J Biochem Tech (2010) 2(4):196-198
ISSN: 0974-2328

A cross reactive study on attenuation of Cobra and Russell's viper venoms cytotoxicity by EDTA using Sp2/0 myeloma cells

U D Bharathirajan, D Sureka, A Mathiyalagan and K Ramalingam*

Received: 11 August 2010 / Received in revised form: 30 August 2010, Accepted: 31 August 2010, Published online: 08 September 2010,

Snake venom is a potentially lethal and complex mixture of functionally diverse proteins. The complex nature of the venom toxin exerts limitations towards the development of toxin targeted therapy. Conventional antivenom/antisera of equine origin also pose the risks of anaphylaxis and serum sickness in the envenomated snake bite victims. Previous investigations have revealed that the active subunits of the toxic venom are potentiated by the non-toxic subunits which act as chaperones, inorder to increase the specificity and stability of the lethal components (Gutierrez et al., 2005; Swenson and Markland, 2005; Kamiguti, 2005; White, 2005). In this context, metallic elements present in the venom function as co factors in the enzymes activity as well as in enabling the cellular infiltration of the venom toxin to cause vascular and circulatory system disorders. In the present study, an attempt was made to determine the effect of a chelator compound like EDTA in the cytotoxic action of Cobra venom (CV) and Russell's viper venom (RV) on Sp_2/0 cell lines. Since these venom toxins exhibit calcium dependent cytotoxicity, (Moral et al 2006) the effect of EDTA on the above property was also determined. EDTA is a polyamino carboxylic acid and its water soluble nature enables it as a best chelating agent capable of combining stoichiometrically almost all divalent and trivalent metal ions. Since calcium plays a key role in the haemorrhage and proteolysis, the neutralizing action of EDTA would be of importance in the attenuation of snake venom toxicity.

Cytotoxicity assay by Tryphan blue exclusion method was carried out using various concentrations of Russell's viper venom and cobra venom (Obtained from Irula's Society, Kancheepuram, Tamil Nadu, India) on Sp_2/O cell lines (Aldred and Cooke 1983 ; Lipps 1999).0.1 million of myeloma cells were added to each well of 96 well plate, along the row in duplicate. To these cells, venom was serially diluted from the concentration of 1500ng to 300ng for CV and 20µg to 2.5µg RV. The cells were incubated for 2 hrs at 37^{o}C. After incubation, the viability of the cells were measured using Trypan Blue. Similarly, the EDTA was standardized and a concentration of 10mM was mixed along with the venom sample for cytotoxicity. To check the chelating property of EDTA, $CaCl_2$ was also added to the cells at the equimolar concentration of the EDTA. The results of cytotoxicity effect of venoms, their property in the presence of EDTA, $CaCl_2$ and/ EDTA + $CaCl_2$ were illustrated.

U D Bharathirajan, D Sureka, A Mathiyalagan and K Ramalingam*

Mediclone Research center, Mediclone Biotech Pvt Ltd, Chennai 600048, India

* Tel: 0091 44 27475050, Fax: 0091 44 24963846
E-mail: kr.research@mediclonebiotech.com

Cytotoxicity of various concentrations of RV and CV

The viability of Sp_2/O cells increases with increase in the venom concentration. Concentration between 20µg to 0.08µg was taken for the viability studies. 100% death was noted at 20µg, LD_{50} value was found at 3.5µg and maximum viability of about 80% was found at 2.5µg. The viability did not show much difference beyond 2.5µg, the viability was between 80-81%. Hence the concentration values between 20µg-2.5µg were used for the further studies. Cobra venom of various concentrations from 15000mg to 300ng was treated on the Sp_2/O cell lines and its viability was noted. Complete cell death was found at 1500ng, LD_{50} value was observed at 450ng. Maximum viability of about 86% was found around 300ng. Beyond 300ng, the viability did not show much variation. Hence, the concentration between 1500ng and 300ng was used for further studies. Table - 1

Optimization of EDTA concentrations

Sp2/O cell lines were treated with various concentration of EDTA ranging between 100mM and 0.08mM. EDTA did not show much effect on the cell lines. Viability showed a gradual increase with decrease in EDTA concentration. The medium became acidic between the concentration ranges of 100mM to 20mM, Neutral pH was maintained at 10mM and beyond which the medium became more alkaline. Hence, in order to maintain neutral pH, 10mM EDTA was used for treating the venom sample.

Russell's viper venom concentration between 20µg and 2.5µg was treated with 10mM EDTA concentration. Increase in viability was noticed when the venom sample was treated with EDTA. The LD_{50} concentration shifted from 3.5µg to 20µg. At 20µg the viability increases from 0% to 58%. The viability at 3.5µg was shifted from 55% to 75%, when compared with neat venom. The viability

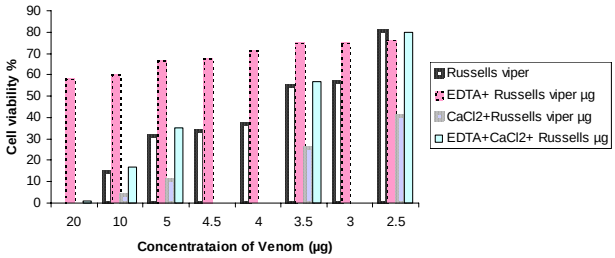

Figure 1: Cytotoxocity of EDTA, $CaCl_2$ and EDTA + $CaCl_2$ on Russel's viper venom

beyond 2.5µg did not show much variation as that of the neat venom. Russel's viper venom was treated with 10mM $CaCl_2$, the cytotoxocity of the venom increased due to the action of $CaCl_2$. 3.5µg which showed 50% viability decreased to 26% and 2.5µg which showed maximum viability of about 81% decreased to 41%. But $CaCl_2$ on its own did not show any change in viability when used as the control.Russell's viper venom was treated with 10mM EDTA and equimolar concentration of $CaCl_2$ together. The venom toxicity was decreased with EDTA treatment and increased with $CaCl_2$ treatment. But when the venom was treated with EDTA and $CaCl_2$ the viability was restored similar to that of venom treated on Sp_2/O cell lines. The LD_{50} value was again restored at 3.5µg.

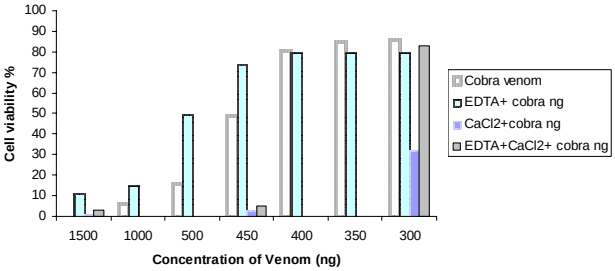

Figure 2: Cytotoxocity of EDTA, $CaCl_2$ and EDTA + $CaCl_2$ on Cobra venom

Different concentrations of cobra venom from 1500ng to 300ng were treated with 10mM EDTA concentration. Gradual increase in viability was noticed when compared with the neat venom sample. At 1500ng, the viability from 0% increased to 10%, the LD_{50} value from 450ng shifted to 500ng. Att 450ng the viability increased to about 74%. Beyond 400ng the viability became constant and maximum of about 80% $CaCl_2$ of equimolar concentration to EDTA (10mM) was used to treat the venom sample. Concentration of Cobra venom that showed maximum, 50% and 0% viability was treated with $CaCl_2$. Cytotoxicity of the venom was increased which resulted in drastic decrease in viability. At 450ng the viability decreased from 5% to about 3% and at 300ng which showed maximum viability (86%) decreased to 32%. The results show that $CaCl_2$ increased the toxicity of the Cobra venom sample. Cobra venom sample was treated with 10mM EDTA and 10mM $CaCl_2$ together. 1500ng, 450ngabd 300ng which showed complete cell death, 50% viability and maximum viability respectively was chosen for the treatment. The above venom concentration whose toxicity decreased with EDTA and which increased with $CaCl_2$ when treated separately restored its property as that of original venom sample. The LD_{50} value was restored at 450ng. 1500ng which showed 0% viability showed 3% viablilty.

Snake venoms are both neurotoxic and haemorrhagic in action (Kamiguti *et al*., 2003; Schneemann *et al*., 2004; Lu *et al*., 2005). Their principal action is on the cell membrane of these tissues resulting in the failure of circulatory and respiratory systems while the lethal effect has been attributed to toxic proteins, other ingredients may help to complement the toxicity of venom and bring tissue deterioration. Various authors have opined that the main cause of cytotoxicity in tissues / cells is the release of reactive oxygen species which induce apoptosis.

In the present study, the cytotoxicity of both cobra and viper venom and the enhancing property of $CaCl_2$ have been observed. Cobra venom was found to be more potent in cytotoxicity than that of viper. It showed 100% cell –death at 1500ng when compared to Russell's viper venom which showed complete death only at 20µg. When both venom were treated with metal chelator EDTA the cytotoxocity of them reduced markedly. The RV venom (20µg) along with the EDTA increased the cell viability to 40% while that of cobra venom (1500µg) increased the same to 90%. Similarly the LD_{50} of the cobra venom was shifted from 450 ng to 50ng and in the case of RV venom it shifted form 3.5µg to 20µg. Our study also revealed that the concentration of 10mM EDTA used maintained the neutral pH and showed less significant mortality of the cells (27%). The results thus reveal that EDTA in spite of its own toxicity, in the venom mixed conditions it increased the viability of the cells significantly. The above chelator action might be due to blockage of Ca^{++} influx into cells by complexing withit. Previous studies have already demonstrated that cardiotoxin induced cardiomyocyte degeneration is suppressed by the blockage of Ca^{2+} influx and by the inhibition of non-lysosomal proteinaseas. (Tzeng and Chen 1988). In the present study $CaCl_2$ added venom showed significantly the cell loss. The probable cause for the may be the disruption of the cell membrane and the consequent influx of Ca^{2+} to cause cell death. Already Mora *et al* (2006) have revealed that the cellular proliferation response depends on a regulated Ca^{2+} influx through the plasma membrane

In the present study, in order to validate that Ca^{2+} ions are responsible for high cytotoxicity and cell death the cobra and RV venom were treated with both EDTA and $CaCl_2$. The results revealed that the Toxicity of the venoms was restored almost to the same level as that of neat venom when treated separately on Sp_2/O cells. The observations suggest that EDTA is a useful tool in understanding the molecular mechanisms involved in the cofactor role of Ca^{2+} in the cytotoxicity property of the venom. The results also construe that EDTA by blocking the membrane potential in cells might prevent the entry of Ca^{2+} ions and the attendant cell death in cells. It is also known that EDTA in binding dissolved metallic elements and removing them reduces abnormal production of oxygen free radical molecules (Senheff, 1999) which are attributed per se for venom toxicity.

Conclusion

Reduction of cytotoxicity of venom in persons victimized by snake bite and envenomation is the scope of antivenom studies. The present observation of EDTA ensuring cell viability in venom reveals its utility as metal chelators and therapeutic value alongside other targeting antivenom agents.

Acknowledgement

The authors (1, 2 & 4) are thankful to the Managing Director, Mr. A. Mathiyalagan, who is instrumental in implementing the ASVS project and for his consisten support in carrying out the cytotoxicity

studies in Mediclone Research Center, Velichai, Chennai – 600048, India.

References

Aldred L F, Cooke B A (1953) The effect of cell damage on the density and steriodogenic capacity of rat testis leydig cells using an NADH exclusion test for determination of viability. J Steroid Biochem 18:411-414

Fohlman J, Eaker D, Karlsson E, Thesleff S (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian snake taipan (oxyaranus. Scutellatus). Isolation characterization, quarternary structure and pharmacological properties. Eur J Biochem 68:457-469

Gutierrez J M, Rucavado A, Escalante T, Diaz C (2005) Hemorrhage induced by snake venom metalloproteinases: Biochemical and biophysical mechanisms involved in microvessel damage. Toxicon. 45:997-1011

Habermann E, Breithaupt H (1978) Minireview. The crotoxin complex- an example of biochemical and pharmacological protein complementation. Toxin 16:19-30

Islet TS, Sykes AS (1960) Diamino- ethane tetra-acetic acid and its complexs. In Analytical Applications of Diamino-ethane-tetra-acetic-acid. 2:9-22

Kamiguti A S, Gallagher P, Marcinkiewicz C, Theakston R D G, Zuzel M, et al., (2003). Identification of sites in the cysteine-rich domain of the class P-III snake venom metalloproteinases responsible for inhibition of platelet function. FEBS Lett. 549:129-134

Kamiguti A S, (2005) Platelets as targets of snake venom metalloproteinases. Toxicon, 45:1041-1049

Lipps BV (1999) Novel snake venom protein cytolytic to cancer cells in vitro and in vivo systems. J Venom Anim Toxin 5(2)

Lu Q, Navdaed A, Clemetson J M, Clemetson K L (2005) Snake venom C-type lectins interacting with platelet receptors. Structure-function relationships and effects of haemostasis. Toxicon. 45:1089-1098

Mirzajani F (1999) Characterisation of venom proteins from Psedocerastes persicus eprints. hec. gov. pk/105/1/105.html. htm

Mora R, Maldonado A, Valverde B, Gutie'rrez JM (2006). Calcium plays a key role in the effects induced by a snake venom Lys49 phospholipase A2 homologue on a lymphoblastid cell line. Toxicon 47:75-86

Schneemann M, Cathomas R, Laidlaw S T, El Nahas A M, Theakston R D G et al. (2004) Life-threatening envenoming by the Saharan horned viper (*Cerastes cerastes*) causing micro-angiopathic haemolysis, coagulopathy and acute renal failure: Clinical cases and review. Q J Med. 97:717-727

Senneff JA (1999) Numb toes and Aching Soles: Coping with peripheral Neuropathy. Med press. San Antanio, Texan: 300 PP.

Swenson S, Markland F S (2005) Snake venom fibrin(ogen)olytic enzymes. Toxicon. 45:1021-1039

Tzeng WF, Chen YH (1988) Suppression of Snake venom Cardiotoxin-induced degeneration by blockage of ca^{2+} influx or inhibition of non- lysomal proteinases. Biochemistry Journal 256:89-95

White J (2005) Snake venoms and coagulopathy. Toxicon 45:951-967