
Rabbit snake-bite model to assess safety and efficacy of anti viper chicken antibodies (IgY)

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Abstract: Infusion of mammalian antivenom is considered to be the best available treatment for snake bites; but, production of cost-effective IgG in pure form is challenging. Purification of egg yolk immunoglobulins (IgY) raised against various toxins has been found to be relatively easier. But to use IgY for therapeutic purpose its efficacy and safety need to be experimentally proven which is hardly done due to lack of an appropriate model. In this study, pure IgY against viper venom was isolated and its efficacy and safety for intravenous infusion was tested in rabbits. Rabbit snake bite model was created by subcutaneous injection of 2x lethal dose₅₀ (LD₅₀) venom. Animals were given intravenous infusion of pure anti-viper IgY and recovery was monitored. Isolated chicken immunoglobulin (IgY) was >90% homogenous and showed 1:32 titre in immunodiffusion experiment. The minimum hemorrhagic dose (MHD) of viper venom was 0.2mg and antihemorrhagic dose (AHD) of IgY was 4x concentration (0.8 mg) of native venom. Subcutaneously injected venom at LD₅₀ resulted in severe local reaction, coagulation abnormality and mortality in rabbits. When anti viper IgY was infused within 2 h of envenomation, the animals survived, clotting parameters were reversed to normal and animals showed steady weight gain like healthy animals. No adverse effect of IgY was noticed on renal or hepatic function. The efficacy of commercially available mammalian IgG was lower than that of anti-viper IgY. Long term stability of the purified and lyophilized IgY was demonstrated. The effective IgY dose required to prevent mortality in the envenomed rabbits was found to be 4x of the injected venom estimated by Lowry's protein assay. It has been demonstrated that rabbit model of snake bite is successfully cured by anti-snake IgY infusion at a specific dose.

Keywords: Antivenom, Chicken IgY, Russell's Viper, Effective Dose, Animal Model

1. Introduction

Snake bite associated morbidity and mortality is a major public health problem in many of the tropical countries. Intravenous infusion of anti venom is the best treatment option for snake bite envenomation¹. Development of mammalian anti venom immunoglobulin (IgG) in equine has been the accepted antivenom production method in many laboratories which need to conform to pharmacopoeia regulations². Since the process of anti venom purification from equine serum is laborious and expensive, fewer efforts

are taken to produce antibodies in pure form. In many of the commercially available products, IgG is a minor fraction; the specific antibody against each toxin is likely to be much lower³. Purer the antivenom, the higher will be the cost of production. So, cost effective method for development of pure antibodies needs more attention.

Avian antibodies (IgY) have been raised against different toxins and hen is considered to be a better host for large scale production of antibodies^{4,5}. The IgY has better stability as compared to mammalian IgG and has better antigen binding efficiency. Antigen-induced immunoglobulins that are

developed in hens get concentrated in the egg yolk. There is less number of proteins in yolk as compared to mammalian serum, but lipid content is very high. Purification of IgY from lipid-free yolk diluted in water is found to be easier⁶. However, if lipid is not removed efficiently, protein-lipid micelle compromises the purity. In this study, we have modified our previous method⁶ to improve the purity of IgY and demonstrated a better *in vitro* reactivity of IgY with venom.

An important problem that is faced world-wide is in testing the potency of antibody in suitable model to calculate the human dose required⁷. So a model mimicking snake bite was proposed to be versatile in identifying the efficacy and safety of intravenous IgY infusion. We demonstrate use of rabbit snake bite model to estimate the effective dose (ED50) which may be appropriate for testing different antibody preparations.

2. Materials and Methods

Venom was collected from a local government serpentarium after required regulatory clearance. Russell's viper venom was milked into ~10x volume of normal saline. The venom milked from 3 animals were pooled, aliquot of 0.5 ml volume was stored at -80°C till the use. Other reagents used for purification of IgY were ammonium sulphate, sodium deoxycholate [SDC] (Sigma Chemicals USA) and Sephacryl 100 (GE Healthcare, USA). For immunodiffusion, molecular biology grade agarose (Sigma Chemicals, USA) was used.

Institutional Animal Ethics Committee (IAEC) approval which conforms to CPCSEA (Govt of India) regulations was obtained for using hens to raise antibodies. To the pectoral muscle of White Leghorn hens, sub lethal concentration of venom (Lowry's protein assayed) mixed with complete adjuvant (Sigma Chemicals, USA) was injected to immunize the bird. The eggs obtained 12 days after primary immunization was processed and collected the yolk. On the 35th day and 70th day, booster dose of venom mixed with incomplete adjuvant was given. Eggs were collected till 90th day after the primary immunization.

Egg yolk was separated from albumin and before breaking the sac, the yolk was washed thoroughly with plenty of distilled water. The sac was punctured to collect the yolk and was diluted with 3x double distilled water. The diluted yolk was frozen for >24 h at -80°C and thawed slowly at 4°C to precipitate the lipid. The suspension was centrifuged at 22,000g in a high speed centrifuge (Hitachi SCR55, Japan). The supernatant was collected carefully, and proteins were precipitated with 70% (NH₄)₂SO₄. Precipitate was collected by centrifugation at 10,000g and dissolved in 50 mM PBS; 5 ml aliquots were stored at 4°C. The (NH₄)₂SO₄ precipitated protein was mixed with 0.2% v/w SDC and was incubated for 1h. Further purification was done by gel filtration using Sephacryl column connected to protein purification system (AKTA Prime GE Health, USA). The eluted first peak- and second peak- fractions were separately pooled and used for

analysis of purity. An alternate purification method adopted was affinity chromatography using 2-mercaptopyridine coupled Sepharose (GE Healthcare, USA) according to the protocol provided by the manufacturer. Before loading the protein on to the affinity chromatography column, the (NH₄)₂SO₄ precipitated protein was dialyzed against the binding buffer. Column (1ml) was equilibrated, the protein solution was loaded, washed and the bound protein was eluted as per the protocol provided by the manufacturer, using AKTA Prime protein purification system.

At various stages of purification, protein was assayed using Lowry's method for calculating the recovery and yield. To test for purity, SDS-PAGE electrophoresis was performed on 8% gel and the bands were developed with Coomassie Brilliant Blue. For the immunodiffusion, 0.8% agarose was melted in barbitone buffer (pH 7.4), and the gel was cast on microscope slides. Using a standard immunodiffusion template, wells were cut, known concentrations of serially diluted IgY was added into the peripheral wells and known concentration of venom was added in the middle well. The slide was incubated for 72h-96h at 4°C in a humidified air-tight chamber. Once the precipitin line was visible, the slide was immersed in plenty of saline for 24 h to remove unreacted proteins. The gel was dried by inverting over several layers of filter paper and dried slides were stained with Coomassie Brilliant Blue.

The test for abnormal toxicity was done as per CFR Sec. 610.11 General Safety. The pure IgY was injected (intra peritoneal) to two mice weighing < 22g and 2 Guinea pig weighing < 400g. In both species, animals were observed for 7 days. Each test animal was weighed and the individual weights were recorded just prior to injecting IgY. Animal weight was monitored for 7 days. Criteria for passing the test were: all animals survive and weigh no less at the end of the test period than at the time of injection.

Adult New Zealand white rabbits weighing >2.5 kg were used for testing the efficacy. Blood was collected on the previous day of experiment and 7/15 days after the experiment for biochemical and hematological analysis. For assay of minimum hemorrhagic dose (MHD) and anti hemorrhagic dose (AHD) as described in the WHO workshop (Theakson 2003), the back of the animal was clipped and shaved. Four to five points were marked on each lateral side and 10 mm diameter circle around the point was marked. Graded concentrations of venom were injected subcutaneously at each marked point. The hemorrhage was measured and graded after 30 min. The minimum dose at which hemorrhage of 10 mm diameter was measured in 30 min is defined as MHD. For anti hemorrhagic assay, MHD concentration of venom was premixed with increasing concentration of IgY, incubated for 30 min at 37°C and were injected at different points marked at the back.

For creating *in vivo* snake bite model, animal (rabbit) back was shaved and the venom (1mg/kg) was injected. Between 2h to 6h after injecting venom, 4x venom concentrations (4 mg/kg body weight) of IgY (test) or IgG (control) was mixed into 15 ml sterile saline and was infused through the

marginal ear vein. Animals were put back into the cage and monitored. Biochemical parameters were assayed using commercially available standard kits and semi automated chemistry analyzer (ERBA Chem-7, Transasia, India), complete blood count (CBC) was taken using automated analyzer (ABX vet model, France) and coagulation parameters were measured using standard reagents from Diagnostica Stago (France) on a Coagulation Analyzer (Stago Start4, France). All laboratory tests are under quality system as per ISO17025 and are accredited by COFAC (France).

3. Results

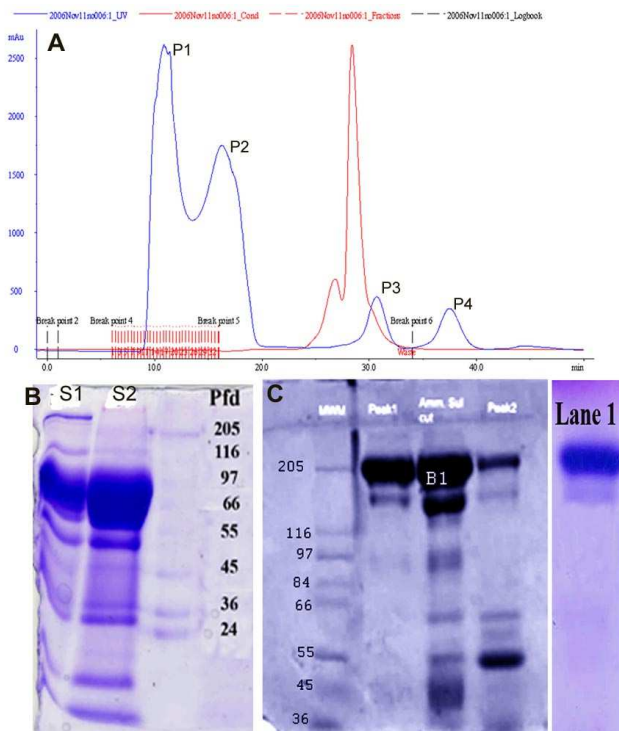


Fig. 1. Results of purification and analysis of IgY. A, Pattern of chromatographic elution of proteins peaks; B, SDS-PAGE pattern of commercial IgG; C, SDS-PAGE pattern of crude and pure IgY. The tracing in the chromatogram is absorbance of protein eluting, the red tracing indicates conductivity. One ml fractions were collected; 1st & 2nd peak were pooled separately. Lowry's protein was detected and used for analysis. IgG from two sources were analyzed and both are shown. Lane1 marked in C is IgY purified by affinity chromatography; NH₂(SO₄) precipitate, peak1, peak2 that were eluted from gel filtration column and MW marker lanes are marked in the figure.

A method for purification of IgY, using gel filtration chromatography was described in previous publication⁶. The modified purification protocol described in this study demonstrated efficient separation of proteins into two major and two minor peaks during chromatography (Fig.1A). Upon SDS-PAGE analysis, separation of IgY into pure form in the first eluted peak was evident. The crude (NH₄)₂SO₄ - precipitated fraction showed several bands ranging from ~200 kDa to 40 kDa molecular weight. The MW of the IgY was confirmed using affinity purified protein, to be 180 to

200kDa (Fig 1C). The protein that eluted in the first peak from gel filtration column also showed mainly the IgY band at ~200kDa MW and a second minor band, very close to it which was not separated by either gel filtration or affinity chromatography. Protein that eluted as the second peak of the chromatogram had IgY band but at lower intensity and other bands corresponding to lower molecular weights were visible. Two sources of commercially available anti venoms were analyzed. In both products, IgG band (150 kDa) was hardly prominent but the albumin band (66kDa) was the most visible one (Fig 1B). It appears that only <5% of the total protein is IgG in both cases. Therefore, purity of commercially available anti venom is considerably low.

Immuno diffusion experiment showed that the titre of IgY was 1:4 after the first immunization (Fig 2A), and titre was > 1:32 (Fig 2B) when eggs were processed and IgY purified after giving booster. The result indicates that in a unit concentration of IgY, the specific immunoglobulin against venom toxins increased when the booster was given. In both commercial preparations, titre was ~1:2 (Fig 2 C&D). The major difference in the case of commercial preparation was that they formed bands at different positions and multiple bands were formed near the wells. In the case of IgY the precipitin line was formed as thick band which suggests that antibody was developed against each antigen with similar response and precipitin lines were formed at an equidistant position between antigen and antibody wells.

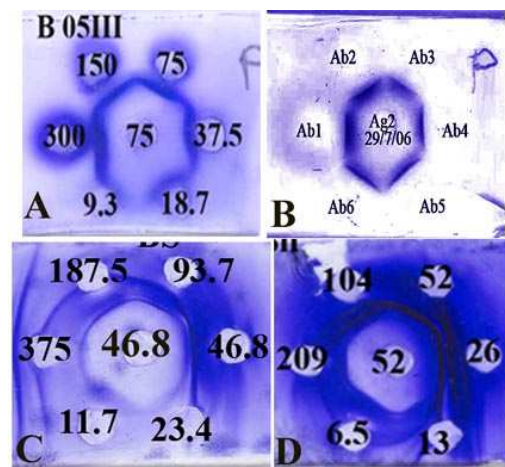


Fig. 2. Ouchterlony's immunodiffusion. A & B, Immunodiffusion of purified IgY after 1st booster and 2nd booster. C & D, Immunodiffusion of IgG from two different sources. Dilution of IgY was done and in B, Ab1(1) is the highest concentration and Ab6(1:32) is the lowest.

Abnormal toxicity test was passed favorably because all animals gained weight, similar to their litter group. For detection of minimum haemorrhagic dose (MHD), 5 different concentrations of venom were injected such as 0.15mg, 0.20mg, 0.25mg or 0.30 mg per site. Dose3 (0.25 mg) created hemorrhage of 10 mm dia in 30 min after injection (Fig 3A). Four sites were used for each dose using replicate animals and the results were found repeatable. For MHD, dose 1 and dose 2 were not hemorrhagic. So, for detection of anti hemorrhagic dose (AHD), dose3 (0.25 mg

venom) was mixed with 4 different concentrations of anti venom such as 1x (0.25 mg), 2x (0.5 mg), 3x (0.75mg), 4x (1 mg), mixture was incubated at 37°C and injected at four points for each composition; one on the left and the other on the right, in 2 such animals as shown in Fig 3B. One site was injected with 4x (1 mg) dose of IgY alone. Hemorrhagic area was measured to identify the effectiveness of anti venom to reverse the hemorrhagic effect of 0.25 mg venom. When IgY was used to neutralize the venom, hemorrhage was the lowest at 4x IgY concentration, when mixed with venom (0.25 mg venom + 1 mg IgY). Injection of IgY alone did not cause any hemorrhage or edema. Replicate experiments were carried out to confirm the observation; separate animals were used to test commercial IgG from each source.

For creation of snake bite model, subcutaneous injection of venom was given. One mg venom per kg body weight was selected because during MHD experiment, total venom injected to all 8 sites together was 2 mg for the animal weighing >2 kg making the total injected dose 1mg kg⁻¹. At this dose, animals were very sick and more than 50% died within 48 h. The observation indicated that 1mg kg⁻¹ can be lethal to rabbits. When 1mg kg⁻¹ venom was used as controls ('no anti venom' group), out of three animals, 2 died within 24h. So it is confirmed that 1 mg kg⁻¹ is lethal to rabbits. In all animals in the experimental groups, a severe hematoma was found at the site of injection within 30 min after injection (Fig4A). Four times (4x) concentration of IgY was given intravenously (Fig.4 B & C). The logic for using this dose of IgY was that in the AHD experiment, venom mixed with 4xIgY showed very minimal hemorrhage (Fig 3B). This dose was effective and all animals survived. Reversal of hematology parameters to normal level was also achieved within a week. Even though there was severe reaction on the second day of the experiment (Fig 5 A), by two weeks the wound healed and there was normal appearance around the wound (Fig 5 C & D). Also when the venom dose was increased to 2 mg kg⁻¹ body weight, all animal survived if anti venom dose was also increased proportionately to

achieve 4x the dose of venom; i.e. increasing the IgY dose to 8 mg kg⁻¹ body weight. This time, three animals out of four survived after the infusion of IgY. One animal died in 48 h, probably because IgY infusion could not be completed due to severe hematoma at the site of infusion (Fig 5B).



Fig. 3. Image of injections sites of MHD & AHD detection A, Sites of hemorrhage 30 min after injection of venom; B, Sites of hemorrhage 30 min after injection of venom-IgY or venom IgG mixtures. The composition injected on left and right sides are similar and are marked in the figure.

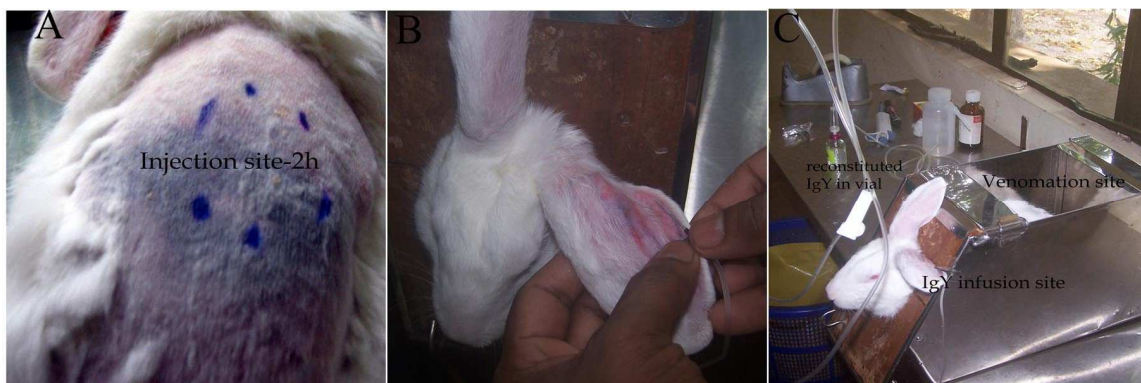


Fig. 4. Representative images of the experiment. A, injection site: B, infusion in progress: C, Set up for infusion. Severe hemorrhage was observed 2h after subcutaneous injection of venom. Smooth infusion of IgY was done through the marginal ear vein.

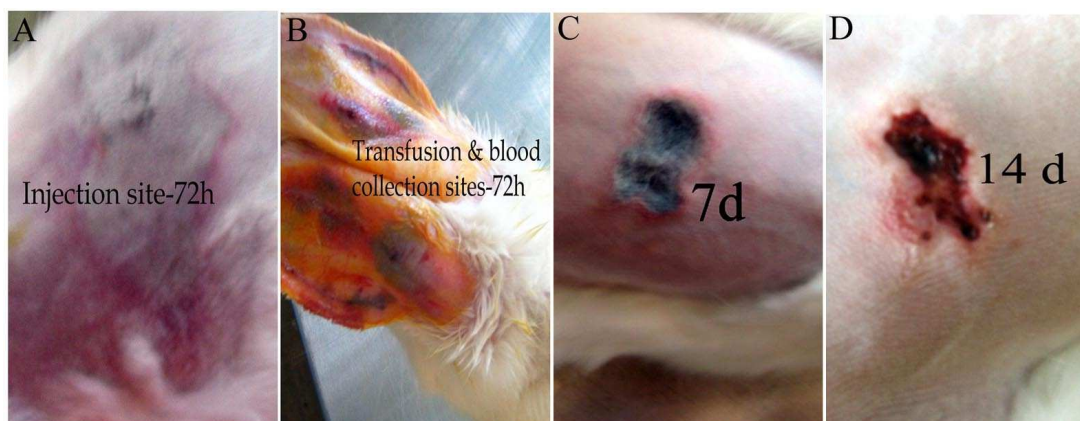


Fig. 5. Representative images showing recovery. A, venom injection site after 72h; B, infusion site after 72; C, injection site after 7 d and D, injection site after 2 wks. Hemorrhage on the ear after 72h is due to the attempt to collect blood for analysis. Severe hemorrhage is seen; so blood could be collected only on the 7th day.

The blood tests that were done include, complete blood count, plasma Hb, prothrombin time (PT), activated partial thromboplastin time ((APTT), clottable fibrinogen, urea, creatinine, serum alanine transaminase (ALT/SGPT), total protein and albumin. In animals that were infused with IgY after envenomation, all parameters including APTT were normal by 7 days, except an initial high level of fibrinogen and low platelet count which was reversed back to normal within 14 days of the experiment. The increase in fibrinogen may be attributed to the inflammation. When IgY was administered without any venom dose, all parameters remained in the same range as the basal level, at all periods of measurement. In the control (commercial IgG) group, only one out of three animals survived with similar dose as IgY. In the survived animal, APTT was very high after 7d (>500s), fibrinogen level was high till 2 weeks and platelet count was 50% of the basal level (count before envenomation). So overall, it was suggestive of poor reversal of hemostasis if IgG was used, whereas 100% recovery on infusing 4x IgY was observed. After IgY infusion at both dose (4mg/kg or 8mg/kg body weight) the renal function and liver function remained steady as confirmed by routine biochemical tests.

4. Discussion

Anti venom therapy is currently indispensable in cases of snake bite envenomation as there is no alternate effective treatment. There are many different species of venomous snakes and there is geographical distinction in their frequency. So, commercialization of a universal preparation that can be effective and pure is far from reality. It is the geographical diversity in the type of venom which makes it difficult to focus on the availability of uniformly pure preparations of anti venom for all snakes. In India and other Asian countries there are 4 major types of venomous snakes and anti venom against all the 4 type together are available commercially and are used for any snake bite. The major problem with these preparations is that majority of them are available in a less pure state. The purification of IgG from mammalian serum is laborious and the purified product may

become more expensive. So to compromise the cost and wide availability, poly specific antibodies in less pure form are being sold. Less pure forms will have less specific antibodies which pose a need for increase in quantity infused. When we analyzed two commercial sources of antibodies, more or less similar protein profile was found in both. In SDS-PAGE, only very minor band is seen in 150 kDa region which correspond to IgG. Upon infusion, the preparation heavily contaminated with other proteins can lead to problems such as serum sickness. So, to solve these side reactions, it is important to develop antibodies in pure form according to the guideline from WHO⁸.

The efficacy and safety profiles of most of the anti venoms that are routinely used in South Asia have not been well established. Up to 80% of patients treated with such anti venoms present one or more adverse effect(s) such as anaphylactic or pyrogenic reactions, or late serum sickness^{9,10}. The risk of severe adverse events must be balanced against the life-saving potential of this treatment. When it is difficult to estimate how much immunoglobulin is present in the preparation, dose calculations become practically impossible. Our major goal was development of pure anti venom that is tested appropriately to give a clear indication to its safety, efficacy and dosage. Our current focus is on Russell's viper venom antibodies; mainly because it is possible to monitor coagulation parameters which indicate the extent of toxic effects of envenomation and possible normalization upon anti venom infusion. Among the true vipers, Russell's viper (*Daboia russelii*) is associated with the highest morbidity and mortality. In south Asia, starting from Sri Lanka to foot hills of Himalayas including Nepal, Bangladesh and Pakistan this species is a major cause for snake bite deaths¹¹.

In recent years it is understood that hen is a good host for producing antibodies. Affinity chromatography is one of the best techniques to obtain the purest protein. In our effort for purification of IgY by affinity chromatography, the yield was relatively very low on affinity purification and insufficient quantity for therapeutic use was obtained. Low yield may be due to poor binding efficiency of the ligand to the column

material. Both gel filtration and affinity chromatography resulted in similar level of purity in our experiment. The first peak in our earlier study was not well resolved and proteins of many sizes eluted together, which could be due to micelle formation⁶. The lipid content in the extract increases the chance for micelle formation. Significant outcome was observed when detergent was used to break micelles. This study demonstrates resolution of proteins into two distinct major peaks, out of which the first one showed good purity and yield. Up to 20mg pure IgY could be obtained from one egg. This method of salt precipitation followed by gel filtration in presence of SDC produced active antibody that showed good titre in Ouchterlony's immuno diffusion experiment.

Once we found that the titre, purity and recovery were improved, the next objective was to identify the effective dose. The AHD which was estimated as per the CPMP protocol showed that 1:4 composition of venom:IgY is sufficient to reduce the hemorrhage when the pre-incubated mixture of venom and antivenom was injected. Out of the two commercially obtained anti venoms, one was effective for reducing the hemorrhage but the other was not. For the efficacy evaluation similar composition was infused in the rabbit model of snake bite.

To create the model, animals were injected with venom which resulted in severe reaction at the site of injection and lethality if no anti venom was infused. On infusion of the IgY, all animals survived whereas with commercial IgG infusion only 33% survival was achieved. The animal which survived was very sick and took long duration to recover from the signs of toxicity. Only a single dose of IgY or IgG could be given due to the haemorrhage at the infusion site, which is a symptom of envenomation. Probably that was the major limitation in this animal model. It may be possible to give multiple dose in human subjects. Similarly, it was difficult to monitor haematology parameter after 24h of subcutaneous injection of venom. So, blood was collected only 7 days after the experiment. We found this a good model to estimate the efficacy dose for treating envenomed victims. It has been estimated and published in literature that during one normal bite by Russell's viper, up to 60 mg venom is injected; so according to the severity of reaction after the bite, the anti venom requirement may be calculated.

The dose of anti venom to be given to the patient is currently not been standardized for IgG. Therefore, many units are given to patients, which could lead to various side effects. So, two major factors that need to be considered before a new anti venom preparation is used clinically are the dose and safety. On the safety point of view, we observed there was no toxic reaction when one human dose was injected intra peritoneal to mice and guinea pigs. In addition, the rabbits maintained stable liver function and kidney function after intra venous infusion of IgY. In IgG infused rabbit, even at 4mg/kg body weight, no adverse reaction was found. It has been reported that when multiple dose of IgG is used in human there is renal failure which leads to hemodialysis. Any how if IgY is used, higher dose may not be

required because at the dose administered, it was effective to reverse the toxic effect of venom. But IgG was not sufficient, so additional dose may be required.

5. Conclusion

Pure IgY against Russell's viper venom was produced using low dose of native venom as the antigen. Good separation of the IgY from other yolk proteins was achieved by gel filtration in the presence of detergent. The anti venom showed good antigen binding *in vitro* in immuno-diffusion experiments. Using rabbit model, minimum haemorrhagic dose of venom and anti haemorrhagic dose of IgY was determined. Using a newly developed preclinical model in rabbits, effective dose was estimated. The AHD and effective dose for reversing the toxic effects of subcutaneous envenomation was similar i.e. 4x concentration of venom dose. Infusion of IgY through intravenous route is found to be safe because no adverse hematological or biochemical effect was noted in the animal with and without envenomation. It is concluded that IgY produced in an almost pure form is safe and efficient for treating snake bite rabbit models which needs to be tested in limited clinical trials for patient use.

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Ethics: Institutional Animal Ethics Committee (IAEC-SCTIMST) approval was obtained for conducting animal experiments (development of antivenom in hens and rabbit animal model of snake bite) which conforms to CPCSEA regulations of Government of India.

Permission was obtained from the Department of Forest and Department of Narcotics, Government of Kerala, India for milking venom from Russell's viper for research purposes

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