ORIGINAL ARTICLE

Cytopathic Effect of Snake *(Echis Carinatus)* Venom on Human Embryonic Kidney Cells

MAHBOOBEH BALALI BAHADORANI[1], ABBAS ZARE MIRAKABADI [2,3*]

[1] Department of Biology, Islamic Azad University, Karaj, Iran
[2] Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Karaj, Iran
[3] Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

Abstract

Background: Snake venom from *Echis carinatus* induces hemorrhage and necrosis locally at the bite site and acute renal failure (ARF) as a consequence of morphological and functional alterations in glomerular and tubular cells. It is not clear if 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 cytopathic effect of *Echis carinatus* venom.
Methods: The effect of *Echis Carinatus* snake venom on HEK 293 cell viability was determined by MTT assay and neutral red uptake assay. The integrity of cell membranes was determined through LDH release and 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 was confirmed by neutral red assay. Crude venom caused changes in the integrity of cell membranes as determined by increases in LDH release.
Conclusions: Based on the results obtained in the present study it may be concluded that the damage induced by *Echis carinatus* venom on kidneys is probably related to direct effects, as well as indirect effects including hypotension, hemolysis, hemoglobinuria, rhabdomyolysis, and myoglobinuria, which may lead to Acute renal failure (ARF).

Keywords: Acute Renal Failure; Cytopathic Effect; Echis Carinatus; HEK 293 Cells; Snake Venom

How to cite this article: Balali Bahadorani M, Zare Mirakabadi A. Cytopathic Effect of Snake (Echis Carinatus) Venom on Human Embryonic Kidney Cells. *Asia Pac J Med Toxicol* 2016;5:88-93.

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. 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, hypotention, 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.

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 Dulbecco's Modified Eagle's medium, high glucose (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- Steptomycin were purchased from Gibco BRL (Life Technologies, Paisley,

*Correspondence to: Abbas Zare Mirakabadi; PhD. Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Karaj, Iran
Tel: +98 02634552006, Email:zareabbas83@gmail.com
Received 14 May 2016; Accepted 29 July 2016

Scotland), MTT(3-(4, 5- dimethylthiazolyl-2)-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 3-4 × 10^4 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 × 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 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 HEK293 cells exposed to the venom increased significantly ($p<0.01$) compared to venom unexposed cells in a concentration-dependent manner. The

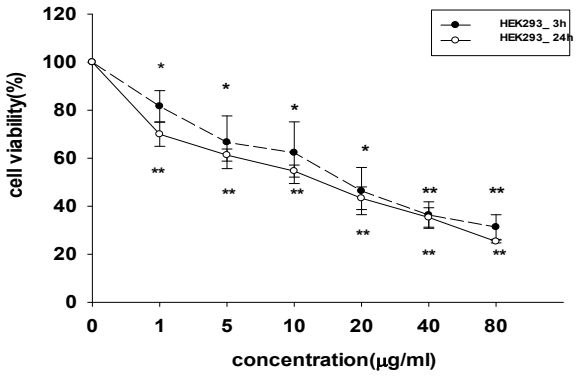

Figure 1. Cytopathic effects of *Echis carinatus* crude venom on HEK 293 cell line cell viability after exposure to various concentrations of venom for 3 and 24 hr. Cell viability was determined by MTT assay. The control value (without venom) was set as 100%. Data are expressed as the mean ± SD. *P 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

maximum cell inhibition was 69% after 3hr and 75% after 24hr in 80 µg/ml concentration exposure, respectively. The least cell inhibition was 19% after 3hr and 30% after 24hr in 1 µg/ml concentration respectively. The IC50value 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.

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 reduced with an IC50 value of 16.66 ± 1.26 µg/mL and 8.43 ± 0.54 µg/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 a concentration-dependent manner. The maximum cell inhibition was 78% after 3hr and 81% after 24hr in the 80 µg/ml concentration exposure to *Echis carinatus* snake venom, and the lowest cell inhibition was 10% after 3hr and 28% after 24hr 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 to40 µg/ml and above, LDH activity in the cultured media increased significantly (P< 0.01). At the maximum concentration of snake venom (80µg/ml), the rise in LDH activity was 3-fold higher than the unexposed cells after 24hr 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 3hr incubation were 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 cytopathic 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 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

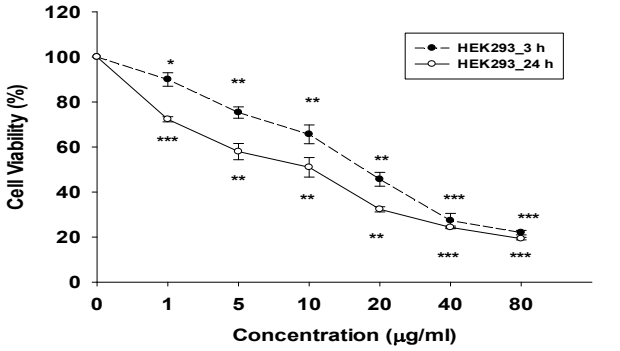

Figure 2. Cytopathic effect of *Echis carinatus* crude venom on HEK 293 cell line cell viability after exposure to various concentrations of venom for 3 and 24 hr. Cell viability was determined using by neutral red uptake assay (NR). The control value (without venom) was set as 100%, data are expressed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

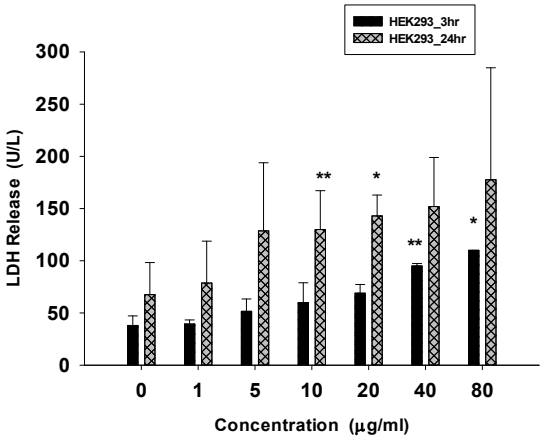

Figure 3. Effect of *Echis carinatus* crude venom on HEK 293 cells, growth inhibition determined by LDH release assay. Values indicate mean ±SD of LDH activity (U/L) (compared to control) for 3 and 24 hr. *P <0.05 and **P <0.01 were considered to be statistically significant, compared with values from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

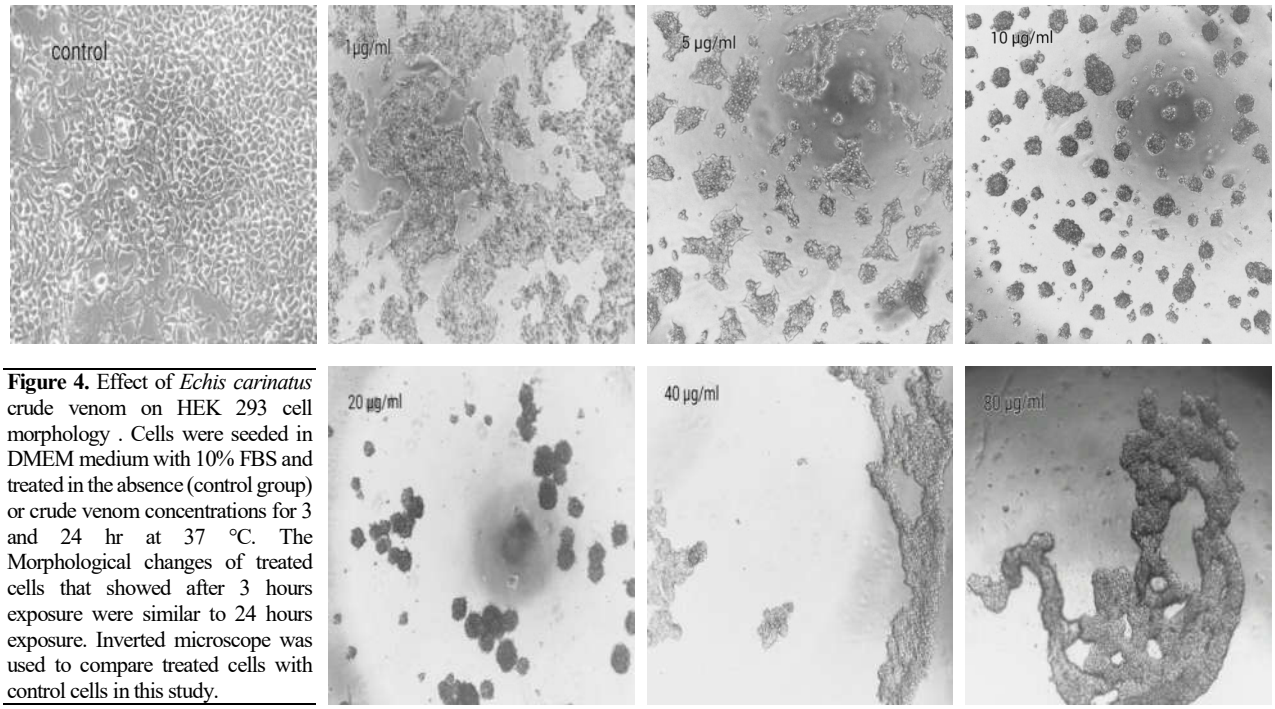

Figure 4. Effect of *Echis carinatus* crude venom on HEK 293 cell morphology . Cells were seeded in DMEM medium with 10% FBS and treated in the absence (control group) or crude venom concentrations for 3 and 24 hr at 37 ℃. The Morphological changes of treated cells that showed after 3 hours exposure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

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 cytopathicity, 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).

ACKNOWLEDGEMENT

The authors thank Razi Vaccine and Serum Research Institute, Karaj, Iran for providing us the lyophilized crude *Echis carinatus* venom and other materials for our research.

Conflict of interest: None to be declared.
Funding and support: None.

REFERENCES

1. Al Qahtani MA, Altheaby A, Al Anazi T, Al Saad K, Binsalih S, Al Helail M..Snake Bite Complicated by Acute Kidney Injury Secondary to Necrotizing Glomerulonephritis. *Saudi J Kidney Dis Transpl* 2014 25:1259-62.
2. Hasson SS, Al-Jabri AA, Sallam TA, Al-Balushi MS, Mothana RA.Antisnake Venom Activity of Hibiscus aethiopicus L. against Echis ocellatus and Naja n. nigricollis. *J Toxicol*2010;2010:837864.
3. Monteiro FN,Kanchan T, Bhagavath P, Kumar GP, Menezes RG, Yoganarasimha K. Clinico-epidemiological features of viper bite envenomation: a study from Manipal, South India. *Singapore Med J* 2012;53:203_7.
4. Wagstaff SC, Harrison RA. Venom gland EST analysis of the saw-scaled viper, Echis ocellatus,reveals novel $\alpha_9\beta_1$ integrin-binding motifs in venom metalloproteinases and a new group of putative toxins, renin-like aspartic proteases. *Gene* 2006;377:21–32.
5. Nanjaraj Urs AN, Yariswamy M, Joshi V, Suvilesh KN, Sumanth MS, Das Det al. Local and systemic toxicity of Echis carinatus venom: neutralization by Cassia auriculata L. leaf methanol extract. *J Nat Med* 2015;69:111–22.
6. Savanur A, Ali SA, Munir I, Abbasi A, Alam M, Shaikh HA. Pharmacological and biochemical studies on the venom of a clinically important viper snake (Echis carinatus) of Pakistan. *Toxicon* 2014;80:47–57.
7. Ramos OH, Selistre-de-Araujo HS. Snake venom metalloproteases--structure and function of catalytic and disintegrin domains. *Comp Biochem Physiol C Toxicol Pharmacol* 2006;142:328–46.
8. Kularatnea SA, Sivansuthanb S, Medagedara SC, Maduwage K, de Silva A. Revisiting saw-scaled viper (Echis carinatus) bites in the Jaffna Peninsula of Sri Lanka: distribution, epidemiology and clinical Manifestations. *Trans R Soc Trop Med Hyg* 2011;105:591–7.
9. Athappan G, Balaji MV, Navaneethan U, Thirumalikolundusubramanian P. Acute Renal Failure in Snake Envenomation: A Large Prospective Study. *Saudi J Kidney Dis Transplant* 2008;19:404-10.
10. Kohli HS, Sakhuja V.Snake Bites and Acute Renal Failure. *Saudi J Kidney Dis Transplant* 2003;14:165-76.
11. Thamarai R, Sivakumar K.Plasma netrophil gelatinase associated lipocalin as an early biomarker of acute kidney injury in snake bite.*J Evol Med Dental Sci* 2014;3:14137-46.
12. Sitprija V. Snake bite nephropathy. Nephrology (Carlton) 2006;11:442-8.
13. Khalid H, MohammedMukhtar M, Konstantakopoulos N.Cytotoxiciy of Naja nubiae (Serpentes: Elapidae) and Echis ocellatus (Serpentes: Viperidae) Venoms from Sudan.*J Toxins* 2015;2015:1-7.
14. Pierce RD, Kim ES, Girton LW, McMurry JL, Francis JW, Albrecht EA Characterization of crude *Echis carinatus* venom-induced cytotoxicity in HEK 293T cells. *J Venom Res* 2011;2:59-67.
15. Fonseka CL, Jeevagan V, Gnanathasan CA. Life threatening intracerebral haemorrhage following saw- scaled viper (Echis carinatus) envenoming-authenticated case report from Sri Lanka. *BMC Emerg Med* 2013;13:5.
16. Otsuka M, Matsumoto T, Morimoto R, Arioka S, Omote H, Moriyama Y.A human transporter protein that mediates the final excretion step for toxic organic cations. *Proc Natl Acad Sci U S A* 2005;102:17923-8.
17. Escalante T, Shannon J, Moura-da-Silva AM, Gutiérrez JM, Fox JW.Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases:A biochemical and immunohistochemical study.*Arch Biochem Biophys* 2006;455:144-53.
18. Bilgrami S, Tomar S, Yadav S, Kaur P, Kumar J, Jabeen T et al. Crystal Structure of Schistatin, a Disintegrin Homodimer from Saw-scaled Viper (Echis carinatus) at 2.5 A° Resolution. *J Mol Biol* 2004;341:829–37
19. Babaie M, Zolfagharian H, Salmanizadeh H, Zare Mirakabadi A, Alizadeh H.Effect of a protrombin activator isolated from Iranian Echis carinatus venom on hemostasis. *Sci J Iran Blood Transfus Organ* 2013; 10:173-81.
20. Kakanj M, Ghazi-Khansari M, Zare Mirakabadi A, Daraei B4, Vatanpour H..**Cytotoxic** Effect of Iranian Vipera lebetinaSnake Venom on HUVEC Cells.Iran J Pharm Res 2015;14:109-14.
21. Collares-Buzato CB, de Paula Le Sueur L, da Cruz-Höfling MA.Impairment of the Cell-to-Matrix Adhesion and Cytotoxicity Induced by *Bothrops moojeni* Snake Venom in Cultured Renal Tubular Epithelia. *Toxicol Appl Pharmacol* 2002;181:124–32.
22. Pathan J, Martin A, Chowdhury R, Chakrabarty D, Sarkar A. Russell's viper venom affects regulation of small GTPases and causes nuclear damage. *Toxicon* 2015;108:216-25.
23. Conlon JM, Attoub S, Arafat H, Mechkarska M, Casewell RN, Harrison AR et al. Cytotoxic activities of [Ser49]phospholipase A2 from the venom of the saw-scaled vipers Echis ocellatus, Echis pyramidum leakeyi, Echis carinatus sochureki, and Echis coloratus. *Toxicon* 2013;71:96–104.
24. Ali G, Kak M, Kumar M, Bali SK, Tak SI, Hassan G et al.Acute renal failure following echis carinatus (saw – scaled viper) envenomation. *Indian J Nephrol* 2004;14:177-81.
25. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability /cytotoxicity. *Nat Protoc* 2008;3:1125-31.
26. Fotakis G, Timbrell JA. In vitro cytotoxicity assays: Comparison of LDH, neutral red,MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 2006;160:171–7.
27. Kouadio JH, Mobio TA, Baudrimont I, Moukha S, dano SD, Creppy EE. Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. *Toxicology* 2005;213:56–65.
28. Weyermann J, Lochmann D, Zimmer A.A practical note on the use of cytotoxicity assays.*Int J Pharm*2005;288:369–76.

29. Stockert JC, Blázquez-Castro A, Cañete M, Horobin RW, Villanueva A.MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochem* 2012;114:785–96.
30. Elmore S. Apoptosis: A Review of Programmed Cell Death.*Toxicol Pathol* 2007;35:495–516.
31. Park EK, Mak SK, Kültz D, Hammock BD. Determination of cytotoxicity of nephrotoxins on murine and human kidney cell lines. *J Environ Sci Health B* 2008;43:71-4.
32. Wang SH, Shen XC, Yang GZ, Wu XF.. cDNA cloning and characterization of Agkistin, a new metalloproteinase from Agkistrodon halys. *Biochem Biophys Res Commun* 2003;301:298–303.
33. Smith SM, Wunder MB, Norris DA, Shellman YG.A Simple Protocol for Using a LDH-Based Cytotoxicity Assay to Assess the Effects of Death and Growth Inhibition at the Same Time. *PLoS ONE* 2011;6:e26908.
34. Kendig DM, Tarloff JB.. Inactivation Of Lactate Dehydrogenase By Several Chemicals: Implications For In Vitro Toxicology Studies. *Toxicol In Vitro* 2007;21: 125–32.
35. Golay V, Roychowdhary A, Pandey R, Singh A, Pasari A, Abraham A.Acute interstitial nephritis in patients with viperine snake bite: Single center experience of a rare presentation.*Saudi J Kidney Dis Transpl* 2012;23:1262-7.