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Chapter

The Effects of Snake Venom (*Bitis arietans*) on Embryonic Development

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Abstract

Venomous snake bites in pregnant women can lead to poor survival rates in both the foetus and mother; early bites can precipitate teratogenesis, miscarriages, preterm delivery, foetal death and antepartum haemorrhage. The chicken embryo poses as a valuable research model for venom research due to its advantages such as ease of availability, economic feasibility and its non-invasiveness. This study evaluates the embryotoxic effects of Puff adder venom (*Bitis arietans*) from Namibia, Kenya, South Africa and non-specified region of Africa at varying concentrations. The venoms were applied to chicken embryos on the fourth day of incubation and assessed on a ninth day, focusing on body weight, heart weight, liver weight and mortality rate. Nile blue staining was also performed to observe the occurrence of apoptosis amongst the venoms at the strongest concentrations. The information provided from our results suggested that there was a regional variation in venom toxicity, with the Kenyan venom producing the largest weight changes, whereas the non-specified African venom proved the most lethal across the concentrations. Further studies to assess venom protein concentrations in comparison with regional diet disparities are required.

Keywords: *Bitis arietans*, chicken embryo, development, embryotoxicity, snake venom

1. Chicken embryo as an animal model for biomedicine

The chick embryo has established itself within biomedical science as a crucial research model due to its similarity on a cellular and anatomical level to human embryos along with its rapid development, easy visibility and manipulation [1]. The cardiovascular system in the avian develops rapidly with the added benefit of the embryo remaining transparent during morphological development of primary organs, allowing visibility of vasculature formation using simple microscopy techniques [2].

1.1 Transgenic chicken egg as a bioreactor

The use of a chick egg as a bioreactor has been promoted over the use of standard large-scale fermenters due to its ability to produce large quantities of complex proteins and cheaper running costs [3]. A 2002 study placed a retroviral vector

based on avian leucosis virus into the chicken genome and noted that biologically active enzymes were secreted into the serum and egg white of four generations of chickens. The levels of enzyme expression remained steady through each successive generation and remained constant for over 16 months in the magnum of hens indicating the expression of this enzyme was stable. This supports the possibility of chicken eggs in the role of a bioreactor for the production of biologically active proteins for therapeutic interest [4]. Human interferon a2b and β1a along with human granulocyte-colony stimulating factor have been successfully harvested from this method [5]. The egg is an ideal model for the recovery of these proteins as the content is sterile and provides a long half-life [6]. Production of human proteins in hens could become the method of choice for proteins that are toxic to mammals, an example of this is human erythropoietin which is damaging to the mammary gland of rabbits but remains inactive in chickens [7].

1.2 Chicken egg used for drug screening and production

The avian chorioallantoic membrane (CAM) refers to the outermost membrane that is highly vascularised for gas exchange and calcium transportation between the embryo and its environment [8]. It has been widely used to discover targets and measure the in vivo angiogenic effects of factors such as vascular endothelial growth [9] and P13 kinase [5]. The chorioallantoic membrane provides advantageous exposure to the embryo with minimal invasiveness [5]. The chicken egg is a reliable candidate in assessing angiogenic responses to drugs as well as being cost and time-effective [10]. In 1984, inactivated rabies vaccine for human use was developed which had been adapted to proliferate in primary chick egg cultures [11]. This vaccine provided similar antigenicity to the human diploid cell strain with the advantages of cheaper and faster production. This purified chick embryo cell culture vaccine has now been licenced in over 60 countries worldwide for 30 years [12]. The influenza vaccine has been developed in the embryonated chicken eggs since the 1940s, the egg provides high titre capabilities and large-scale production opportunity which has led to the streamlined process of today which provides vaccination for millions worldwide [13].

1.3 Chicken egg used in retinal development

The chicken embryo is advantageous in studying retinal development due to its significant similarity to the human embryo at a molecular, cellular and anatomical level and its rapid development [1]. It is an important model in regenerative research as the neural retina can regenerate from the pigmented epithelium as late as stage 24 [14]. The regeneration can occur from the cup margin or patches of pigmented epithelium far from the margin. The lens does not have to be present for the regeneration, and the regenerating tissue develops faster than normal [14]. The chicken model is used in the vision research community to study human retinogenesis and develop new ways to battle human blindness and eye disease by allowing scientists an insight into the complex regulatory network of neurons and glial cells [15]. In America, diabetic retinopathy is the leading cause of blindness amongst over 40-year-olds [16] with all type 1 diabetes developing DR and more than 60% with type 2 [17]. Chickens, like humans, are diurnal with complex colour vision and as their retina is cone-dominant it makes them an ideal model for photoreceptor-degenerative blindness [18]. In a 2014 study, chicken embryos were injected with either streptozotocin or high concentration of glucose at day 11; in both groups, cataracts occurred in varying degrees making them a promising animal model for diabetes research [19].

1.4 Chicken embryo as an animal model for research in Alzheimer's disease

Studies by Murphy and LeVine [20] and Carrodeguas et al. [21] revealed that the chick embryo has an active process for the formulation and degeneration of the amyloid-beta peptide deposits associated with Alzheimer's disease. The distribution of the peptide sequence in chick embryos is similar to humans, predominating in the nervous system. This study also discovered that the chick embryo produces neprilysin (ADAM-17), a protease that degrades the peptide [21]. Due to the easy access of chick embryos, it's an ideal candidate for developing a potential system for drug regulation and regression for this neurodegenerative disease [22]. In a 2003 study, the chick embryo was a model for supporting the use of *Ginkgo biloba* extract as Alzheimer's therapy. The embryo was exposed to cyanide, with and without therapeutic use of the extract. Results show an increase function in cultured neurons from the telencephalon and a reduction in apoptotic damage caused by serum deprivation after 24 hours [23].

1.5 The role of the chicken embryo in oncology research

The chick embryo allows the unique research of oncology in vivo. Along with being highly accessible and easy to manipulate, the CAM is naturally immune-deficient which can support engraftment of both normal and cancerous tissue up until developmental day 18 [24]. The CAM has also been used for research into carcinogenesis [25] and tumour angiogenesis [26]. In 1911, by inoculating the Rouse Sarcoma virus into chick embryos, it was suggested for the first time that viruses could cause cancer [27]. It is now estimated that 15–20% of human cancers worldwide involve tumour inducing viruses, such as T-cell leukaemia virus type 1 and hepatitis C [28]. In 2002, Zijlstra et al. [29] developed a highly sensitive assay to monitor metastatic dissemination of human cancer cells in the chick embryo by using the CAM as an established biological platform. In 2014, Mu et al. concluded that chicken embryo extract may promote the reversion of metastatic phenotypes of osteosarcoma cells, which could lead to tumour reversion through epigenetics [30].

1.6 Role of the chicken embryo in stem cell research

In 1996, Pain et al. displayed that early chicken blastoderm cells or avian embryonic stem cells can be maintained in vitro for long term culture. These cells model traits of murine stem cells in morphology, reactivity to antibodies and high telomerase activity along with the high capability to differentiate into various cell types [31]. When blastoderm embryonic stem cells are grafted onto the CAM, they organise into complex structures such as embryoid bodies and derivatives of the three primary germ layers. These cells can provide an in vitro model of cell differentiation and maturation along with means of targeted genome control [32]. Germline stem cell research is associated with the manufacture of transgenic animals using male spermatogonial stem cells [33] for the production of pharmaceutical proteins [34]. Recently direct reprogramming has been created to convert differentiated somatic cells into pluripotent embryo stem-like cells; this is an advantageous medical method avoiding ethical issues surrounding human egg use [33]. Amniotic stem cells isolated from the amniotic cavity can create clonal cell lines and due to their ease of isolation, are considered a potential source for regenerative medicine application [35].

1.7 Chick embryotoxicity screening test

Developed by Jelinek in 1977 the chick embryotoxicity screening test (CHEST) is a standardised technique to allow administration of small amounts of test

compounds and the quantitative measurement of results. This method is advantageous as it requires limited materials. Fertilised eggs, micropipettes for administration, incubator and a dissection kit are all that is needed. The CHEST test allows the use of a chick embryo to study experimental teratogenicity. The number of dead, malformed, and growth-retarded foetuses (weight <650 mg and with no malformation) are totalled for each concentration and stage of the embryo at the time of exposure. The proportion of affected embryos is plotted against the concentration of test compound, and the embryo-stage of administration. The proportion of effects on particular organs in surviving embryos is considered separately to establish a profile of effects for each compound [36]. The screening test has been used to determine the toxicity of many substances, establishing parameters of dose–response and stage-response [37].

2. Snake venom

2.1 The role of snake venom

Venom is defined as 'a secretion, produced in a specialised gland in one animal and delivered to a target animal through the infliction of a wound, which contains molecules that disrupt normal physiological or biochemical processes to facilitate feeding or defence' [38].

Venom is composed of proteins and polypeptides, it has two primary functions—to paralyse their prey and start the digestive process with hydrolysing proteins leading to tissue necrosis and blood clotting [39]. These venom components can be grouped according to the mode of action:

1. Binding to cholinergic receptors, often leading to respiratory muscle paralysis.

2. Inhibition or increase release of acetylcholine, causing muscle cells to not react to nerve stimulus, leading to spasms, damage of the skin and disruption connective tissues.

3. Cytotoxic and cardiotoxic, which harm cell membranes and disrupt the transport of substances across the membranes [39].

In broad terms, snake venoms are classified as inflammatory, cytotoxic, neurotoxic or haemotoxic [40].

2.2 The venom delivery system

Many snakes use fangs, specialised dentition associated with the venom gland (Duvernoy gland) to introduce venom into prey. Fangs can either be posteriorly (grass snake) or anteriorly (vipers) positioned within the upper jaw [41].

Many poisonous snakes possess different types of dentition; vipers and atractaspidids have a shortened maxilla which rotates allowing the fangs to move; whereas elapids fangs are fixed at the front of the maxilla. Colubrids can possess an enlarged rear positioned fang or no fang at all. The venom gland, also known as the Duvernoy gland in posteriorly positioned fanged species, is innervated by the maxillary branch of the trigeminal nerve, facial nerve and supplied by the internal carotid artery [42]. The Duvernoy gland is positioned posteriorly to the eye, encased in a thin layer of connective tissue and consists mainly of serous cells; a single duct extends from the gland to the base of the fang; whereas the venom gland

is encased in a fibrous sheath varying in position depending on species. The viperid gland is large, isosceles with the longest side along the upper lip directed dorsally; the gland is divided into lobules by the outer sheath with the lumen becoming a primary duct which passes through a mucous accessory duct into a secondary duct extending the length of the fang [43]. In contrast, the elapid venom gland is oval made from many branching tubules, its lumen is narrow and therefore most of the venom is stored in the surrounding cells [44]. The atractaspidid species possess a cylindrical gland extending posteriorly beyond the head, its lumen has a characteristic pattern of unbranched tubules radiating outwards [45].

2.3 Snakebite classification

Bites can be classified as cytotoxic bites characterised by a painful swelling with watery blood leaking from the wound followed by shock, blistering and discolouration. Venomous snake bites in pregnant women can lead to poor survival rates in both the foetus and mother; early bites can precipitate teratogenesis, miscarriages, preterm delivery, foetal death and antepartum haemorrhage [46]. The bite will cause severe pain to the limb affected. Species associated with this bite include the puff adder, Gaboon adder and spitting cobras. Black and green mambas along with non-spitting cobras produce a neurotoxic bite in victims which leads to moderate swelling, cold and clammy extremities, dilated pupils and drooping of the eyelids. Patients suffering these bites will develop swollen lymph glands, vomiting, ptyalism and breathing difficulties. Haemotoxic bites associated with boomslang and vine snakes cause bleeding from the gums, nose, corner of eyes and old wounds and scratches. Certain snake species can produce more than one type of bites such as the *Bitis arietans* that produces a haemotoxic and cytotoxic bite [47].

2.4 Composition of snake venom

Medically significant venomous snakes are all front-fanged and are classified into three families: Atractaspididae, Elapidae and Viperidae; the glands of these snakes are homologous [48], with current evidence suggesting these evolved from non-front fanged venomous snakes [49]. These glands contain the snake venom, made from a combination of different protein families, each containing a variety of toxin isoforms along with carbohydrates, lipids, nucleosides and metals. Although homologous, the venom proteome is not due to the influence of genetic mutations and natural selection [50]. In a 2017 review, 59 protein families were identified in these three species, with four dominant proteins: phospholipase A2s, metalloproteases, serine proteases and three-finger toxins; six secondary families: cysteine-rich secretory proteins, L-amino acid oxidases, Kunitz peptides, c-type lectins, disintegrins and natriuretic peptides; nine minor proteins and 36 rare. Results revealed that elapid venom contained a less diverse range of protein families than the others, mostly consisting of phospholipases and three-finger toxins. Viper venom showed to scarcely contain three-finger toxin [51].

2.4.1 Phospholipase A_2

These enzymes play an important role in the regulation of phospholipid turnover, membrane permeability, cell maintenance and growth, apoptosis and the production of leukotrienes and prostaglandins [50]. Of the four types of PLAs, only type one and two are found in snake venom with several isoforms. These PLAs target the motor nerve terminal and the terminal part of the motor axon, by initiating hydrolysis of the lipids of the outer leaf of the plasma membrane of the nerve

terminal resulting in its depolarization. Synaptic vesicles are also destroyed by the PLA and the products of lipid hydrolysis [52]. Exposure of skeletal muscle to venom PLAs causes a severe inflammatory degenerative response, with the first clinical signs apparent less than 1 hour after inoculation, with affected fibres rapidly depolarising [50].

2.4.2 Metalloprotease

Haemorrhaging is a common clinical sign associated with viper and crotaline snake bites and has been associated with the proteolytic activity of metalloprotease [53]. The proteases induce direct damage of the microvessels [54]. Snake venom metalloproteases are classified into four main groups based on their domain structure: PI—possesses a metalloprotease domain only, PII—consists of both metalloprotease and disintegrin-like domains, PIII—compromised of metalloprotease, disintegrin-like and high-cysteine domains, PIV—in addition to metalloprotease, disintegrin and cysteine also possesses a lectin-like polypeptide [53]. The disintegrin-like domain inhibits platelet aggregation by binding to the fibrinogen receptor in platelet plasma membranes and although the role of the other domains is not clear, venoms with cysteine and disintegrin domains are more active at inducing haemorrhage than enzymes with only metalloprotease domains [55]. Investigations suggest pathogenesis is associated with a per rhexis mechanism whereby endothelial cells of capillary blood vessels rapidly thin and detach from surrounding basal lamina, progressive degeneration of these vessels leads to breaks in the endothelial lining, allowing blood to enter interstitial space [56].

2.4.3 Serine protease

Serine proteases are major components of snake venom, mostly identified in snakes of the Viperidae family and certain Elapidae, Colubridae and Hydrophiidae families [57]. These venom enzymes affect the haemostatic system by acting on the coagulation cascade and possess strong pro-coagulant effects through the activation of platelets, production of thrombin-like enzymes which clot fibrinogen and the creation anti-coagulant enzymes such as protein C [58], these serine protease produced thrombins are not susceptible to hirudin or heparin, other proteases possess kininogenase activity which releases hypotensive bradykinin [59].

2.4.4 Three-finger toxin (3FTs)

This family of non-enzymatic polypeptides exhibit potent toxic effects. Based on their biological properties, they can be classified as postsynaptic neurotoxins targeting the nicotinic and muscarinic acetylcholinesterase receptors; fasciculins targeting acetylcholinesterase; calciseptins and FS2 toxins targeting L-type calcium channels; anticoagulants; β-blockers targeting β_1 and β_2 adrenergic receptors; dendroaspin targeting specific glycoproteins; cardiotoxin A5 targeting integrins and antagonists of α_1A and α_2A adrenergic receptors [60]. A large number of 3FTs are neurotoxic, interfering with cholinergic transmission at post-synaptic sites in the peripheral and central nervous system; mipartoxin-I, a 3FT, is the most abundant protein found in coral snake venom from northern South America and possesses a lethal effect in mice and a clear neuromuscular blockade in avian and mice subjects with an affinity for the cholinergic nicotinic receptor [61]. A group of cardiotoxic 3FTs found only in cobra venom is the second-largest group, at low concentrations they elevate heart rates and at high concentrations, cause death by cardiac arrest [62].

2.5 The use of *Bitis arietans* snake venom in biopharmaceutical research

Biscetin, a platelet adhesion inducer, isolated from the venom of *Bitis arietans* has been shown to activate the binding of the Von Willebrand factor to glycoprotein without altering the binding site; this factor plays a key role in haemostatic response to vascular injury [63]. In a 2015 study, bitistatin a disintegrin isolated from the Puff adder was demonstrated to have an affinity for the integrin receptor avβ3, a marker used for tumour angiogenesis. This can be radiolabeled, injected and then detected on imaging. The ability to detect these receptors via diagnostic imaging has been used to successfully diagnose thrombosis in a canine model [64]. Studies researching strategies to enhance immunological responses against venoms observed the cross-immunoreactivity of antivenoms of the *Echis* and *Bitis* species, supporting the hypothesis that immunising horses with a mixture of *Echis ocellatus*, *Bitis arietans* and *Naja nigricollis* generates enough antibodies that can recognise all medically relevant viper venoms in sub-Saharan Africa [65].

2.6 The Puff adder (*Bitis arietans*)

The Puff adder (*Bitis arietans*) is venomous snake from the Viperidae family found in the Middle East, North and sub-Saharan Africa. The species has a broad, flattened, lanceolate shaped head covered with small keeled scales attached to a narrow neck. Puff adders along with Russell's vipers can make a loud hissing sound by expelling air through their large nostrils. These snakes are distinguishable by their distinctive repeated U or V dorsal pattern [66]. *Bitis arietans* feed on a variety of small animals including hares, hedgehogs, rodents, lizards and birds; they can actively forage by visiting birds' nests to feed on eggs, nestling or the adult birds themselves. This species can also deploy ambush tactics by camouflaging themselves in areas where bird encounters are common, such as water pools [67].

2.7 The occurrence of snake bites

A global review by White in 2000 estimated that between 1.25 and 5.5 million snakebites occur annually [68]. These primarily occur in developing countries where there is a dense population of humans, an abundance of snakes and a lack of medical treatment facilities. In 1987 in South Africa, pregnant women accounted for 0–4% of cases admitted to hospital, in India, they accounted for 1% of admissions [46]. Venomous snakebites may lead to poor foetal development and adversely affect the mother's health; previous studies determined that foetal death occurred in 38–43% of snakebite cases, whereas maternal death accounted for 10% [69]. The African puff adder, along with the carpet viper, is the two most common species responsible for fatalities following bites in Africa. These adder venoms possess cardiotoxic and haemorrhagic effects which can lead to hypovolemic shock, necrosis, systemic haemorrhage and arrhythmias [70].

2.8 The geographical variations in snake venom

Variation in venom composition occurs between species of snake as well as within a species. These variations can have a significant impact on venom toxicity and medical management. Different toxin-encoding genes in the genome attribute to these variations [28], however the mechanisms by which these gene expressions are controlled are poorly understood [71]. Results from Barlow et al. [72] provides evidence for the theory of diet affecting venom composition, by observing *Echis* snake species diet and the LD50 by which to incapacitate their natural scorpion

prey; their study findings suggested that variations in composition are derived from adaptive evolution, driven by natural selection, for different diets. It has more recently been hypothesised by other literature that variation in venom composition is the result of adaption to dietary selection [28]. In a 2015 study, the intraspecific variations of venom activities of *E. ocellatus* snakes from Nigeria and Cameroon were observed; the Nigerian venom showed higher lethality and coagulation in comparison to the Cameroonian venom. The antivenoms produced by both snakes were similarly effective in neutralising the venom, however, the coagulant activity was better neutralised by the Nigerian antivenom than the Cameroonian venom [73]. Further studies in geographical variations involving envenomation by *N. naja* revealed that the median lethal dose in mice differed between northern (0.55 mg/kg), central (0.66), western (0.68), southern (0.62) and Sabaragamuwa (0.7) regions respectively. Histopathological changes of these regional venoms, however, showed a higher affinity for certain bodily systems, with northern venom observing a significantly higher infiltration of inflammatory and necrotic cells into skeletal muscle and central venom demonstrating high cardiotoxic effects, these results confirm venom disparity [74]. Geographical location showed a remarkable degree of variation amongst *Bitis arietans* species originating from the same geographical origin, most evident by the venom components metalloproteinases [71]. These metalloproteinases are responsible for inducing haemorrhaging, myonecrosis and degradation of extracellular matrix components which in turn affects local inflammatory response to a bite [53].

3. Materials and methods

3.1 Ethics and morality of research

In accordance with the European Union Directive 2010/63/EU section nine, embryos were not developed beyond the first two-thirds of development. This also corresponds with mandates of The Institutional Animal Care and Use Committee, Association of New England Medical Centre (Tufts) and the National Institute of Health, USA which dictate that a chick embryo that has not reached the 14th day of its gestation period will not experience pain and therefore can be used for experimentation without ethical restrictions.

3.2 CHEST II

For this research, the chick embryotoxicity screening test was used. This standardised technique allows for the administration of small amounts of test compounds into the fertilised egg on 4th embryonic day (ED4) and the measurement of a quantitative endpoint on ED9.

3.2.1 Materials

1. Fertilised eggs: alive and morphologically normal (breed Lohmann Brown, Hatchery Farm Párovské Háje, Nitra, Slovakia).

2. Incubator (ART 549/A).

3. Dissection kit: including tissue forceps, dissecting scissors and blunt scissors.

4. Micropipette with disposable tips.

5. Stereomicroscope (Olympus SZ 61 with digital camera ARTCAM-300MI).

6. Observing dish with fixing pins.

7. Distilled water: to aid observation.

3.3 Application of snake venom: CHEST II

Eggs were placed in an incubator on day 0 at 37–38°C with a 50–60% relative humidity and rotated periodically until (ED4). Then eggs were removed and blunt end of eggs was cleaned with 70% alcohol and covered by a transparent adhesive tape. Subsequently, using serrated scissors (FST 14071-12), an opening was cut for application of the respective doses of snake venom (100 μl). The tested concentration was applied directly over the embryo on the top of inner shell membrane (*membrana papyracea*). Controls received the same volume of sterile distilled water—100 μl (**Figure 1**). The ranges of concentration as well as the total number of embryos and the days of application are listed in **Tables 1** and **2**. A small hole was then covered by micro-tape and labelled, before being returned to the incubator without rotation. On ED9 the eggs were removed from the incubator, the tape was removed and the survived chicken embryos were removed from the eggs using a crook, weighed and examined under a dissecting microscope. Hearts and livers were dissected from the chicken embryos and they were weighted separately (**Figure 1**).

3.4 Preparation of snake venom concentrations

Venoms from *Bitis arietans* (*B.A.*) of different regions were extracted in the breeding garden Pata near Hlohovec (Slovakia), which had been designed for reptiles' conservation of the gene pool under the veterinary certificate No. CHEZ-TT-01. We used the snake venom of *Bitis arietans* from three different regions (Kenya, South Africa, Namibia). Also, we used one more snake venom sample without specific region determination (B.A. nonspecific). The breeding garden also serves as a quarantine station for imported animals and is an official importer of exotic animals from around the world, having the permission of the State Nature Protection of the Slovak Republic under the No. 03418/06, the trade with endangered species of wild fauna and flora and on amendments to certain laws under Law No. 237/2002.

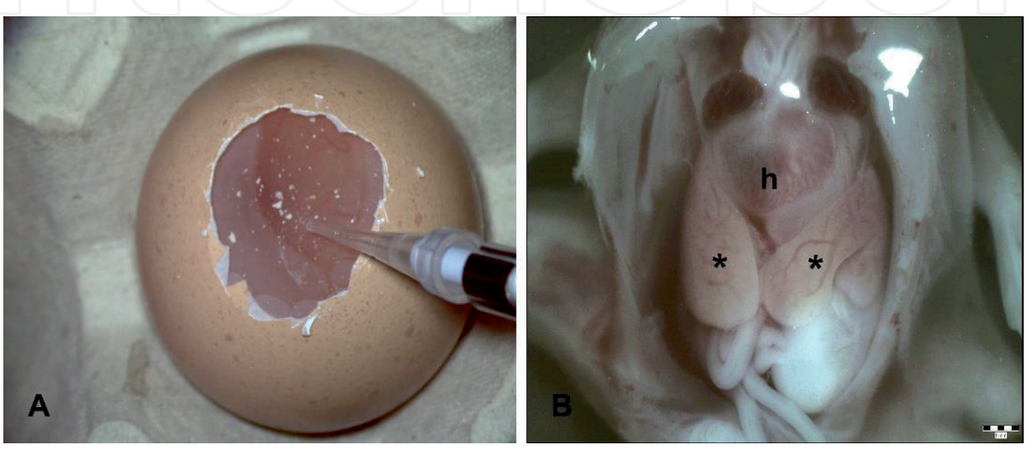

Figure 1.
Graphical illustration of snake venom administration on ED4 (A) and visualisation of developing heart and liver on ED9 (B); asterisk—lobes of liver, h—heart.

Snake venom	Concentrations (mg/ml)			
	E-1	E-2	E-3	E-4
Control (sterile distilled water)				
B.A. nonspecific region	100.00	10.00	1.00	0.10
B.A. Kenya	100.00	10.00	1.00	0.10
B.A. Namibia	100.00	10.00	1.00	0.10
B.A. South Africa	100.00	10.00	1.00	0.10

Table 1.
Concentrations of snake venom used for each venom.

Snake venom	Dose (mg/ml)	*N*	Dead embryos	Mortality (%)	Mean body weight (g)	Mean heart weight (mg)	Mean liver weight (mg)
Bitis arietans—nonspecific region	0	10	1	10	1.73	22.9	31.6
	100	10	7	70	1.42	16.3	20.7
	10	10	3	30	1.66	**18.3**	**20.9**
	1	10	2	20	1.59	17.0	**19.9**
	0.1	10	1	10	1.66	**18.8**	**22.2**
Bitis arietans—Kenya	0	10	1	10	1,53	18.7	28.5
	100	10	2	20	**1.16**	**13.5**	**15.5**
	10	10	4	40	**1.19**	**13.3**	**14.8**
	1	10	1	10	**1.19**	**15.6**	**14.2**
	0.1	10	3	30	1.57	18.3	**20.4**
Bitis arietans—South Africa	0	10	1	10	1.62	18.0	28.8
	100	10	2	20	1.67	18.5	**19.3**
	10	10	2	20	1.62	19.9	**19.6**
	1	10	2	20	1.66	19.9	26.8
	0.1	10	0	0	1.43	**15.4**	**20.7**
Bitis arietans—Namibia	0	10	1	10	1.70	21.0	31.2
	100	10	2	20	1.47	17.3	**20.8**
	10	11	3	27	1.72	20.6	35.3
	1	11	1	9	1.60	20.1	31.4
	0.1	11	2	18	1.54	20.1	30.1
Total		**203**	**41**				

N, number of chicken embryos; numbers in bold represent statistically significant results ($P \leq 0.05$).

Table 2.
Embryotoxic effect of Bitis arietans *venom from different regions of Africa.*

A sterile plastic cup was used for venom extraction with plastic food wrap, and rubber bands fixed the plastic wrap. For the application, we used snake venoms immediately after their extracting. Before use, it was ensured the venoms were continuously kept in cold storage to ensure that they retained their full toxicological

potential. The venoms were diluted with sterile distilled water to give equal concentrations (based on molecular weights) of E-1, E-2, E-3 and E-4 when required (**Table 1**). The composition of tested snake venoms used in our study has been already described and determined in previous studies [75, 76].

3.5 Nile blue staining

For this staining method, embryos with the highest concentration of snake venom (10-1) were used. The blue stain was applied on ED4. The embryos were then dissected on day ED6 and incubated in a 1/8000 solution of Nile Blue A (Sigma) and PBS for 15 minutes at 37°C in an incubator. Following this, embryos were transferred to cold PBS (4 °C) and washed for 4 hours. Embryos were then photographed with a stereomicroscope Olympus SZ 61 with digital camera ARTCAM-300MI and Quick Photo 2.3 software. These embryos were lastly compared with control embryos (sterile distilled water applied only) dissected on ED6.

3.6 Evaluation

The number of dead or growth-retarded embryos (those <650 mg) were totalled for each concentration. The proportion of effects on heart and liver in surviving embryos were considered separately to establish a profile of each concentration. The result of the CHEST was rank venom in order of their teratogenic potency in chickens; these ranges can be compared for an assessment of human risk. For statistical analysis, the programme GraphPad Prism 6.0 was utilised (one-way ANOVA, with a *P*-value of 0.05).

4. Results

The following results were analysed—body weight, heart weight and liver weight. These results were compared between control samples and the venom of *Bitis arietans* from four different regions of Africa (Namibia, Kenya, South Africa and non-specified region).

4.1 Body weight

All living embryos were weighed on ED 9 in grams. **Figure 2** presents the following concentrations E1 (10-1), E2 (10-2), E3 (10-3) and E4 (10-4).

4.1.1 Kenya

The average body weight of the control embryos was 1.73 g, whereas embryos infused with venom concentration 10-1 (E1), 10-2 (E2) and 10-3 (E3) were significantly reduced ($P < 0.05$) in comparison. E1 averaged at 1.15 g in body weight, E2 was slightly increased at 1.18 g and E3 at 1.19 g. There was a large increase between E3 and 10-4 (E4) body weight, with E4 averaging at 1.5 g.

4.1.2 Namibia

The embryos injected with venoms of the Namibian snake showed no statistically significant changes in body weight in comparison with the control embryos. E1 body weight averaged at 1.4 g in comparison with the 1.72 g of control embryos. E2 average body weight was 1.7 g whereas E3 averaged at 1.6 g. E4 embryos body weight was less than E2 and E3 at 1.54 g.

4.1.3 South Africa

None of the embryos impregnated with South African Puff adder venom showed a statistically significant change in body weight compared to the control group. E1, E2 and E3 body weights were all similar, averaging at 1.67, 1.62 and 1.66 g respectively. E4 averaged the smallest body weight at 1.43 g.

4.1.4 Non-specific region

None of the embryos infused with the non-specific venom showed significant differences in body weight compared to the control group. E1 averaged the smallest body weight at 1.42 g, E2 averaged at 1.66 g, E3 at 1.58 g and E4 at 1.66 g.

4.2 Heart weight

Hearts were cut from surviving embryos at the level of the aorta and weighed in milligrams (**Figure 3**).

4.2.1 Kenya

The control heart mean weighed 23 mg; the embryo hearts infused with venom were significantly smaller in E1, E2 and E3. E1 and E2 weighed in at 13 mg, E3 had a slight increase in weight at 15 mg. There was a large increase between E3 and E4 with the latter averaging 18 mg.

4.2.2 Namibia

None of the concentrations had a significant effect on heart weight in comparison to the control group. E1 mean heart weight came in at 17 mg. E2 was the largest weights at 21 mg, E3 and E4 averaged 20 mg.

4.2.3 South Africa

The largest average heart weight was seen in E3 and E2 at 19.8 mg. E1 averaged at 18.5 mg. E4 had the lowest body weight of 15 mg; this result was statistically significant.

4.2.4 Non-specified region

E1 and E3 results were both deemed statistically significant. E1 averaged the lowest heart weight of 16 mg. E2 averaged 18 mg whereas E3 mean was lower than E2 at 17 mg. E4 weighed in with the largest heart weight of 18 mg.

4.3 Liver weight

Livers were cut at the level of the portal vein, cut from surviving embryos and weighed in milligrams (**Figure 4**).

4.3.1 Kenya

All four concentrations of snake venom s were proven statistically significant. The mean control group liver weighed 31.6 mg, larger than all four test groups. E1

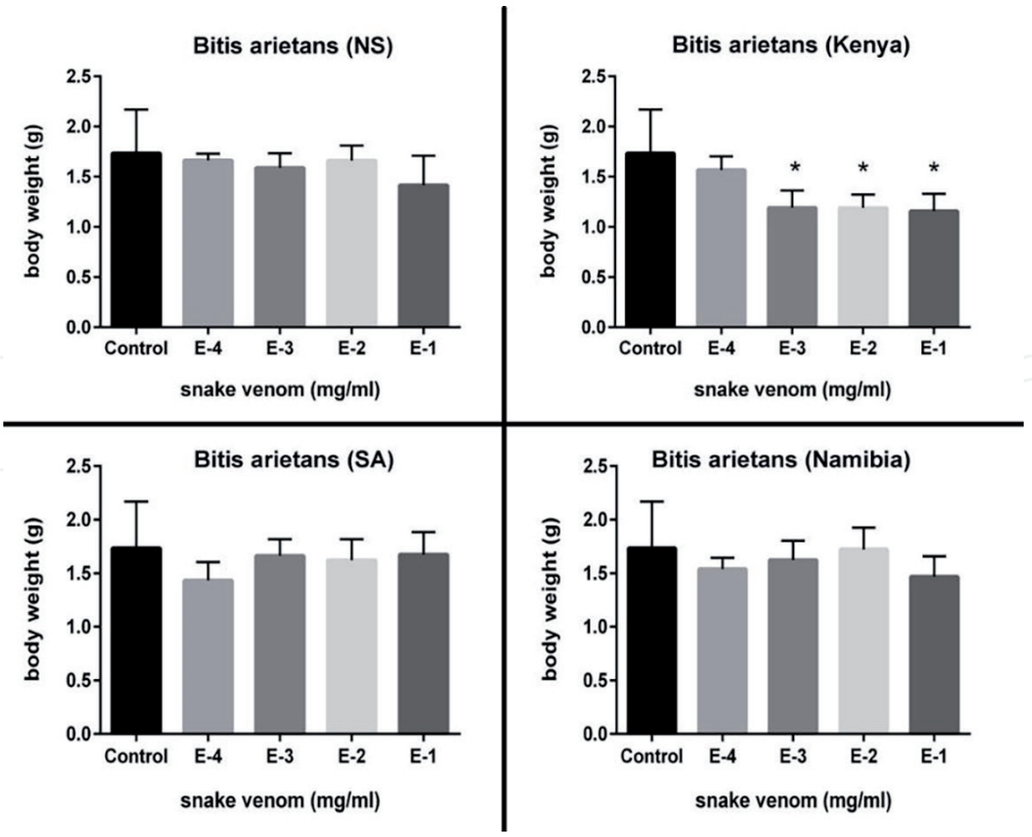

Figure 2.
Graphs depicting body weights of the Bitis arietans *venoms of Kenya, non-specified (NS), South Africa (SA) and Namibia, (*): statistically significant ($P \leq 0.05$).*

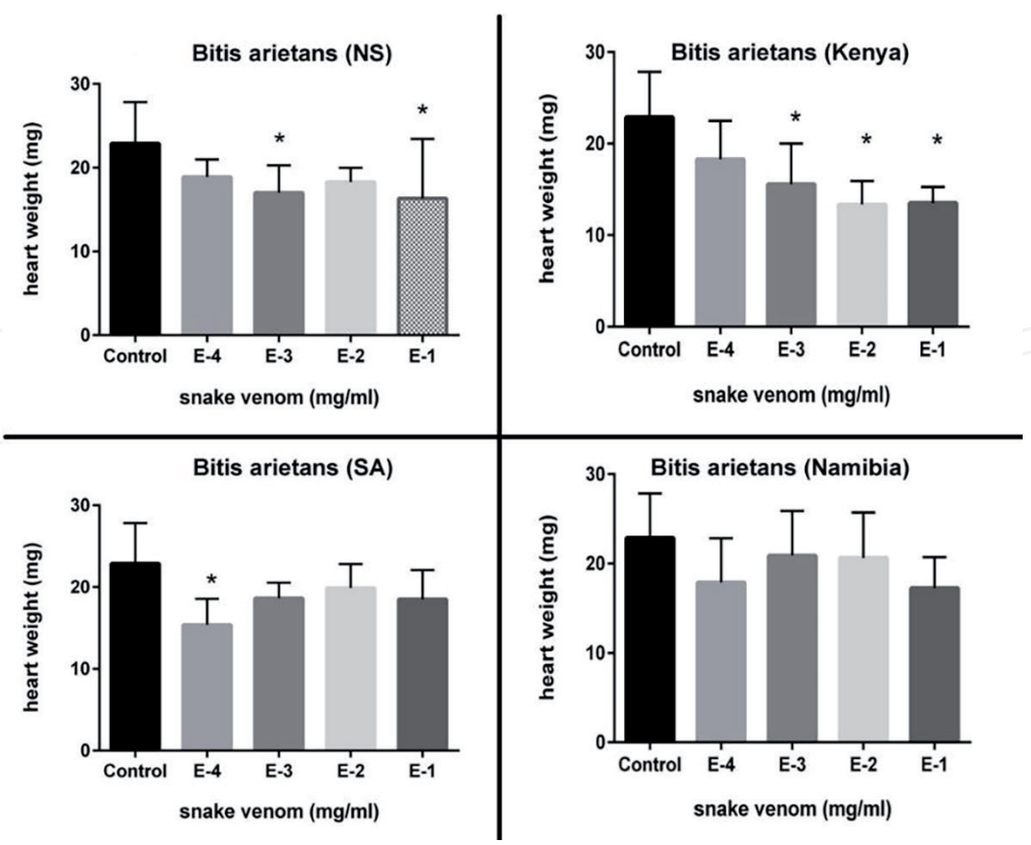

Figure 3.
Graphs depicting heart weights of the Bitis arietans *venoms of Kenya, non-specified (NS), South Africa (SA) and Namibia, (*): Statistically significant ($P \leq 0.05$).*

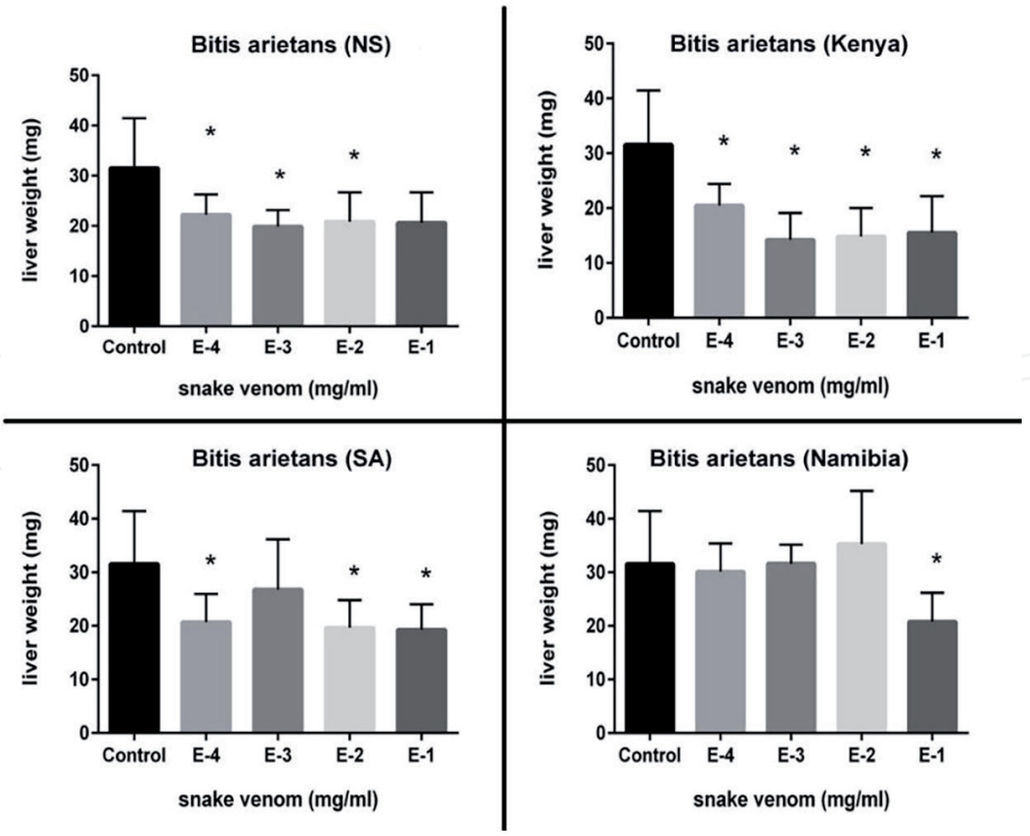

Figure 4.
Graphs depicting liver weights of the Bitis arietans *venoms of Kenya, non-specified (NS), South Africa (SA) and Namibia, (*): Statistically significant ($P \leq 0.05$).*

liver weight mean was recorded as 15.5 mg, E2 weighed in at 14.8 mg; E3 was the lowest average liver weight of 14 mg. E4 average liver weight was the largest at 20 mg.

4.3.2 Namibia

Venom concentration E1 was the only statistically significant result in this test group, with its average weight calculated at 20 mg. E2 liver weight averaged heavier than the control group (31.6 mg) with 35 mg. E3 mean weight was similar to the control at 31.4 mg and E4 averaged 30 mg.

4.3.3 South Africa

E1, E2 and E4 concentrations were proven statistically significant; whereas E3, with the largest liver weight of 26 mg, was not. E1 had the lowest average liver weight of 19.2 mg; E2 was marginally heavier at 19.8 mg. E4 liver weight averaged at 20.7 mg.

4.3.4 Non-specified region

Three of the four concentrations of non-specified venom had a statistically significant effect on the liver weight (E2, E3 and E4). E1 mean result weighed in at 20 mg; E2 was faintly heavier at 20.9 mg. E3 weighed the least with 19.9 mg. E4 had the heaviest weight within the test group at 22.2 mg.

4.4 Mortality rates

The highest mortality rate was seen in the highest concentration, in this maximum venom concentration (E1) the non-specific venom samples had a 70% death

occurrence (embryos not surviving to day 9); the Kenyan, Namibian and South African E1 all had a 20% mortality rate. All groups at each concentration had a fatality, except the South African E4 group.

The non-specified venom produced the highest overall amount of fatalities, with the Kenyan being second, Namibian third and South African venom being the least lethal. The 10 mg/ml (E2) concentration saw the highest mortality rates overall, with the Kenyan test group showing a 40% mortality, the Namibian and non-specific test group perceiving 30% mortality and the South African group a 20% mortality. E3 showed 20% fatality in the South African and non-specific test groups, in Namibia and Kenyan groups only 10% fatality rate was recorded. In the lowest concentration (E4) the highest mortality rate was recorded in the Kenyan group with 30%. No fatalities were recorded in the South African group. The Namibian test group showing a 20% mortality and the non-specific test group a 10% mortality (**Figure 5**, **Table 2**).

4.5 Nile blue staining

The embryos were stained on ED4 and removed from their eggs on ED6; all were envenomed with the strongest concentrations of venom (10-1; **Figure 6**).

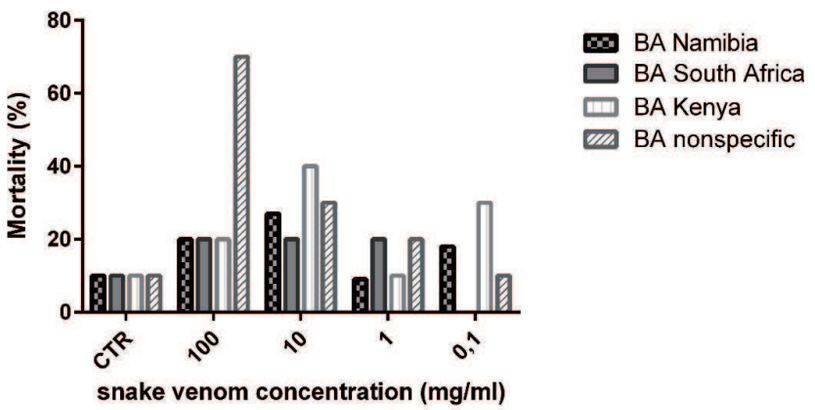

Figure 5.
Mortality rates of chicken embryos against snake venom.

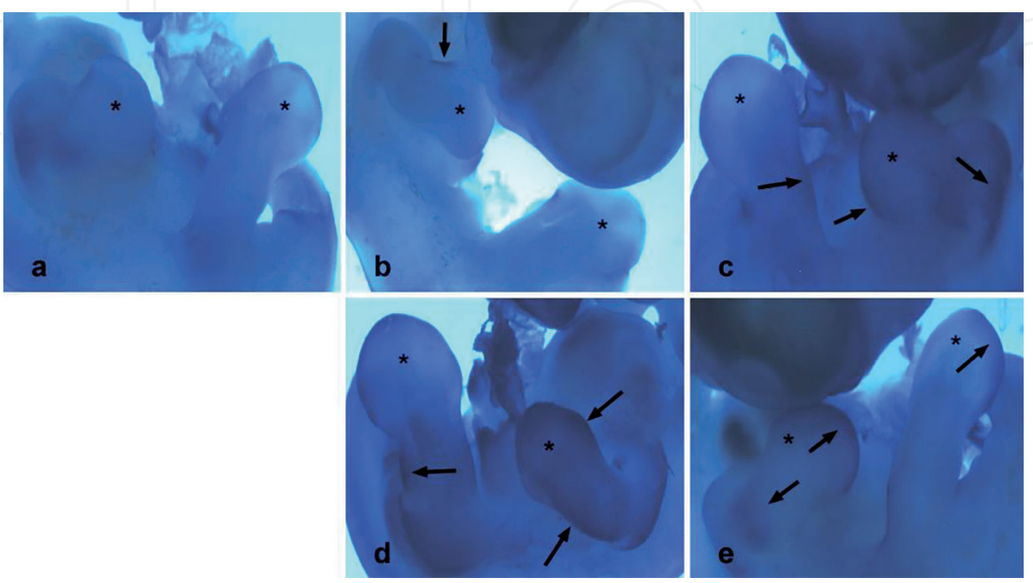

Figure 6.
Nile blue staining results. (a) Control, (b) B.A. nonspecific region, (c) B.A. South Africa, (d) B.A. Kenya, (e) B.A. Namibia; asterisk—wing and leg buds on ED9, arrows—dark coloured areas with apoptotic response.

The arrows on the above images highlight the darkened areas of apoptotic cells. These images show that initial cell death is initiated on the wing buds. The control embryo (a), infused with distilled sterile water only, demonstrates no apoptosis. The nonspecific venom (b) indicates a small amount of apoptosis located on the wing buds. Embryos envenomed with South African (c) and Kenyan (d) B.A. showed significant apoptosis along with the entire wing buds, with the Kenyan spreading to the extremities. The Namibian embryo showed less severe cell apoptosis than (c) and (d) along with the wing buds.

5. Discussion

5.1 Bodyweight

Although the body weights varied amongst regional snakes and concentrations, they all presented similarly which was anticipated as they are the same species of snake. Generalised haemorrhaging was present across all four venom groups, which was expected due to the haemotoxic nature of *Bitis arietans* [47]. The Kenyan test group was the only one to have statistically significant changes in body weight with envenomation (E1, E2 and E3); the average weights for this group were also notably smaller than the rest, suggesting a larger detrimental effect than the other venom groups. The South African, Namibian and non-specific groups had similar mean weights across all four concentrations, which could propose a similar toxicity level across these regions. Further research into regional diet variations could strengthen the hypothesis that venom composition is derived from a natural dietary selection [28].

5.2 Heart weight

All heart weights of embryos envenomed with *Bitis arietans* were reduced, with the Kenyan group showing the highest rate of statistical significance, with E1, E2 and E3 all being significant; this group also presented the lowest mean weights at 13–15 mg. These findings support the proposal that the Kenyan venom has a stronger effect than the other regions. A histopathological study observed that *Vipera raddei*, from the family Viperidae, venom caused cardiomyocyte disruption, fibrosis and blood vessel congestion in post mortem specimens [77]. Another study found *Naja nigricollis* venom injected into pregnant mice caused hepatic and myocardial damage as well as pulmonary vascular congestion and accumulation of blood in the intestinal lumen of the foetus [78]. One of the venom components of *Bitis arietans* is a serine protease, a bioactive protein, which plays a role in the regulation of the blood coagulation cascade and affects the circulatory haemostasis [59]. Disturbances by these proteins could lead to adverse effects on embryonic organ development, which may account for the reduction in heart weights; however, to date, no studies are focusing on the effects of snake venom on the heart function.

5.3 Liver weight

All groups had a minimum of one statistically significant concentration, suggesting the liver was the most affected parameter (in comparison with heart and body weight). In furtherance with the proposal that the Kenyan venom has the highest potency, E1, E2 and E3 all proved statistically significant as well as being of the lowest median body weights of the groups surveyed. All livers exhibited a decrease in mean weight, varying yellow colour change along with increased susceptibility to disintegration on removal; this substantiates the results from

a previous study on the effects of *Echis coloratus* venom on sheep liver, where biochemical and histological alterations were observed. The study reported an elevation in glucose, aspartate aminotransferase, alanine transferase, triglyceride and total bilirubin whilst cholesterol levels were reduced. Histologically, pyknosis, karyorrhexis, cytoplasmic vacuolation, necrosis, fatty changes and hepatocyte atrophy were observed [79].

5.4 Mortality

The highest incidence of mortality was observed in E1 by the non-specified test group, with a 70% mortality; whereas Kenya, Namibia and South Africa observed a 40–10% death rate. This evidence proposes that although the Kenyan venom has the most significant impact on development, the non-specific venom has the strongest potency. Furthers study with a larger specimen pool is needed to confirm these findings.

5.5 Apoptotic cell appearance (Nile blue staining)

A 1993 study observed that haemorrhagic snake venom (*Bitis arietans*) induced apoptosis of vascular endothelial cells whereas neurotoxic venom did not induce programmed cell death; instead it caused necrosis at much higher doses of venom [80]. This corroborates with the findings of the Nile blue staining of this study which revealed varying levels of cell death along with the wing buds and limb extremities of *Bitis arietans* envenomed embryos. The development of limb vasculature may explain why apoptosis was seen the distal limbs, clustered away from the tips of the extremities. Interestingly, apoptosis was primarily seen on the wing buds, which develop later than the legs [81]. Previous studies may have explained the affinity for wing bud cells over leg bud cells due to the principle of non-equivalence meaning that cells in different areas of the body have different intrinsic characters and positional information [82]; however it has more recently been proven that these cells are interchangeable and not restricted in their development [83].

6. Conclusions

In conclusion, it was seen that the test group envenomed by *Bitis arietans* from Kenya exhibited the highest incidence of detrimental effects. Concentration 100, 10 and 1 mg/ml of venom all showed statistically significant changes to the body, heart and liver weight. This venom also produced the smallest average weights in comparison to the other test groups. The highest mortality rate, however, was observed in the non-specific venom, with the strongest concentration resulting in 70% fatalities. This suggests that the most fatal of the four venoms observed was the non-specific African *Bitis arietans*. The induced cell death was observed in all four venom specimens, which correlates to *Bitis arietans* previously documented haemotoxic potential. The results showed that the Kenyan venom, along with having the most significant effect on organ weights of the embryo, also initiated the strongest apoptosis.

These results suggest a geographical variation in potency of *Bitis arietans* venom, however further study is required to determine the cause of this variation. Current hypotheses suggest a natural dietary evolution influencing the venom composition; future studies examining if there is a regional difference in the diet of these snakes along with an analysis of their venom enzymes could aid in confirming this hypothesis.

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