Original Article

Antagonistic Effects of Suramin Against the Venom of Snake, *Echis Carinatus*, on the Circulatory System of Developing Chicken Embryos

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ABSTRACT

Background: The snake, *Echis carinatus,* one of the most venomous snakes in Asia, possesses a deadly hemotoxic venom. It has been reported that suramin, an anti-trypansomiasis drug, can inhibit the toxic effects of some snake venoms. This study was conducted to evaluate the possible antagonistic effects of suramin against the hemorrhagic activity of the venom from an Iranian snake, *Echis carinatus,* in developing chicken embryos.

Methods: One day old fertile eggs (n=250) were incubated for six days at 37ºC and 60% humidity. Paper discs (5 mm diameter) containing different concentrations of *E. carinatus* venom (5, 10, 20 & 30 µg) were placed on the chorioallantoic membrane over the major bilateral vein and were left in place until hemorrage occurred and the embryos died.

Results: We found the standard hemorrhagic dose (SHD) of *E. carinatus* venom to be (5 µg/disc). Various concentrations (5, 10 & 20 µg) of suramin were tested against SHD of *E. carinatus* venom in different protocols. Control experiments were performed with the buffered saline solution, venom and suramin individually. The results demonstrated that suramin at 5, 10 and 20 µg significantly ($P<0.05$) delayed time to death (717, 521 & 208 min) of embryos poisoned with *E. carinatus* venom in a dose-dependent manner.

Conclusion: Suramin exerted protective and inhibitory effects against the deadly *E. carinatus* venom, and therefore, may potentially offer future therapeutic applications against poisoning with *E. carinatus* venom.

Keywords: Antagonistic Property, Chicken Embryos, Embryonal Hemorrhage**,** Suramin, *Echis Carinatus.*

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INTRODUCTION

Snake bite is a serious public health concern especially in rural areas and can be fatal if not treated promptly and properly. Snake venoms with hematoxic effects are rich in different factors including a variety of proteins and peptides that affect hemostasis [1-3]. The venom of *Echis carinatus* (*E. carinatus*) contains proteins and peptides such as metalloproteinase, phospholipase A_2 (PLA_2), L-amino acid oxidase, C-type lectins, disintegrins and other toxins [4]. These components exert both coagulation and anticoagulation effects; reduce most clotting factors, including II, V, VIII, XIII, and through direct activation of prothrombin, can affect blood coagulation [5, 6]. *E. carinatus* is considered to be one of the most deadly snakes in the world, predominantly in Asia and Africa. Severe hemorrhagic diathesis is the leading symptom of the venom. It is believed that this snake could be responsible for more human deaths than cobras, mambas and rattlesnakes together [5, 7]. Although there is little definitive information on the incidence of snake bites or total fatality rate due to *E. carinatus* venom, this snake is certainly responsible for many dangerous bites in Iran [8]. In one report examining 103 poisonous bites in the southern Iran, *E. carinatus* was one of the major causes of snake bite poisonings [9]. The conventional treatment for the poisoning is the administration of anti-snake venom (ASV); however, it is costly and has a high incidence of adverse reactions in the victims [10].

Suramin is a hexasulfonated derivative of napthylurea, is classed as a thrombin inhibitor, and was originally synthesized as an antiparasitic agent [11-13]. Previous studies have shown that suramin can effectively inhibit haemostatic changes caused by *Bothrops jararaca* snake venom, both *in-vitro* and *in-vivo* [12]. In addition, suramin inhibits some presynaptic PLA_2 neurotoxins like β-bungarotoxin and crotoxin *in-vivo* and *in-vitro* [14, 15]. Suramin significantly delays the time to paralysis induced by β-bungarotoxin in mice when administered intravenously 30min before injecting the toxin and delays the blocking of transmitter release *in-vitro* [14]. In 2000, Lin and colleagues reported that suramin protected the murine motor nerves from the toxic effects of the presynaptic Ca^{+2} channel inhibitor, omega-conotoxin MVIIC and omega-agatoxin IVA, and reduced their depressant effects on muscle contraction [16].

Furthermore, suramin has been shown to interfere with the pharmacologic effects of some snake venoms, such as the myotoxic and paralyzing effects of bothrops toxin-I [17] and some crotalid venoms [18]. Moreover,

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one study in 2008 [19] has reported on the ability of suramin to antagonize the cardiotoxic, proteolytic and phospholipase A_2 activities of *Bothrops jararacussu* crude venom on the rat heart. Additionally, *in-vivo* tests have shown that suramin antagonizes the effect of *E. carinatus* venom in mice and increases the survival time after the venom poisoning [20]. Further, Fathi *et al*. [21] have shown that suramin inhibits the early effects of PLA_2 neurotoxins in mouse neuromuscular junctions. In another recent study, Kuruppu and colleagues [22] reported that suramin prevented the *in vitro* neurotoxic effects of the three presynaptic neurotoxins in taipan venoms including taipoxin, paradoxin and cannitoxin.

To date, there has been no study into the potential efficacy of suramin against *E. carinatus* venom. Therefore, this is the first study to investigate the effect of suramin in the neutralization of the hemorrhagic activity of *E. carinatus* venom in the circulatory system of developing chicken embryos.

MATERIALS AND METHODS

Materials and Venom

Suramin was purchased from Sigma-Aldrich Chemicals (Sigma Chemical Co. Ltd., Poole, Dorset, England), and Lyophilized crude *E. carinatus* venom was a gift from the Department of Venomous Animals and Antivenom Products at Razi Vaccine and Serum Research Institute, Karaj, Iran.

One day old fertilized eggs (n=250) were purchased from Dizbad Broiler Breeder Company (Mashhad, Iran). They were incubated in Korean automatic digital egg incubator R-COM20 series (New PX-20D) in vertical position at 37ºC and 60% humidity (Figure 1). A DIY candler device was used to determine the air sac, growth and development of embryos.

Figure 1. The fertilized eggs were incubated in vertical position at 37ºC± 0.5 and 60% humidity.

The holes were covered with parafilm.

Preparation of Discs

Using whatman No. 2 filter papers and a manual puncher, 5 mm diameter blank discs were made. They were kept under UV light for two hours to be sterilized prior to commencing the inoculation of samples. Varying concentrations of suramin, venom or a mixture of both were dissolved in saline solution and were inoculated onto the discs, which were then allowed to dry sufficiently before commencing the tests.

Preparation of Eggs

The eggs were taken out of the incubator on day six, their outer shells were cleaned with 70% ethanol. To prevent any possible damages they candled and marked to determine the location of embryo, air sac and large vessels. Using a sterile scalpel, bend forceps and small scissor; a small hole was made on the marked area of the blunt head of the eggs. For easy access and observation of vessels, the hole was carefully widened to about 2cm in diameter and covered with parafilm. It should be noted that, 90% of embryos remained alive at least for seven days and 50% of chicks hatched. Using a forceps, the impregnated discs were carefully placed on the lateral vein of the chorioallantoic membrane (CAM) and then the open shell hole was carefully covered with parafilm, and eggs were incubated for a certain amount of time.

Statistical Analysis

Mean ± standard deviation of the survival time for embryos were reported in each group. The comparison of survival time between groups was performed, using one way ANOVA followed by post hoc Dunnett's test. *P*-values less than 0.05 were considered significant. All statistical analyses were performed by SPSS software, version 18.

RESULTS

Effect of Saline Solution and Suramin Alone on Survival Time of Chicken Embryos

Prepared discs were impregnated with 10 µl buffered saline solution and placed on the yolk sac membrane over a major bilateral vein (n=6). In another control experiment, 10 µl of buffered saline solution was spread on the same area (n=10). To investigate the effect of suramin alone, the disc containing 10 µg suramin was tested on 10 eggs. The results showed that suramin and saline solution did not exert negative or fatal effects on the growth of the embryos for 8 and 9 days, respectively.

Effect of E. Carinatus Venom on Survival Time of Chicken Embryos

Prepared discs of various concentrations (5, 10, 20 & 30 µg) of *E. carinatus* venom were placed on the yolk sac membrane over a major bilateral vessel and the time to the embryos death (i.e., stopping heart beats) was recorded for each concentration (n=6 to 10). The results showed that *E. carinatus* venom killed all of the embryos in a dose-dependent manner. The average time to death for the 5, 10, 20 and

30 µg/disc concentrations was 115, 72, 56 and 35 minutes, respectively (Figure 2).

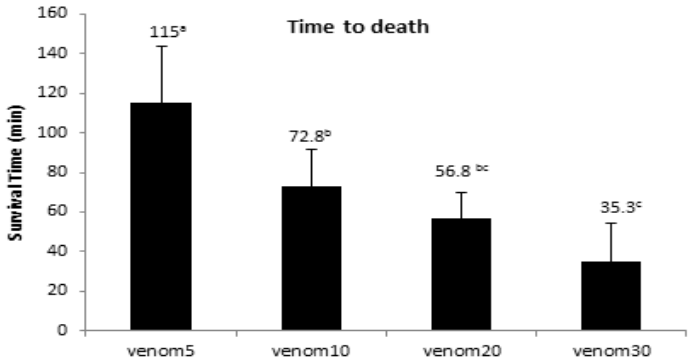

Figure 2. Mean and standard deviation of survival time with different concentrations of venom (µg/disc).
[ab] Values followed by different superscript letters are significantly different (P < 0.05)

Standard Hemorrhagic Dose of E. Carinatus Venom

To determine the SHD of *E. carinatus* venom, the concentration of venom required to create a 2 mm hemorrhagic corona in 120 min, different concentrations (5, 10, 20 & 30 µg/disc) of venom were tested. The discs were impregnated with mentioned concentrations of venom and placed on the yolk sac membrane over a major bilateral vessel. The hemorrhagic coronas were measured with a transparent ruler after 120min and recorded. The tests for each concentration were repeated on at least seven eggs. The average time to cause a 2mm hemorrhagic corona was 28, 50, 69 and 117 minutes for 30, 20, 10 and 5 µg/disc concentrations, respectively (Figure 3). It should be noted that in several tests, embryos death occurred before the hemorrhagic cornea reached 2 mm. Based on this experiment, SHD of *E. cariaitus* venom was confirmed at 5 µg/disc and thus the same concentration was used in all other experiments.

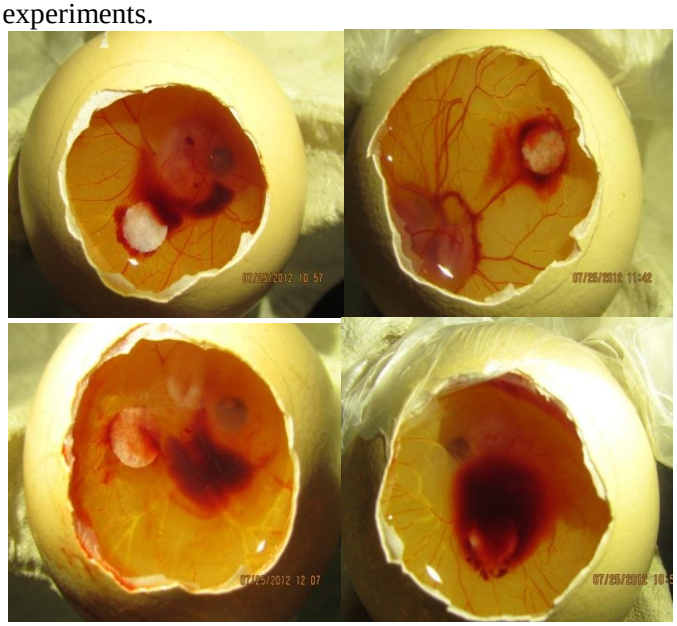

Figure 3. Determination of *E. carinatus* venom SHD, using different concentrations of venom to create a 2 mm hemorrhagic corona within 120 min. *Top row*: left – 5 µg; right – 10 µg. *Bottom row*: left – 20 µg; right – 30 µg of *E. carinatus* venom per paper disk.

Effect of Suramin on SHD of E. Carinatus Venom

Different concentrations of suramin (5, 10 & 20 µg) were mixed with the SHD of *E. carinatus* venom, incubated for 30 minutes and finally used to inoculate individual discs. The impregnated dry discs were placed on CAM large vessel and the time to embryos death was recorded for each concentration (n=6-10). The results showed that suramin at 5, 10, and 20 µg significantly increased the time to death of the embryos to 208, 521 and 717 min, respectively; in comparison with those in the control group (Figures 4 & 5).

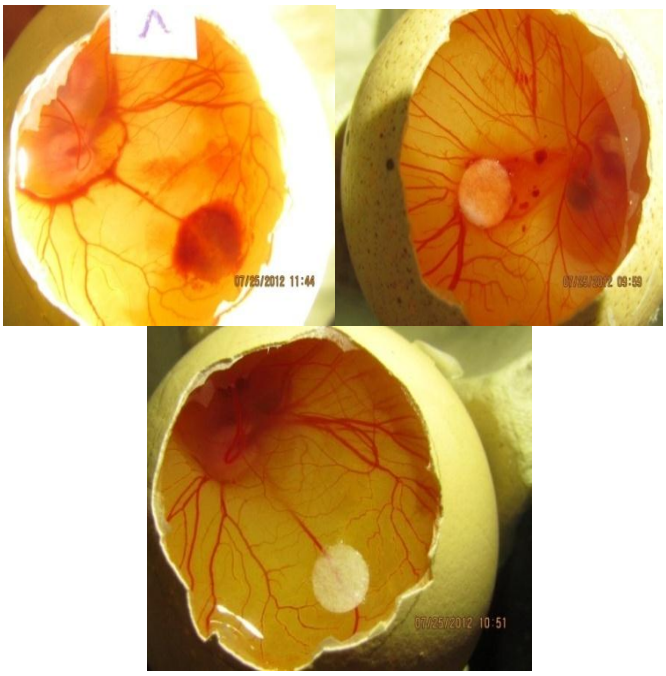

Figure4. Effect of different concentrations of suramin on SHD. Left: 5 µg, Middle: 10 µg and Right: 20 µg, plus 5µg *E. carinatus* venom in each paper disc.

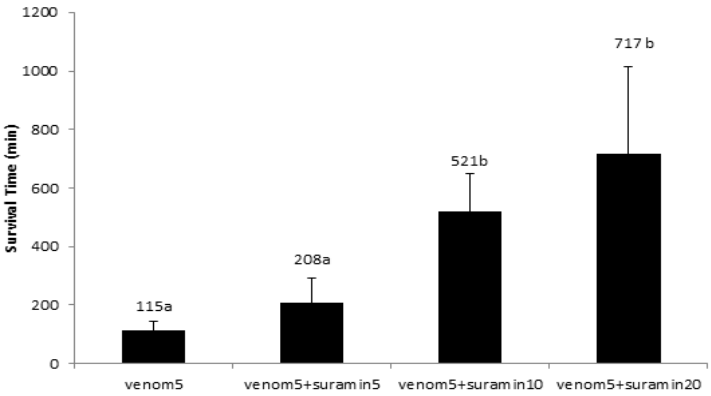

Figure 5. Mean and standard deviation of survival time (min) while using SHD of *E. carinatus* venom (5 µg/disc) accompanied by different concentrations of suramin.
[ab] Values followed by different superscript letters denote significant differences at P < 0.05.

DISCUSSION

Treatment of choice against snake poisoning by *E. carinatus* venom is the use of ASV. The success of this treatment is highly dependent on the administration of ASV as soon as possible after the snake bite. Obviously, patients' prompt access to effective medical care and receiving appropriate anti-venom therapy prevents or delays the development of serious symptoms following the snake bites. A routine and WHO-approved test to establish anti-venom efficacy and to neutralize the induced hemorrhage, is the rodent intra-dermal skin test. However, this test is associated with the use of a large number of animals, often neglecting their misery.

Undeveloped reflex arcs in chicken eggs at an early developmental stage, which is necessary for pain sensation (usually before gestation day 11) along with highly vascularized yolk sac membrane, make this model a suitable, time saving and low-cost assay for the study of hemorrhagic effects of venoms. Also, there is high correlation among the results of the rodent and chicken egg tests [23, 24].

The number of studies on SHD of different hemorrhagic or neurotoxin venoms are limited. For *E. ocellatus* venom, the concentration was 3 µg/1.5 µl, as reported by Asuzu in 2003 [25]. However, in 2006, Ode and Asuzu reported that the SHD of this venom was 2.8 mg/ml that is equivalent to 4.2 µg/1.5 µl [27, 28]. The reported concentration for *Naja naja karachiensis* venom has been 2.9 µg/1.5 µl [28]. In this study, we found that SHD of *E. carinatus* venom was about 5 µg (in 10µl of saline solution) per paper disc. This study is the first of its kind to investigate the ability of suramin to antagonize the heamotoxic effect of *E. carinatus* venom on the circulatory system of developing chicken embryos. The results of this study clearly established that suramin had antagonistic effects against the venom of the Iranian snake, *E. carinatus*.

Previous studies have shown that surmain inhibits the effects of some toxins and venoms, such as pre-synaptic neurotoxins, myotoxins and hemotoxins including *E. carinatus* venom. However, the mechanism of action has not been clearly described [14, 20-22]. Lin-Shiau and Lin, (1999) proposed that suramin could delay the neuromuscular paralyzing effect of β-bungarotoxin and crotoxin. However, they concluded that the action of suramin was not related to its effect on acetylcholinesterase (AChE) activity, compared to neostigmine which blocks this neurotransmitter [14, 29].

In mouse hemi-diaphragm nerve-muscle preparations that are partially paralyzed by high Mg^{+2}, suramin alters the triphasic action of β-bungarotoxin and taipoxin, and inhibits the facilitatory effect of these PLA_2 neurotoxins [21]. It has also been reported that suramin inhibits the PLA_2 activity of *Bothrop sjararacussu* snake venom in a dose-dependent manner [19]. Since *E. carinatus* venom contains PLA_2 [30], it is possible that suramin counteracts this venom in the same manner as it does to other PLA_2 venoms. The mechanism of action of PLA_2 toxins is not well understood, and, therefore, it is not easy to explain the mechanism of suramin's inhibitory action at this point [15].

It is possible that suramin competes with *E. carinatus* venom components to bind to certain receptors in target cells, such as platelets, red blood cells, and vascular endothelia. Arruda *et al*. (2002) has reported that suramin has significant anti-myotoxic effects against some crotalid snake venoms that contain basic myotoxic phospholipase A_2 [18]. They indicated that suramin was less effective in antagonizing the myotoxic effect of some other tested venoms with highly active hemorrhagic property. Their findings are contradictory to ours regarding the suramin's antagonizing effect on the potent hemorrhagic components of *E. carinatus* venom.

Also, it is possible that suramin directly interacts with the toxins present in the venom. Suramin is a polysulfate anionic compound with rich negative molecular charges. These charges can directly interact with positive charges present in proteins and polypeptides molecules of many snake toxins. This could cause structural changes in the toxins' molecule, thus preventing its binding to the target sites.

Suramin has been previously reported to have a protective role against *E. carinatus* venom in mice when injected intra-peritoneally, causing a delay in the time to death of the mice [20]. It was also noted that suramin considerably decreased the internal bleeding and reduced the pathological damages (unpublished results). The effects of venom and suramin were dose-dependent; i.e., by increasing the venom dosage, the survival times of tested mice were decreased, whereas an increase in the suramin dosage prolonged the survival [20]. This is consistent with the results of this study, where the incidence of bleeding increased with a rise in the venom concentration. Suramin reduced the hemorrhagic effect of the venom on tested chicken embryos' blood vessels and prolonged their survival in a dose-dependent manner (Figure 3).

As we concluded in 2010, suramin somehow prevents the *E. carinatus* venom from strongly binding to their receptors, slowing its effect on some tested animals, thus causing improved recovery [20]. It appears that suramin works through the same mechanism of action to interact with *E. carinatus* venom and reduce hemorrhage in the chicken embryos.

For hemotoxic venoms, hemorrhages are principally caused by zinc-dependent metallo-protinase enzymes. They have a cytotoxic effect on endothelial cells by disrupting proteins in microvessels' basement membrane, affecting components of the hemostatic system, demeaning proteins of extracellular matrix and provoking local and systemic hemorrhages [31, 32].

By eliminating the zinc, for example with a chelator, the venom has lost its proteolytic and hemorrhagic activities due to structural alterations [33]. Suramin has many negative charges and may interact with metallo-protinases, remove its zinc ions and changes the

molecular configurations; therefore, causing reduced hemorrhagic activity by *E. carinatus* venom.

CONCLUSSION

The results of this study demonstrated that suramin has protective and inhibitory properties against the fatal effects of *E. carinatus* venom. This drug can postpone the lethal effect of *E. carinatus* venom by slowing down the hemorrhagic effects and; therefore, may have potential therapeutic applications against snake bites poisoning.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in conducting this study.

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