INTRODUCTION

The morbidity and mortality associated with snake bites 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, cause progressive tissue necrosis and permanent physical deformities (4 - 5). Metalloproteases appear to cause lysis of structural proteins including basal lamina (6). It is a well-established fact that some snake venoms, including E. carinatus, 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, and acute renal failure (ARF) (7-8). ARF is mainly observed following bites by snakes which belong to the Viperidae group. (9-10). The ARF which occurs after snake bites 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 filtration 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). This study was performed to elucidate a putative direct cytopathic action of Echis carinatus venom using cultured Human embryonic kidney (HEK 293) cell monolayers as a model.
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) were 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). There were three control groups in each plate for all the assays. The cells in control groups were not exposed to the venom and comparison was between the exposed cells and control groups

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 cytopathic effects of Echis Carinatus crude venom was evaluated using MTT assay. HEK 293 cells were seeded in a 96 well plate at 3×10^4 cells/ well and incubated for 24 hr. 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 × 10^4 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 dye was 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 minutes (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 with the E. carinatus crude venom at various concentrations. Cells were seeded in 96-well plate at a density of 3×10^4 cells/ well in culture medium. After overnight incubation, the media was replaced with serum-free medium containing various concentrations of E. carinatus crude venom and incubated for 3 and 24 hr. Lactate dehydrogenase enzyme was estimated in cultured media using colorimetric assay Kit provided by Pars Azmoon company, Iran.

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

Statistical analysis
Experiments were performed in triplicate with four replicates for each exposure concentration. Results are expressed as mean ± SD. Data were analyzed by Student t-test and an analysis of IC50 (half maximal inhibitory concentration) by fitting the data to log (inhibitor) vs. response equation. A significance level of p < 0.05 was used for statistical testing. All statistical analyses were performed using SigmaPlot 12 software.

RESULTS

Determination of cell viability (MTT assay)
The inhibitory effects of crude venom of Echis carinatuson 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 HEK293 cells exposed to the venom increased significantly (p<0.01) compared to venom unexposed cells in a concentration-dependent manner. The

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Figure 1. Cytopathic effects of Echis 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 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.
maximum cell inhibition was 69% after 3 hr and 75% after 24 hr in 80 μg/ml concentration exposure, respectively. The least cell inhibition was 19% after 3 hr and 30% after 24 hr in 1 μg/ml concentration respectively. The IC50 value of Echis 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.

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 a concentration-dependent manner. The maximum cell inhibition was 78% after 3 hr and 81% after 24 hr in the 80 μg/ml concentration exposure to Echis carinatus snake venom, and the lowest cell inhibition was 10% after 3 hr and 28% after 24 hr in the 1 μg/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 Echis carinatus venom at concentrations ranging from 1 to 80 μg/ml. The effect of venom on LDH release was concentration-dependent. HEK 293 cells were treated with Echis carinatus snake venom at concentrations, 1, 5, 10 and 20 μg/ml for 3 hrs. Snake venom caused LDH release to increase 1.5 to 2-fold as compared to controls, but statistical analysis did not show significance. However when the concentration increased to 40 μg/ml and above, LDH activity was 3-fold higher than the 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 were observed in cells exposed to various concentrations of snake venom; HEK 293 cells lost their common polygonal shape and appeared in the form of numerous roughly rounded cells of variable size. Areas devoid of cells were also recorded. The treatment with 10 μg/ml to 80 μg/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 3 hr incubation were similar to 24 hr incubation (Figure 4).

**Discussion**

Echis carinatus venom is a highly complex mixture of various biological substances including protein and non-protein toxins which degrade tissue structure and promote hemorrhaging (16). In this study, we have demonstrated a direct cytopathic effect of Echis carinatus crude venom by exposing the HEK 293 cells to various concentrations of crude venom for 3 and 24 hr using MTT, Neutral red and LDH assays. The cell line HEK-293 was used in this study. Most cells derived from an embryonic kidney would be endothelial, epithelial, or fibroblasts. Although HEK293 does not clearly represent kidney tissue, the origin of the cells is human embryonic kidney, and can still serve as a related model to kidney tissue. The primary effects of crude venom of Echis carinatus on HEK 293 cells was induction of changes in cell shape and detachment of cells from the...
surface of the plate. Subsequent aggregation was also examined by phase-contrast microscopy. The detachment of cells may be due to the disintegrin in the venom of *Echis carinatus* (17-19). Disintergins are non-enzymatic proteins which bind to integrin receptors, resulting in competitive inhibition of integrin binding to extracellular matrix proteins (15, 19, 20). The results in the present study are in accordance with the results obtained by Hoda Khalid (2015) that recently reported the cytopathic 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 the cytopathic effect of *V. lebetina* crude venom on human umbilical vein endothelial cells (HUVEC), *Bothrops moojeni* crude Venom on MDCK cells, 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 HUVEC, and showed concentration-dependent inhibition of cells (24). Some in vivo studies recently reported the effects of *Echis carinatus* venom on the kidney, and showed the necrotic effect of this venom to cause acute renal failure (25). We used two colorimetric assays, MTT and Neutral red, to determine the cytopathic of the venom (26-27). The results of NR and MTT assays are often comparable (28). It is a well established fact that close correlations between the NR and MTT assays exist (29, 30). In our study, the MTT and neutral red (NR) assays showed that *Echis carinatus* venom has cytopathic effects 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 exposure for 24 hours. This may be due to the necrotic effect of the venom on cells rather than the apoptotic nature of the venom. Susan Elmore (2007) reported that apoptosis is a time-consuming process; hence the results obtained in the present study may reveal the necrotic effect of the venom rather than its apoptotic effect (31).

The cell line HEK-293 was used for cytopathic assay of the venom on kidney cells (32). In order to further characterize crude *Echis carinatus* venom cytopathic, we examined plasma membrane integrity because SVMP have been shown to induce plasma membrane disintegrity (33). We used the LDH assay, as LDH is an ubiquitous cytosolic enzyme which releases if the plasma membrane of cells are injured (34-35). The necrosis of cells may be the cause of ARF in patients. Acute renal failure following vasculotoxic viperine snake bites is very common in South Asia, the region where *Echis carinatus* is well distributed, Acute tubular necrosis and acute cortical necrosis are common findings, whereas acute interstitial nephritis (AIN) is rare (36). The results obtained indicate that the effect of *Echis carinatus* venom on cells was dose-dependent . 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 a high standard deviation (SD) in the results obtained at 24 hours exposure. The release of LDH into the culture medium and the MTT and NR assay results on cell viability with obtained after 3 and 24 hours exposure give an accurate measure of cellular toxicity induced by the venom 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 *Echis carinatus* venom on the kidney is probably related to direct effects, as well as indirect effects including hypotension, hemolysis, hemoglobinuria, rhabdomyolysis, and myoglobinuria of this venom on kidney which may lead to acute renal failure (ARF).

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**REFERENCES**


