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Naja nigricincta nigricincta venom, a murine model. Evaluation of skeletal and cardio-myonecrosis, kidney injury and inflammatory response along with neutralisation efficacy by the SAIMR/SAVP - And EchiTAb-Plus-ICP polyvalent antivenoms

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ABSTRACT

African spitting cobra, *Naja nigricincta nigricincta* (Zebra snake), envenomation is an important cause of snakebite morbidity and mortality in Namibia. The snake is endemic to central and northern Namibia as well as southern Angola. The venom is mainly cytotoxic, resulting in aggressive dermo-necrosis and often accompanied by severe systemic complications. No specific antivenom exists. Rhabdomyolysis, systemic inflammatory response, haemostatic abnormalities, infective necrotising fasciitis as well as acute kidney failure have been documented.

Based on murine models, this study assessed SAVP/SAIMR - and EchiTAb-Plus-ICP polyvalent antivenom neutralisation as well as subdermal necrosis. Additional muscle, cardiac, kidney and lung histology, creatine kinase measurements and post-mortems were performed.

An intravenous median lethal dose (LD50) of Naja nigricincta nigricincta venom was determined at 18.4 (CI: 16.3; 20.52) μg and a subdermal lethal dose at 15.3(CI: 12.96; 17.74)μg. The SAIMR/SAVP polyvalent antivenom median effective dose (ED50) was 1.2 ml antivenom/1 mg venom equating to a potency (WHO) of 1 ml antivenom neutralising 0.63 mg venom and approximately 240 ml (24 vials) needed for initial treatment. The ED50 of the EchiTAb-Plus-ICP was 1 ml antivenom/1 mg venom and a potency of 65 mg venom/ml antivenom (3.3 x LD_{50}), estimating 230 ml (23 vials) for treatment.

Histology and serology (creatine kinase) evidenced venom induced skeletal myotoxicity, which was not prevented by the antivenoms tested. Cardiac myonecrosis, an inflammatory response, direct venom kidney tubular necrosis and cardio-pulmonary failure were documented.

1. Introduction

Snakebite envenomation is a major concern in large parts of the world, and an important neglected public health problem in sub-Saharan Africa (Chippaux, 2011). More so, envenomation can have extremely serious outcomes based on the type of snake, venom, size, age and co-morbidities of the victim as well as time elapsed between bite and initialisation of treatment (Spawls and Branch, 2020; WHO, 2016). One such snake is the zebra snake (*Naja nigricincta nigricincta*), endemic to central and northern Namibia as well as southern Angola. It is responsible for most of the venomous snakebites seen in these areas of Namibia (Griffen, 2001; Marais, 2022).

Like other African spitting cobras, namely *Naja mossambica* and *Naja nigricollis*, *N. n. nigricincta* injects its highly cytotoxic venom into the subdermal fascia layer. Rarely fatal in adults, the necrosis spreads rapidly in a plane between skin and deeper-lying muscles, with the subdermal necrotic spread extending considerably further than the overlying skin discolouration (Griffen, 2001; Buys, 2003; Warrell et al., 1976; Tilbury, 1982). The snake frequents human habitation, both urban and rural, and is commonly found inside dwellings. The majority of bites occur at night while the victims are asleep, often babies and small children who suffer a high mortality (Griffen, 2001; Buys, 2003; Saaiman and Buys, 2019)

The accepted southern African protocol for the treatment of spitting cobra bites *i.e.,* intravenous South African Institute for Medical Research (SAIMR) polyvalent antivenom followed by late debridement, was

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historically part of the routine treatment of *N. n. nigricincta* bites. Even very large doses of up to 200 mL, administered early (within hours) following the bite, were unable to prevent severe disfiguration and function loss. This contrasts with the reported effectivity following *Naja mossambica* envenomation. This protocol was eventually abandoned in favour of a "no antivenom, emergency surgical debridement, antibiotics and supportive treatment" regime, which resulted in much more favourable functional and cosmetic clinical outcomes (Buys, 2003; Tilbury, 1982; Vermaak et al., 2010; Saaiman and Buys, 2022).

It is widely accepted that local tissue damage is poorly neutralised by available antivenoms, and mainly attributed to venom induced vascular damage and oedema, inhibiting antivenom distribution in the affected area (WHO, 2017; Warrell, 2010; Boyer and Fry, 2015; Wood et al., 2016; Gutié et al., 2017a). Children bitten by *N. n. nigricincta* present with severe local signs and symptoms, often accompanied by aggressive and fulminant manifestations of systemic envenomation (Saaiman and Buys, 2022; Saaiman et al., 2023). The etiology of mortality has not been specified and even aggressive supportive management is not always effective. The mortality following envenomation, witnessed in children, emphasize the need for the addition of antivenom targeting mortality and systemic envenomation in this subset of victims. As snakes inject the same volume of venom irrespective of the size (and thus age) of the patient, children require equivalent volumes of antivenom as needed in adults (Buys, 2003; WHO, 2010).

The larger venom exposure per kilogram body weight combined with a smaller vascular volume, result in higher circulating venom concentrations, greater envenomation severity and larger initial antivenom doses needed (Nimorakiotakis et al., 2005; Seifert et al., 2022). Guidelines propose a maximum of 10 ml/kg antivenom (White, 2013). The importance of volume overload in the paediatric snakebite victim dictates the identification of a safe and potent antivenom effecting neutralisation in low volumes.

Most prescribed pharmacological substances in medicine are subject to strict pharmacological safety guidelines. Even though antivenom and the availability thereof are proclaimed to be the corner stone of snakebite management (WHO, 2010), very few antivenoms have been properly validated with pre-clinical efficacy test and clinical trials (Boyer and Fry, 2015; Harrison et al., 2017). Especially in the paediatric population, when confronted with a compromised critically ill patient, safety and specificity are of the utmost importance. Administrating antivenom without proven neutralisation efficacy or administering sub-optimal volumes of effective antivenom to snakebite victims in the expectation of improving outcome, are without scientific foundation as the benefits don't outweigh the risks and cannot be ethically and medico-legally supported (Warrell, 2010; Ahmed et al., 2008).

Identifying a safe and specific antivenom is a matter of priority. But which antivenom? Regarding the SAIMR/SAVP polyvalent there are a few problematic considerations. Previous clinical experience indicated inefficiency against dermonecrosis. Specificity and purity of the SAIMR/SAVP polyvalent is controversial as evidenced by an adverse reactions rate of 42.9%, with children younger than twelve exhibiting a 58.3% chance of developing acute adverse reactions (Blaylock, 2002, 2005; Wood et al., 2009; Pattinson et al., 2018; Moran et al., 1998). The package insert of above polyvalent list *Naja mossambica* as the only spitting cobra whose venom is included in the manufacturing and claims effectiveness against the venom of all spitting cobras. There are however no published data on SAIMR/SAVP- *N. n. nigricinta* efficient dose (ED_{50}) and neither does the producers seem to possess specific data (personal communication with SAVP).

Another antivenom manufactured for the sub-Saharan African market and potentially available is the EchiTAb-Plus-ICP polyvalent antivenom. Recent producer data (March 2022) claim an ED_{50} (4 x LD_{50}) of 0.6 (0.4–0.7) mg/ml [1.42 ml/mg] [LD_{50} = 27.0 (19.1–40.2) μg/18-g mouse] against *N. n. nigricincta* venom (Harrison et al., 2017; Menzies et al., 2022; Petras et al., 2011; Sá et al., 2017) and Manufacturers in formation.

Neutralisation often relies on the immunological cross-reactivity exhibited by the antibodies contained in the antivenom to neutralise the venoms of any additional species phylogenetically related to the species against which the antivenom was developed (Gutié et al., 2012). The venom proteomes of five African spitting *Naja's* (*Naja nigricollis*, black-necked spitting cobra; *Naja katiensis*, Malian or Katian spitting cobra; *Naja pallida*, red spitting cobra; *Naja nubiae*, Nubian spitting cobra and *Naja mossambica*, Mozambique spitting cobra) have very similar chromatographic profiles and compositional trends. Three finger toxins (3FTx) and cytotoxic phospholipase A_2 (PLA_2) molecules account respectively for 67–73% and 22–30% of the total venom proteins (Petras et al., 2011). Therefore, antivenom developed against the venom of one African spitting *Naja* species, in theory, should likely exhibit para-specific neutralisation towards the venom of other African spitting cobra species (Petras et al., 2011), even if not 100% as effective as monovalent antisera developed for the particular species (Gutié et al., 2017a; WHO, 1990; WHO, 2023). Based on WHO pre-clinical efficacy mice-models studies, neutralisation of mortality and different envenomation pathologies following *N. n. nigricincta* envenomation by the SAVP/SAIMR - and EchiTAb-Plus-ICP polyvalent antivenom were evaluated (WHO, 1990).

Identification of ensuing envenomation pathologies as well as clarifying the pathogenesis of mortality will ensure targeted supportive treatment. Rhabdomyolysis, indicated by early onset raised CK, AST. ALT and LD values along with an inflammatory response with high CRP values, abnormal leucocytes and hypoalbuminemia are frequently reported (Bhagwat and Amar, 2013; Chatzizisis et al., 2008; Gounden et al., 2020; Gutié et al., 2003, 2017b; Hunter et al., 2006; Morales De Guzman, 2024; Peralta, 2020; Sauret et al., 2002a; Torres et al., 2015). A clinical bleeding tendency, raised INR values, thrombocytopaenia and positive d-dimers can develop after 12–24 h. Infective necrotising fasciitis, acute kidney failure and sub-arachnoidal bleeding have also been documented (Personal communication Dr's Saaiman and Buys).

In clinical practice, fatal bites in children tend to progress rapidly from striking local swelling, to a severe systemic inflammatory response, shock, cardiopulmonary arrest and death (Namibian clinical experience). Even though a severe inflammatory response can progress to multi-organ failure and cardiac arrest, the cardio-pulmonary arrest following within 36 h after the bite, against the backdrop of systemic myotoxicity, raises the possibility of concomitant direct venom induced cardio-myonecrosis (Namibian clinical experience) (Abdou et al., 2015; Aisenberg and Fred, 2016a; Hoffman et al., 1993; Ghani et al., 2010; Verma et al., 2017). Only a few case reports of skeletal myotoxicity accompanied by cardiac muscle damage following envenomation by Crotalus durissus terrificus (South American rattle snake)

and Micrurus surinamensis (Aquatic coral snake) have been reported (Cupo et al., 1990; De Siqueira et al., 1990; De Paula Gonç et al., 2021).

The aim of this study was to assess the feasibility of adding one or both of these antivenoms to the treatment regimen, as well as the assessment of ensuing envenomation pathologies. For this, the median lethal dose (LD_{50}) for *N. n. nigricincta* venom and the median effective (ED_{50}) dose of both the SAIMR/SAVP- as well as the EchiTAb-Plus-ICP polyvalent antivenoms were determined, using the WHO proposed pre-clinical mouse efficacy assays (WHO, 1990). A subdermal (subcutaneous) venom necrosis assay was undertaken to evaluate subdermal necrotic spread and calculate the sub-dermal median lethal dose ($_{SD}LD_{50}$). Post-mortems, histological examination were performed on the envenomated mice with the aim of shedding light on the total envenomation profile, the possibility of cardiac myotoxicity and corroboration of our clinical findings, *i.e.:* rhabdomyolysis, DIC, kidney injury and a severe inflammatory response.

2. Methodology

The Animal Ethical Committee, Faculty of Veterinary Science, University of Pretoria granted ethical approval (REC215-19). Fresh freeze-

dried venom, from 3 zebra snakes in captivity, was purchased from African Venom and Reptiles (official venom supplier for the South African Vaccine Producers). SAIMR/SAVP polyvalent antivenom was bought from the South African Vaccine Producers and Echi-TAb-Plus-ICP polyvalent antivenom from the Instituto Clodomiro Picado, San Jose, Costa Rica.

This mice model consisted of groups of five, 18–20 g, CD1 male mice. The mice, obtained from Onderstepoort Biological Products (OBP), were weighed, marked via ear notch and given routine Ivermectin. They were single housed in conventional Type II mouse cages with sawdust as bedding, autoclaved toilet rolls, mouse houses, wooden sticks and egg containers for enrichment. Room temperature was kept between 22 and 24 °C and humidity between 40 and 60%. A light cycle of 12 light and 12 dark hours was maintained. The mice were fed EPOL rodent pellets and given reverse osmosis water ad-lib. Local anaesthetic cream (*Emla*®) was applied to the tails for tail vein injection. The mice were placed under an infra-red light to keep warm. The mice were restrained in appropriate restrainers whilst the solution was slowly injected. The mice for sub-dermal injection were anaesthetised with Sevoflurane. Mice were monitored for swelling, dyspnoea and change of habitus. On deterioration or any indication of severe pain, distress, suffering, or impending death, mice were terminated. Animals were euthanised by sevoflurane *(Ultane)* overdose and subsequent cardiac puncture for blood sampling.

The intravenous LD_{50} study entailed 5 μg, 10 μg, 20 μg and 40 μg venom (per group) diluted to 0.2 ml administered in the tail vein. For the median effective dose (ED_{50}) study, antivenom/venom combinations of 80 μg venom mixed with specific antivenom doses were diluted into 0.2 ml aliquots, incubated for 30 min at 37° Celsius before administration into a tail vein. Doses of 60, 80, 100 and 120 μl SAIMR/SAVP, and 40, 60, 80 and 100 μl EchiTAb-Plus-ICP polyvalent antivenom per group of mice were used. The control group was exposed to 80 μg venom only. (SAIMR/SAVP- 3.2 LD50; EchiTab- 3.3 LD50; Control- 3.1 LD50). In the sub-dermal necrosis study, groups received 0.2 ml sub-dermal injections of 25 μg, 50 μg, 100 μg and 200 μg venom as well as 0.1% methylene-blue saline solution (control group). Mice were terminated after 24 h and the skin lesion measured during necropsy.

Creatine kinase (CK) measurements were done upon death or euthanasia (Sevoflurane overdose) on all mice from the median lethal dose (LD_{50}), median effective dose (ED_{50}) and sub-dermal necrosis studies. Blood samples taken via cardiac puncture, were transferred into paediatric clotting tubes. One hour clot formation was evaluated via gentle tilting, followed by serum CK measurement by the Clinical Pathology Department. The mean CK concentration derived from the methylene blue control assay was taken as representative of the normal value.

Histology from skin and tissue surrounding subdermal injection sites, hind limb muscle, kidney and cardiac tissue from the 20 μg and 40 μg IV groups, the methylene blue-saline sub-dermal study as well as the SAIMR/SAVP 80/100, 80/120, EchiTAb-Plus-ICP 80/80 and 80/100 groups as well as lung tissue samples from 20 μg to 40 μg IV groups were collected and processed according to standard operating procedures and assessed by a board-certified veterinary pathologist. Necropsies were performed by a veterinary anatomical pathologist on selected animals from the LD_{50}, ED_{50} and subdermal necrosis studies, one day post venom administration.

Mice reactions following venom administration were closely monitored and documented.

3. Results

3.1. Median lethal dose LD_{50}

3.1.1. Intravenous

Zero mortality was recorded in the 0, 5, and 10 μg groups. One death was recorded for the 20 μg group. All the animals of the 40 μg group displayed signs of severe swelling, dyspnoea and loss of motor-function within 30 min following venom injection.

LD_{50} and ED_{50} doses were calculated by the online AAT Bioquest Inc. Quest graph LD50/ED50 calculator (Quest-Graph 2024). The formula: LD_{50} + the standard error (SE); was used to calculate the 95% confidence interval (Mohammad, 2022). The intravenous lethal dose ($_{IV}LD_{50}$) was determined 1.02 μg venom/gram mouse (95% CI = 0.90; 1.14) and 18.40 μg (CI: 16.30; 20.52)/18-g mouse (Fig. 1).

3.1.2. Sub-dermal

Only one mouse from the 25 μg group survived while all the other died or had to be terminated. The higher the venom dose, the shorter the survival time, with a mean survival time of 1.06 h (range of 0.83–1.26) in the 200 μg group. The animals developed rapid and severe swelling and dyspnoea, with congested blood vessels and seizures.

The $_{SD}LD_{50}$ was calculated at 0.85 μg venom/gram mouse (95% CI = 0.72; 0.98), thus 15.30 μg/18 g mouse (CI: 12.96; 17.74). (Fig. 2).

3.2. Median effect dose (ED_{50})

3.2.1. SAIMR/SAVP polyvalent antivenom

The $_{SD}LD_{50}$ was calculated at 0.85 μg venom/gram mouse (95% CI = 0.72; 0.98), thus 15.30 μg/18 g mouse (CI: 12.96; 17.74) (Fig. 3).

3.2.2. EchiTAb-Plus-ICP polyvalent antivenom

The 80 μg venom represents 3.3 times the LD_{50}. The EchiTAb-Plus-ICP ED_{50} was calculated at 78 μl or 1 μl EchiTAb-Plus-ICP polyvalent/1 μg of *N. n. nigricincta* (1ml/1 mg) (Fig. 4).

3.3. Myotoxicity

3.3.1. Creatine kinase (CK)

Four times the normal CK value was used for estimation of the minimum myotoxic dose (MMD) (WHO, 1990). The CK fraction of elevation above normal were plotted against venom dosages on an excel graph, with the MMD equivalent to the venom dose causing a four-fold increase in CK values over the control calculated at 14 μg/mouse (≈0.54 μg venom/gram mouse).

Values as high as 23.2 times the normal value were recorded in the 40 μg IV group, with 83 times the normal value reported from the 50 μg subdermal group. CK values derived from mice allocated to the sub-dermal injection studies were 3.6–6 times higher than values from mice of near-equivalent intravenous dosages (Fig. 5).

Higher venom dosages and longer survival times resulted in higher CK values. Mean CK concentrations per group of mice that survived were 1.194 times normal, whilst those per group of mice that died were 58.7 times normal (Fig. 6).

Venom only dosages of 80 μg resulted in mean CK values of venom

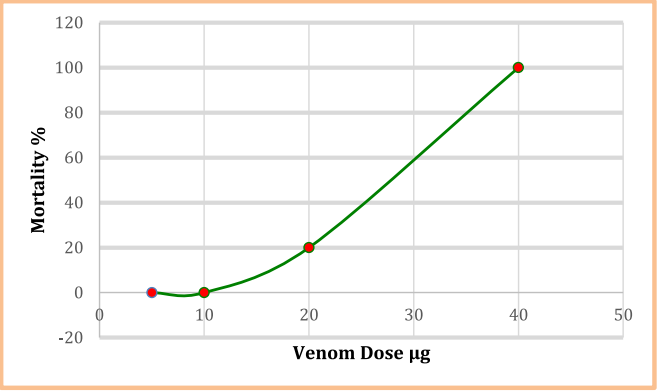

Fig. 1. Percentage mortality (%) plotted against intravenous venom dose in μg.

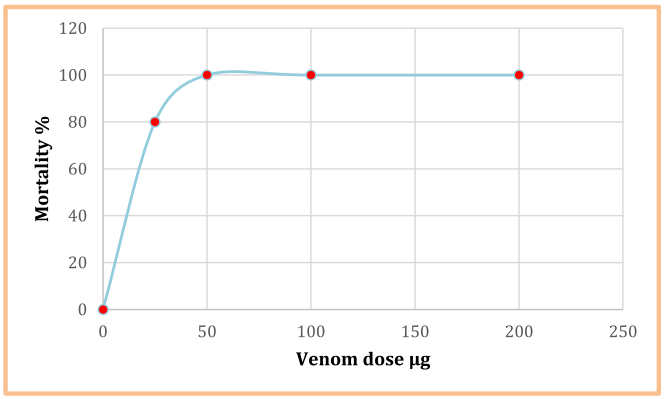

Fig. 2. Percentage mortality (%) plotted against sub-dermal venom dose in µg.

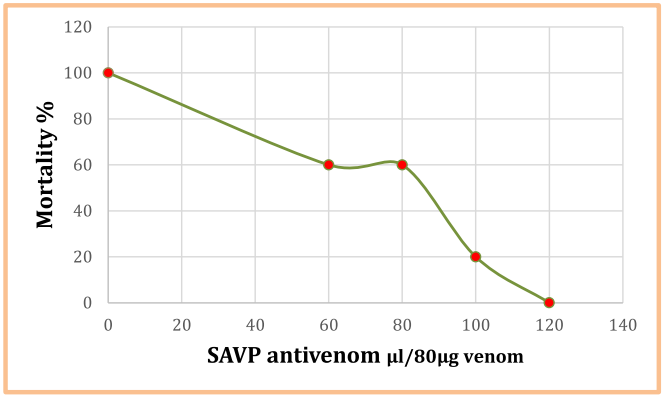

Fig. 3. Percentage (%) mortality plotted against dosage of SAIMR/SAVP polyvalent in µl per 80 µg venom.

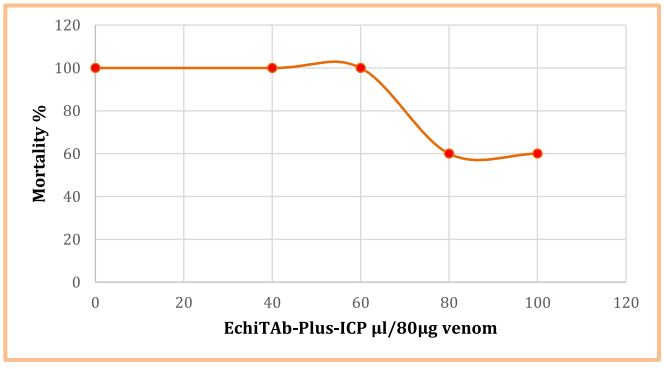

Fig. 4. Percentage (%) Mortality plotted against dosage of EchiTAb-Plus-ICP polyvalent in µl per 80 µg venom.

11.9 times normal. The CK values were plotted against antivenom per 80 µg venom dosages on excel graphs. The SAIMR/SAVP MMD_{50} (the volume of SAIMR/SAVP polyvalent antivenom per 80 µg venom causing a 50% reduction in CK values) equaled 108 µl antivenom per 80 µg venom or 1.35 µl per µg venom. The corresponding Echi-TAb-Plus-ICP MMD_{50} equaled 78 µl antivenom per 80 µg venom or 0.97 µl antivenom per µg venom (Figs. 7 and 8).

CK concentrations measured from the mice that died or were terminated, originating from the SAIMR/SAVP 80 µg/120 µl as well as the Echi-Tab-Plus-ICP 80 µg/80 µl and 80 µg/100 µl groups, were below the 4 times normal limit.

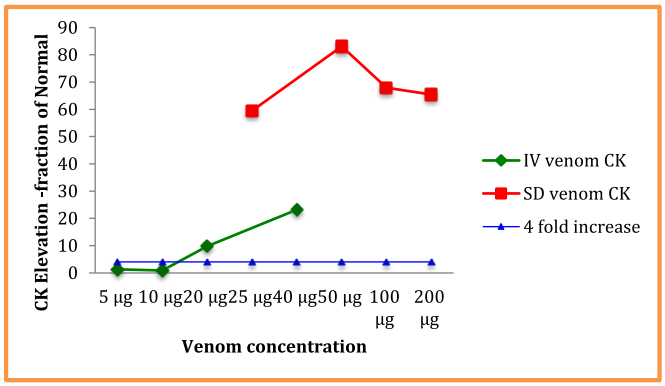

Fig. 5. Plot of multiple of CK value increase above the control group for venom concentration via the intravenous and subdermal route.

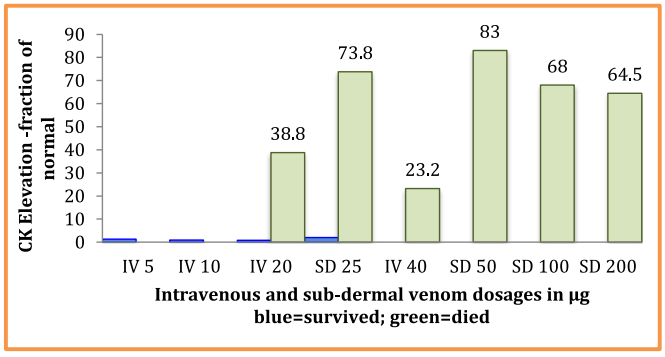

Fig. 6. Histogram: Factor of CK increase; mice that survived vs. mice that died.

3.4. Cardiac and skeletal muscle histology

Histology evidenced venom induced cardiac and skeletal muscle necrosis, also present in the venom-antivenom samples (Fig. 9). Local muscle necrosis was witnessed in the surrounding subdermal injection tissue samples.

3.5. Kidney, lung and skin histology, clot evaluation and post-mortems

Histology and postmortems evidenced venom-induced kidney tubular necrosis. Inflammatory cellular infiltrates, surrounding the subdermal injection and pulmonary leukostasis were reported. Cardiopulmonary failure was implicated as cause of death by both necropsy findings and mice reactions.

Methylene blue subdermal injection resulted in a blue dye skin

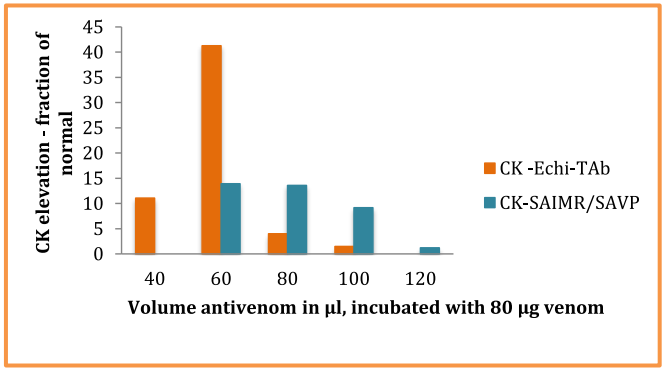

Fig. 7. Histogram: CK elevation per group following intravenous venom and SAIMR/SAVP or Echi-TAb antivenom administration.

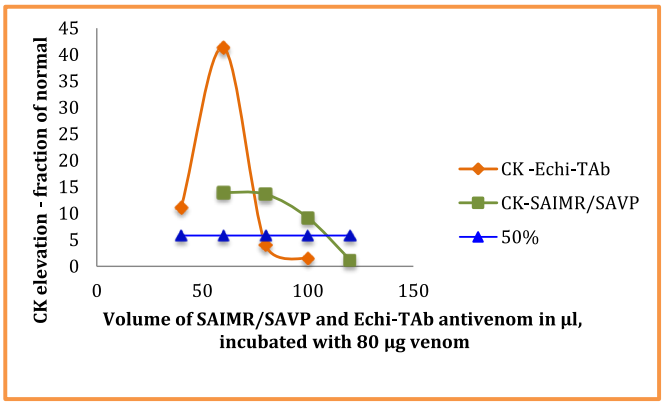

Fig. 8. CK elevation per group following intravenous venom and SAIMR/SAVP or Echi-TAb antivenom administration.

discolouration of 1 cm in diameter (after 24 h). The 200 µg subdermal venom injection study prompted subcutaneous congestion, haemorrhage and oedema, that spread within 15–65 min from the injection site, at the dorsal inter scapular midline, to the lateral thoracic wall with a diameter of 3–4.5 cm.

Histological examination of all the injection sites revealed varying degrees of subcutaneous skeletal muscle necrosis, fibrin-rich oedema, mixed inflammatory cellular infiltrates and occasional areas of free haemorrhage.

Non-clotted blood was reported in two mice from the 40 µg IV venom group. All the blood samples from the sub-dermal study clotted.

4. Discussion and clinical implications

4.1. Lethal dose and neutralisation

The lethal dose of 18.4 µg (CI: 16.3; 20.52) per 18-g mouse as established by this study is less than the doses published for *Naja mossambica at* 22 µg (13–32) and *Naja nigricollis* at 23 µg (20–27) (Petras et al., 2011) and 25.4 µg (20–29) (Theakston and Reid, 1983) (Equation 1). Despite the similar proportions of venom protein types (e.g., 3FTx

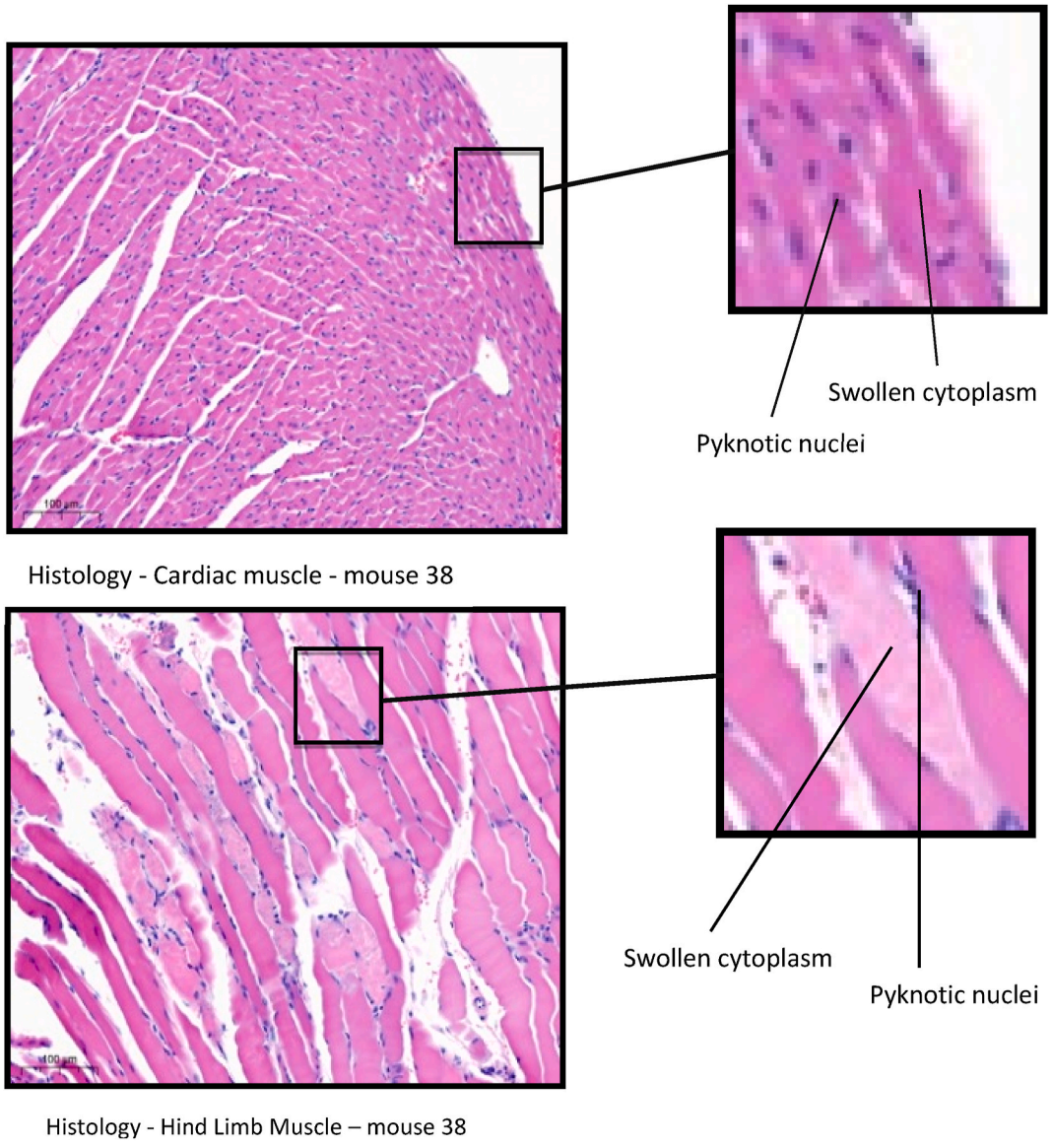

Fig. 9. Histological slide of cardiac and skeletal muscle depiction the swollen cytoplasm and pyknosis.

and PLA_2) demonstrated between African pitting cobras, this difference in lethality highlights the existence of specie specific iso- and proteo-forms within main protein groups, exhibiting distinct toxicological and immunological profiles (Petras et al., 2011) This may also explain the differences seen in the envenomation profiles between individual *Naja* sub-species and the limited specificity and poor cross-neutralisation evidenced by this study (Gutié et al., 2017a).

Equation 1. Potency of the SAIMR/SAVP against Naja nigricincta nigricincta venom

Equation 2. Potency of EchiTAb-Plus-ICP polyvalent antivenom vs. Naja nigricincta nigricincta venom

The median effective dose (ED_{50}) of the SAVP/SAIMR polyvalent antivenom in neutralising *N. n. nigricincta* venom was calculated as 1.2 ml antivenom/1 mg venom (63 mg venom/ml antivenom) (3.2 xLD_{50}). The ED_{50} of the EchiTAb-Plus-ICP polyvalent antivenom was calculated as 1 ml antivenom/1 mg venom (65 mg venom/ml antivenom) (3.3 x LD_{50}),

To determine an initial antivenom dose that would be sufficient to neutralise 100% of the average adult snake venom yield, the World Health Organization (WHO) propose employment of the potency equation (Equation 1) (WHO, 2023; Morais et al., 2010).

Applying the data from this study using an estimated venom yield of 150–250 mg (Marais, 2022; Hayes et al., 2008) an estimated 230 ml (23 vials) EchiTAb-Plus-ICP polyvalent and about 240 ml (24 vials) of SAIMR/SAVP polyvalent antivenom will be required as initial dose (Equation 1). This is nearly double the 100–120 ml of the SAIMR/SAVP polyvalent antivenom advocated for the treatment of Mozambique spitting cobra (*Naja mossambica*) bites (Personal communication with clinicians from Swaziland and South Africa). Both availability and cost, with one vial of the SAIMR/SAVP polyvalent retailing at R2130.00 and a total cost of R51120.00 for 24 vials, may be unattainable (African Snakebite Institute, 2023)

These implicated high volumes of antivenom required to achieve effectiveness create a dilemma, especially in the treatment of the paediatric *N. n. nigricincta* victim. With guidelines proposing a maximum of 10 ml/kg antivenom (Gutié et al., 2017b), the 230 and 240 ml of the antivenoms respectively required, is unsafe in children weighing less than 23/24 kg (average weight of a seven-year-old) (Hunter et al., 2006). The volume issue is further complicated by the advocated antivenom dilution as mandated by most snakebite management guidelines. This follows the high rate of hypersensitivity reactions associated with animal-derived antivenom preparations - reported incidence of early adverse reactions (EAR's) varies between 3 and 88% - and the possible association with infusion velocity. Dilution and slow infusion are proposed as additional safety measures. Comparative studies have, however, failed to link infusion rates to severity of adverse reactions (WHO, 2016; Warrell, 2010; WHO, 2010; White, 2013; Ahmed et al., 2008; Blaylock, 2002; Wood et al., 2009; Gutié et al., 2012; Snakebite Management: South African Consensus Guidelines, 2022; De Silva et al., 2016; Leó et al., 2013; Holstege et al., 2002).

Potency: SAIMR/SAVP polyvalent antivenom against *Naja nigricincta nigricincta* venom

The number of LD_{50}: (n) = 3.2

LD_{50} (mg venom/mouse) = 1.02 µg/g x 24.62 g = 26.44 µg/mouse = 0.02839 mg/mouse

ED_{50} (ml antivenom/mouse) = 99.5µl/mouse;

ED_{50} = 0.099 ml/mouse

$$P = \frac{(n-1)LD50}{ED50}$$

P = [(3.2 – 1) x 0.02839 mg/mouse] ÷ 0.099 ml/mouse

P = 0.63 mg ➔1ml antivenom neutralises 0.63 mg *Naja nigricincta nigricincta* venom

Potency: EchiTAb-Plus-ICP polyvalent antivenom vs. *Naja nigricincta nigricincta* venom

The number of LD_{50}: (n) = 3.3

LD_{50} (mg venom/mouse) = 1.02 µg/g x 23.91 g = 22.67 µg/mouse

= 0.0227 mg/mouse

ED_{50} = 78µl/mouse = 0.078ml/mouse

$$P = \frac{(n-1)LD50}{ED50}$$

P = (3.3 – 1) x 0.0222 mg/mouse ÷ 0.078 ml/mouse

P = 0.65 mg ➔1ml antivenom neutralises 0.65 mg *Naja nigricincta nigricincta* venom

4.2. Dermo- and myonecrosis

The pathophysiology behind the rapid progression of the local dermo-necrosis has not been fully determined; this study investigated the influence of the force of venom expulsion on necrotic expansion (Buys, 2003; Warrell et al., 1976; Tilbury, 1982; Saaiman and Buys, 2019, 2022; Saaiman et al., 2023; Wagener et al., 2017). Following 0.2 ml saline-methylene blue sub-dermal injection the stained area when assessed on post-mortem examination after 24 h, was 1 cm in diameter. This was regarded as being representative of venom spreading caused by the force of injection alone. In contrast, subdermal injection of 0.2 ml of a 200 μg venom concentration resulted in severe envenomation effects, with quick progression of the local reaction that spread from the injection sites to the lateral thoracic walls and death within 1.06 h. This quick progression of the local necrotic and inflammatory reaction up to 4.5 cm in comparison to the 1 cm diameter stain (after 24 h), infer that the force of venom injection does not play a direct role in necrotic progression and unknown intrinsic venom factors are most likely to be blamed.

Necrotic spread can thus only be halted if the venom is either neutralised, the venom depot removed or fully absorbed. With elimination of both the EchiTAb-Plus-ICP and SAVP/SAIMR for clinical use, the strategy of physical venom depot and necrotic tissue excision via early radical surgical debridement as means to limit necrotic spread seems the only alternative (Saaiman and Buys, 2019, 2022; Lin et al., 2022)

The presence of venom induced myotoxicity was evaluated by serum creatine kinase concentrations (at time of death) and muscle histology. Increased serum creatine kinase concentrations, with a minimum myotoxic dose (MMD_{50}) of 14 μg venom/mouse (0.54 μg venom/gram mouse) as well as muscle necrosis reported from both hind-limb and cardiac muscle, confirmed direct venom induced skeletal and cardiac myotoxicity which was not prevented by either antivenom.

Contrary to expectation, direct intravenous venom administration, whereby absorption *de facto* becomes 100%, was associated with 3.6–6 times lower CK concentrations than equivalent sub-dermal venom injections. Local muscle damage following venom deposition into the subdermal fascia layer; as evidenced by the histological evaluation of the skin surrounding injection sites; must therefore be a significant driver of CK changes observed in practice.

With both antivenoms unable to prevent skeletal and cardiac muscle damage, early detection of cardiac damage is vital in order to initiate appropriate supportive treatment. S-troponin measurement is advocated as s-CK-MB is not a reliable indicator of myocardial injury in the setting of concomitant rhabdomyolysis (Benoist et al., 1997; Cabaniss et al., 1990; Junpaparp, 2019; Takagi et al., 2001) Serial ECG's (electrocardiogram) demonstrating conduction and ischemic changes as well as echocardiograms (sonography) that evaluate functional and dimensional card (Verma et al., 2017; Aisenberg and Fred, 2016b).

4.3. Anticoagulation

Severe *N. n. nigricincta* envenomation can result in a clinical bleeding tendency with raised INR values, thrombocytopaenia and positive d-dimers, 12–24 h following the bite. *Ex vivo* studies report African spitting cobra venom causing both pro- and anticoagulation effects (Bittenbinder et al., 2018, 2019; Kazandjian, 2020).

With this study unable to evidence direct anti-coagulant effects, the clinical bleeding tendency noted is most likely due to secondary disseminated intravascular coagulation (DIC) following envenomation sequalae (Buys, 2003; Saaiman and Buys, 2019; Bhai et al., 2022; Boral et al., 2016; Isbister, 2010; Stanley et al., 2023).

4.4. Subdermal LD_{50} and the inflammatory response

Venom toxico-kinetics proclaim sub-dermal venom injection to be characterised by an initial absorption phase, followed by distribution and elimination, with bioavailability ranging from 4 to 81.5%. Lower bioavailability with resultant lower lethality following sub-dermal versus intravenous venom administration would be expected (Nikapitiya and Maduwage, 2018; Sanhajariya et al., 2018) The subdermal median lethal dose LD50 (SDLD50) of 15.3 μg/18-g mouse (CI: 12.96; 17.74) in contrast to the intravenous (IVLD50) dose of 18.4 μg/18-g mouse (CI: 16.3; 20.52) was an unexpected result. The higher lethality associated with subdermal venom administration suggests the presence of secondary venom-evoked complications, unique to the sub-dermal model, contributing towards mortality. One such acknowledged venom induced tissue reaction is the inflammatory response initiated by local snake envenomation (Alsolaiss et al., 2022; An et al., 2016; Hysong et al., 2020; Leó et al., 2011; Moore-Lotridge et al., 2020; Stone et al., 2013; Teixeira et al., 2019; Vogel et al., 2017; Voronov et al., 1999; Zornetta et al., 2012).

The histological presence of inflammatory cells surrounding the subdermal injection sites and pulmonary leukostasis as well as the fever, tachycardia, raised CRP values, abnormal leucocytes, hypoalbuminaemia and hyponatraemia, exhibited with severe *N. n. nigricincta* envenomation, suggest an active role of the inflammatory response in the clinical presentation and mortality in these victims (Namibian experience) (Swart et al., 2011; Udayabhaskaran et al., 2017).

4.5. Kidney injury

The acute kidney tubular necrosis observed in samples from the higher intravenous venom dosages and on postmortem examination, supports clinical experience, where a number of cases with acute kidney injury (AKI), requiring dialysis, following *N. n. nigricincta* envenomation have been documented. This is in contrast to studies from South Africa where renal toxicity is seldom observed (Personal observation by the author) (Wood et al., 2016; Taylor, 2018). The *N. n. nigricincta* victim seems to be at higher risk for acute kidney failure as a result of direct venom pathology, the additional effects of rhabdomyolysis with sequestration of fluid into the damaged muscle cells resulting in severe hypovolemia combined with the direct toxic and obstructive effects of the myoglobin (Torres et al., 2015; Esposito et al., 2018). Snake bite induced haemolysis, haemodynamic instability, micro-thrombi, coagulopathy and non-steroidal anti-inflammatory drugs (NSAIDS) used during treatment, can further compromise kidney function (Sitprija, 2006; Vikrant et al., 2017).

4.6. Mortality

Decompensation, as seen with severe *N. n. nigricincta* envenomations, seem to follow a pattern of deteriorating kidney function, tachycardia, tachypnea, fever, respiratory distress, hypotension and cardio-respiratory collapse (Namibian clinical experience) (Buys, 2003; Saaiman and Buys, 2019, 2022). In this mice study cardio-pulmonary failure as cause of death was reported from post-mortem reports and was further supported by the severe clinical reactions noted in the mice following venom administration.

The possible aggravation and interaction between the cardiac myonecrosis and the complications associated with concurrent rhabdomyolysis may play an important part in the pathogenesis of mortality. Myocardial necrosis translates to a non-infective toxin mediated myocarditis. Depending on the extent of cardiac damage symptoms may range from mild to rapidly progressive hemodynamic compromise, cardiogenic shock, and fatal arrhythmia (Veronese et al., 2018). The resulting complications of a concomittant rhabdomyolysis, *i.e.:* hyperkalaemia, metabolic acidosis and initial hypocalcaemia can induce cardiac arrhythmias and cardiac arrest whilst the sequestration of fluid into the damaged muscle cells may cause severe hypovolemia exacerbated by inflammatory induced vasodilatation and hypovolemia, leading to refractory shock and death (Bhagwat and Amar, 2013; Chatzizisis et al., 2008; Gutié et al., 2003, 2017b; Hunter et al., 2006; Morales De

Guzman, 2024; Torres et al., 2015; Sauret et al., 2002b; Standl et al., 2018; Vahdatpour et al., 2019).

4.7. Limitations

The biggest limitation of this study lies in the translation of this data to clinical practice, as the intravenous administration with pre-incubated venom and antivenom does not represent the clinical scenario where venom is expelled into the sub-dermal fascia area followed by a variable time interval of intravenous antivenom. Important factors, such as rate of venom and antivenom absorption, volumes of distribution, toxicokinetics, time from bite to antivenom administration as well as secondary venom responses are bypassed (Gutié et al., 2017a; Nikapitiya and Maduwage, 2018; Sanhajariya et al., 2018).

This study only measured total serum CK concentrations. Creatine kinase is a dimeric enzyme consisting of two subunits named M and B, with CK-MM found mainly in skeletal muscle, CK-MB in cardiac muscle and CK-BB in brain tissue (Cabaniss et al., 1990; Junpaparp, 2019; Takagi et al., 2001; Nsiah et al., 2011). The specific ratio or contributions of the different CK fractions towards the total CK value in the case of *N. n. nigricincta* envenomation remains undetermined.

5. Conclusion and clinical implications

A median effective dose (ED_{50}) could be demonstrated for both the SAVP/SAIMR and EchiTAb-Plus-ICP polyvalent antivenom. The implicated high volumes required to achieve effectiveness is not practical. This mandates further research in order to establish the clinical safety, efficacy and suitability of these antivenoms in treating *N. n. nigricincta* envenomation.

Aiming to improve treatment extra emphasis must be put on early recognition and aggressive treatment of the confirmed venom induced skeletal, cardiac muscle and kidney tubular necrosis as well as inflammatory activation. In addition, early radical debridement with removal of necrotic and infected tissue remains a viable option to inhibit necrotic spread, remove the venom depot and limit secondary venom evoked sequalae.

Although many questions regarding the pathogenesis of *N. n. nigricincta* envenomation remain and a specific antivenom still elusive, this study will serve as the foundation for future research.

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The authors hereby declare that the material is the authors' own work and own research.

The analysis was done in a truthful manner.

The work has not been previously published elsewhere and is not currently being considered for publication elsewhere.

CRediT authorship contribution statement

Esta L. Saaiman Engelbrecht: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Vinny Naidoo:** Writing – review & editing, Supervision. **Christo J. Botha:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Esta Saaiman reports financial support was provided by Lady Pohamba Private Hospital, Windhoek, Namibia. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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