfoxide) were obtained from Merck (Germany), LDH (Lactate Dehydrogenase) assay Kit were purchased from Pars azmoon, Iran, Human embryonic kidney (HEK 293) cells obtained from the Venomous Animals and Antivenom Production Department of Razi Vaccine and Serum Research Institute, Iran.

Cell culture

Normal human embryonic kidney cells (HEK 293), grown in plastic flasks at 37°C in humidified atmosphere of 5% CO₂/air with DMEM supplemented with 10% Fetal Bovine Serum FBS and 1% penicillin (10,000 IU/ml)/streptomycin (50 mg/ml).

Determination of cell viability (MTT assay)

HEK 293 cells were cultured in DMEM medium in the presence of FBS 10% plus penicillin-streptomycin 1%, and incubated in presence of CO₂ 5% at 37°C. The cytotoxicity of *Echis Carinatus* crude venom was evaluated using MTT assay. HEK 293 cells were seeded in a 96 well plate at 3-4 × 10⁴ cells/ well and incubated for 24 hr to adhere. After discarding the old medium, the cells were exposed in the medium containing various concentrations 1, 5, 10, 20, 40, 80 µg/mL of crude venom. After 3 and 24 hr exposure, 20 µL MTT (5 mg/mL) was added to each well and cells were incubated for another 4 hr. Finally, the culture medium containing MTT solution was removed and the Formazan crystals were dissolved in 150 µL of dimethyl sulfoxide solvent (DMSO). Absorbance was read at 540 nm with a microplate reader (Labsystem Multiskan MS 4.0, Finland). IC₅₀ was calculated using the Sigma Plot 12.0 software.

Neutral red uptake assay

For the neutral red (NR) cytotoxicity assay, the HEK 293 cells were seeded in 96-well plates at a cell density of 3-4 × 10⁴ cells/ well. Following venom exposure (as mentioned above in MTT assay), the media were removed and the culture was washed once with phosphate buffered saline (PBS), pH 7.4. To each well 100 µl of media containing NR (40 µg/mL) was added and the plate was incubated for 3 hr at 37°C. The media containing the dye were removed and each well was washed once for 2-3 min with formol-calcium (40% formaldehyde, 10% anhydrous calcium chloride, w/w) to remove non incorporated dye. Finally, 200 µl of an acetic acid-ethanol (1 ml glacial acetic acid in 100 ml 50% ethanol) was added to each well for 15 min at room temperature and then the plate was read at 540 nm in a microplate reader. The cell viability was determined by comparing the absorbance values of all the wells with the absorbance mean value obtained from the control wells (without venom), which were taken as 100% cell viability.

Lactate dehydrogenase (LDH) release assay

In order to quantify the cell death, lactate dehydrogenase (LDH) released from damaged cells into the cell culture media was measured 3 and 24 hr after treatment by the *E. carinatus* crude venom with various concentrations. Cells were seeded in 96-well plate at a density of 3 × 10⁴ cells/ well in culture medium. After overnight incubation, the medium was replaced with serum free medium containing various concentrations of *E. carinatus* crude venom and incubated for 3 and 24 hr. A colorimetric assay was applied according to (Pars azmoon, Iran) kit, and then the content of LDH released from the cells to the culture medium was calculated according to recipe kit.

Morphological studies

Following overnight incubation of the cells with venom, various morphological alterations and cell damage were qualitatively investigated using a invert microscope and their photos were taken with a digital camera.

Statistical analysis

Experiments were performed in triplicate with four replicates for each exposure concentration and results are expressed as mean ± SD.

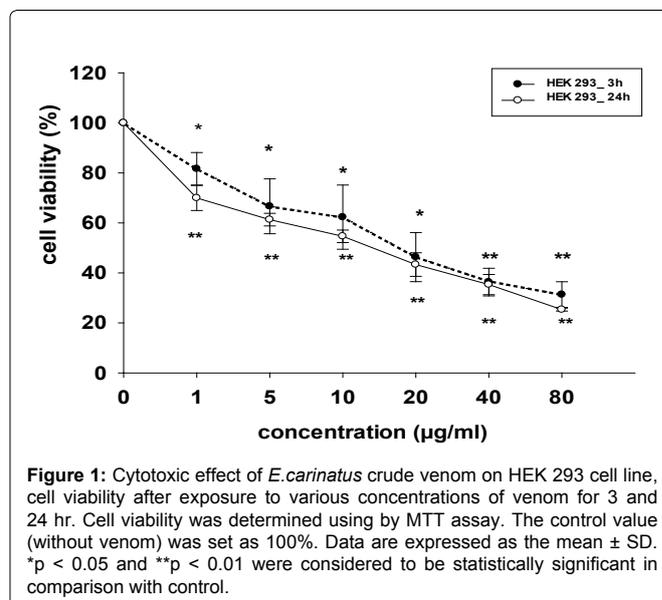


Figure 1: Cytotoxic effect of *E. carinatus* crude venom on HEK 293 cell line, cell viability after exposure to various concentrations of venom for 3 and 24 hr. Cell viability was determined using by MTT assay. The control value (without venom) was set as 100%. Data are expressed as the mean ± SD. *p < 0.05 and **p < 0.01 were considered to be statistically significant in comparison with control.

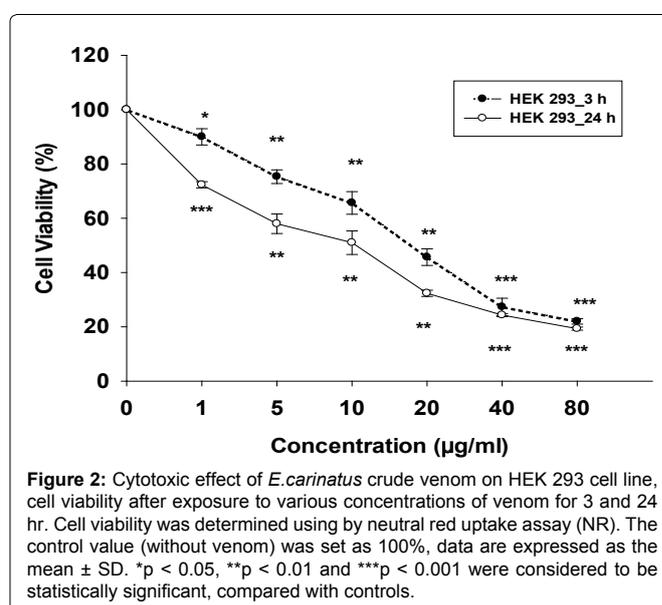


Figure 2: Cytotoxic effect of *E. carinatus* crude venom on HEK 293 cell line, cell viability after exposure to various concentrations of venom for 3 and 24 hr. Cell viability was determined using by neutral red uptake assay (NR). The control value (without venom) was set as 100%, data are expressed as the mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001 were considered to be statistically significant, compared with controls.

Data were analyzed by Student t-test and an analysis IC₅₀ by fitting the data to log (inhibitor) vs. response equation. Significance level of p < 0.05 was used for statistical testing. All statistical analyses were performed using Sigma Plot 12 software.

Results

Determination of cell viability (MTT assay)

The inhibitory effects of crude venom of *E. carinatus* on growth inhibition of HEK 293 cells were tested at various concentrations (1 to 80 µg/ml) for 3 and 24 hr using colorimetric MTT assay. Data analysis showed (Figure 1) that the growth inhibition of HEK 293 cells exposed to the venom were increased significantly (p < 0.01) as compared to venom unexposed cells in concentration-dependent manner. The maximum cell inhibition was 69% after 3 hr and 75% after 24 hr in 80 µg/ml concentration exposure respectively and the least cell inhibition was 19% after 3 hr and 30% after 24 hr in 1 µg/ml concentration respectively. The IC₅₀ (half maximal inhibitory concentration) value of *E. carinatus* snake venom on HEK 293 cell was 18.54 ± 8.96 µg/mL and 14.06 ± 3.17 µg/mL after 3 and 24 hours exposure respectively.

Neutral red uptake assay

Cell viability and effects of cytotoxicity on lysosomal integrity was determined with the neutral red (NR) assay. As seen in figure 2. Following venom exposure cells lysosomal neutral red uptake

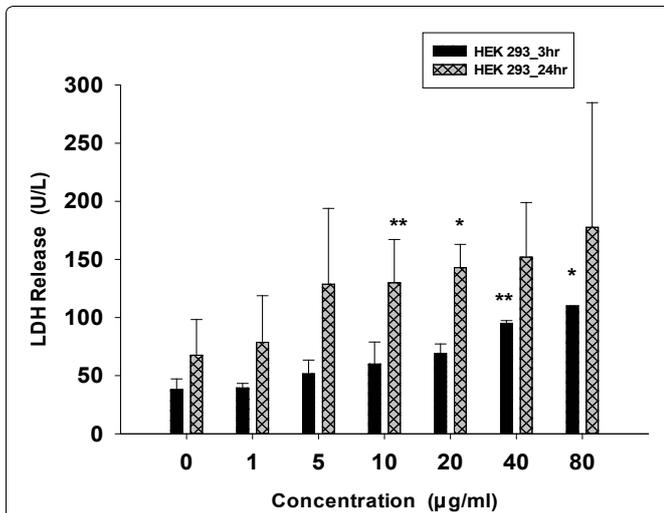


Figure 3: Effect of *E.carinatus* crude venom on HEK 293 cells, growth inhibition determined by LDH release assay. Values indicate mean \pm SD of LDH activity (U/L) (compare to control) for 3 and 24 hr. * $p < 0.05$ and ** $p < 0.01$ were considered to be statistically significant, compared with values from cells incubated in the absence of *E.carinatus* crude venom (controls).

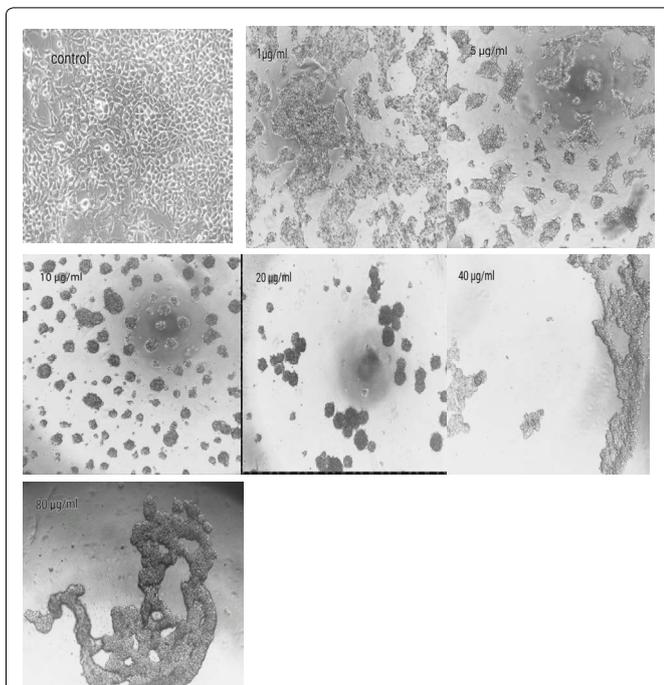


Figure 4: Effect of *E.Carinatus* crude venom on morphology of HEK 293 cells. Cells were seeded in DMEM medium with 10% FBS and treated in the absence (control group) or crude venom concentrations for 3 and 24 hr at 37°C. The Morphological changes of treated cells that showed after 3 hours exposure were similar to 24 hours exposure. Inverted microscope was used to compare with control cells in this study.

reduced with an IC₅₀ value of $16.66 \pm 1.26 \mu\text{g/mL}$ and $8.43 \pm 0.54 \mu\text{g/mL}$ after 3 and 24 hours incubation respectively.

Data analysis showed that the cell inhibition of HEK 293 cells exposed to the venom was significantly ($p < 0.001$) inhibited as compared to control cells in concentration dependent manner. The maximum cell inhibition was 78% after 3 hr and 81% after 24 hr in 80 $\mu\text{g/ml}$ concentration exposure with *E. carinatus* snake venom and the least cell inhibition was 10% after 3 hr and 28% after 24 hr in 1 $\mu\text{g/ml}$ concentration exposure.

Lactate dehydrogenase (LDH) release assay

Figure 3 shows the values of LDH released from the HEK 293 cell line after 3 and 24 hours of incubation with *E. carinatus* venom at concentrations ranging from 1 to 80 $\mu\text{g/ml}$. The effect of venom on LDH release was concentration dependent. Treatment of HEK

293 cells with *E. carinatus* snake venom at concentrations 1, 5, 10 and 20 $\mu\text{g/ml}$ for 3 hrs caused LDH release by about 1.5 to 2 folds as compared to control but statistical analysis did not show significant. However when the concentration increased to 40 $\mu\text{g/ml}$ and above, LDH activity in the cultured media increased significantly ($p < 0.01$). At maximum concentration (80 $\mu\text{g/ml}$) the rise in LDH activity was by 3 folds as compared with unexposed cells after 24 hr exposure.

Morphological studies

Untreated HEK 293 cells were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries. Various morphological abnormalities was observed in cells exposed to various concentration and showed that HEK 293 cells lost their common polygonal shape and appeared in a form of numerous roughly rounded cells of variable size. Areas devoid of cells were also recorded. The treatment with 10 $\mu\text{g/ml}$ to 80 $\mu\text{g/ml}$ of venom led to the aggregation of dense irregular cellular debris. No intact cells were recognized in this medium, which indicates the occurrence of widespread cell death. Interestingly, the morphological changes that showed after 3hr incubation was similar to 24 hr incubation (Figure 4).

Discussion

Echis carinatus venom is a highly complex mixture of a variety of biological substances including protein and non protein toxins which degrade tissue structure and promote hemorrhaging [16]. In this study, we have demonstrated a direct cytotoxic effect of *Echis carinatus* crude venom by exposing the HEK 293 cells to various concentrations of crude venom for 3 and 24 hour using MTT, Neutral red and LDH assay. The cell line HEK 293 was used in this study. Most cells derived from an embryonic kidney would be endothelial, epithelial, or fibroblasts. Although HEK 293 is not clearly represent kidney tissue, but since the origin of the cells is human embryonic Kidney, it can play a role as a related model to kidney tissue. The primary effects of crude venom of *Echis carinatus* on HEK 293 cell was induction of changes in the cell shape and detachment of cell from the surface of plate and subsequent aggregation which examined by phase-contrast microscopy. The detachment of cells may be due to the disintegrin in the venom of *E. carinatus* [17-19]. Disintegrins are non enzymatic proteins which binds to integrin receptors T resulting in competitive inhibition of integrin binding to extracellular matrix proteins [15,19,20]. The results in present study are in accordance with the results obtained by Hoda Khalid (2015) that recently reported the cytotoxicity effect of crude venom of *Echis* on rat skeletal muscle cell line (L6) and evaluated the concentration-dependent inhibition of cells exposed to the venom [14]. Recently cytotoxic effect of *V. lebetina* crude venom on the HUVEC, *Bothrops moojeni* crude Venom on MDCK and Russell's viper venom on human A549 cells were reported by various research workers [21-23]. Also, Michael Conlon (2013) investigated the cytotoxic activities of purified phospholipase A2 (Ser49) from the venom of the *Echis carinatus* on lung adenocarcinoma A549 cells and human umbilical vein endothelial cells (HUVECs) and showed concentration-dependent inhibition of cells [24]. Some in vivo studies recently reported the effect of *Echis carinatus* venom on kidney and showed the necrotic effect of this venom to cause acute renal failure [25]. We used two colorimetric assay MTT and Neutral red to determine the cytotoxicity of the venom [26,27]. The results of NR and MTT assays are often comparable [28]. Vian, et al. (1995) reported close correlations between the NR and MTT assays for most test chemicals [29,30]. In our study, the MTT and neutral red (NR) assay showed that *Echis carinatus* venom has cytotoxic effect on HEK 293 cells in a concentration-dependent manner after 3 and 24 hours exposure. The results obtained from MTT assay after exposure of cells to the crude venom for 3 hours is more or less similar to the results obtained after exposing for 24 hours. This may be due to the necrotic effect of the venom on cells rather than apoptotic nature of the venom. Susan Elmore (2007) reported that the apoptosis is a time-consuming process; hence the results obtained in the present study may reveal the necrotic effect of the venom rather than apoptotic effect [31].

The cell line HEK 293 was used for cytotoxicity assay of the venom on kidney cells [32]. In order to further characterize crude *E. carinatus* venom cytotoxicity, we examined plasma membrane integrity. Because SVMP have been shown to induce plasma membrane disintegration [33]. We used the LDH assay an ubiquitous cytosolic enzyme which releases if the plasma membrane of cells injured [34,35]. The results obtained indicate that the effect of *E. carinatus* venom on cells was dose related. This effect was significant when compared to the control values at 3 hours. However the rise in activity of LDH at 24 hours exposure was non-significant. This can be due to high standard deviation (SD) in the results obtained at 24 hours exposure. The release of LDH into the culture medium and results of cell viability that obtained after 3 and 24 hours exposure with MTT and NR assay gives an accurate measure of cellular toxicity induced by to the venom and correlates well with the severity of cell death and membrane damage observed in this study.

Conclusion

Based on the results obtained in the present study it may be concluded that the damage induced by *E. carinatus* venom on Kidney is probably related to the direct effect as well as indirect effect including hypotension, hemolysis, hemoglobinuria, rhabdomyolysis, and myoglobinuria of this venom on kidney which may lead to Acute renal failure (ARF).

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