Toxinology

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Elisabeth F. Schwartz
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Scorpion Venoms

Springer Reference

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In recent years, the field of toxinology has expanded substantially. On the one hand it studies venomous animals, plants and micro organisms in detail to understand their mode of action on targets. While on the other, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their three interaction with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on human body. Given the huge impact of toxin-based deaths globally, and the potential of venom in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that collects all of the important biochemical, biomedical and clinical insights relating to toxinology.

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Scorpion Venoms

With 115 Figures and 38 Tables

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Series Preface

The term TOXIN is derived from the Greek word *Toeikov* and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On the one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology, etc. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally and the potential of venom in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field is imminent.

Springer has taken the bold initiative of producing this series, which is not an easy target of producing about 11 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

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I would like to sincerely thank the section editors of this volume, Lourival D. Possani, Elisabeth F. Schwartz, and Ricardo C. Rodríguez de la Vega, for the invaluable contribution of their expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to Mokshika Gaur, Meghna Singh, and Audrey Wong for their tireless effort in bringing these volumes to reality.

Singapore

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Volume Preface

Old as the hills are, pictured in the sky, seen from no matter where, or engraved on millennial stones, scorpions have quite obviously fascinated humans for a long time, first and foremost because of the harm the sting of a few species could cause but also due to their unique natural history and for the many biologically active compounds found in their venoms. We have planned this volume to cover all those aspects. The subjects are divided into seven sections starting with an **Introduction** to the general aspects of scorpion biology and ecology, followed by the description of the **Envenomation** pathophysiology, pharmacokinetics, and pharmacodynamics of venoms and their complex interactions with the immune system. The future of antiscorpion venom therapy is then covered by two chapters dedicated to alternatives to the century-old techniques currently used to produce **Antivenoms**. The next section presents a world tour of **Scorpionism** and dangerous scorpion species and their impact on human health. It is worth remembering that envenomation due to scorpion stings is a substantial health hazard in Asian, Middle Eastern, African, and Latin American countries, with over one million people stung by scorpions every year resulting in more than 3,000 deaths. Species-centered overviews of **Scorpion Venoms** are presented in the next section, after which a section details the two main types of **Scorpion Toxins**. The last section covers high-throughput transcriptome and proteome screenings now known as **Venomics**. We have been lucky that many of the top-of-the-line scientists working on scorpions and their venoms have accepted our invitation; our gratitude to all of them. This is obviously more their book than it is ours.

The scope and time to publication differs greatly between reference books and standard academic journals. The road to this volume started as an initiative of our editor-in-chief, Ponnampalam Gopalakrishnakone, back in the middle of 2012. We agreed to act as volume editors by December of that year and started sending invitations, making agreements on the scope of the chapters, and setting up for the reception and reviewing of the chapters. Over the following year and a half, all the chapters were reviewed by at least two independent colleagues. We are indebted to our dozens of anonymous reviewers; their evaluation and constructive criticism have certainly improved the quality of the book. We are also thankful to the editorial team at Springer (Mokshika Gaur, Meghna Singh, and Audrey Wong) for their efforts during the editorial and production phases and general support.

As editors, we have tried to keep the chapters accessible to a wide audience yet deep enough as to provide detailed information to the experts. Being part of a reference book, the chapters are intended to be self-contained and can be consulted independently; nonetheless, all the chapters are cross-referenced to other chapters in the book in order to facilitate browsing through the different topics. The online version contains hyperlinks to indexed terms and references. We wanted this volume to be an accurate account of the state of the art on scorpion venom research. In line with the ambitious project of Springer's *Toxinology* handbook series, we envisioned this volume as a reference book that could be consulted for a long time. We purposely avoided some themes that are deemed controversial or speculative, for which we believe there is enough space in periodical journals. Should a breakthrough become available in the near future, such as the recently published genome draft of a single scorpion species, the online version of the book will be open to the authors to update their chapters, and a new edition could be planned in 3–5 years' time.

February 2015

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P. Gopalakrishnakone, M.B.B.S., Ph.D., F.A.M.S., D.Sc., is presently professor of anatomy and chairman of the Venom and Toxin Research Programme at Yong Loo Lin School of Medicine, National University of Singapore. He is also a consultant to the Defence Science Organization in Singapore and adjunct senior research scientist at the Defence Medical Research Institute. Prof. Gopalakrishnakone is an honorary principal fellow at the Australian Venom Research Unit, University of Melbourne, Australia.

His research studies include structure function studies, toxin detection, biosensors, antitoxins and neutralization factors, toxinogenomics and expression studies, antimicrobial peptides from venoms and toxins, and PLA2 inhibitors as potential drug candidates for inflammatory diseases. The techniques he employs include quantum dots to toxinology, computational biology, microarrays, and protein chips.

Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of *Toxicon*, the society's official journal.

His research awards include the Outstanding University Researcher Award from the National University of Singapore (1998); Ministerial Citation, NSTB Year 2000 Award in Singapore; and the Research Excellence Award from the Faculty of Medicine at NUS (2003).

His awards in teaching include Faculty Teaching Excellence Award 2003/4 and NUS Teaching Excellence Award 2003/4. Prof. Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

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Prof. Lourival D. Possani was born in Brazil, now Mexican by naturalization, completed his college degree in Porto Alegre, Brazil, on subjects related to natural sciences, and obtained his Ph.D. degree in molecular biophysics at the University of Paris in 1970. He spent a postdoctoral training at the Rockefeller University in New York and sabbatical years at the Max Planck Institute in Germany and at the Baylor College of Medicine in Houston.

Since 1974, he is professor at the Institute of Biotechnology of the National Autonomous University of Mexico. The work performed is mainly related to scorpion venom components' structure and function. His contributions to science are printed in 306 publications of international journals listed in the Science Citation Index. He has 40 patents of invention approved in several countries. In his laboratory, 84 students have finished their thesis at levels of college, master's, and Ph.D. degrees. His work is cited more than 7,000 times by other authors. He received many recognitions and distinctions for his work, among which is the Redi Award from the International Society on Toxinology; the Doctorate Honnoris Causa by the University of Debrecen, Hungary; the National Prize of Science in Arts from the Mexican Government; the National Autonomous University of Mexico prize on natural sciences; and numerous recognitions from pharmaceutical companies. He was an international scholar of the Howard Hughes Medical Institute for 10 years and recently received the Carlos Slim prize 2014 for working on health problems of Latin American and Caribbean countries.

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Prof. Elisabeth F. Schwartz, born in Brazil, is a biologist and completed her Ph.D. degree in physiological sciences at the University of São Paulo, Brazil, in 1987. Since then, she has been working with animal toxins. After spending 1 year in postdoctoral training at the lab of Prof. Lourival D. Possani at the Institute of Biotechnology of the National Autonomous University of Mexico, in 2004–2005, her research interest was pointed to arthropod venom toxins, mainly scorpion peptides acting on ion channels. Over the last 10 years, her work – mainly focused on the identification and characterization of scorpion toxins – combined proteomic and transcriptomic approaches. She is professor at the University of Brasilia, Brazil, where she teaches physiology and pharmacology. Her investigative work resulted in about 40 scientific publications, independently cited 400 times. She advised more than 70 students at levels of college, master's, and Ph.D. degrees.

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Dr. Ricardo C. Rodríguez de la Vega is a Mexican scientist holding a college degree in chemistry and a Ph.D. in biochemistry, both from the National Autonomous University of Mexico and both under the supervision of Prof. Lourival D. Possani. He received further postdoctoral training in Germany (European Molecular Biology Laboratory in Heidelberg) and France. He has also been a visiting scientist at the National Natural History Museum in Paris (France). A chemist-turned-evolutionary biologist, he has a long-lasting research interest in whether and how the evolutionary history of biotic interactions has been recorded at the molecular level. Over the last 10 years, his work has been mainly focused on the study of animal venoms and venom components, with combined experimental and computational biology approaches. He has contributed with some landmark evidence and analysis into

two main areas: (1) the molecular and evolutionary basis of target recognition by animal toxins, including the identification of previously neglected interaction modes of potassium channel blocking toxins, and (2) the convergent recruitment of protein folds into animal venoms, including one of the earliest identified venom recruitment events from immune response proteins and a paradigm-shifting analysis of venom evolution. A heavily cited author (over 1,000 independent citations to his nearly 30 papers), passionate for teaching and outreaching, Dr. Ricardo C. Rodríguez de la Vega is currently working in the genetics, ecology, and evolution team at the Paris-Sud University in France.

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Part I

Introduction

Scorpion Diversity and Distribution: Past and Present Patterns

1

Wilson R. Lourenço

Contents

Abstract

The aim of this chapter is to bring general information on the order Scorpiones. The content is in majority addressed to nonspecialists whose research embraces scorpions. Aspects covered include scorpion classification, diversity, and general patterns of distribution, applied both to fossil and extant faunas. Since a global consensus in relation to classification and/or patterns of distribution does not exist among modern experts, a rather broader perspective is proposed here, but several approaches remain open for discussion.

Introduction

Scorpions are Chelicerate arthropods and members of the class Arachnida which is a more or less distant relative of other arthropods such as the Insects, Myriapods, and Crustacea. They represent a very depauperate group with approximately 2,000

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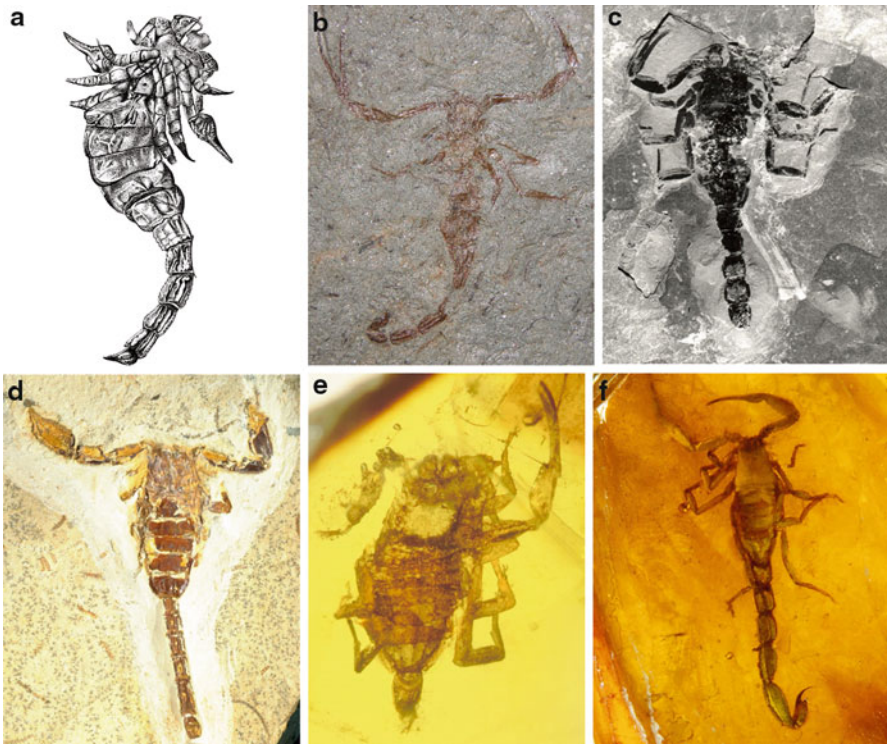

Fig. 1 Fossil scorpions. (**a**) *Allopaleophonus caledonicus* Hunter, Silurian of Scotland (After Vachon 1952). (**b**) *Protobuthus elegans* Lourenço and Gall, early Triassic of France. (**c**) *Gallioscorpio voltzi* Lourenço and Gall, early Triassic of France. (**d**) *Protoischnurus axelrodorum* Carvalho and Lourenço, early Cretaceous of Brazil. (**e**) *Archaeobuthus estephani* Lourenço, early Cretaceous of Lebanon. (**f**) *Palaeolychas balticus* Lourenço and Weitschat, Eocene/Paleocene

known species. These numbers being much less impressive than those known for other groups such as insects with over one million species or spiders with about 40,000 species.

Scorpions can be classified among the most ancestral arthropods both in origin and in body morphology. They first appeared as aquatic organisms during the Silurian (444–416 MYA) and knew rather small morphological changes since (Briggs 1987; Shear and Kukalová-Peck 1990; Sissom 1990; Jeran 2001). Although their apparent conservative form (Fig. 1) led some authors to define them as "living fossils," scorpions most certainly knew major biochemical, physiological, behavioral, and ecological adaptations that have combined to ensure their continued success over the past 450 million years.

The group first appeared as aquatic organisms. In Silurian times large landmasses were emerging from the oceans, forming the shallow estuaries and terrestrial habitats that scorpions and other arthropods would subsequently colonize. In their evolutionary history, scorpions almost certainly evolved from Eurypterida ("water scorpions")

since both groups share several common features, including external book gills, flap-like abdominal appendages, large multifaceted compound eyes, and similar chewing structures on the coxae of the first pair of appendages. The general acceptance that early scorpions were aquatic, maybe marine or amphibious, is supported by the presence of gills but also legs adapted to a benthic existence. Besides, the fact that many of the early scorpions were rather large also strongly suggests that they need water to support their bodies.

Marine and amphibious scorpions most certainly persisted well into the Carboniferous (359–299 MYA), and some species probably reached the Permian (299–251 MYA) and Triassic (251–200 MYA) periods (Briggs 1987; Shear and Kukalová-Peck 1990). The first unequivocally terrestrial (air-breathing) scorpion most certainly appeared on land during the Late Devonian (416–359 MYA) or Early Carboniferous. The earliest terrestrial fossil clearly established is *Palaeopisthacanthus schucherti* Petrunkevitch from the Upper Carboniferous, in which stigmata are preserved (Rolfe 1980). The presence of trichobothria (sensorial hairs) is also fundamental in the definition of terrestrial forms since these structures have no sense in an aquatic environment (Jeran 2001; Lourenço and Gall 2004). The evolution of book lungs in place of external book gills was the capital change associated with the transition from water to land. These paleo-scorpions radiated into several superfamilies and families (see the section on "Fossil Scorpions") now all extinct (Kjellesvig-Waering (1986). They ranged in size from a few centimeters to almost a meter for species such as *Gigantoscorpio willsi* Størmer and *Brontoscorpio anglicus* Kjellesvig-Waering.

Although scorpions can be considered as fascinating animals, the interest shown by people in general is only connected with their negative reputation of a "killer of man." Nonetheless, only a limited number of species, probably less than 50 are actually responsible of serious or lethal incidents. The interest on scorpions as "lethal organisms" was generated by the fact that a relatively small number of species possess venoms with potent toxins capable of killing humans. It is true, however, that scorpions are responsible for an important number of human deaths every year which is only surpassed by those caused by snakes and bees (Polis 1990a; Loret and Hammock 2001). Most deadly species belong to the family Buthidae; however, species belonging to at least two other families, Hemiscorpiidae and Scorpionidae, also contain species posing a threat to humans.

The origin of mammal-specific toxins appears as an important issue in scorpion evolution. Old World lineages of Buthidae with very potent neurotoxic venom, such as the genera *Androctonus* Ehrenberg and *Leiurus* Ehrenberg, share separate mammal- and insect-specialized neurotoxins which are specific for $\mathbf{Na^+}$ channels (Loret and Hammock 2001). Inversely New World genera such as *Centruroides* Marx and *Tityus* C. L. Koch have potent toxins acting on both mammals and insects. It is quite possible that the separate mammal-specific $\mathbf{Na^+}$ toxins could have evolved during aridification of the Palearctic in the Tertiary period, when one of the most important selective factors was rapid radiation of small burrowing mammals (mostly rodents) in arid landscapes. Such newcomers to the scorpion environment as rodents would be a direct competitor for space (burrows) and in

addition important nocturnal predators, as many of them are today (McCormick and Polis 1990). Such pressure explains the emergence of specific mammal-targeting toxins used for defense and not for foraging (Fet et al. 2003).

From the very beginning of scorpion studies on the late eighteenth century and during almost 150 years, researchers focused primarily at descriptive taxonomy, general anatomy, and very rudimentary biogeography, followed by medical research on venom biochemistry. Since the 1970s, however, basic research on scorpions expanded greatly to encompass behavior, physiology, ecology, and evolutionary biology. These studies attested about the scorpion's utility as a zoological model to investigate broader questions of the organismal biology (Polis 1990a; Brownell and Polis 2001).

The aim of this chapter is to provide some general information about scorpion diversity, distribution patterns, and evolution. The targets are readers who possibly use these organisms (or relative ones) in their own research but are not aware of their biological characteristics. For this reason, both the text and illustrations are prepared to ensure clarity, accuracy, and unambiguous communication, hopping that in this way the matter may be accessible to a broad audience.

Fossil Scorpions

Early scorpions were almost all aquatic or amphibian, and the order quickly radiated into an impressive number of superfamilies and families (see, for example, Fig. 1a). All these non-terrestrial fossils scorpions have been placed in the suborder Branchioscorpionina Kjellesvig-Waering by Kjellesvig-Waering (1986). Other fossil scorpions, clearly accepted as terrestrial forms, are classified in a distinct suborder Neoscorpionina Thorell and Lindström together with extant families (Fig. 1b, c). The suborder Branchioscorpionina includes 18–21 superfamilies and 41–47 families according to different authors (Sissom 1990; Fet 2000). These numerous lineages are a clear indication of their early and great success. Moreover, because the fossil record is rather fragmentary, these more than 20 superfamilies are probably only a fraction of the total number that actually existed (Sissom 1990; Jeran 2001). It is clear, however, that only a few, possibly only one, of these lineages survived and radiated into present day. Naturally, all extant scorpions live now in land (see section about extant scorpions).

Families of fossil scorpions are characterized on a few morphological features as defined by Kjellesvig-Waering (1986): the development of the maxillary lobes, the shape of the sternum, the terminations of the tarsi, and the presence or absence of lateral, faceted eyes (see also Sissom 1990). Since these characteristics are quite diverse in fossil scorpions, a rather large number of superfamilies and families have been recognized. In contrast, however, Kjellesvig-Waering (1986) suggested that on the basis of these same characteristics, only three families should be recognized among extant scorpions, rather than the much larger number accepted by neontologists. As suggested by Sissom (1990), this divergence of opinion clearly indicates a taxonomic problem, and the difficulties of this type are often the result of

different approaches in the studies performed by paleontologists and neontologists; the first working from the higher categories down and the second from lower categories up (Sissom 1990).

One question that is often addressed is the one about the age of extant scorpion lineages. Until recently, present scorpion lineages were estimated to have been present since the very early Cenozoic period (Sissom 1990). This estimation was based on very few fossil records available for the Cenozoic and Mesozoic periods. More recent discoveries for both the Cenozoic and Mesozoic periods based on both sedimentary and amber fossils attested that some extant lineages or at least protoelements of these lineages were already present in the Lower Cretaceous (Lourenço 2001, 2002a, 2003, 2012a; Lourenço and Beigel 2011; Carvalho and Lourenço 2001; Santiago-Blay et al. 2004) (Fig. 1c–e).

Patterns of distribution of early scorpions are poorly known since most fossil species are based on single specimens from rather localized sites. In account to the fact that early fossil discoveries took place in the North Hemisphere, it was a current suggestion, in the nineteenth and twentieth centuries, that these first appeared in Laurasia and subsequently "migrated" to Gondwana (Lamoral 1980). Naturally, this theory was largely biased by the early discoveries, but in more recent years, a number of fossils have also been found on sites located in the Southern Hemisphere attesting rather of an early colonization of all landmasses (Carvalho and Lourenço 2001) (Fig. 1d).

Since the Silurian period, with the emergence of major landmasses from the oceans, many shallow estuaries and also appropriate terrestrial habitats started to be formed appearing as ideal habitats for scorpion colonization. This process most certainly took place in all available landmass (Fig. 2) and continued during all the subsequent continental formation, including the Pangaea continental drift which started by the Triassic (251–200 MYA). It was followed by a more passive vicariant process in association with dispersal in response to the progressive fragmentation of Pangaea. This was followed by a continental drift which led to the present configuration of the continents and climates and naturally to the scorpion diversity observed since the Mesozoic period up to present days. The biogeographic patterns observed among modern groups of scorpions (i.e., families and genera) are derived from protofamilies and protogenera elements of Pulmonate-Neoscorpionina which originated in Laurasia and Gondwanaland during Pangaean times (see "Biogeography of Extant Scorpions" section) This suggestion seems to be in accordance with the very poor vagility observed in extant species (Lourenço 1996).

Extant Scorpions

Extant groups of scorpions have a wide geographical range of distribution and live on all major land continental masses with the exception of Antarctica (Fig. 3). Scorpion habitats are extremely diverse. Some opportunistic species were introduced, most certainly by human agency, in some lands originally lacking any scorpion. Examples are New Zealand, England, Indian Ocean islands such as

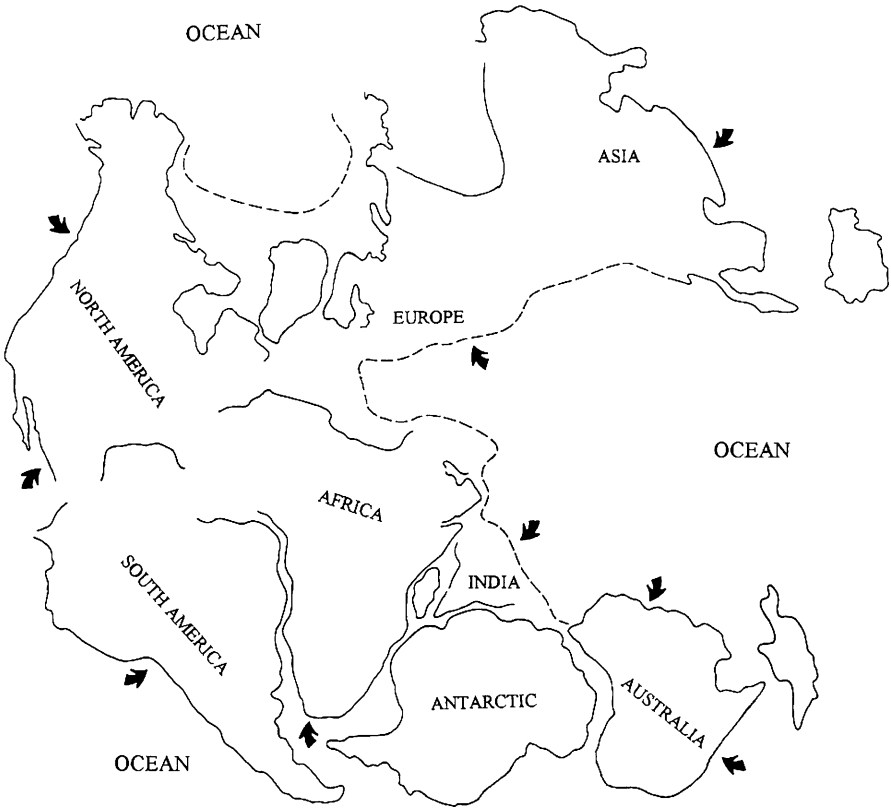

Fig. 2 Configuration of Pangaea about 300/200 MYA, with hypothetical ways of coastal colonization by aquatic/amphibian scorpions (*arrows*)

the "Réunion," and small Pacific Islands. They can be present on very high latitudes from Canada to the extremity of South America's Patagonia. Their tropical to temperate distribution is globally similar to that of other Arachnida. Only some spiders, mites, pseudoscorpions, and opiliones may have wider distributions reaching subpolar regions (Savory 1977). Species can be found in a large diversity of habitats, such as deserts, savannas, grasslands, temperate, subtropical, and rainforests; some are found in the intertidal zone and in organic soil while others on high mountains over 5,000 m in altitude. A number of species live in caves, and one recently described species from Vietnam, *Vietbocap thienduongensis* Lourenço and Pham, was found at depths of more than 2,000 m from the cave entrance (Lourenço 2012b).

The familial classification of scorpions was the subject of numerous studies during the last 150 years. These began in the middle of the nineteenth century with the contribution by Peters (1861) which was followed by several other relevant publications such as those of Thorell (1876), Simon (1879, 1880), Pocock (1893), Kraepelin (1899, 1905) and Birula (1917a, b). A more conservative period followed shortly after the appearance of these contributions about the classification of scorpions.

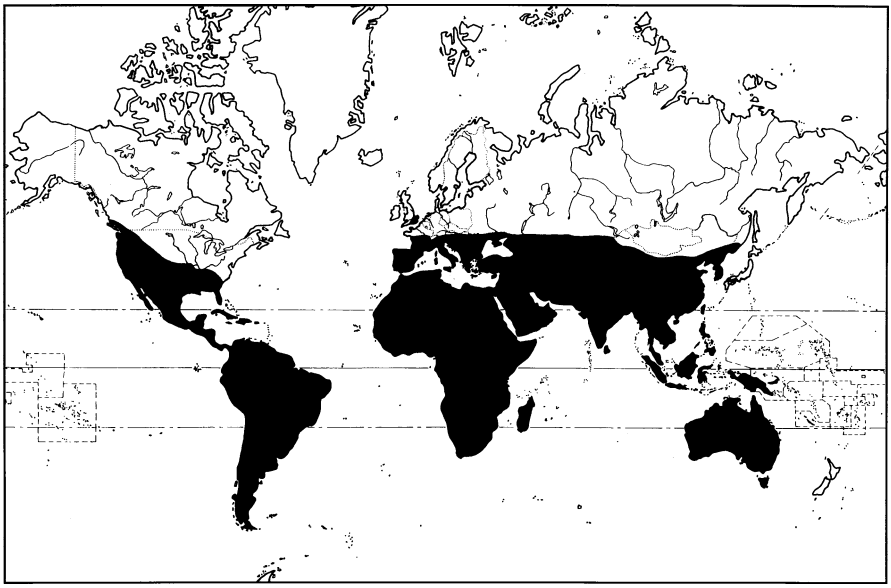

Fig. 3 World distribution of extant scorpions

During this period, only 6 or 7 families were recognized, and the situation remained practically unchanged until the publications of Lamoral (1980) and Lourenço (1989), in which 8 and 9 families were, respectively, accepted.

In more recent years, the familial classification of scorpions remained a subject of preoccupation among taxonomists, in particular since the contribution by Stockwell (1989). Several of these subfamilies were promoted to familial rank, and the position of a number of genera within the different families was revised. After the presentation of this doctoral thesis (which was not published), several authors continued to recognize only the 9 families defined by Lourenço (1989), but, for others, a division of these into several smaller groups appeared to be a real necessity. This new situation led to the recognition of a variable number of families (and superfamilies) according to different authors. The number of families was increased mainly by the promotion of subfamilies to families but also due to the discovery of totally new unsuspected familial groups (Gromov 1998; Lourenço 1998, 2000; Levy 2007).

The indecision of previous authors regarding the division of certain "large families" into smaller groups seems to be associated with two major considerations:

(a) Greater subdivisions of familial groups inevitably lead to the creation of much smaller families containing fewer genera and species.
(b) In several cases, elements belonging to a given familial group may consist of relict species with ruptures in their geographical distributions. These appeared enigmatic to earlier authors but also constitute a problem to some modern ones.

The situation as summarized above in **a** and **b** can be explained better in the light of panbiogeographic concept (Morrone and Crisci 1995). It is important to realize that scorpions are a very ancient group and that the biogeographical patterns observed today constitute only fragments of their original distribution (see "Biogeography of Extant Scorpions" section). The remainder of this incomplete "puzzle" is the consequence of various vicariant processes of delegation (Lourenço 1996).

Stockwell (1989) showed the way to some important decisions, among others the proposition of superfamilies which appeared as a possible need. The supra-familial classification proposed by Stockwell (1989) is convenient since it deals with many fossil forms, although it may led to considerable speculation. These propositions have been partially accepted but with some modifications by Lourenço (1998, 2000). Consequently, only a synthesis of the most recent familial classification is presented here. It comprises the extant but also fossil families included in the suborder Neoscorpionina and the infraorder Orthosternina Pocock, 1911. If all different currents of opinion are combined, one can reach, more or less, to the following classification. The aim of this classification is to serve as a reference to nonspecialists whose research embraces scorpions.

Order Scorpiones C. L. Koch, 1850 (1837)

Suborder Branchioscorpionina Kjellesvig-Waering, 1986

Including all extinct families of scorpions except Palaeopisthacanthidae Kjellesvig-Waering, Protobuthidae Lourenço and Gall, Gallioscorpionidae Lourenço and Gall, and several families found in Cretaceous amber. The suborder includes 18–21 superfamilies and 41–47 families according to different authors (Sissom 1990; Fet 2000; Soleglad and Fet 2003; Prendini and Wheeler 2005). See also the section on "Fossil Scorpions."

Suborder Neoscorpionina Thorell and Lindström, 1885

Infraorder Orthosternina Pocock, 1911

Family Akravidae Levy, 2007 (1 genus, 1 species)

Geographical distribution: Israel

† **Family** Archaeobuthidae Lourenço, 2001 (1 genus, 1 species) (Fig. 1e)

Geographical distribution: Lower Cretaceous of Lebanon

Family Bothriuridae Simon, 1880 (14 genera, 145 species) (Fig. 4a)

Geographical distribution: South America, Australia, and India (Himalaya)

Family Buthidae C. L. Koch, 1837 (86/†6 genera, 990/†18 species) (Fig. 5)

Geographical distribution: All continents with the exception of Antarctica; in tropical, subtropical, and, to some extent temperate regions

Family Chactidae Pocock, 1893 (15/†1 genera, 180/†2 species) (Fig. 6a)

Geographical distribution: South and Central Americas, Mexico (Baja California)

Family Chaerilidae Pocock, 1893 (1/†1 genera, 43/† 1 species) (Fig. 6b)

Geographical distribution: Asia and Southeast Asia

† **Family** Chaerilobuthidae Lourenço and Beigel, 2011 (1 genus, 1 species)

Geographical distribution: Lower Cretaceous of Burma

Family Diplocentridae Karsch, 1880 (9 genera, 116 species) (Fig. 4b)

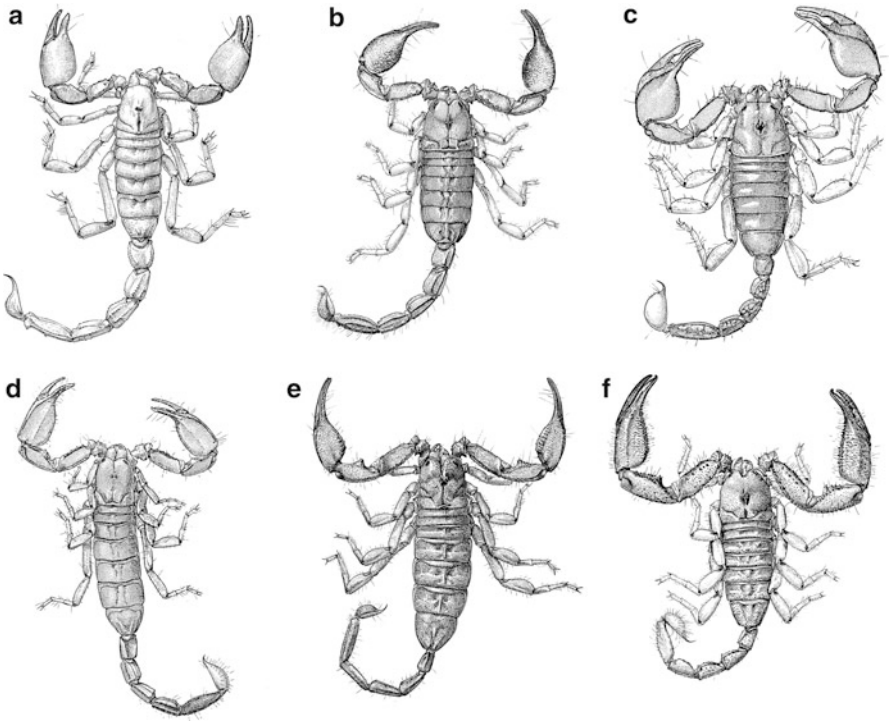

Fig. 4 Miscellaneous scorpions I. (**a**) *Timogenes sumatranus* Simon, Bothriuridae. Male from Argentina. (**b**) *Nébo flavipes* Simon, Diplocentridae. Female from Yemen. (**c**) *Euscorpius carpathicus* Linnaeus, Euscorpiidae. Male from Romania. (**d**) *Hemiscorpius maindroni* Kraepelin, Hemiscorpiidae. Male from Oman. (**e**) *Heteroscorpion opisthacanthoides* Kraepelin, Heteroscorpionidae. Female from Madagascar. (**f**) *Chiromachus ochropus* C. L. Koch, Hormuridae. Male from the Seychelles Islands

Geographical distribution: North and Central America, the north of South America, the Caribbean region, and the Middle East

Family Euscorpiidae Laurie, 1896 (4 genera, 31 species) (Fig. 4c)

Geographical distribution: Mexico and Guatemala, North Africa, southern Europe, Middle East

† **Family** Gallioscorpionidae Lourenço and Gall, 2004 (1 genus, 1 species) (Fig. 1c)

Geographical distribution: Triassic of France

Family Hadogenidae Lourenço, 1999 (1 genus, 20 species) (Fig. 6c)

Geographical distribution: South Africa

Family Hemiscorpiidae Pocock, 1893 (1 genus, 15 species) (Fig. 4d)

Geographical distribution: North Africa and Middle East

Family Heteroscorpionidae Kraepelin, 1905 (1 genus, 6 species) (Fig. 4e)

Geographical distribution: Madagascar

Family Hormuridae Laurie, 1896 (9 genera, 54 species) (Fig. 4f)

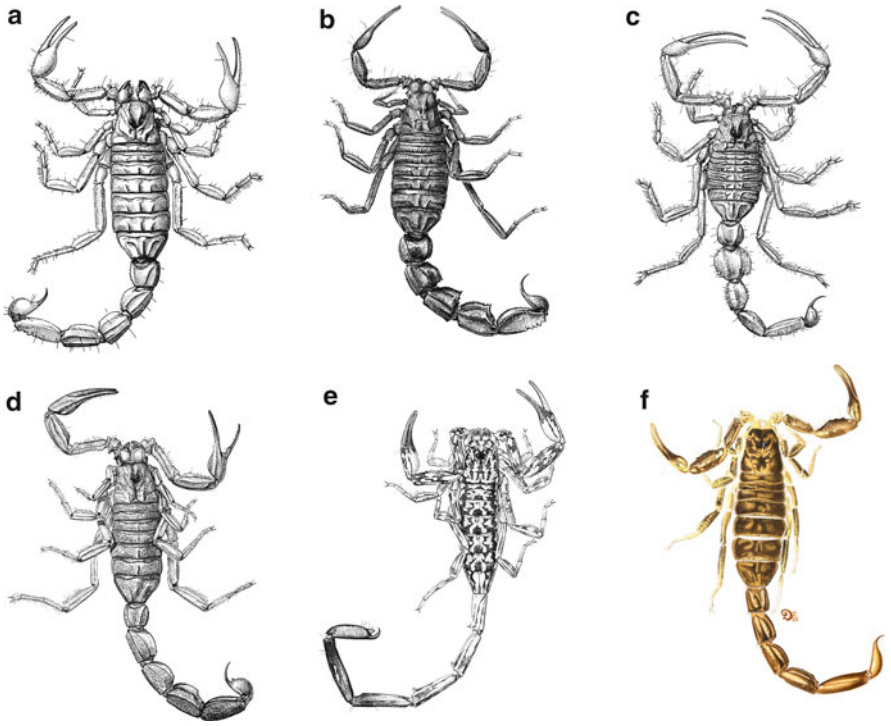

Fig. 5 Buthidae scorpions. (**a**) *Buthus occitanus* Amoreux, Buthidae. Female from France. (**b**) *Androctonus bicolor* Ehrenberg, Buthidae. Female from Israel. (**c**) *Apistobuthus pterygocercus* Finnegan, Buthidae. Female from Saudi Arabia. (**d**) *Rhopalurus agamemnon* C. L. Koch, Buthidae. Male from Brazil. (**e**) *Isometrus heimi* Vachon, Buthidae. Male from New Caledonia. (**f**) *Pseudouroplectes maculatus* Lourenço and Goodman, Buthidae. Female from Madagascar

Geographical distribution: Australia, Africa, Central and South America, the Caribbean region, India, Southeast Asia, the Pacific Islands, Madagascar, and Indian Ocean islands

Family Iuridae Thorell, 1876 (8 genera, 42 species) (Fig. 7a)

Geographical distribution: South America, North America, Asia (Turkey), and Europe (Greece)

Family Lisposomidae Lawrence, 1928 (2 genera, 3 species) (Fig. 7b)

Geographical distribution: South and Southeast Africa

Family Microcharmidae Lourenço, 1996 (2 genera, 16 species) (Fig. 7c)

Geographical distribution: Madagascar

† **Family** Palaeoeuscorpiidae Lourenço, 2003 (1 genus, 1 species)

Geographical distribution: Lower Cretaceous of France

† **Family** Palaeopisthacanthidae Kjellesvig-Waering, 1986 (3 genera, 4 species)

Geographical distribution: Upper Carboniferous.

† **Family** Protobuthidae Lourenço and Gall, 2004 (1 genus, 1 species) (Fig. 1b)

Geographical distribution: Triassic of France

Fig. 6 Miscellaneous scorpions II. (**a**) *Broteochactas delicatus* Pocock, Chactidae. Female from French Guiana. (**b**) *Hadogenes troglodytes* Lourenço and Ythier, Chaerilidae. Female from the Philippines. (**c**) *Chaerilus philippinus* (Peters), Hadogenidae. Male from Mozambique (Photo Jan O. Rain). (**d**) V*ietbocap thienduongensis* Lourenço and Pham, Pseudochactidae. Male from Vietnam (Photo D.-S. Pham)

† **Family** Protoischnuridae Carvalho and Lourenço, 2001 (1 genus, 1 species) (Fig. 1d)

Geographical distribution: Lower Cretaceous of Brazil

Family Pseudochactidae Gromov, 1998 (3 genera, 6 species) (Fig. 6d)

Geographical distribution: South of Central Asia and Southeast Asia

Family Scorpionidae Latreille, 1802 (5 genera, 135 species) (Fig. 7d)

Geographical distribution: Africa, Middle East, South and Southeast Asia, and Indonesia

Family Scorpiopidae Kraepelin, 1905 (6/†1 genera, 56/†1 species) (Fig. 7e)

Geographical distribution: Southern Central Asia (Afghanistan, India, Pakistan) and Southeast Asia (Cambodia, Laos, Indonesia, Malaya, Vietnam)

Family Superstitioniidae Stahnke, 1940 (1 genus, 1 species) (Fig. 7f)

Geographical distribution: Southwestern United States and Mexico

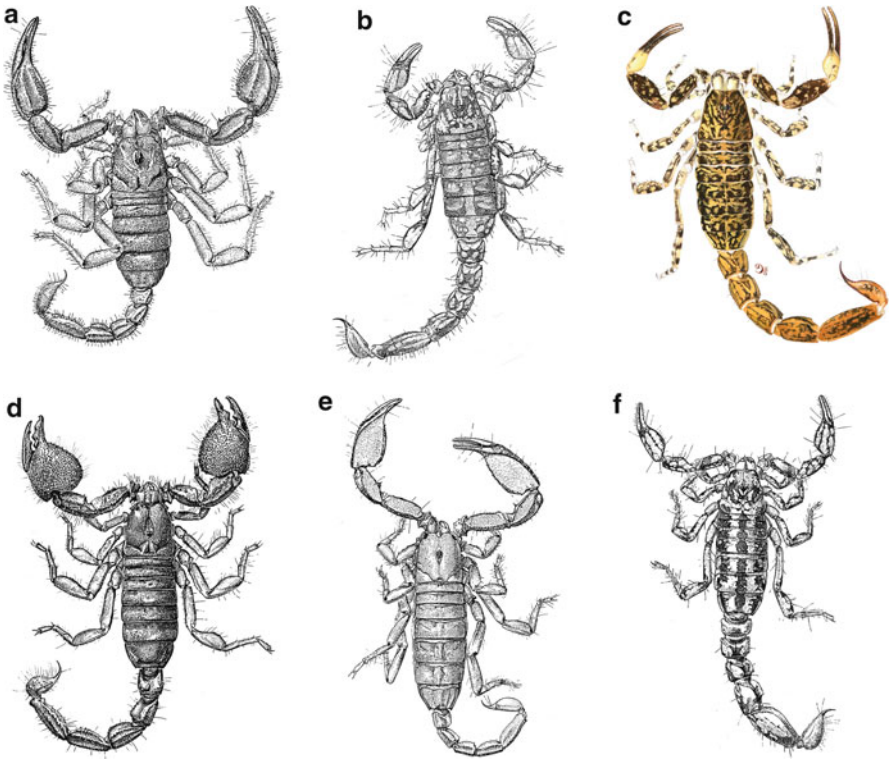

Fig. 7 Miscellaneous scorpions III. (**a**) *Iurus dufoureius* Brullé, Iuridae. Male from Greece. (**b**) *Lisposoma elegans* Lawrence, Lisposomidae. Female from Namibia. (**c**) *Microcharmus variegatus* Lourenço, Goodman, and Fisher, Microcharmidae. Female from Madagascar. (**d**) *Pandinus dictator* Pocock, Scorpionidae. Male from Cameroon. (**e**) *Scorpiops kraepelini* Lourenço, Scorpiopidae. Male from Pakistan. (**f**) *Superstitionia donensis* Stahnke, Superstitioniidae. Female from the USA

† **Family** Palaeotrilineatidae Lourenço, 2012 (1 genus, 1 species)

Geographical distribution: Lower Cretaceous of Burma

Family Troglotayosicidae Lourenço, 1998 (2 genera, 4 species) (Fig. 8a)

Geographical distribution: Oriental Pyrenees (France/Spain), Ecuadorian Amazonia, and Colombian Pacific Rainforest

Family Typhlochactidae Mitchell, 1971 (4 genera, 10 species)

Geographical distribution: Mexico

Family Urodacidae Pocock, 1893 (2 genera, 22 species) (Fig. 8b)

Geographical distribution: Australia

Family Vaejovidae Thorell, 1876 (17 genera, 181 species) (Fig. 8c)

Geographical distribution: Mexico and southern/southwestern United States

As explained at the beginning of this section, the familial classification of scorpions has changed significantly during the last 10–20 years. Specialists now recognize a number of families more than double the total they had accepted until the end of the 1980s.

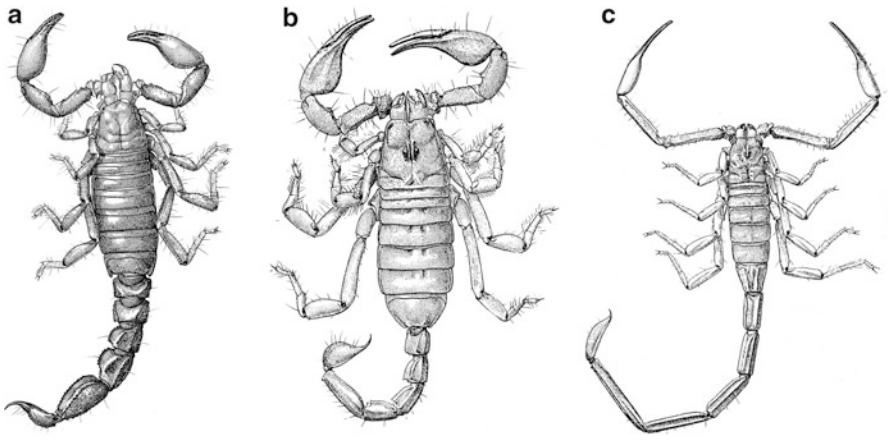

Fig. 8 Miscellaneous scorpions IV. (**a**) *Troglotayosicus vachoni* Lourenço, Troglotayosicidae. Female from Ecuador. (**b**) *Urodacus yaschenkoi* Birula, Urodacidae. Female from Australia. (**c**) *Syntropis macrura* Kraepelin, Vaejovidae. Male from Baja California, Mexico

Obviously, not all scorpion taxonomists agree about a single or unique classification, and different subdivisions may therefore be suggested for the same familial group. These differences can be explained as being based on the personal view of each specialist. Certain authors prefer to reduce the number of families, while others have the tendency to subdivide them. Naturally, these personal views do not explain all the differences of opinion. Some may be the result of the different approaches used in various taxonomic and phylogenetic studies.

Certain authors base their studies exclusively on morphological characters which are usually exclusively external. Even with the appropriate use of cladistics, it is not always easy to define apomorphic or plesiomorphic characteristics with sufficient precision to achieve the necessary synapomorphies. One reason for this is the limited information that can be obtained from fossil scorpions. Sedimentary fossils are often too badly preserved to be really useful, and scorpions preserved in amber still remain rare, even if more and more pieces have been studied in the last 10 years.

There is optimum today regarding new techniques, especially those based on molecular studies and in particular on DNA analysis. This type of approach could undoubtedly contribute to a better definition of natural lineages. Unfortunately in the case of scorpions, one particular problem still exists: the rarity of specimens. While some populations are very numerous, others, in contrast, occur at very low densities. Several species are known only from a single specimen. Some genera and even some families have been defined on the basis of very few specimens. This type of situation is not rare and may persist for several decades. Under such conditions, studies based on molecular biology are far from being simple.

Finally, taxonomy should not remain isolated from other fields of research. Data from biology, in particular from embryology, can certainly help in the clarification of natural lineages. Some preliminary results obtained by Lourenço et al. (1986)

demonstrate that a precise knowledge of embryonic processes and of variations in them can introduce useful information for a better definition of the familial lineages of scorpions. Such biological approaches naturally require a considerable amount of time and energy, but when such information is available, it should not be neglected in phylogenetic studies.

Biogeography of Extant Scorpions

Studies on scorpion biogeography are not recent. Attempts began with the contributions of Pocock (1894), Kraepelin (1905), and Birula (1917a, b). Certain general patterns of distribution were then proposed, even though the viewpoints of the different authors were frequently not in agreement. These early general contributions have been followed by regional biogeographical studies (Mello-Leitão 1945; Vachon 1952; Koch 1977; Francke 1978; Lamoral 1979; Couzijn 1981). Most of these studies, however, were unable to demonstrate precise biogeographical patterns. More recent studies, dealing with Neotropical scorpions, allow the definition of some biogeographic patterns (Lourenço 1986a, b, 1994, 1996, 2002b). The definition of these patterns became possible due to a better knowledge of the phylogeny of several groups, the application of recent hypothesis concerning climatic vicissitudes in tropical biomes during the late Cenozoic and Pleistocene periods, and a much better knowledge of scorpion life history strategies. According to Polis (1990) and Lourenço (1991), most scorpions can be defined as being equilibrium species, presenting therefore very predictable patterns of distribution.

A more detailed biogeographical model was proposed by Lourenço (1996) based on Udvardy's (1981) division of biogeography into three spatiotemporal scales (Fig. 9). This approach proved to be clear and didactic, and three major biogeographical events can be suggested to explain most of the patterns of distribution observed among scorpions today.

The first scale which can be defined as phylogenetic or paleobiogeographic encompasses the evolutionary time of all biota and is limited in space only by the size of the earth (Udvardy 1981). On this scale, only historical factors can be assumed to have taken place since, for almost all ecological conditions, data are largely or totally unknown. At this level, the evolutionary process of biogeography is, to a considerable extent, a tributary of continental drift and plate tectonics. This new view shook the foundations of the theories of many older paleontologists and biogeographers (Udvardy 1981).

Few authors (e.g., Lamoral 1979, 1980; Couzijn 1981; Nenelin and Fet 1992; Lourenço 1996) have taken continental drift into consideration when discussing aspects of regional biogeography. Lamoral's (1980) suprageneric classification of recent scorpions, with discussion on their zoogeography, was an important attempt to explain the general patterns of scorpion biogeography. The zoogeographical suggestions which Lamoral made are generally acceptable: (A) The present global scorpion fauna is derived from elements of the pulmonates (Neoscorpionina) that originated in Laurasia and Gondwanaland during Pangaean times; (B) Protobuthids

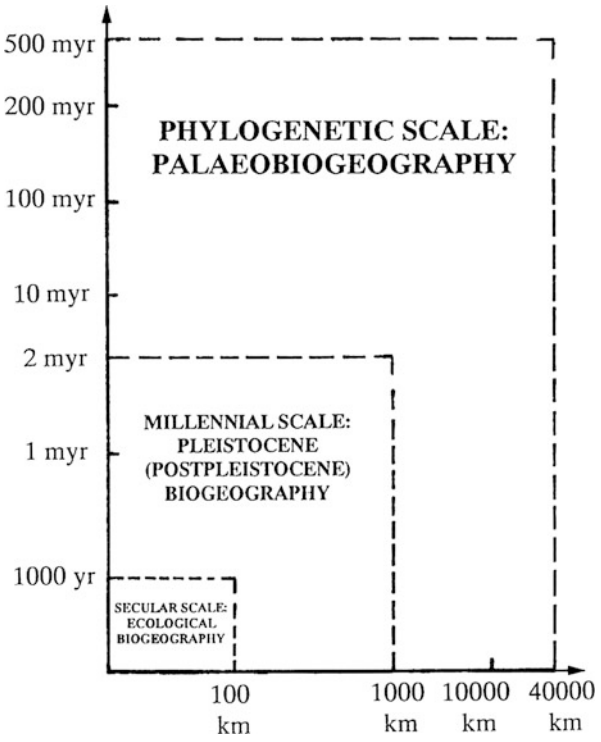

Fig. 9 Division of biogeography into the three spatiotemporal scales (Modified from Udvardy (1981))

were the dominant fauna in Pangaea, and the distribution of present Buthidae is the result of a vicariant process emanating from the fragmentation of Laurasia and Gondwanaland; (C) other early ancestors of scorpions such as the Chaeriloids, Chactoids, Pseudochactoids, and Scorpionoids also evolved in Laurasia and/or Gondwanaland in this past period. The more detailed conclusions of Lamoral (1980) are mainly correlated with vicariance and with continental drift. Lamoral (1980) was unable, however, to explain some important points. He possibly insisted too much on the role of dispersion when affirming that two major factors have influenced speciation and distribution patterns. One is the fragmentation of Pangaea and Gondwanaland and the other is the movement of Laurasian elements to the north of Gondwanaland. This second factor should be reconsidered. The process of "active" dispersion should rather be interpreted as being a more "passive" process in the dispersal sense as defined by Haffer (1981). This argument can be supported by the poor vagility presented in modern species of scorpions. Naturally, it could be suggested that early aquatic scorpions were better able to disperse than terrestrial forms, being therefore able to reach many of the shores of Pangaea before and during the fragmentation process. Present biogeographic patterns should be considered as the result of different vicariant processes and as pieces of an incomplete puzzle. What can be called as the "apparent anomalies in the distribution of some groups of families and genera" have been discussed since Pocock (1894).

Even today the disjunctive distributions of several families and genera of scorpions remain unexplained. The cases of the present disjunctive distribution of some scorpion groups should be regarded as the result of the previous distribution of protoelements of families and genera, followed by a vicariant process. The precise mechanism of these processes is not, however, always known. In conclusion, the main event responsible for the distribution of scorpions on a paleogeographic scale is the fragmentation of Pangaea and subsequent continental drift. The difficulties to explain the discontinuous distribution of some familial and generic groups point not only to the great geological age of these groups but also to the relict faunas and biogeographical patterns which they exhibit at present.

The second scale used in scorpion biogeography can be defined as millennial or Pleistocene biogeography. Between the development of the earth's crust and the Pleistocene epoch several events took place, many of which were related to the continuous drift of the continents. Some examples are mountain building, differential erosion, epicontinental seas, climatic-vegetational fluctuations, changes of world sea level, and the formation of major river systems. These events took place during the Cenozoic over a period of 60 My and have influenced the present biogeographical patterns of scorpions. Climatic-vegetational fluctuation most certainly played a major role, starting on the late Cenozoic period but having a major impact during the Pleistocene time (Haffer 1981, 1993). During many years most contributions concerning tropical regions stated that the biogeographic and diversity patterns observed in these regions could be explained by the long stability of tropical forests over millions of years (Federov 1966; Richards 1969). Subsequent studies on geology, paleoclimates, and palynology, especially in Amazonia and Africa (Prance 1982; Moreau 1963; Livingstone 1975, 1982), demonstrated that this presumed stability was a fallacy. In fact, although the temperatures in tropical lowlands remained "tropical" during glacial periods (3–5 °C lower than today), the forest broke into isolated remnants during cool dry periods (glacial phases). The remnants of forest expanded and coalesced during warm humid periods (interglacial phases). Conversely, nonforest vegetation expanded during glacial phases and retreated during interglacial phases (as at present). Data from geoscience, however, have been insufficient to indicate the precise areas of changing forests and nonforests and, in particular, the areas in which forests remained during arid phases, presumably serving as refugia for animal and plant populations. Nevertheless, in the Neotropical region, studies on the biogeographical patterns of scorpions (Lourenço 1986, 1987) suggested several endemic centers which are well correlated with the conclusions of Prance (1982) on woody plants and Haffer (1969) on birds.

A third scale also used in scorpion biogeography is defined as "ecological biogeography." This scale, however, was globally rejected in pioneer biogeographic studies, mainly because it was biased by two major considerations: (A) an almost total lack of knowledge of life history strategies (more precise data on this subject was only available on the 1970/1980s but was almost the only preoccupation of ecologists) and (B) a generalized opinion, even among modern biologists, according to which scorpions are capable of withstanding radical changes in environmental conditions and, therefore, of being very good colonizers.

This assumption is obviously fallacious. With growing knowledge of scorpion life history strategies, it was clearly evident that many, if not most scorpions, are equilibrium species, which tend to inhabit stable and predictable natural environments, produce single egg clutches, do not store sperm, have long life spans, present low population densities, have a very low r_{max}, show weak mobility, and are highly endemic (Polis 1990b).

In contrast, some scorpion species can be "opportunistic". These include species mainly of the families, Buthidae, Euscorpiidae, and Liochelidae (now Hormuridae). They are marked by ecological plasticity and are readily capable of invading disturbed environments. They may produce multiple clutches from a single insemination, have elaborate sperm-storage capabilities (Kovoor et al. 1987), short embryonic development, short life spans, high population densities, rapid mobility, and are widely distributed. The study of these opportunistic species is, however, of little use for establishing biogeographical patterns.

Opportunistic species evolve mainly in disturbed and unpredictable environments which can be the result of natural causes (e.g., volcanic activity) or are directly associated with human action. Several classical examples can be outlined: The population of the Neotropical species *Centruroides gracilis* (Latreille) are established in the Canary Islands for almost two centuries (Kraepelin 1905; Lourenço 1991); the originally Sri Lankan species *Isometrus maculatus* (DeGeer) has today a worldwide distribution in tropical and semitropical regions; and this species was transported by human agency during the last four centuries. The replacement of species is well illustrated in several islands of Eastern Asia where natural volcanic activity and human impact are important (Vachon and Abe 1988).

In continental regions, opportunistic species can rapidly occupy habitats disturbed by human activities, where the original native species have been selected against, thus leaving their ecological niches vacant. Several examples are known of scorpion experts, but one is well known by biologists in general. It concerns the remarkable expansion of the noxious Brazilian species *Tityus serrulatus* Lutz and Mello during historical times (Lourenço and Cuellar 1995).

In less than a century, the geographic range of *Tityus serrulatus* has increased considerably. This phenomenon was readily observed by ecologists mainly because this species poses an exceptional health problem, due to its rapid expansion in urban areas, rapid proliferation, and great toxicity.

Scorpionism is well known in Brazil and has been documented since the end of the nineteenth century. The first comprehensive study of the phenomenon was published by Maurano (1915). Before this publication, not much had appeared in the literature about scorpion problems in Brazil. This seems curious because of the enormous health problem caused by scorpions there today. In other regions such as in Mexico, scorpionism is also a very severe health problem; it has been, however, cited in the literature since the sixteenth century (Cardenas 1591). Another curious aspect is the fact that Maurano (1915) dealt only with *Tityus bahiensis* (Perty) and also that antivenom serum has been produced from this species since 1915. At the same time no mention exists about *T. serrulatus* which was only described in 1922.

T. serrulatus is most certainly a native species from the Brazilian states of Minas Gerais and Bahia. However, before the beginning of the eighteenth century, its presence was most certainly inconspicuous. At the beginning of the eighteenth century, an important development was engendered by the Portuguese, in their search for gold, with the foundation of new towns such as Curral d'El Rei and Vila Rica de Ouro Preto. Previously metaclimax environments suffered human impact, turning them into a disclimax situation. This favored the previously discrete parthenogenetic population of *T. serrulatus*, an opportunistic species, to explore the newly created disclimax habitat. The expansion of human colonization toward the west and north resulted in a significant expansion of the original population of *T. serrulatus* which usually colonizes urban areas (cities and towns) and can easily be transported by human agency from old to new cities. Brasilia, the new capital of Brazil, was constructed from 1956 to 1960 and has been invaded and colonized by *T. serrulatus* in less than 15 years (Lourenço et al. 1994). Obviously, the condition of obligate parthenogenetic species largely favored *T. serrulatus* geographic expansion. Most certainly this species was a savanna dwelling, where it probably inhabited palm trees. Today, however, since natural savannas have been converted to agriculture and grazing, it is almost restricted to human habitations. For more information on parthenogenetic species of scorpions, the reader can refer to Lourenço (2008).

Conclusion and Future Direction

The first objective of this chapter is to bring general information about scorpions, addressed to nonspecialist people whose research embraces this group. The idea is to demonstrate that the group's diversity and patterns of distribution are much more complex than it seems at first sight, in particular to those having access to a limited number of species. If the group's diversity is important, the same should be applied to the diversity of toxins. Today only a very limited number of distinct taxa retain the attention of toxin experts, but maybe this overview about scorpions may encourage the interest of researchers on biochemistry and molecular biology of venom toxins to expand their research to a broader array of scorpion groups, in particular for those that can be informative on the evolution of complex venoms.

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Introduction to Scorpion Biology and Ecology

2

Roland Stockmann

Contents

Abstract

Scorpions are venomous predatory arthropods with highly effective sensory mechanisms. They are equipped with a telson armed with two venom glands and a sting. Their ecophysiological characteristics have allowed them to adapt at

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many different environments and in particular deserts. Their ecological features reflect several key traits of their strategies and their relationships with their environment.

Introduction

The order Scorpiones belongs to the class Arachnida. It includes about 2,100 species, belonging to 190 genera and 16 (to 19) families as currently defined, two of which (Buthidae and Hemiscorpiidae) include species dangerous to humans (Fig. 1). Scorpions are principally predators of arthropods. They need to detect the prey on which they feed, and they have many very effective sensory organs enabling them to do this. Their venom apparatus is located at the extreme posterior end of the body and consists of two venom glands within a vesicle prolonged into a sharp needle, the aculeus. Through their behavior and their morphological, anatomical, physiological, and biochemical characteristics, scorpions have been able to adapt to many constraints in the surrounding environment, including desert conditions. The ethological features of scorpion reproduction are unique and their ecological features reflect some key traits in their strategies and their relationship with their environment.

Scorpion Morphology

Scorpions belong to phylum Arthropoda: their bodies are divided into segments, they are covered with a cuticle consisting of chitin and tanned proteins and they grow by molting. They belong to subphylum Chelicerata: they have a pair of preoral appendages, the chelicerae, and have no antennae. Like all members of the class Arachnida, they have a pair of pedipalps and four pairs of walking legs. Paleontologists believe that the common ancestors of scorpions were probably eurypterids (Dunlop and Braddy 2001) or xiphosurans (Shulz 2007). The first scorpions date from the Silurian era (about 420 million years ago).

The body of the scorpion has two parts: a prosoma or cephalothorax and an opisthosoma or abdomen, which is itself divided into two parts, a large mesosoma and a narrow metasoma (or tail) ending in the venom vesicle (Fig. 2).

The prosoma is covered on the dorsal side by a “carapace,” which may be trapezoid or rectangular in shape and bears two medial eyes and two to five pairs of lateral eyes, together with the carinae or keels. The prosoma carries six pairs of appendages. At the very front are very small tripartite pincers called chelicerae, located in front of the mouth and armed with teeth. The pedipalps, ended by a chelae, and four pairs of walking legs follow. The ventral face of the prosoma is

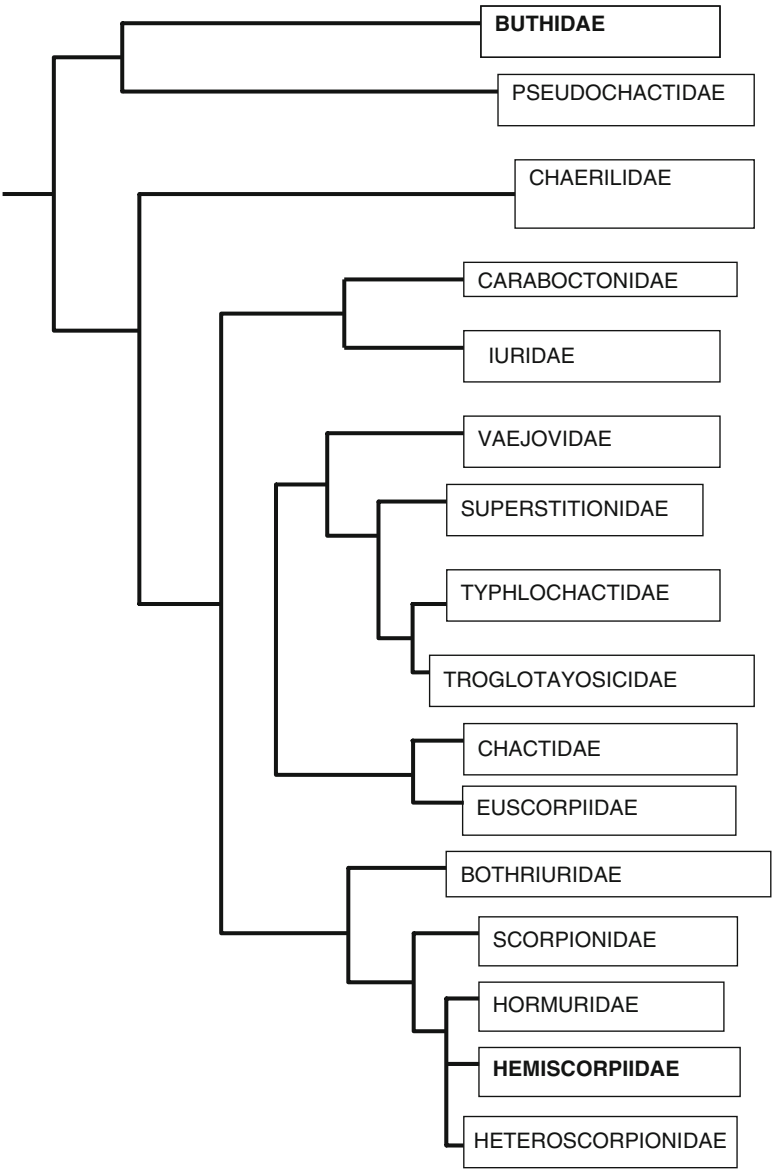

Fig. 1 Classification of the Scorpions

composed of the bases of the legs, the last two of which frame the sternum, a small structure that may be triangular or pentagonal (or may take the form of a transverse bar).

The mesosoma bears seven dorsal plates or tergites, often decorated with carinae. On its vertical side, it bears a genital operculum formed from one or two

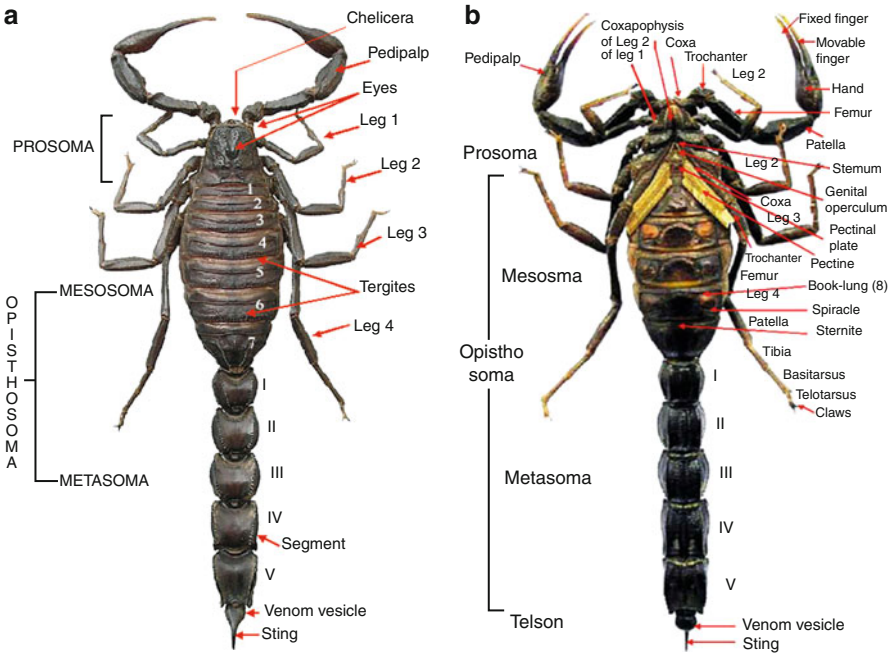

Fig. 2 (**a**) *Androctonus crassicauda* Dorsal view. (**b**) *Androctonus crassicauda* Ventral view (Photos by S. Sanchez)

valves, followed by a medial pectiniferous plate carrying the appendages characteristic of scorpions, the pectines. These paired mesosomal appendages consist of several parts, with variable numbers of laminae or teeth (from 1 to 58). They are followed by five large plates or sternites, the first four of which each carry a pair of slit-like (or rounded) respiratory orifices (spiracles) connected to four pairs of lungs. Flexible articular membranes connect each tergite or sternite to the next one, and pleural membranes connect the tergites and sternites laterally.

The metasoma or tail consists of five narrow, mobile segments without pleural membranes. It ends in the telson or venom vesicle, which itself ends in the aculeus (or sting), a needlelike structure with two subterminal laterodorsal orifices.

The Venom Apparatus

The venom apparatus is located in the telson, at the end of the metasoma (Fig. 3). This positioning is unique among animals and, together with the presence of the pectines, is one of the apomorphic (also known as derived) characteristics of scorpions. In arthropods, the telson is the terminal part of the animal, located behind the anus. In scorpions, it consists of a venom vesicle prolonged ending in the aculeus.

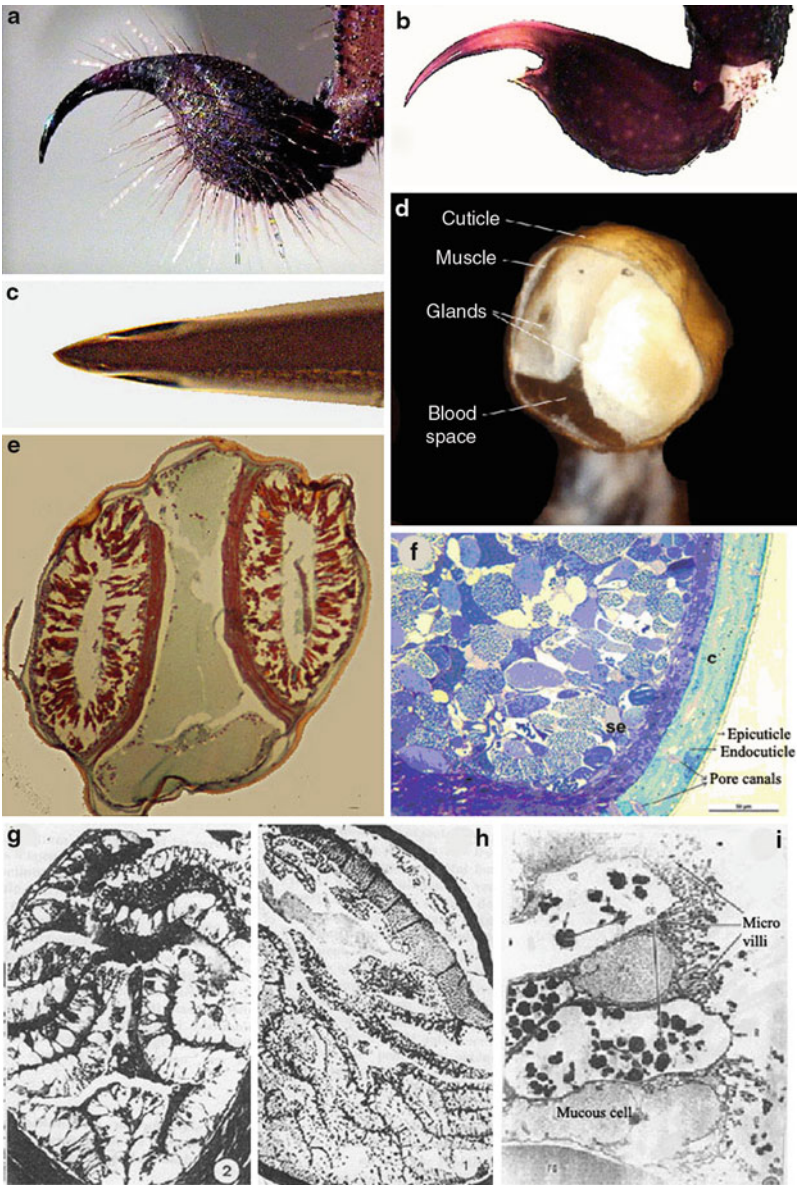

Fig. 3 Venom vesicle and sting. (**a**) Venom vesicle of *Parabuthus transvaalicus* with numerous setae, (**b**) Venom vesicle of *Tityus obscurus* with a sub-aculear protuberance, (**c**) Sting of *Androctonus australis*, (**d**) Section of the venom vesicle of *Euscorpius tergestinus*, (**e**) Histology section of the venom vesicle of *Euscorpius tergestinus*, (**f**) Semi-thin section of a venom gland of *E. mingrelicus* (without folds) (in Yigit and Binli 2008), (**g**) Histology section of the venom gland of *Hottentotta judaicus* with folds (in Kovoor 1973), (**h**) Histology section of the venom gland of *Leiurus quinquestriatus* with folds (in Kovoor 1973), (**i**) Histology section of the venom gland of *Centruroides* sp. at the border of the light (in Keegan and Lockwood 1971)

From the outside, the venom vesicle appears to be a more or less globular, smooth or granular bulge, which may have a tubercle or ventral denticle. It is often covered with fine hairs, which may be numerous. Dissection (or the analysis of sections) has shown that the vesicle consists of two venom glands surrounded by a muscle attached to the cuticle. The two glands are separated by a blood lacuna. The venom vesicle is associated with a powerful, highly mobile, **extrinsic muscle system** consisting of the tail muscles and muscles with insertion points before the vesicle (powerful extensors and flexors of the aculeus) and an **intrinsic muscle system** located around the glands. The extrinsic system makes it possible to reach the prey, to propel the aculeus mechanically, and to ensure its penetration. The intrinsic muscles are organized into a large band of **striated muscles** surrounding each gland; their contraction propels the venom through the aculeus. These muscles are attached to the cuticle dorsally and laterally by tendons. They are type II muscles, displaying short, rapid contractions. The glandular epithelium may be simple (Euscorpiidae) or folded and digitated to various extents (many folds in Buthidae) (Pawlowsky 1913). The number of cell types present in this epithelium differs between species and between studies (Samano-Bishop and Gomez de la Ferriz 1964; Keegan and Lockwood 1971; Mazurkewiecz and Bertke 1972; Kovoor 1973; Gopalakrishnakone et al. 1995; Yigit and Benli 2008; 2009). The glandular epithelium consists of at least one layer of columnar cells with apical microvillosities. The nucleus and organelles are located at the base of the cells. This epithelium often contains support cells that are sometimes considered to be essentially replacement cells, mucous-secreting goblet cells, and serous glandular cells containing secretion granules of different sizes, shapes, and electron densities, in enclosed vesicles. Intercalated nerve cells and dendrites are found in the layer of secretory cells. In certain members of the Bothriuridae, the venom glands are overlaid by an unpaired dorsal gland that is probably pheromonal in nature (Peretti 1997).

The aculeus (or sting) ends in two subterminal laterodorsal orifices, it differs in length between species and may be curved. In some cases (in the Vaejovidae), it is covered, for some of its length, with small spines (serrations). Thermoreceptors and short, possibly mechanoreceptor sensilla have been identified along its length. The aculeus has a thick external cuticle and two canals for the evacuation of venom, bordered internally by an intima formed from cylindrical cells. These canals are surrounded by a loose support tissue. In rare species of a few genera (*Parabuthus, Centruroides, Hadruroides, Urodacus*), the venom may be expelled as a spray (Kästner 1935; Newlands 1974; Stockmann and Ythier 2010).

Scorpion Biology

The Tegument and Fluorescence

Scorpions are covered by an epidermis made up of a single layer of cells that secrete a cuticle consisting of chitin (a carbohydrate) and proteins hardened by phenolic tanning (sclerotin). As in other arthropods, the cuticle has several layers: the

Fig. 4 Ultraviolet fluorescence in *Androctonus crassicauda* (photo by S. Sanchez)

endocuticle, exocuticle, and the waxy epicuticle (from the inside to the outside). The endo- and exocuticles are pierced by transverse canals. In scorpions, the exocuticle contains a hyaline layer with no equivalent in any other arthropod. The cuticle is particularly thick on the dorsal side. In some places as in tubercules, it is strengthened by metals (Zn, Fe, Mn) (Schoefield 2001).

The articular membranes between the segments of the body and the parts of the appendages are flexible and have no exocuticle; high concentrations of resilin, an elastic protein, have been detected (this protein increases the elasticity of the pedipalp chelae joints, allowing the passive opening of the chelae, the mobile part of the chelae having no adductor muscle).

Fluorescence: When illuminated with ultraviolet radiation of wavelength 360–390 nm, the cuticle of scorpions fluoresces (Fig. 4). The fluorescence may be yellow or blue, depending on the species. This property seems to be due to two substances, β-carboline, a substance closely related to tryptamine that forms in the epicuticle (with antioxidant properties, similar to substances formed in human cataract), and 7-hydroxy-4-methylcoumarin, a phenolic substance present in the hyaline exocuticle and the mesocuticle (closely related to substances from the castor sacs of beavers) (Stachel et al. 1999; Frost et al. 2001). Resilin, which is found in the epi- and exocuticle, is also fluorescent. However, the genus *Chaerilus* (from Southeast Asia) is not fluorescent (Lourenço 2012).

The role of this fluorescence, if any, remains a matter of debate. The glow from the stars or the moon, reflected on stones, may facilitate homing. It may also be involved in the identification of sexual partners or play a role in locomotor activity. However, it may simply reflect the formation of a secondary metabolite and have no particular function. Fluorescence does not occur until the second juvenile stage: baby scorpions are not fluorescent. The animals are also nonfluorescent just after molting, fluorescence being linked to cuticular tanning, which occurs progressively. If a scorpion is exposed to UV light for a long time, it loses its fluorescence, but the fluorescence can be restored by ending the UV exposure.

According to Gaffin et al. (2012), the entire cuticle is sensitive to UV light and serves as a transducer, converting the UV light into a green color corresponding to the maximum sensitivity of the eyes (490 nm).

Scorpion fluorescence (the observation of which is currently facilitated by the spread of LED lamps emitting UV light) has led to the discovery of many new species and has, above all, provided ecologists with precious data concerning these essentially nocturnal animals.

Anatomy and Principal Biological Functions

Prey Identification

Scorpions are principally predators of arthropods. Human stings are accidental and constitute a protective reflex by the scorpion. The prey is detected by a large number of diverse sensory organs. Scorpions make little use of vision for prey detection: rather, they mostly use mechanoreceptors and chemoreceptors.

Mechanoreceptors

Trichobothria (from the Greek trichos = hair and bothrion = pit): These are long sensory hairs located exclusively on three articles of the pedipalps – the femur, patella, and tibia (the fixed "hand" and "finger"). They are typically perliform, with a sectional area that is constant along most of their length. Most are longer than the other hairs (but there are also "short" trichobothria, which are much shorter). Unlike other hairs, they all have a large basal cupule. They can twist slightly and move to a precise angle in a single direction. This enables them to react to the currents of air generated by the prey, providing the scorpion with information about the direction and distance of the prey. They detect flying insects passing close by and terrestrial prey animals on the ground (Fig. 5a–c). The number and position of the trichobothria (which do not change during growth) are systematic criteria that can be used to distinguish between taxa.

Mechanoreceptive hairs: These are the hairs found all over the body, but particularly on the appendages. They may be of various lengths and, unlike the trichobothria, they steadily decrease in thickness along their length. Those in contact with the ground detect ground vibration (Brownell 1977). The **slit sense organs** (Fig. 5d) or lyrifissures are slits in the cuticle covered with a fine membrane and bordered by thick rolls of tissue. They are found in isolation over the whole body, but particularly around the joints, where they are oriented perpendicular to the axis of the appendage. They are movement receptors sensitive to vibration. Those on the tarsi pick up transverse ground waves. Working together with the hairs under the tarsi, which pick up longitudinal waves from the surface, they indicate the distance and direction of prey buried in a sandy soil. They can detect movements of the order of a nanometer (10^{-10} m). The time lags between detection of longitudinal and transverse waves by the various legs of the scorpion allow the animal to determine

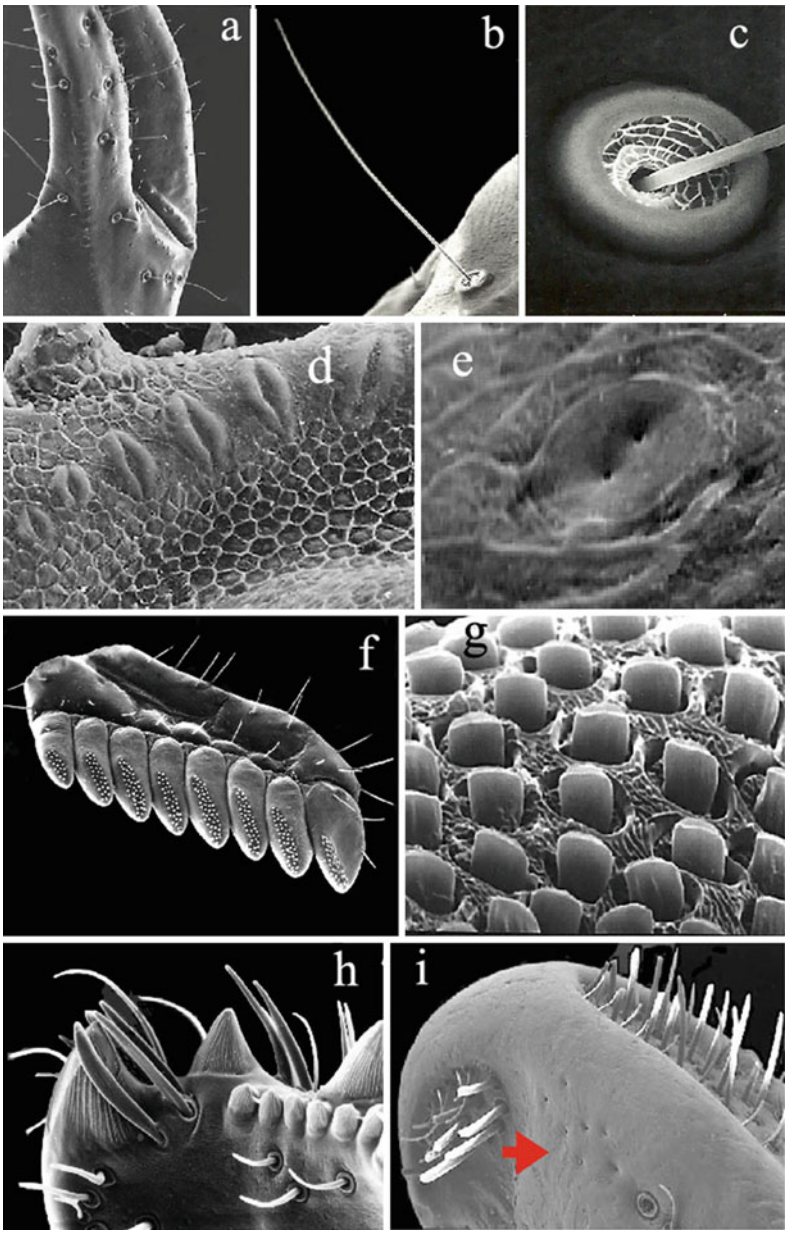

Fig. 5 Sensory organs of Scorpions (**a**) Chelae with numerous trichobothria in *Euscorpius tergestinus*. (**b**) One trichobothria of *E. tergestinus*. (**c**) Detail of the base of a trichobothria of *Hottentotta minax*. (**d**) Slit sense organ on the chelicerae of *H. minax*. (**e**) Tarsal organ of *H. minax*. (**f**) A pectine of a young *E. tergestinus*. (**g**) Sensilla on the pectine of *Butheoloides maroccanus*. (**h**) End of the chelae of *Buthus atlantis* with enlarged setae. (**i**) Constellation arrays at the end of the chelae of *H. gentili*

the distance and direction of the prey (Brownell 1977; Brownell and Van Hammel 2001).

The sensilla on the pectines: There are several hundred (400 per tooth in *Leiurus*) sensilla located on the lamellae (or teeth) of the pectines (Fig. 5g). They evaluate the granulometry of the ground, enabling the scorpion to identify an appropriate place to deposit its spermatophores, in particular (Carthy 1968). The pectines can perceive vibrations and detect ultrasound (200 Hz).

Chemoreceptors

The sensilla on the pectines: These structures are also chemoreceptor (Foelix 1985). One of the points, common to all chemoreceptors, is an opening at the end allowing odorant molecules, or "chemicals" in general, to penetrate. They have a complex structure, with dendrites, a ciliary residue, and microvillosities. They are sensitive to acids, diverse salt solutions, and the pheromones released for reproduction. The number of sensilla and of pectine teeth is closely correlated with the hygrometry in the surrounding environment. Most of the scorpions from very wet environments have very small numbers of pectine teeth, whereas those from very arid regions have very large numbers of teeth on the pectines (Sreenivasa-Reddy 1959).

Chemoreceptive hairs: These hairs may be of various sizes and straight or curved; they are particularly abundant around the chelicerae and the prebuccal cavity. Many curved hairs are distributed evenly over the fingers of the chelae. Small curved chemoreceptive hairs are present on the tail of *Orthochirus* scorpions.

Other chemoreceptors: The "tarsal organs" are pores on the surface of the tarsi of the legs (Fig. 5e). They are also thought to be involved in the perception of pheromones. The **constellation arrays** are very small sensilla, each formed by a short hair surrounded by an areola. There are one to 15 of these structures located at the extreme end of the fixed finger of the claw (Fig. 5i). Their role and precise structure have yet to be studied (Fet et al. 2006a, b). The presence of chemoreceptors in the pharynx has also been reported.

Prey Capture and Predigestion

Once detected, the prey is caught by the pedipalps, which bear multiple sensory organs; they are also covered, along the entire length of the fingers, with rows of denticles that prevent the prey from escaping. Prey capture may or may not be accompanied by a venomous sting. The prey is, thus, brought to the chelicerae, which grind it up into a sort of pulp that is chewed in the prebuccal cavity formed dorsally by the chelicerae, laterally by the base of the pedipalps, and ventrally by the extensions of the first two pairs of legs, the coxapophyses. The coxapophyses are equipped with glands opening via pores and are covered with thousands of barbed spines, in which saliva is mixed with the pulp for predigestion. The rostrum or epistoma is also covered with hairs and occupies the back of this cavity. The pulp flows down channels in the coxapophyses to a minuscule ventral mouth. In this way, only liquids and

very fine particles enter the mouth and are transported to the accordion-like pharynx, the walls of which are covered by powerful muscles fixed to internal cuticular folds (the apodemas). This system is responsible for the action of the pharyngeal pump which culminates in the ejection of an entirely dry pellet (rejection pellets).

The presence of a prebuccal cavity in scorpions and harvestmen (Opiliones) has been used as a criterion for classifying these two orders together in the Stomothecata (Shulz 2007).

Several phases in prey capture, characterized by different postures, have been defined**:** rest, displacement, alert, orientation, claw projection, stinger approach, stinging, stinger withdrawal, claw flexion, prey reorientation, biting by the chelicerae, grinding, transport, and ingestion. Stinging is used if the prey is large or aggressive. In a number of scorpions, the prey is much smaller than the scorpion itself and stinging is not used: this is frequently the case in scorpions with large chelae (e.g., Scorpionidae, Hormuridae, Chactidae). Scorpions can control both the amount of venom injected and its composition (amounts of protein, dilution) (Nisani and Hayes 2011). The stinging movement is very rapid: in *Hadrurus arizonensis* (Ewing 1928), the aculeus takes 0.75 s to move from the subvertical alert position to the stinging position in front of the chelae (Bub and Bowerman 1979). Capture is often preceded or followed by cleaning of the appendages.

Digestion takes place in the digestive diverticula (hepatopancreas or digestive gland) occupying the most part of the mesosoma. These structures contain both cells secreting enzymes for the purposes of external digestion and cells responsible for nutrient absorption.

The waste products of digestion, essentially packets of guanine, are found in a rectilinear ileum and are responsible for most of the **nitrogen excretion** of the animal. Water is reabsorbed in the ileum. At the anterior end of the ileum, the digestive tract is joined by fine, branched Malpighian tubules, which play a minor role in osmoregulation, principally mediated by two coxal glands located in the prosoma. The feces are excreted by a short posterior intestine, ending at the anus, which is located in the middle of four anal valves located anterior to the telson (Fig. 6). Accumulation-based excretion is mediated by large cells distributed around the nervous system in particular: the nephrocytes, which are often found in pairs.

The hepatopancreas plays a major role in scorpion physiology. It stores water and lipids and is also the site of production of enzymes and **hemocyanin**, the copper-based pigment responsible for transporting oxygen in the hemolymph. Electrophoretic studies of the hemolymph revealed biochemical differences between taxa, before studies on nucleic acids, allozymes, and venoms.

Respiration and Circulatory System

Respiration involves four pairs of book lungs that open via four pairs of spiracles located on the mesosoma. These lungs consist of very fine stacked lamella, separated by blood-filled spaces maintained by pillar cells. The air bathes the aerial

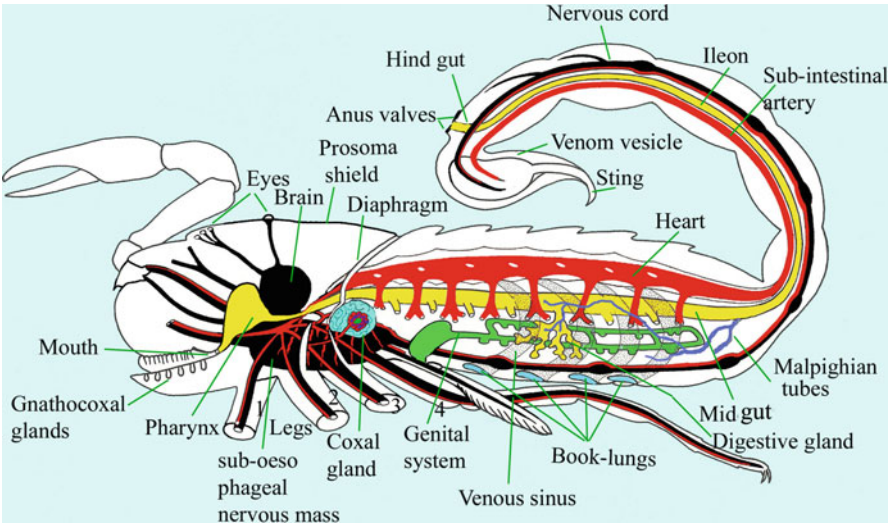

Fig. 6 Anatomy of a Scorpion (after P. Gervais 1935, modified)

spaces, which open onto the eight pulmonary atria connected to the exterior by the spiracles. Lamellar collapse is prevented by cuticular thickening of various forms. The circulatory system is a semi-open apparatus consisting of a heart or dorsal vessel, continuous with a deep arterial network extending into the appendages, the nerve mass, and the metasoma. Venous blood returns to the heart from the lungs via the sinuses and is recuperated by the cardiac ostioles.

Ecology and Ecophysiology

The following descriptions are mainly based on the ecological studies carried out by G. A. Polis in the USA (1979–1989), M. Warburg in Israel (from 1978), L. E. Koch in Australia (from 1978), G. Newlands in South Africa (from 1969), and J. L. Cloudsley-Thompson in the UK (from 1955 to 2013). Many of the original references can be found in Polis G. A (1990).

Inter- and Intraspecific Relationships

Interspecific Relationships

Predators: Scorpions have many predators in the desert, savannah, and forests. They may be eaten by other species of scorpions (intraguild predation), or even, when food is scarce, by members of their own species, with the larger individuals eating the smaller ones. Postnuptial cannibalism has also been observed, with the female eating the male after deposition of the spermatophore (although this is not

the case in all species). Other arachnids, such as the Solifugae (also known as camel spiders or wind scorpions), or spiders (Mygalomorphae or Araneomorphae), centipedes, and ants willingly attack scorpions. The predators of scorpions also include diverse vertebrates, such as toads, many reptiles (lizards, varans, agamas, iguanas, geckos, skinks, and snakes (Colubridae and Viperidae), many wild sedentary or migratory birds (wading birds, serpent eagles, hornbills, passerines, woodpeckers, owls, falcons), domesticated birds (ducks, geese, chickens, turkeys), and mammals (carnivores, e.g., foxes, fennecs, meerkats, cats, mongooses, genets; insectivores, e.g., hedgehogs, shrews; rodents, e.g., rats, porcupines; and bats). Monkeys remove the venom vesicle with their hands before eating scorpions.

When a scorpion encounters a predator, it adopts a defensive posture, with the chelae raised at the front and the tail lifted up above the mesosoma. Some scorpions stridulate when threatened (*Parabuthus, Rhopalurus, Heterometrus, Pandinus, Scorpio*). Various mechanisms of stridulation are used, including rubbing hairs against the tubercles on the hips and coxal blades of the first pair of legs, rubbing the stinger against the granules of the first few metasomal segments of the tail, or rubbing the pectines against the ventral surface of the sternites.

Scorpions of the genera *Mesobuthus, Heterometrus, Pandinus*, and *Androctonus* are eaten by humans in some areas (China, Thailand, West Africa, Morocco, and Egypt). Scorpion hunts, either to eliminate these animals or to obtain the sera required in countries in which scorpions constitute a danger to humans, may decrease population size (more than 400,000 scorpions are sometimes collected in a single season in Tunisia). A large number of scorpions are also collected for sale as exotic pets (three species of *Pandinus* are included in the list of species protected by the Washington Convention).

Several countries have **antiscorpion policies**, involving preventive measures or the elimination of these animals, communicated through the media: there are urban policies (street lighting, removal of waste and rubble, repairing of old walls, paving houses with ceramic tiles) and individual protection measures (not walking barefoot or in sandals, checking of bedding and potential hiding places, wearing of gloves for work in the field, starvation of domestic animals, conservation of the wild fauna, spreading of insecticides in open environments or fumigation of closed environments, etc.).

Prey: Apart from other scorpions, as mentioned above, the principal prey animals of scorpions are animals living on the ground surface, in sand (cockroaches, diverse larvae, woodlice), and even a few flying insects (butterflies). Insects are particularly well represented among the most common prey: Orthoptera (crickets and grasshoppers), cockroaches, termites, beetles, and Hymenoptera in particular. Common prey animals also include spiders, Solifugae, millipedes, woodlice, and a few small vertebrates (lizards, geckos, blind snakes, young mammals). Some large-clawed scorpions eat mollusks and earthworms. A few scorpions are stenophagous, eating only one genus or species of prey: for example, in Australia, the diet of *Isometroides vescus* (Karsch 1880) consists principally of a few species of burrowing spiders. Most scorpions would eat a wide range of prey (they are euryphagous), but have access to a limited number of species. Some can eat insects with a thick integument (darkling beetles and ground beetles), whereas

others reject such prey. Ants and woodlice (which curl themselves into a ball) may also be rejected. When food is scarce, scorpions may attack other species of scorpions, and cannibalism may account for up to 35 % of the prey ingested.

Scorpions often switch between prey animals, as a function of their abundance. The size of the prey depends on the size of the scorpion. Scorpions are thus opportunists. The effects of their predation on prey may be considerable. For example, they are sometimes identified as the principal insectivores in certain environments: *Hadrurus* may ingest 106–160 kg of prey per hectare per year in the USA and *Scorpio maurus* (Linnaeus 1758) is the principal predator of woodlice in Israel, eliminating a mean of 10.9 % of the population in 5 years.

Two different strategies are used to capture prey. Many scorpions adopt a "sit and wait" strategy. In this case, the scorpion waits for its prey either at the entrance to its burrow ("doorkeeping") or at a short distance away. This is the case for scorpions living in or on the ground. Many species practice homing (returning to the burrow to eat), with the prey often eaten within the burrow. The other principal strategy, practiced by **errant scorpions**, involves excursions at some distance from shelter during active hunting periods in which the scorpion seeks its prey, followed by refuge under tree bark or in crevices, without the construction of a burrow.

Parasites and Phoretic Relationships

The most frequent parasites of scorpions are mites of various families, nematodes, Phoridae (scuttle flies), pathogenic *Rickettsia*, and other bacteria, which cause the white marks sometimes seen on scorpions. As in many other arthropods, bacteria of the genus *Wolbachia* have been implicated in feminization phenomena and genital abnormalities in scorpions.

Fungal infections (*Aspergillus*, which causes brown or black marks; *Fusarium,* resulting in the presence of large numbers of hyphae on the surface) are facilitated by the presence of mites.

Phoretic relationships: A few mites of the Acaridae and Trombiculidae and microhymenopteran larvae are carried by scorpions but do not cause lesions.

Symbionts: 23 genera of bacteria have been found in the digestive tract of the Chinese scorpion *Mesobuthus martensii* (Karsch 1879).

Mimicry: A few homomorphs have been identified – the spiny leaf insect [*Extatosoma tiaratum* (Macleay 1827)] and a Ghanaian cicada provide examples of Batesian mimicry, in which the mimic is not dangerous, whereas the model (the scorpion) is venomous.

Scorpions may also play dead (**thanatosis**) in the presence of a predator.

Intraspecific Relationships (Social Behavior)

Most scorpions are solitary, nonsocial animals. However, there is an essential postnatal period during which the first- and second-stage (before dispersal) offspring are kept together on the back of the mother. The mother carries the pullus (the babies) from 1 to 51 days, and the second-stage juveniles until 20 days. The young are arranged on the back of their mother often in no particular order, in several layers. Sometimes, they may face the front or are cross oriented.

Contact pheromones are present in the cuticle of gestating females and females carrying young, attracting the young to their mother and ensuring that they remain on her back. During this period, there seem to be exchanges of water between the mother and her young. In the Scorpionidae and certain members of the Hormuridae, young of all sizes may live with the adults in shared burrows with several exits. The young of *Scorpio maurus* may remain with the adults, in their burrow, for 3 to 4 months, and *Urophonius* adults may share their burrows with their young for up to 6½ months.

Gregarious gatherings are observed in intertidal animals (*Serradigitus littoralis* (Williams 1980)), bark-dwelling animals (*Lychas marmoreus* (Koch 1844)), and overwintering groups of scorpions (*Centruroides exilicauda* (Wood 1863)). Individuals of *Mesobuthus martensii* of the same age orient themselves in the same direction under stones. In *Bioculus caboensis* (Stahnke 1968) from Baja California, scorpions may work together to capture the prey and transport it to the burrow. Thus, in some cases, scorpions may attain a **subsocial state**.

Environmental Relationships and Adaptations

Edaphic factors are of utmost importance. Some species may be classified in terms of the types of substrate in which they live: sand (psammophilic), rock (lithophilic), and soft or compacted soil (soil dwelling). Each environment is associated with morphological adaptations (lengthening of the body and the tail), adaptations of the chelae (in terms of their length and width; burrowing scorpions often have very broad chelae), length of the legs and claws, arrangement and length of hairs on the appendages (long legs, tibial and tarsal combs in psammophilic scorpions), and the contact area of the tarsi (spines or hairs).

Climatic factors may also have an effect on the distribution. There are xerophilic (very dry and desert environments), mesophilic (moderately humid environments, in accumulations of rocks in Mediterranean forests, savannah), and hygrophylic (wet tropical forests, caves) scorpions.

Altitude is also a limiting factor on the distribution. Scorpions are found at sea level, in the intertidal zone under the debris left by the sea, on beaches, and at high altitudes (5,500 m in the Andes, up to 5,000 m in the Himalayas, 2,000 m in the Alps, and higher in the Moroccan Atlas Mountains).

In many families, **cave species** living at various depths (up to −916 m for *Alacran tartarus* (Francke 1982)) have been described; these scorpions often live in environments that are saturated. They are often depigmented and blind, with the medial and/or lateral eyes having disappeared. Some, but not all, are very small (8.5–9 mm for *Typhlochactas mitchelli* (Sissom 1988)), and their pectines generally carry a very small number of teeth.

Most scorpions live on the ground or up to a few meters above the ground under tree bark or on bushes, but some are tree dwellers, living in the canopy (up to 40 m above ground), or on epiphytes in tropical forests.

The **density of scorpion populations** is highly variable and depends on abiotic and biotic environmental factors. In the deserts of the USA, scorpions are "extraordinarily abundant" (Polis and Yamashita 1991). Their biomass may reach more than 50 kg/ha, exceeding that of other arthropods (excluding termites and ants) and even vertebrates. The population density may reach more than 20 scorpions per square meter, over a thickness of 15 cm, in the stony areas of Mediterranean forests (*Euscorpius tergestinus* (Koch 1837)). There may be up to 4,000–5,000 individuals/ha, corresponding to a biomass of 5–20 kg/ha in many environments: deserts, arid prairies, garrigues, tropical forests, and intertidal environments. The population density of a given species differs considerably between microhabitats. For example, in Israel, *Leiurus quinquestriatus* (Ehrenberg 1828) populations may reach a density of 1.12 animals per m^2 (Shulov and Levy 1978), and in South Africa *Cheloctonus jonesi* (Pocock 1892) populations may also reach densities of more than 1 animal per m^2 (Newlands 1978).

Scorpion Activity Rates and Variations of Surface Population Size

Surface locomotion activity is the easiest to evaluate. Scorpions are subject to a **seasonal rhythm**, with variations in their activities with the seasons. They have one or two rest periods during the year. The period during which scorpions are most abundant on the ground is, for most species, the hot season, when reproductive partners come together and scorpions move the furthest away from their burrows, sometimes covering more than 100 m per night in the case of *Smeringurus mesaensis* (Stahnke 1957). Mature males wander much more than females and juveniles, which tend to remain much closer to their burrows. Searching for food accounts for only 1–8.1 % of scorpion activity (Polis 1990).

Scorpions often leave their burrows after heavy rainfall, which leads to plant germination and an abundance of insects, or after invasions of termites or crickets. There is also a strong correlation between surface temperature and population density, with scorpions leaving their burrows even in winter on days when it is warm. **Mating** occurs from April to October in the northern hemisphere and from September to February in the southern hemisphere (except for *Urophonius brachycentrus* (Thorell 1876), which follows the opposite pattern). Tropical scorpions may have reproductive periods spread out throughout the year or at least over a long period.

Most scorpions are less active in winter, but desert scorpions may have two rest periods, one in winter and the other during the hottest months, like a true estivation. In general, **births** peak from May to October for the species of northern temperate countries and from December to February for the species of southern temperate countries, whereas the birth rate remains fairly constant throughout the year for tropical species.

Circadian rhythms: Scorpions have different patterns of activity during the course of the day. Most are nocturnal or most active at dusk. During the day, they

rest in their burrows or shelters, cracks in rocks or tree bark. However, the peaks of locomotor activity differ between species and depend on variations in light levels and temperature. They therefore differ according to season. In most cases, there are two peaks in activity during the nychthemeron, one at nightfall and the other early in the morning. *Urodacus yaschenkoi* (Birula 1903) begins digging its burrows at dusk. Several genera are clearly more nocturnal (*Androctonus, Hottentotta, Leiurus*). *Smeringurus mesaensis* is active between 9 p.m. and 3 a.m. Following a period of active hunting at dusk, scorpions return temporarily to their burrows, resulting in a strong decrease in the density of individuals on the surface. Tropical scorpions tend to be active over a longer period during the day. Scorpion activity may occasionally be diurnal. This is the case, for example, in *Leiurus* and *Scorpio maurus palmatus* (Ehrenberg 1828), following rain or at the start of the spring. Some species of *Parabuthus* (South Africa) tends to be active during the day.

Scorpions do not leave their burrow every day to feed. It is estimated that 20 % of the population, at most, is active, while the other 80 % of the population is resting. *Smeringurus mesaensis* spends 92–97 % of its time in its burrow (Polis 1990).

Many physiological functions also follow a circadian rhythm: enzyme activities in the hepatopancreas and muscles vary through the day; the glucose concentration in the blood is maximal at 8 p.m., when the glycogen concentration in the hepatopancreas is at its lowest in *Heterometrus*; the respiratory activity is maximal at 8 p.m. and 4 a.m. in *Euscorpius tergestinus*, and the activity of the nerve chain also displays circadian variations. The ocular pigment moves under the influence of light, acting as a sunscreen, and the eyes thus have a nocturnal and a diurnal state. These seasonal and circadian variations are controlled by neurosecretory cells in the nerve centers and by neurosecretory nerve endings under the retina of the medial eyes. The lateral eyes, which are more sensitive at night than during the day, also act as a "zeitgeber," synchronizing the rhythms (Fleissner 1974; Fleissner and Fleissner 1985, 2001). The photoperiod, particularly the scotophase (the period of darkness), plays a key role in controlling the rhythm of scorpion activity.

Ecological Strategies r-K

Ecologists have identified different characteristics of animals, in several domains, corresponding to different ecological strategies. A long development period, late sexual maturity, a small number of young, parents caring for their offspring, living in a stable environment, and a low mortality rate are found in animals adopting a selective strategy "K." By contrast, opportunistic species, for which reproductive rates are high but the parents are not involved in rearing the young, mortality rates are high regardless of density and population density fluctuates strongly, following an "r" strategy.

Most scorpions adopt a strategy that is intermediate between these two extremes, with many of the characteristics of a "K" strategy. Indeed, they have a long lifespan, of several years (from 2 to 25 years, with a mean of 4–8 years), they mature late (6 months to 7 years), and they have a long gestation period (1.5 months to 2 years)

and a relatively small number of young (from 1 to 105, with a mean of 25–40), and the females take care of the young by carrying them on their backs and protecting them in their burrows. The K strategy is also that adopted by mammals and birds, and it predominates among scorpions living in protected environments, such as forests, or sites little affected by variations over geological time.

While Buthidae scorpions are also regarded as K selected, many species adopt a strategy closer to the "r" strategy: they live in much less stable environments, with major changes in population density as conditions become favorable or unfavorable (temperature, pullulation, or a shortage of prey). They are able to colonize new biotopes (volcanic islands, urban environments) rapidly. Several factors facilitate this strategy, which prioritizes reproduction: resistance to various environmental factors (lack of food, drought), opportunism in terms of diet and habitat, venomousness, rendering prey capture highly effective, the ability to have several litters of offspring over a short period of time without the need for further mating (long periods of sperm storage), and parthenogenesis in several species.

Resistance to Environmental Factors and Adaptation to Life in the Desert

Resistance to Ionizing Radiation

During French nuclear tests in the Sahara Desert in Algeria between 1961 and 1963, scorpions and beetles were found to be the animals most resistant to irradiation. Experimental irradiation trials with cobalt-60 in three species of *Androctonus* demonstrated radioresistance, with an LD_{50} of 400–820 grays (Gy) over a period of 30 days, which is much higher than that for many other animals (640 Gy for adult *Drosophila*, 15 Gy for tortoise, 9 Gy for mice; a dose of 40 Gy is sufficient to cause instant death in humans and a dose of 2–6 Gy may cause blood and digestive problems). The threshold dose for tolerance in *Androctonus* is 200–250 Gy. Irradiation of *A. mauritanicus* (Pocock 1902) at a dose of 100–200 Gy causes the reversible arrest of gonad cell mitosis, this arrest becoming irreversible at higher doses. There may be multiple reasons for this exceptional resistance. DNA is highly sensitive to irradiation, but mitosis is rare in scorpion tissues other than during molts and other than in the gonads; scorpions have high circulating taurine (a radioprotective amino-acid) concentrations that do not increase following irradiation, low levels of metabolic exchange, low levels of oxygen consumption, and hemocyanin, which has antioxidant enzymatic properties, neutralizing the effects of the radiolysis of water. Desert scorpions from arid areas (Buthidae) seem to be the most resistant (Goyffon 2010).

Resistance to Extreme Temperatures

Many desert scorpions can resist temperatures of the order of 45–50 °C (*Leiurus quinquestriatus*, *Scorpio maurus*, *Hadrurus arizonensis*) provided that they are well hydrated; indeed, scorpions are among the terrestrial animals with the

highest resistance to high temperatures: only a few desert mammals, such as the dromedary, and a few reptiles and insects are as tolerant. This tolerance increases during the summer. These temperatures are close to those at which proteins coagulate and are above the upper limit for enzyme activity and the temperature at which waxes fuse. Biochemical adaptations in the hemolymph, tissues, and tegument are therefore required to survive at these high temperatures. The mechanisms responsible include, as in certain insects, particular molecular conformations of the hydrocarbon chains of the cuticle. It is also probable that genetic factors influence this heat resistance, through heat-shock proteins (HSPs), for example. Scorpions from nondesert environments seem to be tolerant to only lower temperatures. Indeed, the temperature preferences differ between species: 34–35 °C for *Leiurus quinquestriatus* and *Hottentotta judaicus* (Simon 1872) and 24–27 °C for *Heterometrus petersii* (Thorell 1876).

Many species living at high altitude or in deserts are subject to temperatures below 0 °C. Many scorpions from hot deserts may be subject to considerable differences in temperature between the day and the night or between seasons (from −31 to +50 °C for *Pectinibuthus birulai* (Fet 1984) in Turkmenistan). Some scorpions are thus resistant to very low temperatures (supercooling). The concentration of "antifreeze" compounds, such as glycerol, in the blood is too low to account for this cold tolerance. Trehalose (a sugar) concentrations increase during the cold season. The point at which hemolymph becomes supercooled differs between seasons and is higher in the winter than in the summer. Antifreeze compounds (nucleation proteins) preventing the formation of ice crystals have been found in the intestine of *Centruroides vittatus* (Say 1821) from North America. High levels of sodium, the presence of cysteine, and the properties of hemocyanin may account for the resistance of scorpions to freezing. Thus, scorpions may go through a true hibernation period during which their metabolism greatly decreases.

Scorpions also have behavioral adaptations to resist extreme heat or cold. A few scorpions (*Opistophthalmus latimanus* (Koch 1841)) display stilting behavior when the temperature increases, lifting their bodies as high off the ground as possible, providing them with some insulation against the overheated ground, as in certain Tenebrionidae beetles.

Many scorpions dig burrows of various depths and come closer to the surface or descend deeper into their burrows to adjust their body temperature with respect to that of the exterior. *S. maurus* can dig burrows more than 1 m deep and *Hadrurus* can dig to a depth of 2 m. Most burrowing scorpions dig burrows of between 15 and 50 cm in depth or hide in hollow spaces under stones. Burrows may be vertical or spiral and may contain berms or chambers. Their depth depends on the nature of the substrate and the age of the scorpion. Soil temperatures and the temperature of the air at the surface in deserts vary substantially between night and day (the nychthemeron) and throughout the year, but they decrease and become more constant with increasing depth below the surface (in the Sahara, when the ground temperature at the surface is 57.5 °C, the temperature at a depth of 50 cm is 33.4 °C). At a depth of 20 cm below the surface, daily variations in temperature do not exceed 10 °C, and the fluctuation is no more than 2 °C at a depth of 50 cm.

Heat fluctuations are therefore considerably attenuated at the bottom of the burrow. Burrows protect against heat, evaporation, and predators. Some scorpions prefer to occupy the burrows of other animals, such as small mammals or reptiles.

Burrows also provide protection against brush fires. Similarly, their conformation may provide protection against flooding. Burrows are dug differently by different species and as a function of the nature of the terrain. For example, in *Opistophthalmus*, the substrate is excavated by the chelicerae and the first three pairs of walking legs are used to remove the debris by scraping it in batches underneath the underbelly of the animal. In *Cheloctonus*, the large chelae are used as a shovel, to dig into the ground and remove the excavated material. In *Hadrurus*, the first two or three pairs of legs are involved in digging and the chelae are not mechanically involved in burrowing. A number of scorpions use their tail to remove the excavated material.

Resistance to Drought and the Relationship to Water

Some scorpions live in very damp environments, in caves, under the debris left at low tide by the sea, or in equatorial forests. These hygrophilic or mesophilic scorpions obtain their body water by drinking from puddles or from dew deposited on plants. The water in prey is often sufficient for the vital needs of scorpions, and many scorpions from hot regions do not drink. Some scorpions adopt stilting behavior resembling that of desert beetles. *Parabuthus villosus* (Peters 1862) can drink water from mist, by making use of its chelae. *Smeringurus mesaensis* can also absorb water from damp ground.

Scorpions lose water by **evaporation** from the tegument, through the **respiratory spiracles,** and through the **excretion of feces**. Many authors (Hadley 1974, 1990, 1994; Warburg et al. 1980; Gefen and Ar 2004; Gefen 2011) have shown that desert scorpions have the lowest evaporation rates of any arthropod. Buthidae scorpions lose much less water than scorpions from damper environments. The same is true for oxygen consumption. Water loss via the spiracles is also much lower in desert scorpions; water loss by this route may reach 30 % of the loss through the tegument in active *Scorpio maurus fuscus* (Linnaeus 1758)**,** although it is much lower when the animal is resting.

The osmolarity of the hemolymph remains approximately constant with weight loss due to dehydration (most scorpions are osmoregulators). The exception is *Opistophthalmus capensis* (Herbst 1800), in which the osmolarity of the hemolymph decreases with weight loss (osmoconformity). Scorpions subjected to dehydration regulate the volume of the hemolymph and its osmotic concentration, by mobilizing water from glycogen in the hepatopancreas. Scorpions can thus produce “metabolic water.”

Scorpions excrete nitrogen in the form of insoluble products, such as guanine, or even xanthine in *Smeringurus mesaensis*. This makes them more efficient in terms of water economy than insects, which excrete uric acid, a system already more efficient than that in vertebrates, which secrete urea, a highly soluble compound requiring large amounts of water for its elimination.

Metabolism

Scorpions have a very low metabolic rate, which can be measured by determining CO_2 release or O_2 uptake. At 25 °C, the metabolic rate of scorpions is less than a quarter that of other terrestrial arthropods of the same mass, with the exception of ticks. The use of food reserves decreases as temperature increases. This "metabolic depression" may be due to an abnormally low membrane permeability, particularly effective mitochondria, or a strong dependence on the storage of phosphagen. These very low levels of metabolism allow the animal to adapt to difficult environmental conditions, resulting, in particular, in low evaporation rates and high levels of resistance to food shortage. Scorpions can close their spiracles for long periods, thereby decreasing the loss of respiratory water and leading to anaerobic catabolism. They tire rapidly when running. The concentration of lactate increases quickly during physical activity, but less so than in spiders, crabs, or ectothermic vertebrates with more specialized running behavior.

Resistance to a Lack of Food

It has been shown experimentally that scorpions can survive for up to 12 months without water or food (*Buthus occitanus* (Amoreux 1789) and *Androctonus australis* (Linnaeus 1758)). If provided with water but not food, they can survive for up to 3 years (Vachon 1957). In natural conditions, scorpions may spend many days in their burrows without eating, and they do not eat in the inclement season. Their resistance to a lack of food is correlated with their low metabolic rate. The weight of a scorpion may decline by a third in cases of starvation or severe dehydration.

Resistance to Bacterial Infections

Scorpions are particularly resistant to many bacteria. This resistance is due in part to their innate immune processes which are based on oxidation reactions followed by classic phagocytosis, but also to the presence of defensins in the hemolymph (Ehret-Sabatier et al. 1996; Rodríguez de la Vega et al. 2004). These substances are cysteine-rich peptides with a structure very similar to that of the neurotoxins present in venom. They act by creating pores in the bacterial wall. Another circulating peptide, androctonin, also has antibacterial and antifungal effects.

Scorpion Reproduction and Growth

Reproduction

The Female Genital Organs

The female gonads are located in the mesosoma and consist of three or four parallel tubes linked by two to four transverse anastomoses. They are tightly surrounded by the hepatopancreas. They are known as the ovariuterus because they are responsible for the formation of the sex cells (oocytes) and also act as the site of embryonic development. These tubes join two oviducts (sometimes enlarged into two

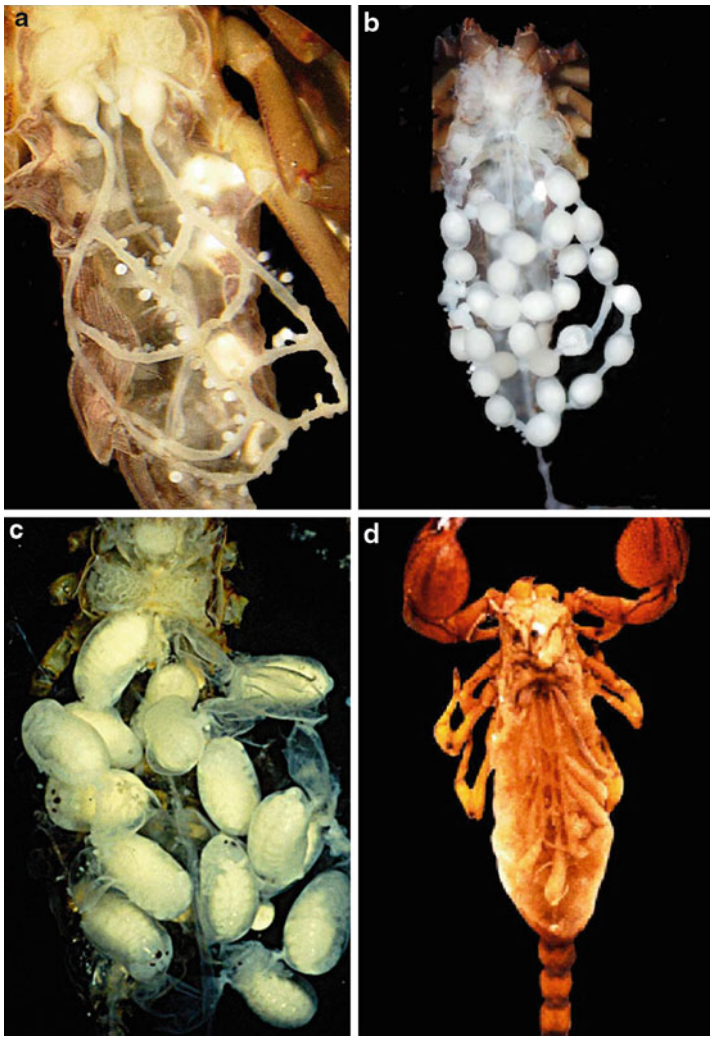

Fig. 7 Female genital system. (**a**) Female genital system of *Euscorpius tergestinus* before fertilization (ovariuterus with oocytes). (**b**) Female genital system of *E. tergestinus* : perliform ovariuterus with embryos. (**c**) Female genital system of *E. tergestinus* just before parturition. (**d**) Female genital system of *Scorpio maurus* with embryos in diverticulum with their appendix

spermatheca), which then join a common atrium opening via a genital orifice protected by one or two genital valves located behind the sternum. The ovariuterus changes in appearance during the course of the life cycle: tubular and cylindrical before fertilization and perliform when embryos are developing within it. In some viviparous families, the embryos are fixed to the ovariuterus, forming embryonic diverticula (Fig. 7).

The Male Genital Organs

The males have two gonads, surrounded by the hepatopancreas. Each consists of two cylindrical tubes in the form of a ladder containing a large number of cysts in which the spermatozoa are produced. Each tube ends in a short spermiduct, one on either side of the animal, leading to the "paraxial organs" consisting of additional glands, one of which secretes a chitin-based structure, the hemispermatophore. During mating, the two hemispermatophores join to form a rod-shaped structure, the spermatophore, which carries the sperm and is deposited on the substrate. The paraxial organs meet in a genital atrium protected by one or two valves, each bearing a small papilla. The morphology of the paraxial organs is a criterion used in taxonomic descriptions.

Mating

Mating in *Buthus occitanus* was described poetically by Jean-Henri Fabre (1907) in his *Souvenirs Entomologiques* which nevertheless did not include a description of the final phase. The mating partners find each other through the emission of pheromones by the female. Both volatile (in *Opistophthalmus*) and contact pheromones have been detected. The contact pheromones are emitted from the cuticle (which has a different composition in the two sexes) and may be transmitted via sand and perceived by the pectines of the male (Farley 2001; Taylor et al. 2012). The site of pheromone emission is not known with certainty. Esters present in the venom glands of males may act as attractants or aphrodisiacs. In the Bothriuridae, a gland located above the venom gland may be pheromonal. In *Tityus*, pheromones are thought to enable the males to distinguish between fertilized and unfertilized females.

When searching for a mate, scorpions walk rapidly, with their chelae to the front, pausing occasionally. The pectines comb the substrate, searching for contact pheromones. The male (and sometimes the female) trembles and makes rapid backward and forward movements with the body, without moving the legs. This movement is known as "**juddering**" and is the first phase of mating. It is possible that the ground vibrations perceived by the sensory organs on the legs trigger a reaction in the other partner. Once the male comes into contact with the female, he tries to grab her with his chelae. He may grab the tail or one of the two chelae of the female and then both chelae. The male then leads the female in a **"promenade à deux"** (literally, walking as a pair) that may last from several minutes to several hours. The male and the female face each other and move backwards and forwards, using their pectines to search for a heterogeneous substrate, in a sort of "**dance.**" Several phases of the "promenade à deux" can be identified and may be present or absent in different species (Fig. 8).

Sexual stinging: Still holding on by the two chelae, the male stings the female in one of the articular membranes of the chelae or mesosoma. The sting contains venom and such sexual stinging may occur several times during mating. As a result, the female, which may be aggressive and reluctant to follow the male, puts up less of a fight. This phase does not exist in the Buthidae.

Fig. 8 Reproduction in scorpions. (**a**) Courtship of *Androctonus mauretanicus*: "embrassades" (Photo E. Ythier). (**b**) Courtship of *Euscorpius tergestinus*: sexual sting. (**c**) Courtship of *Hottentotta minax*; ventral view: "embrassades". (**d**) Spermatophore of *E. tergestinus*. (**e**) Courtship of *Hottentotta minax*; ventral view: "promenade à deux". (**f**) Female of *E. flavicaudis* with their "pullus" on her back. (**g**) A pullus of *Opistophthalmus wahlbergii* with the provisory organs on his back (Photo E. Ythier)

Juddering: or trembling. This phase also occurs during the promenade. It is generated by the male in particular, in a sustained state of excitation.

Touching: The male uses his first pair of legs to touch the genital region of the female.

Trills: The male rapidly moves his first pair of legs over the ground or the genital operculum of the female.

Soil scratching: The male scratches the ground if it is sandy and may create a furrow. All the legs may be required for this.

The "embrassades": During the forward and backward movements, the two partners approach their chelicerae such that they touch or the chelicerae of one partner touch the front of the prosomal shield of the other. This "kissing" occurs, as a rule, in most scorpion species.

Chelicera holding and massage: In some species, the animals hold onto each other by their chelicerae, which open and close, allowing a clear liquid (saliva) to seep out.

The "arbre droit": Facing each other, with their chelae withdrawn, the two partners straighten up the backs of their bodies. The tails, which are held vertically, cross and uncross a number of times, with the venom vesicles withdrawn. In some species (*Pandinus imperator* (Koch 1841)), the "arbre droit" is performed with the tails held horizontally.

Tail movements: During mating, movements of the metasoma generally occur, with the tail being lifted or curved vertically. The tail is often moved to the side, alternating between the left and the right. These movements may be jerky (*Orthochirus*).

Pectine movements: The male pectines are swept across the surface of the substrate, soil, bark or stone. These structures are usually held flat against the ventral side of the animal, but they are held vertically during mating. The pectines are, effectively, chemical receptors, but they are also mechanoreceptors used to evaluate the texture of the substrate.

Spermatophore deposition: The male must find an appropriate substrate – stable and sufficiently hard and heterogeneous – for the deposition of his spermatophore. Deposition may occur on hard ground, agglomerated sand, rock, or tree bark.

The male pulls the female towards him by the chelae. The two partners place their chelicerae against each other, and the male, holding himself flat against the ground, opens his genital valves. His genital papillae thus touch the ground. A drop of transparent "glue" emerges, followed by a chitin-based rod, the spermatophore, consisting of a foot, a shaft, and a flagellum (Buthidae) or a blade (or lever) (other families). At the end of the shaft is a "capsule" carrying a small ball of sperm. The spermatophore is a double structure, made of the two hemispermatophores, which slide and stick to each other. The sperm, released from the testicles, is organized into packages in the glands of the paraxial organs and pushed into the sperm sacs of the spermatophore.

Spermatophore uptake by the female: The male uses a few brusque movements to pull the female backwards so that her genital orifice is over the capsule.

Spermatophore deposition is generally very rapid, taking between a few seconds and 6 min (*Nebo*). Spermatophore collection is also often very rapid, taking only a few seconds, although some scorpions "pump" the spermatophore for several minutes (5 or 6 min in *Opistophthalmus*).

After deposition of the spermatophore, the genital orifice and atrium of the female contain a **mating plug**, the form and origin of which differ considerably between families (spermatophore breakage or sperm and secretions (Mattoni and Peretti 2004; Althaus et al. 2010)). This plug prevents the fertilized female from mating again until the young are born. A male can reform hemispermatophores within a few hours or a few days and can deposit them during new mating events.

Separation of the couple: Once the spermatophore has been collected, the partners separate brusquely and move away from each other.

Eating of the spermatophore: The female or, in some cases, the male may return to the spermatophore to eat it. Otherwise, it remains in position and dries out.

After separation, **postnuptial juddering** frequently occurs, with phases of trembling separated by phases of walking.

The female may display a **swaying** phase**:** She lifts herself up off of the substrate and sways from side to side.

Postnuptial cannibalism: G. Polis (1990) reported that the male was eaten after mating in 39 % of the species for which mating had been described. In nature, however, cannibalism may not necessarily be the rule.

This "dance" occurs throughout the order Scorpiones. Some phases are probably derived, as in many animals, from behavior of no sexual significance. For example, the "arbre droit," in which the tails rub against each other but the venom vesicle is withdrawn, is undoubtedly derived from combat behavior. The massaging of the chelicerae is probably derived from eating behavior. These types of behavior are described as **ritual behavior**. It has proved possible, during the rearing of scorpions in artificial conditions, to obtain hybrids between some species (*Androctonus*).

Development

The Different Types of Embryo Development

Within the ovariuterus, large cells called oocytes differentiate very early during the growth of female individuals. After fertilization, which occurs within the ovariuterus, the type of segmentation (discoidal or equal) differs between families and as a function of the amount of vitellus. Two types of development can be distinguished.

Scorpions displaying "in situ" development (apoikogenic): The oocytes, which are initially internal, move to the outside of the tube, to which they remain attached by a peduncle. They are surrounded by follicular cells and undergo several phases of oogenesis, during which they grow and accumulate reserves. After

fertilization, the egg moves back inside the ovariuterus, where it differentiates into an embryo. The ovariuterus thus bulges and stretches, each round swelling corresponding to an embryo. At the end of embryonic development, all the embryos, the eyes and segmentation of which can be seen due to the transparency of the ovariuterus, are contiguous, the ovariuterus displaying a form like a rosary or string of beads.

A major variant described by R. Farley (2001) occurs in a member of the Vaejovidae, *Smeringurus mesaensis*. In this species, which produces eggs with a low vitelline content, the same process occurs except that a trophic cord is located just above the ovariuterus. This cord is initially fragmented, subsequently becoming continuous and developing into a bilobed structure above the embryo. The embryo remains connected to this nutrient-providing structure while within the ovariuterus.

Scorpions with embryonic diverticula (katoikogenic): This type of development is found for eggs with little vitellus. The eggs develop in a diverticulum, which lengthens during embryo growth. The ovariuterus thus displays expansion known as embryonic diverticula, located within the mass of the hepatopancreas. In the Scorpionidae and Hormuridae, the diverticulum is long. By contrast, in *Urodacus* (Urodacinae), the diverticulum is very short and resembles a cork.

Katoikogenic scorpions may have diverticula of different sizes, corresponding to several successive generations of embryos (two or three generations in *Scorpio maurus*), together with degenerating diverticula (Warburg 2010, 2012). Each diverticulum has several parts: a peduncle, a body, and an appendage. The embryo occupies the peduncle and the body. The appendage evolves into a baby's bottle-like structure. It contains giant cells that lyse and serve as food for the embryo. The appendage, a tube that may be spiral, is inserted between the chelicerae (Vachon 1950) and the base of the chelae (Stockmann 1990), these two organs being completely transformed. These temporary organs equipped with contractile vesicles press against the tube of the "bottle" forming a "teat" and directing the nutritive liquid from the "bottle" towards the mouth. The liquid is absorbed through the pumping action of the pharynx. Some nutrients may be transferred from the hemolymph or the digestive diverticula of the mother closely linked to the genital apparatus, via the appendage or the embryonic tegument.

Embryonic development: The embryo gradually segments. A germinative band forms above the mass of the vitellus and gradually lengthens. During growth of the embryo, the ventral ectoderm (the germ layer furthest towards the outside of the embryo) of the prosoma and mesosoma progresses in a dorsal direction and surrounds the vitellus, until it is completely closed. The prosoma segments first, followed by the mesosoma, with the tail segments appearing later. The appendages appear as buttons that lengthen and differentiate into articles. At the end of development in scorpions without diverticula, the legs are crossed over the ventral surface and the tail is folded towards the front, whereas in scorpions with diverticula, it remains unfolded, in line with the body.

The embryos of many genera, both with and without diverticula, have **temporary extensions** on their dorsal surface. These structures sometimes have dispersed smooth cuticular buttons (*Pandinus*). They are probably sites of oxygen exchange between the mother and embryo and may also mediate nutrient exchange.

Studies of **developmental genes** in *Euscorpius flavicaudis* (DeGeer 1778) and *Smeringurus mesaensis* have demonstrated similarities to the development of crustaceans and insects (*Drosophila*). Genes controlling segment polarity (determining the front, back, dorsal, and ventral areas for each segment; the *engrailed* and *hedgehog* genes) and "gap" segmentation genes (such as *orthodenticle* and *empty spiracle*) have been identified. The *orthodenticle* gene is involved in the organization of the nervous system. The expression of homeotic genes (*Hox*) controlling segment identity has also been demonstrated, particularly for the *ultrabithorax* and *abdominal-A* genes. These studies have made it possible to confirm the existence of a pregenital segment. They have also shown that arthropod genes have been strongly conserved during evolution (Popadic and Nagy 2001; Simmonet et al. 2004; Simmonet and Célérier 2006).

Gestation: The young are born 1.5–22 months after mating. The gestation period is long in species with diverticula (7–22 months). In the Buthidae, the mean gestation time is 5 months (1.5–11.5 months). The duration of gestation depends on many factors, including external temperature, rainfall, and prey abundance. The female spends the gestation period in a secluded place, such as her burrow, underneath a stone or a piece of bark or in a crevice. Gestation often occurs in warm periods, and the season in which it occurs depends on the climate.

Birth (parturition): Births often occur during the night or early in the morning. They generally occur during the warm season, but tropical species give birth throughout the year. At the time of parturition, the female adopts a characteristic position, lifting up the front of her body. The posterior rings of the tail rest on the ground. The rest of the tail curves in on itself or on the mesosoma. The chelae are bent towards the ground, which they almost touch. The first, and often the second, pair of walking legs form a "birth basket" below the genital operculum. The genital operculum opens and releases either embryonic eggs (in ovoviviparous scorpions) covered with a fine envelope or babies (pullus) without envelopes (viviparous scorpions). W. R. Lourenço et al. in 1986 and many authors since, considered all scorpions to be viviparous, because the embryos are fed, in situ, by the mother in all species.

The birth basket receives the eggs, one by one. In a few seconds or minutes, the young free themselves by tearing the fine membranes surrounding them, and they immediately climb onto the back of their mother, by clambering up a chelae (pointing towards the ground) or a leg. The duration of parturition is highly variable. It is rapid in *Hadrurus arizonensis,* in which it takes no more than an hour. For other species, it often takes 2–5 h, but there may be pauses and the entire process may take up to a day and a half. In ovoviviparous scorpions, the "egg" membrane surrounds the "young," which have their tails folded over their ventral surfaces. Parturition generally takes longer in viviparous scorpions, extended over several minutes to a few hours, but sometimes up to 10 days (*Hadogenes*), with the young released in waves of several individuals at a time and with multiple intervals. A gestating female may lose 43 % of her body weight after parturition (*Smeringurus mesaensis*).

Maternal behavior: The mother's posture at parturition enables the young to climb onto her back. If one of the offspring falls, the female adopts a searching position, walks and then stops, directing her front chelae towards the ground so that the young can climb up again, which they generally do with no other assistance. In a few species, the female may help the young by pulling it nearer with her chelae and even by grabbing it and placing it on her back.

Number of young per litter: The number of young per litter varies between 2 and 105: 30–50 in *Buthus occitanus,* 45–80 in *Androctonus australis,* 6–105 in *Centruroides insulanus* (Thorell 1876)*,* 26–91 in *Centruroides gracilis* (Latreille 1804)*,* 15–35 in *Euscorpius tergestinus,* 30–40 in *Pandinus imperator*, and 2 or 3 in *Urodacus yaschenkoi* (Birula 1903).

Iteroparity: Many scorpion species can give birth to several litters of young after a single insemination. These litters are separated by intervals of several months or of one or several years. This phenomenon is called "iteroparity." The interval between litters differs between species. *Isometrus maculatus* (DeGeer 1778) may have 4 or 5 l separated by intervals of 2–3 months, whereas *Smeringurus mesaensis* and *Hadrurus* from North America have 1 litter per year for several years. Thus, some scorpions may reproduce four or five times, or even more frequently (probably about a dozen times for *Pandinus imperator*), during their lifetime, due to the storage of spermatozoa in the genital tract of the female.

Parthenogenesis: Some species appear to have no males. For many species, the sex ratio (number of males/number of females) is not one and there are many more females than males (twice as many in most cases but three or four times as many in some instances). Twelve species have been identified as parthenogenic by Lourenço et al. (1999). In some cases, there are populations with both sexes at some sites and parthenogenic populations at other sites: this situation is described as geographic parthenogenesis. For example, *Tityus columbianus* (Thorell 1876), a species from Colombia, is parthenogenic in the south, whereas two-sex populations are found in the north. The parthenogenic populations inhabit warmer and damper sites than the two-sex populations. The parthenogenic females are smaller and have fewer young per litter than the females of the two-sex population. Four parthenogenic genera have been identified in the Buthidae: six species of *Tityus* (South America), one species of *Ananteris*, one of *Centruroides*, and two of *Hottentotta*. One parthenogenic member of the Hormuridae has been found in the South Pacific: *Liocheles australasiae* (Fabricius 1775).

Parthenogenesis is generally thelytokous (only females develop from the unfertilized eggs), other than in *Tityus metuendus* (Pocock 1897) which displays arrhenotokous (unfertilized eggs develop into males) parthenogenesis.

In *Tityus serrulatus* (Lutz and Mello 1922)*,* an intracellular endosymbiotic bacterium, *Wolbachia,* has been found. This bacterium has been detected in other arthropods (crustaceans and insects) and it induces the feminization of males. It may therefore be involved in the process of parthenogenesis.

The Young at Birth: The "Pullus"

At birth, the baby scorpion is referred to as a "pullus," "neonate," or "stage 1 juvenile." Stage 1 juveniles were previously inappropriately referred to as "larvae." They are generally white or pale yellow, but they may sometimes have a colored back, appendages, or tail. Their tegument is transparent and the medial eyes are large and surrounded by a large zone of black pigmentation. The lateral eyes are also highly pigmented. The pullus has suckers at the end of its legs, rather than the claws of the adult. These suckers enable the pullus to attach itself to its mother's back or to the back of its congeners. The pullus can move by taking a few steps on the back of the female, but it does not descend until after its first molt. The chelicerae are rounded and have no apparent teeth. The chelae are also rounded at the end and have small buttons instead of rows of denticles. There are no trichobothria. The tegument hairs are small, with a raised base. The tail is relatively short and ends in a venom vesicle armed with a nonfunctional aculeus with a wide base and a rounded tip. The pullus is thus enabled to sting. Under its transparent cuticle, the future aculeus and claws gradually appear. In viviparous scorpions, the chelicerae and the base of the chelae present residues of temporary organs (the "teat" organs used to nourish the embryo). The pullus does not eat. The pullus is carried on its mother's back: this provides it with protection against predators and the mother seeks the type of microclimate essential for her young, which have a permeable cuticle at this stage of development.

Growth and molting: Like all arthropods, scorpions molt as they grow. The increasing size of the animal necessitates the replacement of the cuticle. The old cuticle detaches and is abandoned as an exuvia. During its life, a scorpion may molt 4–13 times, depending on the species. Most molt five to seven times. The first molt occurs when the animal passes from the pullus stage to juvenile stage 2. This molt has several unusual features. Firstly, it takes place on the back of the mother and is almost synchronous for all the individuals. It may take place 1–51 days after birth, depending on the temperature and the species. Scorpions with diverticula often have a longer pullus stage than the scorpions of the Buthidae. The pullus tears its fine, transparent cuticle and extracts itself. The young often turn onto their backs with their tails extended for this first molt and a certain number appear to be hanging from the side of their mother. The fine tegument remains stuck to the female's back for some time and is often still visible when the young leave their mother. The stage 2 juvenile closely resembles the adult, but is smaller. The color, proportions of the tail and chelae, and granulosity are different, but the animal has lost its suckers, its claws have formed, the trichobothria are present, and the sharp aculeus is present at the end of the venom vesicle. The animal remains on its mother's back while still soft and unpigmented. It develops pigmentation and hardens over the course of a few days. It leaves its mother, initially only temporarily, remounting at the slightest sign of danger. When the tegument has hardened, the young can catch prey animals (mostly small insects) and feed. The young eventually leave their mother definitively and become autonomous, seeking a refuge of their own.

The other molts generally take place in a refuge: in the burrow, under a stone or a piece of bark, or in a rock crevice. In a few species, molts occur at regular intervals. In most species, they occur in a more irregular manner, with the animal remaining blocked at the same stage for several months. The animal displays a so-called intermolt cycle: following a postexuvial period, the tegument of the animal hardens and becomes pigmented and the animal goes through a period of activity during which it hunts and feeds. When the next molt approaches, the animal begins to "swell." The articular membranes between the sternites and tergites and the pleural membranes distend, by absorbing water. The animal then becomes transparent and stops feeding before undergoing exuviation.

Exuviation: The scorpion is initially immobile, flattened against the ground with the chelae forwards and the legs in a position similar to that during rest periods. The tail is curved over the body. The animal anchors itself to the substrate, to enable it to release itself from its old cuticle. Many species adopt a "stilting" position for molting. Exuviation begins with the rupture of the tegument at the front and sides of the prosoma, opening up a large slit. The scorpion makes jerky upward movements and releases its prosoma by sliding forwards. The mesosoma follows and the chelae and legs gradually come out of the old cuticle, which forms a sort of sheath. There is an alternation of lifting and rest phases, to allow the gradual release of the mesosoma. The "new" chelae come out from underneath the prosoma and are initially oriented towards the back, as are the first pairs of walking legs. They must thus turn on their base, towards the front, for a normal position to be obtained. Finally, the tail is released. This sliding out of the old cuticle is possible only because the animal is soft, part of the cuticle having been digested by the underlying cells and a molting fluid having been secreted. The animal is highly vulnerable at this stage and molting takes place in a secluded, safe place. Exuviation may take several hours (from 20 min to 12 h). The position adopted for exuviations varies and it is not unusual for the scorpion to carry out part of this process with its ventral surface uppermost. In all arthropods, molting is dependent on molting hormones (ecdysteroids) secreted by the molting glands. The location of these glands has been well established in spiders but remains a matter of debate in scorpions (coxal glomus or base of the chelae?).

The chelae display strong, disproportionate, allometric growth, sometimes reaching the same length as some of the tail segments. The number of hairs or sensilla tends to increase during growth. Growth stops in mature males. However, in some genera (*Centruroides, Rhopalurus, Heterometrus, Paruroctonus utahensis* (Williams 1968)), the female remains capable of molting after reproducing (postnuptial molting).

The growth rate (in terms of dimensions) of the hard parts of the body is consistent with that in other arthropods. Between molts, the scorpion increases in size by a factor of 1.26, but the growth rate differs considerably between species and between different parts of the body (from 1 to 1.5). Weight gain is also rapid. For example, a pullus of *Smeringurus* weighing 0.03 g reaches a weight of 1.85 g by adulthood (in 2 years). The weight of a large adult scorpion, such as an 18 cm long *Pandinus*, may reach 60 g.

Sexual maturity is reached when the animal becomes capable of reproducing. In scorpions, this is generally after the fifth molt (variable, between the fourth and seventh molts). In many species, there are several different sizes of male and female capable of reproducing. There are therefore "strong" and "weak" males and "strong" and "weak" females. Sexual maturity is reached between the ages of 6 months (*Buthus occitanus*) and 7 years (but often between 1 and 2 years). **Longevity** also differs considerably between scorpion species, with life spans of 2–25 years, mostly within the range 2–8 years. The males seem to have shorter life spans than the females. In natural conditions, male scorpions wander to a greater extent than females and are, therefore, exposed to a higher risk of predation. Mortality rates may also be high among the young.

Conclusion and Future Directions

Scorpiones is a relatively homogeneous order, the morphological characteristics of which have remained fairly constant since the Silurian era: scorpions have been described as "living fossils." Nevertheless, they have acquired a remarkable plasticity, enabling them to adapt at many environments and to react to major ecological changes. Their preadaptation has made them one of the best-equipped animals for survival in extreme terrestrial environments.

Many biological issues, concerning the cytology of sensory organs, neurophysiology and ecophysiology in particular, remain to be resolved and have been little studied, whereas the field of systematics is currently booming, with the description of several new genera and of many new species over a short period. However, this is continually raising questions about relations between species and evolution.

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Part II

Envenomation

Scorpion Venoms: Pathogenesis and Biotherapies

3

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Contents

Abstract

Accidental pathogenesis, such as that attributed to scorpion stings, required an emergency treatment. Scorpion envenoming (SE) is an accidental disease encountered in tropical and subtropical countries. This accident is considered as a public health problem due to its induced pathophysiological effects, which could be fatal to humans. Observed clinical cases after scorpion stings are often different from one species to another. The severity of the symptoms depends on the health of the victim and the injected amount of the venom by sting and predominantly on patient's age (stung children seem to be the more vulnerable). Some symptoms appear rapidly and could evolve and worsen to multivisceral damage (MVD) leading to the fatal outcome. In regions at risk, immunotherapy

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is the most commonly used approach to treat stung victims. This therapy is associated with symptomatic treatment according to the health state of each patient. The specific treatment may have a limited therapeutic value mainly due to neutralizing capacity of the used antibodies and also to other factors such as the time taken to reach at the health sectors. This review emphasizes SE as a public health problem raging not only in Maghreb regions but also in the rest of the world. Understanding of the induced effects after stings is essential to optimize the treatment.

Introduction

Pathogenesis of scorpion envenoming (SE) is present in the five continents. According to its frequency and severity, this pathology is considered as a real concern for health in many countries of Maghreb regions, Middle East, India, and in America from Southern United States down to Argentina. Medical care of patients taken too late and other environmental factors contribute to the severity of SE. Almost all dangerous scorpions to humans belong to the Buthidae family which is common in arid areas. This family regroups the largest number of named scorpions (1,000), including almost all considered dangerous to humans. Epidemiological data report that more than 2,600 people worldwide die each year (Chippaux 2012).

Scorpion stings can cause various effects ranging from local pain and inflammation to tissue damage. Even though most cases of scorpion stings (95 %) cause only local manifestations dominated by pain, the severe cases can be life threatening. Stung people may exhibit signs and symptoms involving central nervous systems (CNS) and autonomic nervous systems (ANS) and, occasionally, respiratory and heart failure, leading to the death in the most severe cases. Severity of scorpion stings depends on the involved species, its size, age, nutrition, climatic conditions of habitat, amount of injected venom, and the delay in taking care. High severity is observed mainly when the site of sting is a vascularized area, such as the trunk, head, or neck, as well as when patients are children. SE is often accompanied by cardiorespiratory distress including hypertension, bradycardia, tachycardia, hypotension, bronchorrhea, and pulmonary edema (Ismail 1995).

Observed cardiovascular effects after SE usually occurred in two phases, the initial hyperdynamic phase characterized by hypertension and tachycardia attributed to catecholamine secretion from the nerve endings of the postganglionic sympathetic autonomic nervous system and adrenal glands. This phase is followed by another characterized by a prolonged hypokinetic hypotension and bradycardia attributed to cholinergic effects after stimulation by the venom neurotoxins of parasympathetic branch node and postganglionic (Ismail 1995). Cardiorespiratory failure plays a central role in the diagnosis of pulmonary dysfunction.

Immunotherapy associated with symptomatic treatment remains the only means to combat this serious public health problem, in order to neutralize the occurred deleterious effects in envenomed patients.

This review integrates some research developments on the pathophysiological effects in order to better face the risk of the fatality of SE syndrome raging mainly during the hot season in the regions at risk.

Pathogenesis

Scorpions and Envenomation

Accidental pathology attributed to the well-adapted venomous animals to the desert environment may be due to their resistance at the harsh conditions where they live. Scorpions are presented as homogeneous order with around 2,000 species; twenty five of them are the cause of serious injuries for humans. The incidence of SE is higher in adults, although its severity is more important in children. Majority of stung patients (up to 95 % regarding on the species and region) present asymptomatic manifestations such as pain, while the severe cases presenting important pathophysiological effects are less than 20 % (Chippaux 2012).

Scorpions are abundant in rural and also peri-urban areas of dry tropical and subtropical regions of the Maghreb, Middle East, and Central and Southern America. Scorpion species (*Androctonus* and *Buthus*) are found in Maghreb regions and Middle East. *Centruroides* and *Tityus* species are mainly spread, respectively, in North or Central America and South America (Bucherl 1971). The most dangerous scorpion species in Algeria are *Androctonus australis* (Aah), *Androctonus amoreuxi* (Aam), and *Buthus occitanus tunetanus* (Bot), they differ in their size and their lethal effects.

Venoms and Toxicity

Scorpion venoms are very toxic with very fast diffusion and action, explaining the observed early symptoms in envenomed patients. Biochemical analysis of scorpion venoms showed a complex composition with toxic and nontoxic fractions.

The nontoxic fraction is a mixture of mucopolysaccharides, enzymes (e.g., hyaluronidases and phospholipases), protease inhibitors, and bioamines such as serotonin and histamine.

Toxic fraction is the best studied; it consists mainly in highly specific neurotoxins to ion channels of excitable cells. Although having high sequence identity (sodium, potassium, calcium, or chloride) these neurotoxins differ in their degree of toxicity and affinity to specific target of animal species (mammals, crustaceans, and insects), there are also toxins with dual specificity (anti-mammal and anti-insect) (Martin and Rochat 1986). High similarity of toxic effects and, antigenic structures of neurotoxins exist between scorpion species. Venom composition depends on the species and their geographic origins.

Toxic manifestations observed after exposure to scorpion venom are due to its neurotoxins which act on sodium or potassium channels of nerve endings, leading to neurotransmitter release that can induce general symptoms resulting in local pain, inflammation, cardiorespiratory distresses, and neurological disorders.

Neurotoxins and Their Targets

Neurotoxins

The first studies on the composition of scorpion venoms concerned Buthidae family. Neurotoxins were isolated from Buthidae venom by Miranda and collaborators in 1960. Since that period, research on scorpion venoms has continued to advance and allowed the purification of many toxins active on sodium, potassium, chlorine, and calcium channels (Martin and Rochat 1986; Laraba-Djebari et al. 1994; Possani et al. 1999).

Neurotoxins of Buthidae venoms are basic polypeptides of low molecular weight single subunit (3–7 kDa). Their sequence consists of 31–70 amino acid residues cross-linked by 3–4 disulfide bridges that confer their stability. The toxicity of venom is mainly due to its neurotoxins and to their specific binding to their cellular targets, the ionic channels. The main toxic components of Buthidae venoms correspond to neurotoxins acting on sodium and potassium channels.

Neurotoxins active on sodium channels are polypeptides of 63–70 amino acid residues cross-linked by four disulfide bridges. Those acting on potassium channels are peptides of 42 amino acid residues or less cross-linked by three disulfide bridges; they block the various types of potassium channels (Laraba-Djebari et al. 1994). Another group of toxin of 58 amino acid residues cross-linked by three disulfide bridges called birtoxin-like peptides have also been characterized. Birtoxin peptides present almost identical sequences with different activities; some of them act on potassium channels.

Identification and biochemical characterization allowed to the isolation of many neurotoxins. Alpha-type neurotoxins are highly active on sodium channels in mammals (Martin-Eauclaire et al. 2005). These toxins bind to the site 3 of sodium channel of excitable cells and induce prolongation of the potential of action. Affinity of α-toxins to the sodium channel is dependent on membrane potential. The result is a massive influx of sodium ions into the intracellular spaces with intense and sustained depolarization of the cell. Venoms of Buthidae contain also anti-insect toxins and short toxins active on potassium channels, such as kaliotoxin which is a toxin of 37 amino acid residues active on K^+ channel (Laraba-Djebari et al. 1994). Beta-type toxins active on mammals have been isolated from the venoms of American scorpions; they act on another site of the sodium channel activated at a lower action potential and independently of the membrane potential (Chippaux 2012).

Neurotoxin Targets

Toxic effects of scorpion venoms are mainly due to the neurotoxins, acting with high affinity on voltage-gated sodium channels of excitable cells. Activation of these channels is induced by the action potential. Indeed, these channels play an essential role in the initiation and propagation of the action potential through the membrane, increasing the sodium permeability. Neurotoxins activate voltage-gated sodium channels and inhibit potassium channels leading to a prolongation of the action potential in neuromuscular cells, exerting a slower phase of inactivation of

the sodium channel by interaction with the membrane site 3 or 4 of the channel (Gur et al. 2011). These events lead to a large influx of extracellular sodium and release of neurotransmitters, followed by adrenergic blocking of membrane excitability. Increase of sodium permeability induces a large flow of intracellular calcium. Electrolyte imbalance would cause, in the second phase, the activation of phospholipases that are responsible of phospholipid membrane hydrolysis (Catterall 1980; Possani et al. 1999).

Potassium channels play a vital role in the physiological function of different cell types. They regulate the membrane potential, ensuring acceleration function of membrane repolarization, both in excitable (e.g., neurons and muscles) and non-excitable cells (e.g., β cells of pancreas, lymphocytes, and erythrocytes). Potassium channels are responsible for the repolarization phase, which is involved in the genesis of neuronal and cardiac action potential of ion channels. The main function of Kv1.3- and Ca^{2+}-dependent KCa3.1 channels is to maintain negative potential membrane, which facilitates the maintenance of Ca^{2+} signaling in the activation of T cells. They are therefore involved in signaling pathways that control cell proliferation and synthesis of IL-2 (Chandy et al. 2001).

Symptomatology of Scorpion Envenomation

Clinical symptoms observed after SE depend on the sting site, age, weight, and health of the victim. On the other hand, they depend also on scorpion species and the amount of injected venom. The first symptoms evolved from local signs (restlessness, sweating, vomiting, nausea, numbness, and inflammation) to shock (cardiovascular and respiratory disorders), in the absence of treatment.

Classification of clinical manifestations based on the increased severity was proposed in 1998 at the meeting of Pasteur Institutes and Associated Institutes (ACIP) (Krifi et al. 1998). This classification was then revised by a group of experts; three classes based on observed signs and their amplitude have been identified. These signs may be local, moderate, or severe with no chronological order between them (Khattabi et al. 2011).

Induced Pathophysiology After Envenomation

Complexity of biological changes and disturbances caused by scorpion venoms is multifactorial. Induced pathophysiological effects are not only attributed to the neurotoxins, but involvement of other biological mechanisms contributes also to the severity of disorders. In addition to cardiovascular disorders, acute pulmonary edema and tissue damage associated to the metabolic changes observed after severe accidental or experimental SE that have also been reported; the nervous system can also be affected. Neurological disorders have been observed and characterized by seizures, hyperthermia, hypothermia, irritability, restlessness, tremor, paralysis, and coma. These events are sometimes accompanied by biological disorders (leukocytosis, hypocalcemia, and hyperglycemia) (Ismail 1995).

Involvement of the inflammatory process after SE has been demonstrated by several experimental studies (Adi-Bessalem et al. 2008; Sami-Merah et al. 2008; Petricevich 2010; Raouraoua-Boukari et al. 2012; Aït-Lounis and Laraba-Djebari 2012).

Biodistribution of Scorpion Venoms and Their Toxic Components

Conducted studies on pharmacokinetic venom demonstrated that venom distribution from the injection site to the tissues is within 15 min (Hammoudi-Triki et al. 2007). Experimental studies report fast biodistribution (Tmax of 30 min) of scorpion venoms in extravascular compartment explaining the observed symptoms. High concentration of the venom found (70 % of the total dose) in the bloodstream is associated with tissue damage; metabolic and biochemical changes; and inflammatory response. High levels of venom were detected at the site of injection, serum, liver, and heart, while the distribution is late in the lung, spleen, and pancreas (60 min). The venom is not detectable in the CNS, which is likely due to blood–brain barrier (Bessalem et al. 2002). Neurotoxins disappear more rapidly from the bloodstream than the other components of the venom; they are preferentially concentrated in the tissues (lung, kidney, and liver) leading to their alterations. Correlation between pharmacological activity and venom concentration in the tissues showed that the action of venom is located in deep tissue compartments (Ismail 1995). Rapid diffusion of neurotoxins in the plasma is correlated with the majority of evolved clinical signs in stung patients.

Hemodynamic Manifestations Caused by Scorpion Venom

After SE, hemodynamic manifestations are the main causes leading to the death; scorpion venoms affect the cardiovascular system and may cause fatal pulmonary edema and cardiac arrhythmias. The main observed symptoms after envenomation are hypertension in mild cases and hypotension or acute pulmonary edema in severe cases. Marked increase in blood pressure associated with an acute left ventricular failure and elevation of pulmonary edema have been demonstrated by several studies (Fig. 1).

Experimental studies showed that SE-induced an initial hyperdynamic phase characterized by tachycardia and hypertension followed by a phase of hypotension and bradychardia attributed to cholinergic effects. Tachycardia seems to be due to sympathetic stimulation and catecholamine release in the tissue (Ismail 1995; Correa et al. 1997).

Although the mechanisms accounting to the hemodynamic disorders are still not clearly established, the pivotal role of the marked release of catecholamines induced by scorpion toxins seems to be the culprit. However, several findings suggest that additional factors could contribute to hemodynamic disorders following SE. On one hand, scorpion venom can cause important alterations of excitable cells that can induce a change in myocardial function and vascular tone (Teixeira et al. 2001). On the other hand, many of the observed hemodynamic changes in severe SE could be due to the release of endogenous mediators such as the release of various peptides including endothelin-1 and neuropeptides (D'Suze et al. 2003).

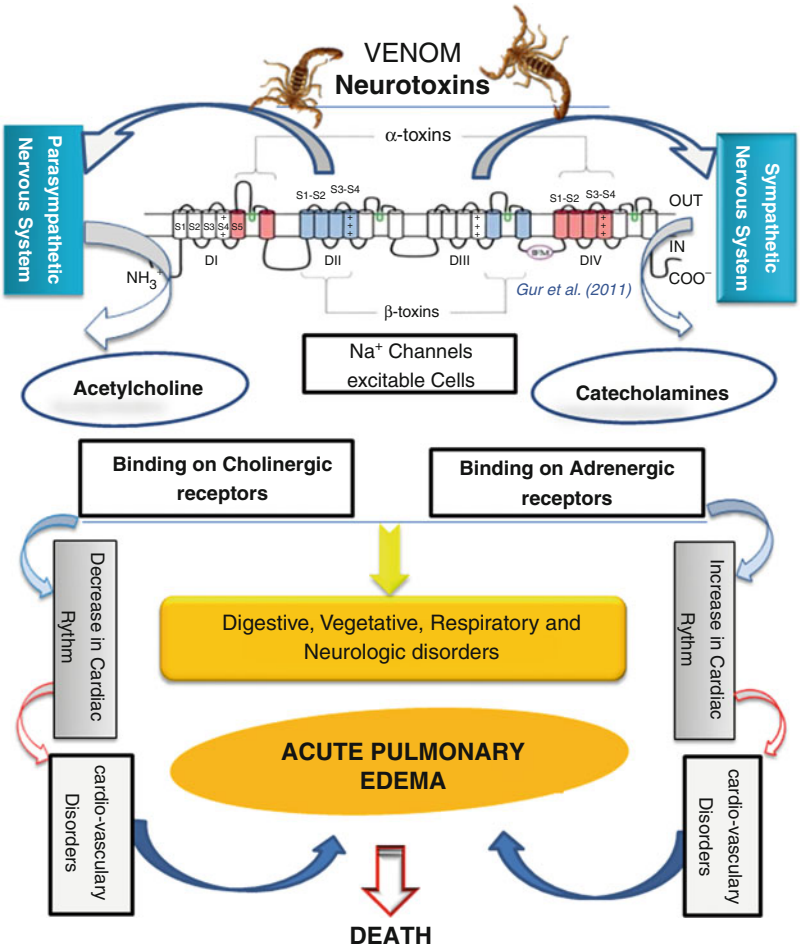

Fig. 1 Pathophysiological effects induced after neurotoxin binding to cell targets

These neuropeptides are involved in the systemic vascular resistance elevation, which is a characteristic effect of SE.

Experimental envenomation induces rapid changes in respiratory rhythm, an inflammatory response, and bronchoconstriction (Hammoudi-Triki et al. 2007; Adi-Bessalem et al. 2008).

In severe cases of SE, pulmonary edema followed by respiratory disorders leads to death (Fig. 1). Pathogenesis of pulmonary edema is dual; the mechanism of acute pulmonary edema has not yet been elucidated. However, two factors seem to be predominant in the pulmonary edema genesis: (i) non-cardiogenic or lesional edema could be due to an increase in membrane permeability resulting from the alveolar–capillary membrane lesion and activation of inflammatory cascade by molecule secretion such as prostaglandins, leukotrienes, platelet-activating

factors (PAF), histamines, and cytokines (Andrade et al. 2004). (ii) Cardiogenic origin of edema could be due to an ischemia state related to catecholamine and acetylcholine discharge or biventricular failure as a response to venom toxicity leading to left ventricular dysfunction (Amaral and Rezende 1997; Teixeira et al. 2001).

Induced Anatomopathological Effects by Scorpion Venoms

Experimental SE induces several tissue damage characterized by severe organ alterations. Tissue damage is observed in almost all organs (including the heart, lungs, kidneys, liver, and spleen), but the most striking effect occurred in the lungs as a diffuse injury of the alveolar–capillary barrier, interstitial and alveolar edema associated with leukocytosis, epithelial transmigration, and marked fibrin deposits responsible for thrombi, wall infarct, and necrosis (Correa et al. 1997; D'Suze et al. 2004; Adi-Bessalem et al. 2008; Sami-Merah et al. 2008). Tissue damage, respiratory distress syndrome, and multiple organ failure (MOF) could be related to the uncontrolled production of cytokines and other products after macrophage and lymphocyte activation (Fig. 2).

Scorpion venoms such as *Androctonus australis* venom caused disorganization of myocardial fibers, alterations in hepatic and pulmonary parenchyma and renal cortex with necrotic areas, interstitial edema, hemorrhage into the interstitial space, and polymorphonuclear cell infiltration. *Centruroides* venom also induced the same disorganization of the myocardial fibers, liver and lung parenchyma, and renal cortex. Kidney plays a major role in the process of filtration and removal of toxic elements and their metabolites; it is therefore a target organ of several toxins. Furthermore, morphological changes in glomeruli and tubules are frequently observed after intramuscular administration of *Centruroides* venom to rats (Bertke and Atkins 1961). Otherwise, serious alterations in structures of the lung accompanied by emphysema, hemorrhage, and pulmonary edema have been also observed (Sami-Merah et al. 2008). Pulmonary edema could be attributed partially to acute left ventricular failure resulting from massive release of catecholamines or myocardial damage induced by the inoculated venom.

Metabolic and Hormonal Dysfunctions

Several studies reported important alterations occurring in cardiovascular, respiratory, digestive, and nervous systems after SE. Induced tissue damage leads to the release of intracellular enzymes into the bloodstream and their level decrease in the organs.

Some seric enzymes or proteins are usually used as a marker in the diagnosis of tissue alterations. Indeed, high seric levels of transaminases (aspartate aminotransferase, AST, and alanine aminotransferase, ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), and troponin I (markers of muscle necrosis) correlate with the observed structural tissue disorganization (Bessalem et al. 2003; Adi-Bessalem et al. 2008).

These enzymatic activities can be correlated with the intensity of tissue damage after SE. Biochemical disturbances are also observed in lipid levels with an increase

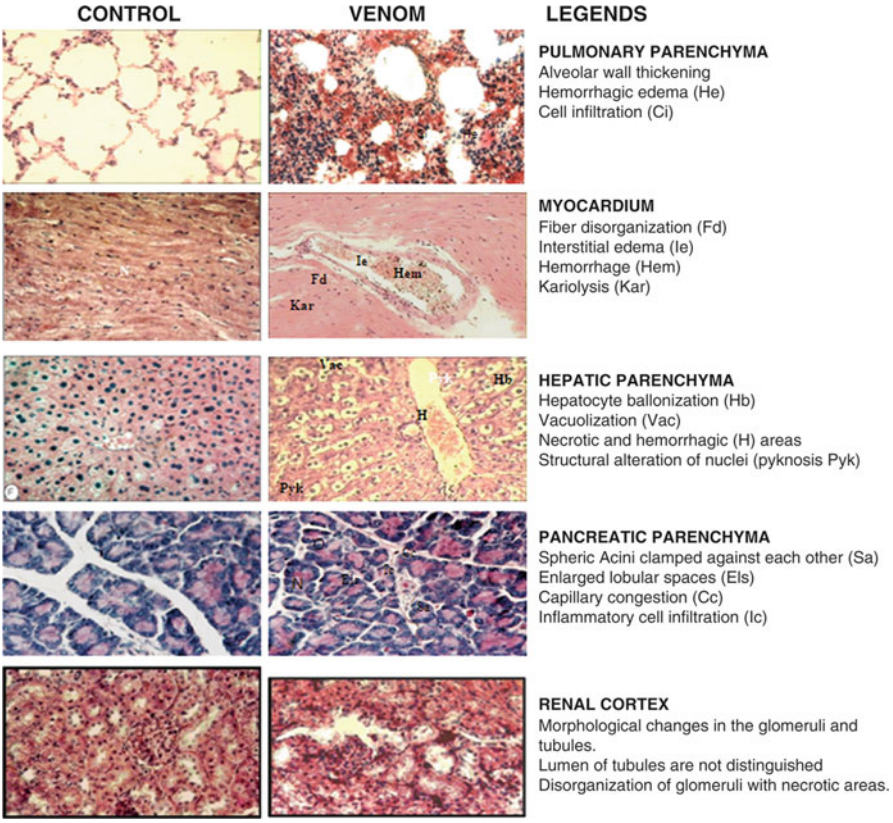

Fig. 2 Tissue damage induced by *Androctonus australis* venom (Bessalem et al. 2003, 2008; Sami-Merah et al. 2008). Magnification × 40; coloration: hematoxylin and Eosin

of circulating free fatty acids. Transient hyperglycemia induced by catecholamines stimulates gluconeogenesis associated with significant increase of urea (Murthy and Hase 1994).

SE is also characterized by an intense discharge of neurotransmitters that explain the intense vasoconstriction, blood pressure increase, and the initial left ventricular dysfunction. Adrenaline and noradrenaline releases are also induced by the venom on peripheral nervous system. Increase of angiotensin, thyroxin, cortisol, and glucagon levels was also reported following SE. In addition, SE leads to an inhibition of insulin secretion, which may contribute to the development of hyperglycemia and hyperkalemia (Murthy and Hase 1994). In hepatic tissue, adrenaline and glucose production increase was reported due to hepatic glycogenolysis, with a deficiency of insulin secretion and glucagon secretion increase. All of these induced disorders following SE can result essentially in a syndrome of energy deficit and inability to use metabolites by vital organs causing MOF and death.

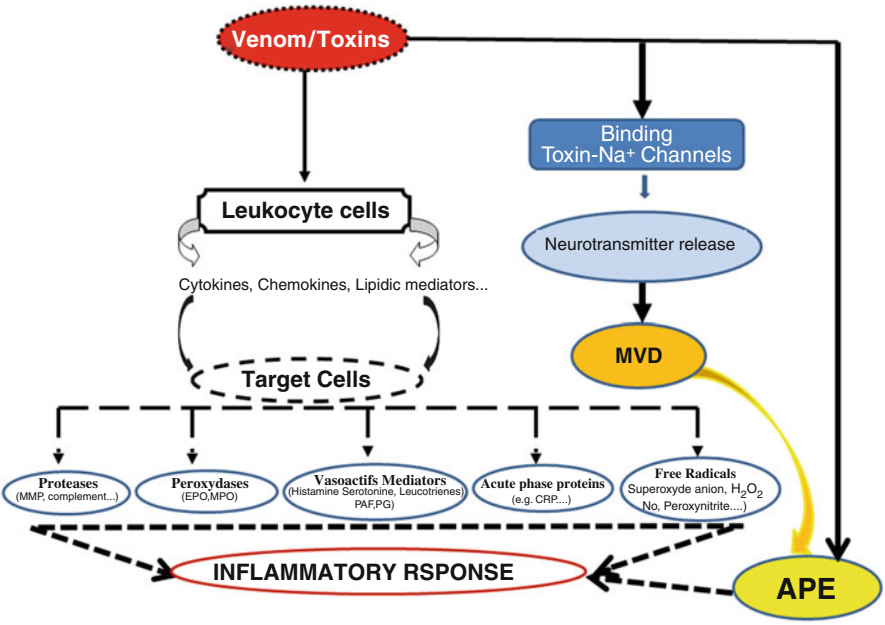

Fig. 3 Induced inflammatory response by scorpion venoms (MVD: Multi-visceral dysfunction; APE: Acute pulmonary edema)

Inflammatory Response

SE is a noninfectious process that can lead to a systemic inflammatory response. It has been reported that scorpion venom can stimulate neuroendocrine immune axes by their ability to mobilize leukocytes and to release catecholamines, corticosteroids, bradykinin, and prostaglandins. Inflammatory mediator release, adhesion molecule expression, platelet-activating factors, cytokines, and immunoglobulins are involved in this response (Magalhães et al. 1999).

Depolarization caused by the toxin binding to its receptor triggers a cascade of events characterized by synthesis and secretion of mediators (e.g., cytokines, lipids, complement, and reactive oxygen species (ROS)), which in turn cause various secondary manifestations that can lead to the death of the stung victim or injected animals with venom or its toxins (Fig. 3).

The release and activation of pro-inflammatory mediators are among the most important factors that appear in this pathogenesis (Fukuhara et al. 2003). Clinical and experimental envenomations induce an inflammatory response characterized by cytokine level increase, such as IL-1, IL-6, TNF-α, IL-4, IL-5, and IL-10, and alterations in tissue structures with hemorrhagic and necrotic areas and interstitial edema in several organs. Involvement of apoptosis markers in the cellular damage induced by scorpion venom is also suggested. These effects are firstly mediated by stimulation of the Autonomic nervous system, sympathetic and parasympathetic systems (Ismail 1995).

Hemodynamic disorders and the release of pro-inflammatory mediators may also be involved in the development of cardiorespiratory disturbances (Fukuhara et al. 2003).

Stimulation of peritoneal macrophages by venom allows their activation characterized by significant release of inflammatory mediators such as hydrogen peroxide (H_2O_2), nitric oxide (NO), myeloperoxidase (MPO), and several pro-inflammatory (TNF-α, IL-1β, IL-6, IL-8, IFN-γ) and anti-inflammatory (IL-4, IL-10) cytokines, which can modulate macrophage response (Fig. 3).

Cytokines play an important role in orchestrating the inflammatory response. Disequilibrium in their production can lead to the serious consequences, hence the need to assess the balance between pro-inflammatory and anti-inflammatory pathophysiological processes such as in the SE (Magalhães et al. 1999). High cytokine levels are related to the severity of SE; they are correlated with the venom concentrations in the blood of envenomed patients or animals (Hammoudi-Triki et al. 2004).

Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α play an important role in the pathophysiology of pulmonary edema (Andrade et al. 2004). Increased levels of the cytokines induced the production of other inflammatory factors such as prostaglandins (PGE2), leukotrienes (LTB4), PAF, proteases, and free radicals (NO) (Fukuhara et al. 2003). PGE2 and LTB4 lipid mediators are also involved in inflammation and several homeostatic biological functions, including vascular permeability and leukocyte influx to the bronchoalveolar fluid. PGE2 is involved in the inflammatory response and in the neutrophil recruitment after *T. serrulatus* SE (Pessini et al. 2006).

Furthermore the intensity of inflammatory response is amplified by the activation of C-reactive protein and complement proteins. These factors contribute to the local and systemic reactions. The activation of the complement system may be involved in tissue damage and pulmonary edema observed in SE resulting in an increase of vascular permeability, chemotactic migration of leukocytes to the inflammatory site, degranulation phagocytic cells, and induction of bronchoconstriction (Bertazzi et al. 2003; Adi-Bessalem et al. 2008). Other inflammatory mediators such as complement factors, PAF, PGE2, and prostacyclin may modulate inflammatory response.

Previous studies have shown that scorpion venom induces lymphocytosis accompanied by tissue damage in several organs, stimulating the inflammatory response and cytokine release (Sami-Merah et al. 2008; Raouraoua-Boukari et al. 2012).

In pulmonary inflammation, recruitment of circulating polymorphonuclears is essential for host defense; it initiates the specific immune response. One pathological hallmark of acute lung injury and acute respiratory distress syndrome is the uncontrolled transmigration of neutrophil into the lung interstitium and alveolar space (Sami-Merah et al. 2008). Thereby, extravasation of leukocytes from vascular system into the tissue is induced by inflammatory mediators. These factors contribute to local and systemic reactions.

Involvement of oxidative stress and proteases was also described in experimental envenomation. Lipid peroxidation occurring in biological membranes induces membrane dysfunction, fluidity decrease, and inactivation of membrane-bound receptors. All of these effects contribute to multiple visceral dysfunctions (MVD).

SE -induced changes in the composition of the biomembranes and phospholipase activation may occur as a result of the oxidative stress produced in cells. Accumulation of free arachidonic acid due to PLA2 and cyclooxygenase activities may induce apoptosis, thereby contributing to the SE pathogenesis. On the other hand, inflammatory cells produce ROS that react with NO to form NO derived (inflammatory oxidants) which induces tissue damage (Dousset et al. 2005). Scorpion venom induced also significant lipid peroxidation since the use of antioxidant allowed a protective effect of the most vital organs. However, the real mechanisms by which the generation of free radicals and lipid peroxidation after SE remains complex, several factors can interact.

Balance between pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-10) after SE could determine the evolution and the extent of the inflammatory response leading to major clinical effects such as cardiac dysfunction, pulmonary edema, and shock (Petricevich 2010). SE severity has been associated with IL-6 level as a marker and also with an imbalance of inflammatory cytokines induced by the activation of Th1 and Th2 cells. Some studies confirmed the Th2 pathway activation; however, the involvement of Th1 pathway in the induced response by SE needs more investigations. Cellular and molecular pathways involved in SE pathogenesis act in concert to lead to cell necrosis and apoptotic events observed in severe cases of envenomation.

SE is a complex multifactorial pathogenesis which involves several signaling pathways leading to multiple and complex disturbances (Table 1). To face this complexity, understanding and control of the mechanisms underlying these pathways may help to better undertake effective therapy in moderate and severe cases of SE (Fig. 4).

Therapies

Treatment of SE: Therapeutic or Preventive Measures

SE constitutes a public health problem in several parts of the world. Several areas were identified as at risk (North-Saharan Africa, Near and Middle East, South India, Mexico, and several countries of South America). Health care of envenomed victims requires an effective therapeutic strategy based on understanding of involved mechanisms in the induced pathophysiological disorders. Many approaches to treat SE are advocated, ranging from symptomatic treatment to antivenom or vaccine. In all cases, the treatment is complex and controversial, especially according the use of antivenom and symptomatic treatments that must be associated.

Symptomatic Treatment

Symptomatic treatment was used to maintain vital functions such as blood pressure and hydration regulation to compensate the water loss due to vomiting and diarrhea. It involves administration of vasodilators, anticholinergics, anticonvulsants, antiemetics, and antipyretics. To regulate the inflammatory response and prevent some

Table 1 Pathophysiological effects and inflammatory response reported after experimental envenomation

	Characteristics	Scorpion species involved	References
Tissue alterations (liver, heart, kidney, lung, pancreas)	Alveolar edema, hemorrhage, cell infiltration	*T. serrulatus*, *A. australis*, *T. discrepans*	Correa et al. (1997), Bessalem et al. (2003), D'Suze et al. (2004), Sami-Merah et al. (2008), Adi-Bessalem et al. (2008), Boussag-Abib and Laraba-Djebari (2011)
	Tissular alterations of hepatic parenchyma		
	Serous acini of exocrine pancreas		
	Degeneration of glomeruli		
Biochemical perturbations	Level increase of metabolic enzymes and Troponin	*T. serrulatus*, *A. australis*, *L. quinquestriatus*	Amaral et al. (1991), Murthy and Hase (1994), Correa et al. (1997), D'Suze et al. (2003), Andrade et al. (2004), Adi-Bessalem et al. (2008)
	Transitional hyperglycemia		
	Hypercreatinemia		
Electrolyte disturbances	Level decrease of Na^+, Ca^{2+}, Mg^{2+}, K^+, Pi	*T. serrulatus*	
Neurohormonal disorders	Increase of plasmatic level of catecholamines (adrenaline; noradrenaline)	*A. australis*, *M. tamulus*	Murthy and Hase (1994), Ait Lounis and Laraba-Djebari (2012)
	Consequent secretion of vasoconstrictor peptides (NPY; ET-1; ANP)		
	Hyperinsulinemia		
Overproduction of cytokines	Pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α)	*A. australis*	Magalhaes et al. (1999), D'Suze et al. (2003), Fukuhara et al. (2003), Hammoudi-Triki et al. (2004), Petricevich (2010), Adi-Bessalem et al. (2008), Raouraoua-Boukari et al. (2012)
	Anti-inflammatory cytokines (IL-10, IL-4)	*T. serrulatus*	
		L. quinquestriatus	
Vasoactive mediators and chemoattractants	Activation of complement and kallikrein–kinin systems	*A. australis*	Meki et al. (1995), Bertazzi et al. (2003, 2010), Pessini et al. (2006), Adi-Bessalem et al. (2008)
	Production of histamine, prostaglandin, leukotriene...	*T. serrulatus*	
		B. occitanus	
Oxidative and nitrosative stress	Lipid peroxidation	*A. australis*	Dousset et al. (2005), Raouraoua-Boukari et al. (2012)
	NO production	*T. serrulatus*	
		L. quinquestriatus	

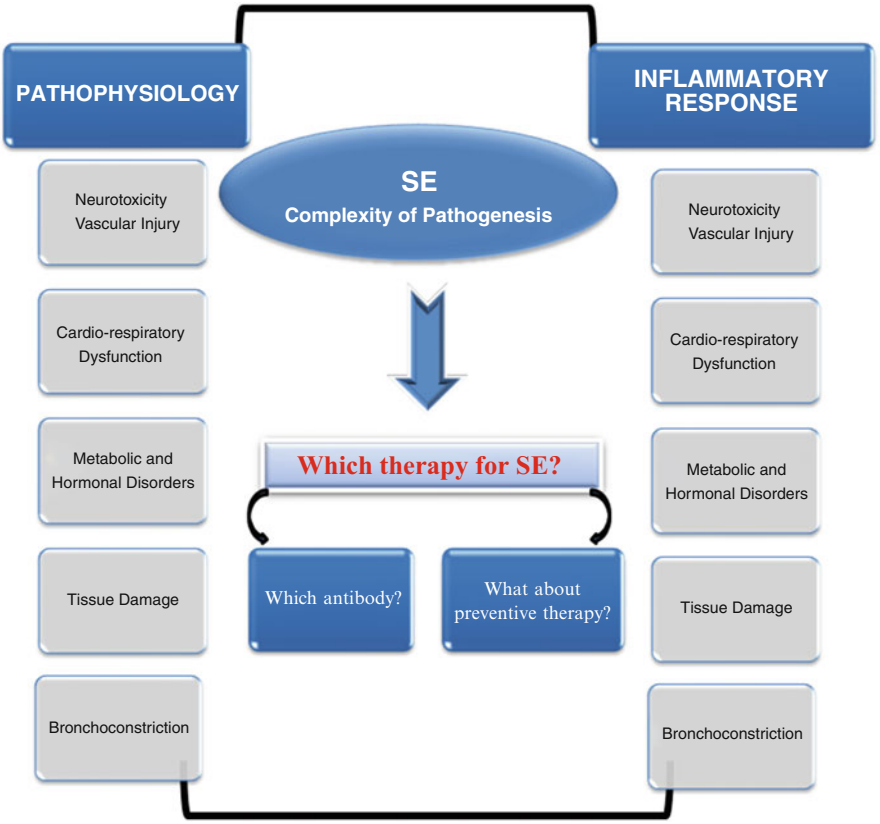

Fig. 4 Multifactorial effects induced after scorpion envenomation

tissue damage, anti-inflammatory drugs (antihistamines, corticosteroids, and aprotinin) are also recommended (Ismail 1995). Administration of a Na^+ channel blocker lidocaine may be more beneficial than treating the aftermath symptomatically or attempting to block the multitude neurotransmitters and modulators released after SE (Fatani 2010).

Immunotherapy Treatment

Severity of SE and the rapid distribution of inoculated venom after stings required specific treatment which is immunotherapy. More than a century ago, Von Behring and Kitasato showed the effectiveness of equine sera, raised against diphtheria and tetanus toxoids in the treatment of these diseases. Calmette (1894) had prepared the first antivenom against bites of cobra for therapeutic use in India. The first produced antiscorpionic serum was performed by Todd in 1909 in Cairo. Other antiscorpionic sera were then produced in Johannesburg and in Algeria by Sergent (Bucherl 1971). Side effects caused by the administration of heterologous proteins were quickly identified. Most current antivenoms are now produced as immunoglobulin

fragments. In SE cases, the used antibodies in immunotherapy are usually from horses and the reactive molecules consist in F(ab′)2 fragments.

Immunotherapy is subject to many controversies requiring its optimization. Produced neutralizing antibodies against the toxic effects of venoms require selection and monitoring of some parameters such as time of antibody administration, route, and dose of antibody injection that could improve this therapy. The choice of animal producers, used antigen for their immunization, the obtained neutralizing molecule, and the conditions of its application are also among these required parameters (Chippaux 2012).

Choice of antigens from toxic fractions of venoms is also a parameter to improve immunotherapy. Neutralizing effects of antibodies appear to be higher after immunization of animals with multiple injections of toxic fraction than those obtained with whole venom.

Choice of Antibody Format

The choice of neutralizing molecule in SE treatment is influenced by its efficiency and clinical safety. The effectiveness of antibodies is linked to their neutralizing capacity but also to their tolerance (IgG is less tolerated than F(ab′)2 and Fab) and their pharmacokinetics: (i) distribution in body compartments (IgG and F(ab′)2 remains mainly in the vascular compartment, while Fab spreads in all compartments of the body except the brain), (ii) elimination rate (very fast for Fab, which requires frequent renewal of administration), and (iii) clearance ways (by immunocompetent cells for IgG and F(ab′)2, kidney for the Fab, which can cause renal failure).

Different methods using a set of techniques are used to obtain the immune serum consisting I_gG, F (ab′)2, or Fab antibodies. Fab and F (ab′)2 fragments are obtained by proteolysis of the I_gG molecule to remove the Fc fragment, which allows best tolerance to the fragments. Indeed, clinical experience showed no anaphylactic reactions after F(ab′)2 injection.

When injected to animals by i.v. route, the pharmacokinetic studies of IgG, F(ab′)2, and Fab fragments showed that I_gG molecules present the same volume of distribution as vascular compartment. However, the Fab fragments have the largest volume of distribution and rapidly reach extravascular compartments.

The elimination of the three antibody molecules occurs in two ways according to their molecular weight. Due to their high molecular weight compared to the glomerular filter, elimination of I_gG molecules (150 kDa) and F(ab′)2 (100 kDa) is by immune system cells, while Fab fragments (50 kDa) are eliminated through renal filtration.

As a result, a mixture of F(ab′)2 and Fab could be of great interest: the former neutralizes the venom early before they bind to receptors, while the latter can act in the organs and try to remove the toxins from their receptors. Mixture of these two fragments F(ab′)2/Fab could improve the efficiency of the immunotherapy. Indeed, histopathological changes and cell migration into the peritoneal cavity were neutralized by the mixture of the two antibody molecules (Sami-Merah et al. 2008). Appropriate dose and best ratio of Fab to F(ab′)2 fragments may thus be beneficial in counteracting the pathophysiological effects induced by scorpion venom.

New forms of molecules (rFab, scFv) have also been developed for therapeutic use. The structures of small fragments scFv (monomeric, dimeric, trimeric, and tetrameric) are formed by two variable domains (VH and VL) of immunoglobulin, covalently associated by a flexible peptide linker. These molecules present an improved pharmacokinetic properties (fast tissue distribution, low immunogenicity, and high neutralizing capacity) compared to classical fragments (Mousli et al. 1999; Aubrey et al. 2004).

Camelid IgG and their derivates (nanobodies) offer potential safe advantages over conventional antibodies used in antivenom production. Nanobodies obtained from immune library and phage display technology seemed to be stable and also able to neutralize toxins (Hmila et al. 2008). Antibodies in laying hens seemed to be also a good approach to produce immunoglobulins in larger amount than in horses. It was reported that the produced antibodies in laying hens are able to neutralize toxic effects induced by snake venoms as equine IgG. IgY, the major immunoglobulin found in avian species, is structurally and functionally related to mammalian IgG. Laying hens have been recognized as a good source of polyclonal antibodies and could represent an alternative method for antiserum production. Seric IgY can be transferred in important amount to egg yolks (Carlander et al. 1999).

Dose of Antibodies

Correlation has been established between venom biodistribution, intensity of clinical signs, and SE severity. Evaluation of antibody dose depends on the distributed amount of venom in the body. The use of adequate doses of antibodies allows a neutralization of induced effects by the venom and to a decrease of toxicity and mortality induced after SE.

Route of Antibody Injection

The route of antibody administration is one of the limiting factors for the effectiveness of antibodies. Recommended route is intravenous, in which the bioavailability of F(ab′)2 fragments is maximal while it is no more than 50 % when the route was intramuscular (Krifi et al. 1998; Hammoudi-Triki et al. 2004).

Intravenous injection of F(ab′)2 or Fab fragments soon after SE allows the recovery of cardiopulmonary disorders; these alterations persist when they are injected intramuscularly.

Based on pharmacokinetic studies, the optimal efficiency has been reported after an intravenous injection with a sufficient dose of antiserum and within rapid delay (Hammoudi-Triki et al. 2007).

Time of Antibody Administration

The delay of immune serum administration is also an important factor after SE. There is time delay limit, for which the action of the venom is irreversible, making the immunotherapy ineffective. Rapid absorption and distribution of neurotoxins of scorpion venoms compared to antibodies require rapid administration of immune sera. However, it has been reported that the administration of a high concentration

Table 2 Improvement of venom immunogenic properties

Scorpion venom species	Process	Observations	References
C. noxius	Detoxification by glutaraldehyde (toxic fraction)	High level of specific antibodies against venom and toxin	Possani et al. (1981)
B. occitanus tunetanus			
A. australis		Immunoprotection against 6 LD_{50} after half and 2 months	
T. serrulatus	Liposome encapsulation (toxic fraction)	Decrease in toxicity (five times)	Chavez-Olortegui et al. (1991)
T. serrulatus	Synthetic peptide covering sequence of TsNPxP no toxic and immunogenic protein	Antipeptide antibodies able to neutralize the venom (13.5 LD_{50})	Alvarenga et al. (2002)
A. australis	Synthetic peptide mimicking the Aah II toxin produced by chemical solid phase	High antibody title Immunoprotection against challenge with 8 LD_{50} of native toxin after 6 months	Zenouaki et al. (1997)
A. australis	Recombinant toxins as fusion proteins (MBP-AaH I, MBP-Aah II, MBP-AaH III)	Protection against 3 LD_{50} of AaH-G50 fraction	Legros et al. (2001)

of immune serum even after a long period of SE could lead to the destabilization of the binding toxin channel and release them from their target.

Immunotherapy Optimization

Antigen Preparation and Immunoprotection

High toxicity of the venom could limit the efficiency of immune serum preparation. Animal producers may have chronic injuries due to the toxic administration during the immunization schedule having an impact on the quality of antivenoms. The use of native antigens required gradual and multiple injections to avoid the toxic effects and is required to produce efficient antibodies (WHO 2008). On another hand, detoxification of antigens could be also an approach that will improve the production and quality of antivenoms.

In order to prevent chronic toxicity to the laboratory animals that suffered during the immunization protocols and to increase the protective capacity, some approaches have been developed (Tables 2 and 3):

(i) Use of liposomes containing antigen, administered intravenously or subcutaneously, to obtain specific immune response allowing resistance to subsequent injection of antigen (Chavez-Olortegui et al. 1991; Zenouaki et al. 1997).

Table 3 Improvement of protective effect of antivenoms

Scorpion venom species	Process	Efficiency	References
A. mauretanicus mauretanicus	Natural anatoxin Amm VIII	Protection against 42 LD_{50} of Aah II toxin	Martin-Eauclaire et al. (2006)
T. serrulatus	Natural anatoxin TsNTxP	Cross-reactivity of anti-TsNTxP antibodies with toxins of Ts	Alvarenga et al. (2002)
			Chavez-Olortegui et al. (2002)
C. noxius	Recombinant peptide Cn5 expressed as a FP		Alvarenga et al. (2002)
C. noxius	Mimotope selected by phage-displayed random peptide libraries	Protection against one LD_{50}	Gazarian et al. (2003)
A. australis	Irradiated Aah venom	Detoxification of antigen reduced ($20 \times LD_{50}$)	Abib and Laraba-Djebari, (2003), Boussag-Abib et Laraba-Djebari (2011)
		Immunoprotection against 10 LD_{50} after 6 months	
A. australis	Nanobody (Nb), single-domain antigen-binding fragments of camelid	VHH, neutralize 10 LD_{50} of Aah I	Hmila et al. (2008)
C. noxius	scFv dimer of human scFvs	New variant scFv (LER) neutralize 2 LD_{50} of Cn2	Rodriguez-Rodriguez et al. (2012)

(ii) Use of recombinant scorpion toxins in the form of fusion proteins as antigens for immunization of animals, as exemplified by the production of protective antisera against the most lethal α-type toxins of *Androctonus australis* (Legros et al. 2001).
(iii) Identification of immunogenic proteins and/or their neutralizing epitopes may lead to their use as immunogens to develop efficient antivenoms or as antigens to assess in vitro antivenom potency (Chavez-Olortegui et al. 2002).
(iv) Immunization of animals with venom attenuated by gamma irradiation; it has yielded immunoprotection against a lethal dose of Aah venom (Abib and Laraba-Djebari 2003).

Good neutralizing antivenom can reach the affected tissues and must bind to toxin; the formed antigen–antibody complex must be eliminated rapidly. Obtained immunocomplex between neurotoxins and antibodies allows circulating venom to decrease in vascular and tissue compartments and therefore their toxicity.

Based on the affinity and the reversibility of the toxin binding to its target, the formed immunocomplex associated to the pharmacokinetic of the neurotoxin

redistribution may lead to the toxin release from their targets. Several studies have been conducted to better improve the production of antisera, their potency, safety, and homogeneity whether (i) by using other animals (sheep, mule) and/or (ii) by producing other immunoglobulin fragments and (iii) by using toxic fraction instead of the whole venom in the immunization of horses for improvement of the immune serum efficiency.

The immune serum consisting of F(ab′)2 fragments of higher molecular weight (100 kDa) compared to that of toxins (4–7 kDa) influences the absorption and distribution of the toxins. To reduce side reactions induced by heterologous proteins, smaller antibody fragments Fab (50 kDa) were used experimentally as well as in clinical studies. These antibodies provide faster kinetics with a larger volume of distribution and a faster clearance than conventional F(ab′)2 fragments. In addition, due to their monovalence, these fragments do not form immune complex, which are responsible for the risk of hypersensitivity type III. The low efficacy is attributed partially to the weak diffusion of F(ab′)2 fragments from the vascular to tissular compartments to neutralize toxins. Indeed in severe cases of envenoming, the treatment failed due to the very fast diffusion of scorpion toxins to their targets.

Effectiveness of immunotherapy is limited by several factors such as the choice of administration route of antivenom, the neutralizing capacity of the different antibody preparations, the quality of antivenom (purity and efficiency), the delay between stings and administration of treatment, and the amount of inoculated venom. It is well established that clinical efficiency of treatment depends also on the conditions of its application.

Immunoprotective Approach

Another approach to prevent the SE syndrome is vaccination which could be an alternative and/or complement to immunotherapy. Studies based on the recognition of antigenic properties of toxins confirmed that immunization with detoxified toxins provides good protection against the harmful effects of venom in different animal models. Immunization with toxic fraction of detoxified *Buthus occitanus tunetanus* venom by polymerization with glutaraldehyde, inactivated AahI and AahII neurotoxins (substitution of cysteine), or detoxified Aah venom by gamma radiation protected animals during 6 months against high doses of venom (Zenouaki et al. 1997; Boussag-Abib and Laraba-Djebari 2011).

Conclusion and Future Directions

Fatality of SE is a major safety concern in some disadvantaged regions at risk. Epidemiological data on the risk of SE place this neglected disease as a health problem. However, physicians who manage with stung patients are often helpless against complexity of the clinical symptomatology.

The increase of prevalence of SE in rural areas could be associated with several environmental factors. Before any therapy, it is beneficial to advise these populations about the risks related to hygiene and various other potential aspects

involving the proliferation of scorpions. On the other hand, better understanding of the mechanisms of induced pathophysiological effects after SE, mainly those attributed to hemodynamic, tissue alterations, and inflammatory response, could lead to develop or adapt new therapeutic strategies or new drugs to better treat and manage with the envenomed patients even when their admission to health structures is too late.

Fatal accidental pathologies due to SE syndrome are multifactorial features where immunotherapy associated with symptomatic therapy remains the currently used approach to treat stung patients. Optimization of this therapy could be enhanced by safe approach using attenuated antigens to produce efficient antibodies and also in vaccine development.

Immunomodulatory therapy and biotherapy (e.g., specific antibodies and anti-inflammatory drugs), especially when used in combination, can cure the moderate and severe cases of envenomed population. Biotherapies targeting the immune-inflammatory response could be beneficial mainly when some population categories are refractory to immunotherapy.

Prophylactic approach could be an alternative to better take care of stung patients. Therefore, it is necessary to develop preventive actions targeting the regions at risk. Attenuated venoms could be used as antigen to enhance the elicited antibodies and also to protect the populations of regions at risk.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ New Insights on the Pharmacokinetics of Venoms and Antivenoms
- ▶ Poultry IgY Alternatives to Antivenom Production
- ▶ Recombinant Neutralizing Antibodies, A New Generation of Antivenoms
- ▶ Scorpion Venom Interactions with the Immune System

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Scorpion Venom Interactions with the Immune System

4

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Contents

Abstract

Scorpion envenomation (SE) is a common medical problem in many countries; it is an important cause of morbidity and mortality, especially among children. In certain cases s*corpion* stings lead to multiorgan failure that may be fatal; the manifestations include acute respiratory distress syndrome and systemic inflammatory response syndrome. Neurotoxins are the most active components of the scorpion venom responsible for the toxic effects induced after SE. They induce a massive release of neurotransmitters during stimulation of sympathetic and parasympathetic of the autonomic nervous system. The pathophysiological disturbances caused by scorpion venom are not exclusively assigned to the released neurotransmitters. The activation and release of inflammatory mediators (cytokines, kinins, eicosanoids, reactive oxygen species, and nitric oxide) may also play an important role in the pathophysiology of envenomation after stings and may be responsible for some of the inflammatory manifestations and

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organ failure. The massive release of these mediators from injured and activated cells promotes the inflammatory response and may be responsible for its exacerbation and its maintenance. The present chapter focuses on the role of inflammatory mediators and on elucidation of the potential mechanisms by which the immune system affects the pathophysiology following SE. Understanding of involved inflammatory cascade in scorpion envenoming syndromes may have future therapeutic and diagnostic benefits.

Introduction

Envenomation by scorpions is widely spread in some countries of Asia, Africa, and the American continent (Chippaux 2012). Scorpion venom is rich in low molecular mass neurotoxins of high diffusibility with large volume of distribution reaching their tissue targets rapidly after envenomation (Hammoudi-Triki et al. 2004).

Scorpion envenomation (SE) severity is mainly due to cardiorespiratory dysfunction associated with formation of pulmonary edema (Ismail 1995). Heart failure, cardiac arrhythmias, and arterial hypertension followed by hypotension, acute pulmonary edema, respiratory failure, and biological abnormalities such as leukocytosis and metabolic perturbations are often observed (Ismail 1995; Hammoudi-Triki et al. 2004; Sami-Merah et al. 2008; Adi-Bessalem et al. 2008).

The pathogenesis of SE is multifactorial; it mainly results from a sympathetic and parasympathetic stimulation of the autonomic nervous system by the neurotoxins (Ismail 1995). Neurotransmitters have a marked and often diverse influence on differentiation and activation states of the immune cells. The cross talk between immune cells and cells of the nervous system is involved in tissue injury either in the central nervous or in the peripheral nervous system. The inflammatory cytokines (IL-1β, IL-6, and TNF-α) produced by innate immune cells in response to venom cross the blood–brain barrier to activate neurons. The release of these pro-inflammatory mediators would also be implied in the genesis of the cardiorespiratory perturbations (Magalhães et al. 1999; Fukuhara et al. 2003). Furthermore, the inflammatory reaction observed after scorpion stings is characterized by the release of various inflammatory mediators, including cytokines, eicosanoids, reactive oxygen species, and nitric oxide (NO) (Magalhães et al. 1999; De-Matos et al. 2001; Fukuhara et al. 2003; Hammoudi-Triki et al. 2004; Raouraoua-Boukari et al. 2012). The intense inflammatory response is further amplified by an intrinsic vascular compartment involving the activation of kinin–kallikrein and complement system. These factors contribute to the local and systemic reactions (Fukuhara et al. 2004; Bertazzi et al. 2005; Adi-Bessalem et al. 2008). In addition, it has been suggested that scorpion venom affects the immune system by mobilizing leukocytes and other inflammatory cells (Magalhães et al. 1999; Matos et al. 1999; Borges et al. 2000). Increased levels of these immune cells and cytokines have been identified in clinical samples, and their potential role in envenomation pathogenesis has been demonstrated in studies using murine models (Sofer et al. 1996; Magalhães et al. 1999; Fukuhara et al. 2003).

Cell- and plasma-derived mediators modulate their own activation and play an important role in developmental-induced acute and chronic inflammation after SE. Systemic inflammatory response induced by SE can lead to multiple organ failure (Meki and Mohey El-Dean 1998; D'Suze et al. 2003).

The aim of this chapter is to describe and analyze the role of various inflammatory mediators and oxidative/nitrosative stress in the pathophysiology of SE. A better understanding of the pathogenic mechanisms in SE may help to identify biomarkers that can be used in the prognosis of severe cases and would make it possible to treat more effectively the potentially fatal outcomes of SE.

Immune–Inflammatory Process in Scorpion Envenoming

Inflammation is now well known to play a key role in initiating and promoting the tissues injury. The activation and release of cytokines, vasoactive substances, and proteases may be important in the pathophysiology of envenomation as they are the molecules responsible for some systemic inflammatory manifestations and organ failure (Table 1) (Sofer et al. 1996; Teixeira et al. 2001; Petricevich 2010). Further, venom affects differentiation and chemotaxis of most immune cells, stimulates antibody synthesis in B cells, and promotes phagocytosis.

Soluble Inflammatory Mediators

During inflammatory processes induced by scorpion venoms, different soluble factors are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction. These soluble mediators regulate the activation of the resident cells (endothelial cells, tissue macrophages, and mast cells) and the newly recruited inflammatory cells (monocytes, lymphocytes, neutrophils, and eosinophils). Some of these mediators are involved in the development of the systemic inflammatory response syndrome characterized by fever, hypotension, tachycardia, synthesis of acute phase proteins, and leukocytosis. The soluble factors that mediate the inflammatory response can be divided into four main categories: (i) a group of cell-derived polypeptides, known as cytokines, which orchestrate the inflammatory response, e.g., they are major determinants of the makeup of the cellular infiltrate, the state of cellular activation, and the systemic responses to inflammation; (ii) cascades of soluble proteases/substrates (complement and kinins), which generate various pro-inflammatory peptides; (iii) vasoactive substances such as histamine, which induce vasodilatation and increase vascular permeability; and (iiii) inflammatory lipid metabolites such as platelet-activating factor (PAF) and numerous derivatives of arachidonic acid (e.g., prostaglandins and leukotrienes), which are generated from cellular phospholipids.

Table 1 Involved mediators in inflammatory response following SE

Inflammatory mediators	Main source	Actions	Incriminated scorpion species	References
Pro-inflammatory cytokines	Macrophages and APC monocytes, activated Th2 cells, macrophages, other somatic cells	Initiation of inflammatory response	Ts, Aah, Aam, Lqq, Td, Cn	Magalhães et al. (1999), Petricevich and Peña (2002), Fukuhara et al. (2003), D'Suze et al. (2003), Hammoudi-Triki et al. (2004), Adi-Bessalem et al. (2008), Raouraoua-Boukari et al. (2012), Aït-Lounis and Laraba-Djebari (2012), Saidi et al. (2013)
IL-1		Pyrogenic, synthesis and release of acute phase proteins, PAF production, chemokine production		
TNF-α				
IL-6				
Anti-inflammatory cytokines	Monocytes/ macrophages, dendritic cells, lymphocytes	Inhibition of antigen presentation, inhibition of T cell proliferation, attenuation of inflammatory gene expression in multiple cell types	Ts, Aah, Cn	Magalhães et al. (1999), Pessini et al (2006), Petricevich et al. (2007), Adi-Bessalem et al. (2008), Raouraoua-Boukari et al. (2012), Zoccal et al. (2013)
IL-10				
IL-4				
IL-1ra				
Phospholipid metabolites	Mast cells and leukocytes	Vasodilatation/vasoconstriction	Ts, MBT	Ferreira et al. (1993), Kanoo and Deshpande (2008), Zoccal et al. (2013)
Prostaglandins		Increased vascular permeability		
Leukotrienes		Chemotaxis, leukocyte adhesion, bronchoconstriction		
PAF				
Thromboxane				
Biogenic amines	Mast cells	Vasodilatation	Ts, BmK, MBT	De Matos et al. (2001), Liu et al. (2007), Dutta and Deshpande (2011)
Histamine		Increased vascular permeability		
		Bronchoconstriction		

(*continued*)

Complement activation	Plasma proteins	Increased vascular permeability	Aah, Ts	Bertazzi et al. (2003, 2005), Adi-Bessalem et al. (2008)
Protein fragments		Leukocyte chemotaxis and activation		
		Release histamine		
Free radicals	Leukocytes	Cytotoxicity, tissue damage	Lqq, Aah, Ts, Bot	Petricevich and Peña (2002), Dousset et al. (2005), Sahnoun et al. (2007), Zoccal et al. (2011), Adi-Bessalem et al. (2012), Raouraoua-Boukari et al. (2012)
Superoxide anion, H_2O_2, hydroxyl radical		Vasoconstriction		
NO	Macrophages, endothelium	Vasodilatation/cytotoxicity		
Proteolytic enzymes	Neutrophils	Cytotoxicity	Aah, Aam, Ts	Coelho et al. (2007), Adi-Bessalem et al. (2012), Raouraoua-Boukari et al. (2012), Saidi et al. (2013)
Metalloproteinase, elastin, myeloperoxidase Cationic enzymes, eosinophil peroxidase, major basic protein	Eosinophils			
Kinin–kallikrein system	Plasma	Vasodilatation, plasma extravasation, cell migration, inflammatory cell activation and inflammatory-mediated pain responses, stimulate release of histamine	Lqq, Ts, MBT	Fukuhara et al. (2004), Pessini et al. (2008), Kanoo and Deshpande (2008), Dutta and Deshpande (2011)

Species abbreviations: Aah: *Androctonus australis*, Aam: *Androctonus amoreuxi*, BmK: *Mesobuthus martensi*, Bot: *Buthus occitanus tunetanus*, Cn: *Centruroides noxius*, Lqq: *Leiurus quinquestriatus*, MBT: *Mesobuthus tamulus*, Td: *Tityus discrepans*, Ts: *Tityus serrulatus*

Pro-inflammatory Cytokines and Chemokines

Cytokines are involved in extensive networks that involve synergistic as well as antagonistic interactions and exhibit both pro- and anti-inflammatory effects on various target cells (Fig. 1). Imbalance of pro- and anti-inflammatory cytokines contributes to shock and multiple organ failure which can be fatal (Sofer et al. 1996; Petricevich et al. 2007).

Some pathophysiological manifestations of envenomation may be mediated by direct action of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IFN-γ, and IL-8) or mediators released indirectly by these cytokines (Fukuhara et al. 2003; Hammoudi-Triki et al. 2004; Adi-Bessalem et al. 2008). All of these mediators correlate with the severity following envenomation. Cytokine release has been reported to be more pronounced in severe rather than in mild envenoming cases (Meki and Mohey El-Dean 1998), suggesting that the intensity of inflammatory response is dependent on venom concentration found in the body.

The first mediators involved during inflammatory response are TNF-α and IL-1, they affect directly organ functions or indirectly through the production of lipid mediators, proteolytic enzymes, and free radicals (Fukuhara et al. 2003; D'Suze et al. 2003; Petricevich 2006; Adi-Bessalem et al. 2008; Aït-Lounis and Laraba-Djebari 2012; Saidi et al. 2013). Interleukin-6 (IL-6) level was also increased after accidental or experimental SE, and it was positively correlated with the production of acute phase reactant proteins including *α1*-antitrypsin (α1-AT) (Meki and Mohey El-Dean 1998; Magalhães et al. 1999; Fukuhara et al. 2003; Petricevich and Peña 2002; Hammoudi-Triki et al. 2004; Adi-Bessalem et al. 2008; Zoccal et al. 2011). This cytokine exerts its effects on multiple cell types and can stimulate the liver to secrete acute phase proteins, activate B lymphocytes to produce immunoglobulin, promote neutrophil mobilization from the bone marrow, and in concert with IL-1 causes T cell activation.

In severe clinical cases of SE, it appears that IL-6 and TNF-α increase in serum and correlate with the severe clinical symptoms (Sofer et al. 1996; Fukuhara et al. 2003; Hammoudi-Triki et al. 2004; Zoccal et al. 2011). As mentioned above, TNF-α induces the production of several pro-inflammatory factors such as prostaglandins, leukotrienes, PAF, proteases, and free radicals (Fukuhara et al. 2003). These factors are powerful spasmogens (bronchoconstriction) and vasoactive and they also promote systemic inflammatory response syndrome causing cardiac dysfunction and respiratory failure (D'Suze et al. 2003; Fukuhara et al. 2004).

Release of IFN-γ from peritoneal macrophages is also observed after envenomation by *Tityus serrulatus* scorpion venom (TsV) and its toxins Ts1 or Ts6 (Petricevich et al. 2007; Zoccal et al. 2013). This pleiotropic cytokine is involved in multiple pathways of immune response, including activation of phagocytic cells to produce effectors such as nitrogen derivatives in macrophages. The production of IFN-γ is inhibited by IL-4 and IL-10 cytokines.

Chemokines participate also in immune and inflammatory responses through the chemoattraction and activation of immune cells such as monocytes, lymphocytes,

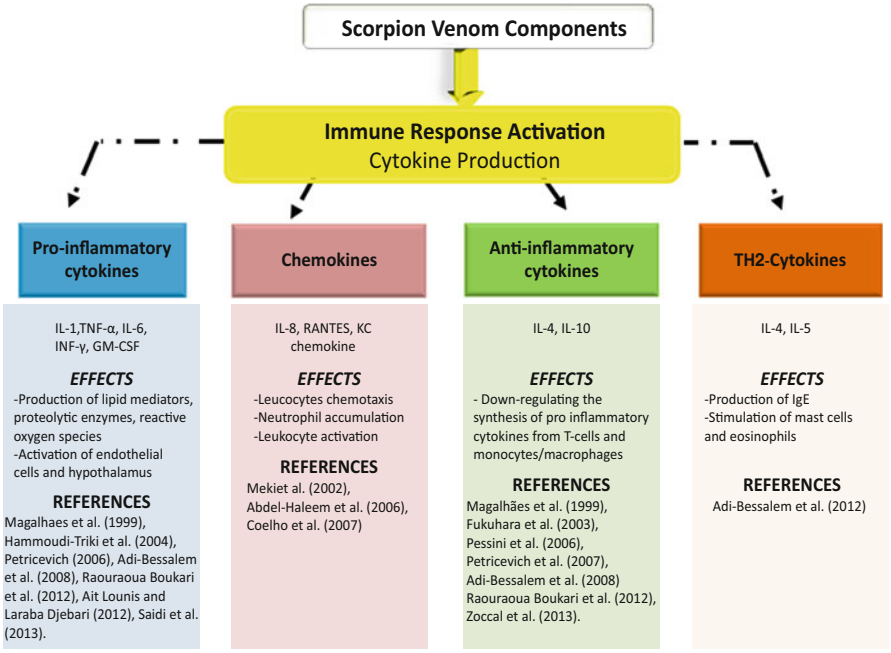

Fig. 1 Inflammatory cytokine release following SE. The inflammatory response protects the organism from the excessive production of mediators by suppressing immune cell functions under pathological conditions. Some immune cells may contribute to pro-inflammatory or anti-inflammatory cytokine production

neutrophils, and eosinophils (Fig. 1). Among inflammatory mediators shown to activate neutrophils and induce their recruitment in vivo after SE, much interest has been placed on the role of IL-8 or KC chemokines (CXC chemokine family), respectively, in humans and murine models (Meki et al. 2002; Coelho et al. 2007). Indeed, high levels of IL-8 were observed in plasma from patients at different grade of SE severity (Meki et al. 2002). In addition to its chemoattraction function, IL-8 also stimulates NO production by macrophages, endothelial cells, and smooth muscle cells. The formation of free radicals due to cellular stress can induce increase of membrane permeability and myocardial injury (Meki et al. 2002). RANTES (CC chemokine family) is among the important chemokines involved in the pathogenesis of SE and is correlated with its severity (Abdel-Haleem et al. 2006). Upon injury, resident macrophages release IL-1 and TNF-α, which in turn promote the release of RANTES by the activated T lymphocytes, giving this chemokine a special role in the maintenance and prolongation of an immune response (Krensky 1999).

Anti-inflammatory Cytokines

Overall, the inflammatory response protects the organism from the excessive production of mediators by suppressing immune cell functions under various

pathological conditions. Some immune cells may contribute to anti-inflammatory cytokine production such as IL-1ra, IL-4, IL-10, and TGF-β (Fig. 1).

IL-10 and IL-4 regulatory cytokines were increased in animals exposed to *Tityus serrulatus* (Ts) or *Androctonus australis* (Aah) or *Centruroides noxius* (Cn) venoms (Magalhães et al. 1999; Petricevich 2006; Petricevich et al. 2007; Adi-Bessalem et al. 2008; Zoccal et al. 2013). The induced inflammatory response after envenomation also depends on the components of the scorpion venom. In the same venom, toxins may exert pro-inflammatory or anti-inflammatory effects. TsV and its toxins Ts1 and Ts6 stimulated the production of NO, IL-6, and TNF-α in a macrophage cell line (J774.1), which were enhanced under lipopolysaccharide co-stimulation. However, in the presence of lipopolysaccharide, Ts2 toxin inhibited NO, IL-6, and TNF-α production and stimulated alone the production of IL-10, suggesting an anti-inflammatory activity by this toxin (Zoccal et al. 2011).

High concentration of IL-10 was also observed in the plasma of patients with both moderate and severe cases of envenomation. In addition to reduce the production of pro-inflammatory cytokines (IL-1 β, TNF-α, and IL-6), this cytokine causes also a decrease in expression of enzymes involved in the inflammatory mediator release (cyclooxygenase and NO synthase) and inhibits cytokine production by Th1 cells. Therefore, it allows to downregulate the exacerbated inflammatory process and the reduction of the alteration intensity (Fukuhara et al. 2003; Petricevich 2010).

IL-10 is both an anti-inflammatory and a regulatory cytokine; its main targets on immune cells are antigen-presenting cells and lymphocytes. On the one hand, IL-10 inhibited the function of dendritic cells by downregulating IL-12 production and expression of major histocompatibility complex class II and costimulatory molecules. On the other hand, IL-10 promoted the development of Th2 cytokine pattern by inhibiting the IFN-γ production of T lymphocytes, consequently impairing cellular immune responses, and regulates Th1/Th2 imbalance.

Thus, the balance between pro- and anti-inflammatory cytokines following SE determines the degree and extent of inflammation, which is associated with the major clinical effects of scorpion venom such as cardiac dysfunction, pulmonary edema, and shock (Fukuhara et al. 2003; Petricevich 2010). SE can induce increased cytokine concentrations and other cell mediators in a similar pattern of systemic inflammatory response syndrome (Fig. 2). The persistence of increased cytokine concentrations reflects the body's inability to downregulate their production or increase their metabolism and at the same time indicates the importance to follow up cytokines to correlate them with the severity and outcome of envenomation.

Complement System and Histamine

Complement system activation may contribute to tissue damage and pulmonary edema observed during SE (Bertazzi et al. 2003, 2005; Adi-Bessalem et al. 2008). Some products of complement activation have potent biological activity, including the ability to increase vascular permeability and to trigger mast cells to release histamine, resulting in vasodilation, the promotion of chemotaxis of inflammatory

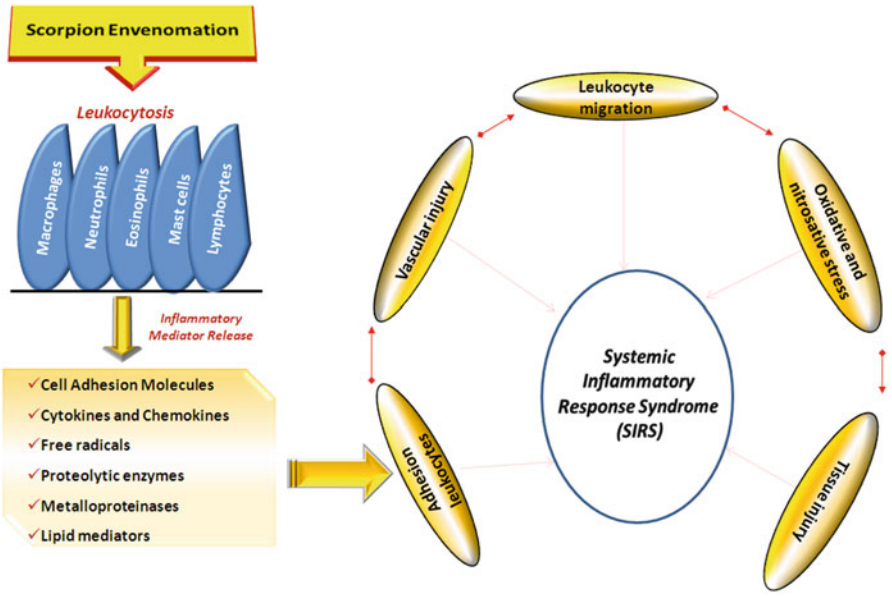

Fig. 2 Involvement of the inflammatory cells in the systemic inflammatory response syndrome (SIRS) induced after SE. Activation of different types of inflammatory cells chemoattracted to the tissue by a signaling network involving a number of growth factors, cytokines, and chemokines can lead to tissue damage and development of systemic inflammatory response syndrome

cells into the site of injury, and induction of bronchoconstriction. The increased vascular permeability may exacerbate the pulmonary edema and cardiac alterations that are mainly responsible for the lethality of SE (Bertazzi et al. 2005).

Two important mediators of the inflammatory reaction, C3a and C5a, are produced as a consequence of complement system activation. C5a fragment may further amplify the inflammatory response by inducing production of macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemoattractant (CINC), monocyte chemoattractant protein (MCP)-1, and pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) (Czermak et al. 1999).

Ts and Aah venoms may induce an increased level of components of complement system as evidenced by the increase of serum lytic activity. This activity can be observed rapidly following envenomation which could be explained by a higher concentration of complement system components in consequence to the dehydration, tissue damage, or even direct action of the venom (Bertazzi et al. 2003; Adi-Bessalem et al. 2008). TsV is able to activate in vitro the classical (CP)/Lectin (LP) complement pathway; the effect on the alternative pathway could be indirect (through the amplification loop by C3b formation via the CP/LP) or direct (through C3 cleavage by venom components) (Bertazzi et al. 2005).

Activated complement components (C3a and C5a) and lysosomal proteins released from neutrophils are responsible of histamine release from mast cells (De-Matos et al. 2001; Liu et al. 2007). Histamine, a vasoactive mediator, is

involved in the initiation of the inflammatory response. This biogenic amine found in *Palamneus gravimanus* scorpion venom (Ismail et al. 1975) or released from mast cells is an important mediator of acute inflammation and also a potent vasodilator. Its involvement during inflammation may be related to its ability to increase vascular permeability and edema after SE (Liu et al. 2007). Indeed, rat pretreatment with a mast cell-depleting agent protected the animals from TsV induced toxicity and prevented the pulmonary edema (De-Matos et al. 2001). Degranulation of mast cells and histamine release were also involved in rat pain-related behaviors and paw edema induced by *Mesobuthus martensii* scorpion venom (Liu et al. 2007).

Lipid Mediators and Kinin system

Arachidonic acid metabolites prostaglandin E_2 (PGE2) and leukotriene B4 (LTB_4) may modulate inflammatory response induced by scorpion venoms (Teixeira et al. 1997; Fukuhara et al. 2004; Kanoo and Deshpande 2008). PGE2 mediates vasodilatation, increases vascular permeability, and enhances pain perception by bradykinin and histamine, and neutrophil chemotaxis. LTB_4 causes the accumulation of inflammatory cells in the inflamed sites and degranulation of polymorphonuclear leukocytes (Teixeira et al. 1997; Pessini et al. 2006; Zoccal et al. 2013). These mediators may contribute also for the scorpion venom-induced lung edema. The generation of pulmonary edema may be due to the altered permeability of alveolocapillary membrane induced by PLA2–prostaglandin pathway that is triggered by B2 kinin receptors (De Matos et al. 2001; Kanoo and Deshpande 2008).

Clinical and experimental studies support the participation of kinin system in almost all phases of the inflammatory process observed after SE (Teixeira et al. 1997; Fatani et al. 1998; Fukuhara et al. 2004). Increased levels of kinins may contribute to local pain, vasodilatation, and vascular permeability changes; promote polymorphonuclear cell migration; and induce the production of mediators such as cytokines, reactive oxygen species, prostaglandins, and leukotrienes (Ferreira et al. 1993; Teixeira et al. 1997). The pharmacological actions of kinins are mediated mainly through B2 receptors, which are also located on the external surface of the leukocytes such as neutrophils (Teixeira et al. 1997). Further, the used of kallikrein inhibitor or a B2 bradykinin antagonist in animals treated with Ts or *Leiurus quinquestriatus quinquestriatus* (Lqq) venoms significantly increases the survival of animals, attenuating the venom-induced effects on the cardiovascular system and blocked the pulmonary edema (Fukuhara et al. 2004). The kinin system is also involved in the mechanical hypernociception, flinches, and paw edema observed after the injection of scorpion venom (Pessini et al. 2008).

PAF is a potent inflammatory mediator with a wide range of biological activities; it is derived from cell membrane phospholipids by the action of PLA_2. Biosynthesis of PAF, by mast cells and neutrophils, activates alveolar macrophages and eosinophils, induces platelet aggregation, and stimulates platelets to release vasoactive amines and thromboxanes synthesis (Borges et al. 2000). This lipid mediator is also a potent chemoattractant of neutrophils and increases vascular permeability, and it

is involved in the genesis of pulmonary edema observed after SE (Matos et al. 1999; Borges et al. 2000; Coelho et al. 2007). However, various studies demonstrate that other inflammatory mediators, in addition to PAF, appear to be involved in the development of lung injury.

Inflammation and Cell-Mediated Immune Response

Scorpion venoms induced leukocyte mobilization and recruitment in various tissues such as the lung, liver, heart, pancreas, adipose tissue, and kidney (D'Suze et al. 2004; Adi-Bessalem et al. 2008; Sami-Merah et al. 2008; Aït-Lounis and Laraba-Djebari 2012). As the inflammation progresses, different types of inflammatory cells are activated and chemoattracted to the tissue by a signaling network involving a number of growth factors, cytokines, and chemokines, which lead to tissue damage and development of systemic inflammatory response (Fig. 2). At the very early stage of inflammation, neutrophils are the first cells to migrate to the inflammatory sites under the regulation of molecules produced by macrophages and mast cells resident in tissues.

Neutrophils and Macrophages

Drastic blood neutrophilia is an important feature of the severe SE and may contribute to the development of tissue injury (Borges et al. 2000; Coelho et al. 2007; Adi-Bessalem et al. 2008). Experimental studies showed that *T. discrepans*, Ts, Lqq, and Aah venoms induce neutrophil infiltration and accumulation in different tissues or in the peritoneal cavity, which can be caused by the massive release of neurotransmitters, mainly catecholamines (Borges et al. 2000; D'Suze et al. 2004; Vasconcelos et al. 2005; Adi-Bessalem et al. 2008; Sami-Merah et al. 2008; Zoccal et al. 2011, 2013).

The influx of neutrophils in tissue is also dependent on the activation of PAF receptor (PAFR) and on PAFR-dependent production of the chemokine KC and activation of CXCR2 (CXC chemokine receptors) on neutrophils (Borges et al. 2000; Coelho et al. 2007). There are several chemotactic signals that have the potential for neutrophil recruitment following scorpion envenoming, including LTB_4, IL-8, and related CXC chemokines. These mediators may be derived from macrophages and epithelial cells, but the neutrophil itself is a major source of IL-8. Thus, adherent neutrophils produce and release oxygen free radicals and enzymes, such as proteases (e.g., elastase and metalloproteinases) and myeloperoxidase (MPO), exacerbating damage to endothelial cells and inducing tissue injury after SE (Coelho et al. 2007; Adi-Bessalem et al. 2012; Raouraoua-Boukari et al. 2012). Polymorphonuclear activation and migration is a result of a cascade of cellular events, in which polymorphonuclear and endothelial cells and macrophages act in concert (Fig. 2).

Macrophages have been shown to be involved in different homeostatic mechanisms and pathological events and may be engaged in complex interactions of cellular organisms. In response to scorpion venom, macrophages secrete mediators

such as NO and inflammatory cytokines (Petricevich et al. 2007). Depending on the microenvironment, macrophages can acquire distinct functional phenotypes. The phenotypes exhibited by tissue macrophages correspond to a M1–M2 polarization state: M1 cells are defined as activated pro-inflammatory macrophages and M2 cells comprise an anti-inflammatory macrophage population. After Aah envenomation, M1-like macrophages accumulation and inflammation accompanied by insulin resistance in adipose tissue are observed in envenomed animals (Aït-Lounis and Laraba-Djebari 2012). Peritoneal cell line macrophages (J774.1) exposed to TsV fraction or to its toxins Ts1 and Ts6 induced release of high levels of pro-inflammatory mediators, reactive oxygen species, and cytokines such as TNF-α, IFN-γ, IL-6, IL-1β, and IL-10 (Petricevich et al. 2007; Zoccal et al. 2011). Ts1 has an important immunomodulatory effect on macrophages because it induced an altered balance of inflammatory cytokines (Petricevich et al. 2007).

Mast Cells and Eosinophils

Mast cells located closely to peripheral nerve endings and associated with blood vessels are considered as important effectors in immunity. These cells are able to release potent inflammatory mediators (histamine, kinin, proteases, chemotactic factors, cytokines, and metabolites of arachidonic acid) that act on vasculature, smooth muscle, connective tissue, mucous glands, and inflammatory cells. Mast cells are implicated in scorpion venom-induced lung edema and nociceptive response (De-Matos et al. 2001; Liu et al. 2007). Activated mast cells can release many mediators contributing to pain responses. There are mainly two effects of mast cell-derived mediators to be recognized: (i) peripheral nociceptors are activated and/or sensitized and (ii) leukocytes are recruited to release algesic substances (Liu et al. 2007).

The important role for mast cells in mediating lung edema responses following administration of TsV has been demonstrated using animals depleted of mast cells. TsV venom induced less pulmonary edema and animals survived longer, suggesting that degranulation of mast cells is an important event induced by scorpion venom (De Matos et al. 2001). There are two possibilities to explain the mechanisms underlying scorpion venom-induced mast cell degranulation. One possibility is that degranulation of mast cells is driven by some neuroendocrine factors such as substance P released from sensory nerve fiber terminals activated by scorpion venom; the other possibility is that degranulation of mast cells is triggered directly by scorpion venom (Matos et al. 1999; Liu et al. 2007). Other possibility is that scorpion venom enhances the synthesis of kinins; kinins activate the mast cells to release histamine to bring about the pulmonary edema (Dutta and Deshpande 2011).

Eosinophils as mast cells are important inflammatory cells observed after SE. Eosinophils can regulate local immune and inflammatory responses, and their accumulation in the blood and tissue is associated with severe inflammation. Immediate hypersensitivity reaction to scorpion venoms associated with pulmonary infiltration and blood eosinophilia has been observed in experimental and accidental envenomation (Shah et al. 1989; Adi-Bessalem et al. 2012). After envenoming

with Aah venom or its toxins, eosinophils stimulated via IL-5 cytokine and complement fractions (C3a and C5a) leads to the release of inflammatory mediators (leukotrienes, PAF) and basic proteins such as eosinophil peroxidase (EPO) (Adi-Bessalem et al. 2012). Eosinophil peroxidase catalyzes the formation of oxygen free radicals (O^-) or bromide (Br^-). The presence of highly unstable oxidizing free radicals can explain the cytotoxic activity observed in lung tissue after SE.

Lymphocytes

Lymphocytes orchestrate immune response in envenomation, mainly the production of TNF-α that exerts its pro-inflammatory effects through increased production of IL-1β and IL-6, expression of adhesion molecules, proliferation of fibroblasts, and procoagulant factors, as well as initiation of cytotoxic, apoptotic, and acute phase responses. Interaction of lymphocytes with antigen-presenting cells is required for activation of T cells. These cells are involved in signaling pathways that control cell proliferation and synthesis of IL-2 (Cahalan and Chandy 1997).

Otherwise, ion channels expressed by T cell play key roles in the control of the membrane potential and calcium signaling, thereby affecting signal transduction pathways that lead to the activation of these cells following antigenic stimulation. Indeed, ion channels modulators have now been shown to be effective in suppressing T cell function and may provide excellent pharmaceutical targets for modulating immune system function. The activity of ion channel can be modulated by direct block, phosphorylation or some other posttranslational modification of the protein, or by altered levels of expression. The voltage-gated K^+ channel encoded by Kv1.3 has been subjected to scrutiny, with the effectiveness of highly selective peptide toxins in an in vivo model proving that channel blocking can suppress the immune response (Cahalan and Chandy 1997).

To better understand the immune response induced by scorpion venom, it is necessary to explore more extensively the Th1, Th2, and Th17 pathways.

Oxidative and Nitrosative Stress

Oxidative stress originates mainly in mitochondria from reactive oxygen and nitrogen species and can be identified in most of the key steps in the pathophysiology of SE (Dousset et al. 2005; Sahnoun et al. 2007; Raouraoua-Boukari et al. 2012).

Oxidative stress parameters revealed that scorpion venoms such as Lqq, Aah, and *Buthus occitanus tunetanus* (Bot) significantly increased the level of lipid peroxidative damage in cardiac tissues and reduced the activity of antioxidant enzymes (Dousset et al. 2005; Sahnoun et al. 2007; El-Alfy et al. 2008). High production of O_2 occurring during oxidative stress compromises the natural antioxidant defenses of cells. The oxidant and antioxidant balance is an important determinant of immune cell function, not only for maintaining the integrity and functionality of membrane lipids, cellular proteins, and nucleic acids but also for the control of

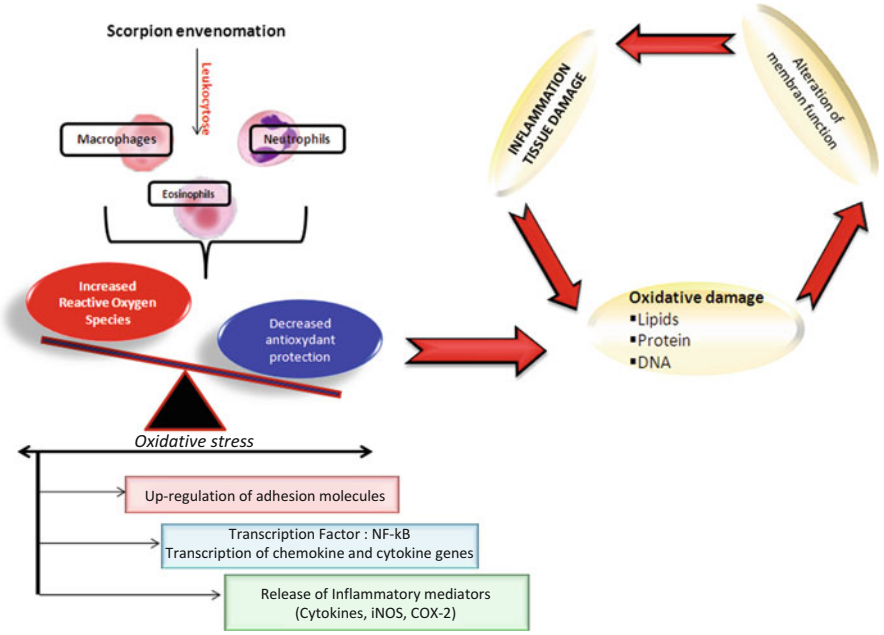

Fig. 3 Imbalance and oxidative stress following SE. Imbalanced defense mechanism of antioxidants and overproduction of free radicals lead to oxidative damage of biomolecules (lipids, proteins, DNA), to alteration of the integrity and functionality of membrane lipids, and to tissue damage (COX-2: cyclooxygenase-2, iNOS: inducible nitric oxide synthase, NF-κB: Nuclear factor kappa-B)

signal transduction and gene expression in immune cells (Petricevich 2010). Reactive oxygen species from inflammatory cells (particularly macrophages and neutrophils) result in several damaging effects including decreased antiprotease defenses such as *α1*-antitrypsin (α1-AT) and secretory leukoprotease inhibitor (SLPI) and activation of NF-κB resulting in increased secretion of the pro-inflammatory cytokines (Fig. 3).

In addition, inflammatory cells produce reactive oxygen species that react with NO to form NO-derived inflammatory oxidants that damage tissues. NO plays a role in immunity and inflammation but also in neurotransmission, muscle relaxation, and vasodilatation. An increase of NO levels in the serum or in peritoneal macrophage culture correlated with production of pro-inflammatory cytokines has been shown after experimental and accidental SE (Meki and Mohey El-Dean 1998; Petricevich and Peña 2002; Pessini et al. 2006; Zoccal et al. 2011; Raouraoua-Boukari et al. 2012). These high concentration of NO may be associated with severe conditions, such as septic shock, hypertension, febrile response, and severe SE (Petricevich and Peña 2002; Pessini et al. 2006; Petricevich 2010).

The NO effects on the inflammatory response are concentration dependent (Petricevich and Peña 2002). Under physiological conditions, NO is produced in small amounts, maintaining the integrity and function of the membrane and participates in neurotransmission, regulating gene expression in immune cells. The

cytotoxic functions of this molecule appear after NO release in high amounts by macrophages and other cells.

The significant high values of NO in the envenomed victims can be explained by the effect of released cytokines, especially IL-1β, on the activation of the inducible isoform of NO synthase enzyme on L-arginine amino acid in the endothelial cells (Meki and Mohey El-Dean 1998). NO synthesis may be also increased by the enhancement of the constitutive isoform of NO synthases by the venom-released acetylcholine, cytokines, and/or bradykinin which has been proven to be released after scorpion stings (Fatani et al. 1998; Fukuhara et al. 2004).

Immunopathology of Scorpion Envenomation and Mode of Action of Scorpion Venom

The specific signs of SE are directly related to the venom components which induce a systemic immune response and multiple organ failure (Fig. 4). Multiple organ failure is considered to be due, in part, to prolonged and excessive activation of inflammatory pathways. The mechanisms by which scorpion venom induces immune response are not completely known. The first event of scorpion envenomation is the recognition of ionic channels by specific neurotoxins. The depolarization caused by the recognition and binding of toxin to its receptor triggers a cascade of events leading to synthesis and secretion of neuroimmune mediators (catecholamines, acetylcholine, cytokines, lipid mediators, free radicals), which in turn cause a variety of secondary manifestations, that can lead to the death of the victims (Ismail 1995; De-Matos et al. 2001; Petricevich et al. 2007) (Fig. 4).

Neurotoxins could affect indirectly the immune system following their binding to sodium channels of neuronal terminals and may lead to depolarization of axonal membrane and release of neuropeptides and neurotransmitters, and these agents proved to induce release of immunological mediators such as cytokines and others (Petricevich 2006). In addition, the activation of the sympathetic nervous system appears to be able to modulate Th1 and Th2 cytokine profiles (Elenkov et al. 1996).

The mechanism involved in the activation of immune cells by scorpion venom could be also explained by the binding of substance P released by nerve cells after their depolarization induced by the neurotoxins of the venom to the tachykinin-1 (NK1) receptors present on inflammatory cells (Matos et al. 1999). There is much evidence demonstrating that substance P can induce the release of inflammatory mediators from several leukocytes, including mast cells and macrophages (Matos et al. 1999), and other cell types, including epithelial cells and fibroblasts (Koyama et al. 1998).This substance may also have a direct effect on the endothelium and alter vascular permeability (De-Matos et al. 2001; Fukuhara et al. 2003).

A high leukocyte level observed after SE is due in one hand to the action of catecholamines, released in response to the scorpion's venom, that are known to induce leukocytosis through the mobilization of marginated cells (Magalhaes

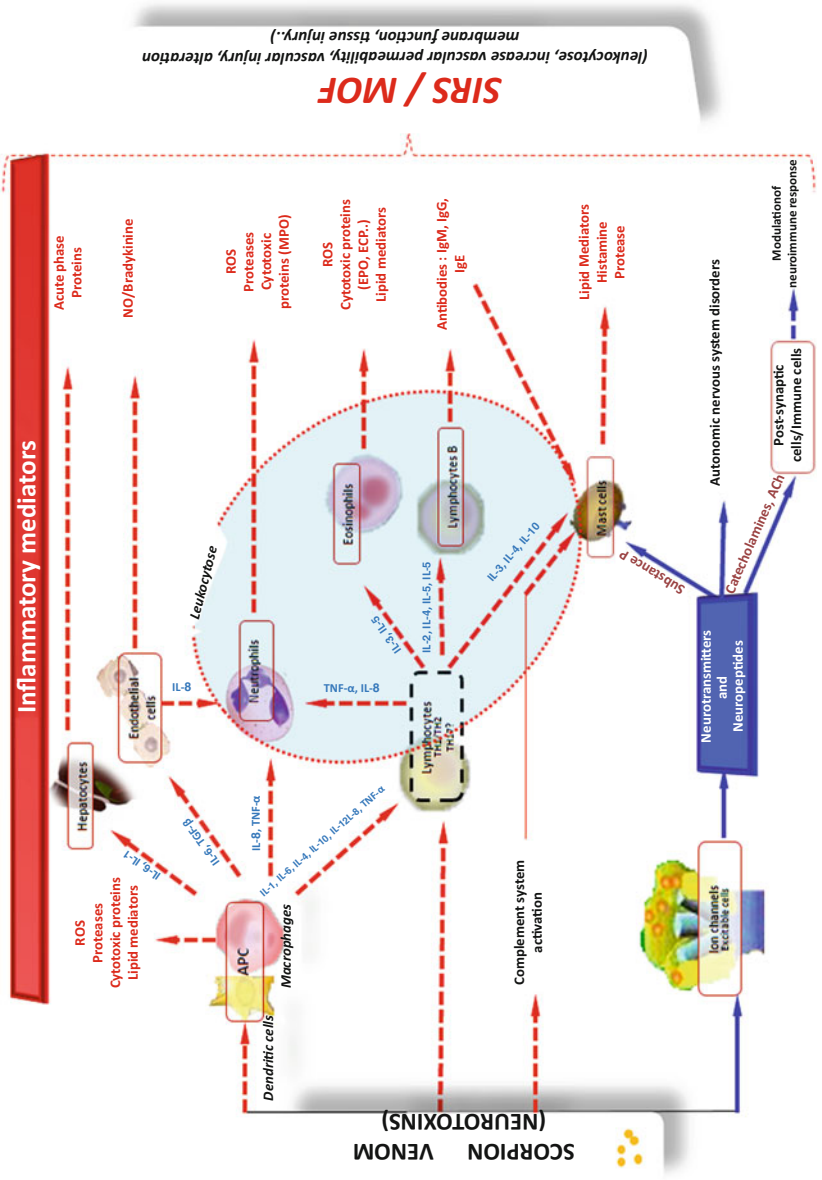

Fig. 4 (continued)

et al. 1999; Borges et al. 2000) and in the other hand by the hematopoietic stimulatory effects of IL-6. The association of leukocytosis and the severity of envenomation can be explained by the fact that the products of these activated leukocytes such as oxygen free radical molecules may further impair the oxygen delivery by their effects on the microcirculation which can induce multiple organ failure (Fig. 4).

Another plausible mechanism is the direct action of scorpion toxins on the ion channels of immune cells, particularly voltage-gated potassium channels Kv1.3 type. It is well known that ion channels play an important modulatory role in the activation of macrophages and lymphocytes, participating in the release of cytokines, proliferation, mitogenesis, elimination of target cells, adhesion, chemotaxis, and activation of inflammatory transcription factors. A major function of Kv1.3 and Ca^{2+}-dependent potassium channels KCa3.1 is to maintain the negative membrane potential, which facilitates the maintenance of a Ca^{2+} signaling in the activation T cells (Cahalan and Chandy 1997).

Conclusion and Future Directions

Systemic and local inflammation with leukocytosis and increased levels of circulating pro-inflammatory mediators have been reported in the pathophysiological manifestations of human and experimental SE. The inflammatory mediators have complex effects, resulting in the recruitment of inflammatory cells from the circulation, bronchoconstriction, vascular changes, and tissue injury. Further studies are needed to determine the best therapeutic protocol that would take into account the mediators being released and how best to combat their deleterious effects.

The development of biomarkers can enable earlier diagnosis as well as help physicians in selecting and monitoring treatment. The identification of the key mediators in the pathogenesis of SE could be also useful for the optimization of therapeutic strategies including the use of more selective anti-inflammatory drugs and sodium channel blockers associated with immunotherapy (Fig. 5).

The end goal should be the ability to prevent multiple organ failure, thus saving peoples' lives by improving their chances of survival following severe SE.

Fig. 4 Potential mechanism by which scorpion venom induces systemic inflammatory response syndrome (SIRS) and multiple organ failure(MOF). Scorpion venom components may exert direct effect on cellular targets including excitable cells and immune cells; these activated cells release various neuroimmune mediators and modulators (neurotransmitters, cytokines, oxygen free radical molecules, lipid mediators, etc.), which in turn exacerbate and promote inflammation causing many immune-pathophysiological effects (*ACh* acetylcholine, *APC* antigen-presenting cell, *ECP* eosinophil cationic protein, *EPO* eosinophil peroxidase, *MOF* multiple organ failure, *MPO* myeloperoxidase, *NO* nitric oxide, *ROS* reactive oxygen species, *SIRS* systemic inflammatory response syndrome)

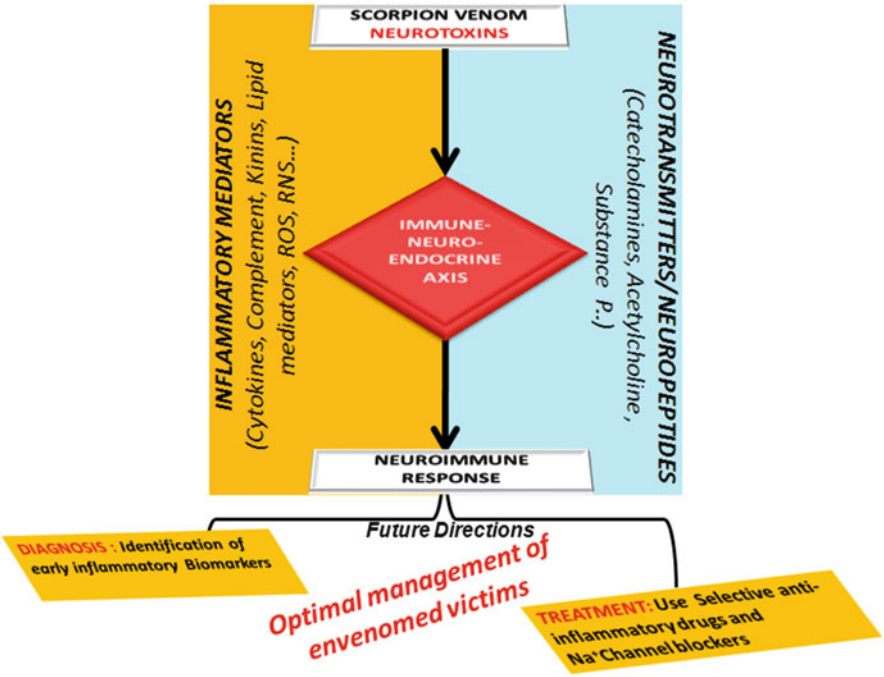

Fig. 5 Neuroimmune response following SE and future directions. The specific signs of SE are directly related to the venom components which induce a neuroimmune response. A better understanding of SE pathogenesis can improve the identification of the key mediators involved in the pathogenesis of SE and could be useful for the optimization of therapeutic strategies

Cross-References

- ▶ Recombinant Neutralizing Antibodies, A New Generation of Antivenoms
- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ New Insights on the Pharmacokinetics of Venoms and Antivenoms
- ▶ Scorpion Venoms: Pathogenesis and Biotherapies

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New Insights on the Pharmacokinetics of Venoms and Antivenoms

5

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Abstract

The development of enzyme-linked immunosorbent assays (ELISA) for venoms and antivenoms with high sensitivity has enabled to characterize pharmacokinetics (PK) of venoms and antivenoms, which in turn allowed modeling their absorption, distribution, and elimination, as well as the adequacy of different therapeutic regimes. Pharmacokinetics is the branch of pharmacology dealing with absorption, distribution, and elimination of drugs in the body; it is fundamental to determine the dose and dosing scheme of a drug. ELISA studies provided evidence indicating that antivenoms (in spite of their large molecular size) are quickly and actively extravasated from blood to tissues. ELISA studies have also enabled to show that heterologous antibodies induce production of antibodies able to interact with antivenoms, modifying their PK and reducing their effectiveness. This has been confirmed using high-resolution deconvolution fluorescence microscopy (HRDFM) of fluorescently labeled antivenoms. HRDFM has also provided evidence showing a complex distribution of antivenoms in the body and has shown that mammalian immunoglobulins (IgG) are transported very differently in the body than avian IgYs, which suggests they must have different PK.

Introduction

Pharmacokinetics (PK) is the branch of pharmacology dealing with absorption, distribution, and elimination of drugs in the body; it is fundamental to determine the dose and dosing scheme of a drug (Gibaldi and Perrier 1982). The amount of drug in the blood producing the effect desired (drug *absolute bioavailability*) depends on the mass (amount) of drug administered but also on the manner it is administered [oral (PO), subcutaneous (SQ), intravenous (IV), intramuscular (IM), etc.], the concentration being administrated, the mechanisms of destruction or elimination from the administration site, or the body in general. Proteinic medicines (such as antivenoms) are destroyed in the digestive tract; the same occurs with drugs which do not tolerate acidic or alkaline environments. Other drugs do not cross or cross in variable amounts through intestinal mucosa. Pharmaceutical formulation also affects bioavailability. Two, apparently equal, formulations of a drug may produce different bioavailabilities (see below), depending on where and how a pill disintegrates (Glazko et al. 1968; Koeleman and Van Oudtshoorn 1973), the kind of drug crystals in the tablets, particle's shape in a suspension, kind of food with which the drug is administered, etc.

There is a wealth of venom and antivenom PK studies [see Gutiérrez et al. (2003) for a review], most of them with methods such as radioisotopes with limited sensitivity or time resolution, especially at short times after venom or antivenom is administered (Ismail et al. 1980, 1983; Pentel et al. 1988; Ho et al. 1990, 42: pp. 260–266; Pépin et al. 1995; Ismail et al. 1996; Pepin-Covata et al. 1996a, b; Rivière et al. 1997, 1998; ; Ismail et al. 1998; Gutiérrez et al. 2003). The resolution needed for PK has been achieved after *enzyme-linked immunosorbent assays*

(*ELISA*) for venoms and antivenoms were devised (Theakston et al. 1977) and achieved high sensitivity (D'Suze et al. 2003; Vázquez et al. 2005). This chapter is not a general review on venom or antivenom pharmacokinetics, and it is limited in space and purpose to venom and antivenom PK for which quantitative information permitting modeling of basic and clinical situations is available; the chapter also includes a summary of recent evidence of direct visualization of fluorescently labeled antivenom distribution in the body using high-resolution deconvolution fluorescent microscopy (HRDFM). Since the chapter assumes no prior knowledge of PK concepts, some of this knowledge is described to target general audiences.

Preliminary Definitions

Molecule Concentrations

Avogadro (1811) made the interesting discovery that for any gas at a pressure and temperature previously defined, a mole always occupies the same volume. With atmospheric pressure at the sea level (1 bar) and a temperature of 0 °C, a mole of **any** gas occupies always the same volume: ≈22.7 l. This means that **no matter which gas** is considered, all gas molecules occupy the same volume. Another useful definition is the *concentration*. A concentration is defined as the amount of substance in a unit volume (such as the liter, abbreviated L). If "amount" is taken as number of molecules or moles, all gases, under the conditions defined above, would have the same concentration.

Counting molecules is difficult; thus, concentration is usually defined in terms of mass of substance in a given volume (e.g., grams in a liter).

Concentration is important since the higher it gets, the more likely it will be for molecules to meet and to react between them. In a living being this means that the higher the concentration of a drug or a toxin within the living being, the easier will it be for drug molecules to react with the body's molecules in a short time. Thus, the effect of the drug or toxin in the body increases with its concentration.

Dose is the name usually given to the amount of a drug or a toxin administered to a living creature. Since the volume of an organism is limited and more or less fixed, the drug's concentration in the body mainly depends on the dose administered. Effects which depend on the concentration a drug reaches in the body depend on the dose administered; such drugs are called *dose dependent*. To determine dose dependency, it is usually necessary to establish the *time of exposure* of the body to the drug. If the drug is administered in a (more or less) continuous manner, exposure time will depend fundamentally on the time during which drug is being administered. It will depend also, however, on the manner the drug disperses from the administration locus to the rest of the body and on how it is eliminated from the body. In some instances elimination may be rather passive, through urine, exhaled air, sweat or feces, etc. That is why breath changes in smell when drinking alcohol, or with foods such as garlic or onions, or urine changes in smell or color with certain foods (e.g., asparagus) or medicines.

Other molecules are not easily eliminated passively as mentioned in the preceding paragraph; such molecules are actively destroyed by the body, for example, in the liver. High MW molecules are not eliminated passively and must be destroyed or eliminated actively. The active destruction process is known as *catabolism* and may be quite complex.

Amino Acids, Proteins, and Peptides

One kind of molecule constituting living beings is the proteins. Proteins are usually chains of a special kind of molecules called amino acids (AA) linked together by a special junction called peptidic bond. Relatively small proteins (MW below ≈20 kDa) are called *peptides*; this definition is somewhat arbitrary. The structure of a peptide (61 AA) is shown in Fig. 1a. Proteins may be formed by groups of peptides called *subunits*. And proteins, especially big proteins, may have parts with special functions; they are called *functional domains*.

Characteristics of Natural Venom

This chapter is not a comprehensive review of venom mode of action. Still, mentioning some venoms in particular, and their PK characteristics, as venom prototypes, is necessary. The selection was made with the hope that it will assist the readers in extrapolating this general discussion to other particular cases of interest.

Potency and Units of Measurement of Natural Venom's Potency

Since molar doses are usually very small mole fractions, they are also expressed with an abbreviated nomenclature: 10^{-3} mol is abbreviated as 1 mmol, 10^{-6} mol would be 1 μmol, and so on. Tetrodotoxin, a paralytic toxin isolated from puffer fishes, exerts its action at 5 nM ($5 \cdot 10^{-9}$ mol/L) concentrations. Botulinum toxin kills half of the animals inoculated with 1 ng/kg (nanogram/kilogram) or 10^{-9} g/kg (Arnon et al. 2001), which is equivalent to 6 fmol/kg! Assuming that a human weights 70 kg on average, and given a world human population of 5,000 millions, 350 g (≈2 mmol), uniformly dosed to all humans, are enough to reduce humanity to half. No wonder that botulinum toxin has been considered a potential chemical weapon.

Although there might be some lack of consensus, the following definitions are used here. *Toxins* are natural substances, or substances produced by living organisms, in contrast to toxic substances from chemicals, which are toxicants. Living organisms producing or using toxins do so as either *venoms* or *poisons*. Venoms are toxins, or more commonly collections of varying toxins, that are used actively (biting, stinging, or by any other mechanism) against prey or predators, most

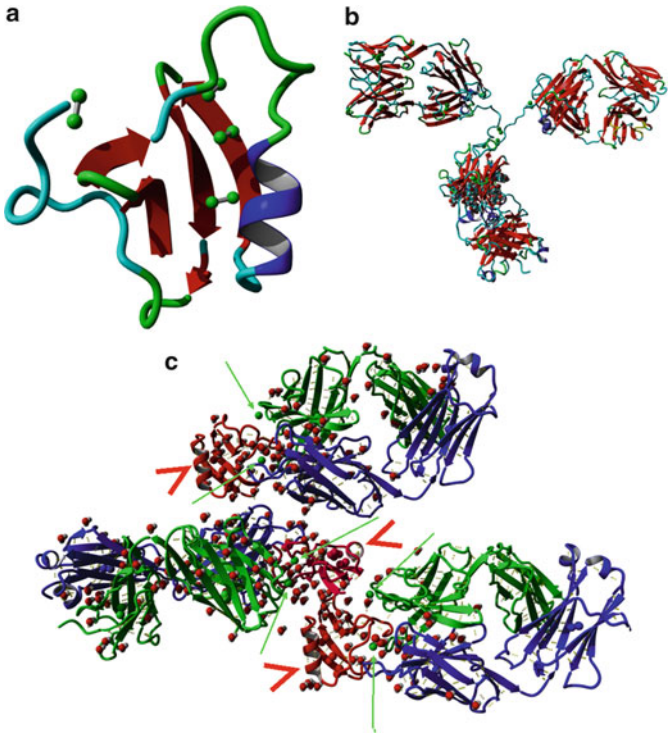

Fig. 1 (**a**) Bactridine 1 secondary and tertiary structure. Bactridine 1 is a toxin isolated from *Tityus discrepans* scorpion venom. The secondary and tertiary structure of bactridine 1, as shown here, is characteristic of scorpion toxins from the Americas, Africa, and Eurasia which modify the physiology of Na^+-channels; bactridine 1 is an antibacterial peptide (Díaz et al. 2009). Its MW is ≈7 kDa and its predicted charge is +1.4. In silico homology modeling with the Swiss-Model server (swissmodel.expasy.org) and Yasara (www.yasara.org) [see Díaz et al. (2009) for details] using template 1npi A (Pinheiro et al. 2003). (**b**) Visualization of recombinant human immunoglobulin Ga2 isoform. When the so-called Fc domain of IgG (*bottom*) is removed enzymatically with pepsin, the two top Fab domains remain together and form an $F(ab')_2$ fragment. If the molecule is hydrolyzed with papain, the two Fab domains are separated such as in Fab antivenoms. IgG2a MW ≈141 kDa and its predicted electric charge is +5.267; $F(ab')_2$ MW is ≈99 kDa and has a predicted charge of +2.464; and Fab MW is ≈50 kDa and has a predicted charge of +1.439. The visualization was built based on the Protein Data Bank 1igt atomic coordinates (Harris et al. 1997). (**c**) Crystal structure of the AaHII-Fab complex. This panel presents a complex formed by three Fab fragments, each of them bound to a molecule of the Na^+-channel-modulating toxin AaHII from the *Androctonus australis* scorpion venom. The molecule was color coded as follows: AaHII toxin molecules in red (pointed by *red arrowheads*), light chains of the 439Fab fragments in *green*, and heavy chain segments preserved in the Fab fragments in *blue*. Short *dotted lines* indicate H bonds. As shown, the highly stable toxin–antivenom complex involves water molecules, H bonds, and ionic interactions with two chloride ions (in *green*, pointed by *green arrows*); not shown are the Van der Waals–London bonds which also stabilize the toxin–antivenom complexes. The visualization was built based on the Protein Data Bank 4aei atomic coordinates (Fabrichny et al. 2012). Except for panel (**c**), the molecules are presented as secondary and tertiary structure sketches color coded as *red*, β sheaths; *dark blue*, α helices; *light blue*, chains without definite structure; and *green*, β turns. Cysteine sulfur atoms participating in disulfide bonds are presented as *green spheres*; these bonds join different parts of the bactridine 1, AsHII, and IgGa2 molecular chains

commonly to subdue, kill, and digest prey or dissuade predators. Poisons of natural origin, that is, containing toxins and used by living organisms, are passive and generally used for defense.

Toxins vary between 100 and 150.000 Da, but most of potent toxins have MW bellow 40 kDa. Toxins from elapid snakes (cobras and corals), invertebrates such as insects and arachnids, and marine animals such as jelly fishes, snails, and certain fishes are able to affect humans and in general have MW below 10 kDa. The largest toxins are bacterial proteins of some 150 kDa, for example, botulinum toxins A, B, C, D, E, and F produced by *Clostridium botulinum* and tetanic toxin or tetanospasmin produced by *Clostridium tetani*.

A More Detailed Consideration of Some Peptidic Venoms

Snake venoms are complex mixtures of peptides and proteins. Snakes dangerous to man belong to two families: Viperidae and Elapidae. The Viperidae family includes notoriously venomous genera such as *Agkistrodon* (cottonmouth, moccasin), *Atheris* (bush vipers), *Bitis* (puff adders), *Bothrops* (fer de lance, jararaca, macaurel, mapanare, nauyaca), *Bothriechis*, *Cerastes* (horned vipers), *Crotalus* (rattlesnakes), *Daboia* (Russell's viper), *Echis* (saw-scaled vipers, carpet vipers), *Lachesis* (bush-master), *Proatheris* (lowland viper), *Pseudocerastes* (false horned vipers), and *Vipera* (palearctic vipers). Their venoms are fundamentally necrotic and/or hemorrhagic, but some South American *Crotalus* also have neurotoxic peptides. The Elapidae family includes various venomous genera such as *Acanthophis* (death adders), *Bungarus* (banded krait), *Dendroaspis* (green and black mambas), *Enhydrina* (beaked sea snake, hook-nosed sea snake, common sea snake, Valakadyn sea snake), *Hydrophis* (Australian sea snakes), *Micruroides* (Sonoran coral snakes), *Micrurus* (American coral snakes), *Naja* (cobras), *Notechis* (tiger snake), *Ophiophagus* (king cobra), and *Pelamis* (yellow-bellied sea snake). Elapidae snakes are notoriously neurotoxic and are also myonecrotic and cardiotoxic. Independently from their taxonomic and toxic variability, venoms of these snakes are mixtures of peptides ranging 3–40 kDa. A classical and remarkable work by Australian researchers demonstrated that elapid venom peptides are absorbed via the *lymphatic system* [see White (1982) for a comprehensive revision of the Australian work]; this has recently been confirmed also for coral snake venom, including a partial pharmacokinetic analysis, by Paniagua et al. (2012).

Spiders dangerous to humans can be grouped also as neurotoxic and necrotic. Dangerous neurotoxic spiders include genera such as *Atrax*, *Hadronyche*, *Illawarra* (Australian funnel-web spiders), *Phoneutria* (South American armed spiders) and *Latrodectus* (black or brown widow spiders), and the severely necrotizing *Loxosceles* (recluse spiders). *Loxosceles* produces slowly progressing local necrotic lesions which may be mild or severe enough to demand reconstructive surgery and in extreme cases may kill the patients; all these effects are produced by an enzyme,

sphingomyelinase D, of 30 kDa (Tambourgi et al. 1998; Cunha et al. 2003; Binford et al. 2005). Except for α-*Latrotoxin* (MW 120 kDa), a protein from *Latrodectus* which produces massive release of neurotransmitters (Südhof 2001), spider venom effect is produced by peptides below 40 KDa.

The *Centruroides* scorpion genus can be found from the Pacific Ocean coastline in Colombia, Central America to North America (up to Arizona, Nuevo México, Texas, Colorado, and Southern California), and in the Caribbean Islands (from Cuba down south to the Los Roques archipelago in Venezuela). *Centruroides* scorpion venoms are uniquely neurotoxic, and some species (e.g., *C. margaritatus*, *C. sculpturatus*, *C. noxius*) may kill humans in less than 2 h (Dehesa-Davila and Possani 1994). Another life-threatening genus of interest for the subject of the chapter is *Tityus*. More than 60 species of *Tityus* are known to exist in Argentina, Brazil, Colombia, Trinidad–Tobago, and Venezuela. Their venom is neurotoxic, but it also induces an inflammatory reaction which manifests its maximum severity as acute pancreatitis, myocarditis, and a scorpionism respiratory distress syndrome (SRDS), sometimes called acute pulmonary edema (D'Suze et al. 1999). *Tityus* venom-induced respiratory distress syndrome is fatal as a rule. *Tityus* venom effect occurs more slowly than *Centruroides* venom effect; in severe cases death occurs in 8–24 h after envenoming. If specific antivenom is not administered IV early and in sufficient amount to neutralize the venom received, SRDS tends to become irreversible after some 8 h. Although peptides with MW above 10 kDa are known (D'Suze et al. 1997; Batista et al. 2006) in both *Centruroides* and *Tityus* venoms, toxins dangerous to humans have MWs between 3 and 8 kDa.

Caterpillars of two moth species, *Lonomia achelous*, in Venezuela, and *L. obliqua*, in Brazil, contain potent hemorrhagic toxins in their hemolymph; these toxins are inoculated through skin after brushing accidentally the spiny hairs in caterpillar's surface. *Lonomia* hemorrhagins have MWs ranging 20–40 kDa (Amarant et al. 1991).

Antivenom Characteristics

Two strategies have been used to eliminate or prevent the effect of natural venoms, vaccines and specific antitoxins (antivenins or antivenoms). Vaccines induce immunity against toxins by inducing specific antibodies in possible victims; for this purpose toxins are modified to eliminate toxicity preserving their immunogenicity, and these modified toxins are often called *toxoids*. Fractions of toxins containing immunogenic epitopes which are later expressed in microorganisms (to massify production) have also been successfully applied in some cases (Smith 2009). Antitoxins are purified antibodies, or antibody fragments, produced in animals with repeated sublethal toxin doses until developing high toxin immunity levels (the

animals are *hyperimmunized*) against a toxin or a venom. These antibodies or their fractions are administered to victims, not to induce antibody production but to produce *passive immunity* neutralizing the venom it has received.

On first sight, *vaccines* seem ideal to protect since this follows the preventive medicine ideal. A properly immunized subject does not have to worry about envenomation beyond the mechanical and psychical trauma (and possible infection or tetanus complication) of bites or stings. Still, vaccines pose their own risks which may not be trivial, which ought to be, but not always are, much less serious than being envenomed. In spite of the high risk once envenomed, from a sanitary viewpoint, it is not justified, economically or due to patient's safety, to vaccinate a population if the likelihood of envenoming is low. Furthermore, for many venoms, the vaccine does not exist and perhaps will never exist. Antitetanic vaccine, a very safe vaccine, is routinely used to protect against tetanus, a disease with 50 % mortality which is easily acquired after a closed penetrating (even trivial) wound or after anfractuous and massive lesions. An antibotulinic vaccine also exists (Smith 2009) but is not used, in the absence of the military risks of biochemical warfare or terrorism, since the average person's chance of intoxication is extremely low. Thus, antitoxins remain as the most common tool to treat intoxications and envenomations.

Modern antitoxins are immunoglobulins G (IgG) produced in hyperimmunized animals, highly purified and usually modified to avoid the dreaded *anaphylactic shock*. Figure 1a shows the structure of bactridine 1, a toxin isolated from the *Tityus discrepans* scorpion venom; bactridine 1 is an antibacterial peptide (Díaz et al. 2009). Its MW is ≈7 kDa and its predicted charge is +1.4. The structure resembles most of the so-called sodium channels toxins isolated from scorpions. Its tertiary structure contains three β-strands (in red) and α-helical part (in dark blue). Figure 1b shows the structure of a monoclonal human IgG2a visualization when the so-called Fc domain (lower part in the Fig. 1b) is removed enzymatically with pepsin; the remaining fragment called $F(ab')_2$ is a safe and efficient antivenom devoid of anaphylactic shock-inducing ability; cleaving with papain, a different enzyme, it is possible to produce Fab residues also able to neutralize venoms. IgG MW is 150 kDa, $F(ab')_2$ weights 110 kDa, and Fab has an MW of 55 kDa. Figure 1c shows a visualization of a complex of three Fab fragments, each of them bound to a molecule of the Na^+-channel-modulating toxin AaHII from the *Androctonus australis* scorpion venom. Figure 1c also illustrates an interesting feature of antigen-antibody interactions in general; although the interaction is extremely stable, it does not involve covalent bonds, and the stability is achieved through multiple weak interactions (H bonds, Van der Waals–London interactions, ionic bonds); in the case illustrated, water molecules and Cl^- ions complete the keylock fit between the molecules. The characteristics of the antigen-antibody interaction shown in Fig. 1c are also typical of the vast majority of drug–receptor interactions, including toxin–receptor binding.

From the preceding discussion follows that except for some large bacterial and spider toxins, IgG, $F(ab')_2$, and Fab have MW which may be ten times larger than the majority of most potent toxins (Fig. 1).

Dynamics of Molecules in Solution

When a substance (solute) is placed in a solvent at a temperature different from the so-called absolute zero (−273.15 °C), its molecules will move. The movement is possible since at any temperature above absolute zero, the system has certain thermic energy (Feynman et al. 1964) which may be described for each mole of a substance as

$$E_T = R \cdot T \tag{1}$$

where R is the gas molar constant (8.31452 $J \cdot mol^{-1} \cdot K^{-1}$) and T is the absolute temperature in Kelvin degrees (K, i.e., the temperature in Celsius degrees plus 273.15). Classical mechanics indicates that a moving body has the following energy (*kinetic energy*):

$$E_k = \frac{m \cdot v^2}{2} = \frac{3}{2} \cdot k \cdot T \tag{2}$$

where m is the mass of the moving body and v is its speed. The right hand term in Eq. 2 is the *kinetic thermic energy* of each isolate particle or molecule. The constant k is the gas constant scaled for each particle. Since a mole of any substance contains the Avogadro number $N = 6.02 \cdot 10^{23}$ atoms or molecules (for substances made by plain atomic or molecular particles, respectively), the energy of each particle is then

$$k = \frac{R}{N} \approx 1.38 \cdot 10^{-23} J \cdot K^{-1}. \tag{3}$$

Equation 2 enables us to predict the average speed of a particle at any given temperature as

$$v = \sqrt{\frac{3 \cdot k \cdot T}{m}} \tag{4a}$$

or

$$v \propto \frac{1}{\sqrt{m}}. \tag{4b}$$

Expressions (4a) and (4b) show that at any temperature, larger particles move (in a solution it is said that they *diffuse*) more slowly and that its mean speed is inversely proportional to the square root of their mass. Equation 4a underestimates the mass effect since it does not include the effect of particle collisions or interactions between particles, which increase in size as the molecular mass increases. In short prose, the following axiom holds: **substances made of molecules with a bigger mass (greater MW) diffuse slower than molecules of lesser size**. The true expression for molecule speed of diffusion when the only energy available is thermal is then

$$v \tilde{<} \sqrt{\frac{3 \cdot k \cdot T}{m}} \tag{5}$$

where the inequality symbol is read *approximately equal or less than*. The diffusion observed under these conditions is called *passive diffusion*.

There are numerous examples where large molecules moving in a living being seem to violate the preceding axiom. In those cases a mechanism has always been found (usually specific for the molecules in question) which assists in the movement of these molecules; the mechanism is called *active transport*. It is a phenomenon which characterizes life since it demands a constant supply of energy, different from thermal energy, to operate. Diffusion facilitated by active transport is called *active diffusion*.

Another feature differentiating active from passive diffusion is the direction in which the diffusion occurs. While passive diffusion occurs in any direction possible in space, *active diffusion is directional*. The additional energy is used to facilitate diffusion in a definite direction: from inside a cell outwards or vice versa, from inside an organism out, from the blood to tissues, etc.

Pharmacokinetics of Venoms and Antivenoms

Active Transport of Antivenoms and Other Immunoglobulins

Generally animal toxins are, as discussed above, smaller than antivenoms; thus it could be expected that they will diffuse and distribute faster and more extensively than their antidotes. But this is not always the case. PK parameters of *Tityus discrepans* venom and its $F(ab')_2$ antivenom have been determined in rams (Sevcik et al. 2004) and appear summarized in Tables 1 and 2.

The time ($T_{½}$) required in order that half of the venom administered passes from the blood to tissues is 46.2 min (Table 1); in contrast with this data in Table 2 shows that $F(ab')_2$ antivenom administered IV diffuses from the blood to tissues with a $T_{½}$ of just 14.2 (9.8, 24.8) min, which is 3.2 times faster than the venom. This is surprising given that venom biologically active components have an MW of less than 10 kDa, while antivenom MW is ≈100 kDa, roughly 11–12 times larger. The only way to explain this paradox is active transport, an active transport "pump," extruding antivenom from plasma to tissues.

The fast outflow of $F(ab')_2$ antivenoms from blood to tissues is not a characteristic exclusive to rams since it has been also observed in healthy human volunteers in the absence of envenoming (Vázquez et al. 2005) and in rabbits (Vázquez et al. 2010a). The nature of the mechanism extruding immunoglobulins from vessels is not exactly known but seems to be properties of *vascular endothelium* (Sevcik et al. 2007, 2013). Vascular endothelium covers between 20,000 and 40,000 km (the Earth's circumference is 40,000 km!) of blood vessels per person; with a 4–8 μm in diameter, capillary surface may be estimated to range 500–1,000 m^2, and taking the endothelial thickness as 0.1 μm, its volume is between 0.24 and 2.4 L (Freitas 1999). Vascular endothelium, thus, has a volume comparable to the blood

Table 1 Pharmacokinetic parameters for *Tityus discrepans* venom determined in rams

Parameter	*Value*	$T_{1/2}$
k_a	1.2 (0.78, 1.8)·10^{-2} min^{-1}	57.8 (38.5, 88.9) min
k_{el}	1.03 (0.56, 3.0)·10^{-3} min^{-1}	673.0 (231.0, 1237.8) min
k_{pe}	1.5 (0.52, 2.7)·10^{-2} min^{-1}	46.2 (25.7, 133.3) min
k_{ep}	4.5 (2.0, 20.0)·10^{-4} min^{-1}	1540.3 (346.6, 3465.7) min

Parameters are k_a, venom absorption rate constant from the inoculation site; k_{el}, venom elimination rate constant; k_{pe}, venom rate constant to flow from plasma to tissues; k_{ep}, venom rate constant to return from tissues to the blood. $T_{1/2}$ are half times needed by the venom to be absorbed, eliminated, exit blood vessels, or return to them, respectively. Data presented as medians and their 95 % confidence intervals (between parentheses) for five experiments

Table 2 Intravenous pharmacokinetic parameters for Venezuelan *Tityus discrepans* scorpion $F(ab')_2$ antivenom determined in rams

Parameters	*Value*	$T_{1/2}$
$k_{fd.pe}$	4.9 (2.8, 7.1)·10^{-2} min^{-1}	14.2 (9.8, 24.8) min
$k_{fd.ep}$	1.2 (0.7, 1.7)·10^{-2} min^{-1}	57.8 (40.8, 99.0) min

Parameters: $k_{fd.pe}$, antivenom rate constant to flow from plasma to tissues; $k_{fd.ep}$, antivenom rate constant to return from tissues to the blood. $T_{1/2}$ are half times needed by the venom to exit blood vessels or return to them, respectively. Data presented as mean and range (between parentheses) of 3 rams (Sevcik et al. 2004)

volume and a very large surface; vascular endothelium has the ability to form vesicles able to trap very large molecules and move them towards the extracellular extravascular space. A recent study using HRDFM in mice has shown that vascular endothelium in kidney, liver, and lungs is loaded with green vesicles 15 min after administering horse IgG or its $F(ab')_2$ fragments labeled with fluorescein isothiocyanate (FITC) (Sevcik et al. 2013).

Modeling the Distribution of Venoms and Antivenoms

PK parameters in Tables 1 and 2 were calculated by transforming the ram in an equivalent or model having three compartments such as that shown in Fig. 2. In the model a rectangle or box stands for the compartment where the venom is inoculated by SQ injection or by a sting or bite in the experiments or in an envenoming accident, a second box stands for blood plasma, and a third box represents the *extravascular extracellular fluid* in which all body cells are immersed. Arrows represent the direction venom (purple arrow) or antivenom (red arrows) move between the different compartments. Green arrows originating from blood plasma or the extracellular extravascular space summarize mechanisms eliminating venom from the body (Sevcik et al. 2004).

Even though the model is a simplification of reality, it permits to calculate speeds of venom and antivenom movements following the arrows connecting the

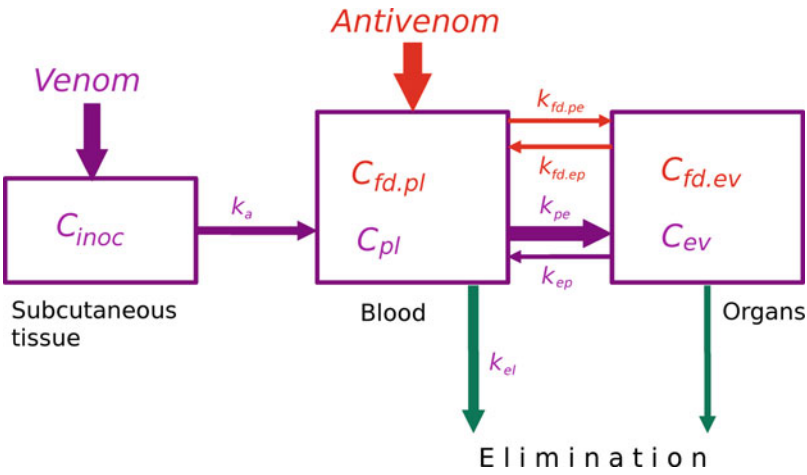

Fig. 2 Kinetic model to understand how venom (*violet arrows*) and antivenom (*red arrows*) are absorbed and distributed in the body. *Rectangles* represent spaces or "boxes" or "compartments" where venom or antivenom is either inoculated or diffused. The leftmost compartment represents subcutaneous tissues where venoms are usually inoculated in envenomed subjects; C_{inoc} is venom concentration in this compartment. A single *violet arrow* indicates that venom flows unidirectionally to the blood (*central box* in the figure). Antivenoms applied IV (*central box* in the figure). Venom and antivenom may be partially eliminated for the blood, either being catabolized or interacting between them (*green arrow*), or they may diffuse to tissues. A *thick violet arrow* represents the venom tendency to flow to extracellular extravascular space (*right box*) where it produces damage and tends to stay there trapped by high-affinity receptors. The figure suggests that venom may also be eliminated from tissues

diverse model boxes and then to predict the amounts (masses) and concentrations of venom in the compartments as function of time elapsed after venom administration to an animal, human or not. The outcome of the model is the parameters in Tables 1 and 2.

Modeling Witchcraft: Using the Models to Predict

If the calculated multicompartment model parameters are used to set a system of equation numerically soluble in a computer, these parameters enable us to predict the distribution of venom and/or antivenom under conditions that cannot be measured directly in an experiment (Sevcik et al. 2004). The first example is seen in Fig. 3 where the blue line represents blood plasma venom concentration (amount or mass of a substance per unit volume), while the red line represents venom concentration in the extracellular extravascular space. The figure indicates that plasma concentration reaches a maximum in ≈1 h and decays afterwards. Tissue concentration, in contrast, rises slowly to reach a concentration plateau lower than plasma during the initial 300 minutes. These data are, notwithstanding, deceiving. Since venom concentration in plasma is higher than in tissues, it seems that plasma concentration determines envenoming severity. To evidence this falsehood, data were recalculated to show ***total venom mass*** (not ***concentration***) within the compartments. Results are presented in Fig. 4.

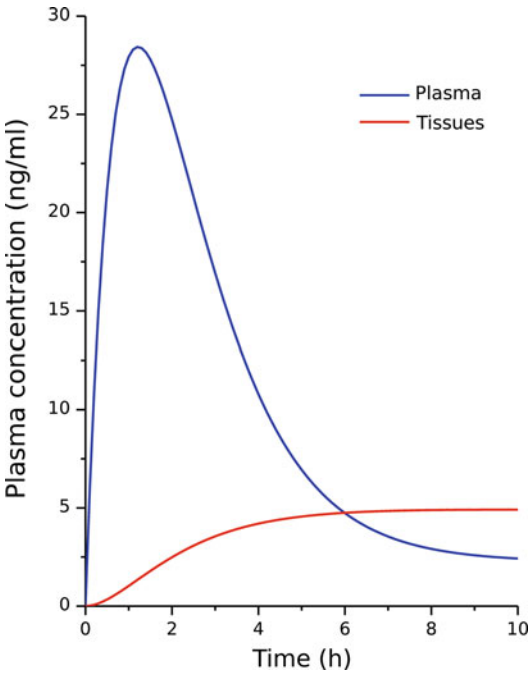

Fig. 3 The figure indicates how venom ***concentration*** changes as times goes by, in blood plasma (*blue line*) and in tissues (*red line*). Both lines were calculated by numerically solving a model using parameters presented in Table 1 assuming that the subject received 1 mg venom at time = 0. Abscissa is venom ***concentration***, in ng/ml; ordinate is time elapsed from venom inoculation, in hours

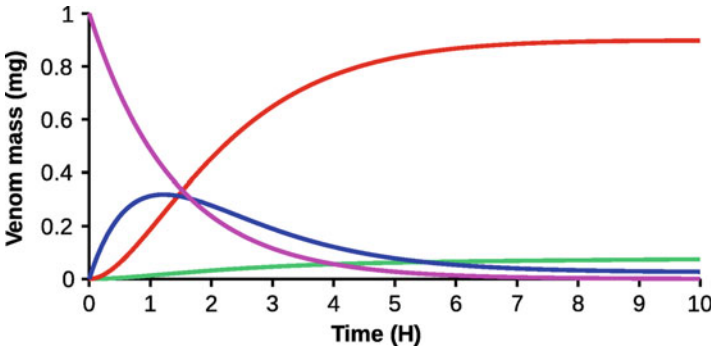

Fig. 4 The figure indicates how venom ***mass*** (not ***concentration*** as in Fig. 3) or ***amount*** of venom at the inoculation site (*fuchsia line*), in plasma (*blue line*), and in tissues (*red line*) and mass is eliminated (*green line*). All lines were calculated by numerically solving a model using parameters presented in Table 1 assuming that the subject received 1 mg venom at time = 0. Abscissa is venom ***mass***, in mg; ordinate is time elapsed from venom inoculation, in hours

In Fig. 4 the fuchsia line is venom mass in the SQ inoculation site, the blue line is venom mass in blood plasma, red line is mass of venom in tissues, and the green line is mass of venom eliminated from the body. The fuchsia line at time zero expresses the total mass of venom administered. Although the blue line's time course is similar to the blue line in Fig. 3, the figure dramatically indicates that there is

never more than 1/3 of the venom in plasma; ***it also shows that venom passes transiently through plasma and that when plasma concentration decays, the vast majority of venom is in tissues, precisely where it hurts most***. The shift of venom mass to tissues is fundamentally due to two factors: first, it is due to the tissue sites "strongly" binding venom (the so-called high-affinity receptors) and second to the slow venom catabolism or elimination from the body as suggested by the green line in Fig. 4.

Therapeutic Modalities and Venom PK

A fact highlighted by pharmacokinetic studies is that drug effects not just depend on the chemical nature and dose of a drug but also on the manner it is delivered, the route used to administer it (oral, subcutaneous, intravenous, intramuscular, rectal, percutaneous, etc.), the administration modality (single dose or repeated doses, continuous infusion, etc.), the pharmaceutical formulation of the product (tablets, capsules, syrups, type of excipient, type of solvent, drug crystal's type or size, release mode, etc.), and even the time of day when it is administered (its *chronopharmacology*).

The route and site of venom administration in accidental envenomings are usually independent variables out of human will control. Antivenom use is a therapist decision and is enormously important to optimally treat the patient to reduce envenoming to the absolute minimum intensity and duration. Pharmacokinetic modeling such as that sketched previously and the parameterization of diffusion and elimination speeds of venoms and antivenoms like in Tables 1 and 2 are essential to analyze precisely and to compare diverse *therapeutic modalities* (Sevcik et al. 2004; Vázquez et al. 2005; Sevcik et al. 2007; Vázquez et al. 2010a).

The Intravenous Route

Given the size of immunoglobulins or their fragments, it is the consensus that they should be administered IV; yet doses, dilutions, and manners of antivenom infusion are sometimes subjects of controversy among clinicians. **The most important point to stress is that the dose of antivenoms, in contrast with other drugs, is not determined by age or patient's weight**. The **purpose of antivenoms is to neutralize venom not patients**, and the dose of venom administered by a poisonous animal is largely an unpredictable variable. **Venomous animals do not gauge patients in neither weight nor size, and a baby or a toddler will get the same amount of venom as an adult of any age**, with the aggravating factor that due to the smaller size, venom concentrations will be much higher in children and the envenoming will, consequently, be more severe. But the amount of venom to neutralize and thus the antivenom to be used are the same. A special situation occurs, however, when the volumes that must be administered are large (say 25–50 ml) and the patient is small; special care has to be taken to avoid expanding blood

volume too quickly. **The antivenom infusion rate, not the antivenom amount, must then be individualized**, following good pediatric practice rehydration guidelines whose details escape the purpose of this article.

The Intramuscular Route

This section will begin with a quote of an International Immunopharmacology Editorial by Leslie Boyer (2010):

> A controversy that has plagued clinical toxinologists for decades, one that affects the life and health of millions of people bitten or stung by venomous creatures in the rural and developing world. Hitherto, the paucity of human data addressing the controversy has greatly hindered professional discourse. Worldwide, most venom injuries – including the majority of clinically important scorpion stings – occur in tropical and developing countries. In most cases, intravenous antivenom, prepared either from whole immunoglobulin or from $F(ab')_2$ fragments targeted against venom of snakes or scorpions, is a long-accepted standard of care. Worldwide shortages of antivenom are ongoing, driven by a combination of adverse economic, geopolitical and practical considerations. The situation has resulted in the designation of venom injury, by the World Health Organization, as a Neglected Tropical Disease. Compounding and confounding shortage issues in remote areas are the problem of access to a safe medical environment for the administration of serum-derived treatments, for which both Type 1 and Type 3 immune reactions are well known. Recent improvements in the safety profile of some antivenoms have reduced these risks substantially; and as a consequence smaller clinics have begun to stock antivenom, regardless of whether these clinics are able to provide intravenous treatment. Unmonitored and unpublished reports suggest that tens of thousands of rural patients may annually be receiving scorpion antivenom by intramuscular injection, with a prevailing belief by providers that such administration is saving lives. This "20,000 Mexicans can't be wrong" theory has understandably generated heated debate between pragmatists and purists; but in the absence of human pharmacokinetic information the argument has involved more anguish than answers.

PK of $F(ab')_2$ administered IM to humans has been studied by Vázquez et al. (2010) using Mexican anti-scorpion (*Centruroides*) antivenom. The results of the study show dramatically and quantitatively the inadequacy of the IM route for the antivenoms. In this work it was shown that IM antivenom produced a maximum plasma concentration (C_{max}) in 45 (33, 74) h [t_{max}, median and its 95 % confidence interval (CI), n = 6] or almost 50 times longer than it takes the venom to reach a peak in the blood (Sevcik et al. 2004) and at least ten times longer than the time it takes for a *Tityus* scorpion venom to become irreversible and 50–100 times longer than the time it takes for some *Centruroides* scorpion venom human victims to die. And t_{max} is neither the only nor the worst PK parameter in Vázquez et al.'s (2010) study to show the inadequacy of the IM route for antivenoms; the mean time it takes an antivenom molecule to be absorbed from muscle (MAT_{im}) was 279 (42, 1166) h (median and its 95 % CI, n = 6), extremely slow and variable. Most venom never got absorbed to the blood from the muscle; this is indicated by the poor IM venom *relative bioavailability*. To understand this, an important PK parameter to consider is the area under the blood drug

concentration curve between time 0 and the duration of the study, $t(AUC_t)$. This parameter is formally defined as

$$AUC_t = \int_0^T C(t)dt \tag{6}$$

or the integral of blood concentration [$C(t)$] from time 0 to time T, the duration of the experiment. AUC_t can also be calculated numerically using the trapezoidal rule and in any of the two cases can be extrapolated to $T = \infty$, if the study was long enough in time, with little error to give AUC_∞ (Blode et al. 2004). Since drug effect is proportional to its concentration and the time it persists in the body, it is intuitively easy to see that AUC_∞ is a good indicator of both factors. Relative bioavailability is obtained by the ratio

$$f_{rel} = \frac{AUC_{niv}}{AUC_{iv}} \cdot \frac{D_{iv}}{D_{niv}} \tag{7}$$

where D is the dose, *iv* stands for intravenous, and *niv* represents any other route. In prose, f_{rel} is the fraction reaching the blood when the drug is administered via any other route with respect to the IV route, and f_{rel} is also expressed as percent. In Vázquez et al.'s (2010a) study, F(ab')$_2$ f_{rel} was found to be 30.0 (15.3, 46.6)% (median and its 95 % CI, n = 6), meaning that after IM administration, most of the antivenom never reached the blood or was destroyed at a very high rate and, thus, had no chance to neutralize venom other than in the site of injection.

This is perhaps a good time to point out that due to diverse reasons, 50–60 % of scorpion stings (D'Suze et al. 2003) or 25 (Thygerson et al. 2006) to 50 % of snakebites (Silveira and Nishioka 1995; Russell et al. 1997) are "dry," meaning that no venom is inoculated, which means that any "treatment" has a high chance of "succeeding" and being hailed as valid by the mere reason that little or no venom was inoculated to the victim; this is certainly the case of the successes in treating patients with antivenom using pharmacokinetically unsound routes, prayers, ointments, etc.

Treating Envenoming Under Desperate Conditions

All PK evidence available indicates that **the route** to administer antivenom in any poisoning is IV. The IV route guarantees 100 % bioavailability and produces the most reproducible results in minimum time. IV **is the only route** that should be used by **capable** medical personnel. And still, the question is always put forwards by lay people, paramedics, and even practicing medical doctors not trained in toxicology: what to do when an envenoming occurs where there is no personnel able to practice a venipuncture and antivenom is available? In Venezuela, long ago, antiviperid ***raw horse antivenom serum*** (*Bothrops* and *Crotalus*, essentially) was commonly injected SQ in the back of the patient. The rationale for this was the large

volume to inject and the fear to anaphylactic shock associated with rapid administration of ***raw horse serum***. There is no information known to us on PK of such SQ administration, but many victims [including one of the authors (CS) of this article] seemingly benefited from this practice; perhaps the apparent beneficial effects of this outdated practice were related to the relatively slow mode of action of viperid venoms. Perhaps slow absorption of antivenoms may be acceptable for slow-acting venoms such as from *Loxosceles* spiders or even for slow-acting snake venoms, but evidence on this subject is lacking.

It was proposed once, for exceptional circumstances, that the *intraperitoneal route* may be useful when venipuncture was not possible (Sevcik and D'Suze 2011). Due to its great surface, most substances are quickly absorbed from peritoneal cavity and pass to the hepatic portal system, liver, and systemic circulation; another fraction probably passes via the thoracic duct directly to venous circulation; the intraperitoneal route also accommodates large volumes of fluid. However, recent experiments carried out in rabbits using ELISA assay and HRDFM observations indicate that no amount of $F(ab')_2$ useful for antivenom therapy is absorbed via the peritoneum (Sevcik et al. unpublished).

Pharmacokinetics of Venom Neutralization

Figure 5 presents modeling results (using parameters in Tables 1 and 2) of *Tityus discrepans* scorpion envenoming in typical situations (Sevcik et al. 2004). Lines in the figure have the same meaning as in Fig. 4. The only difference is that the green line includes venom eliminated through mechanisms discussed in regard to Fig. 4, *plus the venom eliminated by antivenom neutralization*. In the calculi presented here, for reasons of convenience and clinical clarity, antivenom doses are not expressed as mass but as ampules (amp). One ampule of Venezuelan anti-*T. discrepans* scorpion antivenom neutralizes 1 mg of venom (expressed as mg of protein measured by absorbance at 280 nm), and one ampule of this antivenom contains ≈181 mg protein of which ≈85 % are $F(ab')_2$ horse IgG fragments and ≈10 % are Fab'; only ≈2 % of the antivenom is specific for the scorpion venom, and the rest is part of the normal immune system of the horses used to produce antivenom.

For the calculi in Fig. 5a, it was assumed that a subject receiving initially 1 mg of venom received 1 amp antivenom IV 1 h later. PK calculi in Fig. 5a indicate that antivenom neutralizes very quickly the venom arriving to the blood plasma and, almost as quickly, the venom already in the tissues. The rapid venom neutralization in tissues depends critically on the rapid extravasation of antivenoms observed in rams (Sevcik et al. 2004), humans (Vázquez et al. 2005), and rabbits (Quesada et al. 2006; Vázquez et al. 2010b).

Figure 5b is the modeling of a common clinical practice, the slow infusion of antivenom diluted in isotonic saline; *this practice was justified with old antivenoms prone to produce the dreaded anaphylactic shock*, since it allowed to desensitize the patient and to reduce anaphylaxis probability. It is assumed in the figure that 1 amp antivenom is infused at a constant rate from minutes 60 to 120 after

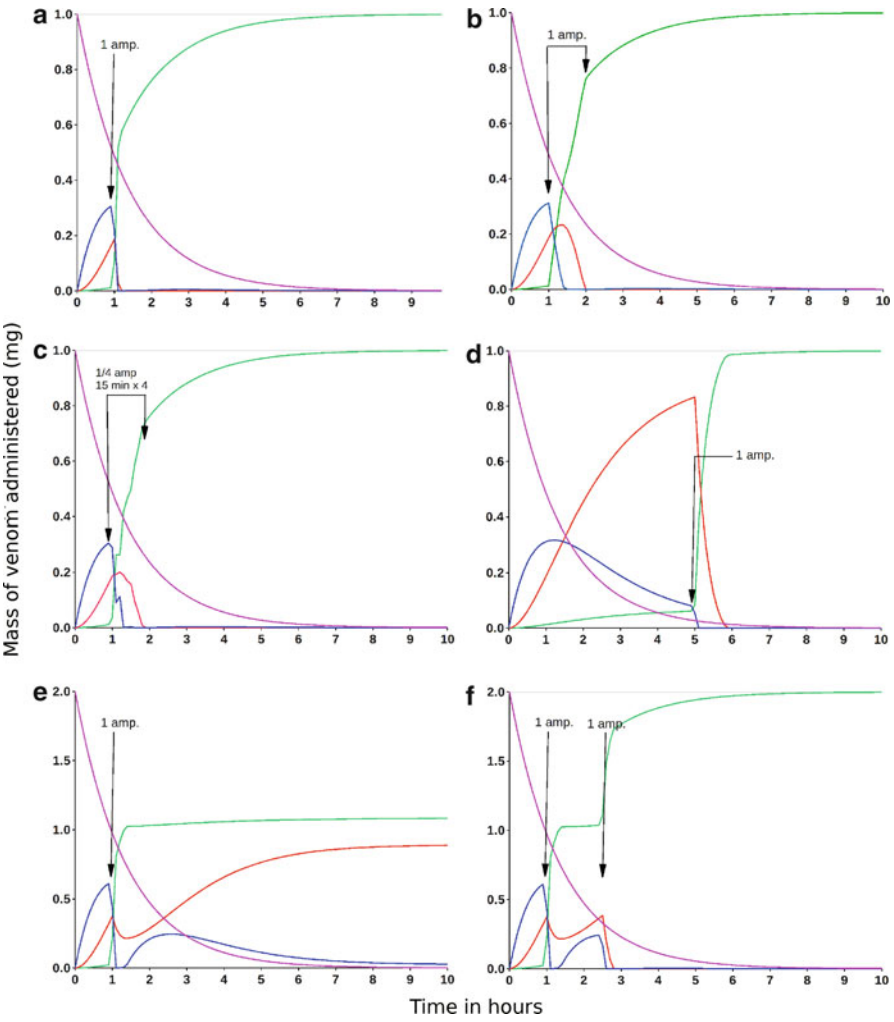

Fig. 5 As in Fig. 4, the figure presents venom ***mass*** changes in the diverse compartments. *Lines* have the same meaning and color as in Fig. 4. Panels (**a**) through (**f**) model predictions calculated with parameters shown in Tables 1 and 2, during different therapeutic modalities of antivenom use. In panels (**a**) through (**d**), the subject is assumed to receive 1 mg at time = 0; in the remaining panels, the subject is assumed to receive 2 mg venom. The therapeutic modalities simulated are the following: (**a**) one antivenom ampule is administered as a single bolus. 1 h after envenoming, (**b**) the same ampule is diluted and passed a constant IV from minutes 60 to 120 after envenoming; (**c**) one antivenom ampule is fractionated into four boluses administered, each one in 15 min intervals starting on minute 60 after envenoming; (**d**) just like (**a**), but the ampule is administered five after envenoming; (**e**) one ampule is administered just like in (**a**), ***but to a subject which received initially 2 mg of venom***; (**f**) like (**e**), but the first ampule is followed by another 1 h later. ***Please notice that only modality A produces a red line which subtends a minimum area, indicating minimum damage to the patient***. Abscissas are venom mass, in mg; ordinates are time elapsed from venom inoculation, in hours

envenoming. As seen in Fig. 5b, venom neutralization is slower than Fig. 5a (green line) and reduction of venom in plasma is slower, and while amounts of tissue venom in Fig. 5a (red line) are only significant during 1 h, in Fig. 5b significant amounts of venom in tissues are observed for up to 2 h. Comparing Fig. 5a, b, it becomes obvious that, given that modern antivenoms of *Fab* and *F(ab')$_2$* types do not produce anaphylactic shock, *administering antivenom by slow infusion is not advised and not necessary*.

Figure 5c presents another common clinical practice, fractionating the antivenom into several partial boluses administered IV. The procedure is equivalent to slow infusion and was justified with similar arguments. The red line in Fig. 5c suggests that the situation of venom in tissues is slightly better than in Fig. 5b; the improvement is minor and very dependent on the precision of volumes and times of infusion of the fractional boluses. Just as the slow infusion, *this modality is not advisable today*.

Figure 5d presents a situation where the subject receives 1 mg of venom and much later 1 amp of antivenom (5 h after envenoming). As indicated by the fuchsia line, in 5 h over 5 % of the initial, venom is still at the inoculation site, and the blue line indicates that ≈10 % is in plasma. In dramatic contrast, 80 % of venom is in the tissues; it hurts. Since the damage produced depends on amount and time it stays in tissues, it is easy to understand that damage to the patient is proportional to the area under the red curve (a mathematician would say, to its integral) from times 0 (time of envenoming) to *T* (when the venom disappears from organs). *The large area under the red line in Fig. 5d suggests severe damage, and what is worse, damage that became irreversible by the late administration of antivenom*. But the figure also suggests that *venom in tissues may still be neutralized*, but more slowly than in Fig. 5a; it took almost 1 h to remove venom already in tissues. PK modeling (and common sense) predicts that the time required to remove venom from the extracellular compartment shortens by triplicating or quadruplicating the amount in the antivenom bolus, although the damage already induced by venom may no longer be reversible; clinically, after 5 h of envenoming, the chance of a fatal SRDS increases rapidly. But Fig. 5d also suggests that venom in tissues is still neutralizable at late times and consequently that it should be neutralized to give the patient a better (though perhaps small) chance of surviving and, in the best of the cases, less irreversible sequelae. *The overall meaning of the figure is that you cannot recover lost time and then the best you can do is to reduce the damage even when the antivenom application is late*.

Figure 5e presents another common clinical situation, the administration of an insufficient dose of antivenom because the dose of venom is unknown and underestimated. In the figure the patient receives initially 2 mg of venom and then 1 amp of antivenom (enough to neutralize only 1 mg of venom) 60 min after. As seen, the initial evolution resembles the initial part of Fig. 5a, and blood venom is initially rapidly and completely neutralized; this is so since 60 min after envenoming, only some 0.6 mg of venom are in plasma which then receives an antivenom bolus able to neutralize 1 mg of venom. The situation changes quickly. First, venom keeps arriving to the blood from the inoculation site where 1.4 mg are

still available for absorption. Second, a concentration gradient now exists between plasma and extracellular extravascular space favoring the return of venom from tissues and the outflow of antivenom from plasma (expressed as an initial decrease of the red line after venom administration). These factors determine that the reduction of venom not neutralized in the tissues begins to increase again (red line) reaching high values and that after a short period during which venemia (venom in the blood) is nil, blood venom begins to increase again (*rebound* effect) since venom that cannot be neutralized keeps coming from the inoculation site. The rebound in plasma is not the serious problem; a small rebound may be observed in Fig. 5a–c where the amount of antivenom was adequate, if the figures are enlarged enough; the problem is the rise in tissue venom which did not happen in Fig. 5a, b nor Fig. 5c. Also modeled (but not shown) was a situation similar to Fig. 5a, but with the antivenom being administered 30 min instead of 60 min after envenoming, this scheme resulted in a significant plasma rebound since the antivenom is quickly mobilized to the extracellular space neutralizing all the venom available, and the free antivenom then returns to plasma where the venom keeps coming from the inoculation site. In this last situation non-neutralized tissue venom is always nil.

Finally, Fig. 5f is similar to Fig. 5e, but here a second ampule is injected IV 2.5 h after envenoming (coinciding with the maximum of the plasma's rebound). As seen the second ampule neutralizes quickly and completely all venom in plasma and tissues. ***The moral is if you were short, do not scare, and do not hesitate to use more antivenom.*** Given the safety of modern antivenoms, giving too much (rationally) is better than giving too little.

The Question of Venom Effects on Antivenom Pharmacokinetics

In the PK work and data that is central to this chapter, venom and antivenom concentrations were determined by ELISA. The high resolution of ELISA assay has, however, a price; you determine either venom or antivenom concentration in blood plasma or serum. A valid question is: would antivenom PK parameters determined in the absence of venom be valid to model PK of venom–antivenom interactions? Or equivalently: does the venom modify vascular permeability or any other factor determining antivenom PK in envenomed subjects?

A definite answer of universal validity may be difficult to find, but an answer is hinted by the work of Quesada et al. (2006). This work was aimed to compare pharmacokinetic parameters of whole IgG equine antivenom in normal rabbits and in rabbits suffering a moderate envenoming by intramuscular injection of the venom of the viperid snake *Bothriechis lateralis*, which induces drastic microvascular alterations. Although originally the venom of *Bothrops asper* was going to be used, it was found that the coagulopathy promoted by this venom makes extremely difficult the repeated bleeding of animals to collect blood for the quantification of antivenom IgG. Therefore, the venom of the arboreal viperid snake *B. lateralis*, which induces a pathophysiological profile very similar to that of *B. asper* and other pit vipers, but without affecting the clotting times, was employed. Anti-*Micrurus*

nigrocinctus antivenom was used, instead of polyvalent (Crotalinae) antivenom, to avoid the formation of toxin–antibody complexes which may alter antivenom pharmacokinetics. It was thus possible to study the effect of vascular alterations, i.e., edema and hemorrhage, induced by the venom on IgG antivenom distribution and elimination.

Interestingly, the pharmacokinetic parameters for IgG antivenom in rabbits envenomed with *B. lateralis* venom did not differ significantly from those of control rabbits, despite the fact that prominent hemorrhage and edema developed at the site of venom injection in the thigh. Quantification of IgG antivenom in the injected muscle revealed a significantly higher extravasation in envenomed rabbits than in control animals. The Quesada et al. (2006) results showed that there are no significant variations in the main pharmacokinetic parameters for whole IgG equine antivenoms in rabbits suffering at least moderate envenoming by a hemorrhagic and edema-inducing viperid snake venom when compared with control, non-envenomed rabbits. This further suggests that the pharmacokinetic studies previously performed in normal animals may be extrapolated to mildly or moderately envenomed animals. These results do not discard the possibility that differences between normal and envenomed animals occur in more severe models of envenoming where systemic hemodynamic disturbances occur.

Immunologic Factors Affecting Antivenom PK and Effectiveness

A factor, often overlooked, is that an envenomed person might have antibodies against the heterologous IgG (or its fractions) used as antivenom. The problem was highlighted by Sevcik et al. (2008) which detected anti-horse, anti-cattle, and anti-chicken IgG in human γ-globulin preparations [5 % human intravenous IgG (50 mg/ml total IgG), "Inmunoglobulina G Endovenosa al 5 %"™, Quimbiotec C.A., Caracas, Venezuela]. The anti-horse IgG levels were low, but the anti-chicken IgG levels were particularly high. The amounts of human IgG detected were 0.017 (0.015, 0.020) mg/ml against horse IgG, 0.37 (0.28, 0.48) mg/ml against cattle IgG, and 1.27 (1.15, 1.40) mg/ml against chicken IgY. The results suggest that a human has a median of ≈780 mg of anti-chicken-IgY IgG, ≈230 mg of anti-cattle-IgG IgG, and ≈11 mg of anti-horse-IgG IgG. Let us now consider, for example, that an ampule of commercial Venezuelan anti-scorpion horse $F(ab')_2$ antivenom contains ≈181 mg protein (Sevcik et al. 2004) and a Mexican anti-scorpion horse $F(ab')_2$ antivenom contains ≈45 mg protein (Vázquez et al. 2005); it becomes evident that if $F(ab')_2$ is able to react with the human IgGs considered in Sevcik et al. (2008), to the same extent as that of heterologous IgGs or IgYs, the human IgGs directed against heterologous immunoglobulins could significantly reduce the efficiency of antivenoms. This problem would be very severe for antivenoms based on IgYs and perhaps also on antivenoms based on cattle IgGs (calculation details may be found in Sevcik et al. 2008). It has recently been shown that anti-horse IgG produced in rabbits after horse antivenom administration indeed modifies subsequent antivenom PK (Vázquez et al. 2013). It is also reasonable to

expect that untoward reactions against antivenoms should be more frequent and more severe if the patient has been previously sensitized against the host where the antivenom was produced.

When heterologous IgG or its fragments are used as antivenoms, they may elicit acquired immunity against these antibodies, and these antibodies in turn alter antivenom PK, reduce its efficiency, and could be the source of untoward reactions. A PK study of Fab, $F(ab')_2$, and IgG antivenoms IV in rabbits by Vázquez et al. (2010b) shows that this indeed occurs. In the study, there was an excellent fit between the blood serum concentration data points and adjusted tri-exponential equations for Fab and $F(ab')_2$ data sets, as well as for the data sets recorded at $t < 120$ h in the case of IgG and IgG(T). After 120 h, however, the points deviated markedly from the curves fitted to the data sets of the IgG isotypes. The discrepancy between the tri-exponential curve fitted after injecting IgG or IgG(T) occurred because the rabbits started producing anti-horse antibodies whose concentration peaked at the 11th day (264 h) and decayed slowly afterward. The Ig fragments were also able to induce anti-horse antibodies. The increase in these antibodies had an onset slower than when whole Igs were used and reached an apparent plateau (it is not known if a decay occurred afterward) after the PK experiments were over. Consequently only the last point of the PK experiment could have been affected by anti-horse antibodies when Ig fragments were used. The data also indicates that Fab and $F(ab')_2$ are equally immunogenic if they are compared at the same molar dose. This suggests that there are two sites where both antigen-binding fractions of a $F(ab')_2$ fragment cannot bind at the same time since they are bound together but may bind when split into two Fab molecules. Also, the immunoglobulins Fc domain must play a role in sensitizing the rabbits, since they induced anti-horse titers $\approx$5 times bigger than the fragments, and the fragments' PK curves remained tri-exponential during the whole observation period. Anti-horse immunoglobulins induced by the IgGs in rabbits were unambiguously identified as IgGs; no traces of IgMs were detected. An open question awaiting further research is if the early production of anti-horse immunoglobulins is a rabbit phenomenon or if it occurs in other species including humans.

Direct Antivenom Observation in the Body Using HRDFM

PK studies of IV antivenoms (Sevcik et al. 2004, 2008; Vázquez et al. 2005, 2010b) have shown that antivenom PK cannot be easily accommodated to fit the classical PK scheme of two exponential phases: one of distribution and one of elimination. Antivenom IV PK follows a multiexponential time course (Vázquez et al. 2005, 2010b), and even classical concepts such as drug *volume of distribution* (Blode et al. 2004) are nonlinear functions of time for antivenoms and some other drugs (Sevcik et al. 2008). To directly observe antivenoms distributing through the body, immunoglobulins and their fractions were fluorescently labeled with fluorescein isothiocyanate (FITC) and their distribution was observed under high-resolution deconvolution fluorescence microscopy.

Standard wide field microscopy is limited by light refraction in the tissues and by optical aberration in the microscope lenses which vary with light's wavelength. These shortcomings may be solved in part by the deconvolution mathematical process. Deconvolution is a computational method used to reduce out-of-focus fluorescence in three-dimensional (3D) microscope images. Compared to other forms of 3D light microscopy, like confocal microscopy, the advantage of deconvolution microscopy is that it can be accomplished at very low light levels. This enables multiple focal-plane imaging of light-sensitive living specimens over long time periods and observing fixed specimens with minimum dye bleaching. 3D deconvolution fluorescent microscopy was used to follow at the subcellular level horse FITC-IgG, horse FITC-F(ab')$_2$, and ostrich (*Struthio camelus*) FITC-IgY injected IV to mice (Sevcik et al. 2012, 2013).

Visualizing IgG and Its Fractions

Sevcik et al. (2012) reported that different FITC-labeled Igs interact differently with erythrocytes and lymphocytes. Both FITC-F(ab')$_2$ and FITC-IgG induce intense erythrocyte fluorescence after 15 min of IV injection, when FITC-IgY is administered and erythrocytes appear very dark in faint maroon fluorescence. The difference between erythrocyte fluorescence when using avian and mammalian FITC-Igs cannot be ascribed to Fc receptors since the Fc domain is lacking in FITC-F(ab')$_2$. Erythrocyte fluorescence induced by mammalian FITC-Igs may be clearly observed for at least 5 h in experimental mice. The functional significance of the erythrocyte-bound Igs is not clear; it is probably a small pharmacokinetic compartment since different studies suggest that horse Igs' initial volume of distribution (*central compartment volume* or VC) is not much larger than blood plasma volume neither in humans nor in other animals (Vázquez et al. 2005, 2010a; Quesada et al. 2006). From these results it is hard to avoid the conclusion that erythrocytes have receptor sites which bind IgG's Fab groups and which are able to differentiate them from IgY's Fab groups. *Adaptive immunity* is capable of recognizing and selectively eliminating specific foreign antigens. Antigen specificity permits the immune system to distinguish between subtle differences among antigens. Antibodies can differentiate between two proteins differing in a single amino acid. Since they produce and display antigen-binding cell-surface receptors, lymphocytes mediate immunologic attributes of specificity, diversity, memory, and self/nonself recognition. Thus, from the point of view of antivenom efficacy and safety, the different binding of avian and mammalian antibodies to lymphocytes may be most significant. Sevcik et al. (2013) results showed that avian Igs bind to lymphocytes. FITC fluorescence was absent from twice as many lymphocytes when F(ab')$_2$ or IgG instead of IgY were administered to mice. Sevcik et al. (2012) results suggest that horse IgG, horse F(ab')$_2$, and ostrich IgY trigger adaptive immunity against them in mice and that IgY is probably more potent in triggering adaptive immunity given its higher tendency to bind to lymphocyte membrane. It is interesting that IgG has an intermediate position as lymphocyte labeler in Table 1 of

Sevcik et al. (2012), which indicates that it may be more active in triggering adaptive immunity than its $F(ab')_2$ fraction and less than IgY. In agreement with this, it has been shown that in rabbits, horse IgG and IgG(T) are more effective in inducing anti-horse IgG than $F(ab')_2$ or Fab (Vázquez et al. 2010a). If the preceding syllogism is true, IgY should be a more efficient inducer of adaptive immunity than IgG and $F(ab')_2$ in mammalians. A higher efficiency of avian over mammalian immunoglobulins to trigger adaptive immunoglobulins could explain that humans which, according to the Food and Agriculture Organization of the U.N. (FAO), feed approximately equally on poultry and cattle have much higher blood plasma concentration of anti-chicken than anti-bovine IgG (Sevcik et al. 2008).

The results of Sevcik et al. (2013) clearly prove that kidney glomeruli are quickly loaded with FITC-IgG and -$F(ab')_2$; the dye is indeed observed within endothelial cytoplasm, near cell nuclei, and also in podocytes, all of which indicates that Igs pass from the blood to the Bowman space and the nephron lumen. When either labeled IgG or $F(ab')_2$ were administered, the whole length of the nephron was loaded with abundant green fluorescent vesicles in 15 min, which by their sheer quick apparition and abundance hints a process of recapture. A recapture mechanism must imply excretion of intact Igs from the nephron back into the body; yet if this excretion occurs, the recovered immunoglobulins do not return via the interstitial renal capillary endothelium which was not labeled green when IgG or $F(ab')_2$ were administered to the mice. Liver actively captured IgG and $F(ab')_2$. Hepatocytes were loaded with green vesicles, more densely packed near sinusoid capillaries. Green fluorescence was also observed within endothelial cells of sinusoids, portal veins and their branches, and hepatic veins and arteries. Portal fields contained green fluorescence which increases towards the bile ductule; at some parts of the portal fields, the fluorescence forms thin green lines which surround bile ductule epithelium and seem to cross between epithelial cells and reach the ductule lumen. The whole picture suggests excretion of Igs from the liver. Lung was the third organ where interesting Igs kinetics was found. Both bronchial and alveolar epithelia were quickly loaded with IgG or $F(ab')_2$. The arterial endothelium is also loaded with FITC-$F(ab')_2$. The images suggest that the labeled horse Igs were extravasated by the vascular endothelium and interacted with the elastic layers.

IgY is Different

Under the same conditions, Sevcik et al. (2013) results suggest that ostrich IgY seems to cross the glomerular capillaries in a manner similar to horse IgG and $F(ab')_2$. But here is just where the similarities stop. Very little FITC-IgY fluorescence was seen within nephron epithelial cells, and the aspect of the green fluorescence there was very different from the fluorescence after FITC- $F(ab')_2$ or FITC-IgG. After FITC-IgY the fluorescence within nephron epithelial cells was merely a few green granules in some cells. FITC-IgY also produced abundant

small brightly green granules in kidney interstitial capillaries endothelial cells and in liver sinusoid capillaries. The vasa vasorum in artery walls also contained green granules after FITC-IgY. The green granules, in all tissues observed, were not detectable until 1 h after administering FITC-IgY IV and were very difficult to find 5 h after that. Lung epithelia, hepatocytes, and portal fields (including the bile ducts) were free of FITC-IgY fluorescence; no FITC-IgY fluorescence was observed in the hepatic central lobular veins which drain into the hepatic vein.

Conclusion and Future Directions

Blood levels of venoms and antivenoms can be measured, thanks to the introduction of highly specific ELISA assays; this enables to determine their PK parameters and, therefore, the simulations of clinical situations which cannot be directly studied for ethical or technical reasons.

PK of antivenoms composed of IgG [or its $F(ab')_2$ and Fab fractions] has much broader implications; the pharmacokinetics of IgG is a key factor in mammalians' physiology. PK of antivenoms composed of IgG [or its $F(ab')_2$] showed that antigen recognition epitopes do not determine antibody PK in the absence of the antigen. The similar PK of IgG and its fractions show that this pharmacokinetics is determined by the Fab groups, not by the Fc domain or the FcR receptors as it was believed.

The complexities of IgG PK, further highlighted by HRDF microscopy, show that immunoglobulins are actively transported and distributed through the body. This strongly suggests that in vitro neutralization of venoms is not enough to propose such antibodies as clinically useful antibodies. This seems particularly true for recombinant antibodies as well as for putative antivenoms based on avian IgY immunoglobulins. IgY immunoglobulins PK has not been and must be studied, but HRDFM suggests that IgYs do not distribute or eliminate from mammalian body in a manner similar to IgG (and its fractions).

Results of Harris et al. (1997) and others, not possible to summarize here for reasons of brevity, prove that it is possible to express full monoclonal recombinant IgGs. Such recombinant complete immunoglobulins must be expected to have PK similar to other IgGs, but quite often recombinant monoclonal antibodies are not complete Igs and cannot be expected to distribute in the body as IgG and its fractions do.

Another point of interest which must be considered, given the active transport of IgG and its fractions, is whether they may be used as carriers of other compounds through barriers; these compounds will normally not cross, or not cross as efficiently, for example, the blood-brain barrier. In any case, results like those summarized in this chapter indicate that a field many gave for definitively and completely studied such as immunoglobulin PK is alive and thriving.

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Part III

Antivenoms

Recombinant Neutralizing Antibodies, A New Generation of Antivenoms

6

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Contents

Abstract

The detailed knowledge of the medically important components within the venoms from poisonous animals has prompted the rational generation of improved antivenoms. The new generation of antivenoms, in the case of scorpion envenoming, will be based on the neutralization of the toxins directed against mammalian ion channels, specifically sodium channels. The neutralization of the major toxic molecules declines the lethality of the whole venom. The next generation of antivenoms will depend substantially on the advancements in the field of antibody engineering. The accumulation of detailed information of antibody structure and function proposes that human recombinant antibodies in combination with phage display and directed evolution as a powerful in vitro biotechnological platform alternative to classical antivenoms or monoclonal

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antibodies for the generation of outstanding therapeutic antibodies. This biotechnological platform has allowed the isolation of safe and efficient recombinant neutralizing antibodies. Many antibody fragments generated using this platform bear exceptional properties that had not been reached using classical approaches. This review describes the great progress that has been achieved on the improvement of antivenoms against scorpion envenoming which have been generated using the already mentioned platform as compared with classical and/or hybridoma approaches.

Introduction

Envenoming syndrome caused by venomous animals is a global health problem in many tropical and subtropical countries. Nowadays, antibodies contained in antivenoms are the most effective molecules used for inhibiting the deadly effects of the toxic components from the venom of poisonous animals. The first report on the preparation of an antivenom was based on the immunization of animals with snake venom (Chippaux and Goyffon 1998). Initially, this practice was very useful; however, the lack of knowledge about the immune response and the heterologous systems used in human therapy caused fatal adverse reactions due to the presence of accessory proteins in serum or associated with the Fc fragment of immunoglobulins.

Essentially, the current way to obtain antivenoms is by hyperimmunization of animals (horse, sheep, goat, cattle, etc.). The matured antibodies from animal serum are recovered and processed by digestion with pepsin to obtain $F(ab')_2$ fragments (Chippaux and Goyffon 1998; Fig. 1). These bivalent molecules, called fabotherapics, are purified for their use in medical treatments. The use of fabotherapics significantly reduces the occurrence of adverse side effects, although some problems associated with these antivenoms are still present. In some cases, hypersensitivity types I and III can occur among other side effects (Chippaux and Goyffon 1998). Despite that these events occur in low proportion, they are still an issue of crucial importance for the safety use of therapeutic antibodies. In spite of these drawbacks, nowadays, the unique effective therapy for envenoming caused by scorpion stings is passive immunization with fabotherapics.

A century after the development of the first antivenom, a great progress in the characterization of venom components and the new technologies for obtaining recombinant antibodies have allowed a substantial advancement in immunotherapy. Furthermore, the requirement of antibodies for the clinical therapy has implicated that these molecules must undergo an extensive research process to ensure their safeness and functionality.

The purpose of this chapter is to present the research advances, in the last 20 years, focusing on the rational strategies that have been developed for obtaining safer antivenom alternatives against scorpion envenoming. The success in this improvement approach has been made possible supported by the detailed characterization of the venoms in terms of the identification of the key deadly components

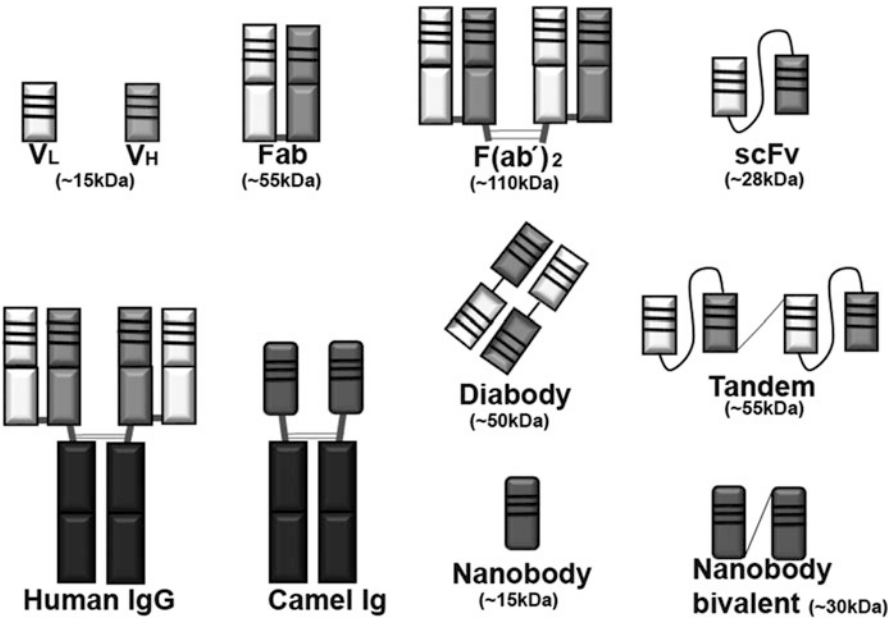

Fig. 1 Antibody formats. Schematic representation of whole human immunoglobulin and camelid Ig, as well as the different fragments obtained by enzymatic digestion or DNA recombinant techniques. Ig means immunoglobulin, V_H and V_L means heavy and light variable domains of antibodies, *Fab* fragment antigen binding, *scFv* single-chain variable fragment

for humans. From these findings, it has been possible to generate neutralizing antibody fragment against them, either through classical immunization and/or by means of the recovery of the immunoglobulin repertoire, its display on bacteriophages, and its maturation by directed evolution. The combination of these methodologies has made possible to generate engineered antibodies that have yielded molecules with remarkable properties against scorpion toxins, which can be the birth and rise of the next generation of antivenoms.

Antibody Fragments

The immunoglobulins (Ig) exhibit a modular structure (Fig. 1) in terms of their functional domains. The molecular biology techniques allowed for the first time the cloning of the genes that encode whole Igs in expression vectors. Furthermore, the modules can be expressed independently maintaining their structure and functionality. This molecular repertoire has allowed the manipulation of Ig genes to express different antibody formats. In order to preserve its recognition properties, all the different formats must maintain the variable regions (Fv), which constitute the antigen-binding site (Fig. 1). Since antibody could be present in different formats, each molecule could bear different pharmacokinetic and stability properties

(Holliger and Hudson 2005). Antibody gene cloning of a repertoire from any organism or source is the first step toward antibody fragment handling, characterization, and/or eventual maturation. An advantage of the cloning of antibody fragments is the possibility to produce them in several expression systems like bacteria, yeast, eukaryotic cells, insects, and plants (Verma et al. 1998). The heterologous expression of recombinant antibodies in bioreactors can be optimized in order to obtain reproducibly high yields of functional products with high purity and homogeneity. When animals are used to obtain antibodies, reproducible quality and homogeneity in these products are not guaranteed. At the end, the bottom line and most important issue is that animals are no longer required for antibody production.

Two of the most widely used recombinant antibody formats include fragment antigen-binding (Fab) (55 kDa) and single-chain variable fragment (scFv) (28 kDa). ScFv consists of a heavy variable domain (V_H) and a light variable domain (V_L) joined by a flexible linker (often 15 amino acid residues) which prevents domain dissociation (Fig. 1). A Fab, additionally to Fv domains, contains the constant heavy and light domains (C_{H1} and C_{L1}), joined by disulphide bonds, which makes unnecessary the presence of a peptide linker (Fig. 1). Fab and scFv fragments usually retain their antigen recognition properties as the parental Ig while showing improved pharmacokinetics specially related to tissue penetration as compared to $F(ab')_2$ (Holliger and Hudson 2005). Other formats have been constructed like dimeric, trimeric, tetrameric, or tandem scFv just to mention some of them (Fig. 1).

Other interesting recombinant antibody fragments have been obtained from different animal sources, like camelids and cartilaginous fishes (de Marco 2011). Particularly, the camelid immunoglobulins (IgG2 and IgG3) are constituted by a single heavy chain, so named heavy-chain antibodies (HCab or V_HH). Despite being constituted by a single heavy chain, they are fully functional in terms of their antigen recognition capacity. Furthermore, V_HH fragments share a high sequence identity with human V_H3 family, the heavy-chain family most abundant in the human antibody repertoire which has been sequenced and reported in antibody repository databases. This property makes these molecules easy to be humanized in order to decrease the anti-camel antibody response. As already mentioned, the modular characteristics of immunoglobulin chains have facilitated the expression of only the V_HH variable domain in yeast or bacteria. These heavy variable domains are called nanobodies (Nb) (15 kDa) (Fig. 1) due to their molecular dimensions which are in the nanometer range. These molecules represent an interesting source of antibody fragments for therapeutic purposes, due to their fast biodistribution and high stability (van der Linden et al. 1999).

Antibody Libraries

By means of molecular biology techniques, it is also possible to generate recombinant antibody libraries with a wide diversity of protein sequences. Such sequence diversity can be obtained from many animal sources including humans.

According to the source and the way in which the genetic material is prepared for the construction of the libraries, they are classified as naïve, immune, or synthetic (McCafferty et al. 1990). Antibody libraries are constructed from a variety of sequences of Ig genes contained in the B lymphocyte repertoire, from either a single organism or a population. The B lymphocytes could be isolated from lymphoid organs such as the thymus, spleen, and bone marrow or even from peripheral blood. Total RNA from B cells containing mature antibody transcripts is used as the source to amplify the DNA segments that encode antibody variable regions. The cDNA is generated by RT-PCR (reverse transcription polymerase chain reaction), which allows to copy the sequences encoding the V_H and $V_{L\lambda}$ or $V_{L\kappa}$ from RNA. The information contained in the cDNA is amplified using oligonucleotides specific for V_H and V_L families. The sequences can be randomly combined and cloned to constitute a library of antibody fragments, usually scFv or Fab formats. The libraries of DNA segments encoding the repertoire of antibodies are cloned into display or expression vectors (Fig. 2). The libraries of recombinant antibodies have the advantage that the time required to produce an antibody fragment is significantly lesser in comparison to the hybridomas (weeks compared with several months). In addition, high-quality libraries can be generated which can be subjected to in vitro selection methods to isolate antibodies specific for any antigen, turning antibody libraries into efficient sources of therapeutic antibodies.

Antibody Displaying Systems and In Vitro Selection

One of the main advances in the development of recombinant antivenoms has been the introduction, 20 years ago, of the in vitro display technologies for the selection of antibodies. The most used methods is antibody phage display (McCafferty et al. 1990) and yeast display systems (Boder and Wittrup 1997; Fig. 2). The principles behind displaying methods show that all of them are similar, in which a repertoire of proteins are expressed on the surface of phage or yeast. The phages or yeasts become a carrier system containing the genetic information for expression, while exposing the protein of interest fused to a phage coat protein or yeast surface protein while retaining the ability to replicate. The display techniques are based on the coupling of genotype (the encapsulated DNA encoding the displayed antibody) and phenotype (the displayed protein). These coupled genotype and phenotype elements allow enrichment selections from which antibodies with high specificity and affinity are isolated from large libraries encoding millions of variants. Phage display is relatively simple in terms of manipulation and does not require special equipment and is inexpensive. For isolating specific variants, biopanning processes are performed (Fig. 2). The biopanning procedure involves the searching of specific clones that recognize a particular antigen. The antibody library is brought into contact with an antigen either immobilized or in solution. Exhaustive washing is performed to eliminate nonspecific binders to finally obtain only specific antibodies than bind the antigen. During the biopanning

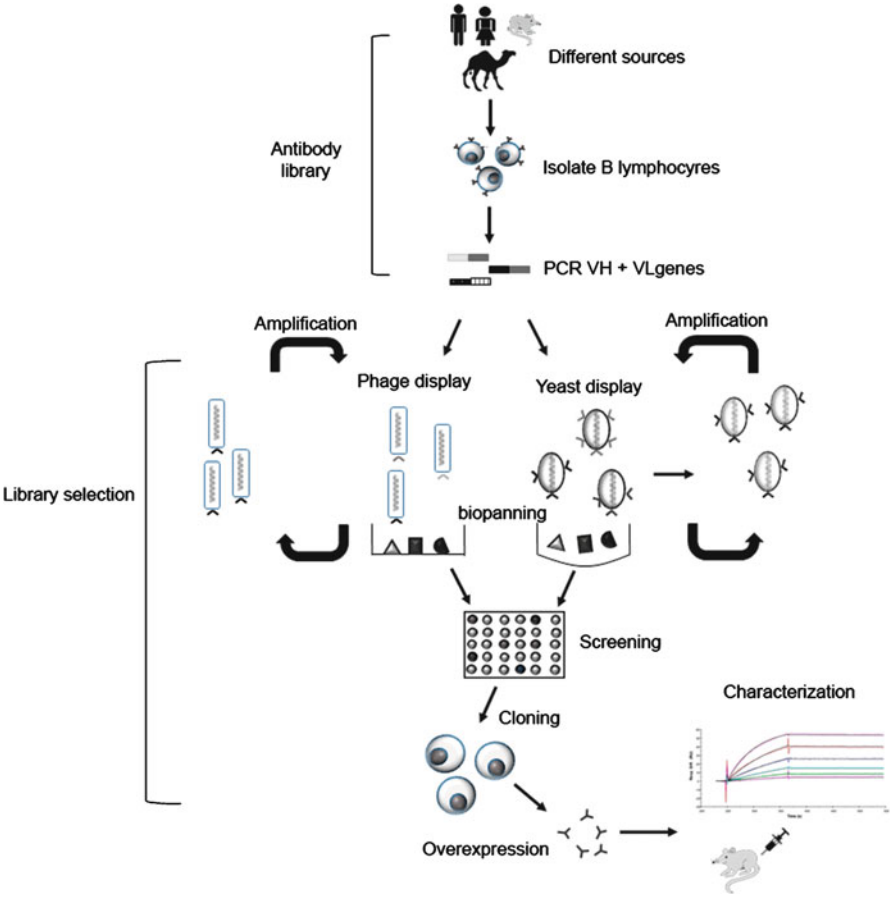

Fig. 2 Schematic representation of the basics of a library construction and isolation of specific antibodies. The different steps of selection and characterization of an antibody by phage or yeast display are shown. The biopanning conditions can be conveniently modified as mentioned above

steps, different conditions can be tested, which include variation in antigen concentration, the use of denaturing agents, or incubation at high temperatures to be successful in selecting the best binders.

Afterward, the specific phage-antibody population is amplified and subjected to a new biopanning round. After performing several rounds of panning, it is necessary to screen individual clones to be tested in antigen recognition assays. Thereafter, the isolated antibodies must be expressed in a soluble form to perform their characterization in terms of affinity, stability, and neutralization capacity (Fig. 2). If the properties of an isolated antibody (neutralization capacity and/or stability) do not fulfill the desired requirements, it can be maturated by directed evolution.

Essentially, the maturation process by directed evolution and phage display consists in the construction of a mutant library by random (error-prone PCR) or

site-specific mutagenesis from which a determined clone that shows improved recognition for its target can be isolated. Using phage display, high-diversity libraries can be evaluated. The new set of variants isolated from the mutant library are again subjected to biopanning processes. In vitro selection of the best antibodies can be successfully performed by including astringent conditions, as already mentioned. This process is repeated several times in order to obtain the best binders (Fig. 2). The most used antibody format in phage display is the single-chain one (scFv) in which the variable region of a heavy chain is joined to the variable domain of a light chain by means of linker peptide (15 amino acids long; see below for more details). This format does not require posttranslational modifications like glycosylation.

Yeast display has some advantages over phage display. Codon usage is similar to mammals. Eukaryotic proteins from different sources can be folded as in their respective cells of origin. Biopanning is performed into a cell sorter by flow cytometry methods (fluorescein-activated cell sorter, FACS), which is very efficient for isolating molecules of interest with determined properties. This system is very useful for displaying mammalian proteins like whole antibodies (with translational modifications like glycosylation) and for the selection of antibody fragments from small libraries (3×10^5 cells) with important features like affinities in the femtomolar range (Boder et al. 2000). However, the use of this display system for the isolation of antibodies against scorpion venoms has not been reported.

Antibody Fragments Against Scorpion Neurotoxins

Scorpion venoms are a complex mixture of components mainly proteins and toxic peptides (Possani et al. 1999; Rodriguez de la Vega et al. 2013). As mentioned above, the current production of scorpion antivenom is by hyperimmunization of horses. This process is carried out using water extracts from macerated telsons that contain the whole scorpion venom. All the immunogenic, toxic, and nontoxic molecules stimulate the animal immune response to generate polyclonal antibodies. Therefore, the obtained sera will contain antibodies that recognize toxic and nontoxic components in the venom. This nonspecific response makes that the amount of heterologous antibodies administered to a patient be high. Pucca and collaborators (2011) reported that equine serum antivenom only contains 1–2.5 % of specific antibodies, which means that a large portion (97.5 %) are non-neutralizing. Likewise, variations in concentration and biochemical characteristics of the components of the venoms, even intraspecies, have been observed. Consequently, the generated antibodies bear different specificities thus decreasing the therapeutic efficiency of antivenom (Chippaux and Goyffon 1998).

In the past 40 years, there has been a great advance in the characterization of scorpion venoms. These studies showed that within the most dangerous scorpion venoms for humans, neurotoxins are the main components of medical importance due to their high toxicity and relative abundance. Furthermore, in some cases but at a lesser proportion, the presence of some enzymes is also medically important

(Venancio et al. 2013). Neurotoxins bind to specific ion channels, mainly sodium and potassium channels (Na_v and K_{av}) with affinities in the nanomolar range (Possani et al. 1999). This binding causes modifications of the mechanisms of channel gating and hence the functions of the central and peripheral nervous system, preventing the transmission of nerve impulses, which affect vital physiological activities, resulting in fatal sting accidents. Particularly the mammal sodium channel toxins exhibit a molecular weight around 6,000–7,500 Da composed by 60–76 amino acid residues, with a high amino acid identity. The regular structure of these toxins is a highly conserved cysteine-stabilized α/β (Cs-α/β). The action of these toxins is similar. They bind to the sites 3 or 4 of the Na_v affecting the channel gating mechanisms (Possani et al. 1999; Rodriguez de la Vega et al. 2013).

This knowledge has prompted the generation of specific antibodies against the major toxic components. At this point, it is important to mention that it has been observed that neutralizing the toxic effects of main neurotoxins, the venom toxicity is eliminated (Licea et al. 1996; Mendes et al. 2008).

The modern approaches to obtain optimized antivenoms will be focused on the specific neutralization of the main venom components against humans. The neutralization capacity would be based on the binding to specific epitopes of the main toxins with appropriately optimized antibodies. The antibody variable domains should recognize the toxic epitopes or neighbor regions to prevent channel binding of the neurotoxins and thus eliminating their toxic effect. Key residues which interact with the sodium channel modifying the gating mechanism have been identified. In summary, it is important to remark that the antibody fragments exhibit good specificity, high affinity, fast biodistribution, fast toxin elimination, and full neutralization.

Nowadays, there are great examples of monoclonal and recombinant antibody fragments which neutralize key neurotoxins from venoms of the most poisonous members of the Buthidae family of scorpions. Those examples are described below.

The Case of *Androctonus australis* Scorpion

Buthidae scorpion family is one of the largest and most widely distributed. Several members of this family are highly toxic. The notoriously dangerous species are among the genera *Androctonus, Centruroides, Tityus, Leiurus,* and *Parabuthus*.

Androctonus australis is one of the most dangerous scorpion species of North Africa, with an average of 540 stings per 100,000 inhabitants, being this species responsible of 80 % of the envenoming cases (di Tommaso et al. 2012). The venom of the scorpion *A. australis* is one of the most toxic with a 50 % lethal dose (LD_{50}) of 3 μg of venom per 20 g of mouse (C57BL/6 strain) by subcutaneous route (s.c.). This venom contains two main neurotoxins which affect mammal sodium channels named AahI and AahII (Bahraoui et al. 1988; Clot-Faybesse et al. 1999).

The efficiency of the horse antivenom $F(ab')_2$ format (fabotherapic) against this venom was not satisfactory because it did not completely reverse the envenoming syndrome. A nonoptimal neutralization capacity has been noted due to a deficient standardization of envenomation levels and prompt administration by intravenous route of an adequate amount of antivenom (Abroug et al. 1999; Hammoudi-Triki et al. 2004). Looking for an alternative to optimize the current antivenom immunotherapy, the use of a combination of $F(ab')_2$ and Fab antibody fragments, obtained by immunization and their respective purification and digestion processes, showed significant advances for eliminating envenomation symptoms (Sami-Merah et al. 2008). However, other factors are involved in using this combination of antibody fragments, as are the ways of administration (subcutaneous or intravenous), depending on the level of envenomation, which turned it impractical prompting necessary to improve immunotherapy (Hammoudi-Triki et al. 2007).

In order to achieve an improvement of the medical treatment of scorpion envenoming, hybridomas from mice were used to generate specific monoclonal antibodies (Mab) to neutralize the most toxic neurotoxins. Two monoclonal antibodies were obtained (4C1 and 9C2) with a great affinity and neutralizing capacity against AahI and AahII toxins (see Table 1) (Bahraoui et al. 1988; Clot-Faybesse et al. 1999). Individually, they had the capacity of neutralizing the venom at low doses ($2LD_{50}$), but in combination, they could neutralize the whole venom at $4LD_{50}$ (Bahraoui et al. 1988; Clot-Faybesse et al. 1999). From these antibodies the authors generated recombinant formats in an *Escherichia coli* expression system, like Fab (Aubrey et al. 2004), scFv (Devaux et al. 2001; Mousli et al. 1999), tandem scFv (Juste et al. 2007), and diabody (Aubrey et al. 2003; di Tommaso et al. 2012). These formats maintain their recognition properties against toxins; however, their neutralization capacity can be affected. Recently, the crystallographic structure of antibody Fab 4C1 in complex with AahII and the model by docking of 9C2 with AahI was determined. This work provides useful information about the immunological determinants of these toxins and how the antibodies exert their neutralizing capacity based on the recognition of specific sites that previously had been defined as key interaction surfaces between the toxin and the sodium channel. Additionally, in this contribution, it was suggested how small amino acid side chain variations in the antibody and distinctive orientation of toxin determine the binding specificity and therefore the absence of cross-reactivity with very similar toxins. Finally, the conformational structure differences within the interaction site of antibody in complex with the toxin or non-bound to it were shown (Fabrichny et al. 2012).

Pharmacokinetic studies of envenoming have allowed a better understanding of immunotherapy. Biodistribution studies showed that the low molecular weight toxins (approx. 7 kDa), like AahI and AahII, showed a faster distribution as compared to some of the antibody formats (Hammoudi-Triki et al. 2007). The DNA sequence encoding the variable domain of Mab 9C2 and 4C1 was converted to scFvs (28 kDa) (Devaux et al. 2001; Mousli et al. 1999). These scFvs showed better pharmacokinetic properties than the whole IgG. 9C2 scFv antibody fragment gave reasonable yields (1–2 mg/L) but showed a propensity to form dimers and a lesser neutralizing capacity as compared with its corresponding Mab.

Table 1 Antibodies generated against *Androctonus australis* and *Tityus serrulatus* toxins

Name	Affinity (nM)	Venom neutralizing capacity	Antigen	Source	Fragment	References
4C1	0.8	+−	AahII	Mouse	scFv	Bahraoui et al. 1988
9C2	0.15	+−	AahI	Mouse	scFv	Clot-Faybesse et al. 1999
Db9C2	0.08	ND	AahI	Mouse	Diabody	Aubrey et al. 2003
Db4C1op	ND	+−	AahII	Mouse	Diabody	Aubrey et al. 2003
T94H6	0.1	+	AahI and AahII	Mouse	Bispecific tandem scFv	Juste et al. 2007
NbAahI′22	55.8	ND	AaHI′	Camel	Nanobody	Hmila et al. 2008
NbAahI′22	44.8	ND	AaHI′	Camel	Nanobody bivalent	Hmila et al. 2008
NbAahI′-Fc	1.21	ND	AaHI′	Camel	Nanobody-human Fc	Hmila et al. 2008
NbAaHII10	0.49	ND	AaHII	Camel	Nanobody	Abderrazek et al. 2009
NbAaHI′F12	0.31	ND	AaHI′	Camel	Nanobody	Hmila et al. 2010
NbF12-10	19.2 4.91	+	AHI′ and AaHII	Camel	Nanobody bispecific	Hmila et al. 2010
Nb22-10	0.56 2.15	ND	AHI′ and AaHII	Camel	Nanobody bispecific	Hmila et al. 2010
2A	ND	ND	Ts1	Human	scFv	Pucca et al. 2011
15e	ND	+−	Ts1	Human	scFv	Amaro et al. 2011

+ Absence of envenoming symptoms
− Presence of envenoming symptoms and death
ND Not determined

Based on these results, some efforts have been focused on the development of alternative formats, like nanobodies, to improve their immunotherapeutic properties related to their smaller size and consequently their faster tissue penetration. In a previous work consisting in immunizing dromedaries with the toxic fraction of venom called AahG50 which contained AahI and AahII toxins, it was shown that it was possible to obtain neutralizing antibodies against those toxins. Based on this precedent, the Tunisian group from Institut Pasteur repeated the process. Subsequently, the genetic material from lymphocytes of these animals was extracted. Following the procedures already described

("Antibody Libraries" section), this group constructed a library of nanobodies (Nb) with the aim of isolating specific nanobodies against the main toxins from *A. australis* venom.

Nanobodies are the smallest in size (15 kDa) (Fig. 1), therefore turning them interesting molecules. This antibody version presents high stability and diffusion capacity compared to the other reported formats.

From the immune nanobody library, using phage display technology, some antibody fragments specific for Aah toxins were isolated (Hmila et al. 2008). The first approach was conducted to perform the biopanning steps against the toxin AahI′. This toxin differs by only one amino acid residue as compared to AahI but conserves the same antigenic and structural motifs, which allowed to expect that the selected antibodies could have recognized both toxins (AahI′ and AahI). After several rounds of biopanning, a nanobody called AahI′22 was isolated (see Table 1). Using this nanobody, the authors generated other formats to improve stability and antigen binding by avidity effects. The formats were a bivalent tandem Nb (two V_HH domains joined by a peptide linker) and a chimeric Fc-human-Nb (HCAb) which consisted in a V_HH domain fused to human constant domains. These antibodies, particularly the bivalent tandem Nb exhibited a superior neutralizing capacity as compared to all previously antibody formats reported (Hmila et al. 2008).

In the same way, a new immune nanobody library was generated and subjected to biopanning against the most toxic component from *A. australis* venom, the AahII toxin. The authors isolated a set of antibodies with nanomolar and subnanomolar affinities (Abderrazek et al. 2009). However, most of them bound to a non-neutralizing epitope. These results show the importance of generating antibodies that recognize neutralizing epitopes that interact with the sodium channel. The message here is that high affinity values (K_D) of an antibody against a toxin are not necessarily related to the antibody-neutralizing capacity. The selected nanobody NbAahII10, despite showing the best neutralizing capacity, did not bear the best affinity (see Table 1), but showed the slowest dissociation velocity ($K_{off} = 5.69 \times 10^{-4}$). These results make us learn that a slower dissociation kinetics is critical for a better neutralizing capacity. It is important to mention that the NbAahII10 had the capacity of neutralizing $7LD_{50}$ of AahII toxin in a molar ratio of 1:4 toxin to antibody (Abderrazek et al. 2009). In a subsequent report, this group isolated other Nb (NbAahI′1 F12) against AahI′ following the same strategy. This nanobody showed the best neutralizing properties highlighting the significant improvement in affinity (0.31nM versus 55.8 of Nb AahI′22; see Table 1) (Hmila et al. 2010).

According to previous reports (Hmila et al. 2008; Juste et al. 2007), the complete neutralization of the *A. australis* venom required at least two different neutralizing antibodies against AahI and AahII toxins. This antibody combination had the ability of neutralizing the whole venom analogously to the polyclonal antivenom used in current therapy. Based on these results, a bispecific Nb (NbF12-10) was constructed combining the best nanobodies isolated previously (Hmila et al. 2010). The recombinant antibody fragment generated is a very promising molecule. This bispecific

Nb bears a fully neutralizing capacity of $5LD_{50}$ of whole venom of *A. australis* scorpion, while the commercial antivenom $F(ab')_2$ only protected 50 % of animals with $4LD_{50}$ using more than 15 times with respect to the bispecific antibody. Moreover, an experiment in which it was simulated a real envenoming situation after scorpion sting (rescue test) was performed. Mice were injected subcutaneously with scorpion venom and when the animals showed envenoming symptoms (from mild to severe), antibodies where then administered. In this case, 100 % of mice survived after being envenomed during 15 min with $1.5LD_{50}$ of venom and rescued with the tandem nanobody administered intravenously. This process was repeated with the same animals 20 days after, previously demonstrating the absence of nanobodies in the serum or anti-nanobodies response by ELISA test (Hmila et al. 2010). This test showed an advantage in terms of safety that can provide the nanobody format. These results showed that these molecules exhibited superior neutralizing properties as compared to the commercial antivenom and some other murine formats previously generated to neutralize scorpion venoms from Africa. More importantly, these nanobodies already in preclinical tests exhibit better times of distribution and elimination (5–9 times faster) as compared to antivenoms in their $F(ab')_2$ format. Therefore, nanobodies prevented effectively the occurrence of organ damage or vital signs affectation (heart rate and arterial pressure changes) observed during critical stages of envenoming (Hmila et al. 2012). Although, nanobodies are nonhuman in origin, nowadays, this is the most important alternative antivenom against *A. australis* stings.

The Case of *Tityus serrulatus*

In Brazil approximately 45,000 cases of scorpion sting are reported annually. *Tityus serrulatus* scorpion is the most dangerous species of this country with a 1 % of mortality in children. This venom contains one relevantly toxic component, Ts 1 or gamma toxin. The Ts1 toxin is a very dangerous mammalian neurotoxin. Therefore, the development of antibodies that neutralize this toxin could prevent venom toxicity (Mendes et al. 2008). However, as already mentioned, there are important concerns about the use of heterologous antibodies.

Using phage display technology, two research groups undertook the task of obtaining human antibody fragments against Ts1 toxin. A research group from Sao Paulo University, using an academically available human scFv library (Griffin I), was capable to isolate an scFv called A2, after 4 biopanning rounds (Pucca et al. 2011). This antibody fragment resulted specific for Ts1 toxin. Its in vitro neutralizing capacity was tested by patch clamp experiments. These tests allowed to evaluate the effects of the toxin on ion channels expressed in mammalian cells through the ability of inhibiting Ts1 binding to the channel in the presence of A2 scFv antibody (Pucca et al. 2011). This preliminary report indicates the possibility of using human antibody fragments to neutralize Ts1 neurotoxins (see Table 1).

Similarly, Amaro and collaborators (Amaro et al. 2011) panned a human scFv library (Riano-Umbarila et al. 2005) against Ts1 toxin. Four cycles of biopanning

were performed. Two scFv were isolated; however, only one (15e) could be expressed in soluble form. The scFv 15e was evaluated in in vivo neutralizing tests and cross-reactivity. This antibody had the property of binding to the toxic peptides of two species of genus *Tityus*: *T. pachyurus* (Tpa2) and *T. cambridgei* (Tc49b) (Possani et al. 1999; Rodriguez de la Vega et al. 2013), which are two lethal toxins for mice. It is important to mention that these species are responsible, although to a lesser extent, for fatal cases in Colombia and Panama. Cross-reactivity of antibodies that recognize scorpion toxins can be understood in terms of the high amino acid sequence identity of these neurotoxins, in this case 60 % of identity (Amaro et al. 2011; Rodriguez de la Vega et al. 2013). ScFv 15e might have recognized a determined 3D structure segment (epitope) of the toxins which might be similar in various related toxins. This phenomenon has been observed in other South American scorpion species using antivenom generated with different scorpion venoms (D'Suze et al. 2007). The authors mentioned that scFv 15e is a recombinant human antibody capable of recognizing related toxins from the venom of some species of the genus Tityus. The scFv 15e could have been subjected to in vitro maturation by directed evolution and phage display in order to obtain fully neutralizing variants.

In terms of neutralizing capacity, scFv 15e protected 70 % of experimental mice when using $1LD_{50}$ of toxin and delaying the appearance of envenoming symptoms as compared with the control mice.

It is important to be aware that it is common to isolate antibodies with a low affinity and consequently non-neutralizing when naïve libraries are panned. If the library contains a large diversity, the probability of isolating specific antibodies with high affinity is higher. Being aware that it is not possible to immunize humans with toxins by ethical reasons, there are two alternatives: one is to immunize transgenic humanized mice whose sequences encoding Ig have been replaced by their corresponding human genes. The second option is the in vitro maturation of antibodies that present low affinity and neutralizing capacity by directed evolution and phage display. This last strategy is very powerful allowing to perform in vitro the equivalent of a natural process (somatic hypermutation) to accelerate the improvement of several antibody properties like thermodynamic stability and affinity.

The Case of *Centruroides* Scorpions

The development of human antibody fragments that neutralize the venom of different species of scorpions of genus *Centruroides* has been one of the most extensive and successful cases.

Mexico is one of the three regions with the highest number of accidents caused by scorpion stings worldwide. In this country, the species of scorpions of the genus *Centruroides* are lethal to humans. Three of these species, *C. noxius*, *C. suffusus suffusus,* and *C. limpidus limpidus*, stand out due to the high toxicity of their

Table 2 Murine antibodies generated against Cn2 toxin of *Centruroides noxius* scorpion

Name	Affinity (nM)	Venom neutralizing capacity	Source	Fragment	References
BCF2	ND	ND	Hybridoma	IgG	Zamudio et al. 1992
BCF2-Fab	ND	+	Hybridoma	Fab	Licea et al. 1996
Ch-BCF2	ND	+	Recombinant	Chimeric	Selisko et al. 1999
scFv BCF2	1.10	–	Recombinant	scFv	Juarez-Gonzalez et al. 2005
scFv-G5	0.43	–	Directed evolution	scFv	Juarez-Gonzalez et al. 2005
scFv-B7	0.71	–	Directed evolution	scFv	Juarez-Gonzalez et al. 2005
scFv-Triple mutant	0.018	+	Site-specific mutagenesis	scFv	Juarez-Gonzalez et al. 2005

+ Absence of envenoming symptoms
– Presence of envenoming symptoms and death
ND Not determined

venoms causing a significant number of accidents related to the total number of accidents reported (more than 250,000 annually). Some of the neurotoxins isolated from this scorpion venom are Cn2, Css2, and Cll1 and Cll2 toxins from *C. noxius, C. s. suffusus,* and *C. l. limpidus,* respectively (Possani et al. 1999; Rodriguez de la Vega et al. 2013). These toxins have high amino acid sequence similarity (at least 85 %) and function as sodium channel modulators (Possani et al. 1999; Rodriguez de la Vega et al. 2013). As mentioned above, neutralizing these molecules, the toxicity of whole venom is eliminated (Riano-Umbarila et al. 2011; Riano-Umbarila et al. 2005; Zamudio et al. 1992).

In the beginning, using hybridoma technology, monoclonal antibodies were generated against Cn2 toxin (Zamudio et al. 1992). The Mab called BCF2 was obtained. This antibody protected 40 % of experimental mice when using $7.5LD_{50}$ and delayed the appearance of envenoming symptoms in in vivo neutralization tests. Starting from the whole BCF2, Fab fragments were generated by trypsin digestion of antibody. In vivo tests revealed that the Fab format showed a better neutralizing capacity than the whole antibody. One mg of Fab fragments was capable of neutralizing $43LD_{50}$ of *Centruroides noxius* scorpion venom in comparison with whole antibody BCF2 which neutralized only $28LD_{50}$ (Table 2) (Licea et al. 1996). Based on these results and using molecular biology techniques, a recombinant chimeric version of Mab BCF2 was generated. In the chimeric molecules, the DNA segments encoding the Fab were a combination of heavy and light variable murine domains joined to human heavy and light constant domains. This construction was expressed in bacterial systems, with good yields (Selisko et al. 1999). However, this product presented a low protection. Afterward, the scFv format from BCF2 antibody was produced, but this antibody fragment lost its neutralizing capacity compared with the parental whole antibody

(Juarez-Gonzalez et al. 2005). The generation of some antibody formats from an Ig can modify its neutralization properties. Some examples of these effects have been already observed by other authors (Poon et al. 2002). In the case of BCF2 scFv format, the authors showed that the drawback is caused by a lower stability of the scFv fragment. This affectation can be intrinsic to BCF2 scFv sequence but not a general problem of scFv format. The scFv BCF2 was subjected to directed evolution. The process allowed to perform three important mutations which in the context of the scFv BCF2 resulted in a recovered full neutralizing capacity being even superior to the parental BCF2 antibody and also showing a very good stability (Juarez-Gonzalez et al. 2005). This antibody has important properties; however, it is still murine in origin.

A significant advance was achieved with the construction of nonimmune human scFv library, which was subjected to biopanning against Cn2 toxin using phage display (Riano-Umbarila et al. 2005). Two scFv variants named C1 and 3F were isolated. These antibodies bound to Cn2 at different epitopes; however, they were not neutralizing due to a low affinity for the target (Table 3). Their affinities were comparable to the ones corresponding to antibodies produced early in the immune response. The scFv 3F was subjected to three different cycles of maturation by directed evolution and phage display. Each of the variants isolated in every round was characterized (see Table 3). Finally, scFv 6009F was obtained which showed a high affinity and full neutralizing capacity against Cn2 toxin (Table 3). This was the first reported human recombinant antibody fragment capable of neutralizing, by itself, a whole scorpion venom (Riano-Umbarila et al. 2005). It has been shown recently that scFv 3F recognizes several scorpion toxins from the genus *Centruroides*, like Css2 toxin. This behavior was expected, as already mentioned, due to the high amino acid sequence similarity among scorpion toxins from the same genus.

ScFv 3F was also subjected to three maturation cycles by directed evolution and phage display against Css2 toxin. The best fragment isolated was called 9004G (Riano-Umbarila et al. 2011). The comparison between both maturation processes (against Cn2 and Css2) allowed to identify key amino acid residue changes. The mutation (V101F) located at CDR3-V_H domain of scFv 6009F was incorporated into scFv 9004G using site-specific mutagenesis. The resulting scFv was called LR, which conferred total protection against $3LD_{50}$ of whole venoms from *C. noxius* and *C. s. suffusus* in preincubation neutralizing tests using a low molar ratio (1:3 toxin to antibody). Furthermore, this antibody was capable of rescuing 90 % and 100 %, respectively, of mice previously envenomed with $3LD_{50}$ of *C. noxius* and *C. s. suffusus* venoms (Riano-Umbarila et al. 2011; Table 3). It is important to notice an emphasis raised by authors on the importance of using fresh venoms to avoid the loss of toxic activity during lyophilizing and storage steps. It is noteworthy that this is the first report about exploiting the cross-reactivity of an antibody that allowed this human scFv antibody fragment to neutralize two different scorpion venoms. The antibody cross-reactivity properties prompted the authors to exploit it in order to optimize the neutralization of toxins from different species.

Table 3 ScFv human fragments against toxins from *Centruroides* scorpion venoms

Name	Affinity (nM)	Neutralizing capacity (for toxin)	Antigen	Venom neutralizing capacity	Fragment	References
C1	540	–	Cn2	ND	scFv	Riano-Umbarila et al. 2005
3 F	12.50 16.60	–	Cn2 and Css2	ND	scFv	Riano-Umbarila et al. 2005
6 F	16.80	–	Cn2 and Css2	ND	scFv	Riano-Umbarila et al. 2005
610A	1.04	–	Cn2 and Css2	ND	scFv	Riano-Umbarila et al. 2005
6009 F	0.21 0.54	+	Cn2 and Css2	++	scFv	Riano-Umbarila et al. 2011; Riano-Umbarila et al. 2005
9004G	0.21	+	Cn2 and Css2	++	scFv	Riano-Umbarila et al. 2011
LR	0.05 0.09	+	Cn2 and Css2	++	scFv	Riano-Umbarila et al. 2011
D4	0.60	+	Cn2	++	Diabody	Rodriguez-Rodriguez et al. 2012
LER	0.03	+	Cn2	++	ScFv	Rodriguez-Rodriguez et al. 2012
202 F	8.10 25.10	+	Cn2 and Cll1	ND	scFv	Riaño-Umbarila et al. 2013

+ Absence of envenoming symptoms
– Presence of envenoming symptoms and death
ND Not determined

Other strategy was conducted in order to improve the properties of human antibody 6009F through the generation of a dimeric scFv. The diabody was obtained by shortening peptide linker from 15 to 5 amino acid residues (Holliger and Hudson 2005). Diabody 6009F lost some of its original neutralizing capacity, as revealed by the fact that despite that deaths were not detected in in vivo tests, some envenomation symptoms were observed (Rodriguez-Rodriguez et al. 2012). In order to recover full neutralization capacity, directed evolution techniques were performed into diabody 6009F. Diabody D4 variant was isolated after a single cycle

of directed evolution and phage display. Only one mutation located at FW2 of V_H (E43G), resulted in the full recovery of the neutralizing capacity and a significant improvement of the stability of D4. Diabody E43G mutation was inserted into scFv LR (Riano-Umbarila et al. 2011). The resulting antibody, called LER, presented a high stability: Tm 62.8 °C compared with 54.7 °C from diabody 6009F and a significant refolding capacity (60 %). These properties only have been observed in camelid nanobodies (Perez et al. 2001; van der Linden et al. 1999). The most important property of LER scFv antibody is the capacity to neutralize $2LD_{50}$ of Cn2 toxin at a molar ratio of 1:1 toxin to antibody (Rodriguez-Rodriguez et al. 2012).

Other interesting contribution to the understanding of toxin neutralization at atomic level was reached by co-crystallization of the antibody 9004G with Cn2 (Canul-Tec et al. 2011). This study allowed to observe that some residues that conform the epitope of toxin Cn2 overlap with residues already determined as essential for Cn2 binding to target channel (e.g., Y14, E15, L17, L19, N22, Y24, among others). The binding of the antibody to Css2 toxin involves mainly residues that are also present in the complex with Cn2 toxin and correspond to key residues detected during the maturation processes that led to the increase of affinity of the antibody. The information emerged from 3D structure determinations and bio-computational technologies can be used as rational or semi-rational approaches to design and mature antibodies (Barderas et al. 2012, 2008).

Finally, scFv C1 mentioned above (Riano-Umbarila et al. 2005) was subjected to an exhaustive biopanning process against Cll1 and Cll2 toxins from *C. l. limpidus* scorpion (Riaño-Umbarila et al. 2013). A couple of maturation processes were necessary to isolate scFv 202F. This antibody resulted from the combination of random and site-specific mutational approaches. The scFv 202F is very interesting, despite its low affinity compared with the other scFv derived from scFv 3F reported (see Table 3). It was able to inhibit completely the toxic effect of Cll1 and Cn2 toxin, allowing the survival of mice challenged with each one of these toxins (Riaño-Umbarila et al. 2013). The authors commented that they will continue performing maturation processes to improve the affinity of scFv 202F and looking for new specific variants against Cll2 toxin from *C. limpidus limpidus* scorpion. Considering the composition of the venom of *C. l. limpidus* that contains at least two important neurotoxins, it means that more than one antibody will be required to completely neutralize this venom, as is the case of *A. australis* venom. Alternatively, the isolation of an antibody having high cross-reactivity against these two toxins could be the solution to this issue.

Conclusions and Future Directions

The optimization of antivenoms is experiencing an important development supported by research advancements on both venoms and antibody engineering. As a consequence, the aim of inhibiting the toxic effect of venom components has been simplified by the generation of antibodies with high specificity and affinity directed against each medically important component of the different venoms.

Based on the characteristics of scorpion toxins against humans (very toxic, highly abundant, and low weight molecular proteins), antibody fragments in scFv or Nb formats can be considered as excellent candidates to constitute the next generation of antivenoms due to their good therapeutic properties as already mentioned: high specificity, affinity, and fast body distribution.

Due to the relative low complexity of scorpion venoms in terms of the number of medically important toxins present, this has allowed the isolation of antibodies with just those properties. The described examples presented in this review point to the fact that one or two different antibodies bearing those properties can be the components of next generation of antivenoms for a determined scorpion species.

It is also important to know the details of each toxic component in terms of the number of neutralizing epitopes present in them. Safer antivenoms will be those which contain at least two different neutralizing antibodies against each toxic component of the venoms.

Therefore, in some cases, the participation of a set of different antibodies is necessary in order to reach a complete neutralization of a certain venom. Preferentially, this small cocktail of antibodies, with high specificity and affinity, should be human in origin to avoid secondary effects.

Advancements in antivenom optimization have been reached not only in scorpion issues but also against other medically important poisonous animals such as snakes (Chavanayarn et al. 2012), spiders (Bugli et al. 2008), and bees (Funayama et al. 2012).

However, these venoms are more complex in terms of the number of toxic components for humans specially snake venoms. It will be necessary to obtain neutralizing antibodies against each toxic medically important venom component. In order to reach that purpose, the main goal is the standardization of generation of high-quality libraries and high-throughput pipeline selections to enable the generation of specific antibodies more efficiently against several antigens simultaneously (Hust et al. 2011; Mersmann et al. 2010).

It is important to mention that this approach of production of recombinant antivenoms changes the use of animals (immunization, feeding, and care) by biological reactors (fermenters), which is advantageous due to the homogeneity and reproducibility of production and much more economic in terms of time and costs.

In summary, the in vitro generation of fully neutralizing antibodies of human origin with high affinity and specificity, fast biodistribution, and clearance of the toxic components of the scorpion venoms will be the most important issues concerning the next generation of antivenoms.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ Scorpion Venom Research Around the World: *Tityus serrulatus*
- ▶ Scorpionism and Dangerous Species of Mexico

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Poultry IgY Alternatives to Antivenom Production

7

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Abstract

Scorpions are venomous arthropods of the order Scorpiones, an old and very homogeneous group, comprising about 2,000 species worldwide. The genus *Tityus* is recognized as the most diverse and widely distributed in South America (Venezuela, Colombia, Brazil, Argentina, Panama, Costa Rica, Trinidad, etc.). In Venezuela, there are about 54 species of the genus *Tityus*. Scorpion's sting in Venezuela is an endemic and regional public health problem which brings medical interest. The hyperimmune serum that comes from horses is recognized as an effective treatment for scorpion envenoming. In Venezuela, IgY technology is being used for experimental production of polyclonal antibodies against scorpion venom of clinical and epidemiological importance. Although not yet applied at the healthcare level, studies have shown therapeutic utility at the experimental level. The IgY against this venom has potential value and possible future application in the therapy against accidents caused by these animals. Actually, due to the legal requirements for its use in medicine, IgY antibodies have not been used as a therapeutic tool, but it is important to note that there are advantages and potential for its use in scientific research.

Introduction

Scorpion envenoming is a major public health problem in tropical and subtropical areas of the world. This is due to its frequent occurrence and potential severity (Chippaux and Goyffon 2008).

Scorpions are among the oldest venomous arthropods, with no major morphological changes except for a decrease in size (González-Sponga 1992). However, differences have been described in the composition of venom from different species suggesting the existence of regional variations (species-specific) as a result of selective pressure of toxins (Borges et al. 2008). The scorpions are solitary and nocturnal, especially during the hot season. They often live in houses or residential areas, which explains the high incidence of scorpion stings that mainly affects children in many parts of the world (Chippaux 2012).

These arthropods are a very homogeneous group comprising about 2,000 species worldwide, out of which a few dozens are considered dangerous to humans, all belonging to the family Buthidae. These species, which are known to occur globally, are important from a medical point of view. The genera involved in the majority of deaths due to scorpionism are *Androctonus*, *Buthus*, and *Leiurus* in North Africa and the Middle East; *Parabuthus* in South Africa; *Mesobuthus* in India; *Centruroides* in Southern USA and Mexico; and *Tityus* in South America (Borges et al. 2006; Chippaux and Goyffon 2008).

Of the genus *Tityus*, approximately 197 species are known (González-Sponga 2004; 2006a, b; Gonzalez-Sponga and Wall-González 2007; Rojas-Runjaic and De Sousa 2007; Rojas-Runjaic and De Armas 2007; Rojas-Runjaic et al. 2008). *Tityus* is acknowledged as the second most diverse in the continent (González-Sponga

2004; Rojas-Runjaic and De Sousa 2007) and for being an exclusively Neotropical genus. *Tityus* species have been described in Venezuela, Colombia, Brazil, Argentina, Panama, Costa Rica, and Trinidad (Borges et al. 2006; Rojas-Runjaic and De Sousa 2007).

In Venezuela, about 54 species of the genus *Tityus* have been described, accounting for the largest number of species for a genus in the Venezuelan scorpionic wildlife. Most *Tityus* live in densely populated regions favoring accidental contact (Rojas-Runjaic and De Sousa 2007; Gonzalez-Sponga 2012). Scorpion stinging in Venezuela is of medical interest and is an endemic and regionalized public health problem (De Sousa et al. 2000; Quiroga et al. 2000).

The scorpion venom apparatus consists of a bladder comprising a pair of glands in the telson together in the last segment of the metasoma. This bladder is surrounded by a striated muscle layer which facilitates and regulates the ejection of venom. This capability could explain in part the variation in severity of symptoms as it will allow "dry" stings, that is, without the inoculation of venom (Chippaux 2012). Scorpion venom is a complex mixture of different active substances such as polypeptides and enzymes. Neurotoxins are the components of greatest medical importance, constituted by peptides capable of blocking important channels vital for homeostasis (Chippaux 2012). The classification of these toxins is based on four different criteria which involve the following: (1) the ion channels (in particular sodium, potassium, chloride, and calcium), (2) toxin's receptor site on the ion channel, (3) three-dimensional structure of the toxins, (4) and type of response induced that blocks and/or modifies the mechanism of opening and closing the ion channels of excitable membranes (Possani et al. 1999; Tan et al. 2006).

Once the venom is deposited deep into the subcutaneous tissue after the sting, complete absorption would occur in 7–8 h (70 % of the maximum concentration of poison in the blood is reached 15 min after the stinging). The severity of the envenoming is related to age (higher mortality is observed in children under 4 years old) and the size of the scorpion. The clinical manifestations can be divided into local and systemic.

Hospital treatment for scorpion envenoming, particularly of the *Tityus* genus, is basically aimed at maintaining vital functions. Mostly, it requires the use of antivenom to neutralize circulating toxins (Parrilla 1999; Mota and Sevcik 1999). One of the therapeutic strategies for neutralizing the toxic effects exerted by molecules present in venoms of medical importance is the administration of venom-specific antibodies. The administration of such antibodies modifies the bioavailability of active venom molecule(s) through complex formation with the toxin(s) for later disposal. The successful use of such antibodies depends, among other factors, on the ability of these antibodies to modify the interaction between toxins and their pharmacological receptors and the kinetic properties of antibodies, toxins, and of antigen-antibody complexes.

Intravenous administration of equine antivenom is the only scientifically validated treatment for envenoming (Alagon 2002; Theakston et al. 2003). The active principles of the immune sera are polyclonal antibodies. The first antivenom was developed in the late nineteenth century in France (Phisalix and Bertand 1894;

Calmette 1894). From these investigations it began the development of antivenom in different parts of the world by using Calmette's protocol. Its production was quickly established at the Butantan Institute, Brazil, in 1905. In subsequent decades, other regional laboratories began producing these immunobiological products, and currently, there are producing centers in Mexico, Costa Rica, Colombia, Venezuela, Ecuador, Peru, Bolivia, Brazil, Argentina, and Uruguay. The World Health Organization (WHO) includes in its "Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins" the list of antivenoms and species whose venoms are neutralized by different antivenoms (Gutierrez et al. 2007).

Various antivenom formats are available either using purified immunoglobulins (antibody IgG), different fragments of immunoglobulins ($F(ab')_2$, Fab, scFv), or different arrangements of these fragments (scFv dimer, tandem, nanobodies, human Fc nanobody) (Espino-Solis et al. 2009). Currently, antivenoms are very pure fragments of high-power neutralizing antibodies, which, if properly purified and stabilized, are very well tolerated by patients. The manufacture of antivenoms has benefited from advances in biochemistry and immunology in the last decades. The technologies used in production of these antivenoms vary according to region. In general terms, the production of antivenoms is based on three types of processes: (a) treatment with pepsin and ammonium sulfate precipitation, which generates antivenom composed of immunoglobulin's $F(ab')_2$ fragments; (b) ammonium sulfate precipitation to generate full-IgG preparations; and (c) caprylic acid precipitation to purify complete IgG molecules (Gutierrez and Leon 2009). In Latin America, the vast majority of producers use the horse for immunization with venoms. In the case of Bolivia, donkeys and llamas are used due to their better adaptability to the altitude of La Paz, where the producing laboratory is located (Gutierrez et al. 2007). In Venezuela, the antidote for envenoming by scorpion is produced by the *Biotecfar* (Center of Biotechnology of the Faculty of Pharmacy, Central University of Venezuela). This antivenom is produced by hyperimmunization of horses (*Equus caballus*) basically with venom of *Tityus discrepans*. Hyperimmune plasma is processed to purify the horse immunoglobulin G. The antibody then is subjected to enzymatic treatment with pepsin to obtain $F(ab')_2$ and F (ab') (Poggioli De Scannone 1996).

To ensure the potency and biological safety of these products, the greatest efforts must be made always maintaining the recommendations of the WHO (2010). This should include the development of new research and production laboratories as well as improving the current test systems. Today, there are different protocols for evaluating the potency of antitoxins that are used by different countries. The recommendation would be to try to develop standardized protocols or uniformity at least within the same region or country (Theakston et al. 2003). In general, the preparation of antivenoms from animals has represented trauma and suffering during blood collection, as well as the need for rapid processing to ensure their viability. This situation has led to the search for alternative methods (Lomonte et al. 2009; De Andrade et al. 2013).

Though the immunotherapy is used worldwide for the treatment of the envenoming by scorpions, in some cases it becomes necessary the application of several doses of the antivenom to neutralize the effect of the venom. This might be explained due to the fact that the size of the therapeutic compound exceeds the size

of the toxin. It is assumed that at least a fraction of this $F(ab')_2$ fails to diffuse readily from the vascular compartment and cannot reach and neutralize the toxin distributed over the various tissues (Chippaux 1998).

Nowadays, new alternatives are explored for the production of antivenoms against scorpion and snake venoms. The use of monoclonal antibodies and the preparation of recombinant ones are some of new alternatives that have been developed for substitution of immunotherapy. Immune and nonimmune antibody libraries, displayed on filamentous phages, have been used for the isolation of specific recombinant antibodies (Bahraoui et al. 1988; Clot-Faybesse et al. 1999; Mousli et al. 1999; Devaux et al. 2001; Alvarenga et al. 2005; Juste et al. 2007). The following necessary step after obtaining specific antibodies against toxic components of the venom is to study the possible side effects that could produce their injection into model animals.

Hmila et al. (2010) have obtained a bispecific nanobody from an immune library and phage display technology. Due to the nanobody's molecular structure and small size, it is stable and distributes very fast and easily into injected animals. Also, the potency of this nanobody resulted very high. The authors considered that this nanobody is an excellent lead for the next-generation antiscorpion venom therapy that might replace the current horse-derived serotherapy in the near future. Finally, they propose to humanize this antiscorpion toxin, according to validated protocols.

At the *Instituto de Estudios Avanzados* (Institute for Advanced Studies, IDEA) in Venezuela, experimental production of polyclonal antibodies against venoms of scorpions and snakes of clinical and epidemiological importance is being pursued by the alternative technology known as "IgY technology." It involves obtaining immunoglobulins from egg yolk from immunized hens, previously known as Y immunoglobulins (IgY). Obtaining antibodies by this methodology has attracted considerable attention as a means to prevent and control diseases, as it has a number of advantages compared to treatment with mammalian IgG, including cost-effectiveness, convenience, and high yield (Carlander et al. 1999; Xu et al. 2011). In addition, it presents great benefits, such as the following: (a) the reduction of numbers used in animals (chickens produce more antibodies than small mammals such as rodents and lagomorphs), (b) animal suffering is avoided, (c) obtaining highly specific antibodies, and (d) reduction in the maintenance budget of animals. All these advantages would be framed within the specifications of the European Centre for the Validation of Alternative Methods (ECVAM), which recommends the use of egg yolk antibodies due to animal welfare reasons (Schade et al. 2005).

IgY Technology

IgY Antibodies: Historical Perspective

An alternative that is not yet applied at health care level but has shown its usefulness at the experimental therapeutic level is the immunization of chickens to obtain IgY from egg yolk (Thalley and Carroll 1990; Carroll et al. 1992; Kiem

2000). For many years, the predominant monomeric immunoglobulin in serum from birds was known as IgG by homology with that of the mammals. However, given that this has a different structure, Clem and Leslie (1969) termed it IgY. Klemperer (1893) showed that mice treated parenterally with extracts of derived of egg yolk of immunized hens with tetanus toxin were protected against subsequent lethal doses of the same toxin administered parenterally. Polson et al. (1980) showed the advantages of using IgY and proposed a relatively simple method to obtain them from the egg yolk. The development of the poultry industry and the creation of laying hens (1 egg per day) also contributed positively to the development of science and technology of obtaining avian IgYs (Chacana et al. 2004). Since 1980, the use of IgY antibodies has increased significantly, in part due to the availability of commercial secondary reagents for fast and simple purification of IgY, to the availability of commercial secondary reagents for fast and simple purification of IgY, including IgY standards and specific anti-IgY antibodies labeled with fluorescein, alkaline phosphatase, or peroxidase.

Since 1996, the production and use of IgY antibodies is known as "IgY technology," name proposed by Staak in 1995 and now used internationally as a standard terminology (Chacana et al. 2004). In 1999, IgY technology was approved by the Swiss Government Federal Office as an alternative method aimed at improving animal welfare. The ethical approach in experimental animals led to the tenets of Russell and Burch (1959) in their paper entitled "The principles of experimental human art" whose philosophy is based on the application of the 3Rs (refinement, replacement, and reduction) of Russell. This means optimizing the search for new alternatives and reducing the number of animals being tested on for development.

The preparation of antivenoms based in IgY antibodies extracted from egg yolk chickens began in the 1990s with the publication of the works of Thalley and Carroll, who immunized hens with venom of USA's western diamondback rattlesnake (*Crotalus atrox*) and yellow scorpion (*Leiurus quinquestriatus hebraeus*). This demonstrated that avian antivenom purified by affinity from of egg yolk has 100 % protective ability against experimental envenoming and is useful in the detection of cross-reactivity with other venoms on nitrocellulose membranes, suggesting that the source of antibodies is a good alternative to the use of antibodies of mammalian origin. Carroll et al. 1992 found that when comparing the affinity and efficacy of avian antibodies (IgY) with commercial horse IgG crotalic antivenom, the IgYs responded immunologically very well to the wide range of toxins in the venom of the *Crotalus* and that these antibodies purified from hens proved to be significantly potent in neutralizing lethality as compared to current antivenoms.

A significant number of researchers have experimented with IgY antibodies as a possible alternative for the production of antivenoms against the venoms of different kinds of snakes from different countries. These contributions have provided important information for the development of protocols where laying hens are used as an economic source of polyclonal antibodies as an alternative to the treatment of snake bites because they can neutralize the effects of toxins as effectively as equine IgG antibodies (Paul et al. 2007; Meenatchisundaram et al. 2008a, b; Almeida et al. 2008; Araujo et al. 2010; De Andrade et al. 2013).

Additionally, these IgY antibodies can be extracted and purified at low cost (Brunda et al. 2006). In summary, immune IgY's extraction and purification could become an alternative method to the production of mammalian antisera.

Physicochemical and Pharmacological Properties

The IgY is an immunoglobulin, having an MW of 180 kDa (7.8S) somewhat greater than the mammalian IgG (7S), because the heavy chain contains an additional constant domain and larger amounts of carbohydrates. In addition it lacks the hinge region that gives the flexibility to mammalian IgG. The IgY molecule is structurally the same as the IgG molecule with two heavy chains (encoded by V_H genes for the variable region and $C\gamma - C\nu$ for the constant region, respectively) and two light chains (encoded by the V_L genes for the variable region and C_L for the constant region). The main difference between these two immunoglobulins is the number of constant regions in the heavy chain, as the IgG has three regions $C\gamma$ ($C\gamma_1 - C\gamma_3$), while the IgY presents four C constant regions ($C\nu_1 - C\nu_4$) (Davalos et al. 2000; Narat 2003; Raj et al. 2004).

Another important difference is the value of the isoelectric point (pI) for the IgY is 5.4–7.6 (Sun et al. 2001), whereas the IgG is 6.1–8.5. This means that under extreme acidic pH, a greater percentage of IgY loses its activity than IgG. Regarding the thermal stability, the IgY can withstand up to 74 °C and IgG 3 °C less. In general terms and under normal conditions, IgY preparations are stable for 10 years at 4 °C for 6 months at room temperature and for 1 month at 37 °C without loss in activity (Nilsson and Larsson 2007).

The functional characteristics of the IgY are homologous to its mammalian IgG equivalent including its role in secondary immune responses. Immunoglobulin Y is transferred from the blood serum of the hen to the egg yolk through processes that take approximately 5 days and that are receptor mediated. The amount of IgY present in the yolk is proportional to the concentration of immunoglobulin in the serum of the chicken (Schade et al. 2005).

Neutralizing Capacity of IgY Antibodies Against the Whole Venom of Scorpions from *Tityus* Genus in Venezuela

In Venezuela there is no statistical reliable information in cases of scorpion envenomation. This is due to the fact that often, these are not informed to the hospitals and traditional home treatment is often used instead. However, data of scorpionism incidence indicates that the most highly affected states are Miranda, Delta Amacuro, Monagas, Sucre, and Merida (De Sousa et al. 1997; Mejías et al. 2007; Fragoza 2012; Vásquez-Suárez et al. 2012). The species implicated in severe and fatal accidents from different endemic regions of Venezuela are *T. discrepans*, *T. isabelceciliae*, *T. pittieri*, and *T. carabobensis* at the north central region; *T. caripitensis*, *T. arellanoparrai*, *T. monaguensis*, *T. surorientalis*, *and T. nororientalis* at the northeast region; *T. falconensis*, *T. ivic-nancor*, *and*

Fig. 1 *Tityus caripitensis*

T. sanarensis at the west central region; *T. valerae* and *T. zulianus* at the Andes region; *T. breweri* at the south region; and *T. perijanensis* at the Perija mountain (Borges and De Sousa 2006).

IgY antibodies against the venom of four species of *Tityus* genus from Venezuelan north central (*T. discrepans*), northeast (*T. caripitensis*), Andean (*T. zulianus*), and Colombia's western border (*T. perijanensis*) regions have been developed. All venoms induced an evident antibody response in chicken models from week five postimmunization demonstrated by the ELISA assays. Although there are few reports about IgY antibody production against scorpions (Thalley and Carroll 1990), similar results have been already observed with IgY anti-*Echis carinatus* snake venom (Paul et al. 2007).

It is important to highlight that the Monagas state (northeast region) groups the highest mortality records from scorpion envenoming (57.2 %) in Venezuela, being *Tityus caripitensis* (*Tc*) responsible for most accidents in this region (Fig. 1; De Sousa et al. 2005; Borges and De Sousa 2006). The amount of venom produced by a scorpion is variable, being for *T. caripitensis* about 1 mg by electrical stimulation (Parrilla et al. 1993).

In a partial characterization by SDS-PAGE electrophoresis of *Tc* (*T. caripitensis*) CV (complete venom), it was demonstrated that the venom is made up of 10 protein bands with molecular weights between ~10 and 136 kDa (Fig. 2), being of medical importance the ones of low molecular weight (~3 to 8 kDa) (Fig. 3; Borges et al. 2006). Toxins between ~3 and 4 kDa found in the majority of the *Tityus* scorpion genus are constituted of short-chain peptides (30–40 amino acids) and are well known that they act on potassium channels (Borges and De Sousa 2006).

Other proteins that have been studied and are evident in the electrophoretic profile of *Tc* CV are the low molecular weight toxins of ~6 to 8 kDa (Alvarez 2011), which are presumably neurotoxins studied in other species of scorpions, consisting of long-chain peptides (66–79 amino acids) which act on sodium channels, having a high affinity for excitable tissue and cells (Rodriguez de la Vega et al. 2013). Alvarez and Cabrera (1992) demonstrated that toxins of *Tc* CV produce decreased activity of Na^+ and K^+ channels.

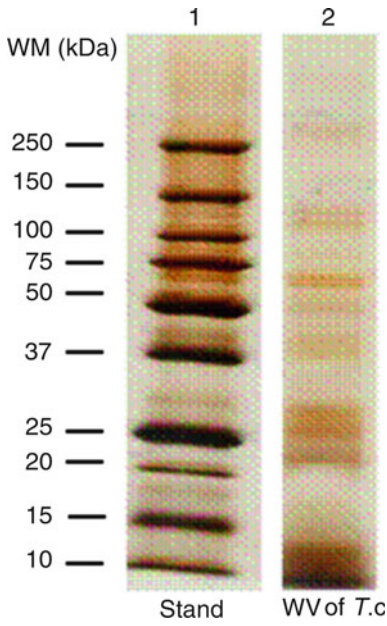

Fig. 2 Electrophoretic run of *Tc* CV on an SDS-PAGE (8–20 % gel colored with silver staining): (*1*) accuracy plus molecular weight standard in a range from 10 to 250 kDa (5 µl) (Bio-Rad, California, USA), (*2*) *Tc* CV (4 µg)

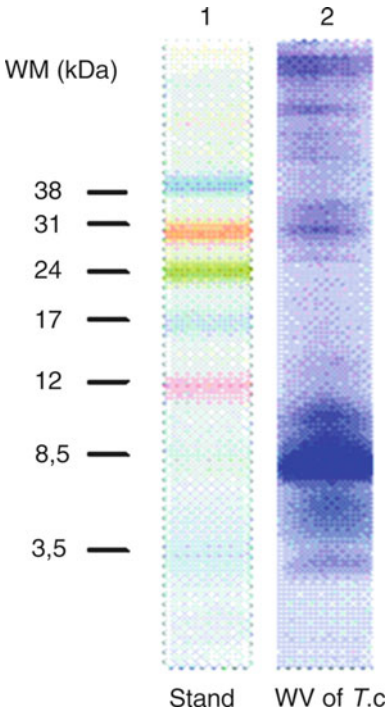

Fig. 3 Electrophoretic run of *Tc* CV on glycine gel SDS-PAGE (15 %, stained with Coomassie Blue): (*1*) low-range rainbow molecular weight standard, ranging from 3.5 to 38 kDa (10 µl) (Amersham, GE healthcare companies), (*2*) *Tc* CV (15 µg)

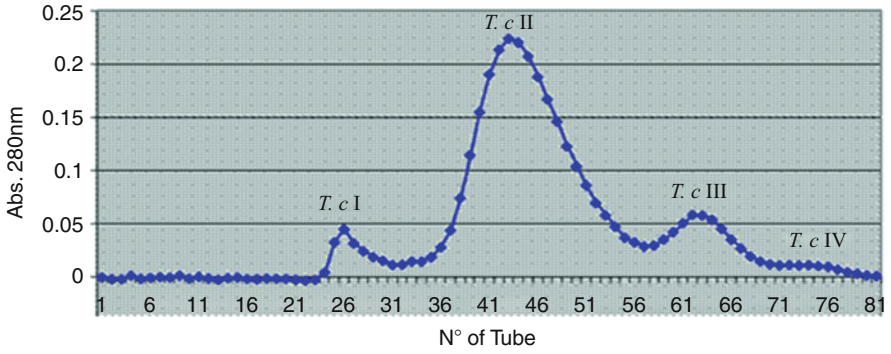

Fig. 4 Chromatographic profile of *Tc* VC (10 mg) run into a column (1.85 × 1 cm) with Sephadex® G-50 using as elution buffer 0.02 M ammonium acetate pH 4.7

The molecular exclusion low-resolution liquid chromatography of *T. caripitensis* venom presents four fractions, of which fraction II is the most abundant and responsible for the neurotoxic effect (Alvarez 2011; Fig. 4).

Another aspect analyzed in the characterization of these arthropods' venom was determining average lethal dose (LD_{50}). This value expresses the degree of toxicity of a substance as the concentration or amount able to cause death in 50 % of a population of experimental animals thus allowing to standardize antidotes (IMDG Code, 2006). The LD_{50} of *Tc* CV was 2.25 mg/kg, demonstrating high lethality intraperitoneally in C57/BL6 mice, under the method of Spearman and Karber (Hamilton et al. 1977; Alvarez 2011).

The toxic effects observed in mice by injecting *Tc* CV are hyperactivity quickly followed by hypoactivity, epiphora, salivation, lacrimation, increased peristalsis, diarrhea, urination, dyspnea, abnormal walking, dragging hind legs, respiratory arrest, and, in many cases, convulsions and death with stretching of the front and hind legs resembling a opisthotonus. These effects are consistent with those observed by other researchers for both the same species (Hudefe 2007) and for different scorpion species (Freire-Maia et al. 1994; Gomez et al. 1995).

Among immunological methods used for the characterization of IgYs is the indirect ELISA assay, which assesses the kinetics of antibody production against the venom. Alvarez (2011) showed that *Tc* CV is immunogenic and that the produced anti-*Tc* CV IgY antibodies exhibited a significant increase after 10 days following the second inoculation.

The antibodies are capable of specifically recognizing a wide variety of antigens with different affinities. All the characteristics related to antigen recognition reflect the antibody's variable regions properties; these can be considerably specific regarding the antigen, able to distinguish small differences in the chemical structure (Abbas et al. 2001). In order to evaluate the simultaneous recognition of IgYs against different antigens, a multiple antigen MABA assay (multiple antigen blot assay), developed by Noya and Alarcón (1998) in Venezuela, is performed,

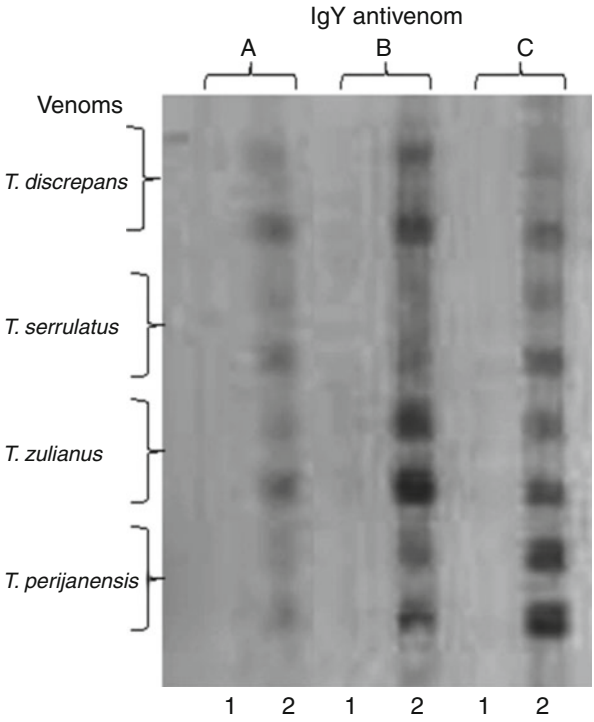

Fig. 5 Immunogenicity and cross-reactivity of IgY antivenoms: *A anti-T. discrepans*, *B* anti-*T. zulianus*, *C* anti-*T. perijanensis* evaluated by MABA. The nitrocellulose paper was sensitized with 1 μg//mL of the venoms: primary antibodies were used at a dilution of 1:1,000. IgY: preimmune (1) and postimmunization (2)

which verifies their ability to recognize whole venom. This technology consists in immobilized antigens (venom) on a nitrocellulose membrane in parallel lanes delineated by a template (multiscreen apparatus). Strips are then cut perpendicularly to the antigen lanes, individually immersed in immune sera (IgY antibodies antivenom), developed with a secondary rabbit anti-IgY antibody conjugated to peroxidase and exposed to a chemiluminescent substrate (Noya and Alarcón 1998).

It has been shown that against *T. discrepans*, *T. zulianus*, and *T. perijanensis* venoms are specific against their antigens. However, showed cross-reactivity with other species of *Tityus* genus (Fig. 5), a fact that may be explained by the presence of homologous toxins in these venoms (Borges et al. 2010).

Several studies have shown that Tityus species are very diverse from the venom components antigenicity viewpoint (Borges et al. 2008). However, neurotoxic components of low molecular weight (3–5 and 7–8 kDa) are shared by scorpions of this genus, which have a high affinity toward pharmacological receptors of excitable cells (voltage ionic channels) and of the immune system (Alami et al. 2001; Gazarian et al. 2005). For this reason it was necessary to establish the capacity of the anti-*Tc* CV IgY antibodies to recognize proteins of that mass range; Western blot assays revealed that the antibodies recognize *Tc CV* toxins of clinical importance both in its native and denatured form. These toxins must be rapidly neutralized in humans envenomed by species of the genus *Tityus* to avoid fatal consequences (Alami et al. 2001; Gazarian et al. 2005).

In a recent study, it was demonstrated that IgY anti-*T. caripitensis* venom was capable of neutralizing $2LD_{50}$ (2×2.25 mg/Kg), with an ED_{50} of 5 mL (225 mg) neutralized 2.3 mg of venom (5 mL/$2DL_{50}$) or 97.8 mg of IgY neutralized 1 mg of *T. caripitensis* venom (Alvarez et al. 2013). Additionally, competitive inhibition assays demonstrate the specificity and selectivity of the anti-*Tc* CV IgY antibodies, where recognition was inhibited by preincubating the IgY antibodies with their respective antigen, including the low molecular weight bands of medical importance. Although several authors have described the neutralization of toxins present in snake venoms using IgY antibodies with satisfactory results (Araújo et al. 2010; Paul et al. 2007; Almeida et al. 1998; Akita et al. 1998; Hatta et al. 1993), there are few reports about the efficacy of IgY antibodies in neutralizing scorpion venoms lethality (Thalley and Carroll 1990).

Nevertheless, it is necessary to perform clinical trials for IgY tolerability and safety for future human applications. One strategy could be the evaluation of egg allergy with diagnostic tests measurement of egg-specific IgE using the skin prick test before applying IgY antivenom to rule out the possibility of inducing an immune response (IgG/IgE), more than a tolerance, in individual with food hypersensitivity reactions against eggs. Accumulated evidence shows that oral tolerance can be mediated by orally activated humoral and cellular factors (Gomes-Santos et al. 2012; Zemann et al. 2003). It is important to highlight that the reactions of hypersensitivity take place against food processed antigens. This processing may destroy existing epitopes on a protein or may cause new ones to be formed (neoallergen formation) as a result of change in protein conformation (Vojdani 2009). In the case of IgY antivenom, it is less probably that hypersensitivity reactions might occur because it is a crude antigen.

The results reviewed above further support that chickens are a convenient and inexpensive source of antivenom. It has been reported that 18 immunized chickens were capable of producing the same amount of antivenom as 1 horse per year (Araújo et al. 2010). The high amount of antivenom and the low maintenance cost make the IgY production an attractive alternative to produce large stocks of antivenom for therapeutic applications to scorpionism.

Conclusions and Future Directions

IgY technology has many applications in the fields of research, development, and innovation, particularly in the production of polyclonal IgY antibodies against numerous antigens. So far they are only used for research purposes and have proven effective in tests where IgG have commonly been used (immunoenzymatic assays, immunoelectrophoresis, Western blotting, immunohistochemistry, ELISA, etc.), and it complies with the three laws of Russel, on the use of experimental animals (refinement, replacement, and reduction). Although this is an alternative not yet applied to the healthcare level, it has exhibited therapeutic utility experimentally by immunizing hens to obtain neutralizing antibodies from the egg yolk.

When developing antivenoms against scorpions of the genus *Tityus*, it is important to consider that the components present in the venom have great antigenic diversity, probably related to changes in regional biogeography. The application of this IgY technology has been increasing in the scientific community and has demonstrated many advantages, not only for its lower cost of production, but in terms of performance and an absence of cross-reaction with mammal antibodies. Assessments by Venezuelan researchers to IgYs obtained against complete venom (CV) of the species *T. caripitensis* (*Tc*) (of great medical importance in Venezuela) allowed to show that the venom induces a response in the immune system of the hen and that these IgY proved to be able to recognize with sensitivity and specificity whole *Tc* CV and also the ~8 and ~3.5 kDa bands corresponding to clinically important toxins, thus managing to neutralize $2LD_{50}$. The IgY antivenom displays cross-reactivity with other species of the genus *Tityus*, showing that these have potential value and possibly a future applicability in the therapy against stings by other scorpions, in addition to support an integrated production and characterization of regionalized genera and species.

While it is true that avian antibodies will not completely replace the mammalian antibodies, it is important to note the potentialities of the IgY technology disseminate its virtues and inform the scientific community of the benefits. Their use entrails in some scientific fields such as the development of polyclonal antibodies against scorpion venoms that strengthen future studies of the prophylactic or therapeutic use of IgYs.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ New Insights on the Pharmacokinetics of Venoms and Antivenoms
- ▶ Scorpion Venoms: Pathogenesis and Biotherapies
- ▶ Scorpionism and Dangerous Species of Venezuela

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Part IV

Scorpionism

Scorpionism and Dangerous Species of Jordan

8

Zuhair Sami Amr

Contents

Abstract

Distributional data were given for 18 species of scorpions known to occur in Jordan. At least five species are considered dangerous (three species of the genus *Androctonus* and two species of the genus *Leiurus*), while the others have low-to-moderate effect on human. The epidemiology of scorpion sting accidents in Jordan was discussed based on previous studies and recent data. Males were more vulnerable than females, and children less than 15 years old constituted the highest percentage of victims. Stings were more frequent in the summer, peaking in July. Most accidents occurred at night, between 21.00 and 01.00 h. Scorpion stings occurred outdoors (66 %) and at the patients' residence (32.2 %). Fingers and toes were the main sites of stings. Symptoms associated with scorpion stings among hospitalized cases in Jordan were outlined. LD_{50} for most species known in Jordan and the Middle East were tabulated. Further studies on the taxonomy and systematics of the scorpion of Jordan are needed to resolve taxonomic status of obscure species.

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Introduction

Jordan, officially the Hashemite Kingdom of Jordan, is a small country located in the heart of the Middle East. The total area is about 98,000 km^2 stretching over four main biogeographical zones (Mediterranean, Irano-Turanian, Saharo-Arabian, and Afrotropical). Arid deserts constitute about 75 % of the total area and cover the southern and the eastern parts of the country (Amr 2012).

The climate of Jordan is characterized by a hot and dry summer, a cool and wet winter, and brief spring. Annual rainfall ranges from 700 to 100 mm annually in the northern mountains and the extreme eastern desert, respectively. Three main geographic and climatic regions prevails: the Jordan Valley that extends along the Jordan River, Dead Sea basin, and Wadi Araba reaching Aqaba at the Red Sea, the Mountain Heights Plateau that comprises the mountain series extending from Irbid in the north reaching Petra mountains in the south, and the eastern desert that stretches eastward of the mountain series. Population as of 2012 was estimated as 6.5 million.

At least 18 species of scorpions have been recorded from Jordan, where as five are known to be venomous and life threatening to human. In this communication, scorpions of Jordan are presented with their current known distribution, and the epidemiology of scorpion sting accidents is revised with additional new data.

Scorpions of Jordan

A total of 18 species and subspecies representing 10 genera within two families (Buthidae and Scorpionidae) have been recorded in Jordan (Vachon 1966; Levy et al. 1973; Kinzelbach 1984; Amr et al. 1988; Amr and El-Oran 1994; Stathi and Mylonas 2001; Lourenço et al. 2002, 2010; Kovařík 2003, 2012; Kovařík and Whitman 2004).

Family Buthidae includes eight genera (*Androctonus, Birulatus, Buthacus, Buthus, Compsobuthus, Hottentotta, Leiurus,* and *Orthochirus*) and 15 species and subspecies. Family Scorpionidae is represented by two genera (*Nebo* and *Scorpio*) with three species and subspecies.

Family Buthidae C. L. Koch, 1837

Triangular sternum is the prominent feature of representatives in this family. Three to five eyes are usually present, and the telson is sometimes equipped with accessory spines. This family includes most of the scorpions dangerous to humans.

Androctonus amoreuxi (Audouin, 1826)

Diagnosis: Olive to chocolate brown or yellow in color. Prosoma: densely granulated. Mesosoma: with distinct crests, the seventh sternite with four smooth or granulated crests. Metasoma: first segment of the tail wider than long, while segments 2–5 are longer than wide. Pectines 27–33 in males and 18–29 in females.

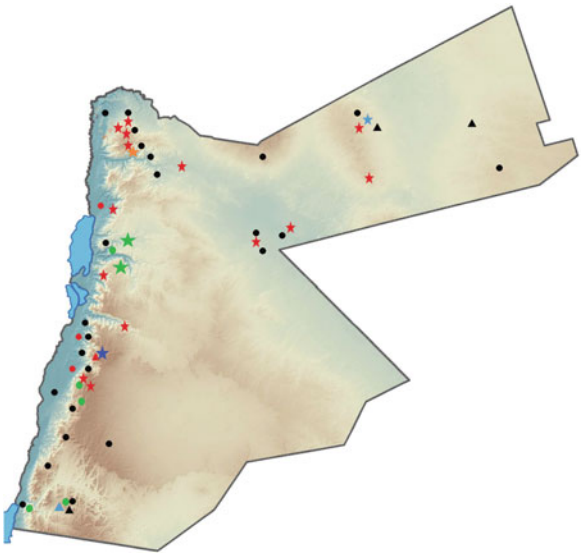

Fig. 1 Distribution of species of the family Buthidae in Jordan. *Androctonus amoreuxi* (*red circles*), *Androctonus bicolor* (*green circles*), *Androctonus crassicauda* (*black circles*), *Buthacus leptochelys* (*black triangles*), *Buthus amri* (*blue triangle*), *Birulatus haasi* (*red triangle*), *Compsobuthus carmelitis* (*orange star*). *Compsobuthus jordanensis* (*green stars*), *Compsobuthus levyi* (*blue star*), *Compsobuthus schmiedeknechti* (*purple star*), and *Compsobuthus werneri* (*red stars*)

Measurements: Total length up to 9 cm (average 5.5 cm), prosoma 5–8 mm, mesosoma 11.7–17.7 mm, and metasoma 18.9–34.4 mm.

Habitat and distribution: It has a wide distribution along the coastal plains of Palestine and Sinai (Levy and Amitai 1980). Its habitat is similar to *A. crassicauda*, recorded from Wadi Araba and the lower Jordan Valley (Amr and El-Oran 1994). Figure 1 shows the distribution of *A. amoreuxi* in Jordan. The taxonomic status of this species remains dubious in the Middle East that requires further investigation. Examination of specimens collected from the Middle East confirms the presence of certain morphometric variability (Lourenço 2005).

Androctonus bicolor Ehrenberg, 1828

Diagnosis: Dark brown to olive black in color. The color of the terminal segments of the legs and pedipalps is light brown. Prosoma: crests are distinct. Mesosoma: densely granulated with distinct tergal crests. Metasoma: median lateral keels of segments two and three are developed and possess few granules. Pectines with 23–29 teeth in males and 19–24 in females (Fig. 2a).

Measurements: Total length may reach 9 cm, prosoma 6.9 mm, mesosoma 15.6 mm, and metasoma 33.2 mm.

Habitat and distribution: This species is known from few localities. El-Hennawy (1988) reported it from Ma'an, Aqaba, and Petra, and Amr and El-Oran (1994) indicated a locality from Karak and Kovařík and Whitman (2004) from Wadi Rum. Figure 1 shows the distribution of *A. bicolor* in Jordan.

Androctonus crassicauda (Olivier, 1807)

Diagnosis: Black to dark brown in color. Prosoma: covered by distinct granules. Mesosoma: with moderately distinct granules. Metasoma: segments thick and

Fig. 2 Scorpions of the family Buthidae. (**a**). *A. bicolor*. (**b**). *A. crassicauda*. (**c**). *B. leptochelys*. (**d**). *C. jordanensis*. (**e**). *H. judaicus*. (**f**). *L. jordanensis*. (**g**). *L. quinquestriatus*. (**h**). *O. scrobiculosus*. The inside scale represents 1 cm

wider than long. Lateral keels of the second and third segments are reduced to only a few granules. Pectines 27–32 in males and 23–27 in females.

Measurements: Total length up to 10 cm (average 8.5 cm), prosoma 10.3–11.5 mm, mesosoma 19.6–23.9 mm, and metasoma 42.1–49.9 mm (Fig. 2b).

Habitat and distribution: This is a desert-adapted species with wide distribution in the eastern desert and Wadi Araba to Aqaba (Levy and Amitai 1980; Amr et al. 1988; Amr and El-Oran 1994), from Azraq, Jerash, and North Shuna (Stathi and Mylonas 2001), and from Wadi Rum (Kovařík and Whitman 2004). Figure 1 shows the distribution of *A. crassicauda* in Jordan.

It was also collected from the Mediterranean region with dense forests but in low numbers. It lives in horizontal burrows in dry soil in desertic regions or in rodent burrows. *A. crassicauda* is one of the most venomous species in the Middle East.

Birulatus haasi Vachon, 1974

Diagnosis: This is a small-sized scorpion, with an average length of 20 mm. Prosoma: heavily granulated. Median eyes small separated by two ocular diameters, lateral eyes absent. Body basically pale yellowish, median eyes surrounded by black pigment. Mesosoma, vesicle, chelicera, pedipalps, and legs yellowish.

Measurements: Average total length 2 cm, carapace 2.8 mm, metasomal segment I length 1.4 mm, metasomal segment V length 2.3 mm, vesicle width 0.5 mm, and length of movable finger 2.9 mm. Measurements based on the female holotype (Lourenço 1999).

Habitat and distribution: This species was originally described from the Tafilah area (Vachon 1974). The species was redescribed by Lourenço (1999). He suggested that this species is a cave-dwelling scorpion. We were unable to collect further specimens of this species. Perhaps the specimen collected from southern Jordan represents a relict population with limited distribution. Two additional species of the genus *Birulatus* have been described recently: *Birulatus israelensis* and *Birulatus astariae* from the Middle East (Lourenço 2002; Stathi and Lourenço 2003). Figure 1 shows the reported distribution of *B. haasi* in Jordan.

Buthacus leptochelys (Ehrenberg, 1829)

Diagnosis: Yellow to yellowish brown in color. Prosoma: entirely smooth. Mesosoma: smooth without crests. Metasoma: tails segments with hairs and telson with many hairs. Pectines with 27–35 teeth in males and 18–29 in females (Fig. 2c).

Measurements: Total length may reach up to 6 cm (average 4.1 cm), prosoma 4.4–4.6 mm, mesosoma 9–10.3 mm, and metasoma 22.7–25 mm.

Habitat and distribution: This species is known from southwest Jordan (Kinzelbach 1984) and from Wadi Rum (Stathi and Mylonas 2001). It was collected from rodent burrows in extreme desertic conditions near Al-Jafr (Amr and El-Oran 1994). Kinzelbach (1984) revised the systematic position of *Buthacus leptochelys nitzani* and suggested synonymy with *Buthacus leptochelys*. Lourenço (2006) concluded that *Buthacus macrocentrus* is distinct from *B. leptochelys* and could be found in Jordan.

The records of Kovařík and Whitman (2004) of *Buthacus arenicola* and *Buthacus tadmorensis* from Wadi Rum are considered as *Buthacus leptochelys* in this study until further validation of the status of species of the genus *Buthacus* in the Middle East. Figure 1 shows the distribution of *B. leptochelys* in Jordan.

Buthus amri Lourenço, Yagmur & Duhem, 2010

Diagnosis: Yellowish to pale yellow in color. Prosoma: intensely spotted with small granules with visible crests. Mesosoma: three longitudinal stripes are distinct, the median one narrower than the lateral. Metasoma: segments yellowish with some diffuse spots over crests and spots better marked on the fifth segment. Pectines with 27–29 teeth in males and 21–23 in females.

Measurements: Small scorpion with a total length of 44 mm in males and 50 mm in females.

Habitat and distribution: This species was collected from Wadi Rum. It inhabits hard sandy soil with sparse bushes (Lourenço et al. 2010).

Lourenço (2003) revised the genus *Buthus* and stated that the true *"Buthus occitanus"* is confined to France and Spain. All previous records of *Buthus occitanus* such as that of Kinzelbach (1984) from Jordan require validation, since they could be either *Buthus israelis* or the newly described species *Buthus amri*. Figure 1 shows the distribution of *B. amri* in Jordan.

Compsobuthus carmelitis Levy, Amitai & Shulov, 1973

Diagnosis: Coloration usually light yellow to light yellowish brown. Prosoma: with fine granules and visible crests. Mesosoma: tergites with small granules. Metasoma: second segment with eight complete crests. Pectines 23–25 in males and 20–22 in females.

Measurements: Total length 32–40 mm.

Habitat and distribution: A single record from Wadi Zarqa (North of Sihan) was indicated by Stathi and Mylonas (2001). Figure 1 shows the distribution of *C. carmelitis* in Jordan.

Compsobuthus jordanensis Levy, Amitai & Shulov, 1973

Diagnosis: Yellow to light brown in color. Prosoma: densely granulated. Mesosoma: each tergite equipped with three rows of crests extending beyond the tergite's margin. Metasoma: with narrow segments. Pectines 18–23 in males and 16–21 in females (Fig. 2d).

Measurements: Total length of the adult approximately 3 cm.

Habitat and distribution: This species was collected from deserts around Wadi Dabaa' (southeast of Amman) and Al-Hasa toward Ma'an (Levy et al. 1973). Figure 1 shows the distribution of *C. jordanensis* in Jordan.

Compsobuthus levyi Kovařík, 2012

Diagnosis: Yellow to yellowish brown in color. Prosoma: covered with granules. Mesosoma: tergites I–VI bear very strong denticulate lateral crests. Metasoma: all metasomal segments of both sexes slender and longer than wide. Pectines 18–23.

Measurements: Total length 28–38.4 mm.

Habitat and distribution: This species was collected from Qasr Burqu, in the eastern desert of Jordan (Kovařík 2012). Figure 1 shows the distribution of *C. levyi* in Jordan.

Compsobuthus schmiedeknechti Vachon, 1949

Diagnosis: Yellow to yellowish brown in color. Prosoma: with distinct granules. Mesosoma: each tergite with three rows of crests extending beyond tergite margins. Metasoma: with narrow segments and telson usually orange to dark yellow in color. Pectines 15–18.

Measurements: Adult specimens may reach up to 3 cm.

Habitat and distribution: Not much is known about the habitat of this scorpion. It was reported from Jordan by Vachon (1949). Records from Jordan include Bonifica and Petra (Kovařík and Whitman 2004). Figure 1 shows the distribution of *C. schmiedeknechti* in Jordan.

Compsobuthus werneri (Birula, 1908)

Diagnosis: Light yellow in color. Prosoma: smooth except for small granules in front of the ocular crest and lateral eyes. Mesosoma: each tergite with three rows of crests extending beyond the tergite margins. Metasoma: intermediary crests of the third segment not distinct with few granules. Pectines 18–23 in males and 16–21 in females.

Measurements: Total length 2.5–3.8 cm (average 3.4 cm), prosoma 3.3–4.2 mm, mesosoma 7.1–8.9 mm, and metasoma 14.6–15.5 mm.

Habitat and distribution: It was collected from Petra, Wadi Al-Hasa, Shaumari, Wadi Sheib, Amman, Bqueuiah, and Quasr Burga (Kinzelbach 1984; Amr and El-Oran 1994; El-Hennawy 1988; Kovařík 2003). This species was recovered from pellets of the Eagle Owl in the Eastern Desert of Jordan (Rifai et al. 2000). Other records are from Ajloun, AI Manshiyya, AI-Mazar al-Janubi, West Azraq, Jerash, North Shuna, Um Quays, Wadi Musa, Zai National Park (Stathi and Mylonas 2001), and Petra (Kovařík and Whitman 2004). Figure 1 shows the distribution of *C. werneri* in Jordan.

Lourenço et al. (2009) stated that *C. werneri* is distributed in Sudan, Egypt, and some parts of Sinai, and records from the Middle East are ascribed as misidentifications. Further studies should reveal the identity of "*werneri*" in Jordan.

Hottentotta judaicus (Simon, 1872)

Diagnosis: Black to dark brown in color. Prosoma: heavily granulated. Mesosoma: with densely granulated tergites. Metasoma: dorsal segments smooth, while the lateral and ventral sides are granulated. Sole of tarsi with small spines. Pectines 27–33 in males and 22–27 in females (Fig. 2e).

Measurements: Total length 5–7 cm (average 5.9 cm), prosoma 6.4 mm, mesosoma 16.6–19.9 mm, and metasoma 27.5–38 mm.

Habitat and distribution: This species was reported from Irbid and Salt (Wahbeh 1976; Kinzelbach 1984); Amman (El-Hennawy 1988); and Ajloun, Jerash, and North Shuna (Stathi and Mylonas 2001). It seems that this species is confined to mountainous areas of Jordan with relatively high rainfall (Amr and El-Oran 1994). It is quite common in the Ajloun mountains and associated with the *terra rossa* soil, where it coexist with *Scorpio maurus palmatus*. It constructs burrows that are usually located under stones and also found under rocks without burrows. Figure 3 shows the distribution of *H. judaicus* in Jordan.

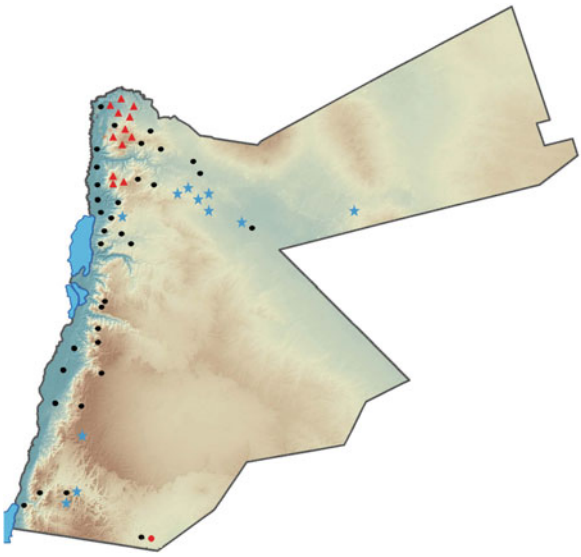

Fig. 3 Distribution of species of the family Buthidae in Jordan. *Hottentotta judaicus* (*red triangles*), *Leiurus jordanensis* (*red circle*), *Leiurus quinquestriatus* (*black circles*), and *Orthochirus scrobiculosus* (*blue stars*)

Leiurus jordanensis Lourenço, Modry & Amr, 2002

Diagnosis: Body coloration generally blackish brown. Prosoma: black and heavily granulated. Mesosoma: five keels present on the first and second tergites. Metasoma: crests are strongly marked and inter crestal spaces smooth to shagreen. Ventral side of tarsi with numerous setae not arranged in straight rows. Pectines 30 in females (Fig. 2f).

Measurements: Total average length 7.4 cm, carapace length 0.85 cm, length of metasomal segment I 0.6 cm, length of metasomal segment V 1.06 cm, vesicle width 0.34 cm, and length of movable finger 1.36 cm.

Habitat and distribution: The species was described from a desert habitat composed of sandstone cliffs surrounded by flat sand dunes from southern Jordan on the basis of a female specimen (Lourenço et al. 2002). According to Lourenço et al. (2002), the species distribution appears limited to an enclave within the area in which its most related species (*Leiurus quinquestriatus*) is distributed. Figure 3 shows the distribution of *L. jordanensis* in Jordan.

Leiurus quinquestriatus (Ehrenberg, 1828)

Diagnosis: Yellow in color. Prosoma: granulated. Mesosoma: the first two tergites have five keels. Metasoma: segments 1–4 yellow in color, segment 5 black. Pectines 31–36 in males and 26–32 in females (Fig. 2g).

Measurements: Adult specimens may reach 9 cm in length (average 6 cm), prosoma 3.8–9.6 mm, mesosoma 16.8–19.8 mm, and metasoma 19.3–42.4 mm.

Habitat and distribution: This is the most common species in Jordan. Wahbeh (1976) reported that *L. quinquestriatus* constituted 85 % of the scorpions collected from 13 different localities. Warburg et al. (1980) noted that *L. quinquestriatus* is quite common in the northern Jordan Valley. It was collected from Mafraq (Levy et al. 1970): Wadi Dabaa' (Levy and Amitai 1980): Wadi Musa, Wadi Al Mujib,

Aqaba, Wadi Ram, and Jabal Nebo (Kinzelbach 1984); Azraq and Wadi Sheib (El-Hennawy 1988); and Deir Alla, Bireen, Dana Reserve, Wadi Musa, Shemakh (Shoubak), Tafilah, Karak, Mashreh Dam, Al-Karameh, Dair El-Warak (Mafraq), Wadi El-Yotom (Aqaba), Okader, and Irbid (Amr and El-Oran 1994). Other localities include southern part of the Dead Sea, Manshiyya, AI-Mazar al-Janubi, Dana Nature Reserve, Wadi Zarqa (Dead Sea), North Shuna, Wadi Zarqa (N of Sihan), Petra, Tygrat al Jubb, SE of Um Quays, Zai National Park, King Talal Dam, Wadi Rum (Stathi and Mylonas 2001), and Wadi Rum and Petra (Kovařík and Whitman 2004). Figure 3 shows the distribution of *L. quinquestriatus* in Jordan.

Leiurus quinquestriatus has rather scattered populations. It was collected from Dana area (between Shoubak and Petra), where it was the only scorpion species with dense populations. Similar observations were seen near El-Hemma in the North, Wadi Al-Walah, Madaba area, and Karak. We have few collections from southern Jordan (Amr and El-Oran 1994). High number of individuals was also collected from Wadi Al Mujib Nature Reserve. It is usually found under stones or rocks with no definite burrows. Stone walls are preferred hiding places for this species. Several specimens were brought from houses in Irbid and surrounding villages. This is the most poisonous species in the area (Amr et al. 1994). Warburg (1997) stated that this scorpion penetrated deeper into the Mediterranean biotope in Palestine.

Orthochirus scrobiculosus (Grube, 1873)

Diagnosis: Black in color. Prosoma: entirely smooth. Mesosoma: densely granulated. Metasoma: tail segments covered with small depressions. Pectines 19–20 in males and 15–18 in females (Fig. 2h).

Measurements: Total length can reach up to 3 cm (average 2.6 cm), prosoma 3 mm, mesosoma 7.6 mm, and metasoma 15.2 mm.

Habitat and distribution: Wahbeh (1976) reported this species from Madaba area. Amr and El-Oran (1994) listed several localities for this species from arid and mid-regions in Jordan. Collected also from Azraq, Jerash, and Tygrat al Jubb (Stathi and Mylonas 2001) and from Wadi Rum (listed as *Orthochirus innesi*) by Kovařík and Whitman (2004). Figure 3 shows the distribution of *O. scrobiculosus* in Jordan.

Orthochirus scrobiculosus negebensis is the known subspecies occurring in Jordan, Palestine, and Sinai (Levy and Amitai 1980), while other subspecies occur in Iraq, Iran, and Turkestan. This is a desert inhabitant, where it is usually found in small crevices under stones and burrows. High population densities were noticed in Azraq area. Specimens from Wadi Rum were found in deep sand burrows that extend more than 50 cm deep. Other specimens were observed during the early morning hours basking on small shrubs, perhaps to absorb humidity.

Family Scorpionidae Latreille, 1802

The pentagonal sternum is the prominent feature of this family. Species of the genus *Scorpio* lack subaculear tubercle on the telson, while present in *Nebo*. In the Middle East, members of this family are not considered dangerous.

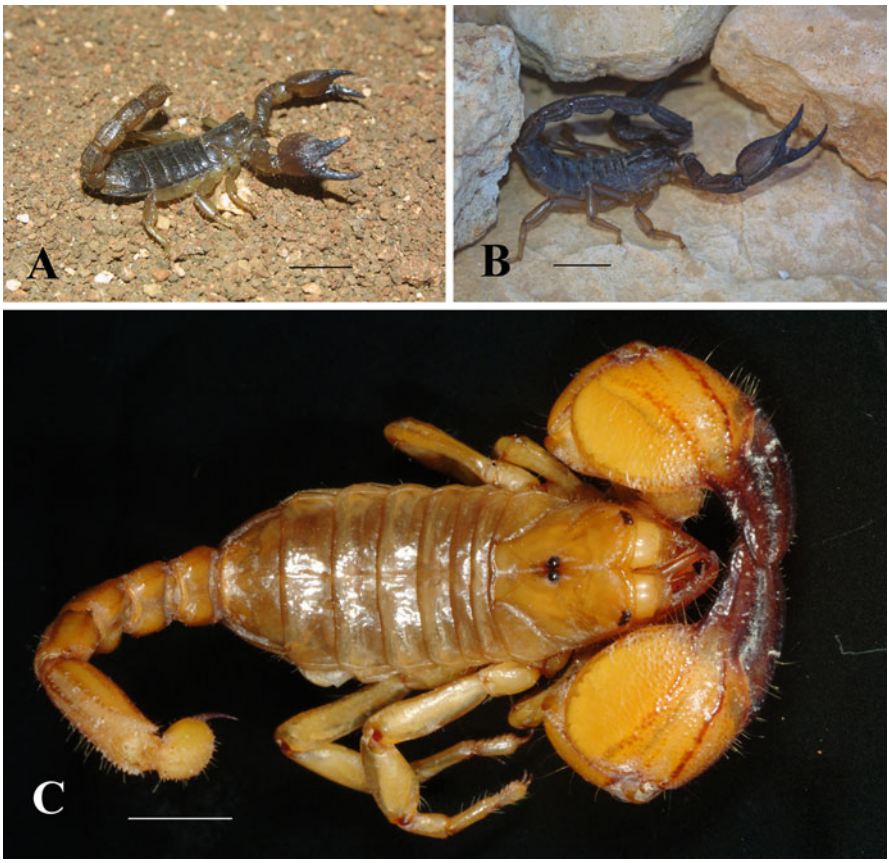

Fig. 4 Scorpions of the family Scorpionidae. (**a**). *Nebo hierichonticus*. (**b**). *Scorpio maurus fuscus*. (**c**). *Scorpio maurus palmatus*. The inside scale represents 1 cm

According to Vachon and Kinzelbach (1987), three subspecies of the genus *Scorpio* occur in Jordan, namely, *Scorpio maurus fuscus* distributed in the north, *Scorpio maurus palmatus* south-western Jordan, and *Scorpio maurus kruglovi* occurring in the eastern desert. Traditionally, *Nebo hierichonticus* was placed under the family Diplocentridae; however, Soleglad and Fet (2003) abolished this family and downgraded it to a subfamily rank in the family Scorpionidae. Having *Nebo* within the family Scorpionidae is consistent with recent publications, although it should be noted that high-level scorpion systematics are a matter of ongoing debate (see Prendini and Wheleer (2005) and the chapter by Lourenço in this book).

Nebo hierichonticus (Simon, 1872)

Diagnosis: Dark brown in color. Pedipalps: thick and long. Prosoma: smooth with granules on the lateral side. Mesosoma: entirely smooth. Metasoma: tail segments long and narrow, subaculear tubercle present on the telson. Pectines 14–22 in males and 11–17 in females (Fig. 4a).

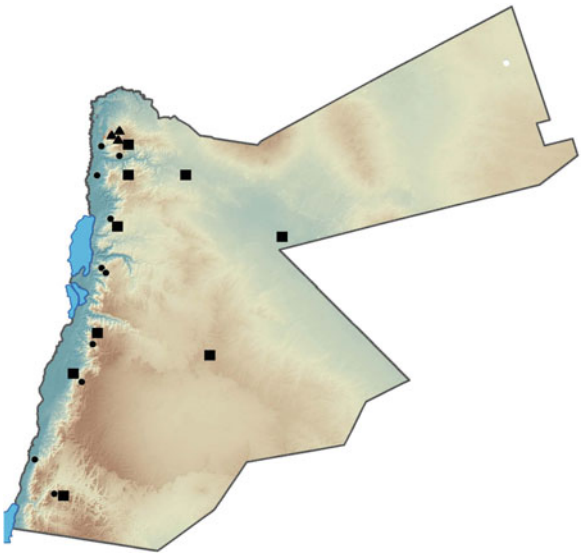

Fig. 5 Distribution of species of the family Scorpionidae. *Nebo hierichonticus* (*circles*), *Scorpio maurus fuscus* (*squares*), and *Scorpio maurus palmatus* (*triangles*)

Measurements: Adult may reach 11 cm (average 7.3 cm), prosoma 7.1–12.9 mm, mesosoma 17.4–35.2 mm, and metasoma 20.2–47.4 mm.

Habitat and distribution: This species is endemic to Syria, Palestine, Lebanon, Jordan, and Arabia (Francke 1980; Vachon and Kinzelbach 1987). Wahbeh (1976) collected this species from Madaba and Karak. Levy and Amitai (1980) reported other localities in Amman and Petra. Kinzelbach (1984) collected specimens from Petra. Other records in our collection are from several localities in Wadi Araba, Jordan Valley, and near Jerash (Amr and El-Oran 1994). Also collected from Wadi Karak, Al-Mazar al-Janubi, and Wadi Rum (Stathi and Mylonas 2001) and from Wadi Rum (Kovařík and Whitman 2004). *Nebo hierichonticus* has a scattered distribution. The localities indicated represent a wide range of biotops. It constructs its own burrows and could be found under rocks and between crevices. Figure 5 shows the distribution of *N. hierichonticus* in Jordan.

Scorpio maurus fuscus (Ehrenberg, 1829)

Diagnosis: Dark brown in color. Pedipalp claw similar to the lobster. Prosoma: entirely smooth. Mesosoma: smooth and without crests. Metasoma: tail segments yellow brown with scattered hairs and telson is usually yellow in color. Pectines 9–11 in males and 6–10 in females (Fig. 4b).

Measurements: Total length may reach 8 cm (average 6.), prosoma 6.5–9.1 mm, mesosoma 18.6–20.3 mm, and metasoma 19.1–25.7 mm. Pectines 9–10.

Habitat and distribution: This species constructs its burrows either under stones or in the *terra rossa* soil. It was collected from areas with high rainfall and cold winters. It is usually found in dense populations within the same area (Amr and El-Oran 1994; Stathi and Mylonas 2001). At Zubya, an oak-forested area, over 15 specimens were collected within an area of about 500 m^2 (Amr and El-Oran

1994). However, Warburg (1997) stated that this oakwood scorpionid, formerly the most abundant scorpion in the Mediterranean region, showed a marked decline in numbers. Figure 5 shows the distribution of *S. maurus fuscus* in Jordan.

***Scorpio maurus palmatus* (Ehrenberg, 1829)**

Diagnosis: Yellow to light olive brown in color. Pedipalp claw similar to the lobster. Prosoma: entirely smooth. Mesosoma: smooth and without crests. Metasoma: tail segments yellow brown with scattered hairs. Pectines 9–13 in males and 7–13 in females (Fig. 4c).

Measurements: Total length 5–7 cm (average 5.25 cm), prosoma 7.6–8.3 mm, mesosoma 14.9–18.6 mm, and metasoma 18.9–22.9 mm.

Habitat and distribution: *Scorpio maurus palmatus* is of African origin that penetrated into southern Jordan. It was reported from Wadi Musa, Theban, Amman, and Ajloun (Wahbeh 1976; El-Hennawy 1988; Amr and El-Oran 1994). This species was recovered from pellets of the Eagle Owl in the Eastern Desert of Jordan (Rifai et al. 2000) and from Wadi Rum (Kovařík and Whitman 2004). Figure 5 shows the distribution of *S. maurus palmatus* in Jordan.

General Zoographical Analysis for the Scorpions of Jordan

Buthacus leptochelys, S. maurus palmatus, A. bicolor, A. crassicauda, and *A. amoreuxi* are considered xerophilic species as suggested by their distribution. They are found in the Saharo-Arabian region. This type of habitat is characterized by low rainfall that does not exceed 10 mm annually. Soil varies from sandy to limestone and sandstone. As indicated by Vachon and Kinzelbach (1987), *S. maurus palmatus* is of African origin that penetrated into southern Jordan. Vachon (1979) reported this species from southern Arabia along the western coasts of the Red Sea. *Buthacus leptochelys* distribution is restricted to the southern deserts of Jordan as well as to the north-eastern desert. It occurs in the extreme deserts of Saudi Arabia (Vachon 1979). Although the majority of *A. crassicauda* specimens were collected from dry regions, it was collected as well from the Mediterranean territory.

Both *H. judaicus and S. maurus fuscus* are truly Mediterranean species. *Orthochirus scrobiculosus* was collected from the three main biogeographical zones as well as *N. hierichonticus* of which showed a varied habitat preference.

Birulatus haasi was described from Tafilah area. No further specimens were collected from this area. It is suggested here that *B. haasi* is a relict species with restricted distribution. It seems that is a Mediterranean form as two other species were described from Palestine and Syria (Lourenço 2002; Stathi and Lourenço 2003).

Compsobuthus werneri was collected from the Irano-Turanian ecozone. Most of our collection originated from basalt and granite deserts; however, other few were collected from steppe regions (Karak area). This is a problematic genus that requires further studies.

Leiurus quinquestriatus was the most common species all over Jordan. It prefers steppe regions, although several locations represent the Mediterranean ecozone,

few specimens were collected from the eastern desert or from Wadi Araba. We have no records from Zubya or Ajloun, both are typical Mediterranean areas. Previous reports indicated its presence in very dry regions (Kinzelbach 1984; El-Henawy 1988). It has a rather scattered population, where it was collected from the Dhana area (between Shoubak and Petra), and seems not to coexist with other scorpions. Similar observations were made in other areas. Warburg et al. (1980) indicated that *L. quinquestriatus* distribution is restricted to areas with low precipitation.

Scorpionism

Scorpionism is an endemic public health problem in Jordan encountered by health providers in all parts of Jordan. Epidemiological data are incomplete and the exact number of accidents remains largely unknown.

Scorpions are mostly nocturnal; they often slip into shoes, bedding or cracks, and under logs or stones. Stings are generally attributable to carelessness or negligence when, for instance, the victim puts his shoes on without first looking inside. Houses in most villages in Jordan have the toilets as a separate structure, located several meters away from the house. Toilets are usually inhabited by scorpions. When family members visit the toilet during the night, scorpions may be provoked into stinging. Fissures and cracks around the doors of the house itself provide easy access for scorpions; once inside they slip into bedding and clothing of children while they are asleep. In fact, most of sting accidents occur among children at night, when scorpions get into their night clothes.

The rocky terrain of northern Jordan offers an especially suitable habitat for scorpions. Here children, while flipping over stones and rocks, receive scorpion stings. Field stone fences are also very common in Jordan, notably in the mountainous areas of Irbid, Jerash, Karak, Ajloun, and Tafilah where they offer a perfect habitat for *L. quinquestriatus*. In the desert terrain, scorpions were observed using desert rodent burrows; *A. crassicauda* was seen in many rodent burrows around Azraq and the Desert Highway.

Most cases were reported from agricultural areas in the northern part of the country (Irbid and North Shuna) and the scattered towns bordering the eastern desert (Mafraq and Zarqa). It is suggested here that the rocky terrain provides a suitable habitat for scorpions to flourish. In addition to this, the north is more densely populated and consists of villages that depend on farming, thus increasing the exposure to scorpion envenomization.

Epidemiology

Several studies addressed the epidemiology of scorpion stings since 1965. The earliest report on scorpion stinging accidents was published by Wahbeh (1965). Amr et al. (1988) reported on 547 cases of scorpion sting during 1982–1985, with two fatalities. Amr et al. (1994) reported a total of 338 cases of scorpion stings in

Table 1 Scorpion sting accidents according to sex treated at Princess Haya Hospital in Aqaba, southern Jordan

Year	Male	Female	Total
2006	13	6	19
2007	8	4	12
2008	9	3	12
2009	4	2	6
2010	3	1	4
2011	4	1	5
2012	6	9	15
Total	47	26	73

Table 2 Scorpion sting accidents according to sex reported to the National Drug and Poison Information Center

Year	M	F	Total
2009	3	1	4
2010	6	13	19
2011	9	6	15
2012	11	4	15
Total	29	24	53

Ma'an Government hospital and out-patient clinics in the Irbid area between 1989 and 1992 with no fatalities. Mutair et al. (2001) reviewed medical records for 96 patients admitted to three hospitals after scorpion sting accidents during 1997–1999, with two fatalities (2.1 %).

During the Gulf War in 1990, an influx of refugees fled Kuwait and passed through Jordan. At the Jordanian-Iraqi border, El-Rwaished, a camp site was established to control the deportation of foreign nationals. At this small desert town, a total of 799 cases of scorpion stings were treated at the Rwaished Hospital during September of 1990. No data on the sex or age of the victims attended by Rwaished Hospital (Amr unpublished data).

Records of the 338 sting cases seen at the Ma'an Government hospital and out-patient clinics in the Irbid area between 1989 and 1992 showed that most cases were males (61.8 %) and children were particularly at risk (48.7 % of the cases were $<$16 years old and 24.7 % $<$6 years).

Data were obtained from Princess Haya Hospital (PHH) in Aqaba, southern Jordan from 2006 to 2012. A total of 73 cases (47 males 26 females) were referred to the emergency unit from Aqaba and the surrounding regions (Table 1). Hospitalization required 1–3 days among admitted patients with no fatalities.

Data were also obtained from the National Drug and Poison Information Center (NDPIC), Jordan University Hospital, covering the years 2009–2012. A total of 53 cases (29 males and 24 females) were reported to NDPIC during 4 years (Table 2). Seventeen cases occurred at patients' residence (32.2 %), 35 outdoors (66 %), and one case (1.8 %) at the workplace. Fingers and toes were the main sites of stings.

Figure 6 shows age groups for scorpion stings reported to NDPIC. Similar to previous studies, children up to 10 years old were the most affect group.

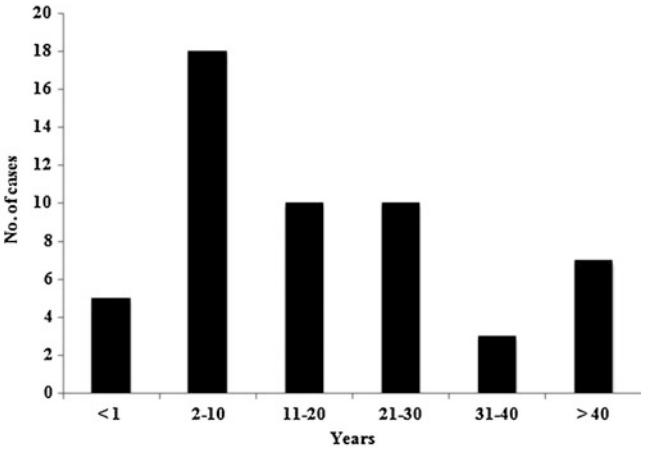

Fig. 6 Scorpion sting accidents according to age reported to the National Drug and Poison Information Center during 2009–2012

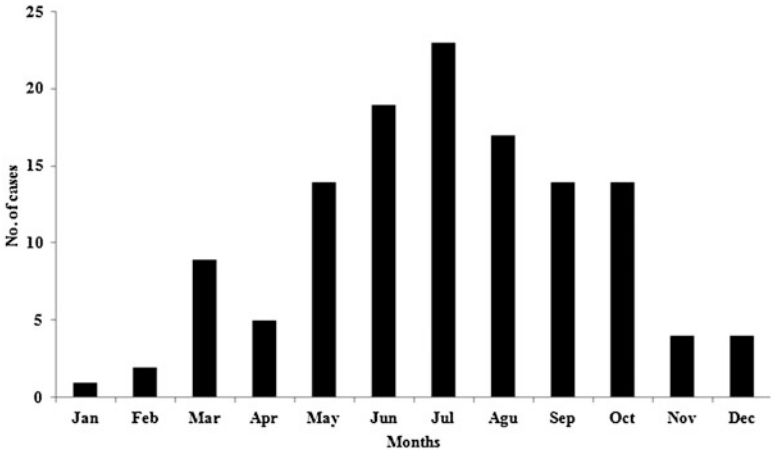

Fig. 7 Seasonal distribution of the scorpion stings based on data from NDPIC and PHH

Seasonality

The incidence of scorpion stings is related to the seasonal activity of scorpions in Jordan. Our field experience indicated higher activity of scorpions during the summer, especially at night, where scorpions become more active to avoid the high temperatures of the daytime and search for food. Stings were more frequent in the summer, peaking in July (Amr et al. 1988, 1994). Combined data obtained from NDPIC and PHH for seasonal sting incidents showed that most accidents occurred in July (Fig. 7).

In a study conducted on scorpion sting accidents in Fidan, a small village in Wadi Araba, with a total of 36 cases, indicated that most occur at night, between 21.00 and 01.00 h (Amr et al. 1994).

Symptoms and Associated Complications

Local pain at the site of the sting was the most common symptoms in all patients, followed by local erythema. Cardiovascular involvement included sinus tachycardia and bradycardia, hypertension, and pulmonary edema, while arterial fibrillation, hypotension, and heart failure were less common. Neurological manifestations included irritability, hypersalivation, and excessive sweating (Mutair et al. 2001).

Clinical study of 15 scorpion sting patients referred to the Jordan University Hospital revealed the following symptoms: tachycardia, abdominal pain, dizziness, leukocytosis ranging from 10,450 to 20,000 WBC/mm 3, dyspnea, proteinuria, and electrocardiographic evidence of myocarditis. Two patients died (9- and 16-year-old girls) due to acute heart failure secondary to toxic effects of scorpion venom on myocardial fibers. These two patients were autopsied and histological examination of the heart revealed evidence of focal myocarditis (Amr et al. 1988).

Wahbeh (1976) summarized symptoms associated with stings of *L. quinquestriatus*, including a sudden rise in blood pressure and increasingly rapid heartbeat. This is followed by bradycardia. Yarom (1970) reported that the EKG of patients following the scorpion sting resembled an early myocardial infarction like pattern and that the urine contained high amounts of both catecholamines and their metabolites. Other symptoms consist of sweating, thirst, burning pain at the site of the sting, vomiting, salivation, and dyspnea.

Wahbeh (1965) reported that "erected penis" (priapism) was one of the symptoms observed while treating a child in Ramallah Hospital on the West Bank of Jordan who was stung by *B. occitanus*. This species is restricted to Europe and perhaps it was misidentified for either *Buthus israelis* or *Buthus amri*. Amayreh et al. (2009) reported a case of necrotizing fasciitis as a complication of a scorpion sting in an 18-day-old male neonate who was managed at PHH in Aqaba. Symptoms include perfused extremities and extensive redness over the right anterior chest wall, marked tachycardia, and respiratory distress with a pulse rate of 190 beats per minute and a respiratory rate of 65 breaths per minute. He later developed blackish discoloration which started to appear over the anterior chest wall.

Lethal Dose of Scorpions of Medical Importance in Jordan

All scorpions are dangerous; however, few of the known species have powerful venom that can cause death among humans. Deadly species in Jordan are members of the family Buthidae under three genera (*Buthus, Leiurus,* and *Androctonus).* The other species of the family Buthidae have mild venoms that have not been reported to cause deaths. All species of the family Scorpionidae are not considered dangerous. Venom of scorpions of the genus *Androctonus* is discussed in this book (Scorpion Venom Research Around the World: *Androctonus* Species, 364647).

Lethal dose 50 (LD_{50}) varies among venomous species (Table 3). *Androctonus crassicauda* has the lowest LD_{50} (0.08–0.5 mg/kg) followed by *L. quinquestriatus*

Table 3 Reported toxicities of some scorpions of medical importance

	LD_{50} (mg/kg)	Method	References
Androctonus amoreuxi	0.75	sc	Habermehl (1981); Hassan (1984); Watt and Simard (1984)
	1.050	sc	Ismail et al (1990)
	0.88 mg	im	Ghazal et al. (1975)
Androctonus crassicauda	0.08–0.50	sc/iv	Zlotkin et al. (1976); Hassan (1984)
	0.87–1.5	sc/iv	Al Sadoon and Al Asmari (2000)
	2.68	sc	Ozkan et al. (2006)
	0.64	sc	Ismail et al. (1994)
Androctonus bicolor	1.21	iv	Hassan (1984)
Buthus occitanus	0.90–1.44	sc/iv	Zlotkin et al. (1976)
	5,750 ug	im	Hassan (1984)
Buthacus leptochelys	5.62	iv	Hassan (1984)
Compsobuthus acutecarinatus	0.75	iv	Hassan (1984)
Hottentotta judaicus	7.94	iv	Hassan (1984)
Leiurus quinquestriatus	0.16–0.50	sc/iv	Zlotkin et al. (1976); Hassan (1984)
	0.46–0.42	sc/iv	Al Sadoon and Al Asmari (2000)
	0.5	ip	Alawi and Jeryes (1982)
Scorpio maurus palmatus	200	iv	Lazarovici and Zlotkin (1982)
Scorpio maurus	9.37 inactive	iv	Hassan (1984)
	141.6 inactive	sc	Habermehl (1981)

All doses are expressed in mg of venom per kg of mouse
im intramuscular injection, *ip* intraperitoneal injection, *iv* intravenous injection, *sc* subcutaneous injection

(0.16–0.50 mg/kg). The other two species of the genus *Androctonus* are still considered venomous with an LD_{50} as low as 0.75–1.21 for *A. amoreuxi* and *A. bicolor*, respectively.

For the local scorpions in Jordan, two papers addressed the LD_{50} for five species. Wahbeh (1976) determined LD_{50} for *L. quinquestriatus*, *Hottentotta judaicus*, and *Androctonus crassicauda* in mice injected with crude venom intraperitoneally, with values of 62.5, 125, and 125 ug/20 g in male Swiss mice, respectively. He stated that both *Nebo hierichonticus* and *Scorpio maurus* are not venomous. Alawi and Jeryes (1982) determined the LD_{50} for *L. quinquestriatus* crude venom collected from different localities in Jordan (Table 3). *Nebo hierichonticus* is the largest scorpion species known to occur in Jordan. Its venom has a negligible effect on human (Rosin 1969a) and has a hemolytic effect (Rosin 1969b). On the other hand, Annobil et al. (1991) reported acute pulmonary edema, fundal hemorrhages, temporary blindness, and deafness following a *N. hierichonticus* scorpion sting in a 3-year-old boy in Saudi Arabia.

Conclusion and Future Directions

The taxonomic status of scorpions of Jordan requires up-to-date revision. For example, species of the genera *Buthus, Buthacus*, and *Compsobuthus* are not well documented and thus require a thorough revision. Further collection and identification of the scorpions of Jordan employing molecular identification would be of great assistance.

The need for documentation of scorpion sting accidents should be mandatory by all health providers countrywide. It was difficult to obtain data on scorpion sting accidents, since most "sting" cases are not filed according to the causative animal. Further studies should focus on the determination of the LD_{50} for the Jordanian scorpions.

Protocol for scorpion sting accidents treatment should be formulated by the Ministry of Health and circulated overall health centers in the country.

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Scorpionism and Dangerous Species of Mexico

9

Alfredo Luis Chávez-Haro and Ernesto Ortiz

Contents

Abstract

The incidence of scorpionism in Mexico is one of the highest around the world. With more than 250,000 accidents reported every year, it has become a serious health problem. Seven scorpion species belonging to the *Centruroides* genus are the main culprits of the cases of human envenomations in this country. The experience acquired after more than 40 years of handling scorpion-stung patients in the Red Cross Hospital of León, Guanajuato, Mexico, is here detailed. Nationwide statistics from the Mexican Secretary of Health are also contributed. Envenomations due to scorpion sting are divided into three categories in

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accordance to the seriousness of the symptoms. Patients with moderate and severe symptoms are immediately treated with specific anti-scorpion polyvalent antivenoms (generically denominated "fabotherapeutic") made in Mexico, with outstanding results. The nationwide application of the Norma Oficial Mexicana for the treatment of scorpion stings has resulted in a dramatic reduction of deceases due to scorpionism.

Introduction

Mexico belongs to the group of countries with the highest morbidity indexes due to scorpion stings (scorpionism). More than 250,000 cases are reported to medical authorities every year, which catapults scorpionism to the 14th place within the most frequent ailments in Mexico. The states with the largest number of reported cases are Jalisco, Guerrero, Morelos, Michoacán, Guanajuato, Puebla, Nayarit, Colima, Estado de México, Sinaloa, Durango, Querétaro, Sonora, and Oaxaca, in that order (Dirección General de Epidemiología, DGE). Figure 1 shows the data reported by the Centro Nacional de Programas Preventivos y Control de Enfermedades (CENAPRECE), belonging to the Secretary of Health (SSA), corresponding to the nationwide incidence of scorpionism over the course of the twenty-first century. It is worth noting that those numbers are only a fraction – probably a half – of all the incidents, since many people, usually healthy adults, do not go to the hospital when stung by a scorpion. These numbers have been slowly but steadily growing. This could be the result of the expansion of human settlements and cultivated areas to scorpion-inhabited ecosystems, leading to a higher number of incidents. Other contributing factors cannot be excluded, such as the invasion and colonization of man-altered environments by opportunistic species (Lourenço et al. 1996) or simply the increment in the percentage of reported incidents due to the increased accessibility to health-care facilities for the general population and the impact of educational campaigns.

The high incidence of scorpion stings and the presence of several scorpion species that pose a real threat to human health and life have derived in the management of these accidents as emergencies in Mexican hospitals. Immunotherapy is the only effective treatment available to physicians. There is solid evidence which demonstrates that the use of immunotherapy at the earliest stages of intoxication reduces the effect of the venom, appearance of complications, and hospital stay. The availability of the anti-scorpion antivenom has resulted in many saved lives.

Venom Components of Mexican Scorpions

Specific book chapters of this edition will be describing in details the composition and function of scorpion-venom components, in general. Please refer to chapters "▶ Potassium Channel Blocking Peptide Toxins from Scorpion Venom" and

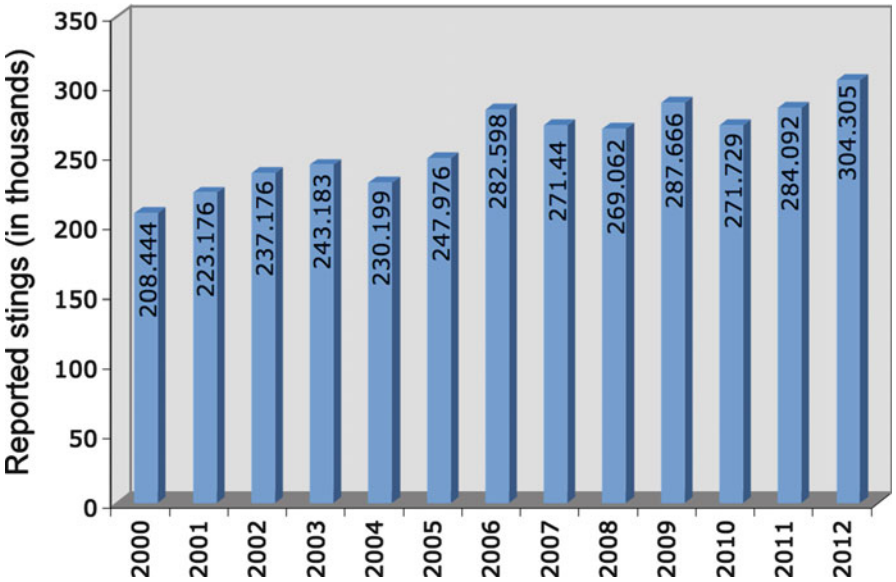

Fig. 1 Total number of reported scorpion stings in Mexico in the twenty-first century (Source: CENAPRECE (http://www.cenavece.salud.gob.mx/))

"► Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels." Here a brief outline of the major findings related to Mexican scorpions will be presented. The venom components of Mexican scorpions are a complex mixture of proteins, peptides, nucleotides, biogenic amines, carbohydrates, amino acids, lipids, and mucopolysaccharides (reviewed in Possani et al. 1999). The most abundant and pharmacologically important are the peptides capable of recognizing ion channels, causing an anomalous depolarization of cells, which can eventually lead the stung individual to death. There are short chain peptides of 30–40 amino acids in length, usually known to block K^+ channels function (for review see Rodríguez de la Vega and Possani 2004), longer peptides of 59–75 amino acids known to affect the gating mechanism of Na^+-channels (reviewed in Rodríguez de la Vega and Possani 2005), and peptides such as hadrucalcin, capable of interfering with the ryanodine-sensitive Ca^{2+} channels (Schwartz et al. 2009). Hundreds of components were isolated and characterized chemically and functionally, but also the genes coding for some of these peptides were cloned, sequenced and analyzed, and even heterologously expressed (for specific reviews see Quintero-Hernández et al. 2011; Rendón-Anaya et al. 2012; Pedraza-Escalona and Possani 2013; Rodríguez de la Vega et al. 2013). In addition, an important effort has been dedicated to the development of single-chain human antibodies, capable of neutralizing the envenomation symptoms caused by major toxic components of some Mexican scorpions (Riaño-Umbarila et al. 2005 and Chap. 6, "► Recombinant Neutralizing Antibodies, A New Generation of Antivenoms").

Epidemiology of Scorpionism in Mexico

In one medical institution alone, the Red Cross Hospital in the city of Leon, Guanajuato, one of the first to start a rigorous data collection and statistics generation system for scorpionism in Mexico, the total number of stung patients from 1971 to 2012 was 274,143. From them, 57.8 % were males and 42.2 % females. The distribution by age was as follows: 1 % were infants younger than 1 year old, 8 % were 1–4-year-old toddlers, 22 % were 5–14-year-old young children, 29 % were 15–24-year-old adolescents, 27 % were 25–44-year-old young adults, 9 % were 45–64-year-old middle-aged adults, and 4 % were 65-year-old and older adults (Fig. 2a.). This distribution is similar to the statistics reported nationwide by other institutions. The DGE reports 26 % for 25–44 years old and 24 % for 15–24 years old, as the groups with a higher incidence, and 9 % for children under 5 years old. It is interesting to notice that, for the last lustrum, the DGE statistics for the whole country reveal that the distribution of stings by gender is 50.9 % for females and 49.1 % for males, which is closer to the general population distribution (51.5 % females and 48.5 % males, Instituto Nacional de Estadística y Geografía, INEGI).

There is a clear seasonal peak for the frequency of stings in the spring and early summer months of March, April, May, and June. These 4 months alone account for 57.8 % of the reported cases. The largest number of accidents takes place inside the houses (80 %), of which 58 % occur in the bedroom, 30 % in the courtyard, 11 % in the kitchen, and 1 % in the bathroom (Fig. 2b.). About 60 % of all cases occur at night and the rest during the day, but with a clear tendency towards dusk.

With respect to the anatomical region affected, 42 % of the stings were in the lower limbs, 40 % the upper limbs, 11 % the torso, and 1 % the head and neck (Fig. 2c.). The Instituto Mexicano del Seguro Social (IMSS) reports the following national statistics on the number of stings a patient has received: 65 % were stung for the first time, 17 % for the second, 4 % for the third, 3.4 % for the fourth, 4 % for the fifth, and 1.6 % for the sixth, and the remaining were stung seven or more times. The same statistics from the Red Cross Hospital in Guanajuato is very similar, but discerns patients stung for the seventh and more times up to the seventeenth: 62 % stung for the first time, 18 % for the second, 5 % for the third, 3.4 % for the fourth, 3 % for the fifth, 2 % for the sixth, 3 % for the seventh, 1.6 % for the eighth to the tenth, 1 % for the eleventh or twelfth, 0.8 % for the thirteenth to the fifteenth, and 0.2 % for the sixteenth and the seventeenth. There is a female patient who has been stung 17 times by scorpions and twice by black widows (*Latrodectus mactans*).

At the national level the mortality index has been steadily decreasing. In the 1980s the mortality rate was roughly 0.4 %. In the 1990s it went down to 0.2 %, and nowadays it is lower than 0.01 % (Sistema Nacional de Información en Salud, SINAIS). In the Red Cross Hospital in Guanajuato, the mortality has been zero for the years between 1971 and 2012, thanks to the employment of the antivenom (see below for standard treatment). Also, no anaphylactic reactions or serum sickness events were reported in that period (Archivos Cruz Roja Mexicana. Delegación León, Guanajuato).

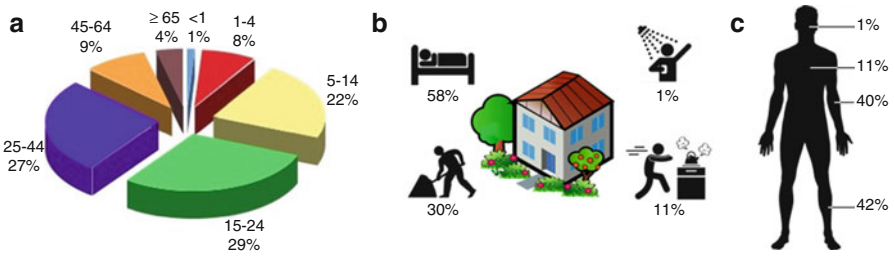

Fig. 2 Distribution of scorpion-related accidents: (**a**) by age, (**b**) place of occurrence in the houses, and (**c**) the affected anatomical region. All clip arts were downloaded from http://www.openclipart.org/ and bear Creative Commons licenses

Risk Factors Associated with Scorpion Stings

In order to tackle the clinical profile that a stung patient faces, it is necessary to consider a number of risk factors concerning the scorpion and the patient:

On the scorpion's side:

- The offending species. Of the more than 220 species identified in the Mexican territory, only seven are dangerous to humans: *Centruroides elegans*, *C. infamatus infamatus*, *C. limpidus limpidus*, *C. tecomanus, C. noxius*, *C. sculpturatus*, and *C. suffusus suffusus*, all belonging to the *Centruroides* genus of the Buthidae family. These species are endemic to areas located towards the Mexican Pacific Coast (Fig. 3).
- The amount of injected venom. It is well documented that, since venom is metabolically expensive, scorpions can regulate the injected amount in a defensive situation based on perceived risk. Under perceived low-threat conditions scorpions can deliver dry (without envenomation) stings and if the threat persists they will then inject the venom. The quality (toxicity) of the venom can also be regulated, with a higher probability of injecting a more toxic venom after repeated stings (Nisani and Hayes 2011). The amount of venom also depends on the age and size of the scorpion.

On the patient's side:

- Age. The Norma Oficial Mexicana NOM-033-SSA2-2002, for the surveillance, prevention, and control of the envenomation due to scorpion stings, determines that for any infant from endemic areas with a sudden and persistent cry and displaying any of the moderate symptoms of envenomation (see below), a sting should be suspected. It also puts the old adults at a high risk of complications.
- General state of health before and at the moment of the sting. NOM-033-SSA2-2002 puts patients with a history of diabetes or hypertension and pregnant

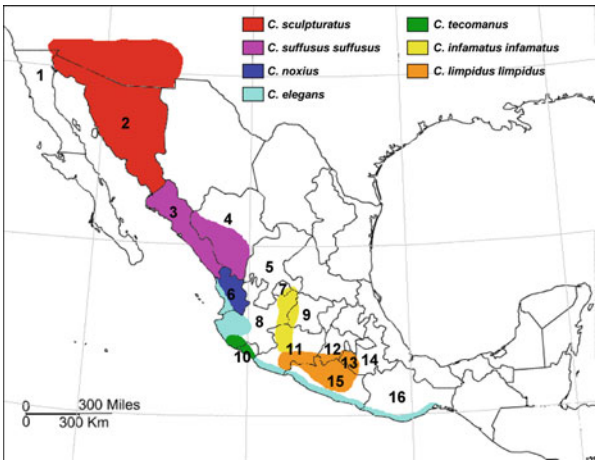

Fig. 3 Approximate geographic distribution of the scorpion species that are dangerous to humans in Mexico. The states identified with numbers are 1-Baja California, 2-Sonora, 3-Sinaloa, 4-Durango, 5-Zacatecas, 6-Nayarit, 7-Aguascalientes, 8-Jalisco, 9-Guanajuato, 10-Colima, 11-Michoacán, 12-Querétaro, 13-Morelos, 14-Puebla, 15-Guerrero, and 16-Oaxaca. This is an approximate map; for the precise distribution of these and other species belonging to the Buthidae family in Mexico, please see http://www.ibiologia.unam.mx/html/buthidae.html

women within the high risk of complications group. Stung patients with alcoholic intoxication and those that display a rapid progression towards systemic manifestations and vital sign alterations also pose a higher risk.

- Weight. For a fixed amount of injected venom, a lower body mass (or volume, strictly speaking) will translate into a higher effective venom concentration. This is particularly relevant for little children, who are more vulnerable to complications and develop the symptoms of envenomation very quickly, demanding a prompt medical response. While in an adult the envenomation symptoms usually appear 20 min to 2 h after the sting, in an infant they can manifest within 14 min or even instantly (LoVecchio and McBride 2003).

Clinical Profile of Scorpion Envenomations

The clinical profile due to envenomation following a scorpion sting can be divided in three stages with increasing severity in accordance with the following symptomatology:

Light envenomation: Local pain and/or paresthesias (pins and needles) at the site of envenomation, pruritus (itching) or inflammation at the affected site, slight agitation.

Moderate envenomation: In addition to local findings, pain and/or paresthesias remote from the site of the sting, persistent cry in infants, anguish, headache, epiphora (overflow of tears), conjunctivitis, nose, mouth and throat pruritus,

sialorrhea (drooling) or rhinorrhea (runny nose), sneezing, dry mouth, foreign body sensation in the throat, dysphagia (difficulty in swallowing), tongue fasciculations (muscle twitch), cutaneous hyperesthesia (hypersensitivity), abdominal distention (swelling), diarrhea, dyslalia (speech impairment), and intense abdominal and muscular pain.

Severe envenomation: Presence of the moderate symptoms plus arterial hypertension or hypotension, fever, miosis or mydriasis (contraction or dilation of the pupils), photophobia, nystagmus (involuntary eye movement), severe involuntary shaking or jerking of the extremities, emesis (vomiting), tachycardia or bradycardia (accelerated or slowed heart rate), arrhythmia (irregular heartbeat), temporal amaurosis (vision loss), dyspnea (shortness of breath), peribuccal cyanosis (bluish purple discoloration around the mouth), chest pain, priapism (persistent penis erection), oliguria (low output of urine), unconsciousness, pulmonary edema (fluid accumulation in the lugs), heart failure, and death.

Besides those symptoms, paraclinic studies can show gastroparesis (delayed gastric emptying) and ileus (bowel obstruction), elevated levels of blood serum amylase, hyperglycemia, electrolyte disturbances without any specific pattern, and cardiopathy (atrioventricular conduction abnormalities, arrhythmias, repolarization disorders, expanded QT interval, interventricular conduction abnormalities) (Calderon-Aranda et al. 1996; Maraboto-Martínez et al. 1997; González-Romero et al. 1991; NOM-033-SSA2-2002).

Treatment of Scorpion-Stung Patients

Scorpions are among the most ancient terrestrial animals. They have inhabited the Earth long before man, and, since they are very successful predators, they became widely spread. Our physical interaction with them frequently results in stings. Because of that, there is a huge amount of information regarding traditional treatment methods, most of them based on serendipity, anecdotes, and oral tradition. Practices that fuse religion with magic and employ a vast array of alternative medicines (of herbal or animal origin) are traditionally exerted by witches, healers, and shamans. It would be necessary to write a different chapter to cover them, since almost every community has its own methods, some of them even very awkward for the uninitiated. Traditional treatments will not be reviewed here since, as demonstrated by the mortality statistics given above, it is evident that none of them have been very helpful in diminishing the lethality of scorpion stings.

Historically, the procedures followed by doctors to treat stung patients have varied and have been dictated by their personal experience. The measures adopted have been basically directed at ameliorating the symptoms that each patient displays. In Mexico, modern-day treatments are based on the use of the scorpion-specific antivenom, which is a mixture of antibody fragments $F(ab')_2$

(hence the generic name "fabotherapeutic"), generated by the purification and enzyme digestion of the immunoglobulins obtained from the polyclonal serum of immunized horses. The widespread use of the antivenom starting in the 1990s has drastically reduced the mortality by scorpion stings, as shown above. The protocols described here have demonstrated their effectiveness in the treatment of more than 270,000 stung patients at the Red Cross Hospital in León, Guanajuato, in more than 40 years of experience. They constitute the basis for the protocols implemented by the IMSS from 1996 (with more than 700,000 patients treated) and of the Norma Oficial Mexicana NOM-033-SSA2-2002 issued by the Mexican Secretary of Health for the treatment of scorpion stings, as part of the National Health System.

The history of scorpion antivenoms in Mexico dates back to 1904 (for a beautiful and touching review, see Boyer 2013) when a physician named Daniel Vergara Lope obtained the first ever scorpion-specific antiserum and demonstrated its effectiveness in pigeons and rodents and reportedly cured two stung adults and one baby in Morelos. Then, in 1926, Drs. Isauro Venzor and Carlos León de la Peña produced the first anti-scorpion antivenom to be used in humans. It consisted of the whole serum from immunized horses and was specific for Durango. They immediately reduced the mortality by 80 %. But this antivenom raised at the time the concerns of many physicians who feared the adverse reactions to horse serum. Their fears were justified. These reactions, known as serum sickness, are the consequence of a hypersensibility produced by foreign proteins from nonhuman sera when injected into humans. The human body produces antiserum immunoglobulins that end up triggering an inflammatory process that can lead to serious effects including shock and death. Notwithstanding the beneficial effects of the antisera in treating seriously envenomed patients, the fear of the serum sickness remained and was the main obstacle for the widespread use of the new generation of antivenoms in Mexico. For the production of the antivenoms used today, the horse immune serum is subjected to a process of enzymatic digestion that cleaves the Fc fragment from the immunoglobulins. This fragment is responsible for the activation of the complement system, eliciting effector responses. The $F(ab')_2$ fragment mix is then purified from all the irrelevant components of the digested serum and used as the antivenom. The dangers of developing a serum sickness-related reaction are therefore reduced to the minimum. It is so that, in the Red Cross Hospital in Guanajuato, not a single adverse reaction has been reported in more than 40 years, even in patients that have been stung and received several doses of the antivenom in more than one event. In the same time the mortality has been reduced to zero. These statistics translated into a nationwide educational campaign in the 1990s that resulted in the adoption of the antivenom by the greatest majority of doctors and its widespread use today.

When a stung patient with no symptoms of envenomation is admitted to the Red Cross Hospital in Guanajuato, only analgesics are dispensed: intravenous Metamizol for adults (35 mg/kg) and rectal acetaminophen for children (20 mg/kg). The patient is then put under observation and, if no symptoms develop, discharged. When a patient shows symptoms of envenomation, five early

diagnostic symptoms work as alarm signals to start the treatment with the antivenom: tongue fasciculations, foreign body sensation in the throat, sialorrhea, nystagmus, and abdominal distention. In this case the protocols issued as NOM-033-SSA2-2002 by the SSA are put to work. This Norma Oficial Mexicana establishes the following.

Care and Treatment

The management and treatment of envenomations due to scorpion stings should be specific, with the anti-scorpion antivenom applied to all age groups and pregnant patients during the first 30 min after the sting, avoiding the waiting for the appearance of serious manifestations to start the treatment.

1. Drugs
   - 1.1. Anti-scorpion antivenom, in all its varieties: serum or fabotherapeutic.
     - 1.1.1. Serotherapy and fabotherapy are the specific treatments for scorpion sting envenomation and therefore the resource of first choice.
     - 1.1.2. The lyophilized anti-scorpion antivenom is preserved under refrigeration (2–8 °C) up to 6 years, or up to five without refrigeration, if the temperature does not rise above 35 °C, in which case its viability will be 6 months. When used, the lyophilized serum requires dilution with 5 ml of sterile diluent.
   - 1.2. Other drugs

   Other drugs useful in the symptomatic treatment, which are not substitutes for the anti-scorpion serum, are:
     - 1.2.1. Metamizol-type analgesics or diclofenac in adults and acetaminophen in children. In case of severe pain, 0.5 % or 1 % lidocaine hydrochloride (Xylocaine) can be applied locally.
     - 1.2.2. Antihistamines of the diphenhydramine type which are to be used with caution in children.
     - 1.2.3. A cardiac glycoside of the *Digitalis* type in case of cardiac failure and acute pulmonary edema.
2. Dosage and administration route
   - 2.1. The anti-scorpion antivenom, besides protecting life, reduces hospital stay, medical expenses, relieves pain and discomfort, and prevents various complications. The anti-scorpion antivenom is applied according to the following criteria:
   - 2.2. In children under 5 years, initially two vials, intravenously, with observation for 20 min. If there is no improvement, another vial should be applied.
   - 2.3. In adults, one vial should be applied intravenously, with observation for 20 min. If there is no improvement, another vial should be applied.
   - 2.4. The anti-scorpion antivenom should be applied intravenously and, in those patients for whom it is not possible to use this route, should be used intramuscularly.

2.5. Although there is no limit to the number of antivenom vials that can be employed, it is recommended that a maximum of five vials per patient be used. It is a dose high enough to neutralize a major dose of the venom.
2.6. Patients with heart disease, asthma, kidney disease, cirrhosis, alcoholics, diabetics, and pregnant women should be treated according to the clinical situation simultaneously with the anti-scorpion antivenom.

3. Adverse Reactions
3.1. Although virtually no adverse reactions to the anti-scorpion antivenoms currently used in Mexico have been reported, any history of hypersensitivity to heterologous sera should be investigated before application.
3.2. Patients with hypersensitivity to the antivenom may exhibit the following symptoms: nausea, vomiting, and, rarely, anaphylactic shock, which should be treated with 1/1,000 (1 mg/ml) adrenaline, administering 0.5 ml every 15 min, depending on response, subcutaneously or intramuscularly, plus oxygen therapy, corticosteroids, and other drugs that according to the doctor's opinion are required.
3.3. Eight days after the anti-scorpion antivenom is administered, serum sickness symptoms may appear due to the production of anti-equine immunoglobulins. The symptoms are urticarial syndrome, tissue edema, joint pain and fever, headache, vomiting, and light lymphadenitis. Treatment consists of antihistamines and topical corticosteroids.
3.4. The general measures of care are permanent monitoring of vital signs, bed rest, keeping airways and a vein permeable, oxygen if necessary, and fasting for six hours, or until the disappearance of pharyngeal symptoms.

4. Contraindications
For patients envenomed due to a scorpion sting, the following drugs are contraindicated:
4.1. Meperidine, codeine, morphine, and other opioids, in general any inhibitor of the respiratory center.
4.2. Calcium gluconate since the serum calcium in patients is elevated.
4.3. Atropine, it adds to the effect of the poison and favors the development of ileus.

5. Community care personnel attending patients with scorpion sting poisoning.
5.1. In order to make the specific treatment of envenomations due to scorpion stings (anti-scorpion serum or fabotherapeutic) available to rural populations without access to health services or when those are more than 30 min away from the localities, community personnel will be trained to apply anti-scorpion antivenom intramuscularly, upon presentation of a case of envenomation. This strategy has contributed significantly to the decline in mortality due to scorpionism in Mexico.
5.2. The community worker or a trained volunteer, besides administering the treatment to the patient, records the event and makes the simplified epidemiological study.
5.3. For patients with a history of diabetes, hypertension, or asthma, as well as for pregnant and lactating women, the community worker will administer the anti-scorpion antivenom and then direct the patient to the nearest medical facility.

General Recommendations

The effectiveness of the specific antivenom therapy is inversely proportional to the time it takes for a stung patient to receive medical attention. Most reported deceases occur because the treatment is started too late, when the patient arrives to the hospital with severe symptoms and complications. A study from 2007 showed that in localities with a population of less than 2,500, the mortality rate was 11.8 times higher than for those with more than 20,000 inhabitants (Celis et al. 2007). This is in direct connection with the availability of health-care systems in the vicinity that could adequately tackle the envenomation syndrome with the standard protocols. Myths, beliefs, and traditions can also pose dangers for the victims since they do nothing but delay professional medical care.

When young children from endemic areas present any of the symptoms that can be associated with envenomation due to a scorpion sting, the treatment with the antivenom should be started immediately, even when the offending scorpion is not found. The same applies to pregnant women, independently of the gestation time, to reduce the possibility of abortion or premature delivery.

Patients with non-related conditions, infectious diseases, and chronic degenerative or metabolic illnesses should receive the required specific medications besides the antivenom. If any complications appear in a stung patient, pancreatitis, heart disease, pulmonary edema, hyperglycemia, etc., the specific treatment should be applied while keeping the administration of the needed amount of antivenom. No patient should be discharged while tongue fasciculations are still present. The experience indicates that this is usually the earliest symptom of envenomation to appear and one of the last to recede.

One of the outcomes of scorpion venom's action at the cellular level is the massive acetylcholine release as a consequence of the modification of the functioning of sodium and potassium channels, which results in the delay of action potentials in the neurons of the autonomous nervous system (Gomez et al. 1973). In the Red Cross Hospital in León, antihistamines are thus administered not because of fear of allergies, but to take advantage of their antimuscarinic effect (Kubo et al. 1987). Steroids are avoided since no medical justification is seen for their use.

The experience in the Red Cross Hospital in León is that roughly 60 % of the admitted scorpion-stung patients respond appropriately to symptomatic treatments and do not show signs beyond those of light envenomation. This could be the result of a dry sting or the injection of a very low amount of venom. Thirty-eight percent of the patients require the application of a single dose (a vial) of the anti-scorpion fabotherapeutic, and only 2 % require two doses or more.

There are two different brands of anti-scorpion fabotherapeutics available in Mexico. One is produced by the state-owned company Laboratorios de Reactivos y Biológicos de México S.A. de C.V., BIRMEX (http://www.birmex.gob.mx/) under the name "Faboterápico Polivalente Antialacrán," and the other is manufactured by the Instituto Bioclon (http://www.bioclon.com.mx/) under the trademark Alacramyn®. Both are very effective for the treatment of envenomations, but the latter requires a lower number of doses. In the Red Cross Hospital in León, up to ten

doses of the BIRMEX antivenom have been needed per patient (four on average), while three doses of Alacramyn® have been the maximum administered per patient.

With these practices, the average hospital stay for stung patients at the Red Cross Hospital in León is one hour. Patients who come to the hospital facilities late and with already severe symptoms of envenomation stay for a maximum of 3 h.

Conclusion and Future Direction

From 1979 to 2003 a total of 6,077 deaths due to scorpionism were registered in Mexico, a mean of 243.1 per year (Celis et al. 2007). Taken together, the periods of 1979–1982 and 2001–2003 saw a mortality reduction of 86.5 %. Nevertheless, in 3 years, 2001–2003, the number of deceases was 206, a mean of 68.7 per year. The Norma Oficial Mexicana NOM-033-SSA2-2002 for the surveillance, prevention, and control of the envenomation due to scorpion stings was issued by the SSA in 2003. From 2006 to 2012, a 7-year interval, the number of deaths was 209, a mean of 29.9 per year (SINAIS). These numbers continue to decrease year after year. These statistics validate the effectiveness of the treatment adopted by Mexican physicians under the guidance of the Secretary of Health.

Cross-References

► Potassium Channel Blocking Peptide Toxins from Scorpion Venom
► Recombinant Neutralizing Antibodies, A New Generation of Antivenoms

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Scorpionism and Dangerous Scorpions in Central America and the Caribbean Region

10

Adolfo Borges

Contents

Abstract

This chapter assesses scorpionism and noxious scorpions in Central America and the Caribbean area, scarcely surveyed previously despite the presence of potentially dangerous scorpions of the genera *Centruroides* and *Tityus*, family Buthidae. In Central America, most scorpion stings in Guatemala, Belize, El Salvador, Honduras, Nicaragua, Costa Rica, and Panama are caused by *Centruroides* spp. which are mildly toxic to humans, including *C. edwardsii*,

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C. granosus, and *C. gracilis*, with the highest regional morbidity corresponding to Panama. Lethal *Tityus* species from Panama are *T. asthenes*, *T. festae*, *T. pachyurus*, *T. cerroazul*, and *T. championi*, the last three shared with Costa Rica, where at least one death has been reported. In the West Indies, *T. trinitatis*, endemic to Trinidad and Tobago, is the only species accountable for human deaths. In the Greater Antilles, synanthropic species are responsible for mild accidents, such as *C. gracilis* and *Rhopalurus junceus* (Cuba), *C. insulanus* (Jamaica), *C. griseus* (Puerto Rico/Virgin Islands), *C. margaritatus* (Jamaica/Cuba), and probably *C. nitidus* (Hispaniola). In the Lesser Antilles, mild envenomations are produced by *C. barbudensis* and *C. testaceus*. The regional distribution of scorpions, and scorpionism thereof, is the result of physiographic, environmental, and anthropic factors. The latter have contributed with extinction of Lesser Antillean *Tityus* spp., which niches are now occupied by opportunistic *Centruroides* spp., whereas the former explains the phylogenetic and toxinological relatedness of Panamanian/Costa Rican and Trinidad/Tobago *Tityus* spp. to congeners from northern South America.

Introduction

Scorpionism, or the accident in humans derived from envenomation by noxious scorpions, is a worldwide public health problem restricted to tropical and subtropical areas, which mainly affect children under 10 years of age (Amitai 2005). In the Americas, species in genera *Centruroides* Marx and *Tityus* C.L. Koch, both belonging to the family Buthidae, are responsible for systemic scorpionism in the Pacific versant of Mexico, southeastern Brazil, the Amazonia, and northern South America (Chippaux and Goyffon 2008). Central America (the land located between the Isthmus of Tehuantepec (Mexico) and the Colombian Andes range) and the Caribbean region (the island area positioned southeast of the Gulf of Mexico and north of South America) are also inhabited by scorpions in these two genera (Fig. 1), but scarcity of data has hampered the toxicological/clinical evaluation of local dangerous species and scorpionism (Borges et al. 2012). This contrasts with the ongoing taxonomic survey of the regional scorpion fauna, which is one of best catalogued in the Americas (Armas 2001; Lourenço 1987; Santiago-Blay 2009; Teruel and Kovařík 2012). Reports on the apparently low toxicity of Caribbean and Central American scorpion taxa are known since the sixteenth century (Fernández de Oviedo y Valdés 1535), but a different picture emerged in modern times when stings by *Tityus* spp. from Panama and Trinidad/Tobago were proven lethal to humans (Borges 2013; Borges et al. 2012). This review is based on the available faunal distribution and epidemiological data to explain the distinct scorpionism picture prevalent in the Caribbean and Central America, a consequence of the distribution of their scorpion species, evolutionary derived from centers of endemism located in Mexico and northern South America.

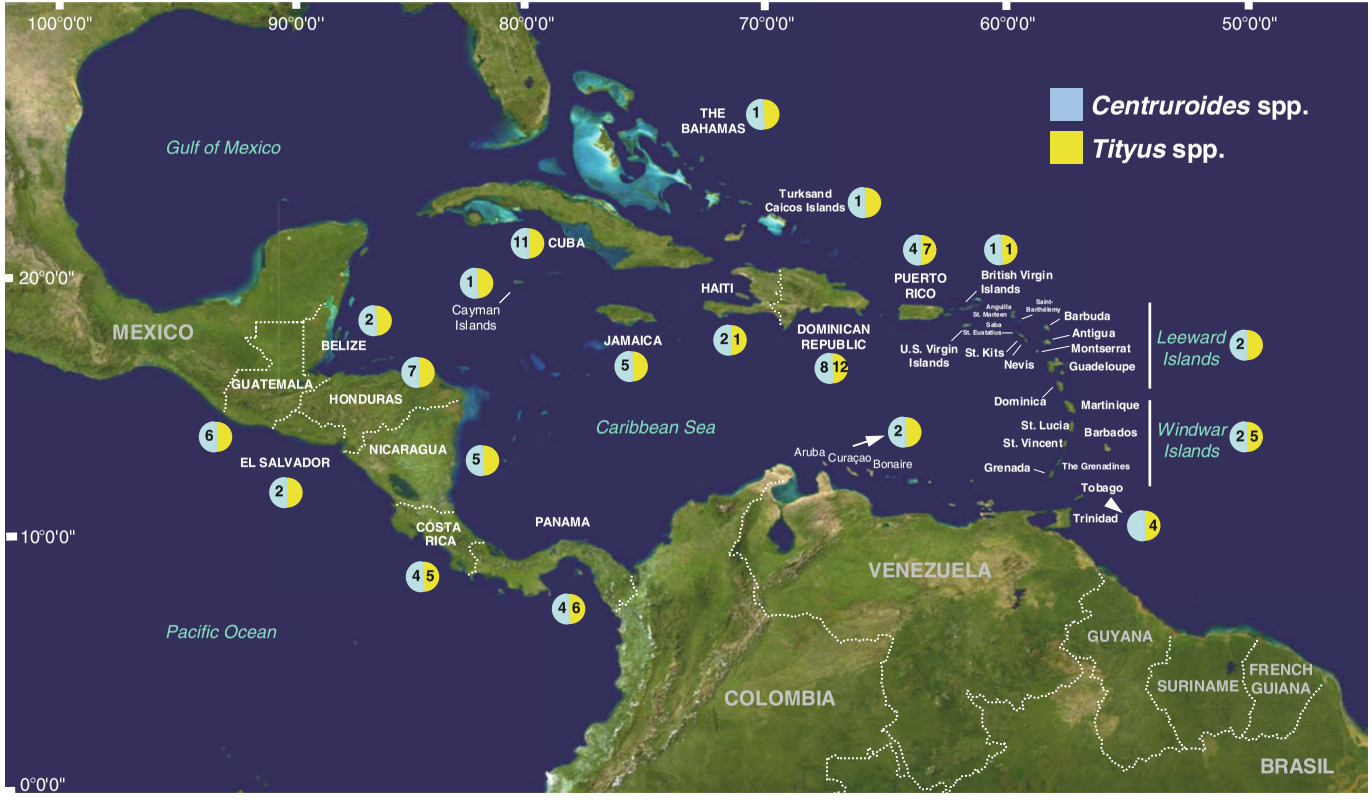

Fig. 1 Map of Central America and the Caribbean area, showing distribution and species number (per country or island group, in *colored circles*) in genera *Centruroides* Marx and *Tityus* C.L. Koch

The Caribbean

The Caribbean region includes several distinct island groups which support abundant, diverse, and largely endemic populations of terrestrial invertebrates, including scorpions. The higher degree of endemism showed by the Caribbean scorpion fauna compared to most of the Neotropical territories has been explained as due to the prolonged isolation of the islands (Armas 2001). The larger islands of Cuba, Jamaica, Hispaniola (encompassing Haiti and the Dominican Republic), and Puerto Rico make up the Greater Antilles, with the Cayman Islands to the south of west-central Cuba (Fig. 1). The Lesser Antilles include the smallest islands of the Caribbean, the Windward and Leeward Islands. The smaller islands extending to the east of Puerto Rico make up the Leeward Islands (the British and the US Virgin Islands, Anguilla, Antigua, Barbuda, Saba, Sint Eustatius, Sint Maarten/Saint Martin, Montserrat, Saint Barthélemy, Guadeloupe, and Dominica), from the eastern end of which the Windward Islands (Martinique, Saint Lucia, Saint Vincent, the Grenadines, and Grenada) extend to the south (Fig. 1).

As a whole, the Antillean scorpion fauna includes upwards of 100 described species belonging to 12 genera and 3 families (Buthidae, Diplocentridae, and Ischnuridae), one of the best studied in the world. The family Buthidae is represented in the Caribbean by six genera: *Centruroides*, *Microtityus* Kjellesvig-Waering, *Rhopalurus* Thorell, *Tityus*, *Alayotityus* Armas, and *Tityopsis* Armas, of which the last two are endemic to the region. Since most accidents due to scorpion stings in the Caribbean are due to *Centruroides*, *Rhopalurus*, and *Tityus* (Armas 1988), this review will focus on species within these genera (Tables 1 and 2).

The Greater Antilles

The Greater Antillean scorpion fauna has been reviewed monographically by Armas (1988, 2001), and updates for Cuba, Hispaniola, and Puerto Rico have been published recently (Armas 2010; Pérez-Gelabert 2008; Santiago-Blay 2009; Teruel and Kovařík 2012), with the exception of Jamaica, whose scorpions, particularly the Buthidae, remain poorly studied. The Caymanian fauna has also been reviewed taxonomically (Hounsome 1994). The most diverse and widespread genus in the region is *Centruroides*, and taxa in this genus are probably responsible for most stings to humans in the Caribbean, followed by *Rhopalurus* (Thorell) (Armas 2001). *Centruroides* contains 15 % of the Greater Antillean species, as well as three of the four non-endemic species: *Centruroides gracilis* (Latreille), *Centruroides guanensis* Franganillo, and *Centruroides margaritatus* (Gervais). Some of its members are the most common scorpions in the following islands: *C. guanensis*, in the Bahamas; *C. gracilis*, in Cuba; *Centruroides insulanus* (Thorell), in Jamaica; *Centruroides nitidus* (Thorell), in Hispaniola; and *Centruroides griseus* (C. L. Koch), in Puerto Rico and the Virgin Islands (Armas 2001). The following is an account of the known cases of scorpionism in the Greater Antilles, including the Bahamas, and the Turks and Caicos Islands (which scorpion faunas

Table 1 Distribution of species in the genus *Centruroides* Marx in the Caribbean region. Species distribution per island was compiled from the following sources: **Antigua** (Lourenço 1987), **Aruba** (Armas et al. 2011b), **Bahamas** (Armas 2001), **Barbuda** (Lourenço 1987), **Bonaire** (Armas et al. 2011b), **Cayman Islands** (Hounsome 1994), **Cuba** (Armas 2001; Teruel and Kovařík 2012), **Curaçao** (Armas et al. 2011b), **Dominica** (Armas 2001; Lourenço 1987), **Dominican Republic** (Armas 2002), **Guadeloupe** (Lourenço 1987), **Haiti** (Armas 2002), **Jamaica** (Armas 2001; Armas et al. 2011a; Teruel 2008), **Martinique** (Lourenço 1987, 2013), **Navassa** (Armas 2001), **Puerto Rico** (Armas 2010; Santiago-Blay 2009), **Saba** (Armas 1988; Lourenço 1987), **Sint Eustatius** (Armas 1988; Teruel 2011b), **Saint Barthélemy** (Lourenço 1987; Questel 2013), **St. Kitts and Nevis** (Lourenço 1987; Questel 2013), **Sint Maarten/Saint Martin** (Lourenço 1987), **Turks and Caicos Islands** (Armas 2001), and the **British and the US Virgin Islands** (Muchmore 1987)

Centruroides species	Antigua	Anguilla	Aruba	Barbados	Bahamas	Barbuda	Bonaire	Cayman Islands	Cuba	Curaçao	Dominica	Dominican Republic	Grenada	Grenadines	Guadeloupe	Haiti	Jamaica	Montserrat	Martinique	Navassa	Puerto Rico	Saba	St. Eustatius	St.Barthélemy	St: Kitts and Nevis	St. Lucia	St. Maarten	St. Vincent	Tobago	Trinidad	Turks and Caicos Islands	British and US Virgin Islands
C. alayoni Armas												X																				
C. anchorellus Armas									X																							
C. arctimanus Armas									X																							
C. bani Armas and Marcano												X																				
C. barbudensis Armas	X	X				X									X			X	X			X	X	X	X		X					
C. edwardsii (Gervais)[a]									X																							
C. farri Armas																	X															
C. gracilis (Latreille)								X	X								X		X													
C. griseus Armas																					X	X										X
C. guanensis Franganillo					X				X																							
C. insulanus (Thorell)																	X															
C. jaragua Armas												X																				
C. jorgeorum Santiago-Blay																					X											
C. luceorum Armas																				X												
C. marcanoi Armas												X																				
C. margaritatus (Gervais)									X								X															
C. mariaorum Santiago-Blay																					X											
C. melanodactylus galano Teruel									X																							

(*continued*)

Table 1 (continued)

Centruroides species	Antigua	Anguilla	Aruba	Barbados	Bahamas	Barbuda	Bonaire	Cayman Islands	Cuba	Curaçao	Dominica	Dominican Republic	Grenada	Grenadines	Guadeloupe	Haiti	Jamaica	Montserrat	Martinique	Navassa	Puerto Rico	Saba	St. Eustatius	St.Barthélemy	St: Kitts and Nevis	St. Lucia	St. Maarten	St. Vincent	Tobago	Trinidad	Turks and Caicos Islands	British and US Virgin Islands
C. m. melanodactylus Teruel									X																							
C. navarroi Teruel												X																				
C. nigropunctatus Teruel												X																				
C. nitidus (Thorell)												X				X																
C. platnicki Armas					X																										X	
C. pococki Armas											X				X			X							X							
C. robertoi Armas									X																							
C. sasae Santiago-Blay																					X											
C. simplex (Thorell)			X																													
C. spectatus Teruel									X																							
C. stockwelli Teruel									X																							
C. tenuis (Thorell)[b]												X																				
C. testaceus (De Geer)							X			X																						
C. underwoodi Armas																	X															
C. zayasi Armas[b]																X																

[a]The presence of *C. margaritatus* (= *C. edwardsii*) in Bonaire and Trinidad/Tobago has been questioned (Armas et al. 2011a)
[b]Confirmation of taxonomical status of these species is still pending (Armas 2002)

Table 2 Distribution of species in the genus *Tityus* C.L. Koch in the Caribbean region. Species distribution per island was compiled from the following sources: **Dominican Republic** (Armas 2001; Armas and Abud Antun 2004; Teruel and Armas 2006), **Grenada** (Teruel 2011b), **the Grenadines** (Teruel 2011b), **Haiti** (Teruel and Armas 2006), **Martinique** (Lourenço 1995), **Puerto Rico** (Armas 2010; Santiago-Blay 2009), **St. Lucia** (Armas 1988; Lourenço 1987), **St. Vincent** (Teruel 2011b), **Trinidad and Tobago** (Prendini 2001), and the **British and the US Virgin Islands** (Santiago-Blay 2009)

Tityus species	Antigua	Anguilla	Aruba	Barbados	Bahamas	Barbuda	Bonaire	Cayman Islands	Cuba	Curaçao	Dominica	Dominican Republic	Grenada	The Grenadines	Guadeloupe	Haiti	Jamaica	Montserrat	Martinique	Navassa	Puerto Rico	Saba	St. Eustatius	St.Barthélemy	St. Kitts and Nevis	St. Lucia	St. Maarten	St. Vincent	Tobago	Trinidad	Turks and Caicos Islands	British and US Virgin Islands
T. abudi Armas												X																				
T. altithronus Armas												X																				
T. angelesae Santiago-Blay																					X											
T. anasilviae Armas and Abud												X																				
T. atriventer Pocock													X	X																		
T. bahoruco Teruel and Armas[a]												X				?																
T. bellulus Armas												X																				
T. clathratus (C.L. Koch)																														X		
T. crassimanus (Thorell)												X				X																
T. dasyurus Pocock[b]																					X											X
T. ebanoverde Armas												X																				
T. estherae Santiago-Blay																					X											
T. exstinctus Lourenço[a]																			X													
T. insignis (Pocock)																										X						
T. juliorum Santiago-Blay																					X											

(*continued*)

Table 2 (continued)

Tityus species	Antigua	Anguilla	Aruba	Barbados	Bahamas	Barbuda	Bonaire	Cayman Islands	Cuba	Curaçao	Dominica	Dominican Republic	Grenada	The Grenadines	Guadeloupe	Haiti	Jamaica	Montserrat	Martinique	Navassa	Puerto Rico	Saba	St. Eustatius	St.Barthélemy	St. Kitts and Nevis	St. Lucia	St. Maarten	St. Vincent	Tobago	Trinidad	Turks and Caicos Islands	British and US Virgin Islands
T. marechali Lourenço																			X													
T. melanostictus Pocock																														X		
T. michelii Armas																					X											
T. neibae Armas												X																				
T. obtusus (Karsch)																					X											
T. ottenwalderi Armas												X																				
T. pictus Pocock																												X				
T. portoplatensis Armas and Marcano												X																				
T. quisqueyanus Armas												X																				
T. riverai Teruel and Sánchez																					X											
T. septentrionalis Armas and Abud												X																				
T. smithii Pocock													X	X																		
T. tenuicauda Prendini																														X		
T. trinitatis Pocock																													X	X		

? the presence in Haiti not yet determined (the species might be present according to their distribution in Dominican Republic; Teruel and Armas 2006)

[a]*T. bahoruco* has been reported from the mountainous region in western Dominican Republic and presumably exists in Haiti

[b]No recent records of these species exist

are derived from Cuba and Hispaniola, respectively), the Cayman Islands, and also the British and the US Virgin Islands, which scorpion species are shared with Puerto Rico.

Cuba

The majority of scorpion sting cases in Cuba are due to envenomation by *Centruroides gracilis*, *C. guanensis*, *Centruroides anchorellus* Armas, and *Rhopalurus junceus* (Herbst) (Suárez-Hernández et al. 1997; Fig. 3). The casuistic attributed to individual species is not known, but all the above taxa are reported to be synanthropic throughout the island (Armas 1988). In a study including 28 patients stung by scorpions in the Ciego de Ávila province, central Cuba, all cases presented with pain at the sting site and local erythema. Tongue numbness and cramps in the afflicted limb were reported in ten of the cases. Recovery times elapsed from 3–4 h to 7 days, and all patients were discharged without further complications (Suárez-Hernández et al. 1997). In a more recent study, symptomatology recorded in 22 patients envenomed by the widespread *R. junceus* included pain at the sting site, numbness and decreased muscle strength of the affected limb, and in some cases sialorrhea. *R. junceus* venom is not toxic to mammals at doses 40 times higher than other classical toxins from Buthidae scorpions, confirming its low clinical importance (García-Gómez et al. 2011). Instead, the most abundant peptide in *R. junceus* venom, RjAa12f, is a 64-amino-acid-long insect toxin compacted by 4 disulfide bridges, similar to many other insect toxins described in scorpion venoms, which is consistent with the crude venom higher toxicity to crickets compared with mice (García-Gómez et al. 2011).

In vivo toxicity of venoms from local *Centruroides* species has not been determined despite their epidemiological importance. Anecdotic reports refer the low toxicity to humans of species within the *C. gracilis* and *C. margaritatus* morphological groups, widespread along the Mexico's Caribbean versant (Armas 1988). In the case of *C. guanensis* (Fig. 3h), a species of ample ecological plasticity, envenomation in humans produces a prolonged local stinging and burning sensation with tenderness at the sting site for several days, and a 1–2 mm necrosis area may develop around the site. In nearby Florida, where *C. guanensis* is found along with *C. gracilis* (Fig. 3b) and *Centruroides hentzi* (Banks), none of these scorpions is expected to cause systemic effects (Muma 1967).

The Bahamas

In comparison with the rest of the Antilles, the Bahamas possess a reduced number of *Centruroides* species, most probably of Cuban origin (Armas 2001). *C. guanensis* is the most amply distributed species in the archipelago, shared with western Cuba, the Turk Islands, and the southernmost tip of the Florida peninsula and adjacent keys (Armas 1988). In the Bahamas, *C. guanensis* can be found in Bimini, Exuma, Berry Island, Andros, San Salvador, Crooked, Cat, Eleuthera, Rose, and Long Island (Armas 1988). *Centruroides platnicki* Armas (Fig. 3f) inhabits some islands in the easternmost part of the Bahamas archipelago such as Mayaguana (Armas 1988). No records of envenomation by this species or any other scorpion have been reported from the Bahamas.

Fig. 2 Representative *Tityus* species inhabiting the Greater and Lesser Antilles. (**a**) *T. tenuicauda* (male) from Mount El Tucuche, northern range, Trinidad (Picture by Michael Rutherford); (**b**) *T. trinitatis* (male) from Trinidad (picture by Jan Ove Rein); (**c**) *T. smithii* (male) from Chatham Bay Trail, Union, Saint Vincent and the Grenadines (Picture by Father Alejandro J. Sánchez); (**d**) *T. obtusus* (male) from Toro Negro State Forest, central Puerto Rico (Picture by Father Alejandro J. Sánchez); (**e**) *T. insignis* (female) from Maria Major Island, off southeastern Saint Lucia (Picture by Father Alejandro J. Sánchez); (**f**) *T. atriventer* (male), Chatham Bay Trail, Union, Saint Vincent and the Grenadines (Picture by Father Alejandro J. Sánchez); (**g**) *T. crassimanus* (male) from Rabo de Gato, southwestern Dominican Republic (Picture by Father Alejandro J. Sánchez); (**h**) *T. riverai* (male) from Rio Grande, northeastern Puerto Rico (Picture by Father Alejandro J. Sánchez)

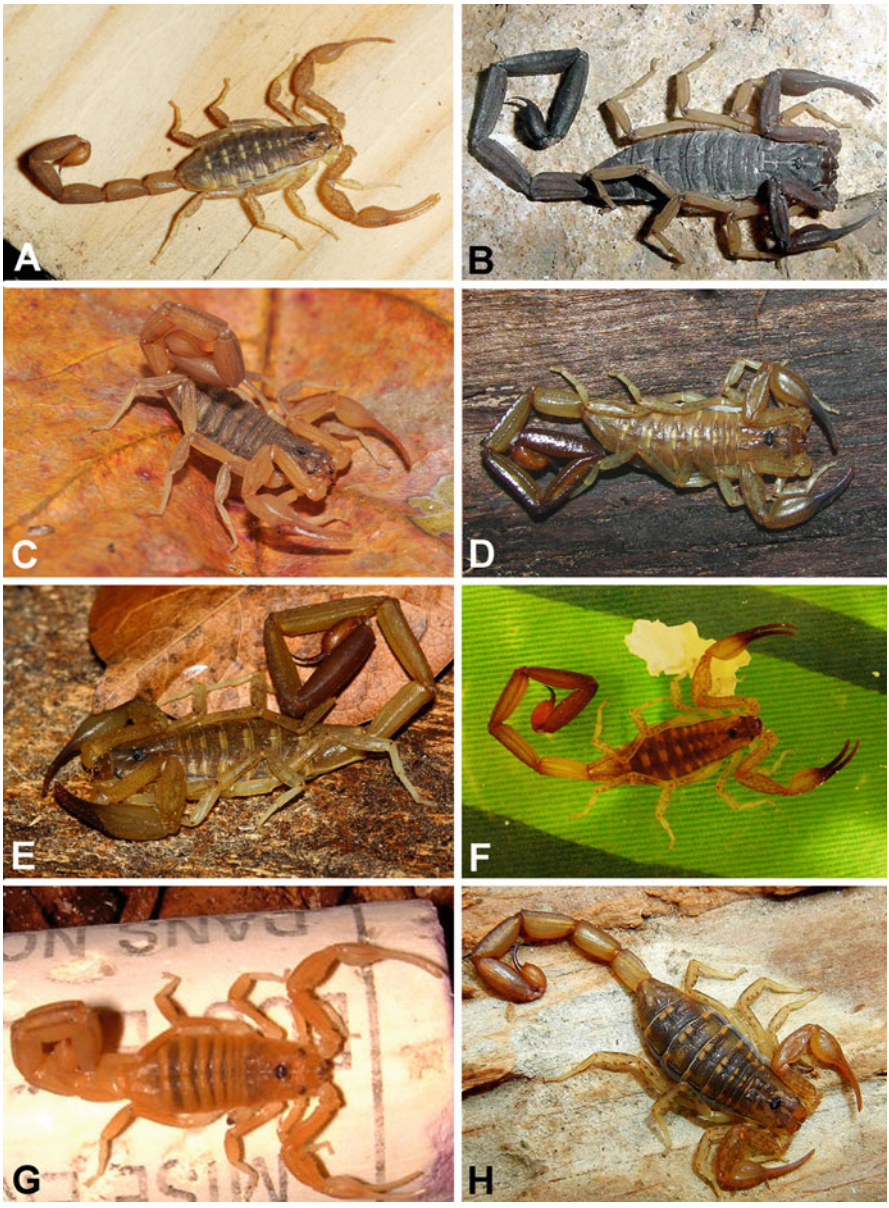

Fig. 3 Representative *Centruroides* species inhabiting the Greater and Lesser Antilles. (**a**) *C. barbudensis* (female) from St. Barthélemy (Picture by Karl Questel). (**b**) *C. gracilis* (male) from Cuba (Picture by Rolando Teruel). (**c**) *C. pococki* (female) from Guadeloupe (picture by Karl Questel). (D) *C. nitidus* from the Dominican Republic (Picture by Martin Koppov). (**e**) *C. griseus* (male) from Guánica State Forest, Puerto Rico (Picture by Father Alejandro J. Sánchez). (**f**) *C. platnicki* (male) from Turtle Cove, island of Providenciales, Turks and Caicos (Picture by Kristian Jones). (**g**) *C. testaceus* (female) from Bonaire (Picture by Ruurd van der Zee). (**h**) *C. guanensis* from Cuba (Picture by Rolando Teruel)

Turks and Caicos Islands

The scorpion fauna of Turks and Caicos Islands, located 190 miles north of the Dominican Republic, appears to be of Hispaniolan origin (Armas 2001). *C. platnicki* has been recorded from Salt Cay, Turks Islands (Armas 2001). Sting by local *Centruroides* sp. is painful to humans and results in a small local swelling that vanishes quickly.

Jamaica

Despite that Jamaica is known for its diverse and highly endemic biota, local arachnids, including scorpions, remain largely unsampled. Two large-sized species of *Centruroides* are confirmed as occurring in Jamaica: the introduced *C. gracilis* and *C. margaritatus*, which are very common all over the island (Armas et al. 2011a). Particularly, *C. margaritatus* was recognized since the nineteenth century as the most abundant scorpion in and around Kingston (Duerden 1896), where it is probably sympatric with *C. gracilis* (Teruel 2008). The only scorpion sting records available are those published in the 1950s on accidents by *Centruroides insulanus* (Thorell), a synanthropic species prevalent throughout the parishes of the eastern half of the island, including St. Catherine, Clarendon, and St. James. *C. insulanus* sting causes sharp pain and tongue numbness (Baerg 1954). *C. gracilis* has probably been imported to Jamaica in shipments from the United States in view of the similar morphology of specimens from the Kingston area and the Florida populations (Teruel 2008), whose stings only produce local symptomatology (Muma 1967). Despite reports of Jamaica being inhabited by *Tityus* species such as *Tityus crassimanus* (Thorell), no recent collections have been conducted in the island to confirm its presence (Teruel and Armas 2006).

Cayman Islands

There are no records of deaths from scorpion stings in the Cayman Islands (Hounsome 1994). *C. gracilis*, also probably imported through commercial travel as in the case of Cuba and Jamaica, is the prevalent species in Gran Cayman, whereas an undescribed *Centruroides* sp. is endemic to Little Cayman (Hounsome 1994). The Cayman Islands Department of Environment reports that stings by *C. gracilis* in the Gran Cayman can produce spreading feelings of pain and numbness around the affected area. Swelling, tightness, and burning at the sting site may be experienced, along with some inflammation and numbness of lips and tongue, symptoms that may last for about an hour.

Puerto Rico

The scorpion fauna of Puerto Rico consists of 17 species in the families Buthidae and Scorpionidae (subfamily Diplocentrinae), including 4 *Centruroides* spp. and 6 *Tityus* spp. (Armas 2010; Santiago-Blay 2009; Tables 1 and 2). *Centruroides griseus* Armas (Fig. 3e) is the most common domiciliary scorpion in coastal Puerto Rico and the islands immediately east, including Vieques and Culebra, and immediately south, such as Desecheo Island, whereas *Tityus obtusus* (Karsch) (Fig. 2d) is the commonest scorpion in the mountain ranges above 300 m (Santiago-Blay 2009). *T. obtusus*, however, has been collected at lower elevations and at urban

regions, usually in connection with accidental transportation of plant material or agricultural products from the mountainous regions, including the cloud forest of El Yunque (Santiago-Blay 2009). *T. obtusus*, an aggressive scorpion which stings repeatedly when threatened, has also been found in the coastal xerophytic and semideciduous forests of Puerto Rico (Santiago-Blay 2009).

Mild cases of scorpionism in Puerto Rico have been reported due to stings by *T. obtusus* and *C. griseus*. In a series of 11 scorpion sting cases, Santiago-Blay (1987) recorded 4 envenomations by *T. obtusus* from Cayey, southeastern Puerto Rico, and Río Piedras, with pain, heat, and redness around the sting site and numbness as the main symptoms. In the cases where *C. griseus* was involved ($n = 7$; all from Coamo, also in southeastern Puerto Rico), symptoms included pain at the sting site, fever, inflammation, and numbness of the affected limb. One of the patients envenomed by *C. griseus* experienced dizziness, cold sweating, and fainted (Santiago-Blay 1987). *T. obtusus* venom should contain vertebrate-active toxins, at least against amphibians, as predation of the frog *Eleutherodactylus coqui* Thomas (Anura: Leptodactylidae) by this scorpion has been reported from El Yunque National Park (Villanueva-Rivera et al. 2000). There are no reports of envenomation by the remaining species of *Centruroides* and *Tityus* inhabiting the island. *Tityus riverai* Teruel and Sánchez (Fig. 2h), from eastern Puerto Rico, and *Tityus michelii* Armas, from the southwest, are morphologically very related to the Hispaniolan *Tityus crassimanus* (Thorell) (Fig. 2g), which has been taken by Teruel and Sánchez (2009) as indication that the "*crassimanus*" group of Antillean *Tityus* did probably diversify in Puerto Rico before the separation of this island from Hispaniola.

The British Virgin Islands and the US Virgin Islands

The most prevalent species in the British and the US Virgin Islands (including St. John) is *C. griseus*, shared with Puerto Rico (Muchmore 1987). Sting by this species in the island of Guana, north of Tortola (British Virgin Islands), is reported painful but not life-threatening. A predominantly arboreal species, *C. griseus*, is particularly abundant in the drier offshore islands of Virgin Gorda, east of Tortola, including Mosquito, Prickly Pear, Eustatia, and Necker, where its sting is painful but not fatal (Muchmore 1987). *Tityus dasyurus* Pocock is the only *Tityus* species ever found in the Virgin Islands (St. Thomas) and also reported from Puerto Rico, but has not been recorded since the nineteenth century and presumably became extinct (Santiago-Blay 2009).

Dominican Republic

The Hispaniolan scorpion fauna is one of the most diverse in the Antilles (see Tables 1 and 2). A list of the current scorpion taxa described for the island is provided by Pérez-Gelabert (2008). Eight species are recognized from the Dominican Republic in the genus *Centruroides*, 12 in the genus *Tityus*, and 3 in the genus *Rhopalurus* (Pérez-Gelabert 2008; Tables 1 and 2). Despite the high diversity of the local fauna, scorpion stings are not considered medically relevant in the Dominican Republic (Armas 1988). Spanish records dating from the sixteenth century already noticed that stings by scorpions from La Española were very painful, even more that than of wasps, but not life-threatening (Fernández de Oviedo y Valdés 1535).

Centruroides nitidus (Thorell) (Fig. 3d) is the most amply distributed scorpion in the Dominican Republic, prevalent in 20 of the 29 provinces of the republic, including Santiago Rodríguez, San Juan, Barahona, Salcedo, Sánchez, La Altagracia, Monte Cristi, Dajabón, Santiago, Valverde, Espaillat, La Vega, Azúa, and Peravia, and is probably the species involved in most accidents (Armas 2002). A species with high ecological plasticity, *C. nitidus* is found from the coastal line up to mountainous regions 2,400 m above sea level. It inhabits crevices and under the bark of fallen logs and also inside bromeliads. Both *C. nitidus* and *Centruroides marcanoi* Armas have been reported to be synanthropic (Armas 2002). Hispaniolan *Tityus* species are less abundant than *Centruroides* spp., and domiciliary habits have only been reported in the case of *Tityus ebanoverde* Armas. All *Tityus* species are restricted to the three main mountainous systems of the country: the Septentrional Cordillera, the Central Range, and the Neiba Cordillera, in an area of 25,000 km^2 (Armas and Abud Antun 2004). In his subdivision of *Tityus* into subgenera, Lourenço (2006) assigned the Hispaniolan *Tityus* into the subgenus *Caribetityus*, but this has been recently contested due to the lack of diagnostic characters to truly segregate subgenera (Armas and Abud Antun 2004). Instead, it has been suggested to keep the previously known species groups "*androcottoides*," "*asthenes*," "*bahiensis*," "*clathratus*," "*crassimanus*," and "*quisqueyanus*," the last two encompassing Hispaniolan and Puerto Rican species (Armas and Abud Antun 2004; Teruel and Armas 2006). Members of the "*crassimanus*" group (*Tityus crassimanus* (Thorell), *Tityus ottenwalderi* Armas) represent an exception within *Tityus* as they almost exclusively inhabit xerophyte shrub areas or dry forests, excluding *T. ottenwalderi*, which is present in upper montane rain forest (Teruel and Armas 2006). Species in the "*quisqueyanus*" group (*Tityus abudi* Armas, *Tityus altithronus* Armas, *Tityus bellulus* Armas, *T. ebanoverde*, *Tityus elii* Armas and Marcano Fondeur, *Tityus neibae* Armas, *T. ottenwalderi*, *Tityus portoplatensis* Armas and Marcano Fondeur, *Tityus quisqueyanus* Armas, *Tityus septentrionalis* Armas and Abud Antun) are all cloud forest-dwelling species, like its South and Central American congeners (Armas and Abud Antun 2004).

Haiti

The Republic of Haiti is almost entirely devoid of forests. Only about 1 % of the original forest covers remains, with drastic effects on the country's biodiversity, including its arachnid fauna, in comparison with their Hispaniolan partner, the Dominican Republic. Only two *Centruroides* species, the amply distributed *C. nitidus* (presumably inhabiting the border with the Dominican Republic) and *Centruroides zayasi* Armas (from Les Cayes, Département du Sud), have been reported from Haiti, although the taxonomic validity of the latter is yet to be confirmed (Armas 2002). *Tityus* is thus far only represented in Haiti by *T. crassimanus*, collected at Grande Anse, Département du Sud (Teruel and Armas 2006). It is presumed that other *Tityus* spp. and *Centruroides* spp. reported from the Dominican Republic should inhabit Haiti (Armas and Abud Antun 2004). No reports of scorpion envenomation have been made, probably because no arthropods toxic to humans are known to inhabit Haiti.

The Lesser Antilles

The Lesser Antillean scorpion fauna was amply studied by Lourenço in the 1980s (Lourenço 1987), and more recent updates have been produced for particular islands such as Sint Eustatius, Saint Barthélemy, Martinique, Grenada, and also Saint Vincent and the Grenadines (Questel 2013; Teruel 2011b; Lourenço 2013; Tables 1 and 2). Westward from Grenada and off the western Venezuelan coast are the islands of Aruba, Curaçao, and Bonaire. Their scorpion faunas, initially characterized by Bakker (1963), have also been partially revised recently as part of a redescription of some species in the genus *Centruroides* (Armas et al. 2011b). The scorpion fauna of the islands of Trinidad and Tobago, located southwest of Grenada and just off the coast of Venezuela, has been reviewed by Prendini (2001).

The Leeward Islands

Most of the Lesser Antilles are colonized by one or two opportunistic species of the genus *Centruroides*, namely, *Centruroides barbudensis* (Pocock) (Fig. 3a) and *Centruroides pococki* Sissom and Francke (Fig. 3c). The most prevalent scorpion, and possibly the most involved in local accidents, is *C. barbudensis* (Fig. 2), widespread over 15 of the Leeward Islands (records exist from Antigua and Barbuda, Sombrero, Anguilla, Sint Maarten, Saint Barthélemy, Saba, Sint Eustatius, Saint Kitts, Nevis, Montserrat, Barbuda, Guadeloupe, and Dominica) (Armas 2001; Questel 2013). The species has domiciliary habits in Barbuda and Guadeloupe (Lourenço 1987). *C. barbudensis* sting only produces local symptomatology at least in the case of the population inhabiting Saint Barthélemy (Questel 2013). *C. pococki* is present in five of the islands, Guadeloupe, Desiderade, Marie-Galante, Les Saintes (all within the Département d'outre-mer de Guadeloupe), and Dominica (Lourenço 1987), but no reports of envenomation by this species have been made.

The Windward Islands

The pattern of distribution of *Centruroides* is interrupted in the Windward Islands since no species in this genus is to be found in the southern islands of Saint Lucia, Saint Vincent, Grenade, the Grenadines, or even Trinidad and Tobago (Lourenço 1987). Instead, *Tityus* occurs at the limit between the Windward and the Leeward Islands, with the species *Tityus exstinctus* Lourenço known to exist in the northern range of Martinique, at least up to 1884 when it was last recorded (Lourenço 1995). The niche of *T. exstinctus* in Martinique, an equilibrium species as most Caribbean and northern South American *Tityus*, was occupied by the ecologically plastic *C. barbudensis* and *C. pococki*. Originally associated with *T. trinitatis* (Lourenço 1995; Fig. 2b), *T. exstinctus* is more related morphologically to *Tityus insignis* Pocock (Saint Lucia; Fig. 2e), *Tityus pictus* Pocock (Saint Vincent), and *Tityus smithii* Pocock (Grenada and the Grenadines; Fig. 2c). They all form a morphologically compact group of species which shows no clear affinities to any other species group but possibly to the "*crassimanus*" and "*quisqueyanus*" groups from Hispaniola and Puerto Rico (Teruel 2011b). A recently described species from Martinique, *Tityus marechali* Lourenço, sets the northern limit of *Tityus* distribution in the Lesser Antilles (Lourenço 2013).

Tityus spp. from the Windward Islands are less toxic to humans than their mainland congeners, reminiscing the case of Hispaniolan and Puerto Rican *Tityus* spp. (Armas 1988). It is surprising that scorpion stings in Saint Lucia were once considered as severe as bites inflicted by the local "rat-tailed" *fer-de-lance* snake, *Bothrops caribbeaus* (Garman), which together with Martinique's endemic *Bothrops lanceolatus* Bonnaterre (Serpentes: Viperidae) are the only venomous snakes inhabiting the Windward Islands (Wüster et al. 2002). The English physician John Davy reported on the presence in Saint Lucia of "scorpions of large size, the sting of which is believed by the natives to be not less virulent than the bite of the snake, if not more so" and that local doctors confirmed that many slaves had died of it more often than those bitten by the "rat-tailed" snake (Davy 1854). In Barbados, a similar report was made by the Swiss physician Felix Spoeri who wrote in 1661 that "scorpions, which are very white in color, are found in old trees and also, occasionally, between the walls of houses, and when they sting a man his life is placed in grave danger" (Gunkel and Handler 1969). Recent Barbadian scorpion fauna only includes the diplocentrid *Heteronebo scaber* (Pocock) and the buthid *Isometrus maculatus* (De Geer) (Lourenço 1987), which are not toxic to humans (Armas 1988). Since no recent records exist of scorpion envenomation in Barbados or Saint Lucia, the venomous species reported by Davy and Spoeri have probably vanished, as did *T. exstinctus* and *T. dasyurus*. *T. smithi*, endemic to Grenada and the Grenadines, and *T. pictus*, endemic to St. Vincent, prefer to inhabit forest areas and are rarely synanthropic (Teruel 2011b), perhaps explaining their infrequent encounters with humans.

Trinidad and Tobago

Scorpionism in Trinidad and Tobago has recently been reviewed (Borges 2013). Whereas the rest of the West Indies are oceanic islands, Trinidad and Tobago are typical continental islands. That is, they show only slight endemism as they closely resemble comparable nearby mainland habitats in their biotic composition and diversity including their scorpion fauna. It has long been recognized that most fatalities by venomous fauna in Trinidad and Tobago are due to scorpion envenomation. Their scorpion fauna (Tables 1 and 2) contains nine species, belonging to the families Chactidae ($n = 3$) and Buthidae ($n = 6$), of which four buthids are shared with mainland Venezuela, *Ananteris cusinii* Borelli, *Tityus clathratus* C.L. Koch, *Tityus tenuicauda* Prendini, and *Tityus melanostictus* Pocock, and one with the Guianas (*T. clathratus*) (Borges 2013; Prendini 2001). Among the local endemic species, *Tityus trinitatis* Pocock (Fig. 2b) stands as the most venomous West Indian scorpion as it is the only species accountable for human deaths in the region (Bartholomew 1970; Waterman 1950; Waterman 1938). *T. trinitatis* is the dominant scorpion of both Trinidad and Tobago, commonly found under coconut husks, logs, and forest debris; in sugar cane fields; in banana, cocoa, and coconut plantations; and in domiciles. There are approximately 175 stings and eight human deaths annually in Trinidad attributed to *T. trinitatis*. Its venom lethality (DL_{50}) in mice has been estimated as 2.00 mg venom/kg of body weight using subcutaneous injection, comparable to other *Tityus* venoms of medical importance (Borges 2013). The recent deaths of two toddlers due to envenomation by *T. trinitatis*, from Cedros

(near Point Fortin, Siparia) and also near San Fernando, attest to the current prevalence of these accidents in Trinidad, and probably Tobago, where systemic complications have also been reported (Borges 2013).

Local scorpions had been recognized as toxic to humans since the nineteenth century. The British naturalist E. L. Joseph referred that "[in Trinidad] we have two kinds of scorpion, one of a brownish colour, the other black. Both their stings are severe, that of the latter especially" (Joseph 1838). These two supposedly kinds are in fact male and female specimens of *T. trinitatis*: upon maturation, the female becomes quite black and the males dark reddish brown, with the last three segments of the tail black (Waterman 1950). Intersexual differences in venom potency have been noticed in the phylogenetically related species, *Tityus nororientalis* González-Sponga, from northeastern Venezuela, which female venom is significantly more toxic to mice, explaining Joseph's observations (De Sousa et al. 2010). Human casualties due to scorpion envenomation in Trinidad were first noticed in the 1920s by K. U. A. Innis, Surgeon General at the Colonial Hospital, Port-of-Spain, who reported fatalities in the Manzanilla district, eastern Trinidad, and described its symptomatology: "The annual average number of deaths for the last five years [1921–1926] was 4.8. During the last three years 71 cases were treated, and deaths among them include two adults. The burning pain of the sting soon passes off, leaving no local effects; but, usually within half an hour, a general envenomation is made manifest in nausea, intractable vomiting, difficulties of respiration, and cardiac and epigastric distress. There is always great restlessness, and in cases of one class this may pass into convulsions and unconsciousness, or even into maniacal disorder. Frequently there is a considerable, but transient, rise of temperature, and it is not uncommon to find sugar in the urine" (Inniss 1927). James Waterman, also from the Colonial Hospital, was the first to associate severe envenomations with *T. trinitatis* and identified the cane fields of southern Trinidad, from Couva to Siparia, and the cocoa plantations of the northeastern part as the regions reporting the majority of fatalities. In a series of 698 sting cases reported during the period 1929–1933, he reported 33 deaths, mostly in 1–5-year-old children ($n = 22$), with a group mortality rate of 25 %. The symptomatology presented in most cases included profuse salivation, nausea, and vomiting, accompanied by profuse perspiration, with the cause of death generally being cardiac or respiratory failure (Waterman 1938), a syndrome later known to be the consequence, at least in part, of the depolarizing action of scorpion toxins on presynaptic terminals, chiefly producing massive release of acetylcholine, catecholamines, and peptidergic neurotransmitters (Amitai 2005). Significantly, Waterman described in his series two cases of acute edematous pancreatitis, two of hemorrhagic pancreatitis, and 12 of pancreatic pseudocysts, all of which were found at laparotomy following stings by *T. trinitatis*, the first scorpion species ever reported to produce a pancreatotoxic venom. Courtenay Bartholomew, from the University of the West Indies Medical Hospital, later confirmed Waterman's findings in a series of 30 sting cases, mainly from Arima and Sangre Grande areas, where 8 cases presented serum amylase levels above 500 units/mL without abdominal pain, indicating that acute painless pancreatitis as a result of scorpion envenomation may occur (Bartholomew 1970).

To elucidate *T. trinitatis* pancreatic secretagogue effect, Bartholomew and coworkers showed in rat pancreatic slices that the venom-induced amylase release was partially abolished with atropine, suggesting that the venom exerts its secretory effect, at least in part, through a cholinergic mechanism involving muscarinic receptors (Sankaran et al. 1977). In anesthetized dogs, the venom induces exocrine secretion in both the isolated and intact pancreas and causes contraction of the isolated sphincter of Oddi, reinforcing the hypothesis that outflow obstruction is part of the pathogenesis of scorpion pancreatitis. Later work showed, in the case of *T. serrulatus* venom, that the partial blockade of the venom pancreatic effects by atropine is indicative of the of venom-stimulated release of non-cholinergic neurotransmitters, such as substance P, involved in acinar cell activation (Borges 2013).

Theodosius Poon-King, from the San Fernando General Hospital, reported in 1963 that *T. trinitatis* venom was also capable of inducing myocarditis. In a series of 45 patients stung by *T. trinitatis*, 34 presented electrocardiographic evidence of myocarditis indicated by inversion of the T waves in several leads, significant deviation of the RST segment, prolongation of Q-T_c, and conduction defects with complete restoration to normal in 3–6 days (Poon-King 1963). Morphological confirmation of myocarditis was later presented by Daisley and coworkers, showing myocardial lesions in two fatal cases: microscopic examination revealed diffuse areas of myocardial necrosis with a mixed inflammatory infiltrate comprised of polymorphonuclear leukocytes, histiocytes, and lymphocytes interspersed between necrotic and hypereosinophilic, damaged myocardial cells (Daisley et al. 1999). Such leukocyte infiltration in organs targeted by *Tityus* scorpion venoms is a hallmark of the envenomation syndrome which was later determined to be triggered by generation of inflammatory cytokines and arachidonic acid-derived lipid mediators (Amitai 2005). To this date, the venom of *T. trinitatis* remains uncharacterized from either biochemical or molecular standpoints.

Scorpionism is still a pediatric emergency in the country, particularly in southern Trinidad. The incidence in this region is particularly high, with stings by scorpions ($n = 580$) accounting for 44.9 % of the patients seen by venomous animal injuries (for the period 2002–2004), followed by bites by centipedes ($n = 230$, 17.9 %), bees and wasps ($n = 222$, 17.2 %), non-specified bites of insects ($n = 147$, 11.4 %), and snake bites ($n = 86$, 6.7 %) (Borges 2013). A recent proposal for partitioning of the Venezuelan territory into scorpion toxinological provinces, including criteria such as the envenomation clinical outcome, phylogenetic (mitochondrial DNA-based) affinities, and immunological cross-reactivity of venoms from prevalent species, has suggested that Trinidad and Tobago are part of the northeastern Venezuelan province (Borges et al. 2010b). In fact, previous phylogeographic research has shown that *T. trinitatis* is a sister species to a clade formed by Venezuelan *Tityus* species prevalent in the northeast such as *T. nororientalis* and *Tityus neoespartanus* González-Sponga, responsible for casualties in the Turimiquire massif (Sucre, Monagas, and Anzóategui states) and Margarita Island, respectively (Borges et al. 2010a). *T. trinitatis* is also present in Tobago, where at least one case of severe envenomation has been reported. The use of atropine to treat this latter patient may have exacerbated the venom-stimulated adrenergic response since

atropine is indicated only in the case of envenomation by the North American *Centruroides sculpturatus* Ewing, where symptomatology is mainly of cholinergic nature (Amitai 2005). No cases of envenomation by *T. tenuicauda* (Fig. 2a) in Trinidad or in the neighboring Venezuelan Paria Peninsula, where the species is also prevalent, have been reported thus far (Borges 2013).

Aruba, Curaçao, and the Netherlands Special Municipality of Bonaire

In Curaçao and Bonaire, the most abundant scorpion species is *Centruroides testaceus* (DeGeer) (Fig. 2g), a species with domiciliary habits which is also prevalent in the neighboring Venezuelan islands of Los Roques and Tortuga (Armas et al. 2011b; Bakker 1963). Local scorpions in Bonaire (and probably also in Curaçao) are regarded as nondangerous. In an envenomation case that took place at São Paulo airport, Brazil, involving a 6-year-old girl traveling from the former Netherlands Antilles, the scorpion involved was a *C. testaceus*, possibly imported from Curaçao. Symptomatology was only local, involving pain and erythema at the sting site (Lobo et al. 2011). The prevalent scorpion in Aruba is *Centruroides simplex* (Thorell), a senior synonym of *C. testaceus arubensis* (Bakker), which has been recently elevated to species level (Armas et al. 2011b). Even though *C. simplex* also has domiciliary habits in Aruba (Bakker 1963), no cases of envenomation by this species have been documented.

Central America

Central America, which harbors the republics of Guatemala, Belize, Honduras, El Salvador, Nicaragua, Costa Rica, and Panama, is inhabited by a scorpion fauna belonging to 6 families and 11 genera: Buthidae (genera *Ananteris* Thorell, *Centruroides*, *Tityus*, and *Isometrus* Ehrenberg), Chactidae (genera *Broteochactas* Pocock and *Chactas* Gervais), Euscorpiidae (genus *Plesiochactas* Pocock), Diplocentridae (genera *Diplocentrus* Peters and *Dydimocentrus* Kraepelin), Hemiscorpiidae (genus *Opisthacanthus* Peters), and Vaejovidae (genus *Vaejovis* C.L. Koch). Genera *Centruroides* ($n = 11$), *Tityus* ($n = 8$), and *Diplocentrus* ($n = 7$) contain 70 % of de described Central American species (Armas and Maes 1998; Borges et al. 2012; Fig. 1). Scorpionism in the area, due to envenomation by species belonging to genera *Centruroides* (Table 3) and *Tityus* (Table 4), has been reviewed recently (Borges et al. 2012), but in the light of new revisions of the regional fauna (Armas et al. 2011a, b; Armas and Trujillo 2010), including species of medical importance (Teruel 2011a), this review updates the list of Central American taxa and includes new references on scorpionism for the area. Particularly, Central American populations of *C. margaritatus*, once thought to be amply distributed in the region and probably responsible for the majority of sting cases in the region, are now classified as *Centruroides edwardsii* (Gervais), a species prevalent in El Salvador, Honduras, Nicaragua, and Costa Rica, and also present in the Colombian Caribbean coast, the Magdalena river basin, including the departments of Cundinamarca and Antioquia (Armas et al. 2011a). *C. margaritatus*

Table 3 Distribution of species in the genus *Centruroides* Marx in Central America. Species distribution per country is updated from that presented previously by Borges et al. (2012) and was compiled from the following sources: **Guatemala** (Armas and Trujillo 2010), **El Salvador** (Armas and Maes 1998; Armas and Trujillo 2010), **Honduras** (Armas and Trujillo 2010), **Belize** (Armas and Maes 1998), **Nicaragua** (Armas and Maes 1998), **Costa Rica** (Víquez 1999), and **Panama** (Miranda 2011; Teruel and Cozijn 2011)

Centruroides species	Guatemala	El Salvador	Honduras	Belize	Nicaragua	Costa Rica	Panama
C. bicolor (Pocock)						X	X
C. edwardsii (Gervais)	N.D.	X	X	N.D.	X	X	N.D.
C. exilimanus Armas	X	X	X				
C. fallassisimus Armas and Trujillo	X	X	X				
C. gracilis (Latreille)	X	X	X	X			
C. granosus (Thorell)							X
C. koesteri Kraepelin	X	X	X		X	X	
C. limbatus (Pocock)			X		X	X	X
C. mahnerti Lourenço[a]					X		
C. schmidti Sissom	X	X	X		X		
C. tapachulaensis Hoffmann	X	X	X				
C. thorelli (Kraepelin)	X	X	X				

N.D., the presence in these countries not yet determined (the species might be present according to their distribution in neighboring countries)
[a]*C. mahnerti* has been synonymized with *C. koesteri* although no basis has been provided for such change (Borges et al. 2012)

Table 4 Distribution of species in the genus *Tityus* C.L. Koch in Central America. Species distribution per country was compiled from the following sources: **Costa Rica** (Teruel 2011a; Víquez 1999) and **Panama** (Miranda 2011; Teruel and Cozijn 2011)

Tityus species	Guatemala	El Salvador	Honduras	Belize	Nicaragua	Costa Rica	Panama
T. asthenes Pocock							X
T. cerroazul Lourenço						X	X
T. championi Pocock						X	X
T. dedoslargos Francke and Stockwell						X	
T. festae Borelli							X
T. ocelote Francke and Stockwell						X	X
T. pachyurus Pocock						X	X
T. tayrona Lourenço						X	X

is now known to be restricted to northwestern South America (Colombia, including the Cauca and the Patía Valleys, Ecuador, and Peru) and has been introduced in the Greater Antilles (Jamaica and Cuba) (Armas et al. 2011a). This finding may have clinical implications as the conflicting reports of *C. margaritatus* toxicity appearing in the literature (Gómez et al. 2010b; Marinkelle and Stahnke 1965) could be the result of the use of venoms derived from different species. In this respect, Central American populations of *C. edwardsii* may produce venom with a higher toxicity, at least in a murine model (Gómez et al. 2010a).

Guatemala

Species in the genus *Centruroides* ($n = 7$) are distributed throughout the country. Reports of *C. margaritatus* for Guatemala probably correspond to *C. edwardsii*, although there are no reliable records for the country (Armas et al. 2011a). For the period 2005–2010, 38 cases of scorpion envenomation were reported, most of which ($n = 22$, 57.8 %) were from the department of Izabal, on the border with Belize and Honduras, in the Caribbean coastal region (Borges et al. 2012); no species has been associated with these accidents. In an epidemiological assessment of scorpionism in the department of Suchitepéquez, southern Guatemala, main symptoms in 20 cases were pain and edema at the sting site, numbness of the affected limb, and significantly the feeling of a foreign object in the throat (*globus pharyngeus*) (García-López 1995), a manifestation found after envenomation by the Mexican *Centruroides tecomanus* Hoffmann (Chowell et al. 2006). In Suchitepéquez, the prevalent species is *Centruroides tapachulaensis* Hoffmann, which is highly synanthropic (Armas et al. 2011a).

Belize

Centruroides gracilis (Latreille), presumably the most common scorpion in the country, has been reported with certainty from the Cockscomb Basin Wildlife Sanctuary, Maya Mountain Range, southern Belize. Its sting, although briefly painful, may produce aftereffects taking up to 24 h to disappear completely (Borges et al. 2012). Other *Centruroides* spp. probably inhabit the country, such as the domiciliary *Centruroides ochraceus* Pocock, which produces mild cases of envenomation in the neighboring Mexican state of Quintana Roo (Armas and Maes 1998).

Honduras

It is difficult to assess the magnitude of the scorpion envenomation problem in Honduras as the country's health statistics report these accidents, as well as that inflicted by other arthropods, as "sting by unknown animal" (Borges et al. 2012). Some of the nine *Centruroides* species that inhabit the country, including the

widespread *C. edwardsii* (Table 3), also occur in neighboring Guatemala and Belize, where they have been responsible for mild envenomations; thus, it is likely that similar cases take place in Honduras.

El Salvador

For the period 2006–2011, the Salvadoran Ministry of Health reported 61 cases of scorpion envenomation with no fatalities, occurring predominantly inside the domicile ($n = 32$, 52.4 %). Most accidents took place in the provinces of La Unión ($n = 21$), Usulatán ($n = 13$), and San Miguel ($n = 10$), in the eastern section of the country. Only local manifestations such as pain and burning sensation are produced after envenomation by local species (Borges et al. 2012). Eight *Centruroides* species are reported to inhabit El Salvador (Table 3), but they have not been associated with any accidents.

Nicaragua

Scorpions are the leading invertebrates responsible for animal envenomation in Nicaragua, at least during the period 2005–2011, with 392 scorpion sting cases recorded for that period. Manifestations included pain, numbness of the tongue, and paresthesia in hands and feet. Patients are usually discharged without further complications. Treatment generally involves analgesics and antihistamine drugs. Most sting cases are reported from the southwest, in the province of Rivas ($n = 226$, 57.7 %), on the border with Costa Rica, and also in the province of Managua ($n = 152$, 38.7 %) (Borges et al. 2012). The scorpionism incidence in Rivas is known since the 1940s, when Woke (1947) reported on the domiciliary habits of *Centruroides* spp. in Los Pochotes and San Juan del Sur, where scorpions were found in most types of dwellings, in bed clothing, clothing, and shoes. Scorpionism by *Centruroides* spp. was also reported for the province of Chinandega (Woke 1947).

Costa Rica

Leveridge (2000) reported that scorpion stings are frequent in Costa Rica: in 1998–1999, 611 envenomations were reported to the National Center for Envenomation Control. Clinical manifestations included pain (44.7 %), numbness (26.7 %), and local inflammation (13.8 %). Most stings were reported from the capital's metropolitan area, including San José, Alajuela, Heredia, and Cartago, with 91 % of the accidents occurring in domiciles (Leveridge 2000). These areas are inhabited by *Centruroides* species ($n = 4$, Table 3) (Víquez 1999), although no association has been reported between taxa and clinical outcome. *C. margaritatus*, the most widespread species in the country and presumably accountable for the majority of accidents, has been reported as of poor toxicity to humans (Leveridge 2000). Costa

Fig. 4 Representative *Tityus* species from Panama. (**a**) *T. asthenes*, (**b**) *T. cerroazul*, (**c**) *T. pachyurus*, (**d**) *T. festae* (Bars, 1cm) (Reproduced from Borges et al. (2012) with permission of the *Journal of Venomous Animals and Toxins including Tropical Diseases*)

Rican populations of *C. margaritatus* are now classified as *C. edwardsii*, also prevalent in El Salvador, Honduras, and Nicaragua (Table 3; Armas et al. 2011a). Despite its apparent low toxicity, Gómez et al. (2010a) reported a DL_{50} in mice of 5.19 mg/kg for *C. edwardsii* venom (within the range of species of medical importance), implying a difference in susceptibility between mice and humans to scorpion venoms. The only death reported thus far from Costa Rica is that of a 3-year-old girl who was stung inside his house in Golfito, Puntarenas province, near the border with Panama, who presented a symptomatology reminiscent of systemic scorpionism (Leveridge 2000). The Pacific southwestern section of the country, including Puntarenas, is inhabited by *Tityus championi* Pocock, previously classified as *Tityus asthenes* Pocock and whose distribution range encompasses the southern versant of the Talamanca range, in Costa Rica, and the Tabasará range in the neighboring Panamanian province of Chiriquí, where the type locality, Bugabá, is located (Teruel 2011a). DL_{50} in mice for *T. championi* venom has been determined as 4.14 mg/kg (Gómez et al. 2010a). Costa Rica is the northernmost limit of distribution of the genus *Tityus* in the Americas and is also inhabited by the endemic *Tityus dedoslargos* Francke and Stockwell, restricted to the province of Puntarenas, and *Tityus pachyurus* Pocock, a species shared with Panama and Colombia (Fig. 4c) and

present predominantly in the Caribbean versant of the country, province of Limón (Víquez 1999). Envenomation by the latter species in Costa Rica has not been reported so far.

Panama

The Panamanian scorpion fauna contains three species in the genus *Centruroides* (the presence of *C. edwardsii* is yet to be confirmed) and seven *Tityus* spp. (Miranda 2011; Teruel and Cozijn 2011). It has significant reduction in the number of *Centruroides* species from Guatemala to the Panamanian isthmus, whereas all Central American *Tityus* species are restricted to Panama and Costa Rica (Tables 3 and 4), some of them shared with northern South America, such as *T. pachyurus*, *Tityus festae* Borelli (Fig. 4d), and *Tityus tayrona* Lourenço (with Colombia) and *Tityus asthenes* Pocock (with Colombia, Ecuador, and Peru) (Fig. 4a). Panama has the highest incidence of scorpionism among all Central American nations (Borges et al. 2012). The national morbidity rate for 2007 was 52 cases per 100,000 inhabitants, mainly encompassing the provinces of Coclé, Chiriquí, Panamá Oeste, and Colón (Borges et al. 2012), a rate only surpassed in the Neotropical region by some Mexican states, such as Colima, with an incidence of 1,350 stings per 100,000 inhabitants reported for 2000–2001 (Chowell et al. 2006). In a series of 229 cases attended at the Children's Hospital, Panama City, Coronado et al. (2007) found that 34.9 % of envenomations were mild, 34.5 % moderate, and 19.2 % severe, and the main complications associated with systemic scorpionism, due to *Tityus* spp., were arterial hypertension (16.2 %) and lung edema (5.7 %). Twenty-three fatalities were reported for the period 1998–2006, mostly 1–14-year-old children from rural localities of difficult access (Coronado et al. 2007).

Centruroides species are accountable for most scorpion stings in Panama, which generally only involve local symptomatology (Borges et al. 2012). For example, *Centruroides limbatus* Pocock, a species with strong domiciliary habits, frequently hides in towels and shoes, and its envenomation produces an intense pain that completely disappears in less than 2 h (Quintero 2005). The most common urban scorpion in Panama was originally thought to be *C. margaritatus*, but local populations are now assigned to the species *Centruroides granosus* (Thorell) (Armas et al. 2011b). Sting by *C. granosus* in Panama produces an intense pain that lasts several minutes and salivation and partial paralysis of the tongue only in hypersensitive subjects (Quintero 2005). The majority of fatalities are due to envenomation by *T. pachyurus*, which is increasingly becoming synanthropic (Miranda 2011). *T. pachyurus* was recognized as a dangerous species in the country only recently, when deaths of children in the Colón province were linked to envenomation by this species (Borges et al. 2012). Other *Tityus* species have been also identified as responsible for human deaths in the country: *T. festae*, in Chepo, El Llano-Cartí, west of the province of Panama; *Tityus cerroazul* Lourenço, in the Antón Valley, province of Coclé; and *T. asthenes*, also in the province of Panama (Borges et al. 2012).

Use of Scorpion Antivenom in Central America and the Caribbean Region

Regarding the use of scorpion antivenoms in Central America, the equine-derived Alacramyn® (from Laboratorio Silanes, Mexico), prepared against venoms from several Mexican *Centruroides* species, has been registered for use in El Salvador and Guatemala (Borges et al. 2012), but its efficacy in these countries has not been documented. Although toxins from dangerous Mexican *Centruroides*, such as *Centruroides noxius* Hoffmann, *C. limpidus*, and *C. tecomanus*, amply share antigenic epitopes (Zamudio et al. 1992), venoms from species outside their range, such as *Centruroides meisei* Hoffmann and *Centruroides elegans* (Thorell), may exhibit decreased antigenic cross-reactivity (Chase et al. 2009; Zamudio et al. 1992). The antivenom currently used in Panama is produced in Venezuela (Biotecfar, Central University of Venezuela) in horses immunized against *Tityus discrepans* (Karsch) venom, which has proven efficacious in neutralizing the toxic effects derived from envenomation by at least Panamanian *T. pachyurus* populations (Borges et al. 2012; Coronado et al. 2007). The use of scorpion antivenoms in the Caribbean has not been documented with the exception of Trinidad and Tobago, where the antidote produced against *Tityus serrulatus* Lutz and Mello and *Tityus bahiensis* (Perty) by Instituto Butantan (São Paulo, Brazil) seems to be effective against *T. trinitatis* envenomation (Borges 2013). A significantly higher immunochemical reactivity of *T. trinitatis* neurotoxic fraction is achieved, however, upon reaction with the Venezuelan *T. discrepans* antivenom, probably the consequence of the higher phylogenetic affinity between the Trinidadian and Venezuelan *Tityus* species (Borges et al. 2010a). Notwithstanding the presence of potentially dangerous *Tityus* species in Costa Rica, it has not been determined whether available *Tityus* antivenoms are efficacious in neutralizing their venom toxicity.

Conclusion and Future Direction

The distribution of scorpion species, and scorpionism thereof, in the Caribbean and Central America, is the result of a combination of physiographic and, more recently, of anthropic factors. The revision outlined above indicates that noxious species in the genus *Tityus*, most probably derived from South America, are accountable for all systemic scorpionism cases in these areas, particularly in Trinidad, Panama, and Costa Rica. Notwithstanding their epidemiological importance, stings by Central American and Caribbean *Centruroides* spp. are not life-threatening, in contrast to their Mexican congeners. Hoffmann (1938) was probably the first to realize the abrupt split in the toxicity to humans that occurs within the spatial distribution of Mexican *Centruroides* when noxious species, restricted to the Pacific versant of the country, from Sonora to Oaxaca, and divided into two areas by the Trans-Mexican Volcanic Belt, are compared with congeners inhabiting outside their range. Reinforcing Hoffmann's observations, Teruel et al. (2006) found that a "Mexican" mitochondrial DNA clade containing *Centruroides balsasensis* Ponce and Francke, *Centruroides infamatus*

C.L. Koch, *Centruroides limpidus* (Karsch), *Centruroides exilicauda* (Wood), and *C. sculpturatus* is well supported statistically and diverges significantly from the less toxic Cuban (*C. anchorellus*, *Centruroides robertoi* Armas, *C. nigropunctatus* Teruel) and Dominican (*Centruroides bani* Armas and Marcano) congeners, the North American *Centruroides vittatus* (Say), and the introduced *C. guanensis* and *C. gracilis*. The hypothesis has been put forward that different evolutionary lineages might exist within this genus (Teruel et al. 2006), but no explanation has been provided for the shift toward decreased vertebrate toxicity in *Centruroides* venoms south and east of Mexico's Pacific versant. Considering that Na^+ channel-active toxins (NaScTx) are responsible for the most dangerous neurotoxic effects observed during human envenomation (Guerrero-Vargas et al. 2012), a lower abundance of these components could explain the reduced toxicity to vertebrates of Caribbean and Central American buthids. Significantly, a proteomic approach (Guerrero-Vargas et al. 2008) has revealed that venom of a *C. margaritatus* population from Colombia (Patía Valley, Cauca province), known for its poor toxicity to vertebrates (DL_{50} = 59.9 mg/kg by subcutaneous injection in mice; Marinkelle and Stahnke 1965), is remarkably enriched (54 %) in K^+ channel-blocking toxins (KScTx; 2–6 kDa), followed by peptides of masses of $\leq$2 kDa (33 %). Only 11 % corresponded to NaScTxs, in contrast to the known toxin content of Neotropical buthids of medical importance such as *Tityus stigmurus* (Thorell) (52 % KScTx versus 42 % NaScTx components; Batista et al. 2007). In addition, toxins prevalent in these venoms may have different pharmacological targets compared to noxious buthids: for instance, the putative NaScTx Cg1 from *C. gracilis*, a poorly toxic scorpion widespread in the Gulf of Mexico area, the Caribbean, and Central America, is most similar to toxin Cll9 from *C. limpidus*, a 66-amino-acid-long peptide that is not toxic peripherally (up to 50 μg per mouse) but induces sleep by central injection. In fact, Cg1 has been hypothesized to constitute, together with Cll9, a novel NaScTx subfamily which diverges from other peptides targeting the voltage sensor of sodium channels (Corona et al. 2003). Future research should explore whether there is a correlation between the different lineages predicted within *Centruroides* by Teruel et al. (2006) and the expression of different toxin repertoires by these groups, which may provide a framework to explain the variations in toxicity across the genus. Given the role that landscape history has played in scorpion diversification (Borges et al. 2010a; Bryson et al. 2013), opportunities for speciation of Central American and Caribbean scorpions have undoubtedly resulted from the regional complex geological history, and geographic isolation has been shown to be one of the processes that drive evolution of scorpion venom variability (Ruiming et al. 2010).

Humans and environmental factors have also played a part in the current distribution of *Tityus* and *Centruroides* species in the region. *Centruroides* spp. such as *C. gracilis*, *C. margaritatus*, *C. edwardsii*, *C. barbudensis*, and *C. pococki* are opportunistic species widespread in the region due largely to recent commercial travel. Prior to European colonization at the end of the fifteenth century, *Tityus* most certainly had a wider range of distribution in the Lesser Antilles, including the now vanished *T. exstinctus* and *T. dasyurus*. The absence of this genus from several islands of the Lesser Antilles can be explained by human impact on the

environment, including widespread logging and conversion of natural vegetation to agriculture, and also by natural catastrophes such as volcanic eruptions (Lourenço 1995). Newly created habitats which are unfavorable to equilibrium species, as most *Tityus* spp., have been colonized by ecologically plastic *Centruroides* spp. Systemic scorpionism in the areas reviewed here is due exclusively to noxious *Tityus* taxa associated with South American elements, such as the endemic *T. trinitatis*, phylogenetically related to northeastern Venezuelan species of medical importance, and *T. pachyurus* and allied Central American species (Borges et al. 2012). Other neighboring regions (e.g., Puntarenas and Limón provinces, Costa Rica) are likely to become endemic in light of the reports of dangerous species inhabiting areas where touristic and industrial activities are increasing (Borges et al. 2012). The apparent lack of toxicity of Lesser Antillean, Hispaniolan, and Puerto Rican *Tityus* (e.g., *T. obtusus*; Santiago-Blay 1987) posits the question of whether these taxa, as probably also occurs in *Centruroides*, evolved toxin repertoires with different specificities in comparison with their noxious congeners in Trinidad and the mainland Americas. Our grasp of the tempo and mode of evolution of scorpion toxins and scorpion venom variability will surely benefit from future research in the West Indies and Central America as laboratories for studying the regional dynamics of toxinological diversity.

Cross-References

- ▶ Scorpion Diversity and Distribution: Past and Present Patterns
- ▶ Scorpionism and Dangerous Species of Mexico
- ▶ Scorpionism and Dangerous Species of Colombia

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Scorpionism and Dangerous Species of Colombia

11

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Abstract

Scorpionism is the disease caused in human beings by a scorpion sting. Depending on the severity of the sting, it can produce multiorgan system failure and death and is a public health problem worldwide with a high incidence and varying degrees of severity. But that in Colombia is a neglected health problem. The lack of awareness of the risks connected with scorpion accidents in Colombia has brought as a consequence the underestimation of this type of accident from the person who suffers it to the health professionals themselves. This too is worryingly reflected in the forming of mistaken diagnoses, in the administering of inadequate therapeutic measures, and in the lack of government programs aimed at preventing it. The systemic clinical symptoms associated with the envenomation caused by Colombian scorpions have been observed in some experimental studies, reports, series of cases, and observational studies and are related to processes of neurotoxicity, characterized by hyperactivity and intense and persistent depolarization of the autonomic fibers with the consequent massive release of neurotransmitters. These elements have been described previously for other species of scorpion of medical importance worldwide, such as *Leiurus quinquestriatus*, *Androctonus australis*, *Tityus serrulatus*, *T. discrepans*, and *Centruroides exilicauda*, among others. Severe scorpionism in Colombia is caused not only by *T. pachyurus* but also by seven species *C. edwardsii*, *C. margaritatus*, *T. asthenes*, *T. forcipula*, *T. fuhrmanni*, *T. pachyurus*, and *T.* n. sp. aff. *metuendus*, and the scorpion *T. columbianus* is ranked as a dangerous species.

Introduction

Scorpionism is the disease caused in human beings by a scorpion sting. Depending on the severity of the sting, it can produce multiorgan system failure and death (Chippaux and Goyffon 2008; Khattabi et al. 2011). The severity of the scorpion accident or the scorpionism depends on a number of factors including the species of scorpion, the aggressiveness of the scorpion that may lead to a number of simultaneous stings, the capacity for production of venom in its gland, the amount and

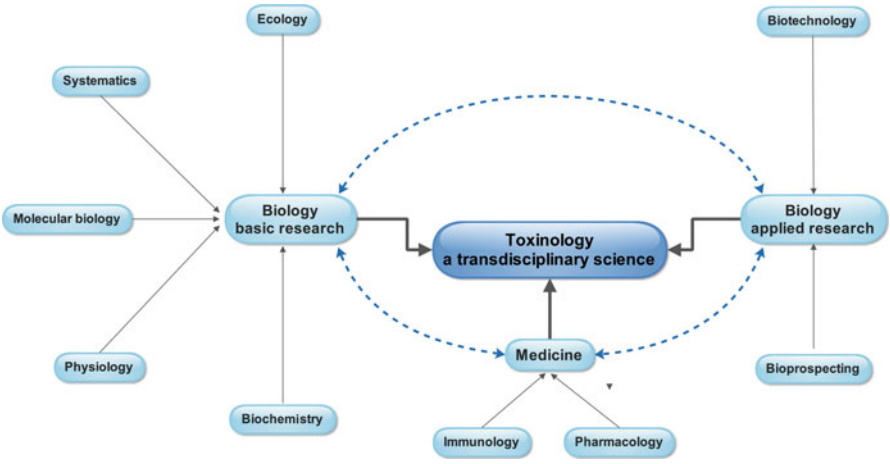

Fig. 1 Toxinology – a transdisciplinary science. The transdisciplinary approach involves such major areas as biology sciences (systematics and taxonomy, ecology, biochemistry, physiology, and molecular biology), applied sciences (biotechnology and bioprospecting), and medicine (immunology and pharmacology)

concentration of venom injected, and the age and individual response of the victim (Inceoglu et al. 2003; Chippaux and Goyffon 2008; D'Suze et al. 2011).

For a better understanding of scorpionism and any other type of accident caused by poisonous animals, it is essential to know what is toxinology, which can be defined as an interdisciplinary science of biology that deals with the study of special metabolic products known as venoms, their toxic and nontoxic molecules, as well as their biotechnological applications (Guerrero-Vargas et al. 2008). Toxinology should be considered as a transdisciplinary science that in addition to nourishing itself in the biological sciences is further strengthened through contributions from medicine, immunology, and pharmacology (see Fig. 1).

Scorpions – known in Latin America as *alacranes* – are a species-rich group. There are currently 15 known families of scorpions around the world, containing about 1900 described species, in which the family Buthidae is the most diverse with 87 genera and 919 species. The scorpion species with the most powerful venom belong to this family, and they are liable to cause severe to fatal scorpionism in humans (Stockmann and Ythier 2010). In Latin America, the family Buthidae has two genera responsible for severe scorpionism, the genus *Tityus* with about 130 species and the genus *Centruroides* with 46 (Fet et al. 2000). In Colombia, the family Buthidae comprises five genera: *Ananteris* with 13 species, *Centruroides* with four species, *Microtityus* with two species, *Rhopalurus* with a single species, and *Tityus* with 30 species (Flórez 2001; Lourenço 2002; Botero-Trujillo and Fagua 2007; Botero-Trujillo et al. 2009; Botero-Trujillo and Flórez 2011; de Armas et al. 2012).

However, research on scorpionism, the toxinology of scorpion venom, and scorpion biology and taxonomy in Colombia is scarce, with much of the literature still unpublished (relating to undergraduate or master's theses in biology and medicine).

The present work seeks to raise awareness about the realities of scorpionism, the medical treatments applied, and the particularly dangerous species and to bring into the light what little research has been done on this topic in Colombia.

Scorpionism in Colombia

Scorpionism is a public health problem worldwide with a high incidence and varying degrees of severity. Nevertheless, research on the biology, geographical distribution, ecology, and the toxinology of scorpion venom in Colombia is only starting to emerge. This has a direct impact on the lack of knowledge of the effects caused by the envenomation following a sting from scorpion species with a geographical distribution in this country.

It is worth pointing out that only from 2008 does Colombia appear on the global list of countries affected by severe scorpionism (Chippaux and Goyffon 2008), caused mainly by the *Tityus pachyurus* species (Barona et al. 2004; Otero et al. 2004).

The lack of awareness of the risks connected with scorpion accidents in Colombia has brought as a consequence the underestimation of this type of accident from the person who suffers it to the health professionals themselves, causing underreporting of this disease. This too is worryingly reflected in the frequency of mistaken diagnoses, in the administering of inadequate therapeutic measures, and in the lack of government programs aimed at preventing, recording, and improving the medical care of patients suffering scorpionism in Colombia.

In the first paper on scorpionism, work made by Marinkelle and Stahnke (1965), with the *Centruroides margaritatus* scorpion venom from Cali at Colombian southwestern. The authors reported that the most common and important symptoms observed in a retrospective study of 820 medical records and direct observation of 31 envenomed patients were pain, local edema, and fever 1–20 h after the sting. People working indoors and people of the age group 4–20 years are stung most frequently. They determined that this venom presents low toxicity for mice, with an LD_{50} of 59.9 mg/kg.

In the late 1990s, Pineda and Castellanos (1998) conducted a retrospective study of 25 cases of scorpionism treated in 1994 in the San Rafael Hospital in Girardot, Cundinamarca, in central Colombia, in which they showed that accidents are most frequent during the night (73 %) and in women (56 %). The parts of the body most commonly affected are the limbs, upper (30 %) and lower (35 %). Medical treatments were not the most appropriate, being limited to antihistamines (88 %), corticosteroids (32 %), and adrenaline (32 %). In the same year in northwestern Colombia – in Chigorodó, Antioquia – the death of a child under 4 years of age by cardiorespiratory arrest as a result of severe scorpionism was recorded. The same paper also highlights that children under 10 years of age are the section of the population at greatest risk of systemic poisoning (Otero et al. 1988).

Efforts to better understand the problem of scorpionism continue and in the first decade of 2000 Otero et al. (2004) determined the clinical and epidemiological characteristics of the scorpion accident in two departments of Colombia – in Antioquia, located in the northwest, and Tolima in the midland of the country.

Over the course of a year, 129 cases were studied (51 in Antioquia and 78 in Tolima): 41 cases were in children under 15 years of age with 70.5 % of sting occurring inside houses; 51 cases were caused by the scorpion *T. pachyurus*, 31 by *Centruroides gracilis*, 29 by *T. fuhrmanni*, seven by *T. asthenes*, and one by *Chactas* spp; and 10 cases with no causative species identified. Systemic envenomation was significantly more common in children than in adults. The overall calculated incidence for this study was of 4.5 cases per 100,000 inhabitants and is significantly higher in Tolima (12.5/100,000 h in Tolima and 2.3 cases per 100,000 h in Antioquia).

Records of cases of scorpionism are sadly lacking. It is nevertheless quite clear that the *T. pachyurus*, *T. asthenes*, *T. fuhrmanni*, and *C. gracilis* species found in central northwestern Colombia all cause serious accidents in humans (Gómez and Otero 2007). In the Mutatá region (Antioquia), the epidemiological, clinical, and toxinological aspects of the *T. asthenes* scorpion were analyzed based on interviews which were carried out among the local population by Gómez et al. (2010). Of the 80 scorpionism cases, only 14 people (17.5 %) went to the hospital. Systemic envenomation was more common in children, who were featured in 67 % of cases. The venom of *T. asthenes* was neutralized with antivenom imported from Mexico and Brazil.

The geographical complexity in Colombia ensures that cases of scorpionism have a wide geographical and altitudinal distribution. Accidents caused by *T. asthenes* occur between 0 and 500 m above sea level (masl) on the Pacific coast for the departments of Antioquia, Cauca, Chocó, Nariño, and Valle del Cauca; scorpionism from *T. columbianus* (2,200–3,000 m) causes accidents in the Magdalena River basin between the Central and Eastern Andes, including the Cundiboyacense high plains in the departments of Boyacá, Cundinamarca, and Santander, as well as in Bogotá, DC; *T. forcipula* (1,200–2,000 m) causes accidents in Cauca River basin between the Central and Western Andes of the departments of Cauca, Caldas, Quindío, Risaralda, and Valle del Cauca; *T.* cf. *ecuadorensis* (600–1,700 m) causes accidents to the east of the Central-Eastern Andes in the departments of Cauca, Putumayo, and Nariño; *T. fuhrmanni* (1,400–1,800 m) causes accidents only in the department of Antioquia; and *T. pachyurus* (400–1,700 m), with a wide distribution in Colombia along the Magdalena River Valley, is responsible for severe cases of scorpionism in the departments of Antioquia, Caldas, Cauca, Cundinamarca, Huila, and Tolima (Guerrero-Vargas and Rodríguez 2011).

Izquierdo and Rodríguez (2012) reported a severe case of scorpionism in a 12-year-old in the Magdalena Valley, caused by *T. pachyurus*: a matter of some minutes after being stung by the scorpion, the child was admitted to the local hospital in Tolemaida with emesis, progressing to hematemesis, sweating, mild respiratory distress, hypertension, and tachycardia. The patient was treated with sodium nitroprusside and prazosin. However, his blood pressure increased and he was referred to the Central Military Hospital in Bogotá. Thirty hours after the accident, the child was admitted to the pediatric intensive care unit with respiratory distress that progressed to respiratory failure and clinical signs of pulmonary edema. He suffered cardiac arrest and was revived and treated with five vials of antivenom imported from Mexico. He responded well to the treatment and after 10 days of hospitalization was discharged.

According to the Center for Information, Management, and Research in Toxicology (CIGITOX) of the Faculty of Medicine at the National University of Colombia, between 2006 and 2010, 1,783 cases of accidents with venomous animals were reported, of which 47 % related to cases of snake bite (a notifiable event in Colombia), 25 % involved scorpions, and spiders accounted for 11 %. The departments with the highest incidence of these accidents were Antioquia, Valle del Cauca, and Cundinamarca. It is important to note that in this same time period scorpionism was the second most prevalent accident after snake bite in Colombia, in addition to the reported five cases of child deaths caused by venom from the *T. pachyurus* scorpion (Rodríguez-Vargas 2012).

Diversity and Geographical Distribution of Scorpions of Medical Importance in Colombia

Colombia is a country with one of the richest biotic diversities of the planet, the greatest number of endemic species, and at the same time one of the most threatened, by different factors (Kattan et al. 2004). Evidence for this significant diversity is based on data on the number of species of plants and vertebrate animal groups. For other groups such as arthropods, the figures are still emerging due to a lack of information. Slowly, however, the knowledge gained of certain groups of insects and arachnids is beginning to assume greater importance.

A particular case is that of diversity of scorpions in Colombia, knowledge of which has doubled in the last two decades from 40 species (Flórez and Sánchez 1995) to 80 species (unpubl. data). These are grouped into 14 genera and five families, Buthidae being the most diverse of them. The family Buthidae contains the genera with the species that pose a risk to human health in Colombia: *Centruroides* and *Tityus* (Barona et al. 2004; Otero et al. 2004; Gómez et al. 2010), which are distributed in the dry forests of the Andean valleys of the Magdalena and Cauca rivers and in the Caribbean region. However, some species are seen as having more medical importance because of the frequency of accidents due to their synanthropic habits in densely populated areas, rather than the toxicity of their venoms.

Taxonomic Recognition of the Species of Medical Importance in Colombia

The scorpions of the family Buthidae are easily recognized because they have pincers on their pedipalps that are long and slender, a triangular-shaped sternum, and a thorn under the stinger (see Fig. 2). They are usually species of opaque colorations, in contrast to the scorpions of the other families living in Colombia, which are brightly colored, with robust and moderately sized pincers, a subpentagonal sternum, and they do not have the thorn beneath the stinger.

The genera *Centruroides* and *Tityus* differ from one another by the number of oblique rows of denticles that they have on the inner edges of the fingers of the

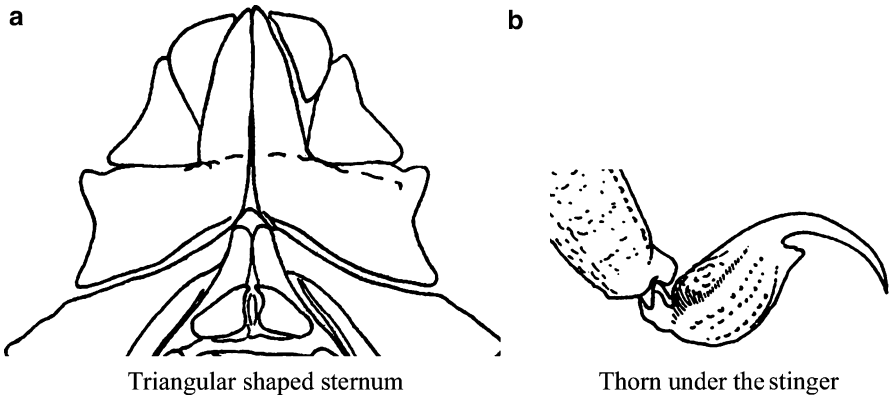

Fig. 2 Sternum and stinger in the Buthidae family. (**a**) Triangular-shaped sternum. (**b**) Thor under the stinger

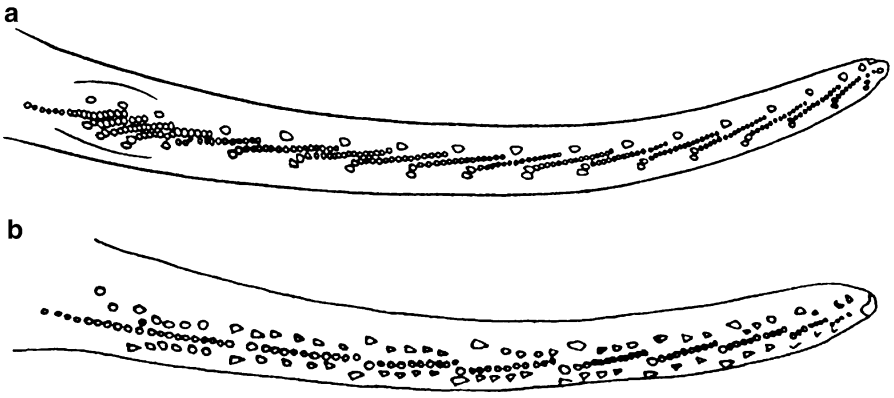

Fig. 3 Oblique rows of denticles in the genera *Centruroides* and *Tityus*. (**a**) Oblique rows of denticles in *Tityus* genus. (**b**) Oblique rows of denticles in *Centruroides* genus

pincers. The species of the genus *Tityus* have between 12 and 17 rows (Fig. 3a) and *Centruroides* between 8 and 9 (Fig. 3b). Furthermore, *Centruroides* species have thinner, brown bodies and yellowish-brown legs, while those of *Tityus* tend to have darker colors and reddish-brown or black palps.

List of scorpions potentially dangerous to human health in Colombia:

Genus *Centruroides* Marx, 1980
Centruroides margaritatus (Gervais, 1841)
Centruroides edwardsii (Gervais, 1843)

Genus *Tityus* C.L. Koch, 1836
Tityus asthenes Pocock, 1893
Tityus columbianus (Thorell, 1876)

Fig. 4 *Centruroides edwardsii* (Gervais, 1843)

Tityus forcipula (Gervais, 1843)
Tityus fuhrmanni (Kraepelin, 1914)
Tityus pachyurus (Pocock, 1897)
Tityus n. sp. aff. *metuendus*

Species of the Genus *Centruroides*

The genus *Centruroides* enjoys its greatest diversity (about 40 species) across southern North America, Central America, and the Caribbean, and its range extends meridionally to Ecuador and northern Peru, where five species are distributed. In a recent review of the genus *Centruroides* in Colombia, de Armas et al. (2012) established that there are four species of this genus; however, only two of them can be considered to date as dangerous to human health. These are *C. edwardsii* (Fig. 4), which is distributed in the Caribbean and in the Magdalena River Valley (north and east-central Colombia), and *C. margaritatus* (Fig. 5), located in the Cauca river valley (southwestern Colombia). Both species exhibit synanthropic habits and live in arid ecosystems and areas considered as dry forest, including densely populated urban centers (e.g., Barranquilla, Santa Marta, and Cali). The species *C. edwardsii* differs from *C. margaritatus* in having very hairy pedipalp segments, in addition to the previously noted geographical separation (see Fig. 6).

Species of the Genus *Tityus*

Most of the species of the genus *Tityus* involved in envenomation accidents of some consideration correspond to the subgenus *Atreus* (Lourenço 2006) whose species are characterized by being the larger ones (60–110 mm), with darker colorations,

Fig. 5 *Centruroides margaritatus* (Gervais, 1841)

ranging from brown to black, and usually with reddish-brown palps; they have an acute thorn under the stinger, have between 18 and 26 pectinal teeth, and have 16–18 rows of granules on the inner edges of the fingers of the pedipalps.

- *Tityus* (*Atreus*) *asthenes* (Fig. 7). It is a dark-brown species, with weak keels on the caudal segments, and exhibits a marked sexual dimorphism in the palps, these being longer and thinner in the males. *T. asthenes* is distributed along the Pacific coast of Colombia (Fig. 8), and human accidents are usually due to the fact that it is a species that often finds its way into the rural and tourist housing in the region.
- *Tityus* (*Atreus*) *forcipula* (Fig. 9). This is a dark-colored scorpion with reddish-brown palps and having keels formed by spiny granules over the caudal segments, with the granules behind each segment progressively increasing in size. It is one of the species with the widest distribution ranges in Colombia, located as it is in the mountainous regions of central and western Andean ranges in southwestern Colombia, taking in the departments of Caldas, Quindio, Risaralda, Cauca Valley, and Cauca (Fig. 8).
- *Tityus* (*Atreus*) *fuhrmanni* (Fig. 10). This species is limited to the department of Antioquia (northwest Colombia), in the city of Medellin and its surroundings (Fig. 8). It is dark in color and is characterized by a very strong spiny granule at the apical end of dorsolateral keels of caudal segments II to IV. Although the toxins of this species are not as powerful, the accidents are relatively common, as its habitat coincides with an area densely populated by man.

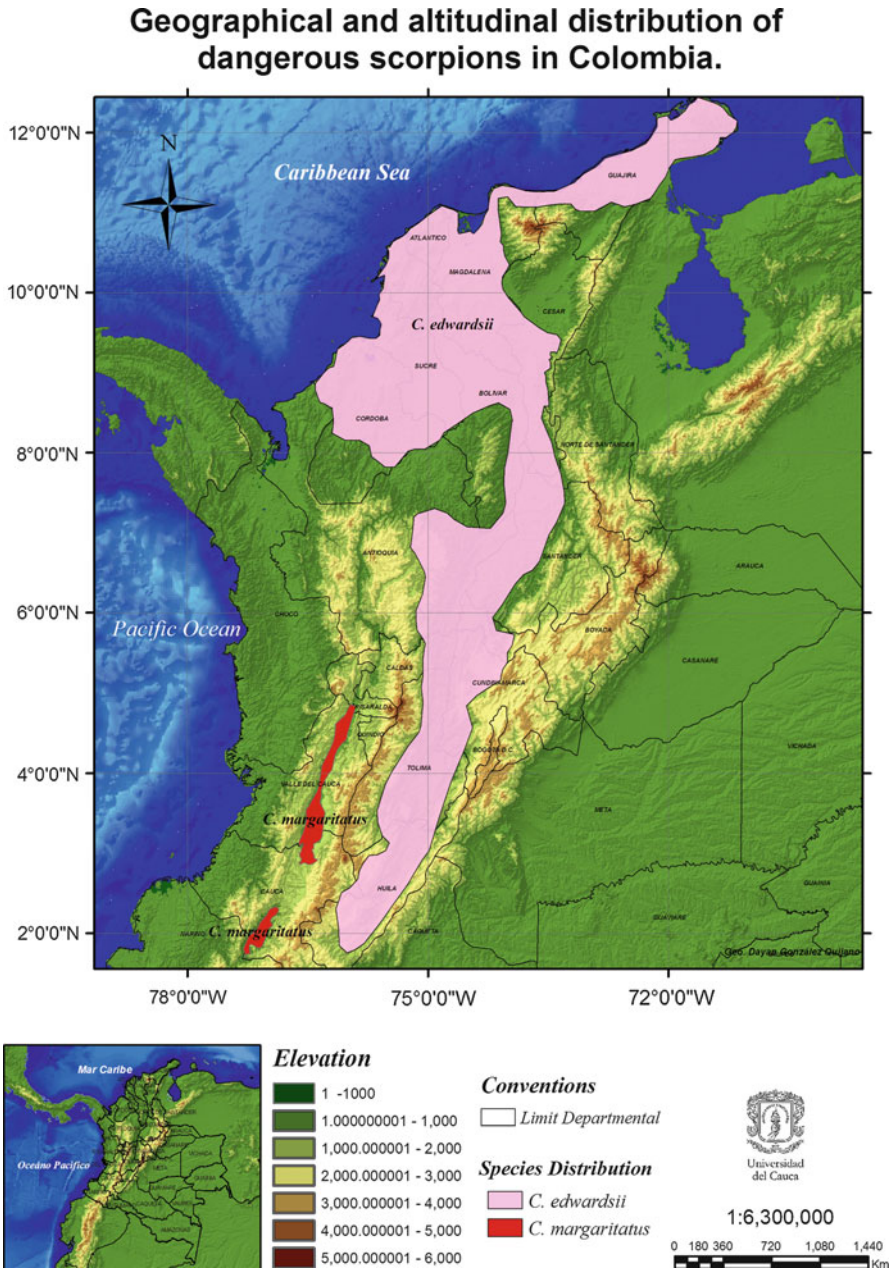

Fig. 6 Geographical and altitudinal distribution of *C. edwardsii* and *C. margaritatus* in Colombia

Fig. 7 *Tityus asthenes* Pocock, 1893

– *Tityus* (*Atreus*) *pachyurus* (Fig. 11). This species is related to *T. forcipula*, which differs by exhibiting a less granulation on the dorsal keels of the metasoma. It inhabits dry forests in the middle sector of the Magdalena River Valley (central Colombia), across the departments of Tolima, Cundinamarca, Boyacá, Antioquia, and Huila (Fig. 8). It is one of the species that have the most toxic venoms in Colombia and is the cause of one of the highest percentages of patient admissions in hospitals and health centers in the country. Notwithstanding, this species shares much in common with *C. edwardsii*, which also has significantly toxic venoms, the reason human populations in this region are more susceptible to envenomation by scorpions. Special care must be taken in the administration of antivenoms, since for this you ought to have previous evidence of the specimen that caused the accident.
– *Tityus* (*Atreus*) *n.* sp. *aff. metuendus* (Fig. 12). This species is related to *T. forcipula* and *T. pachyurus*, differing from them by exhibiting less granulation in the metasomal segments and having fewer pectinal teeth (16–20), while *T. pachyurus* and *T. forcipula* usually exhibit 19 or more pectinal teeth. It inhabits southern Colombia in the urban area of the city of Popayan (Cauca) (Fig. 8) where it causes frequent accidents in the human population.

Besides the normally larger *Atreus* species, *T. columbianus* (Fig. 13) is a species that belongs to the subgenus *Archaeotityus*, this group being characterized by smaller species of less than 45 mm. These generally are gray-brown in color with scattered white markings. They have 11–18 pectinal teeth, 11–15 rows of granules on the inner edges of the fingers of the pedipalps, and a rhomboid thorn under the stinger. *T. columbianus* inhabits the Andes region of Colombia, between 2,200 and 2,800 masl (Fig. 8), featuring sexual populations in Boyacá department but

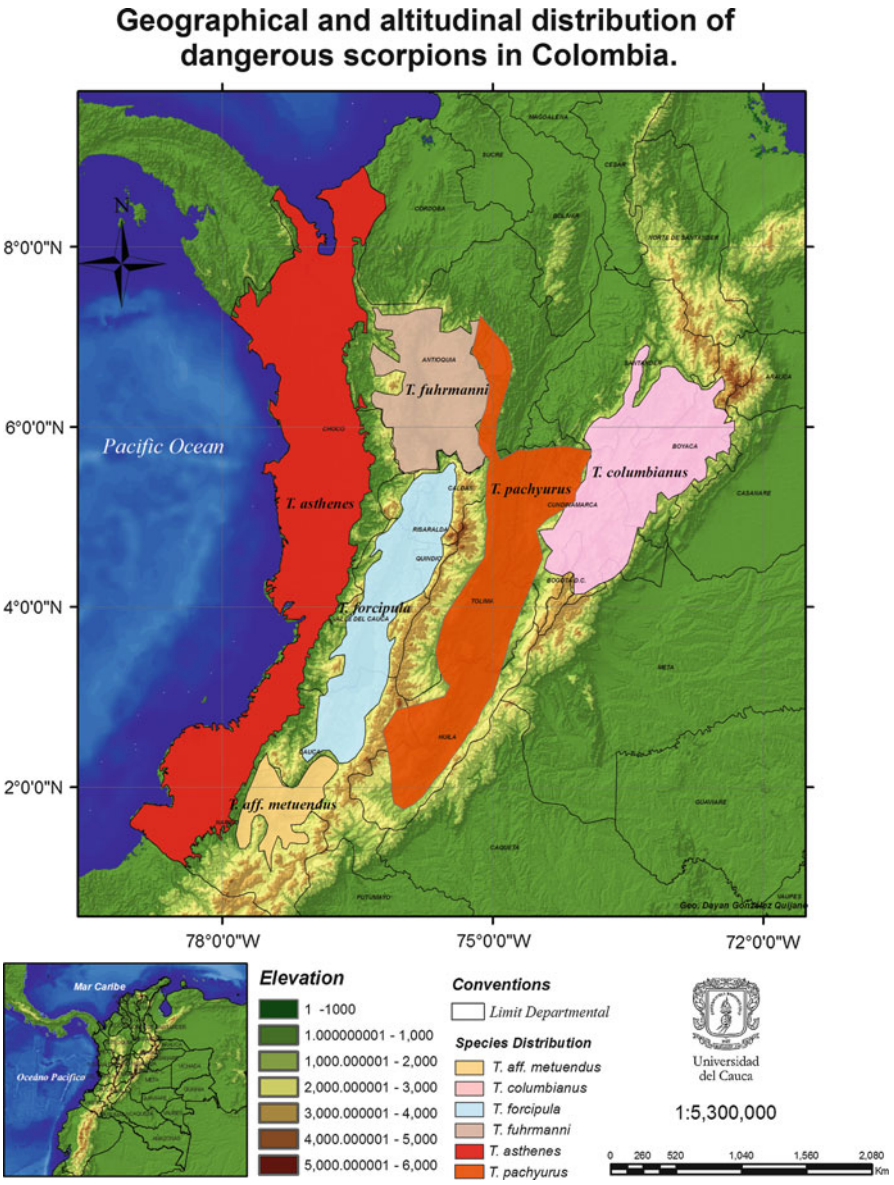

Fig. 8 Geographical and altitudinal distribution of *T. asthenes*, *T. columbianus*, *T. forcipula*, *T. furhmanni*, *T. pachyurus*, and *T.* aff *metuendus* in Colombia

parthenogenetic populations in Cundinamarca. Because abundant populations of *T. columbianus* are found on Bogota's moorland, causing a high incidence of scorpionism, the species is included among Colombia's dangerous species, even though its venom does not cause severe scorpionism.

Fig. 9 *Tityus forcipula* (Gervais 1843)

Fig. 10 *Tityus furhmanni* Kraepelin, 1914

Taxonomic Key to the Species of Scorpions of Medical Importance in Colombia

1. Inner edge of the fingers of the chela has eight to nine oblique rows of denticles; yellowish-brown colorations – g. *Centruroides*. . .2.
1' Inner edge of the fingers of the chela has 16–18 oblique rows of denticles; dark-brown coloration – g. *Tityus*. . .3.
2. Pedipalps have abundant pilosity; dorso-marginal keel on the hand of the chela shows profuse pilosity – *C. edwardsii*.
2' Pedipalps have little pilosity; dorso-marginal keel on the hand of the chela shows no pilosity – *C. margaritatus*.

Fig. 11 *Tityus pachyurus* Pocock, 1897

Fig. 12 *Tityus* n. sp. aff. *metuendus*

3. Small-sized species (under 45 mm), bodies with gray-brown colorations and with scattered white markings, with a rhomboid thorn under the stinger – *T. columbianus*.
3' Large-sized species (over than 60 mm), bodies with dark or reddish-brown colorations, with acute thorn under the stinger. . .4.

Fig. 13 *Tityus columbianus* (Thorell, 1876)

4. Dark-brown coloration (including the pedipalps); males with pedipalps longer and thinner than those of females – *T. asthenes*.
4' Dark coloration, with reddish-brown pedipalps; palps of the males similar to those of the females. . .5.
5. Caudal segments with lateral dorsal keels provided with conspicuous spiny granules. . .6.
5' Caudal segments with lateral dorsal keels provided with moderated spiny granules. . .7.
6. Dorsolateral keel granules gradually increasing in size toward the rear end – *T. forcipula*.
6' Dorsolateral keel granules increasing in size toward the rear end, with the last granule much higher than those of the rest – *T. fuhrmanni*.
7. Pectines provided with 19–24 denticles – *T. pachyurus*.
7' Pectines provided with 16–20 denticles – *T.* n. sp. aff. *metuendus*.

Works About the Toxinology of Scorpion Venom in Colombia

Toxinological Characterizations of Some Venoms of Scorpions with a Geographical Distribution in Colombia

– *Centruroides margaritatus* Venom

The venom of the *C. margaritatus* scorpion from the department of Cauca in southwestern Colombia was investigated. This venom has an LD_{50} in mice of 42.83 mg/kg and causes the following symptoms: excitability, salivation, piloerection, and dyspnea. The whole venom sample was fractionated in a size exclusion column, where 9 fractions were obtained. The most abundant fractions – II, III, IV, and V – were inoculated into a preparation of amphibian gastrocnemius muscle, yielding the result that fractions II, IV, and V inhibit muscle contraction (Guerrero-Vargas et al. 2007).

Dueñas-Cuellar et al. (2009) evaluated the cytotoxic and genotoxic effects of *C. margaritatus* venom on mice, using the micronucleus (Mn) assay, and the rate

of polychromatic erythrocytes (PCE)/1000 cells/animal was counted; the mice were treated by intraperitoneal administration with saline solution (0.9 %) as negative control and three experimental venom doses (9.32, 18.65, and 37.31 mg/kg) and cyclophosphamide (50 mg/kg) as positive control. The cytotoxic effect expressed in PCE, decreased directly related with the time of treatment and genotoxic effect, was only evidenced in the higher dose at 40 h after treatment.

In 2010, in an undergraduate thesis on venom of the scorpion *C. margaritatus*, injecting three groups of juvenile rats at sublethal doses, a cardiotoxic effect was triggered, characterized by tachycardia and electrocardiographic changes. Between 30 and 40 min after the inoculation of the venom, the rats of the three experimental groups registered a higher number of irregular heart rhythms, frequently occurring in the groups treated with the highest concentration of venom. In the experimental groups, records were found with irregular RR distances and replicated F waves pertaining to atrial fibrillation, whose frequency of occurrence was higher in the groups exposed to higher doses. ECG records were found with wide and deformed QRS complexes, irregular with changes in their morphology characteristic of a polymorphic ventricular tachycardia. In addition, the experimental groups revealed the presence of wide, deformed, and polymorph QRS complexes called ventricular systoles or premature ventricular complexes (PVC) (Arenas 2010).

– *Tityus fuhrmanni* Venom

The earliest known work in the line of toxinological characterization dates from 2002, with the venom of the *T. fuhrmanni* scorpion endemic to the department of Antioquia, in northwestern Colombia. This venom has an LD_{50} determined on mice corresponding to 3.9 mg/kg, and the symptoms of envenomation that it causes, also in mice, are salivation, piloerection, diaphoresis, somnolence, and systemic complications such as tachypnea, ataxia, and seizures (Gómez et al. 2002).

– *Tityus pachyurus* Venom

Barona et al. (2004) investigated the venom of the *T. pachyurus* scorpion from the Magdalena River Valley in central Colombia. Symptoms of envenomation presented by mice were sialorrhea, dyspnea, diaphoresis, ataxia, behavioral changes, and hyperglycemia. The LD_{50} was determined to be 4.8 mg/kg. The neutralizing capacity for this poison by three antivenoms imported from Brazil, Mexico, and Venezuela was also determined.

In an experimental study undertaken as a toxicology master's thesis, which objective was to evaluate the effects of scorpion venom inoculation on target organs, a variety of statistically significant abnormalities were observed in the histopathological analysis of the different organs of mice exposed to varying doses (0 %, 25 %, 50 %, and 75 % of LD_{50}) of *T. pachyurus* venom, showing dependence between the dose of poison administered intraperitoneally and the severity of disorders such as pulmonary, hepatic, splenic, and renal

congestion, hepatic vacuolar degeneration, and pulmonary alveolar hemorrhage ($p < 0.05$). Other conditions (e.g., cardiac, suprarenal, and pancreatic congestion, pulmonary edema, and pulmonary microthrombosis) pointed to a lesser degree of dependence regarding the severity of the damage with respect to the exposure dose ($p < 0.1$). It should be noted that in two of the specimens exposed to 75 % of LD_{50}, it was possible to observe the presence of localized necrosis of the hepatocytes (Rodríguez 2008). Figure 14 indicates the histological alterations in the lung, liver, spleen, and kidney.

In a technical report on research conducted by Beltrán et al. (2011), the cardiotoxic effect was evaluated by recording the electrocardiographic and histopathological changes induced by the pool venom of *T. pachyurus*, 3 h following envenomation in rats inoculated with sublethal doses. Electrocardiographic records were obtained that were characterized by unusual rhythms, with a delay in the activation of the right ventricle, showing typical QRS morphology in V1 rSR', indicating the presence of incomplete right bundle branch blockage (RBBB), voltage variations, and elevation of the T wave. The levels of plasma cardiac enzyme CK and isoenzyme CK-MB increased significantly and are directly proportional to the amount of venom injected. In the histopathological analysis of the hearts of rats treated with 20 %, 40 %, and 80 % of the LD_{50}, interfibrillar edema of the heart muscle was identified, with a severity proportional to the inoculated dose. Additionally, in the group of animals inoculated with 80 % of the LD_{50}, alterations were observed such as cytoplasmic vacuolation, hypertrophy of myocytes, and moderate congestion of the ventricles.

– *Tityus* n. sp. aff. *metuendus* Venom

Also in 2010, the preliminary toxinological characterization of the venom from the scorpion *T.* n. sp. aff. *metuendus* was performed. In this work, the biological activity of venom on the muscular contraction (MC) of the frog *Rhinella marina* and the antimicrobial activity on three pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) and three pathogenic fungi (*Candida albicans*, *Candida krusei*, and *Cryptococcus neoformans*) was researched. The venom of the *T.* n. sp. aff. *metuendus* scorpion showed a strong effect on MC and on the contraction time (CT); this effect is dose dependent and the major alteration of the MC was observed in the dose equivalent at 50 % (0.288 mg/kg). Regarding the antimicrobial activity, the venom in all the concentrations inhibited the growth of *P. aeruginosa*, and the most effective dose was given at 1 mg/ml; the growth of *S. aureus* was also inhibited in all concentrations being the most effective at 0.5 mg/ml; in the case of *C. albicans*, the growth was inhibited in the venom concentrations of 0.016–1 mg/ml showing a better effect at 1 mg/ml; the *C. krusei* growth was inhibited by venom concentrations of 0.016–1 mg/ml and that of 1 mg/ml showed the major effect. However, for *E. coli* and *C. neoformans*, the concentrations of the venom did not have a significant effect (Morales-Duque et al. 2010).

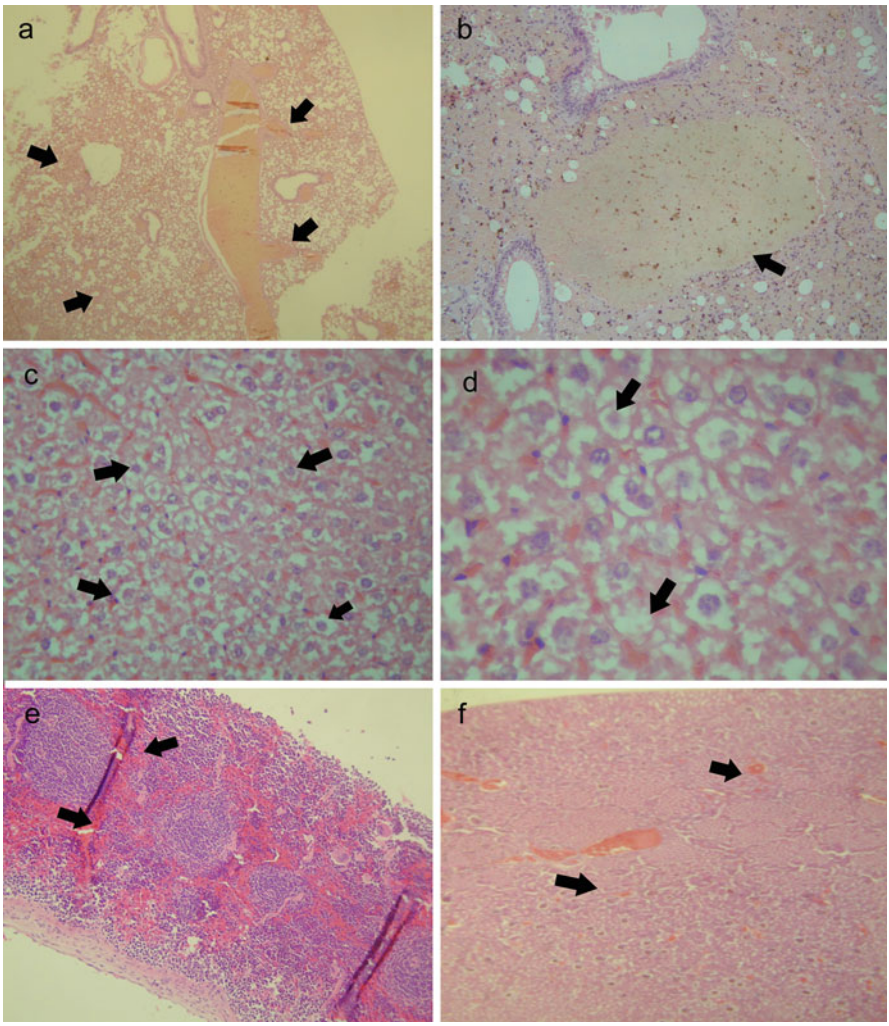

Fig. 14 Histopathological effect of *T. pachyurus* venom on the lung, liver, and spleen of mice. (**a**) and (**b**) show the histological alterations in mouse lung, in panoramic view (*left*). It is possible to observe the presence of diffuse organ congestion, foci of edema, and hemorrhage. At higher magnification (*right*), note the presence of parenchymal necrosis. (**c**) and (**d**) show the histological changes in mouse liver. (*Left*) Marked vacuolar degeneration can be seen, concluding in loss of cytoplasm. Higher magnification (*right*) reveals the absence of nuclei in some hepatocytes, which is compatible with necrosis. (**e**) Histological changes in mouse spleen, in panoramic view (*left*). It is possible to see the marked congestion of the organ with an increase in density of the *red* pulp with respect to the white pulp. (**f**) Histological changes in mouse kidney, in panoramic view (*right*). Note the congestion of the organ

Identification and Characterization of Toxins from Various Venoms from Scorpions with a Geographical Distribution in Colombia

As regards the venom of *T. pachyurus*, Barona et al. (2006), using HPLC and mass spectrometry, reported that this venom comprises at least 104 components with different molecular masses. They further chemically characterized and determined the biological activity of two new toxins: Tpa1 (a potassium channel blocker), containing 32 amino acid residues and a molecular weight of 2,900 Da, and Tpa2, of molecular weight 7,522.5 Da and 60 amino acid residues, toxic in mammals and classified as a β-sodium scorpion toxin (βNaScTx).

In a comparative study seeking to identify new putative sodium scorpion toxins from *T. pachyurus* collected in Colombia and *T. obscurus* in Brazil, Guerrero-Vargas et al. (2012) reported, following construction of a cDNA library of venom glands, five new putative Na^+ toxins for *T. pachyurus*, called Tpa4, Tpa5, Tpa6, Tpa7, and Tpa8 and 15 for *T. obscurus*. Tpa8 is the first toxin identified and classified as β-excitatory anti-insect in a scorpion of the New World. Toxins Tpa4, Tpa5, and Tpa6 presented a high percentage of identity with toxins classified as αNaScTx, and putative toxins Tpa7 and Tpa8 are sequences related more to βNaScTx. These authors also constructed a phylogenetic tree using the NaScTx they had identified and NaScTx from scorpions of the genus *Tityus* deposited in different databases. This phylogenetic analysis indicated a marked geographical separation between the scorpions of the genus *Tityus* living north of the Amazon River and those distributed to the south of this basin.

In another work concerned with venom of the scorpion *C. margaritatus*, Guerrero-Vargas et al. (2009), also using HPLC and MS, reported 91 components of different molecular weights. Here, 54 % of the molecular weights were between 2.5 and 6.0 kDa, where toxins that affect the channels K are usually found; 13 % of the molecular weights were in the range of 6.5–8.0 kDa, the range in which toxins that modulate voltage-dependent Na channels can be found; and 33 % of the molecular weights were small compounds of 2.0 kDa. Two new toxins were also isolated and characterized biochemically: MgTx2 with 24 amino acid residues, three disulfide bridges, and molecular weight 2.6 kDa that displayed a high similarity to toxins classified as alpha-KTx, and MgTx3, containing 30 amino acid residues, three disulfide bridges, and 3.38 kDa molecular weight that showed no similarity to any other toxins present previously described.

Pathophysiology of Scorpion Accidents in Colombia

When a scorpion sting occurs, the venom is primarily inoculated into the subcutaneous space, moving quickly to the central circulation where it is distributed to tissues and such organs as the kidneys, muscles, intestines, liver, lungs, and heart

(D'Suze et al. 2004). This poison, when inoculated in mammals – in our case humans – acts primarily at specific locations of the sodium channels of excitable and non-excitable cells, causing a depolarization of the nerve endings, triggering the release of acetylcholine, epinephrine, and norepinephrine. These abnormally released neurotransmitters are responsible for the majority of the clinical symptoms observed in the course of the scorpionism (Chippaux and Goyffon 2008; D'Suze et al. 2011).

The poisons of scorpion species present in Colombia, especially those of the genus *Tityus*, have an LD_{50} close to those obtained for other scorpions of importance in Latin America and lower than those calculated for other species of importance such as *Tityus discrepans*, the most clinically important species in Venezuela, a country that reports an average of 800 accidents/year (Teixeira et al. 2001). Nevertheless, in Colombia no official data exist on the impact of this envenomation on morbidity and mortality in our country.

The systemic clinical abnormalities associated with scorpion envenomation have been observed in experimental studies, reports and case series, and observational studies (Gómez et al. 2002, 2010; Pineda 2002; Barona et al. 2004; Otero et al. 2004) and are related to processes of neurotoxicity, characterized by hyperactivity and intense and persistent depolarization of the autonomic fibers with the consequent massive release of neurotransmitters (Gwee et al. 2002). These elements have been described previously for other species of scorpion of medical importance worldwide, such as *Leiurus quinquestriatus*, *Androctonus australis*, *T. serrulatus*, *T. discrepans*, and *Centruroides exilicauda*, among others (Rodríguez 2008).

The findings reported by Rodríguez (2008) are consistent with the exposure of the different individuals to venom with a low histotoxic and necrotizing power, which produces mainly neurohormonal changes that lead to the onset of hemodynamic disturbances such as increased mean arterial pressure and pulmonary arterial pressure, decreased cardiac output, and progressive increase in myocardial oxygen consumption, and are compatible with the clinical presentation of commitment diastolic compromise, left ventricular dysfunction, cardiovascular collapse, and increased frequency of sudden death associated with ventricular arrhythmias observed in case reports and described in other genera and species of the family Buthidae (Murthy 2000; Gómez et al. 2010).

In addition, several alterations associated with the presentation of lung injury and how congestion, edema, and alveolar hemorrhage are viewed in the histopathological analysis that are important components of the acute respiratory distress syndrome observed in case reports have been reported (Yugandhar et al. 1999; Murthy 2000; Rodríguez 2008; Izquierdo and Rodríguez 2012).

Other events such as the activation of the renin-angiotensin-aldosterone system by stimulation of the sympathetic system may be related to the reduction of effective blood flow due to the passage of intravascular fluid into the interstitium driving the emergence of peripheral circulatory failure that can be seen in the experimental and clinical descriptions of congestion of organs such as the lung, liver, spleen, and kidney (Yugandhar et al. 1999; Rodríguez 2008; Izquierdo and Rodríguez 2012).

Finally, it is possible to find deterioration of liver function, brought on by the processes of congestion and hypoxia, secondary to cardiocirculatory and respiratory dysfunction, which together with other processes such as the increase in the flow of intracellular Ca^{2+} contribute to the presentation of events such as vacuolar degeneration. The combination of all these circumstances may also contribute to the establishment of cellular anaerobic metabolism leading to multiple organ damage (Rodríguez 2008).

All these changes are clinically compatible with the onset of multisystem organ dysfunction syndrome, in which changes such as increased respiratory rate and dyspnea can be observed within the clinical framework of a variety of victims of moderate and severe envenomations with *T. pachyurus*, *T. asthenes*, and *T. furhmanni* that may be accompanied by other neurological disorders such as hypoactivity, drowsiness, spastic movements, twitches, muscle weakness, lacrimation, salivation, priapism, and even ejaculation and central neurological disorders such as ataxia, motor incoordination, as well as convulsions (Gómez et al. 2002, 2010; Otero et al. 2004; Izquierdo and Rodríguez 2012).

Clinical Characteristics of the Scorpion Accident in Colombia

Scorpion accidents often occur in the evening or at night, and their occurrence has been associated in some descriptions – such as that carried out on the hillsides of El Volador in Medellin (Antioquia) – with the discovery of these animals in areas of clutter featuring items of everyday use in different places inside and outside the home such as on floors, among utensils, in clothes, in shoes, on walls, or in gardens under logs or leaf litter (Gómez et al. 2002).

Regarding the frequency of accidents according to the body area affected, there was a preponderance of stings on the feet and lower limbs, followed by the upper limbs, abdomen, thorax, and head (Gómez et al. 2002, 2010; Barona et al. 2004; Otero et al. 2004). This type of accidents occurs most frequently in the productive ages (between 15 and 45 years), followed by accidents in children under 15 years, for whom a higher morbidity and mortality are a characteristic, especially in children under 6 years (Otero et al. 2004).

Among systemic clinical changes observed in case series and observational and experimental studies, tachycardia, vomiting, diaphoresis, abdominal pain, tachypnea, salivation and dysphagia, cyanosis, dehydration, hypertension, and bradycardia can be mentioned (Gómez et al. 2002, 2010; Rodríguez 2008). Clinical frameworks accompanied by hyperglycemia, urinary retention, hyperkalemia, hyperamylasemia, hypocalcemia, and hyponatremia have also been described (Gómez et al. 2002; Izquierdo and Rodríguez 2012).

In an effort to understand and better classify scorpionism at the global level, in January 2007, a group of 16 experts from four continents (America, Africa, Asia, and Europe) was formed. More details of their work methods and results obtained can be consulted in the work of Khattabi et al. (2011). This meeting of experts

Table 1 Clinical classification of the scorpionism accident for Colombia

Local manifestations	Mild systemic manifestations	Moderate systemic manifestations	Severe systemic manifestations
Paresthesias	Headache	Confusion	Ventricular arrhythmias
Localized pain	Nausea	Psychomotor agitation	Hypotension
Sweating	Pallor	Ataxia	Bradycardia
Local ecchymosis (variable)	Sialorrhea	Diarrhea	Cardiovascular collapse
Erythema	Isolated emesis Rhinorrhea	Dystonia	Shortness of breath
Hyperesthesia	Sweating	Myoclonus	Pulmonary edema
Burning sensation	Odynophagia	Gastrointestinal bleeding pancreatitis	Neurological compromise (coma)
Bullous rash (rare)	Local twitching	Bronchospasm	Convulsive status
		Priapism	Neuromuscular compromise
		Recurrent emesis	

proposed classifying scorpionism in three categories: class I shows only local manifestations, class II are generally lesser systemic manifestations that do not put life at risk, and class III correspond to systemic manifestations that suggest risk to the life of the patient.

However, taking account of the changes observed in different studies and considering that some of the clinical disorders listed as class II by the expert group can be considered as severe due to their life-threatening nature (e.g., convulsions, encephalopathy, severe degrees of arterial hypertension, dysthermias, ataxia, anisocoria, or presentation of pancreatitis), the following classification is proposed here for scorpion sting accidents in Colombia, with a view to increasing diagnostic accuracy and facilitating decision-making by health teams (see Table 1).

The main prognostic factors related to the severity of the scorpion accident are:

- Degree of toxicity of venom injected.
- Age of patient: the most severe cases and deaths have been reported primarily in children under 7 years (Pineda 2002; Otero et al. 2004).
- Species and size: the genera *Centruroides* and *Tityus* are most often related to the occurrence of systemic effects and even death. In some cases, the size of the scorpion causing the sting is related to a greater amount of venom injected and therefore to the effects produced in the victim (Barona et al. 2004).
- Time between accident and access to hospital care. A stronger possibility of deleterious effects was found to be associated with an increase in the delay in obtaining assistance (Pineda 2002; Otero et al. 2004).
- Onset of symptoms such as vomiting. It appears that the onset of vomiting and its intensity in the first 2 h can point to the gravity of the envenomation (Otero et al. 2004).

– Lethality of venoms: there are differences between the potency and lethality of different scorpion venoms even within the same genus, according to the LD_{50} reports available, among which are *T. fuhrmanni* (3.9 μg/g) (Gómez et al. 2002), *T. asthenes* (6.1 μg/g mouse) (Gómez et al. 2010), *T. pachyurus* (4.8 μg/g in 2004 and 6.5 μg/g in 2008) (Barona et al. 2004; Rodríguez 2008), and *C. margaritatus* (42.83 μg/g) (Guerrero-Vargas et al. 2007).

In one case report by Izquierdo and Rodríguez (2012) relating to a presentation of severe scorpionism, cardiovascular compromise and cardiac arrest, secondary to pulseless ventricular tachycardia, were observed in a 12-year-old patient in the town of Tolemaida, 2 h from Bogota. In this case, it was possible to observe the presence of an intense stimulant effect on the sympathetic nervous system with cardiovascular impact expressed in ventricular relaxation disorder, ischemic compromise, and secondary ventricular dysfunction which predominated in the echocardiographic controls following the accident.

Suggested Medical Treatment

The management of scorpion accidents is generally oriented toward stabilizing the victim and controlling the symptoms of the envenomation while neutralizing the poison. It is important to note that moderate accidents require the continuous monitoring of the patient and severe accidents require hospitalization at third-level institutions where the patient can be treated in intensive care. Measures used most often as part of the general support of the patient with scorpion envenomation include the following.

General Management

– Treatment of pain: in common with the rest of the countries around the world, the emphasis in Colombia has been on pain management with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and the use of local anesthetics and non-opioid analgesics (Pineda 2002; Otero et al. 2004). Regarding corticosteroids on the other hand, since there is no conclusive clinical evidence that either supports or restricts their use, this intervention has not been included in the recommendations for the clinical care of patients.
– Hemodynamic support: this is required in cases in which the patient may present changes suggestive of distributive shock resistant to hydration management, or heart failure, and includes the treatment of the hypertensive crisis, in which a number of alternatives have been used that include the α1 blockers such as prazosin to control the hypertensive crises and the use of inotropes such as

Table 2 Recommended use of antivenoms (Adapted from Ayerbe and Rodríguez 2008)

Severity of scorpionism	Treatment
Local symptoms	Observation for 6 h
Mild systemic	Observation for 12 h
Moderate systemic	Two vials intravenously, observation for 12 h
Severe systemic	Four vials intravenously, 24 h minimum observation

dopamine: and calcium gluconate in the treatment of myocardial dysfunction: and hypocalcemia if it occurs (Pineda 2002; Izquierdo and Rodríguez 2012).

- Ventilatory support: used in severe cases of neurological compromise, status epilepticus with respiratory compromise, or presentation of pulmonary edema (Pineda 2002).
- Maintenance of normoglycemia: because in Colombia there are very few case reports of scorpion accidents occurring with critical elevations of blood glucose levels, treatment has been directed mainly to the maintenance of normal levels with measures such as the administration of isotonic fluids and continuous monitoring (Otero et al. 2004; Izquierdo and Rodríguez 2012).

Use of Antivenoms

It is important to note that treatment of scorpion accidents globally has been the subject of a range of controversies and their effectiveness can vary from report to report.

In Colombia, it has not been possible to establish clearly the conditions under which specific serum therapy ought to be used, and there is no domestic production of antivenom. However, currently, a specific fabotherapeutic antivenom imported from Mexico is used.

Although this antivenom is produced from scorpion species of the genus *Centruroides* present in Mexico, some authors reported cross-reactivity for venoms of scorpions of other genera, and in experimental testing, a reduction was observed on the lethal effect of *T. pachyurus* venom in mice (Otero et al. 2004; Izquierdo and Rodríguez 2012).

Currently, the Institute for Food and Drug Monitoring (INVIMA) has approved the import of specific antivenom for therapeutic use in scorpion accidents with a systemic effect (Otero et al. 2004; Izquierdo and Rodríguez 2012). It is necessary to add that in the case of the severe scorpion envenomation from Tolemaida in the department of Tolima, five vials of specific antivenom were administered during cardiopulmonary resuscitation along with inotropic and ventilatory support and the replenishment of calcium necessary to control the hypocalcemia that the patient suffered. These combined interventions allowed the return to the spontaneous circulation of the patient and his progressive recovery from a cardiovascular collapse (Izquierdo and Rodríguez 2012). The recommendations for the use of antivenoms are reported in Table 2.

Complications

Among the major complications, several authors have found the onset of hypertensive emergency, heart failure, and cardiac arrest by malignant ventricular arrhythmias (Izquierdo and Rodríguez 2012), acute pulmonary edema and respiratory distress syndrome in adults (Izquierdo and Rodríguez 2012), acute pancreatitis (described in a case of a child stung by *T. asthenes*) (Otero et al. 2004), bowel bleeding (Gómez et al. 2010), and kidney failure (Rodríguez 2008; Izquierdo and Rodríguez 2012).

In some areas of the country (e.g., in the municipality of El Patía, Cauca Department), the development of diabetes mellitus has been observed in black individuals with no family history or other risk factors, in whom only a previous event of a scorpion accident serves as a nexus. However, studies are needed to corroborate these observations.

Finally, it is important to increase interdisciplinary efforts in Colombia that establish mandatory surveillance of all accidents by venomous animals, including scorpion accidents, in order to measure their actual impact on the health of the Colombian population, develop prevention strategies and appropriate treatment, and form networks of information and cooperation to facilitate access to the antivenoms in cases that warrant it.

Conclusion and Future Directions

The study of venomous animals and their venoms is a complex theme that ideally should be carried out within the field of toxinology, a transdisciplinary science.

Toxinology sciences are in the early stages of development in Colombia and require serious investment by the state organizations for science and technology in order to advance to a respectable world positioning.

Looking beyond the fine efforts of Khattabi et al. (2011) to unify the indications and symptoms of scorpionism, a new global consensus is still lacking. For this reason, a system of classification of scorpionism for Colombia is proposed here, comprising four different degrees – local manifestations, mild systemic manifestations, moderate systemic manifestations, and severe systemic manifestations.

Severe scorpionism in Colombia is caused not only by *T. pachyurus*, as has been reported (Chippaux and Goyffon 2008). Following exhaustive review, it was shown that severe scorpionism is caused by seven species: two from the genus *Centruroides* (*C. edwardsii* and *C. margaritatus*) and five from the genus *Tityus* (*T. asthenes*, *T. forcipula*, *T. fuhrmanni*, *T. pachyurus*, and *T.* n. sp. aff. *metuendus*).

The species *T. columbianus* does not cause severe scorpionism. Events recorded are rather of moderate scorpionism but with a high incidence, principally in Bogota D.C. As such, it is ranked as a dangerous species.

Scorpionism and scorpion species have an important, wide altitudinal and geographical distribution in Colombia – from 0 to 2,800 masl and all cardinal points, respectively.

Colombia currently has to import its scorpion antivenom from Brazil and Mexico. It is recommended that government health authorities promote the manufacture of polyvalent antivenom, produced from pools of venoms of the country's most dangerous scorpions.

Cross-References

- ▶ Scorpion Venom Interactions with the Immune System
- ▶ Scorpion Venoms: Pathogenesis and Biotherapies
- ▶ Scorpionism and Dangerous Scorpions in Central America and the Caribbean Region
- ▶ Scorpionism and Dangerous Species of Mexico
- ▶ Scorpionism and Dangerous Species of Brazil
- ▶ Scorpionism and Dangerous Species of Venezuela

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Scorpionism and Dangerous Species of Venezuela

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Abstract

All 54 recognized *Tityus* species in Venezuela are located at the mountain ranges, from sea level to 2,000 m above sea level, with rain forest as predominant habitat. The species comprised in *Tityus* genus are considered dangerous to humans, with similar LD_{50} among their venoms. Scorpionism is endemic coinciding with the rainy season. Its character is regional, being more severe in five country areas. Scorpionism clinical reports in Venezuela agree with an initial cholinergic phase, which can be followed or not by an adrenergic phase. The most serious complications developed by moderate or severe scorpionism in the country are pancreatitis, myocarditis, acute pulmonary edema, or acute lung injury and its final outcome, the respiratory distress syndrome, the most severe, lethal, and usually untreatable. The complication will depend on the scorpion species involved into the accident. The pathology changes in different Venezuelan regions. Scorpionism in the North Central region is predominantly cholinergic with severe gastrointestinal complications and some cases developing acute lung injury or respiratory distress syndrome. In other regions, scorpionism is predominantly adrenergic with severe cardiopulmonary complications developing acute pulmonary edema and myocarditis. Complications after scorpion envenomation would depend on the particular venom involved in the accident, since it varies intra- and interspecies over the year and in some species, even between females and males. Scorpion envenomation in Venezuela is one noninfectious process inducing pro-inflammatory cytokine release, which can lead to a generalized inflammatory process. Venezuelan scorpion antivenoms are also discussed, as well as diverse reports showing their effectiveness against *Tityus* envenoming. The state of the art of 83 Venezuelan *Tityus* toxins, with their total or partial sequences and their activities, is discussed in detail.

Introduction

Given the diversity of dangerous scorpion species inhabiting Venezuela and despite the efforts of experts in scorpionism and scorpion venoms, the limited knowledge about venom characteristics, action mechanisms, and physiopathology makes difficult the scorpionism diagnosis and therefore the application of the appropriate treatment. Moreover, many health centers in rural areas are geographically isolated, and both medical and paramedical staffs need to make therapeutic regime decisions by themselves. Envenoming by poisonous scorpions in Venezuela constitutes a great challenge in public health care, since the response time, diagnosis, and treatment are still key factors in patients' risk of death. This chapter discusses the state of the art about Venezuelan *Tityus* toxins and their antivenoms, so as the regional physiopathology and clinical manifestations of scorpionism in Venezuela.

Cases of Scorpionism in Venezuela

In the American continent, the Buthidae family has two important genera from the human health point of view: *Tityus* genus, located in South and Central America including some Caribbean islands, and *Centruroides* genus, located in North America, Central America, and in the North of South America. Venezuela occupies the first South American country in scorpion diversity, with 5 families (Buthidae, Chactidae, Euscorpiidae, Hemiscorpiidae, and Scorpionidae), 17 genera, and 184 species; the *Tityus* genus alone comprises 54 species, all considered dangerous to humans (Gonzalez-Sponga 2011). They are found at the mountain ranges, from sea level to 2,000 m above sea level, with rain forest as the predominant habitat (Fig. 1) (Gonzalez-Sponga 2011). Species of this genus are responsible of scorpion envenoming in this country, and they are a cause of medical emergency especially in children. According to statistics from the Hospital "Leopoldo Manrique Terrera," 3,409 cases of scorpionism were received only in this hospital between 1990 and 1997 (Fig. 2). Patients who developed severe or moderate systemic symptomatology represented the 13 % of all cases in urban sites where patients reached the hospital during the first hour after the accident; this percentage increased in rural areas (Guinand et al. 2004; D'Suze et al. 2003, 2011). The distribution of the number of cases with severe or moderate envenoming depended, among other things, on the distance between the site of accident and the nearest hospital.

Physiopathology and Clinical Manifestations of Scorpionism in Venezuela

The venom composition of *Tityus* species from Venezuela varies intra- and interspecies throughout the year and, in some species, even, between females and males (D'Suze and Sevcik 2010). Workers in the field found that the 50 lethal dose (LD_{50}) used to compare venom potency is similar among nine venom species commonly involved in scorpion accidents in Venezuela [*Tityus discrepans* (*Td*), *T. caripitensis* (*Tca*), *T. clathratus* (*Tcl*), *T. falconensis* (*Tf*), *T. funestus* (*Tfu*), *T. isabelceciliae* (*Tic*), *T. ivic-nancor* (*Tin*), *T. pittieri* (*Tp*), *T. zulianus* (*Tz*)]. However, it is important to note that even though *Tcl* venom in terms of LD_{50} is as potent as the others, this species has never been involved in severe scorpionism, probably due to the small amount of venom produced (D'Suze et al. 2011).

Scorpionism is endemic and it coincides with the rainy season between May and July and from October to November. It has also a regional character, more frequently and severe in five regions: (1) the North Central region (Dtto. Capital, Miranda, Aragua, and Carabobo states), (2) the North Eastern region (Monagas, Sucre, and Anzoategui states), (3) the West Central region (Lara, Yaracuy and Falcon states), (4) the Andean region (Mérida, Táchira and Trujillo states), and (5) the Southern region (Bolivar, Amazonas and Delta Amacuro states) (reviewed by Mazzei de Davila et al. 2011). All *Tityus* species are potentially lethal; however,

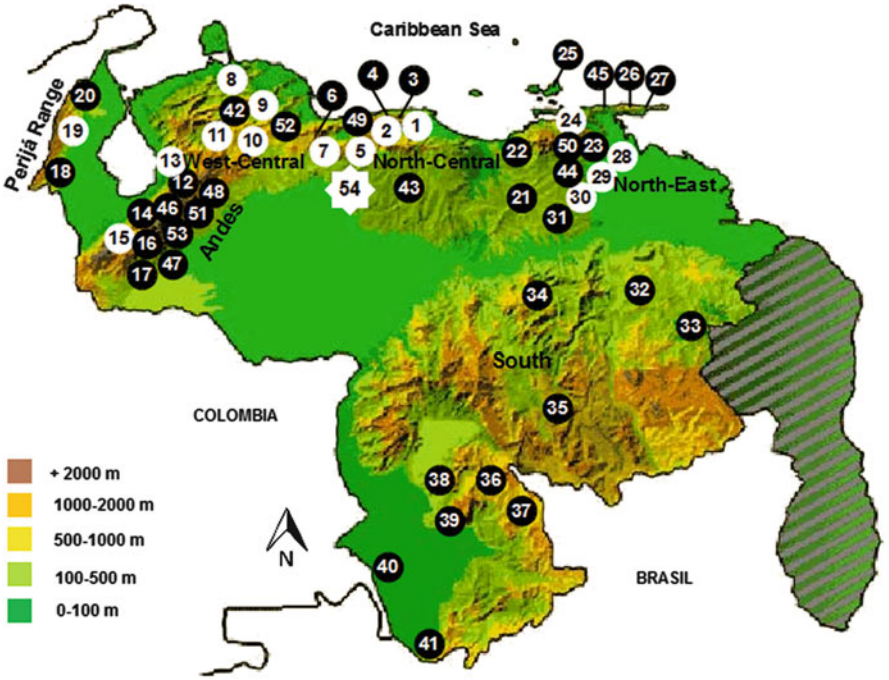
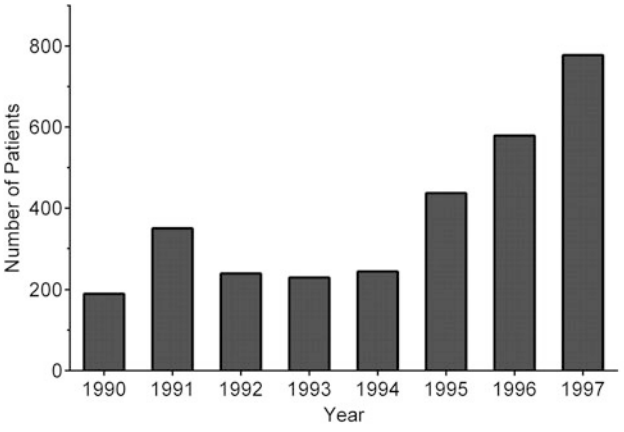


Fig. 1 *Geographical distribution of the 54 Tityus species described in Venezuela.* All *Tityus* species in Venezuela are located at the mountain ranges, from sea level to 2,000 m above sea level, with rainforest as the predominant habitat. Species highlighted in white circles are those involved in severe and fatal accidents, highlighted in black circles those not yet reported involved in scorpion accidents. Only *T. clathratus* is highlighted in star shaped because it is located in almost all country. (*1*) *T. discrepans*, (*2*) *T. isabelceciliae*, (*3*) *T. lancinii*, (*4*) *T. osmanus*, (*5*) *T. pittieri*, (*6*) *T. rojasi*, (*7*) *T. carabobensis*, (*8*) *T. falconensis*, (*9*) *T. barquisimetanus*, (*10*) *T. sanarensis*, (*11*) *T. ivic-nancor*, (*12*) *T. boconoensis*, (*13*) *T. valerae*, (*14*) *T. rubosus*, (*15*) *T. funestus*, (*16*) *T. pococki*, (*17*) *T. surmeridensis*, (*18*) *T. meridanus*, (*19*) *T. zulianus*, (*20*) *T. perijanensis*, (*21*) *T. gonzalespongai*, (*22*) *T. tamayoi*, (*23*) *T. arellanoparrai*, (*24*) *T. nororientalis*, (*25*) *T. neoespartanus*, (*26*) *T. cachipalensis*, (*27*) *T. irapaensis*, (*28*) *T. monaguensis*, (*29*) *T. quirogae*, (*30*) *T. caripitensis*, (*31*) *T. surorientalis*, (*32*) *T. breweri*, (*33*) *T. venamensis*, (*34*) *T. riocaurensis*, (*35*) *T. sarisariñamensis*, (*36*) *T. dupouyi*, (*37*) *T. anduzei*, (*38*)T. *culebrensis*, (*39*) *T. urbinai*, (*40*) *T. filodrendon*, (*41*) *T. siriana*, (*42*) *T. rusmelye*, (*43*) *T. guaricoensis*, (*44*) *T. walli*, (*45*) *T. uquirensis*, (*46*) *T. obispoi*, (*47*) *T. nematochirus*, (*48*) *T. mucusunamensis*, (*49*) *T. melanostictus*, (*50*) *T. elizabethbravoi*, (*51*) *T. dulceae*, (*52*) *T. dorae*, (*53*) *T. ahincoi*, (*54*) *T. clathratus*

only few species, highlighted in white circles in Fig. 1, are involved in severe and fatal accidents; they are as follows: *T. discrepans (Td), T. isabelceciliae (Tic), T. pittieri (Tp),* and *T. carabobensis* (*Tcar*), at the North Central region; *T. caripitensis (Tca), T. quirogae* (*Tq*), *T. arellanoparrai* (*Tar*), *T. monaguensis* (*Tm*), *T. surorientalis* (*Tsu*), and *T. nororientalis* (*Tnor*) at the North Eastern region; *T. falconensis (Tf), T. ivic-nancor (Tin),* and *T. sanarensis* (*Tsa*) at the West Central region; *T. valerae* (*Tv*) and *T. zulianus (Tz)* at the Andean region; *T. breweri* (*Tb*) at



	1990	1991	1992	1993	1994	1995	1996	1997	Total
Total	190	350	240	230	245	437	579	778	3409

Fig. 2 *Case report of the Toxicology Center Hospital "Leopoldo Manrique Terrera," Caracas.* Bar graph showing statistics of scorpionism cases from the Hospital "Leopoldo Manrique Terrera" received between 1990 and 1997 (Source: Epidemiological Surveillance Department, MSAS)

the Southern region; and *T. perijanensis* (*Tpe*) at the Perija mountain ranges near Colombian border.

Tityus sting can induce in few hours severe gastrointestinal, cardiovascular, and/or pulmonary complications, depending on the species involved. Most patients of scorpionism recover after receiving treatment with Venezuelan commercial antiscorpion antivenin together with a correct management of the specific symptoms developed (reviewed by Mazzei de Davila et al. 2011). Mortality is directly related to blood venom concentration, age, and patient's body surface. Fatal cases occur generally when the antivenin is injected too late, being the severe complications hardly neutralized with the antivenom. Scorpionism clinical reports in Venezuela agree with an initial cholinergic phase, which can be followed or not by an adrenergic phase (reviewed by Mazzei de Davila et al. 2011). The predominance of parasympathetic or sympathetic events seems to be related to the species involved in the accident (De Sousa et al. 2000).

Subclinical scorpionism with only local symptomatology is the most frequent; it is characterized by pain at the inoculation site, burning sensation, and radiation to the rest of the affected limb and generally without local signs of inflammation. In a study of 164 cases of scorpionism who developed only local symptoms, D'Suze and coworkers showed that these patients had high levels of IL6, TNF-α, and IL1-α in plasma during the first hours after the accident; the authors suggested that the absence of systemic symptoms was not equivalent to the absence of deleterious physiological changes (D'Suze et al. 2003). Local manifestations may be followed by a systemic symptomatology.

Systemic symptomatology usually starts with a cholinergic phase, which can be followed or not by an adrenergic phase. *The cholinergic symptoms* are abdominal pain, vomiting, sialorrhea, bronchorrhea, sinusal bradycardia, miosis, and increased levels of amylase and glycemia. This phase is usually self-limiting and can progress to necro-lethal hemorrhagic pancreatitis (Rosillo et al. 1999). Hyperglycemia is not common; however, one case was reported at the North Central region, presenting persistent high glucose serum levels due to endocrine pancreas damage (Ghersy de Nieto et al. 2004). *The adrenergic symptoms* are piloerection, mucocutaneous pallor, hypertension, sinus tachycardia, cardiac arrhythmias, serious pulmonary compromise, and cardiogenic shock (Mazzei de Dávila et al. 2002).

The most serious complications developed by moderate or severe scorpionism in Venezuela are pancreatitis, myocarditis, acute pulmonary edema or acute lung injury, and its final outcome, the respiratory distress syndrome, the most severe, lethal, and usually untreatable. The complication will depend on the scorpion species involved in the accident.

The pathogenesis of lung damage by scorpionism is still controversial and is under research; it may involve cardiac or noncardiac factors. Pulmonary edema is described as fluid accumulation in the lung air spaces and parenchyma, which occurs either after a failure of the heart left ventricle ("cardiogenic pulmonary edema") or after an injury to the lung parenchyma or vasculature of the lung ("noncardiogenic pulmonary edema").

The "cardiogenic pulmonary edema" was explained by Mazzei de Dávila and coworkers (2011) as a sequence starting with a sustained activation of the sympathetic nervous system, expressed by high norepinephrine release, leading to systemic vessel and pulmonary vasculature constrictions with pulmonary hypertension. This induces fluid passage to the alveolar space inducing acute pulmonary edema. A persistent and accentuate sympathetic hyperactivity (due to high blood venom concentration or to a delay of antivenin administration) will increase the ventricular afterload and microvasculature spasm, compromising the left ventricular function. Both mechanisms accentuate pulmonary edema inducing arrhythmias, systemic hypotension, and finally a shock. All these refer to a pulmonary edema predominantly cardiogenic, which can be developed by envenomation by *T. zulianus* (Andes region), *T. isabelceciliae* (North Central region), *T. falconensis* (West Central region), and *T. caripitensis* (North Eastern region). Cardiovascular disorders can appear quickly and can be measured through an increase of plasma creatine phosphokinase (CPK-MB), an isoenzyme correlated with acute coronary syndrome, used as early marker of cardiac damage (De Sousa et al. 1995; Guinand et al. 2004; reviewed by Mazzei de Davila et al. 2011).

The "noncardiogenic pulmonary edema" is correlated with a continuum pathological response to pulmonary parenchymal injury known as acute lung injury (ALI). Although ALI is formally considered different from acute respiratory distress syndrome (ARDS), it is usually just a precursor to ARDS and both are considered as an inflammation syndrome, which sometimes results in injury and organ dysfunction followed by endothelial injury. There is convincing evidence that leukocyte–endothelium interactions resulting from inflammatory reaction play

an important role in the pathogenesis of ALI and ARDS. Yet, a critical link between coagulation and inflammation in ALI and ARDS is neutrophil accumulation associated with intravascular and intra-alveolar fibrin deposition. This kind of lung injury is developed mainly by *Td* venom at the North Central region. The "noncardiogenic pulmonary edema" is the most severe and usually untreatable.

Workers in the field have demonstrated that the lung injury induced by *T. discrepans* venom in experimental animals involves a significant alveolar space reduction due to intravascular, intra-alveolar, and interstitial fibrin deposition so as a marked neutrophil sequestration, all of which resulted in nuclei and parenchyma increases (D'Suze et al. 2004a). These features are characteristics of ALI. Activated neutrophils migrating through the epithelium into the alveolar space have been observed in experimental envenoming with *Td*, suggesting the activation of inflammation in situ. A similar event is frequently found in the early phase of ALI and ARDS. *T. discrepans* venom produces an important damage in several organs, but the most striking effect occurs in the lungs as a diffuse injury of the alveolar capillary barrier, interstitial and alveolar edema associated with a marked leukocyte infiltration, epithelial transmigration, and marked fibrin deposits responsible for thrombi, wall infarct, and necrosis (D'Suze et al. 2004a). Other factors also seem to have an important role in the genesis of this damage: the platelet-activating factor (PAF) promotes lung damage, and heparin seems to prevent it (Freire-Maia and Matos 1993). It has been suggested that chemotactic substances, produced elsewhere, can induce lung neutrophil sequestration, and this seems to be closely related to alveolar fibrin deposition and alveolar damage (D'Suze et al. 2004a). Experimental research with sheep envenomed with *T. discrepans* venom (40 μg/kg, 5 h) showed histological lung sections with a marked erythrocyte accumulation in lung alveolar walls which resulted often in wall infarction, a consequent reduction of the alveolar space, and necrosis. Fibrin deposits were found only at places where a marked neutrophil accumulation occurred. Neutrophil accumulations are able to express clotting tissue factor, triggering the clotting cascade by forming a complex with factor VII/VIIa, producing thrombin, which converts fibrinogen to fibrin (Nemerson 1988).

One of the most frequent complications of scorpion evenomation in Venezuela is the acute pancreatitis; this can be developed by the venom of species from the North Central (*Td, Tp, Tic*), West Central (*Tin, Tf, Tsa*), and North Eastern region (*Tc*) and the Andes region (*Tva*); it is never observed in envenoming with *Tz* from the Andes region (reviewed by Mazzei de Davila et al. 2011; Rosillo et al. 1999). The pancreatitis in humans and other animals is induced by a cholinergic pathway involving muscarinic receptors. Experimental research with isolated rat pancreas demonstrated that *T. ivic-nancor* venom induces acetylcholine release, which activates muscarinic receptors and promotes the conversion of zymogen granules into active enzymes. In parallel, *T. ivic-nancor* venom induces depletion of calcium intracellular reservoirs. Animals suffering experimental envenomation with *T. discrepans* venom developed pancreas cytoplasmatic edema, mitochondrial degeneration, acinar cell vacuolization, and a relevant digestion of Langerhans islets (Blanco et al. 1999; D'Suze et al. 2004a). Pancreas histological sections from

sheep envenomed with *T. discrepans* venom (40 μg/kg, 5 h) showed a marked cell degranulation, vacuolization, interstitial inflammation, necrosis, and changes of Langerhans islet structure (D'Suze et al. 2004a). Also, disintegration signs of blood vessels were observed in the vicinity of necrotic acini, particularly near the pancreatic duct, with strong infiltration of neutrophils and macrophages (D'Suze et al. 2004a).

The scorpion envenomation in Venezuela is one of the noninfectious processes inducing massive release of proinflammatory cytokines, especially IL6, IL1-α, and TNF-α, which can lead to a generalized inflammatory response. IL6 level increases in direct relation with envenoming time and venom concentration. Experimental envenomation indicates that *T. discrepans* envenomation produces a severe and generalized inflammatory syndrome remarkably quick in rams, ≤5 h (D'Suze et al. 2004a). *T. discrepans* and *T. falconensis* venoms induce in human a significant increase of plasma IL6 and TNF-α with maximum values at 60 min after envenomation (D'Suze et al. 2003). When IL6 plasma levels increase three to six times above normal, production of acute phase proteins may occur, complicating further the pathology (Steer and Meldolesi 1988). These pro-inflammatory cytokines can contribute to spread inflammation and can also induce acute lung injury. When does this inflammatory syndrome become irreversible is not known; therefore, the onset of antivenom treatment during the first 4 h of envenomation is imperative.

State of the Art About Venezuelan *Tityus* Toxins

Tityus venoms are a mixture of inorganic salts, lipids, amino acids, peptides mucopolysaccharides, low molecular mass (MW) proteins, and high MW proteins, some of them metallo- or serine protease-like enzymes (Batista et al. 2006; Brazón et al. 2009, 2013). Among the Venezuelan *Tityus* species, *T. discrepans* has been the most studied. Using MALDI-TOF and nano-ESI-ITMS, the presence of 205 components with molecular masses from 272 to 57,908 amu was demonstrated (Batista et al. 2006). The MW distribution in this venom showed a high proportion of low-MW peptides or toxins (<5 kDa); most of the peptides within this MW range are the ones blocking K^+ channels (2,500–4,500 Da). Peptides having MW around 5–6 kDa are scarce in *T. discrepans* venom. Peptides modifying the gating mechanism of Na^+-channels have MW between 6,000 and 8,000 Da. In *T. discrepans* venom, these toxins are the most abundant in concentration but not necessarily in diversity.

A total of 83 peptides have their sequences totally or partially determined by Edman sequencing of the native peptides purified by HPLC and/or determined by nucleotide sequence analysis of genes; the complete sequence is known in 34 of these toxins (Batista et al. 2006; Borges et al. 2006; Díaz et al. 2009; Diego-García et al. 2007; D'Suze et al. 1996, 1997, 1999, 2004b, c, 2009, 2010; Peigneur et al. 2012). Table 1 shows the UniProt accession numbers of the 34 toxins, with complete amino acid sequences and their custom names. The other 49 toxins with incomplete sequences are not shown here; however, it is possible to find them in the references cited above. Among the partial sequences are a group of toxins with an important homology with already reported toxins, for example, bact-3, bact-4, bact-5,

Table 1 UniProt accession numbers with their custom names of the 34 toxins, with complete amino acid sequences, isolated from Venezuelan scorpions

Name	UniProt	Class
Ard	P0C1X7	Insect NaScTx
Bact-1	P0CF39	Bacterial, insect NaScTx
Bact-2	P0CF37	Bacterial, mammal NaScTx
TdNa1	C9X4J9	Putative NaScTx
TdNa2	C9K4K0	Putative NaScTx
TdNa3	C9X4K1	Putative NaScTx
TdNa4	P0C1X7	Putative NaScTx
TdNa5	C9X4K3	Putative NaScTx
TdNa6	C9X4K4	Putative NaScTx
TdNa7	C9X4J8	Putative NaScTx
TdNa8	C9X4K6	Putative NaScTx
TdNa9	C9X4K7	Putative NaScTx
TdNa10	C9X4K8	Putative NaScTx
Td1	Q1I180	Putative NaScTx
Td2	Q1I179	Putative NaScTx
Td3	Q1I177	Putative NaScTx
Td4	Q1I174	Putative NaScTx
Td5	Q1I169	Putative NaScTx
Td6	Q1I167	Putative NaScTx
Td7	Q1I164	Putative NaScTx
Td8	Q1I163	Putative NaScTx
Td9	Q1I178	Putative NaScTx
Td10	Q1I176	Putative NaScTx
Td11	Q1I173	Putative NaScTx
Td12	Q1I172	Putative NaScTx
Disc	P84777	αKTx
TdK1	P59925	αKTx
TdK2	P0C1X5	αKTx
TdK3	P0C1X6	αKTx
TdKIK	Q0GY43	Putative βKTx
TdiβKTx	Q0GY44	Putative βKTx
Tdd	P0CF77	Putative defensin
Tz1	Q2NME3	Putative NaScTx
Tz2	Q1I165	Putative NaScTx

bact-6a, and bact-6b have high homology with TdNa7, Td7, ardiscretin, TdNa6, and Td1 respectively; however, it would be incorrect to assign the same name because they still lack the carboxyl-terminal region, and one can not rule out posttranscripcional cuts which are common among the carboxyl-terminal region of scorpion toxins.

Most of the isolated toxins modulate or block ion channels. Regardless of size and function, they have a similar folding pattern (Fig. 3). Toxin 3D structures have been determined by NMR in only one toxin, discrepin (Prochnicka-Chalufour et al. 2006), while other toxins in 3D structures have been modeled in silico (Díaz et al. 2009;

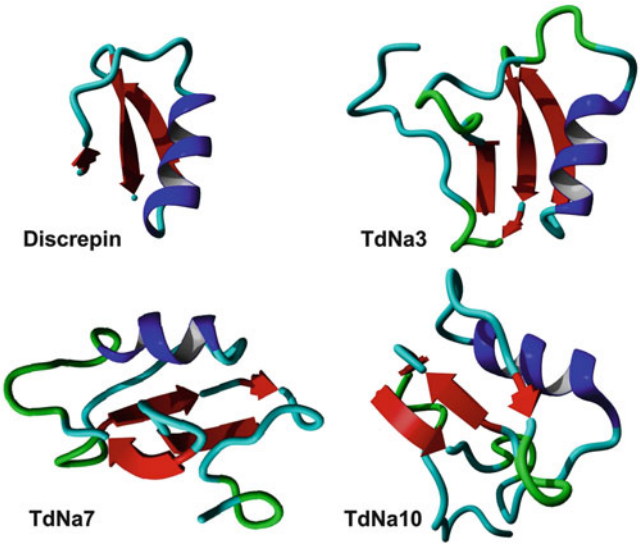


Fig. 3 *Three-dimensional structure of four T. discrepans toxins*. Discrepin 3D was determined by NMR (PDB code: 1axk), and TdNa3, TdNa7, and TdNa10 3D structures were modeled in silico . In all four panels, the secondary structure is colored as follows: *blue*, α-helix; *green*, β-turns; *red*, β-sheets; *cyan*, unstructured segme3nts

D'Suze et al. 2009). They are globular proteins adopting a cysteine-stabilized α/β motif, formed by one α-helix and two or three parallel or antiparallel β sheets stabilized by three or four disulfide bridges. Similarly to most scorpion toxins, two disulfide bridges are strictly conserved, cross-linking an α-helix segment of the peptide with the second strand. These toxins show a high percentage of charged amino acids distributed over the surface of the protein. Their spatial configuration is conserved despite the highly diversified sequences of amino acids. It is generally assumed that having multiple isoforms would confer evolutionary advantages to the species.

Venezuelan *Tityus* Neurotoxins

Neurotoxins have the property of modulating the conductance and/or other properties of ion channels in excitable and non-excitable tissues. The most studied are the long-chain peptides (59–76 residues long) known to modulate voltage-gated Na^+ channels (Na_V). Toxins active on potassium channels also induce depolarization of the cell membrane due to block of K^+ channels. These toxins are not responsible for the lethality induced by short-term envenoming, but probably they would, in a longer time period since they could be altering the physiology of many cell types. Potassium channels represent the largest family of ion channels in living organisms; the K^+ toxins are also numerous in scorpion venoms. Despite having similar three-dimensional structures, they differ in the surface of interaction with the channels (Rodríguez de la Vega et al. 2003).

Table 2 Effect of whole venom from five *Tityus* species on $Na_V1.4$

Species	%	$V_{0.5}$		*k*	
		Ctr	+ Venom	Ctr	+ Venom
T. perijanensis	84	−34 ± 0.4	−44 ± 1.5	3.6 ± 0.2	1.9 ± 0.2
T. zulianus	82	−36 ± 1.6	−35 ± 3.2	2.7 ± 0.4	1.5 ± 0.3
T. discrepans	48	−35 ± 0.7	−40 ± 1.7	3.4 ± 0.3	2.2 ± 0.3
T. quirogae	26	−40 ± 0.9	−39 ± 2.1	3.3 ± 0.3	1.9 ± 0.3
T. caripitensis	72	−39 ± 0.8	−24 ± 1.1	3.2 ± 0.3	2 ± 0.2

The percentages are the reduction in peak current. The $V_{0.5}$ and *k* values were calculated with the Boltzmann equation used to fit the activation curves and shown in the legend of Fig. 4. $n = 3–6$

Modifying Voltage-Dependent Sodium Channels

Whole Venom Effect on Nav1.4

The effect of whole venom from five *Tityus* species (*T. discrepans*, *T. quirogae, T. caripitensis, T. zulianus,* and *T. perijanensis*) from different Venezuelan regions was studied using whole-cell clamp, on the skeletal muscle sodium channel 1.4 ($Na_V1.4$) expressed in HEK 293 cells. These results are original and were selected to illustrate in this chapter the differential effects of diverse species from four Venezuelan scorpionism endemic regions. Currents were measured after applying a double pulse protocol, with a conditioning pulse in the middle to −10 mV in order to prime the channels. Table 2 shows the reduction in peak current percentages, $V_{0.5}$ and k values which were calculated from the Boltzmann equation used to fit the activation curves. In Fig. 4 the current/voltage (I/V) curves (left) and their corresponding activation curves (right) are shown. All studied venoms at the same concentration (1 ng/μL) induced different effects on I/V and activation curves. No effect of the conditioning pulse was observed for any of the studied venoms. *T. perijanensis* and *T. zulianus* decreased by 84 % and 82 %, respectively, the peak sodium currents and shifted the activation curves to the left by ≈10 mV. Td decreased the peak currents by 50 % and left shifted the activation curves by 5 mV. The effect of the North Eastern region venom species, *T. quirogae* and *T. caripitensis*, differ from those described above; *T. quirogae* venom did not shift the activation curve and *T. caripitensis* right shifted activation curves by 15 mV and decreased the currents in a voltage-dependent manner. *T. quirogae* venom decreased the peak current by 26 % and *T. caripitensis* venom decreased the currents by 72 %. The functional effects on I/V and activation curves on $Na_V1.4$ channels are more similar between the nearest geographical species and their effectiveness on $Na_V1.4$ channels diverge from those that inhabit further away.

Isolated Toxins Effect on Voltage-Dependent Sodium Channels

One of the principal targets of scorpion toxins is the voltage-dependent sodium channel. Roughly, depending on their mode of action, toxins are classified as alpha toxins if the inactivation process is affected and beta toxins if the activation process is modified. There are few toxins isolated from Venezuelan *Tityus* venoms whose

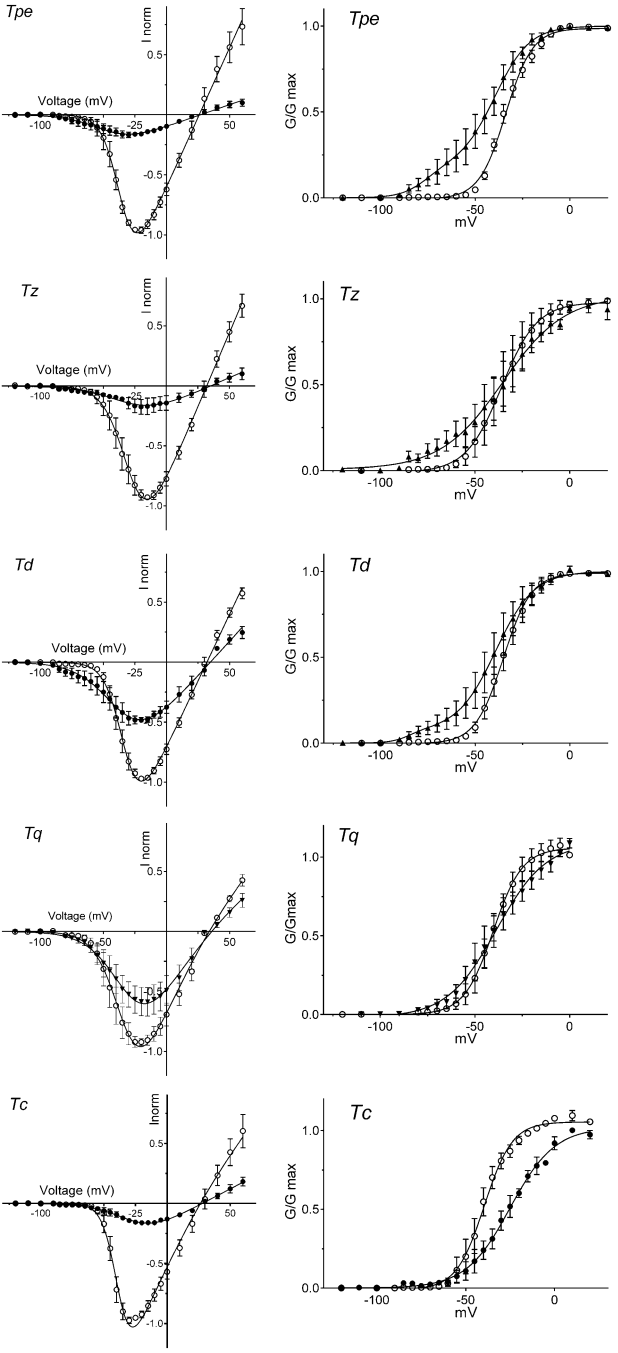


Fig. 4 *Effect of five different Tityus species venoms on Nav1.4*. Whole-cell clamp experiments were performed in HEK293 cells expressing constitutively Nav1.4 channels. Three-step voltage

targets have been studied. Most of the functionally studied isolated toxins are from *T. discrepans* and only one from *T. zulianus*. Bactridines (bact-) are a group of peptides isolated from *T. discrepans* that were first described as antibacterial toxins (Díaz et al. 2009). Bact-1 (MW 6,916 Da) and bact-2 (MW 7,362 Da) are comprised of 61 and 64 amino acids, respectively. Two-thirds of the amino acid sequences of Bacts-3–6 are reported, and their molecular masses are 7,226, 7,011, 7,101, and 7,173 Da, respectively (Díaz et al. 2009; Peigneur et al. 2012). The biochemical characteristics of these peptides share a similar scaffold with most scorpion toxins. They are also capable of altering differentially the activation of various Na_V isoforms expressed in *Xenopus oocytes* ($Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.5$, $Na_V1.6$, $Na_V1.7$, and $Na_V1.8$), insect *Drosophila melanogaster* $DmNa_V1$, and bacterial NaChBac channels (Peigneur et al. 2012). All bactridines except bact-1 were able to modify differentially the channels studied with the exception of NaChBac channels. Most of them exerted a β-type toxin effect, by shifting to the left the voltage dependence of the activation and reducing the peak current. The latter did not occur in $Na_V1.6$ channels. One μM of bact-5 only reduced by 10 % the peak currents of $Na_V1.7$. Bact-6, on the other hand, had effect on channels $Na_V1.3$, $Na_V1.7$, and $DmNa_V1$. Interestingly, on $Na_V1.3$ bact-6 reduced the peak currents by 36 % and left shifted the voltage dependence of the activation curves, thus acting as a typical β-type toxin. In $Na_V1.7$ ~20 % of the conductance remains activated at potential positive to −50 mV, while in $DmNa_V1$ ~50 % of the conductance remains activated. In the same peak were bact-6 eluted; other 2 toxins are co-eluted; these were isolated, characterized, and sequenced. The major component was renamed bact-6a, and the other two were called 6b and 6c. Even though no data were presented, bact-6a and bact-6b (MW 7567.3 Da) had a similar effect

Fig. 4 (continued) protocol applied to elicit Na^+ currents: from a holding potential of −120 mV, the protocol was applied in three successive stages, the first stage from −120 to −90 mV with 10 mV steps, second stage test potentials from −85 to 0 mV with 5 mV steps, and third stage with test protocols from +10 to +50 mV with steps 10 mV steps. A central pulse to −10 mV for 50 ms was used as a conditioning pulse to prime the channels. The segments of 50 ms at −140 mV ensured recovery from inactivation. (*Left*) Normalized current voltage plots in control condition (*open circles*) and with 1 ng/μL of the indicated venom (*filled circles*). Data were fitted to $I = G^*(V-V_{Na})/(1 + \exp(-(V-V_{0.5})/k_G))$ where G is the maximal conductance, V is the step potential, V_{Na} is the sodium reversal potential, $V_{0.5}$ is the voltage of half activation, $kG = RT/zF \approx 25.4/z$ at 22 °C, z is charges per molecule, F is the Faraday constant, R is the gas constant, and T is the absolute temperature. Conductance/voltage (g/V) relationships were calculated from peak current/voltage (I–V) relationships using the equation $g = I/(V\text{-}V_{rev})$, where I is the peak Na + current, V is the test potential, and V_{rev} is the reversal potential. (*Right*) Activation curves in control conditions (*open circles*) and the presence of the venom (*solid circles*). Normalized g/V relationships for channels in the absence and presence of toxin were fitted with a two-state Boltzmann distribution, $G/Gmax = 1/(1 + \exp[-(V-V_{0.5})/k_G])$ where V is the potential of a given pulse, $V_{0.5}$ is the voltage of half activation, and k_G has the same meaning as in the above equations. Data are shown as the mean ± SEM, $n = 3$–6. Solutions: external in mM, NaCl 65, cholineCl 35, $CaCl_2$ 2, KCl 5, TEA-Cl 20, Hepes 10, mannitol 50, pH 7.4 (adjusted with NaOH); pipette: NaCl 35, CsF 105, EGTA 10, Hepes 10, pH 7.4 (adjusted with CsOH)

on $Na_V1.3$, $Na_V1.7$, and $DmNa_V1$. Bact-6a and bact-6b had effect on $Na_V1.3$, $Na_V1.7$, and $DmNa_V1$. Bact-6c had no effect. Most probably bact-6b is the same molecular entity as Td1, based on the fact that their molecular masses are similar and bact-6b first 36 residues are identical to the first 36 amino acids of Td1. Interestingly, bact-6a and bact-6b have primary structural features of β-toxins but display an α-like activity without modulating the activation. These toxins were classified as βα-toxins, belonging to a group of ancestral toxins that further diversified, allowing the emergence of toxins with the β structural features but displaying an α-like activity. All bactridines were also tested in cricket DUM neurons (Forsyth et al. 2012). In these neurons 150 nM of bact-1, bact-4, and bact-6a reduced the peak sodium currents without affecting the voltage dependence of activation. Bact-1 is a specific insect toxin, yet bact-4 and bact-6a are mammalian and insect toxins. Most of the bactridines shifted the voltage inactivation dependence without delaying it. Most interesting, bactridines increased an amiloride-sensitive conductance in DUM neurons (Forsyth et al. 2012). This target could be related to the bactridine-induced sodium outflow in *Yersinia enterocolitica* (Díaz et al. 2009). The amiloride-sensitive current is under study.

The only toxin reported from *T. zulianus* with sequence and electrophysiological function studied is Tz1, the most abundant component from *T. zulianus* venom (Borges et al. 2004; Leipold et al. 2006, 2012). Tz1 molecular mass is 7367.16 Da and has been classified as a typical β-toxin because it shifts the half-maximal activation curves toward the left. It has a strong preference for $Na_V1.4$ skeletal muscle channels compared to $Na_V1.2$ and $Na_V1.6$ where 10 μM is able to modify the activation of fewer channels. On the other hand, $Na_V1.5$ and $Na_V1.7$ are practically insensitive to the same concentration (Leipold et al. 2006). In a recent work, Leipold and coworkers (2012) explained the mode of action of Tz1. This toxin acts in an excitatory mode, i.e., increase the sodium currents when channels are stimulated at a frequency of 2 Hz, while if channels are stimulated at a 0.1 Hz, Tz1 acts in a depressant mode in $Na_V1.4$ channels. On $Na_V1.5$ it only acts in a depressant mode independent of the frequency applied. Similarly as Tz1, Bact-2 from *T. discrepans* is most active on $Na_V1.4$ (Peigneur et al. 2012; Leipold et al. 2006, 2012). These two toxins differ in only one amino acid, Asn56 in bact-2 is changed with an Asp56 in Tz1. It is very interesting that a change of only one aa rendered different affinities between these two toxins. Bact-2 (100 nM) shifted by −23 mV the activation midpoint potential in $Na_V1.4$ expressed in oocytes, while 1 μM Tz1 shifted −10 mV in $Na_V1.4$ expressed in HEK293 cells (Peigneur et al. 2012; Borges et al 2004).

Ardiscretin was the first insect toxin from Venezuelan *Tityus* venoms which was completely sequenced, cDNA cloned, and biological activity reported (D'Suze et al. 2004b). It is composed of 61 amino acids and has a MW of 7103.8 Da. It is specific for arthropods with no effect on mammals at doses 10–100 higher than that of other scorpion toxins specific for mammals. In squid axons, 30 μM of Ardiscretin produced repetitive action potentials similarly as DDT [1,10(pchlorobenzyl) 2-trichloretane] and slows down the rate of rising of the action potential indicating that it inhibits sodium currents.

Previous work on *T. discrepans* venom identified four toxins named TdII-1, TdII-2, TdII-3, and TdII-4 (D'Suze et al. 1996) in which *N*-terminal sequence was determined. The short fragments revealed, nonetheless, unambiguous matches to *Tityus* sodium channel toxins. These peptides were active on sodium currents. TdII-1 and TdII-2 were toxic to crustaceans and mammals. TdII-3 and TdII-4 were only toxic to mammals. TdII-1, TdII-2, TdII-3, and TdII-4 reduced the resting membrane potential of toad *sartorious* muscle. TdII-1, TdII-2, and TdII-3 increased the frequency of miniature end plate potentials of the neuromuscular preparation. Borges and coworkers (1990) determined the amino acid composition of four *Td* toxins named TdIV, TdV, TdVIII, and TdIX. From these, TdVIII was lethal to mice; it has 56 aa residues and an MW of 6,140. Nine years later in a work by Tsushima et al. (1999), the function of TdVIII was described in $Na_V1.4$ expressed in *Xenopus* oocytes.

Toxins That Interact with Voltage-Dependent K^+ Channels

Toxins that block voltage-gated potassium channels are composed of 23–60 amino acidic residues and are divided in short- (23–40 aa) and long-chain toxins (60 aa). Until now only 4 K^+ channel toxins on Venezuelan *Tityus* venoms have been functionally studied, even though many more have been identified by cDNA cloning as putative K^+ toxins. Two long-chain putative K^+ toxins have been identified by cDNA molecular cloning and their presence was confirmed in the venom (Diego-García et al. 2007). Their biological function is not well known, i.e., they were considered orphan peptides. All of these toxins have been isolated from *T. discrepans* venom and gland.

Tdk1 (also named as α-KTx4.3) was the first K^+ blocking toxin isolated from Td venom; it contains 37 aa and has a MW of 3,817 Da. It blocks reversibly Shaker B K^+ channels expressed in SF9 cells with a Kd of ≈280 nM (D'Suze et al. 1999). Discrepin (also named α-KTx15.6), a toxin with 38 aminoacids and a MW of 4177.7 Da, is folded through three disulfide bridges. It blocks preferentially the IA currents of the voltage-dependent K^+ channel of rat cerebellum granular cells in culture (D'Suze et al. 2004c). Discrepin blocks irreversibly these channels with 50 % inhibitory effect at 190 nM. TdK2 (also named as α-KTx18.2) has an MW of 3,451 Da and blocked reversibly Shaker B K^+ channel with a Kd of 1.5 μM. TdK3 (MW 3,832 Da) has a low specificity on Shaker B, since 3 μM only blocks 10 % of Shaker B currents (Batista et al. 2006).

Venezuelan *Tityus* Toxins with Antibacterial and Antifungic Activity

Scorpions are exposed daily to microbial infections. In order to defend themselves against the hostile environment, they have developed potent defensive mechanisms that are part of innate immunity. Some scorpions spray themselves with their own venom; it has been argued that it may help them to get rid of opportunistic fungal and bacterial infections.

Antibacterial Active Toxins

Bactridines are six peptides isolated from *T. discrepans* venom with antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria. They induce a complete bacterial growth inhibition at concentrations from 20 to 80 μM depending on the bacteria and bactridine tested. Bactridines are not typical antibacterial peptides; they are hydrophilic, nonamphipathic, and positively charged polypeptides stabilized by four disulfide bridges. Bactridines (0.3 μM) induce leakage of sodium ions on *Yersinia enterocolitica*, indicating a change in bacteria Na^+ membrane permeability. Amiloride (10 μM) and mibefradil (25 μM), drugs that affect Na^+ and Ca^{2+} currents, respectively, block bactridines effect, suggesting that their effect is mediated by an amiloride- and mibefradil-sensitive sodium current. In a posterior work, authors demonstrated the ability of bact-1 to modulate insect Na_V channels (Forsyth et al. 2012) and that bact-2 to bact-6 can modulate with differential affinities mammal Na_Vchannels (Peigneur et al. 2012). Prior to the report of Díaz et al. (2009), scorpion toxins affecting sodium channels were supposed to act only on eukaryotes. Bactridines emerge as an interesting toxin group with a dual effect. They have differential effects on pathogens and eukaryotic cells and are potential research tools to understand sodium channel isoform structure–function relationships.

Antifungic Active Toxins

Phytopathogenic fungi are major agents infecting plants consumed by humans causing 35 % worldwide crop loss. *T. discrepans* venom inhibits *Macrophomina phaseolina* growth in a concentration-dependent manner. Seven toxins (F1-F7), six of them peptides, were isolated and its antifungal effects on *M. phaseolina* characterized (Joya et al. 2011). *M. phaseolina*, a globally distributed Ascomycota, infect a wide range of crops including soybean, corn, and common bean and a wide range of citrus. This fungus survives in soil or on infected plants as microesclerotium and has become resistant to many chemical fungicides in current use.

The antifungal toxins affected *M. phaseolina* viability by three different mechanisms: (1) F2, F3, and F5 decreased fungus esterase activity, an enzyme involved in tissue decomposition, important for fungus feeding; (2) peptides F3 to F6 altered fungus Na^+ membrane permeability; and (3) F1, F5, F6, and F7 altered wall sterol biosynthesis either by inhibiting ergosterol biosynthesis or by producing ergosterol analogues. It seems that interfering with sterol synthesis is the most important mechanism behind the fungicidal effect. However, the toxins antifungal effects at short times are indicative of a direct esterase inhibition, which, with the increased membrane leakiness to Na^+, makes the fungus unviable. Sterol composition of plasmatic membranes is critical for membrane fluidity, and membrane fluidity is in turn critical for the proper function of transmembrane molecules, such as ionic channels. The most potent toxin was F7 with a minimal inhibition concentration of 0.4 μg/μL and a concentration for 50 % inhibition of 0.13 μg/μL. Venom components with antifungal qualities were isolated by following their biological activity. Previous HPLC studies of *T. discrepans* venom on C18 columns (Batista et al. 2006)

have shown that peaks eluting at similar retention times are clusters of compounds with similar size and activity. The molecular masses (Da) of the purified active components were F1, 1060.1; F2, 7328.9; F3, 7288.3; F4, 7268.5; F5, 7104.6; F6, 6924.6; and F7, 6823.3. The MS analysis resulted in six positive identifications; only F1 remains unidentified. F1 produced no amino acids under tryptic digestion. It is not known if F1 is a small peptide or some other kind of organic molecule (Joya et al. 2011). All of these fungicidal compounds, except F1, have masses between 6823.3 and 7328.8 Da, typical of scorpion toxins acting on sodium channels. F5's mass matched that of ardiscretin, an arthropod-selective sodium channel toxin (D'Suze et al. 2004b). Moreover, the two tryptic peptide sequences from F5 matched perfectly with part of the ardiscretin sequence, suggesting strongly that F5 is ardiscretin. The tryptic peptide sequence from F6 matched partially both ardiscretin and bactridin 1 sequences (Díaz et al. 2009; D'Suze et al. 2004b). Still, F6's molecular mass was the same as that of bactridin 1, suggesting that F6 may be bact-1. F5 and F6 are proteins able to change the fungal sodium ion membrane permeability. Amino acid sequences of F2 tryptic peptides allowed the identification of this toxin as a putative neurotoxin named Td11 (Borges et al. 2006), and the tryptic peptide sequences of F3 and F4 matched another putative neurotoxin named Td3. The effects of Td3 and Td11 have not yet been studied. F7's mass matched the molecular mass of TdiβKTx (Diego-García et al. 2007; D'Suze et al. 2009), the effects of which are also unknown, but which was classified as a β-KTx-like peptide on the basis of its nucleotide and amino acid sequences. These toxins emerge as new antifungal agents gentle to the environment. It is evident that scorpion venoms are a rich source of biodegradable peptides able to interact with cell membranes of living species from diverse taxa.

Venezuelan *Tityus* Toxins with Curarizing Activity

TdI-1 is a large MW toxin of approximate 48,000 Da, isolated from *T. discrepans* venom. This toxin studied on neuromuscular preparations of toad *sartorious* muscle blocks miniature end-plate potentials (MEPP's) and abolishes or reduces end-plate potentials (EPP's) below action potential threshold (D'Suze et al. 1997). The toxin is a potent reversible non-depolarizing muscle relaxant that blocks more than 95 % of the EPP at a low concentration of 2 μM. On a molar basis, TdI-1 is as potent as or more than many muscle relaxants since, at the concentration used, the toxin suppressed more than 95 % of the EPP. The TdI-1's amino terminal sequence of the first 23 residues is known. TdI-1 is the only scorpion toxin described with curarizing activity.

Venezuelan *Tityus* Toxins Inducing Hemostasis Alterations

For more than 35 years, it has been known that some scorpion venoms can affect mammalian blood coagulation. Envenoming by *Centruroides sculpturatus*

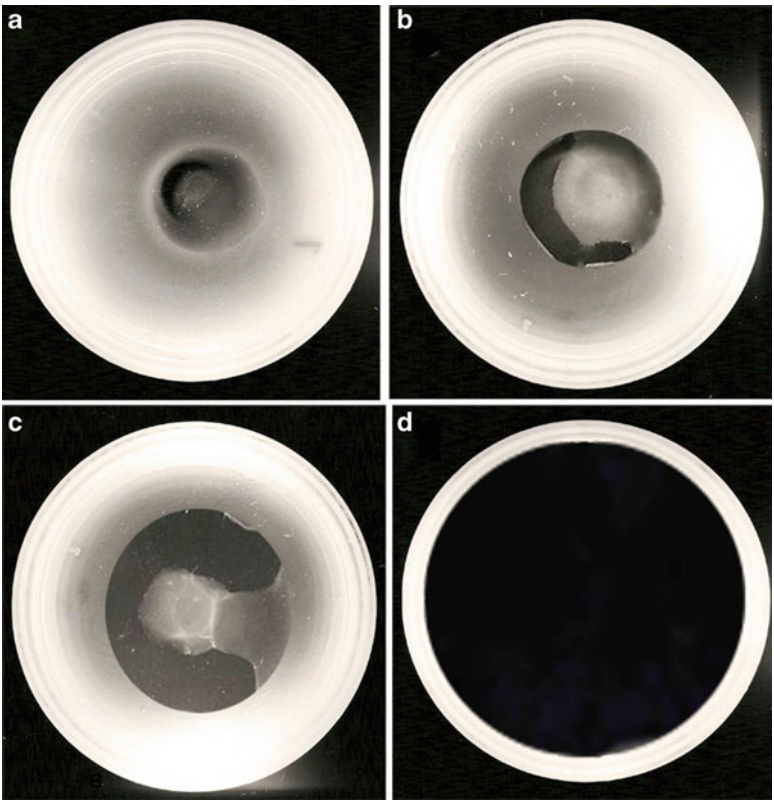


Fig. 5 *Fibrinolytic activity of Tityus discrepans whole venom.* Fibrinolytic activity of *T. discrepans* venom (TdV) using plasminogen-rich fibrin plate. (**a**) TdV (30 μg/10 μL). (**b**) TdV(100 μg/10 μL). (**c**) TdV (300 μg/10 μL). (**d**) t-PA (tissue plasminogen activator – 0.1 μg/10 μL)

(Longenecker and Longenecker 1981), *Buthus tamulus* (actually renamed as *Mesobuthus tamulus*) (Reddy et al. 1972), *B. martensii* (actually renamed as *Mesobuthus martensii*) (Song et al. 2005), *T. discrepans* (D'Suze et al. 2003), and *T. falconensis* (Guinand et al. 2004) has shown the ability of their venoms to perturb the hemostatic system. Clinical studies of scorpionism from the Northeast, North Central, and West Central region in Venezuela have reported patients with alterations of prothrombin and partial thromboplastin time, developing one of them a digestive hemorrhage (De Sousa et al. 1995; D'Suze et al. 2003; Guinand et al. 2004).

Experimental envenomation with *T. discrepans* venom suggested the presence of toxins able to induce alveoli fibrin deposition and platelets activation. In vitro studies on plasminogen-rich fibrin plate showed an irregular lysis area surrounding a non-lysed area; this effect was dose dependent (Fig. 5). The non-lysed central area (Fig. 5b, c) suggested the presence of a fibrinolytic inhibitor, which after isolation and characterization was called discreplasminin. The inhibitory activity of discreplasminin is similar to aprotinin (Brazón et al. 2009). The lysed area on the

plasminogen-rich fibrin plates suggested the presence of fibrinolytic compounds with an activity similar to that of tissue plasminogen activator (t-PA). The fibrinolytic activity could be associated with the anticoagulant effect observed in scorpionism victims with *Tityus* species (D'Suze et al. 2003; Guinand et al. 2004).

Tityus Procoagulant Toxins

Some components of *T. discrepans venom* promote a platelet procoagulant response. This venom stimulates a platelet integrin αIIbβ3-dependent aggregation, downstream of Src kinase activation. The pattern of platelet tyrosine phosphorylation increased in a similar manner to that induced by the collagen receptor GPVI affecting FcRγ-chain, Syk, and PLCγ2 (Brazón et al. 2011). Confirmation of GPVI activation by this venom was achieved by a reporter assay on human GPVI expression in chicken DT40 B cells. The venom also activated mouse platelet deficient in GPVI/FcRγ-chain complex through an Src kinase-dependent pathway. Authors conclude that *T. discrepans* venom activates platelets through two different mechanisms: (1) It activates GPVI receptor and (2) another unidentified Src kinase-dependent pathway mechanism (Brazón et al. 2011). Furthermore, in the same venom, procoagulant activity similar to factor Xa was demonstrated (Brazón et al. 2013).

Tityus Anticoagulant Toxins

Anticoagulant compounds of *T. discrepans* venom induce the inhibition of factor Xa amidolytic activity (Brazón et al. 2013) and fibrinogen degradation (Fig. 6). The latter was demonstrated after 2 h fibrinogen incubation with venom when a slight increase of fibrinogen electrophoretic mobility was observed. As shown in Fig. 6a, this effect was potentiated at 24 h incubation, remaining unchanged after that. Also, some venom components degraded fibrinogen Aα-chains, starting at 2 h venom incubation and achieving a complete degradation at 24 h; fibrinogen Bβ- and γ-chains were unaffected (Fig. 6b); and no degradations products were observed. Venom components responsible of fibrinogenolysis could be serine- or metallo-proteases since its fibrinogenolytic activity was abolished in some case by serine-protease and others by metallo-proteases inhibitors.

Venezuelan *Tityus* Toxins with Antineoplastic Activity

Some scorpion venoms inhibit the growth of various types of cancers, but only few toxins have been found to be responsible for these anticancer effects. These venoms exert their actions by three different mechanisms: (1) blocking a specific ion channel, (2) inhibiting cancer cell invasion, and (3) activating intracellular pathways inducing apoptosis. Recent evidence indicates that antineoplastic drugs induce a chain of events where MAP kinases, FasL, matrix metalloproteinases, P38, P53, or BcL-2 are involved in tumor cell death. Neopladine 1 (Neo-1) and neopladine 2 (Neo-2) purified from *Td* venom were found to kill human breast carcinoma SKBR3 cells (D'Suze et al. 2010). Neo-1 and Neo-2 molecular masses

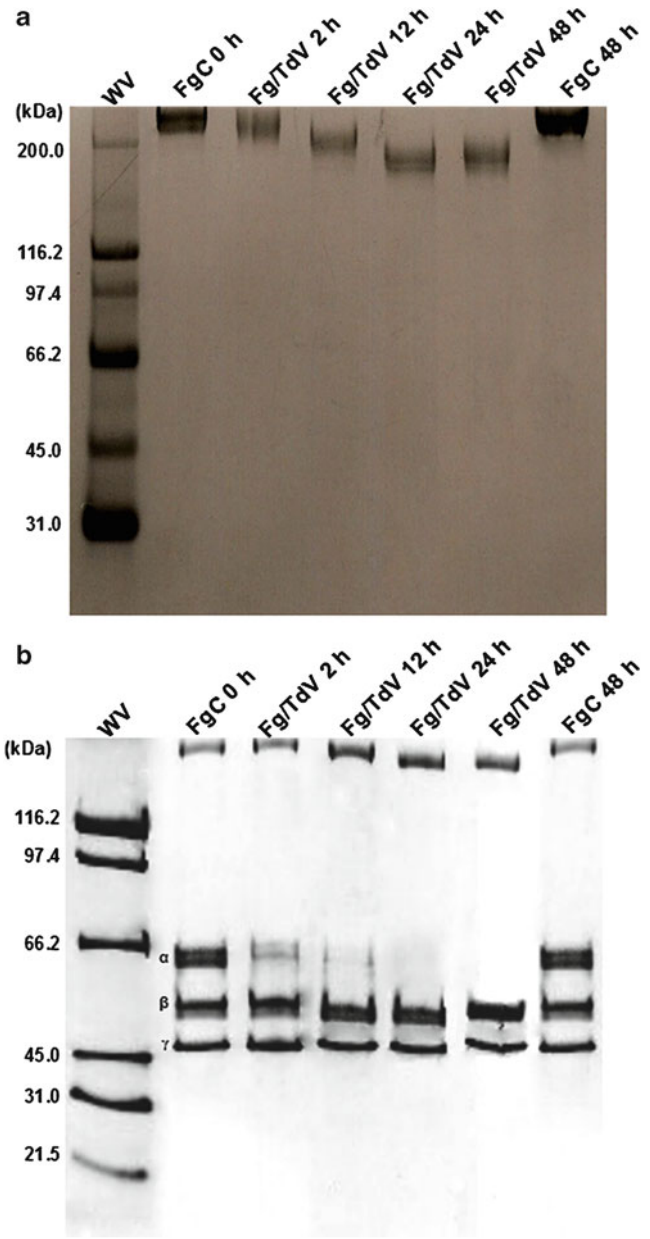


Fig. 6 *Fibrinogen degradation by Tityus discrepans venom*. Fibrinogen degradation (30 μg) by *T. discrepans* venom (1 μg) at 37 °C and different incubation times. SDS-PAGE using Tris-Tricine system. Standard proteins were used as a reference to determine the molecular weight (MW). Gels were stained with R-250 Coomassie Blue. (**a**) Fibrinogen molecule degradation using 5 % gel under nonreducing conditions. (**b**) Fibrinogen chains degradation using 7.5 % gel under reducing conditions. Fibrinogen consists of three polypeptide chains Aα, Bβ, and γ. FgC 0 h: Fibrinogen

are 29,918 and 30,388 Da, respectively; their *N*-terminal sequences were determined by Edman degradation. These peptides induced apoptosis of SKBR3 cells but had a negligible effect on nonmalignant MA104 monkey kidney cells, suggesting a specific activity for neoplastic cells. Neo-1 and Neo-2 induced 6.3 % and 4.1 % of SKBR3 apoptosis, respectively, in 5 h exposure; the effect was larger with more prolonged exposures. Immunohistochemistry experiments demonstrated that neopladines bind to SKBR3 cell surface inducing FasL and BcL-2 expression. Neopladines induced apoptosis in human cancer breast cell lines in a manner resembling the effects of vinblastine, cisplatin, or paclitaxel, which are drugs currently used in chemotherapy.

Antivenoms in Venezuela

Scorpion envenomation is an important health problem in Venezuela since it is a country with a high incidence of scorpion accidents. Yet, there is no reliable statistical information about scorpion envenomation. Nevertheless, isolated studies from diverse country regions showed that in Miranda State (North Central region, Fig. 1) 667 cases were recorded during 2005–2008 period (Fragoza 2012). In Monagas state (North Eastern region, Fig. 1) were recorded 298 cases from 1987 to 1993 (De Sousa et al. 1997). In the Delta (North Eastern region, Fig. 1), from 2002 to 2006, the incidence rate of scorpion accident was 30 cases per 100,000 inhabitants (Vásquez-Suárez et al. 2012). In Merida state (Andean region, Fig. 1), from 1994 to 2003, 5,878 cases with 11 deaths were reported. The annual national incidence is 40 scorpion stings per 100,000 inhabitants, and the mortality rate is 0.04 per 10,000 inhabitants (Chippaux and Goyffon 2008). Epidemiological scorpionism data in Venezuela reflect only partially the real status, so further epidemiological studies are needed.

Venezuela Antivenom Production

Scorpion envenomation is the second most important reason for antivenom production (the first one being snake envenomation) due to the high morbidity and potential mortality in children. Nowadays, the only treatment is immunotherapy based on the administration of antibodies produced by horses, previously immunized with scorpion venom. In Venezuela, the commercial scorpion antivenom is produced by Biotecfar (Center of Biotechnology, Pharmacy Faculty, Universidad Central de Venezuela) by horse hyperimmunization with *T. discrepans* venom. Pooled

Fig. 6 (continued) control without incubation. *Fg/TdV 2 h* fibrinogen incubated with *T. discrepans* venom by 2 h, *Fg/TdV 12 h* fibrinogen incubated with *T. discrepans* venom by 12 h, *Fg/TdV 24 h* fibrinogen incubated with *T. discrepans* venom by 24 h, *Fg/TdV 48 h* fibrinogen incubated with *T. discrepans* venom by 48 h, *FgC 48 h* fibrinogen control incubated by 48 h

hyperimmune plasma is processed to purify horse immunoglobulin G. Then, antibody is treated with pepsin to obtain $F(ab')_2$ and F (ab′) (Poggioli de Scannone 1996). The term fabotherapeutic is used in reference to antivenom made of (Fab′) or $F(ab')_2$. Biotecfar fabotherapeutic is composed of 72.3 % $F(ab')_2$ and 16.6 % F(ab′), and 1 mL neutralizes 0.2 mg of venom. The use of immunoglobulin fragments has increased antivenom tolerance and efficiency. Fabotherapeutics retain the ability to inactivate the venom, even though they are smaller and lack the immunogenic portions. They have a lower mean distribution and elimination times and a larger volume of distribution than IgG, suggesting a better tissue penetration (Sevcik et al. 2004).

Recently, some research groups are introducing variations to the classical methods for the production of antivenoms, providing evidence on the usefulness of poultry (hens and quails) IgY. In favor of the use of IgY are the easiness and low cost of handling poultry and the fact that IgY reactivity with human FC receptors is low, which should result in a lesser chance of severe adverse reactions. An experimental IgY anti-*T. caripitensis* venom has been developed, which has been found able to neutralize the venom toxic activity in mice.

As important caption note, a factor often overlooked, is that an envenomed person might have antibodies against the heterologous IgG (or its fractions) used as antivenom. The problem was highlighted by Sevcik et al. (2008) which detected anti-horse, anti-cattle, and anti-chicken IgG in human γ-globulin preparations; the anti-horse IgG levels were low, but the anti-chicken IgG levels were particularly high. If $F(ab')_2$ is able to react with the human IgGs, to the same extent as that of heterologous IgGs or IgYs, the human IgGs directed against heterologous immunoglobulins could significantly reduce the efficiency of antivenoms.

Effectiveness of the Anti-scorpion Antivenom in Venezuela

Experimental data and clinical observations have confirmed the effectiveness of fabotherapics made in horse with *T. discrepans* whole venom and used against any *Tityus* envenomation, with the disappearance of symptoms in a period between 8 and 24 h after the accidents (reviewed by Mazzei de Davila et al. 2011). Though the antivenom is produced against *T. discrepans*, its efficiency has been demonstrated against other *Tityus* species venoms. Different reports showed that the T. discrepans antivenom is also effective for the treatment of T. neoespartanus (Margarita island; De Sousa et al. 2007); T. falconensis (West-Central region; Guinand et al. 2004); T. nororientalis, T. caripitensis and T. breweri (North Eastern and Southern regions; De Sousa et al. 2005; Borges et al. 2010), and also T. zulianus from the Andean region (Fig. 1) (Mazzei de Davila et al. 2011). Clinical experience has shown that high doses of antivenom are needed only when scorpionism patients receive delayed treatment (reviewed by Mazzei de Davila et al. 2011). The most important condition is the time between the sting and the antivenom application. Studies in vitro have reported cross-reactivity of anti-*T. discrepans* antivenom against *Tityus* species venom from different regions spanning almost the totality of Venezuela (D'Suze et al. 2007).

A waveform particularly interesting to toxinologists is the elution profile obtained while purifying a venom, say, using a high-performance liquid chromatography (HPLC) column. Given a constant set of chromatographic conditions, the elution profile is a fingerprint of the venom composition. Data on the venom of 15 scorpion species listed by increasing value of elution profile complexity demonstrated that that these species are able to express or repress some venom components more efficiently (D'Suze and Sevcik 2010). If this capability is real, it could provide competitive advantages to species with more plastic or variable venoms; more plasticity might result in better adaptation. A plastic venom may resemble venom toxic components of more species making it a better choice to produce antivenoms with wide protective spectrum. Authors propose that this variability can result in broader antigenicity and more protective antivenoms if the venom of the variable species is used as antigen for hyperimmunization.

Conclusion and Future Directions

Tityus venoms are rich in a variety of toxins with different targets in mammals, insects, bacteria, fungi, etc. The venom most studied is that from *T. discrepans;* it is recently that other *Tityus* venoms are beginning to be studied. The richness in scorpion varieties in Venezuela opens a wide field of study where promising toxins that could be used as therapeutic agents (antibiotics, fungicides, antineoplastics, fibrinolytics) are waiting to be described. Even though the envenomation accidents caused by different *Tityus* species produce lung injury with diverse physiopathologies, the genesis of the mechanisms involved is yet to be established.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ New Insights on the Pharmacokinetics of Venoms and Antivenoms
- ▶ Potassium Channel Blocking Peptide Toxins from Scorpion Venom
- ▶ Scorpion Venom Interactions with the Immune System
- ▶ Scorpionism and Dangerous Species of Brazil
- ▶ Scorpionism and Dangerous Species of Colombia

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Scorpionism and Dangerous Species of Brazil

13

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Abstract

Brazil is a country with a large territorial extension (approximately 8.5 million kilometers squared), which is composed of 26 states and a Federal District. The scorpion fauna of Brazil is also quite vast, highlighting the *Tityus* genus. The species that is considered the most dangerous and that is accounted for the highest number of accidents is *Tityus serrulatus*. One of the unique features of this species is its ability to reproduce by parthenogenesis, which enabled its rapid proliferation across the country. According to the records of the Ministry of Health, during the years (2000–2012), accidents related to scorpions have increased dramatically all over the country and are recognized as a public health problem by the government. In this period, 482,479 cases of scorpion envenomation have been reported in Brazil, with the occurrence of 749 deaths for a lethal rate of 0.15 %.

Introduction

Scorpion stings are considered an important public health problem and an actual cause of emergency in many countries of the world. Scorpions are invertebrate animals belonging to the Arthropoda phylum, Chelicerata subphylum, Arachnida class, and Scorpiones order (Carvalho and Lourenço 2001; Whitfield 1885).

So far, about 2,000 species of scorpions have been described in the world (Prendini and Wheeler 2005; Soleglad et al. 2005); however, solely around 34 species belonging to the Buthidae family are regarded as potentially dangerous to human, which involves species mainly from the genera *Tityus, Centruroides, Mesobuthus, Parabuthus, Leiurus, Buthus, Hottentota,* and *Androctonus*. Besides these genera, at least one non-buthid, *Hemiscorpius,* belonging to Hemiscopiidae family, is also considered dangerous to human (Chippaux and Goyffon 2008; Lourenço 2002).

Presently, the scorpion envenomation is recognized as an important problem in many tropical/subtropical areas of the world including north-Saharan Africa, Near East, Middle East, Mexico, Brazil, South Africa, East Africa, South India, and the Amazon Basin (Guyanas, Venezuela, and northern Brazil), due to the fact that there is a high number of scorpion incidence and/or envenomation severity in these areas (Chippaux and Goyffon 2008).

Epidemiology of Scorpionism in Brazil

In Brazil, the federal agencies responsible for collecting the information regarding the accidents with venomous animals are SINAN (Sistema de Informação de Agravos de Notificação), SINITOX (Sistema Nacional de Informações Tóxico Farmacológicas), and RENACIAT (Rede Nacional de Centros de Informação e Assistência Toxicológica). These organizations analyze, systematize, compile, and disseminate information of accidents caused by venomous animals and also spread information about the treatments and prevention of these accidents throughout the country (Bocher and Struchiner 2002).

Table 1 Scorpionism, death and mortality per year in Brazil

Year of occurrence	Scorpionism	Deaths	Mortality (%)
2000	12,552	13	0.104
2001	17,944	39	0.217
2002	22,341	48	0.215
2003	24,146	49	0.203
2004	29,722	40	0.135
2005	35,395	45	0.127
2006	36,965	24	0.065
2007	37,370	61	0.163
2008	40,285	85	0.211
2009	50,234	90	0.179
2010	51,736	77	0.149
2011	59,918	84	0.140
2012	63,871	94	0.147
Total	**482,479**	**749**	**0.155**

Source: SINAN/SVS/MS (2011 dates will be reviewed; 2012 data are partial)

The notifications sent by the municipalities to the Ministry of Health (MS, *Ministério da Saúde*) of Brazil through SINAN, in the period between 2000 and 2012, are presented in Table 1. During this period, a total of 482,479 cases of scorpion envenomation have been reported in all the regions of Brazil, with the occurrence of 749 deaths for a lethal rate of 0.15 %. Moreover, during the last years, significant increases in this type of accident have been reported (Fig. 1). However, the same was not observed for lethality, which may represent a breakthrough in the care and prognosis of scorpion envenomation in Brazil.

In Brazil, the *Tityus* genus is involved in most of the notified scorpion accidents. There are several *Tityus* species with case reports of accidents including *T. aba, T. anneae, T. bahiensis, T. brazilae, T. carvalhoi, T. confluens, T. costatus, T. cylindricus, T. fasciolatus, T. kuryi, T. maranhensis, T. martinpaechi, T. mattogrossensis, T. melici, T. metuendus, T. neglectus, T. obscurus, T. pusillus, T. serrulatus, T. silvestris, T. stigmurus, and T. trivittatus* (Brasil 2009; Lourenço and Leguin 2008; Marcussi et al. 2011); however, *Tityus serrulatus* is considered the most dangerous of them all (Table 2 and Fig. 2).

Other non-*Tityus* genera such as *Bothriurus bonariensis* (south), *Bothriurus araguayae* (southeast), *Ananteris balzanii* (midwest), *Bothriurus asper* (northeast), *Isometrus maculatus* (northeast), *Rhopalurus agamemnon* (northeast), and *Rhopalurus rochae* (northeast) are also present in Brazil. However, because of their low venom toxicity, they are considered of low toxicological interests.

Data provided by the MS demonstrate that in the last decade, the number of scorpion accidents, the incidence, and the deaths dramatically increased (Fig. 1 and Table 3). The improvement of data collection after the implementation of the Mandatory Reporting of Scorpion Accidents in the country in 1988 is probably the main factor explaining the large increase in the number of accidents by scorpion

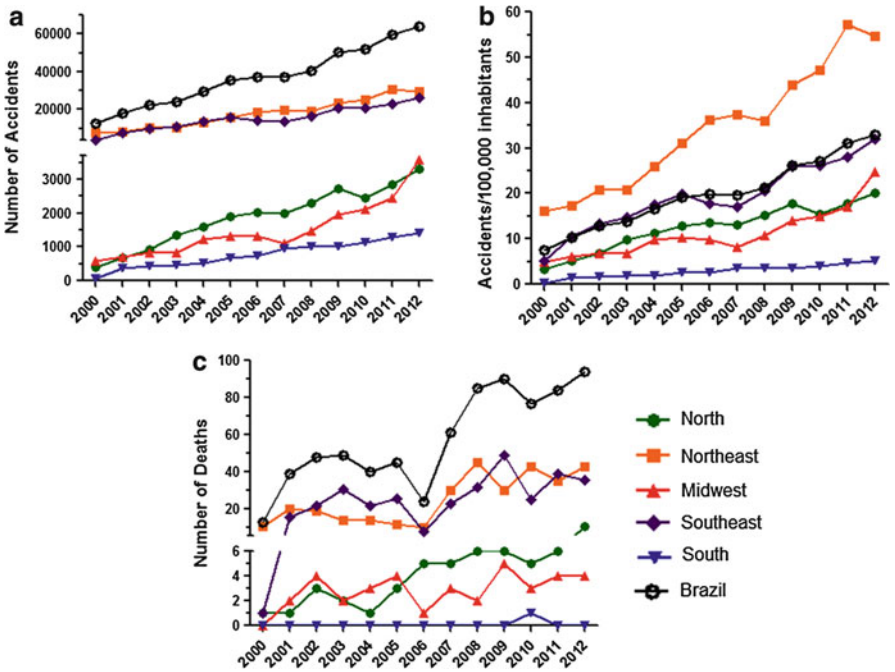

Fig. 1 Epidemiological data from scorpion accidents in Brazil's regions from 2000-2012. (**a**) Number of accidents, (**b**) Incidence and (**c**) Deaths

stings. However, other causes also contributed to this increase, including high demographic density; the expansion of urban areas without planning; the scorpion population growth (especially in the suburbs and newly constructed housing) due to the accumulation of garbage and construction debris, which constitute appropriate housing for scorpions and cockroaches (their main food); and the human behavior that increases their contact with these animals.

In 2012, 63,871 scorpion accidents were registered in Brazil; the incidence was 32.9 accidents/100,000 inhabitants with 94 deaths. Among these accidents, the age group with the greatest number of records was between 20 and 49 years. One- to 9-year-old children were in the group with the highest number of deaths, especially those who, in the course of the systemic framework, received care 6 h or more after the sting (Portal Da Saúde 2012). However, although records of accidents have been better reported after the Mandatory Reporting of Scorpion Accidents implementation, the data is still strongly underestimated.

Possessing a large territorial extension of about 8.5 million kilometers squared, Brazil has 26 states and a Federal District (where Brasília, the capital, is located). The states are divided into five regions: north, northeast, midwest, southeast, and south. The scorpion species and the scorpionism reports for each region are presented below.

Table 2 ***Tityus*** **species of Brazil**. Binomial name, first described by, color characteristics and geographical distribution

Species	Color characteristic	Geographical distribution
Tityus aba (Candido, Lucas, de Souza, Dias & Lira-da-Silva, 2005)	Legs and pedipalps are yellow. Mesosoma and metassoma are brownish with longitudinal dark stripes over the tergites	**BA**
Tityus anneae (Lourenço, 1997)	Legs and pedipalps are yellowish or reddish. Mesosoma and metassoma are yellowish or reddish. Blackish regions are never present	**BA, PE**
Tityus bahiensis (Perty, 1833)	Legs and pedipalps are yellow with dark brown spots. Mesosoma is dark brown and metassoma is reddish brown	**ES, GO, MG, PR, RJ, RS, SC, SP**
Tityus brazilae (Lourenço & Eickstedt, 1984)	Legs and pedipalps are reddish yellow with dark spots. Mesosoma and metassoma are reddish yellow with three longitudinal dark stripes over the tergites	**AL, BA, ES, PB, PE, SE**
Tityus carvalhoi (Mello-Leitão, 1945)	Legs and pedipalps are yellowish with diffuse dark spots. Mesosoma and metassoma are reddish yellow with three longitudinal dark stripes over the tergites	**MT**
Tityus confluens (Borelli, 1899)	Legs and pedipalps are dark yellow. Mesosoma is brown and metassoma is dark yellow	**MG, MT, MS, PR**
Tityus costatus (Karsch, 1879)	Legs and pedipalps are yellowish brown with dark spots. Mesosoma and metassoma are yellowish brown	**BA, ES, MG, MT, MS, PR, RS, RJ, SC, SP**
Tityus cylindricus (Karsch, 1879)	Legs are light yellow and pedipalps are yellow-reddish. Mesosoma and metassoma are reddish yellow	**BA**
Tityus fasciolatus (Pessôa, 1935)	Legs and pedipalps are yellowish brown with dark spots. Mesosoma and metassoma are yellowish brown with three longitudinal dark stripes over the tergites	**FD, GO, MT**
Tityus kuryi (Lourenço, 1997)	Legs and pedipalps are reddish brown with dark spots. Mesosoma is dark brown and metassoma is reddish brown with dark spots	**BA**
Tityus maranhensis (Lourenço, Jesus-Júnior & Limeira-de-Oliveira, 2006)	Legs and pedipalps are yellowish with dark brown varied spots on all segments. Mesosoma and metassoma are yellowish to reddish yellow	**MA**

(*continued*)

Table 2 (continued)

Species	Color characteristic	Geographical distribution
Tityus martinpaechi (Lourenço, 2001)	Legs and pedipalps are light yellowh with dark spots. Mesosoma and metassoma are light yellow with three longitudinal stripes over the tergites	**BA, CE, PB**
Tityus mattogrossensis (Borelli, 1901)	Legs and pedipalps are yellowish brown. Mesosoma and metassoma are yellowish brown. It is totally dark sppoted	**BA, CE, GO, MG, MT, PB, SE, SP**
Tityus melici (Lourenço, 2003)	Legs and pedipalps are light to dark yellow without any diffuse spots. Mesosoma and metassoma are basically yellowish with a longitudinal stripe between ventral keels of metassoma	**BA**
Tityus metuendus (Pocock, 1897)	Legs and pedipalps are black with yellowish spots. Mesosoma and metassoma are dark red or almost black	**AC, AM, PA, RO, RR**
Tityus neglectus (Mello-Leitão, 1932)	Legs and pedipalps are yellowish or reddish brown. Mesosoma and metassoma are yellowish or reddish brown	**AL, BA, PB, PE, RN, SE**
Tityus obscurus (Gervais, 1843)	Legs and pedipalps are dark reddish brown or black. Mesosoma and metassoma are dark reddish brown or black	**AM, AP, MA, MT, PA**
Synonym		
Tityus cambridgei, Tityus paraensis (Pocock, 1897/ Kraepelin, 1896)		
Tityus pusillus (Pocock, 1893)	Legs and pedipalps are light yellow. Mesosoma is brown and metassoma is light yellow	**AL, BA, PB, PE, PI, SE**
Tityus serrulatus (Lutz & Mello, 1922)	Legs and pedipalps are yellowish brown. Mesosoma and metassoma are yellowish brown. It is totally dark sppoted	**BA, CE, ES, FD, GO, MG, MT, PE, PI, PR, RJ, RN, RO, RS, SC, SE, SP**
Tityus silvestris (Pocock, 1897)	Legs and pedipalps are yellowish brown with diffuse dark spots. Mesosoma and metassoma are yellowish brown with darl spots	**AC, AM, AP, GO, MT, PA, RP, TO**
Tityus stigmurus (Thorell, 1876)	Legs and pedipalps are light yellow. Mesosoma and metassoma are yellow with a longitudinal dark stripe over the tergites	**AL, BA, CE, PB, PE, PI, RN, SE**
Tityus trivittatus (Kraepelin, 1898)	Legs and pedipalps are dark yellow. Mesosoma and metassoma are dark yellow with three longitudinal stripes over the tergites	**MG, MS, PR, RJ, RS**

Fig. 2 *Tityus* species distribution in Brazil

Tityus serrulatus: The Main Dangerous Species of Brazil

T. serrulatus is an endemic species with a wide distribution in Brazil. It is responsible for the highest number of accidents and also the most severe cases in the country (Chippaux and Goyffon 2008). It measures between 60 and 70 mm in adulthood and it is more commonly known as yellow scorpion, because of its yellowish or dark brown color (Table 2). Its Latin name "serrulatus" is related to the presence of granules modified as spines (3–5) on the posterior region of the dorsal keels of the metasomal segments III and IV. Moreover, its distribution in Brazil has increased in the last years (Table 2 and Fig. 2) including in the regions where other species previously predominated. The increase and dispersal of the population of *T. serrulatus* have occurred in many urban environments, which can be explained by its reproduction strategy that facilitates its dispersion, besides to be opportunistic and generalist (Lourenço and Eickstedt 2009).

T. serrulatus reproduces by parthenogenesis. Parthenogenesis (from the Greek *parthenos* = virgin, *genesis* = birth) is a form of reproduction in which the egg develops without being fertilized. It is considered a rare phenomenon among Chelicerates, being

observed in some scorpions and spiders species (Lourenco 2008). Curiously, *Tityus serrulatus* was the first scorpion species described as able to proliferate by parthenogenesis (Matthiesen 1962). The major advantage of parthenogenetic species is the ability to single-handedly generate a new colony without a member of the opposite sex, not to mention that this attribute can also circumvent extinction (Cuellar 1994; Lourenco 2008). *T. serrulatus* parthenogenesis and the urban growth, the garbage accumulation, and the proliferation of cockroaches (preferred food of these scorpions) are all factors that help to explain the great increase of cases of scorpion stings in Brazil.

Studies conducted between 1980 and 2000 in the state of Bahia have shown that its envenomation is frequently characterized by local symptoms (pain, dormancy, edema, erythema, paresthesia, hyperemia, itchiness, ardor, prickly heat, and intense heat), general disturbances (headache, sudoresis, cold extremities, hypothermia, lachrymosis), digestive disturbances (vomiting, nausea, abdominal pain, and sialorrhoea), neurological disturbances (tremor, agitation, dizziness, contracture, blurred vision, somnolence, malaise, neurotoxic facies, tearing), cardiovascular disturbances (tachycardia, bradycardia, hypertension, and arrhythmia cardiac), and breathing disturbances (dyspnea, tachypnea, rales/wheezing). However, symptoms variability is found according to the region.

Scorpions and Scorpionism in the North of Brazil

The north region covers about 45 % of the Brazilian territory but has only 7 % of the total population of the country. Seven states compose the northern Brazilian: Acre (AC), Amazonas (AM), Rondônia (RO), Roraima (RR), Amapá (AP), Pará (PA), and Tocantins (TO). Most of the Amazon forest is localized in this region of the country where the scorpions *T. obscurus* (AM, AP, and PA), *T. metuendus* (AC, AM, RO, RR, and PA), and *T. silvestris* (all states except RR) are found. These species have allopatric distribution, with a probable meeting zone in the Pará state. *T. obscurus* is found in eastern Pará, Amazonas, and Amapá, while *T. metuendus* appears further west, on the border between Pará, Amazonas, and Acre, with records in Rondônia and Roraima. The *T. serrulatus* scorpion is found in the Rondônia state; see Table 2 and Fig. 2 (Brasil 2009; Costa 2012; Maestri-Neto et al. 2008; Pardal et al. 2003).

The scorpion species with medical importance in the Brazilian Amazonian region and from northern South America is *Tityus obscurus* Gervais, 1843, a senior synonym of both *Tityus paraensis* Kraepelin, 1896, and *Tityus cambridgei* Pocock, 1897 (Lourenço and Leguin 2008).

T. obscurus is a large scorpion, with a total length of 75–100 mm. Its coloration is uniformly blackish with only some pale zones on the sternites. Metasomal segments I to V and telson are uniformly blackish. A strong spinoid subaculear tooth is present (Brasil 2009).

The LD_{50} (mg/kg) value of the *T. obscurus* venom is 12.14 and this value seems rather high when compared to 1.16 of *T. serrulatus* or 1.06 of *T. bahiensis* (Nishikawa et al. 1994). Despite of that, lethal incidents relating to *T. obscurus* have been reported and the symptoms induced in humans by this venom include local pain, profuse

sweating and cyanosis, hypersensitivity, salivation, and vomiting. In fewer cases were observed numbness of the legs, muscle contractions, convulsions, collapse and semicomatose can be eventually observed (Cozijn 1843). Moreover, different to the symptoms caused by scorpions from the other regions of Brazil, the *T. obscurus* venom effects include central neurotoxicity (Asano et al. 1996).

The first mass fingerprint of the venom of *T. obscurus* describes the isolation of 60 different components and characterization of 26 peptides (Batista et al. 2004). Recently, other studies showed the existence of at least 102 distinct peptide components and, among them, were identified 15 distinct sequences of sodium channel toxins or NaScTxs (Guerrero-Vargas et al. 2012). The NaScTxs are responsible for the most dangerous neurotoxic effects observed in scorpionism in human. In 1997, the death of a 7-year-old child stung by *T. obscurus* was registered in French Guiana (Hommel et al. 2000).

The signs and symptoms observed after scorpion stings in accidents occurring in the northern Brazil are quite different from those observed across the rest of the country. Pardal and colleagues (1999) reported that patients stung by scorpions in Itaituba city in the Pará state described a feeling of widespread shock throughout the body, besides the traditional local manifestations (Pardal 1999). Subsequently, Pardal and colleagues (2003) reported the presence of local (92 %) and systemic (99 %) symptoms in 72 cases of scorpion envenomation that occurred in the Santarém city, also in the Pará state. These symptoms were described as electric shocks through the body (89 %), myoclonus (89 %), dysmetria (86 %), dysarthria (81 %), and ataxia (71 %). Nonetheless, most of the accidents (76 %) were moderate and all patients were cured. The etiologic agent was identified only in 8 % of these cases as *T. obscurus* (Pardal et al. 2003).

According to Maestri-Neto et al. (2008) and Costa (2012), the major scorpions responsible for envenomation in the state of Pará are *T. obscurus* and *T. silvestris* (Costa 2012; Maestri-Neto et al. 2008).

T. metuendus is also responsible for human accidents in north of Brazil. It is a rain forest species distributed mainly in western Amazonia between Brazil and Peru. This species has a total length of 70–90 mm. The coloration is dark red or almost black with discrete yellowish spots on the trunk and legs. The metasomal segments IV and V are thicker as compared to others species (Table 2).

T. metuendus is easily found in Manaus (Amazonas capital) and surrounding regions, inhabiting mainly in leaf sheaths of palm forests. In contrast, *T. obscurus* scorpions have also been found in the urban area of Novo Airão city in the Amazonas state, including inside of the houses, and there are registered accidents in this region (Brasil-MCT 2008).

In the vicinity of Manaus, Brazil, specifically in the *Reserva Florestal Adolpho Ducke*, the populations of *T. metuendus* are strictly sexual with a sex ratio of 1/1 (Lourenço 1997). However the same species collected in the Amazonian region of Peru, near Iquitos (town of Jenaro Herrera), was considered arrhenotokous parthenogenetic in which unfertilized eggs develop into males (Lourenço 2008).

In northern Brazil, 24,533 scorpion stings were reported to the MS of Brazil in the last years (2000–2012). The number of cases increased from 414 in 2000 to 3,298 in

Table 3 Epidemiological data from states and regions of Brazil of 2002 and 2012

	Number of accidents		Incidence Accidents/ 100,000 inhabitants		Number of deaths		Mortality (%)	
States	2002	2012	2002	2012	2002	2012	2002	2012
Rondônia	63	171	4.4	10.8	0	2	0	1.17
Acre	16	165	2.7	21.7	0	0	0	0
Amazonas	41	340	1.4	9.5	0	3	0	0.88
Roraima	19	81	5.5	17.3	0	0	0	0
Pará	622	1,823	9.6	23.4	3	6	0.48	0.33
Amapá	56	157	10.8	22.5	0	0	0	0
Tocantins	118	561	9.8	39.6	0	0	0	0
North	**935**	**3,298**	**6.9**	**20.2**	**3**	**11**	**0.32**	**0.33**
Maranhão	63	358	1.1	5.3	0	0	0	0
Piauí	180	819	6.2	25.9	0	0	0	0
Ceará	363	1,887	4.7	21.9	1	2	0.28	0
Rio Grande do Norte	1,254	2,973	44.0	92.1	0	4	0	0.13
Paraíba	344	2,519	9.8	66.0	0	1	0	0.04
Pernambuco	1,223	5,541	15.1	62.0	0	12	0	0.22
Alagoas	2,349	5,526	81.3	174.6	3	0	0.13	0
Sergipe	28	723	1.5	34.3	0	2	0	0.28
Bahia	4,391	9,144	33.0	64.5	15	22	0.34	0.24
Northeast	**10,195**	**29,490**	**20.9**	**54.7**	**19**	**43**	**0.19**	**0.15**
Mato Grosso do Sul	43	1,064	2.0	42.5	0	0	0	0
Mato Grosso	122	797	4.7	25.6	1	2	0.82	0.25
Goiás	545	1,302	10.5	21.2	3	2	0.55	0.15
Federal District	114	426	5.3	16.1	0	0	0	0
Midwest	**824**	**3,589**	**6.8**	**24.9**	**4**	**4**	**0.49**	**0.15**
Minas Gerais	6,241	14,336	34.0	72.2	19	27	0.30	0.19
Espírito Santo	288	2,149	9.0	60.1	1	6	0.35	0.28
Rio de Janeiro	128	314	0.9	1.9	1	0	0.78	0
São Paulo	3,300	9,270	8.6	22.1	1	3	0.03	0.03
Southeast	**9,957**	**26,069**	**13.4**	**32.0**	**22**	**36**	**0.22**	**0.14**
Paraná	268	1,051	2.7	9.9	0	0	0	0
Santa Catarina	124	212	2.2	3.3	0	0	0	0
Rio Grande do Sul	38	161	0.4	1.5	0	0	0	0
South	**430**	**1,425**	**1.7**	**5.1**	**0**	**0**	**0**	**0**

2012 (partial data), meaning an increase of almost eight times in the number of scorpion accidents, at least in part related to the improvement in the notification of the cases. The regional incidence in 2012 was 20.2 per 100,000 inhabitants (Fig. 1, Table 3).

This region of the country accounted for only 5.08 % of the scorpion stings that occurred mainly between the months of March and June and declined sharply between August and December, corroborating with the observations made by

Oliveira et al. (2009) for the 2000–2007 period in the northern region and by Pardal et al. (2003) in 2000–2001 in Santarém in Pará (Oliveira et al. 2009; Pardal et al. 2003). Since the year 2008, the higher incidence of accidents occurred during the months of August, October, and November (Costa 2012).

The seasonality of scorpionism during different years may reflect changes in the rainy season and floods in the rivers of northern Brazil. The rainy season in this region of the country is between February and June, with higher precipitation in February. In Belém, capital of Pará, a maximum rainfall of 450.8 mm was registered in February 2008 and a minimum value of 422 mm for the same month in 2009 (Campos et al. 2011). Scorpion stings increase in the rainy and warm season.

Of the total deaths reported in Brazil (2000–2012), 55 (7.34 % of the total) occurred in this region. The mortality rate (0.22 %) was above the national rate (0.15 %). Most of the fatal victims were 1–9 years (37.5 %) and 20–59 years (31.25 %) old. It is known that children under 10 years old account for this risk group grievance certainly because of the lower body mass index and the smaller amount of blood, which can facilitate the spread of venom in the body and therefore the action of neurotoxic peptides on ion channels, quickly triggering the onset of signs and symptoms of envenomation (Dhawan et al. 2002; Mendes 2007; Rodriguez de la Vega et al. 2003; Rodriguez de la Vega and Possani 2005). In addition, a greater permeability for scorpion toxins through the blood–brain barrier of young rodents was evidenced (Clot-Faybesse et al. 2000; Juhng et al. 1999; Nunan et al. 2003). These data may help elucidate the severe clinical manifestations frequently observed in young children stung by scorpions.

The high incidence of death in economically active individuals (20–59) can also be justified by the fact that this population is frequently in contact with the scorpions, especially because of the high number of people that work in the extraction of natural resources inside the forests. In fact, Maestri-Neto and colleagues (2008) demonstrated that scorpion accidents primarily affect men in the urban area and working age, although the mortality is more common in children (Maestri-Neto et al. 2008).

The proportion of accidents involving scorpions by area of occurrence shows a predominance of 1:2 (urban area to forests area) in northern Brazil, an exception to the rest of the country, where the inverse relationship is observed (Oliveira et al. 2009).

In addition, the distance and difficult access to health centers in this region of the country can account for severe envenomation and deaths by scorpion stings. Comparing with the midwest region, the north region presents a smaller number of accidents but a higher number of deaths (Fig. 3).

Scorpions and Scorpionism in the Midwest of Brazil

The Brazilian midwest comprises the states of Mato Grosso (MT), Mato Grosso do Sul (MS), Goiás (GO), and the Federal District (FD). In this region, the occurrence of *Tityus* genus scorpions is *T. fasciolatus* (GO, MT, and FD), *T. mattogrossensis* (MT and GO), *T. bahiensis* (GO), *T. carvalhoi* (MT), *T. silvestris* (GO and MT),

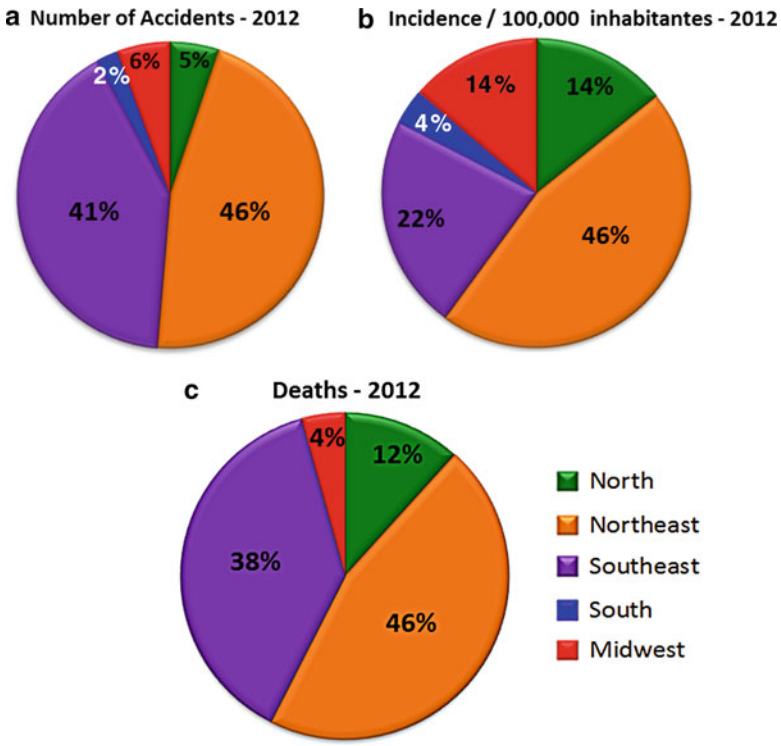

Fig. 3 Epidemiological data from scorpion accidents in Brazil's regions during 2012. (**a**) Number of accidents, (**b**) Incidence and (**c**) Deaths

T. confluens (MT and MS), *T. costatus* (MT and MS), *T. obscurus* (MT), *T. serrulatus* (MT, DF, and GO), and *T. trivittatus* (MS); see Table 2 and Fig. 2. Among these mentioned, the most dangerous species is *T. serrulatus* (Brasil 2009).

An urban epidemic of *T. serrulatus* species was registered in Goiás, and an increasing incidence in Federal District (Andrade and Pasqualetto 2002; Barraviera 1995; Motta 2006).

In the late 1950s, a process of environmental devastation, expanding land area, and urbanization began in the middle of the Brazilian savannah, in the center of the country, where Brasília, capital of the country, was built and inaugurated 10 years later (Lourenço 1986; Motta 2006). It was probably during the inhabitation of Brasília that the dispersion and subsequently increasing of the population of *T. serrulatus* occurred in the Brazilian savannah. This species rapidly occupied the ecological niche of native species *T. fasciolatus*, endemic to the Cerrado (Knox 1997). According to Lourenço, an intense seizure of scorpions was done in the Brasília region in 1970, which detected other species (*Bothriurus araguayae*, *T. fasciolatus*, and *Ananteris balzanii*), but not *T. serrulatus* (Lourenço 1975).

Interestingly, it has been described a regional variation for the toxicity of *T. serrulatus* venom. For the animals occurring in Minas Gerais, in southeastern

Brazil, the LD_{50} of the venom is 26 μg/mouse (Nishikawa et al. 1994; Oliveira et al. 2013), while in Federal District, it is 51.6 μg/mouse (Oliveira et al. 2013) and, therefore, almost twice (1.98) less toxic than the venom of animals from Minas Gerais. In Bahia, northeastern Brazil, the venom LD_{50} was 96.16 μg/mouse (Silva et al. 2005b), indicating that the venom of animals from this state is, at least, about four times less toxic than the venom of animals from southeast Brazil. The single published report on Federal District scorpion accidents described these accidents as mild and indicated there were no death records between the years 1991–2000 (Yoshizawa 2002). However, during the last years (2000–2012), 2–5 deaths were registered per year in the midwest region, alongside an increase of accidents and incidence (Fig. 1). Recently, a one-and-half-year-old child died after being stung by *T. serrulatus* in Brasília (data not published).

It has been extensively described that *T. serrulatus* venom can induce acute pulmonary edema, which is the leading cause of death after scorpion envenomation in humans (De-Matos et al. 2001; De Matos et al. 1997; Matos et al. 1999; Oliveira et al. 2013). All these previous reports were conducted with *T. serrulatus* venom from southeastern Brazilian specimens. On the other hand, the venom obtained from Bahia (Silva et al. 2005a, b) and Federal District (Oliveira et al. 2013) populations was not able to induce acute pulmonary edema on rats.

The second scorpion species that is responsible for a high number of accidents in the midwest of Brazil is *T. fasciolatus*, an endemic species from Cerrado. This species is termitophilous and shelters exclusively in the mounds of *Armitermes euamignathus* (found in this region), while most Buthidae scorpions are considered opportunistic.

T. fasciolatus measures between 45 and 70 mm in adulthood. It is colored in a yellowish brown with three longitudinal stripes on the dorsal trunk and spots on the legs and pedipalps. The LD_{50} value of *T. fasciolatus* venom was 3.65 mg/kg, which is less toxic than the venom from *T. stigmurus* (0.77 mg/kg), *T. bahiensis* (1.06 mg/kg), *T. serrulatus* (1.16 mg/kg), and *T. costatus* (1.59 mg/kg), otherwise more toxic than *T. obscurus* venom (12.14 mg/kg) (Nishikawa et al. 1994).

The central west Brazil accounted for 19,517 (4.0 %) of scorpion stings recorded in the country between 2000 and 2012. The incidence of scorpion envenoming in 2012 in the midwest was 24.9 per 100,000 inhabitants. An increase of about 600 % in the scorpion stings between the years 2000 (573) and 2012 (3,589) was observed, keeping in mind that the data from 2012 are still partial (Fig. 1).

Midwest accidents occur mainly between the months of October and March, with increment in the last 3 months of the year (Oliveira et al. 2009). During this time of year, the highest rainfall occurs in the Brazilian region. The increase in accidents during the raining season can be attributed to the flooding of the natural habitat of these animals, leading them to invade residences, allowing more contact with humans.

During the period between 2000 and 2012, there were 37 deaths in this region, which represent 4.9 % of registered deaths in Brazil. The mortality rate in this region of the country (0.18 %) was high compared to the nationally recorded rate (0.15 %).

The fatal victims were 1–9 years (45.4 %) and 20–39 years (31.8 %) old, being the last similar to north region of the country. Throughout Brazil, children under the

age of 10 years are the most vulnerable group (Oliveira et al. 2009); however, in this region, the group of 20–39 years shows more vulnerability.

In 2011, two deaths were recorded in Mato Grosso do Sul, one of them was caused by *T. serrulatus* and led to the death of a 3-year-old child. After that, the medical community stayed on alert for the accidents caused by *T. serrulatus* in this region.

Rodrigues (2006) reported the presence of *Tityus carvalhoi* in Mato Grosso, though no accidents were report by this animal.

Scorpions and Scorpionism in the Northeast of Brazil

The northeast region is the third largest region of Brazil and the largest in number of states (nine), Maranhão (MA), Piauí (PI), Ceará (CE), Rio Grande do Norte (RN), Paraíba (PB), Pernambuco (PE), Alagoas (AL), Sergipe (SE), and Bahia (BA), which occupy 18 % of the total area of the country. The flora of the northeast region is characterized by savannah-estepica, arid caatinga of the hinterland with its disjunctions vegetation, rain forest (Atlantic and Amazon – Maranhão), open rain forest, semideciduous forest, deciduous forest, and savannah. The vegetation diversity reflects the diversity of the scorpion fauna with 36 species, distributed in 3 families – family Chactidae, 2 species; family Bothriuridae, 4 species; and family Buthidae, 30 species – and 8 genera (*Hadrurochactas*, *Bothriurus*, *Anateris*, *Isometrus*, *Physoctonus*, *Rhopalurus*, *Troglorhopalurus*, and *Tityus*). See Table 2 for *Tityus* sp. and Table 4 for other genus (Brasil 2009; Brazil and Porto 2010; Lourenço 2002; Porto et al. 2010).

Fifteen species of the genus *Tityus* are found in the northeast of Brazil: *T. aba* (BA), *T. anneae* (BA and PE), *T. brazilae* (PB, PE, AL, SE, and BA), *T. costatus* (BA), *T. cylindricus* (BA), *T. kuryi* (BA), *T. maranhensis* (MA), *T. martinpaechi* (CE, PB, and BA), *Tityus mattogrossensis* (CE, PB, SE, and BA), *T. melici* (BA), *T. neglectus* (RN, PB, PE, AL, SE, and BA), *T. obscurus* (MA), *T. pusillus* (PI, PB, PE, AL, SE, and BA), *T. serrulatus* (PI, CE, RN, PE, SE, and BA), and *T. stigmurus* (PI, CE, RN, PB, PE, AL, SE, and BA) (Brazil and Porto 2010; Lourenço 2002; Porto et al. 2010).

The first records of scorpion stings in the northeast were published by Eickstedt (1983/1984) and Nunes and Rodrigues (1987), with the establishment, in 1980, of the Antivenom Information Center of Bahia (CIAVE, from the Português Centro de Informações Antiveneno), indicating the *T. stigmurus* as the most commonly involved species of this region (Eickstedt 1983/1984; Nunes and Rodrigues 1987). The first case of a clinical and therapeutic experience with this species was reported by Figuerôa and Barbosa (1984) (Cupo et al. 2007). Scorpion stings in the northeast were recorded to ten species: *Bothriurus asper*, *Isometrus maculatus, Rhopalurus agamemnon, Rhopalurus rochae, T. brazilae, T. mattogrossensis, T. neglectus, T. pusillus, T. stigmurus*, and *T. serrulatus* (Albuquerque et al. 2009; Biondi-de-Queiroz et al. 1996; Brasil 2009; Brazil and Lira-da-Silva 2010; Brazil et al. 2009; Eickstedt 1983/1984; Figuerôa and Barbosa 1984; Lira-da-Silva et al. 1997, 2001; Nunes and Rodrigues 1987). Among these scorpions, the species with medical

Table 4 Northeast non-*Tityus* scorpion genera. Binomial name, first described by and geographical distribution

Species	Geographical distribution	Species	Geographical distribution
Ananteris balzanii (Thorell, 1891)	**BA**	***Bothriurus asper*** (Pocock, 1893)	**AL, BA, CE, MA, PB, PE, PI, RN, SE**
Ananteris bianchinii (Aguiar-Neto & Limeira-de-Oliveira, 2009)	**MA**	***Bothriurus rochai*** (Mello-Leitão, 1932)	**AL, BA, CE, MA, PB, PE, PI, RN, SE**
Ananteris bonito (Lourenço, 2012)	**PI**	***Bothriurus sooretamensis*** (San Martin, 1966)	**BA**
Ananteris evellynae (Lourenço, 2014)	**BA**	***Isometrus maculatus*** (DeGeer, 1778)	**BA, PE, RN**
Ananteris franckei (Lourenço, 1982)	**BA, PE**	***Physoctonus debilis*** (C.L. Kock, 1840)	**BA, CE, MA, PE, PI**
Ananteris kuryi (Giupponi, Vasconcelos & Lourenço, 2009)	**BA**	***Rhopalurus agamenon*** (C.L. Kock, 1839)	**BA, CE, MA, PE, PI, SE**
Ananteris maranhensis (Lourenço, 1987)	**MA**	***Rhopalurus guanabiensis*** (Lerducci, Pinto-da-Rocha & Lucas, 2005)	**BA**
Ananteris mauryi (Lourenço, 1982)	**BA, PB, PE, RN**	***Rhopalurus lacrau*** (Lourenço & Pinto-da-Rocha, 1997)	**BA**
Bothriurus araguayae (Vellard, 1934)	**BA, PI**	***Rhopalurus rochau*** (Borelli, 1910)	**AL, BA, CE, PB, PE, PI, RN, SE**

importance are *T. serrulatus* and *T. stigmurus*, and although there is occurrence of *T. obscurus* in Maranhão, no accident was recorded by this species.

T. stigmurus has been reported as the main etiologic agent of the scorpionism of northeast Brazil due the high number of accidents, which is caused by its large geographic distribution (occurs in all states with the exception of Maranhão) and high ecological plasticity and proliferation capacity. It is an opportunistic species and considered an urban plague in some neighborhoods of metropolitan regions, with a high demographic density, a disordered population growth, lack of basic sanitation, and great number of unfinished residences (Amorim et al. 2003; Brasil 2009). The envenomation provoked by this species is characterized by local symptoms (pain, dormancy, edema, erythema, paresthesia, hyperemia, ardor, itching, papule, rubor, anesthesia, cramp, prickling), general disorders (headache, sudoresis, cold extremities, hypothermia, cyanosis, and congested eyes), digestive disorders (vomiting, nausea, abdominal pain, and sialorrhoea), neurological disorders (tremor, agitation, dizziness, difficulty in walking, contracture, blurred vision, pallor, and somnolence), cardiovascular disorders (tachycardia, hypertension, and hypotension), and breathing disorders (dyspnea). Most of the cases are mild and end up being cured. The severity of the accidents is directly related to age, since all

serious accidents occurred with children less than 8 years (Brasil 2009; Lira-da-Silva et al. 2000, 2009). Recently, Albuquerque et al. reported three confirmed fatal cases of children stung by *T. stigmurus* in state of Pernambuco from 2006 to 2010 (Albuquerque et al. 2013). These children (2 and 3 years old) experienced vomiting and torpor, which were followed by pallor, sweating, dyspnea, tachycardia, tachypnea, agitation, somnolence, muscle hypertonia, cyanosis, and pulmonary edema. All of them were treated with scorpion antivenom but died within 4 h, 8 h, and 30 h after of the accidents. *T. stigmurus* venom is the most toxic (DL_{50} = 0.773 mg/kg) when compared to *T. serrulatus* and *T. bahiensis* (Nishikawa et al. 1994; Venancio et al. 2013), although the proteomic profile places *T. stigmurus* and *T. serrulatus* as very close species and *T. bahiensis* the less related species in the *Tityus* genus (Nascimento et al. 2006). One hundred distinct components have been described in *T. stigmurus* venom, showing molecular masses from 216.5 to 44,800 Da (Batista et al. 2007). Among these components, we can find the toxins named Tst-1, Tst-2, and Tst-3. They are considered toxic to mice and act on Na^+ channels through different modes of action (Becerril et al. 1996). Almeida et al. (2012) identified six known protein families in the venom gland transcriptome, including potassium channel toxins (subfamilies α and β), sodium channel toxins (subfamilies α and β), hypotensins, antimicrobial peptides, and five atypical types of venom peptides and proteins (such as lectins, anionic peptides, metalloproteases, hypothetical secreted peptides, and cysteine-rich peptides).

T. serrulatus is the species that also causes the most severe accidents in the northeast. Most of the cases are mild but 7.5 % of accidents are moderate to severe (Lira-da-Silva et al. 2009). Silva et al. (2005b) indicated that this species in the metropolitan region of Salvador (Bahia) has low venom toxicity when compared with specimens from other regions of Brazil and does not induce pulmonary edema resulting in death (Silva et al. 2005b).

T. brazilae occurs in undisturbed and disturbed areas of the ombrophilous Atlantic rain forest of the Paraíba, Pernambuco, Alagoas, Sergipe, and Bahia states (Bertani et al. 2008; Porto et al. 2010). This species causes mild accidents, and even without the treatment with scorpion antivenom, no deaths have been recorded. Its envenomation is characterized by local symptoms (pain, dormancy, edema, erythema, paresthesia, itchiness, and papule), general disturbances (headache, sudoresis, dizziness, and nervousness), and neurological disturbances (agitation and difficulty of locomotion) (Lira-da-Silva et al. 2009).

T. pusillus occurs in natural areas of the ombrophilous Atlantic rain forest of the Piauí, Paraíba, Pernambuco, Alagoas, Sergipe, and Bahia, especially in refuges under stones. Albuquerque and coworkers reported two scorpion accidents in which patients presented pain, dormancy, vomiting, dizziness, headache, and chills (Albuquerque et al. 2009).

Rhopalurus agamemnon is a large scorpion found in the Cerrado (savanna) biome, and it is very abundant in many localities in Maranhão, Piauí, Ceará, Pernambuco, Sergipe, and Bahia. The species inhabits open savanna environments and is commonly found inside termite mounds. However, it disappears from places where the native vegetation has been removed. The accidents present symptoms of

envenoming such as local pain of high intensity, paresthesia, intense itching, salivation, agitation, tachycardia, blurred vision, tearing, muscle spasms, and hypotension. Records of accidents by *B. asper*, *I. maculatus*, *R. rochai*, *T. mattogrossensis*, and *T. neglectus* related the occurrence of only local pain, mild cases, and no indication of serum therapy (Lira-da-Silva et al. 2009).

Data provided by Information System (SINAN, Sistema Nacional de Informação de Agravos de Notificação), between 2000 and 2012, shows a total of 231,527 accidents and 326 deaths in the northeast, with an incidence of 54.7 cases per 100,000 inhabitants in 2012. This region of the country is the one with the highest percentage of scorpion sting accidents, deaths related to them, and incidence (46 %) (Fig. 3).

Bahia was the state with the highest number of cases. From 2000 to 2012, Bahia registered 72,079 cases, followed by Pernambuco (47,619) and Alagoas (36,633). The higher incidence in 2012 was recorded for the state of Alagoas (174.6 cases/100,000 inhabitants), followed by Rio Grande do Norte (92.1 cases/100,000 inhabitants) and Paraíba (66.0 cases/100,000 inhabitants). In 12 years, the amount of accidents in the northeast tripled, from 7,713 cases in 2000 to 29,490 cases in 2012. This increase may be due to the absence of a control program by scorpions and accidents and the expansion of Poison Centers by the MS, despite the absence of these services in the states of Maranhão and Alagoas. The northeastern state that had the highest increase in accidents related to scorpions was Ceará, with an increase of 235 times (8 cases in 2000 and 1,887 cases in 2012), followed by Sergipe who recorded an increase of 120 times (6 cases in 2000 and 723 cases in 2012).

Three hundred twenty-six (326) deaths were recorded from 2000 to 2012, in the northeast. Two hundred one (201) occurred in the state of Bahia and 43 in Pernambuco. The highest lethality rates were recorded in Bahia (0.27 %), Piauí (0.17 %), and Maranhão (0.17 %). In 12 years, the number of deaths almost quadrupled, from 11 in 2000 to 43 cases in 2012 (Fig. 1).

These data show that the northern region of Brazil is the most affected with scorpion stings, which can result in death, particularly in children, the most vulnerable to scorpion envenoming.

Scorpions and Scorpionism in the Southeast of Brazil

Formed by four states, the southeast region occupies about 11 % of the Brazilian territory and is home to the largest population of the country, almost 65 million people. The high demographics of the region justify why, after the northeast, the southeast region has the second highest number of scorpion accidents (41 %), with 26,069 cases reported in 2012 (Figs. 1 and 3). The southeastern region comprises the states Minas Gerais (MG), Espírito Santo (ES), Rio de Janeiro (RJ), and São Paulo (SP).

Minas Gerais (MG) is the state that presents the highest number of scorpion accidents in Brazil, as well as the highest number of deaths. The *T. serrulatus*, *T. bahiensis*, *T. trivittatus*, *T. mattogrossensis*, *T. confluens*, and *T. costatus* are the *Tityus* species related with the registered cases (Figure 2 and Table 2). However, no toxic species, such as *Bothriurus araguayae*, are also observed. Although the state

houses a greater number of *Tityus* species, it is *Tityus serrulatus* the most dangerous and studied species in MG. Studies show that *T. serrulatus* was native from the state and occupied a restricted area in Minas Gerais about 300 years ago and then spread to other states in the country (Lourenço et al. 1996). In 2012, MG registered 14,336 scorpion accidents, for 72.2/100,000 inhabitants rate, and accounted for 27 deaths (data from Ministry of Health, MS). By comparing the data from the past 10 years (Table 3), an increase in reported cases accidents, incidence, and death is notable; however, there was a decrease in mortality. This can be explained due to the fact that deaths were always notified in the past – 2002 (severe envenomation cases); however, the majority of mild envenomation cases were omitted. Nowadays, the increased number of developed hospitals and the widespread use of computer and Internet propitiate that the accidents started to be more notified, resulting in a decrease of mortality proportion. Regarding the number of deaths of 2012, Minas Gerais presented the highest number, surpassing even the state of Bahia – responsible for the highest incidence of accidents. The number of fatalities can be justified to the local predominance of the *T. serrulatus*. As in other regions, children from MG are frequent victims of scorpion stings, probably because they do not know that scorpions are dangerous. A clinical and epidemiological study of 325 hospitalized children and adolescents (from 2 months to 15 years, median of 4.9 years), who suffered by scorpion stings at the city of Montes Claros (located at the north of MG), showed 1.5 % of mortality (Horta et al. 2007). In this city, the scorpions *T. serrulatus* and *T. bahiensis* are mainly responsible for the accidents. The data obtained from the research showed that clinical manifestation of this age group can be frequently serious (14.8 % mild cases, 55.4 % moderate cases, and 29.8 % severe cases). A time interval greater than 3 h between the accident and hospital attendance was one variable associated with severity of the cases. As a result, MG is the state with highest number of scorpionism of the country and is the main responsible for the scorpion public health problem in the southeast of Brazil.

The state of Espírito Santo (ES) possesses a small territorial extension (see Fig. 2); nevertheless, it presents a high number of scorpion accidents as well as high scorpion incidence. Interestingly, the accidents reported in a period of 10 years (Table 3) increased approximately seven times with a proportional increase of the incidence. Once again, an improved control of the records could justify the increase. Moreover, in 2012, the incidence of scorpion accidents was not much lower than Minas Gerais, which is the state with the highest number of scorpion accidents. The state registered scorpion accidents caused by *T. serrulatus*, *T. bahiensis*, *T. brazilae*, and *T. costatus*, highlighting to *Tityus serrulatus*. In total, during 2012, the ES state presented 2,149 accidents, incidence of 60.1/100,000 inhabitants, and 6 deaths (data from Ministry of Health, MS).

The Rio de Janeiro (RJ) state presented the lowest number of cases and incidence of scorpion stings in the southeastern region, notwithstanding that it possesses a territorial extension similar to ES. The species found in RJ are *T. serrulatus*, *T. bahiensis*, *T. trivittatus*, and *T. costatus*. In 2012, 314 accidents were registered with incidence of 1.9/100,000 inhabitants and no death (data from Ministry of Health, MS).

Among the states of southeastern region, São Paulo (SP) ranks second in number of scorpion accidents. The species involved are *T. serrulatus*, *T. bahiensis*, *T. mattogrossensis*, and *T. costatus*. Accidents caused by *T. mattogrossensis* and *T. costatus* are rare, since *T. mattogrossensis* is mainly found in the midwest of Brazil (MT and GO), Bahia, and Minas Gerais. *T. costatus* is found all over the south and southeast of Brazil. Regarding the city of São Paulo (state capital), *T. bahiensis* is a native species that causes the most envenomation accidents; however, they are rarely lethal even among children. Although *Tityus serrulatus* is not the species responsible for the majority of the accidents in SP city, its number is continuously increasing and is considered today the most dangerous species of the state and the country. Studies suggest there is a competition between the two species in the cities of the SP state, with *T. serrulatus* dominating for the majority except for some areas such as São Paulo city.

Epidemiological data from 2012 showed 9,270 scorpion accidents in the state of SP, with an incidence of 22.1/100,000 inhabitants and 3 deaths (data from Ministry of Health, MS). The reported deaths and mortality are low comparing to Minas Gerais and this is mostly due to a large number of accidents in SP involving *T. bahiensis*, since the envenomation caused by them is usually less severe than that caused by *T. serrulatus*. Together, Minas Gerais and São Paulo mainly contribute for the scorpionism of the northeast of Brazil.

Scorpions and Scorpionism in the South of Brazil

The southern region is the smallest in Brazil, covering approximately 7 % of the Brazilian territory. It houses the states of Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS), where 22 million Brazilians live.

The south of Brazil presents a low count of scorpions and, consequently, a low number of scorpion accidents (Figs. 1, 2 and Table 2). Nevertheless, during the past 10 years, the number of accidents as well as the incidence increased (Table 3). Deaths caused by scorpion envenomation were rare in the south (Fig. 1 and Table 3). The cold weather of the southern region also contributes to the low number of scorpions.

In Paraná (PR), accidents caused by *T. bahiensis*, *T. serrulatus*, *T. costatus*, *T. confluens*, *T. trivittatus* (Fig. 2 and Table 2), and also the nontoxic *Bothriurus bonariensis* are reported. Among the south states of Brazil, Paraná presented most of the scorpion stings cases. In 2012, Paraná registered 1,051 scorpion accidents, the incidence was 9.9 accidents/100,000 inhabitants, and no death was reported. Regarding the location, PR is the southern state closest to the southeastern region, which can explain why most southern cases are reported in Paraná. The scorpions of Minas Gerais and São Paulo probably migrate to the neighbor state (Fig. 2).

Although few cases were reported, the scorpions *T. serrulatus*, *T. bahiensis*, and *T. costatus* are responsible for the scorpion envenomation in Santa Catarina (SC). In 2012, SC presented 213 accidents, 2.2 accidents/100,000 inhabitants, and no deaths. The cold climate and the distance from the southeast also explain the absence of scorpion stings.

In Rio Grande do Sul (RS), the scorpion fauna is represented by a small number of species. The common accidents are caused by the nontoxic scorpion *Bothriurus bonariensis*. Four *Tityus* species are observed: *Tityus bahiensis*, *T. costatus*, *T. trivittatus*, and the yellow scorpion *T. serrulatus*. The latter species is very rare, but one accident was reported in Porto Alegre (Torres et al. 2002) and two species were found in Uruguaiana city (Bortoluzzi et al. 2007). *T. serrulatus* specimens in Rio Grande do Sul may have been brought from other states of the country with the road network, which contributed to the expansion of the original distribution of the species. In 2012, 161 cases of scorpion envenomation took place, with 1.5 accidents/100,000 inhabitants, and no deaths were recorded. Although there are other Brazilian states with a lower number of scorpion-related accidents, like Rorâima and Amapá, Rio Grande do Sul is the third with the lowest number of scorpion accidents, mainly due to the cold temperature which is not best suited for scorpion species.

Syndromic Classification

The scorpion venom is considered toxic to humans. It presents a rapid distribution in the extracellular compartment, which explains the early onset of clinical symptoms.

Knowing that *Tityus serrulatus* is the most dangerous scorpion in Brazil, the clinical manifestations as well as the treatment are based on this species. However, the same protocol is used for all the other species.

In most cases, the scorpion sting triggers local pain with variable intensity (**mild envenomation or stage Ia**) and may be accompanied by some signs and symptoms such as nausea, sweating, tachycardia, fever, and stirring (**mild envenomation or stage Ib**). The **moderate envenomation or stage II** is characterized with symptoms such as sweating, cramps, vomiting, hypotension, diarrhea, bradycardia, epigastric pain, pulmonary obstruction, and dyspnea (Chippaux and Goyffon 2008; Cupo et al. 1994; Venancio et al. 2013).

The **severe envenomation or stage III**, which often occurs in children, may present several types of manifestations and is potentially lethal (Chippaux and Goyffon 2008; Cupo et al. 1994; Venancio et al. 2013).

Renal failure can occur depending on the concentration of circulating venom and renal clearance (Gutierrez-Mendoza et al. 2011; Malhotra et al. 1978). Studies have demonstrated that mice injected with the *T. serrulatus* venom have renal congestion and hemorrhage (Correa et al. 1997), decreased glomerular filtration, and consequently a decrease in urinary volume (de Sousa Alves et al. 2005).

Cardiac disorders have also been described in cases of severe scorpion envenomation (Bawaskar and Bawaskar 1991; Sundararaman et al. 1999). The α-NaScTxs are probably able to block the fast inactivation of the specific cardiac channel (Nav1.5) and to induce strong depolarization of the cardiac myocytes. Studies in rats suggest that the direct effect of the venom of *Tityus serrulatus* in cardiomyocytes may be responsible for inducing cardiac arrhythmias and

contraction effects (Teixeira et al. 2001). In humans, children seriously envenomed by *T. serrulatus* venom showed different degrees of left ventricular contractile dysfunction, probably caused by ischemia-induced cardiac adrenergic hyperstimulation (Cupo et al. 2007).

In relation to pulmonary edema, which is common and often lethal in scorpion envenoming, it is the result of several factors such as heart failure and inflammatory cascade activation induced by the release of lipid mediators such as platelet-activating factor (PAF), leukotrienes, and prostaglandin induced by the venom (Amaral et al. 1993; De-Matos et al. 2001).

General Treatment

In Brazil, the doctor Vital Brazil Mineiro da Campanha was the pioneer of the studies in the production of antivenoms. His first work in the area involved the antivenom production against the snakes of *Bothrops sp*. and *Crotalus sp*. (rattlesnake). He was the first to propose that antivenoms were monovalent since the serum produced specifically for *Bothrops sp*. venom did not neutralize *Crotalus sp*. venom and vice versa. He was also the one responsible to introduce the immunological term antigen specificity (Craps 1993; Hawgood 1992).

Since 1989, the antivenom is considered the specific treatment for envenoming caused by venomous animals. Today, there are two commercial sera in Brazil capable of neutralizing the venom of scorpions: the scorpion antivenom and the arachnidan antivenom. Scorpion antivenom consists of a solution of heterologous immunoglobulins obtained from horses hyperimmunized with venom of scorpions of the *Tityus serrulatus* species and is currently considered the treatment advised for Brazilian scorpion stings. Arachnidan antivenoms are also heterologous and should only be administered in the absence of scorpion antivenom or in cases of doubt about the animal causing the accident (scorpion or spider).

Currently, the symptomatic treatment for scorpion accidents is based on pain relief by infiltration with 2 % of lidocaine without vasoconstrictor (1–2 mL for children and 3–4 mL for adults) at the sting site or by using dipyrone or other analgesics, orally or parenterally. It is practically the only treatment performed in most of the mild cases (90 %).

In cases of severe scorpion envenomation or stage III, the scorpion antivenoms should be administered as early as possible by intravenous route and in doses that depend on the severity of the accident. The scorpion antivenoms (SAE, from Portuguese *soro antiescorpiônico*) or arachnidan antivenoms (SAAr, from Portuguese *soro antiaracnídeo*) are essential in children under 7 years and in adults with previous health problems (such as hypertension and cardiovascular problems) even if they present mild or moderate clinical manifestations. Anyway, independent from the envenomation stage, even if it is mild, it is mandatory for all the patients to be maintained under monitoring in the hospital for 4–6 h after the accident occurs (Marcussi et al. 2011).

In Brazil, the Butantan Institute (São Paulo city), the Ezequiel Dias Foundation (Belo Horizonte city), and the Vital Brazil Institute (Rio de Janeiro) are the main suppliers of scorpion antivenoms.

Conclusion and Future Direction

This chapter presents a literature review of published books and journals and materials provided by the Ministry of Health. We concluded that the main species of medical importance in Brazil are *Tityus bahiensis*, *Tityus serrulatus*, *and Tityus stigmurus*, with *Tityus serrulatus* being responsible for the greatest number of accidents and also the most severe. This species has a wide distribution in the country due to its parthenogenetic reproduction. During the last years (2000–2012), there was an increase in the number of scorpion sting accidents, which may be explained mainly by the implementation of the Mandatory Reporting of Scorpion Accidents, the high demographic density, and the loss of scorpion habitat by human action, leading the species to acquire synanthropic behavior. Therefore, today, scorpionism is considered a public health problem in Brazil and scorpions are regarded as a pest in many cities across the country.

Cross-References

► Scorpion Venom Research Around the World: *Tityus serrulatus*

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Part V

Scorpion Venoms

Scorpion Venom Research Around the World: Turkish Scorpions

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Contents

Abstract

Pharmacological diversity of animal venoms has made them valuable sources of highly specific molecular tools in drug discovery research. Scorpion venoms contain a number of biologically active compounds, where peptides and proteins play a primary role as novel pharmacologically active molecules. In Turkey, there are 27 different species of scorpions described belonging to the Buthidae, Iuridae, Scorpionidae, and Euscorpiidae families. Despite the long history of venom research in the world, the venom of only few Turkish scorpion species

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has been investigated. Several health-threatening scorpions are found in Turkey, all of them belonging to the Buthidae family: *Androctonus crassicauda, Buthacus macrocentrus, Leiurus abdullahbayrami, Mesobuthus eupeus*, and *Mesobuthus gibbosus* species. Envenomations are characterized by local pain, hyperemia, swelling, burning, hypotension, hypertension, dry mouth, thirst, and sweating. Envenomated patients require medical attention, some of which might be fatal. This chapter gives an overview of peptide research done on the venom of Turkish scorpions and contains some revisions of earlier reports according to newly described scorpion species which was previously incorrectly identified. Up to date, only three medically important scorpion venoms from Buthidae family have been deeply investigated by high-performance liquid chromatography separations, mass spectrometry analysis, and amino acid sequences by direct Edman degradation in conjunction with gene codes obtained from cDNA libraries and electrophysiological records. Eight peptides have been identified from *A. crassicauda* and named as Acra1 to Acra8, only one peptide from *B. macrocentrus* named as Bu1, and four peptides from *M. gibbosus* named as MegKTx1 to MegKTx4. Additionally, electrophoretic profiles of *L. abdullahbayrami* and *Mesobuthus eupeus* (Buthidae) and *Protoiurus kraepelini* and *Iurus kinzelbachi* (Iuridae) venoms are reported. Also in vivo effects and in vitro cytotoxic and gelatinolytic activities of the *A. crassicauda and M. gibbosus* crude venom are reported.

Introduction

Scorpion venoms are complex mixtures of bioactive components which contain enzymes, peptides, nucleotides, lipids, mucopolysaccharides, biogenic amines, and other unknown substances with various pharmacological and physiological properties (Possani and Rodríguez de la Vega 2006). Envenomation due to scorpion stings is a problem of growing medical importance in many countries including Turkey. In 2005, 24, 261 scorpion sting cases were reported: 30.4 % accidents were in Southeastern Anatolia, 24.9 % in Mediterranean Sea, 23.5 % in the Aegean, 11.4 % in Central Anatolia, 5.5 % in East Anatolia, 3.5 % in Black Sea, and 0.8 % in Marmara Regions. Especially Southeastern Anatolia region of Turkey represents high number of stings, which were recorded in the provinces of Şanlıurfa (22.6 %), Adıyaman (19.9 %), Mardin (18 %), Gaziantep (13.9 %), and Batman (13 %). In the Southeastern Anatolia region, especially *Androctonus crassicauda, Leiurus abdullahbayrami, Buthacus macrocentrus, and Mesobuthus eupeus* have been considered dangerous to humans (Ozkan et al. 2007a).

Scorpion venom peptides and proteins have been widely recognized as an important group of neurotoxic components. These peptides are responsible for the symptoms developed during envenomation by interacting with ion channels (Possani and Rodríguez de la Vega 2006). Different types of toxins have been described according to numerous criteria such as their molecular size (long-chain and short-chain toxins), their specificity (to mammals, insects, and crustaceans), mechanisms of action (neurotoxins and cytotoxins), and toxins with or without

disulfide bridges (Zhijian et al. 2006). Toxins stabilized by disulfide bridges can be further divided into four different groups which specifically interact with ion channels: Na^+ channels (Rodríguez de la Vega and Possani 2005), K^+ channels (Rodríguez de la Vega and Possani 2004), Cl^- channels (DeBin et al. 1993), and Ca^+ channels (du Plessis et al. 2008). This chapter provides information about the scorpion venom research in Turkey. Despite the fact that epidemiological and clinical characterizations of Turkish scorpion venom are documented by several authors, their peptide content has not been studied in detail by means of biochemical and molecular biological approaches (Caliskan et al. 2012a). Since a small number of peptides have been isolated and biochemically characterized, this chapter provides more information of the biochemical properties of Turkish scorpion venoms.

Scorpion Species

Scorpion fauna of Turkey is represented by 27 species belonging to four families: Buthidae, Iuridae, Scorpionidae, and Euscorpiidae. Buthidae family has been considered to have medical importance because they cause human envenomation. In Turkey this family comprises 12 species belonging to 7 genera: *A. crassicauda*, *B. macrocentrus*, *Compsobuthus matthiesseni*, *C. schmiedeknechti, Hottentotta saulcyi*, *L. abdullahbayrami*, *M. eupeus*, *M. phillipsii*, *M. nigrocinctus, M. gibbosus, M. caucasicus*, *and Orthochirus zagrosensis* (Yagmur et al. 2009, 2012). Despite that non-buthid scorpions have telson (last postabdominal segment containing the stinger and a pair of venomous glands), they are not considered as dangerous scorpions for public health due to their weak toxicity on mammals. Among them the family Iuridae consists of nine species belonging to four genera: *Calchas birulai*, *C. kosswigi, C. anlasi, C. nordmanni, Neocalchas gruberi, Protoiurus asiaticus, P. kadleci, P. kraepelini*, *and Iurus kinzelbachi* (Soleglad et al. 2012). The family Euscorpiidae is represented by five species in one genus: *Euscorpius avcii*, *E. rahsenae*, *E. lycius*, *E. mingrelicus*, and *E. italicus*. Additionally, six more subspecies of *E. mingrelicus* have been described: *E. m. mingrelicus, E. m. ciliciensis, E. m. phrygius, E. m. ollivieri, E. m. legrandi*, and *E. m. uludagensis* (Yagmur and Tropea 2013). *Scorpio maurus* is the only species of the Scorpionidae family.

Sources of Scorpion Venom

The Public Health Institution (PHI) of Turkey (earlier named as Refik Saydam Hygiene Center) of the Health Ministry of Turkey and the Venom Research Laboratory (VRL) of Eskişehir Osmangazi University are the two main centers of the government that maintain scorpion housing facilities. Additionally, a private company Vetal Sera Production Ind. Trade. Co. Ltd. (VSP) is also housing scorpions (http://www.vetalserum.com.tr/en/raw-materials-supply/scorpions/). These centers collect scorpions using ultraviolet light at night or during the day under

stones from rural areas. In Turkey, the main scorpion venom research is conducted by the two governmental centers mentioned above; also some studies have been made at the universities.

Crude venom at the Public Health Institution has been obtained since 1942 by maceration of telson of animals (Ozkan 2005). Recently, they start using electrical stimulation of scorpions (known as milking the scorpions). After the collection, the pooled venom is dissolved in distilled water and centrifuged at 14,000 *g* for 15 min to remove the insoluble substances; the supernatant is freeze-dried for storage (Caliskan et al. 2006).

Isolation and Primary Structure Determination of the Peptide Toxins

Scorpion venoms contain a variety of bioactive peptides, and toxicity of the venom is attributed to the presence of these peptides. New studies using effective strategies are developing on methods of generating antivenom. Using the knowledge obtained through molecular and biochemical characterization studies gave the chance to design specific antibodies that will be useful for clinical studies on scorpionism.

To date, only three Turkish scorpion species have been investigated concerning their protein and peptide composition: *A. crassicauda*, *B. macrocentrus*, *and M. gibbosus*. Their venom components obtained by electrical stimulation have been characterized by classical biochemical isolation until homogeneity by sequential steps of reverse-phase (RP) or ion-exchange high-performance liquid chromatography (HPLC) followed by determination of their primary structure using direct Edman degradation in a protein sequencer. Pure peptides that were isolated have been enzymatically digested and further separated by HPLC. Each peptide fraction has been subjected to sequence analysis in order to complete the full amino acid sequences. Complementary data has been confirmed with mass spectrometry measurements to complete the peptide primary structure. Additionally, individual cDNA libraries constructed from venomous glands total RNA were used in conjunction with sequence data to identify and clone the genes coding the toxins (Caliskan et al. 2012b, 2013a; Diego-Garcia et al. 2013).

Venom Research Background of the Turkish Scorpions

Although preliminary studies about physiological and toxic effects of the Turkish scorpion venoms were published around the 1960s (Tulga 1960), the first peptide was isolated and biochemically characterized 40 years later (Caliskan et al. 2006). Up to date, only a small number of peptides from the medically important scorpion species have been isolated and biochemically characterized (Adiguzel 2010; Caliskan et al. 2012b). Additionally, some preliminary studies have been performed with the venom of *P. kraepelini* and *I. kinzelbachi*. In the following section, studies conducted with three species of Turkish scorpion will be discussed.

Fig. 1 Photographs of the whole bodies of *Androctonus crassicauda* (**a**), *Buthacus macrocentrus* (**b**), *Leiurus abdullahbayrami* (**c**), *Mesobuthus eupeus* (**d**), and *Mesobuthus gibbosus* (**e**). The inside scale represents 1 cm

Androctonus crassicauda

A. crassicauda from the Buthidae family is one of the most dangerous species with medical importance in the Middle East. Its geographic distribution comprises Armenia, Azerbaijan, Bahrain, Egypt (Sinai), Iran, Iraq, Israel, Jordan, Kuwait, Libya, Oman, Saudi Arabia, Syria, Turkey, United Arab Emirates, and Yemen (Kaltsas et al. 2008). *A. crassicauda* is commonly found in the Southeast of Turkey: Adıyaman, Elâzığ, Diyarbakır, Gaziantep, Malatya, Mardin, and Şanlıurfa provinces. Color varies from brown to black depending on the geographic area and can reach a body length of 8–10 cm (Fig. 1a). These scorpions are generally found inside the farmhouses due to their well-known extreme anthropotolerance features (Vignoli et al. 2003).

Table 1 Multiple sequence alignment of *A. crassicauda* toxins

Toxin	Sequence	Experimental Molecular Mass (Da)	Accession numbers
Acra1	ADVPGNYPLDSSGNKYPCTVLGDNQSCIDVCKKHGVKYGYCYSFK-----CWCEFLEDKNVSI----------	6497	P0C292
Acra2	KKDGYIVDSN GCAPECFPTN---GC…	7849	-
Acra3	DRDGYPVHDGTNCKYSCDIREKWEYCTPLCKRRNAKTGYCYAFA-----CWCIGLPDEVKVYGDDGIFCKS[a]	7260	ADK12684
Acra4	VRDGYIVDDKN-CVYHCIPP-----CDGLCKKNGGKSGSCSFLVPSGLACWCKALPDNVPIKDPSYK-CHKR[a]	6937	JQ975126
Acra5	VRDGYIMIKDTNCKFSCNIFKKWEYCSPLCQSKGAETGYCYNFG-----CWCLDLPDDVPVYGDRGVICRTR	undetermined	JQ975127
Acra6	VRDGYIRRKDEFKFK-CYVDGKD--CDDVCKSEGGSAGYCTALGFL---CYCAGLPDDKAWKPTSS------	undetermined	JQ975128
Acra7	VRDGYIVKPTN-CVIHCIPFSPG--CDKDCKEKGAASGYCQAFGKHGNGCWCIDLPDKVPIKDPNQD-CTR-	undetermined	JQ975129
Acra8	VRDGYIVDDKN-CTFFCGRNAY---CNDECKKKGGESGYCQWASPYGNACWCYKLPDRVPIKEKGR--CNGR	undetermined	JQ975130

The cysteines of each sequence are shown shaded. The sequence of Acra2 has not been completed. The symbol (a) indicates a C-terminal amidation. Acra5–8 were obtained as a putative sequences from cDNA.

Although *A. crassicauda* causes the majority of human fatalities and venom components among the other scorpion species in the country are well known, the knowledge of *A. crassicauda* venom composition and function still remains relatively limited.

The first biochemical and molecular biological characterization of the venom components of *A. crassicauda* was reported in 2006 (Caliskan et al. 2006). The soluble venom of the scorpion was toxic to CD1 strain mice, and the LD_{50} (lethal dose that kills 50 %) determined intraperitoneally was 17 μg/20 g mouse body weight. The insect lethality showed that the venom was lethal to crickets (*Acheta domesticus*). HPLC of the soluble venom yielded at least 44 different fractions. The components were then subjected to fingerprint mass analysis using a mass spectrometer. This analysis identifies 80 distinct molecular mass components, varying from 267 to 44,541 Da.

Caliskan et al. (2006) shown that components eluted from RP-HPLC during 10–20 min are low-molecular-weight substances, from 20 to 40 min eluted components with molecular masses lower than 10 kDa. Components eluted at retention time (RT) from 20 to 28 min correspond mostly to the K^+ channel-specific toxins with molecular masses around 3,200–4,500 Da. These peptides are known to modify potassium permeability through cell membranes (Batista et al. 2002). Components eluted with RT from 28 to 35 min correspond to the Na^+ channel-specific toxins, supporting the clinical findings of intoxication with molecular masses around 6,000–7,500 Da. They are mainly responsible for neurotoxic effects via modifying the gating mechanism of sodium channel. They are modulators of the function, either prolonging the kinetics of closing time (known as alpha-scorpion toxin) or opening the channels at less depolarized potentials (known as beta-scorpion toxin) (Rodríguez de la Vega and Possani 2005).

This assumption can explain the high prevalence of human envenomations caused by this scorpion. To date only eight peptides have been identified from *A. crassicauda* as given in Table 1. The first toxic peptide from *A. crassicauda*

isolated and named Acra1 (abbreviated from *A. crassicauda* toxin1) was fully sequenced. It contains 58 amino acid residues cross-linked by six cysteines forming three disulfide bridges, with a molecular mass of 6,497 Da. A second purified peptide named Acra2 was lethal to mice and partially sequenced with a molecular mass of 7,849 Da. Additionally, a new type of peptide was identified with odd number of cysteines (seven), allowing the formation of heterodimers with molecular masses in a range of 16,000 Da (Caliskan et al. 2006).

The third neurotoxic peptide identified from this venom was named Acra3. In vivo lethality test was determined using mice (BALB/c), crickets (*Acheta domesticus*), and crayfishes (*Procambarus clarkii*), showing that Acra3 is toxic to mammals. The peptide was purified by several chromatographic steps of separation, and its chemical structure was determined by Edman degradation and mass spectrometry analysis and confirmed by cloning the gene that codes this peptide. Acra3 has an amidated serine in C-terminal region, which is a posttranslational modification. The peptide has a 7,260 Da molecular weight, with 66 amino acid residues packed by four disulfide bridges which very likely correspond to long-chain peptides that recognize Na^+ channels. The fact that Acra3 is lethal to mammals, the effect of Acra3 on electrophysiological studies, using six different subtypes of Na^+ channels (Nav1.1–Nav1.6), was reported without any clear effect (Caliskan et al. 2012a). The sequence comparison of Acra3 showed that the peptide is >65 % identical to AaHSTR1 (natural anatoxin), AaH6 (a glycosylated "weak" anti-insect toxin) of *A. australis*, and LmaTx32.5 (Na^+ channel inhibitor) of *Lychas mucronatus*. The molecular target of this toxin is still missing and assays of the toxin over other subtypes of Na^+ channels are pending. It can be suggested that Acra3 may be highly specific to only one subtype of channel such as a potent mammalian Cn2 toxin isolated from the scorpion *Centruroides noxius* specific to Nav1.6. On the other hand, despite the sequence similarities of Acra3 with AaHSTR1, AaH6, and Lmatx32.5, the main target of Acra3 is not specific to Na^+ channel and the main target of the peptide is a different receptor.

Recently, a new alpha-scorpion toxin was reported from *A. crassicauda* and named Acra4. The final purification amount of Acra4 indicated that this component is around 2 % of the venom total protein. The LD_{50} of Acra4 was 50.5 ng/20 g mouse body weights by intracranial injection. Based on the amino acid sequence of the peptide, obtained by direct sequencing and mass spectrometry of the purified peptide, a specific primer was designed and used with a universal primer for obtaining cDNA library. By screening of the cDNA library, four additional genes were obtained and putative toxins, which have similar conserved residues with Acra4, were cloned and named Acra5 to Acra8 (see Table 1).

Acra4 contains 64 amino acid residues closely packed by four disulfide bridges with a molecular mass of 6,937 Da. Comparison of the theoretical and experimental molecular mass of the amino acids showed that Acra4 is expected to undergo posttranslational modifications at the C-terminal region same as Acra3 peptide according to the well-known mechanism found in several toxins of the family Buthidae (Becerril et al. 1996).

```
      1         10        20        30        40        50        60      %I   Accession
      :         :         :         :         :         :         :           Numbers
Acra4 VRDGYIVDDKNCVYHCIPPCDGLCKKNGGKSGSCSFLVPSGLACWCKALPDNVPIKDPSYKCHKR  100   AGE83103.1
AaH3  VRDGYIVDSKNCVYHCVPPCDGLCKKNGAKSGSCGFLIPSGLACWCVALPDNVPIKDPSYKCHSR   89   P01480.3
AaH4  GRDGYIVDSKNCVYHCYPPCDGLCKKNGAKSGSCGFLVPSGLACWCNDLPENVPIKDPSDDCHKR   84   P45658.2
Ac4   VRDGYIVDFKNCVYRCVPPCDGLCKKNGGKGGSCSFLIGSGLACWCNALPDNVPIKDPLHKCPKR   84   ADE42765.1
AaH1´ KRDGYIVYPNNCVYHCIPPCDGLCKKNGGSSGSCSFLVPSGLACWCKDLPDNVPIKDTSRKCTR    83   AAA29947.1
Ac1   VRDGYIVYPNNCVYHCIPACDGLCKKNGGTSGSCSFLIGSGIACWCKDLPDNVPIKDPSQKCTR    81   AAB30626.1
       ******   **** * * *********   *** **  ** ****  ** ******    *
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Fig. 2 Multiple sequence alignment of the sequence of toxin Acra4 with those related toxin sequences obtained by protein BLAST. Sequence references are accession numbers. The percentage of identity (I %) and the different amino acids (*bold*) with respect to the toxin Acra4 are indicated. Acra4 = alpha-toxin of *A. crassicauda,* AaH3, AaH4, and AaH1 = alpha-toxins of *A. australis hector*, Ac4 and Ac1= alpha-toxins of *A. crassicauda* (Weinberger et al. 2010). The eight cysteines of each toxin are shown shaded. Conserved amino acids indicated by asterisk

Electrophysiological studies of Acra4 were performed by patch-clamp assays. Acra4 was shown to modify sodium ion currents of Na^+ channels expressed in F11 cell in the culture, similar to other alpha-toxins (α-NaScTxs). Acra4 sequence was found to be highly similar to other described peptides from the *Androctonus* genus as given in Fig. 2 (Caliskan et al. 2013a).

Sequence of Acra5 was similar to LmNaTx35.2 (63 % identity) of *L. mucronatus* and with Acra3 (58 % identity) which are known to be toxic to mammals. Interestingly, Acra5 showed 57 % identity with anti-insect beta-toxin Aah6 of *A. australis hector*. The sequence of Acra6 containing six cysteines (three disulfide bridges) is dissimilar with other Acra peptides. On the other hand, Acra6 showed similarities with insect toxins: LqhIT2-13 (57 % identity) and LqhIT2-53 (50 % identity) from *Leiurus quinquestriatus hebraeus* and AaHIT5 (53 % identity) from *A. australis hector*. The sequence of Acra7 is also similar to other alpha-toxin sequences specific for Na^+ channels with four disulfide bridges, which belong to the long scorpion toxin superfamily: 62 % identity with Aam3 of *Androctonus amoreuxi*, neurotoxin 1; 61 % identity with alpha-neurotoxin 10 of *A. mauritanicus mauritanicus*; and 58.2 % identity with G-TI toxin of *A. australis garzonii*. The sequence of Acra8 showed high similarities and >80 % identical to potent alpha-mammalian toxins: Lqq5 toxin (92.4 % identity) of *L. quinquestriatus quinquestriatus*, neurotoxin Amm5 (87.9 % identity) of *A. mauritanicus mauritanicus*, neurotoxin AahP1005 (87.9 % identity) of *A. Australis hector*, and toxin-3 (81.8 % identity) of *M. eupeus*. Until now, these are the only toxic peptides described from the Turkish scorpion *A. crassicauda*. Three of them have been fully sequenced (Acra1, Acra3, Acra4), one (Acra2) is partially sequenced, and other four are putative (Acra 5–8). These records confirm the medical findings for widely distributed species of scorpion in Sinai Peninsula, the entire Middle East, and the Arabian Peninsula countries, which has toxic peptides that can cause human hazard, some of which might be fatal.

Several studies also reported that scorpion venoms contain some enzymes such as acetylcholinesterase, alkaline phosphatase, hyaluronidase, phospholipase A2, and proteases (Almeida et al. 2002). Despite the fact that mass fingerprint analyses of the *A. crassicauda* soluble venom indicated that components with molecular masses close to reported scorpion phospholipases elute between 34.91 and 39.53 min in HPLC separation, no phospholipase activities were recorded from these fractions. The specific activity of hyaluronidase determined for the soluble venom was 4,000 units/mg. Proteins over 40,000 Da mw were considered to have hyaluronidase activity; unfortunately, the activity of fractions was not observed due to possible instability of the enzyme by the acidic RP-HPLC buffers. Several authors reported that proteolytic enzymes are present in low levels or completely absent from the venom of the genus *Androctonus*. Surprisingly, proteases with gelatinolytic activity were determined in the soluble venom and its HPLC fractions by zymogram assay. Among the components, at least 10 different bands with gelatinolytic activity were detected and a peptide toxin with a serine metalloproteinase activity was reported (Caliskan et al. 2009a).

Moreover, three major peptides with molecular mass of 16,356 Da, 7,422 Da, and 16,328 Da from the soluble venom of the *A. crassicauda* were further investigated for their cytotoxic effects using tetrazolium salt cleavage (MTT) on BC3H1 mouse brain tumor cells. According to these results, it has been suggested that these peptides may have potential antitumor properties (Caliskan et al. 2009b). In order to investigate the biological effects, Acra3 was tested for its cytotoxicity on BC3H1 cells using MTT and lactate dehydrogenase (LDH) activity assays. The results showed that Acra3 exerted strong cytotoxic effect on BC3H1 cells with an IC_{50} value of 5 μg/ml. Exposure of the cells to 0.1 and 0.5 μg/ml was resulted in very strong appearance of the apoptotic morphology in a dose-dependent manner. Caspases 3 and 9 activities were slightly decreased with Acra3, whereas DNA nucleosomal fragmentation was not observed after treatment of the cells. Results from flow cytometry and LDH activity assays indicate that Acra3 exerts its effects by inducing a stronger necrosis than apoptosis in BC3H1 cells (Caliskan et al. 2013b). These findings will be useful for understanding the mechanism of cell death caused by venom in vitro .

In addition to the specific studies conducted with pure peptides of this scorpion, general pharmacological effects of the whole venom were also reported in the literature. Pharmacological effects of *A. crassicauda* crude venom on laboratory animals and antagonistic effects of streptomycin to most of these effects were reported by Altınkurt and Altan (1980). These authors investigated the effects of crude venom on isolated organs and reported that the constrictor effect of the venom is potentiated with serine. On the other hand, the effect was inhibited by Arfonad (trimethaphan camsylate), atropine, morphine, and streptomycin. The constrictor effect on the rectus abdominis muscle of frog is antagonized by streptomycin and D-tubocurarine. Importantly, the hypertensive effect was inhibited strongly by priscol, partially by reserpine and streptomycin. They also determined that streptomycin prevents significantly the bronchoconstrictor effect of the venom. With this study the preventive effect of streptomycin against the side effects of the

venom was confirmed, but no significant effect was determined related to toxicity. Another pharmacological study was performed by Ay et al. (1996) on the effects of *A. crassicauda* crude venom on rabbit thoracic aorta and the contribution of endothelium to venom-induced alterations. They reported three different effects of *A. crassicauda* crude venom on rabbit thoracic aorta: a contractile effect associated with adrenergic stimulation; potentiation of acetylcholine-induced contractions, related to the release of a contracting prostanoid; and increase of acetylcholine-induced relaxations. Additionally, histopathological studies of heart tissue of rabbits showed that *A. crassicauda* crude venom may cause myocardial injury. It has been suggested that cardiac troponin I may be used as a follow-up criterion in the scorpion envenomation for early establishing cardiac involvement (Bakir et al. 2012).

Buthacus macrocentrus

The genus *Buthacus* (Birula, 1908) is widely distributed in Africa (Algeria, Chad, Egypt, Eritrea, Libya, Mauritania, Morocco, Nigeria, Senegal, Sudan, Tunisia) and Asia (Afghanistan, Bahrain, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Oman, Qatar, Pakistan, Saudi Arabia, Syria, United Arab Emirates). *Buthacus macrocentrus* (Ehrenberg, 1828) was recorded from Akçakale, Birecik, and Harran towns of Şanlıurfa Province of Turkey and is suggested to be more abundant along the Syrian border of Turkey. They are known by their yellow-greenish-colored body with length of 4–6 cm as shown in Fig. 1b. Despite the fact that *A. crassicauda* preferred to be inside houses, *B. macrocentrus* can be often found in open lands in the same habitat (Caliskan et al. 2012b; Yagmur et al. 2008).

The first report concerning on *B. macrocentrus* venom components was a general characterization of its venom and the description of a potent mammalian Na^+ channels alpha-toxin (Caliskan et al. 2012b). Studies on toxicity of the venom indicated that the venom was lethal to mice, confirming the earlier clinical records of "yellow scorpion" from Şanlıurfa Province. At least 70 distinct fractions were obtained from the soluble venom with several steps of chromatographic separations. Mass fingerprint analysis of the fractions showed that the venom consists of molecular mass components varying from 648 to 44,316 Da. *B. macrocentrus* venom is rich in short- and long-chain toxins, corresponding to, as described earlier, K^+- and Na^+-specific peptides, respectively. A lethal fraction obtained from HPLC separations was purified and characterized. The full amino acid sequence and the nucleotide sequence of the corresponding cloned gene were obtained for a lethal peptide named Bu1 (abbreviated from *Buthacus macrocentrus* toxin1). The work was performed using the same biochemical and molecular methods described for Acra peptides above. Bu1 contains 65 amino acid residues, closely packed by four disulfide bridges with a molecular weight of 7,263 Da. The gene encoding mature Bu1 translates into a 67-amino acid residue peptide, where the two last residues are eliminated posttranslationally for production of an amidated C-terminal arginine. As shown in Fig. 3, the sequence was found to be highly similar to the toxins from

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     1        10         20         30         40         50         60     %I   Accession
     :         :         :          :          :          :          :           numbers
Bu1  GVRDAYIADDKNCVYTCAKNSYCNTECTKNGAESGYCQWLGKYGNGCWCIKLPDKVPIRIPGRCR 100   P0DJH8.1
Lqq4 GVRDAYIADDKNCVYTCGSNSYCNTECTKNGAESGYCQWLGKYGNACWCIKLPDKVPIRIPGKCR 94    P01489.1
Lqh4 GVRDAYIADDKNCVYTCGANSYCNTECTKNGAESGYCQWFGKYGNACWCIKLPDKVPIRIPGKCR 92    P83644.1
     ****************  ******************** ***** ***************** **
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Fig. 3 Multiple sequence alignment of Bu1 toxin and related toxin sequences from the genus *Leiurus* obtained by protein BLAST and sequence references are accession numbers. The percentage of identity (I %) and the different amino acids (*bold*) are indicated. Bu1 = alpha-toxin of *Buthacus macrocentrus*, Lqq4 = alpha-toxin of *Leiurus quinquestriatus quinquestriatus*, Lqh4 = alpha-toxin of *Leiurus quinquestriatus hebraeus*. The eight cysteines of each toxin are shown shaded. Conserved amino acids indicated by asterisk

the species *L. quinquestriatus*. However, very little biochemical data is available for the venom of *B. macrocentrus* and almost nothing is known about their enzymatic activity. Scorpion sting cases in Şanlıurfa Province and its towns have been reported for *Mesobuthus eupeus* due to its yellow color and distribution of the species. Unfortunately, wide distribution of yellow-colored *B. macrocentrus* in the same region is not considered in previous studies (Ozkan and Kat 2005). But description of Bu1 peptide indicates that this species is dangerous to humans, having an epidemiological interest of the Turkey.

Leiurus abdullahbayrami

L. abdullahbayrami is a newly described species from Southeastern Turkey which was previously misidentified and reported as *Leiurus quinquestriatus*. *L. abdullahbayrami* was incorrectly identified as *L. quinquestriatus*/*Buthus quinquestriatus* in earlier studies conducted from Adıyaman, Hatay, Kilis, Kahramanmaraş, Mardin, and Gaziantep provinces of Turkey. Recently, it has been reported that all known Turkish populations of *Leiurus* were examined and found to belong to a new species (Yagmur et al. 2009). The specimens are 5–7 cm total length with yellowish coloration. Metasomal segment V is typically with dark coloration as black or blackish brown (Fig. 1c). The species can be often found in steep and rocky areas.

Like the other species of the Turkish scorpion, limited data is available for *L. abdullahbayrami* venom . Preliminary studies are focused on the lethality and antigenic properties of the venom (Tulga 1960). Recently, the lethal potency and in vivo effects of the venom were reported (Ozkan et al. 2011a). The LD_{50} of the soluble venom determined by subcutaneous (s.c.) injection was 3.9 μg/20 g mouse body weight. These results showed that *L. abdullahbayrami* venom is 4–5 times more toxic than *A. crassicauda* soluble venom, indicating that it is the most dangerous scorpion in the country. Interestingly, electrophoretic studies showed

that *L. abdullahbayrami* soluble venom has only two major protein bands which were strongly observed between 3 and 6 kDa molecular weight. Molecular range of the bands was in the expected weights of known low-molecular-weight scorpion neurotoxins. On the other hand, high-molecular-weight proteins in scorpion venoms are mainly enzymes which were not observed in the *L. abdullahbayrami* soluble venom. More strength analysis and better molecular, biochemical, and proteomic analyses need to be done with this highly potent venom. In vivo experiments in mouse showed that *L. abdullahbayrami* soluble venom caused the following symptoms of envenomation: hyperexcitability, agitation, aggressive behaviors, squeaking and fighting, tachypnea, convulsions, weakness, paralyses, and finally death due to cardiac and respiratory failure. *L. abdullahbayrami* is a widespread yellow-color species of the Southeastern Turkey, where it co-occurs with *B. macrocentrus and M. eupeus*. Medical records in hospitals are based on scorpion colors, not particular based on taxonomical characteristic of the species. Therefore, although effect of this scorpion for the human is yet not clear, it will be discussed within epidemiological and clinical aspect below.

Mesobuthus eupeus

M. eupeus is a widespread species in the Georgia, Azerbaijan, Turkmenistan, Uzbekistan, Tajikistan, Kazakhstan, Mongolia, China, Iraq, Iran, Afghanistan, and Pakistan. According to recent findings, *M. eupeus* is commonly found in Eastern, Southeastern, and Central Anatolia: Adıyaman, Ağrı, Aksaray, Artvin, Bingöl–Elâzığ Province boundary, Elâzığ, Erzurum, Kars, Kayseri, Konya, Malatya, Manisa, Mardin, Nevşehir, Niğde, Şanlıurfa, Van, Hatay, and Iskenderun and throughout Turkish–Syrian border (Karataş and Karataş 2003). This scorpion is also described as having a yellow color with 4–5 cm of total length (Fig. 1d). Due to the wide distribution of the species to several countries, well-studied biochemical and molecular reports are available in the world literatures. Up to date, individual variations and molecular masses of protein bands of *M. eupeus* venoms were investigated by means of electrophoresis according to geographic origins, gender, and species of the scorpions in Turkey. Samples from Niğde Province gave six main protein bands between 29 and 100 kDa, whereas only three protein bands were observed as 29, 47, and 74 kDa in samples from Osmaniye Province (Ozkan et al. 2011b). Despite of the limited knowledge on venom components of the *M. eupeus,* several studies concerning the epidemiological and clinical effects of the species were performed. According to the medical records from Şanlıurfa Province, *M. eupeus* is responsible of the local symptoms (pain, hyperemia, swelling, burning, itching, and numbness) and the systemic symptoms (dry mouth, thirst, sweating, hypotension, nausea, hypertension, difficulty in breathing, tachycardia, increase of bronchial secretion, and cyanosis) (Ozkan and Kat 2005). Unfortunately, taxonomic identification of the specimens was not performed. Authors assumed that these scorpions belong to the *M. eupeus* for its yellow color and distribution in the abovementioned area.

Table 2 Multiple sequence alignment of *M. gibbosus* toxins

Toxin	Sequence	Experimental Molecular Mass (Da)	Accession numbers
MegKTx1	GSPLTYPCHSAQ-CEQP-----CKDANMRFGX-CMN…	undetermined	B3EWY0
MegKTx2	VGCEECPMH--CKGKKALPTCDYGCE-----CND	undetermined	B3EWX9
MegKTx3	FGLIDVKCSASRECWVA-----CKKVTGSGQGKCQNNQCRCY	4066.6	B3EWY1
MegKTx4	GKEIPVKCKHSGQCLQP-----CKDAGMRFGK-CMNGKCNCTPK	undetermined	-

The cysteines are in shown shaded. The amino acid sequence of MegKTx1 is incomplete and that of MegKTx4 was obtained as a putative sequence from cDNA.

Mesobuthus gibbosus

M. gibbosus (Burullé, 1932) is widely distributed in the Balkan Peninsula (Albania, Montenegro, Macedonia, and Greece) and Anatolia (the greater part of Turkey, except for the Black Sea coast in the north). For this reason *M. gibbosus* was described as a scorpion species endemic to the Balkan–Anatolian region. Total lengths of the specimens are 6–8 cm and color is generally brownish yellow (Fig. 1e). *M. gibbosus* is a ground-dwelling scorpion, locating relatively as dense populations mostly on red soils (Crucitti 1999).

Venom components of this species can be considered as second well-studied soluble venom in the country. Recently, molecular, biochemical, and electrophysiological characterization of the soluble venom of the *M. gibbosus* was reported (Diego-Garcia et al. 2013). Chromatographic separations of the soluble venom show at least 40 distinct fractions. Molecular mass determination and screening of the activity on potassium ion channels were reported for 8 selected fractions which were eluted at 44–60 min RT of the chromatogram. From the fractions, molecular masses obtained between 2,948 and 4,454 Da correspond to the common mass values of short-chain scorpion toxins, which are known to block a variety of K^+ channels (KTx), as expected from the family Buthidae (Rodríguez de la Vega and Possani 2004). The electrophysiological properties of the eight selected fractions were examined in terms of heterologous expression of ion channels (K_v 1.1–1.6) in *Xenopus laevis* oocytes by using the two-electrode voltage clamp technique. Complementary molecular characterization of *M. gibbosus* was achieved. In order to obtain the complete deduced sequence of the active peptides, a cDNA library from the telson was constructed and specific screening of precursors was performed. Three new α-KTx peptide sequences were obtained and named as MegKTx1, MegKTx2, and MegKTx3 (abbreviated from *Mesobuthus gibbosus* K^+ channel toxin number 1–3) which belong to different α-KTx subfamilies: α-KTx3.x, α-KTx9.x, and α-KTx16.x, respectively (Table 2). Screening of cDNA library using a subfamily primer allowed us to obtain a new sequence named MegTx4 which has similar conserved amino acid residues with MegTx1.

Additionally, *in vitro* cytotoxic and gelatinolytic activities of the *M. gibbosus* crude venom were reported, which suggests that its active components might have a potential in cancer treatments. Cytotoxic effects of the crude venom were examined in 5RP7 (H-*ras*-activated mouse fibroblasts), CO25 (N- *ras*- activated mouse myoblasts), and A549 (human adenocarcinomas cells) cell lines by MTT assay (Incesu et al. 2005). The number of viable 5RP7 cell was significantly decreased after incubation with the crude venom for 48 h and IC_{50} was found to be 200 μg/ml, whereas the venom does not affect the viability of CO25 cells. It can be concluded that transformed cells with active H-*ras* were more sensitive to the venom than cells with active N-*ras* oncogene. Furthermore, morphologic examinations showed that *M. gibbosus* venom-treated 5RP7 cells were smaller and rounded in shape, and nuclei stained with Giemsa were denser than their normal counterparts. Cytoplasmic blebs around the cells and vacuole formation were reported. On the other hand, the number of viable A549 cells was also decreased about 40 % at the lowest dose (10 μg/ml) after 24 h incubation, and the rate of this inhibition was elevated up to 60 % in a dose-dependent manner. The crude venom and the fraction eluted at 37.8 min RT of the RP-HPLC separation of the crude venom showed a significant gelatinolytic activity by zymogram assay. Cytotoxic effect of the crude venom might be due to several enzymatic (proteolytic/apoptotic) activities of its components. Beside, monoamine oxidase inhibitory activities (which may be responsible for the anxiety) of the venom and a fraction which is eluted around 14 min RT were also reported. A purified peptide was lethal to mice and *Musca domestica* larvae. Molecular mass of the peptide was 28,000 Da in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (Ucar et al. 2005). Results showed that the venom contains several peptides with molecular masses between 6,500 and 210,000 Da. Interestingly, another connected study reported that the same fraction is also mainly responsible for a specific acetylcholinesterase inhibitory activity of the *M. gibbosus* venom on human erythrocytes (Ucar and Tas 2003).

Individual peptide variation profiles of *M. gibbosus* soluble venom from eight specimens have been reported by means of SDS-PAGE. A common peptide was observed only between 66 and 84 kDa. On the other hand, the two protein bands around 30 and 98 kDa were common in six venom samples but not in the other venoms. Also two different protein bands of 28 and 45 kDa were observed in seven venom samples. Interestingly, three protein bands around 38, 60, and 78 kDa were observed in the venom of one sample. These results showed that nearly all over Anatolia (west of the Anatolian Diagonal, except for shores of the Black Sea and Sea of Marmara) contain the species *M. gibbosus,* which shows intraspecific variation (Ozkan and Ciftci 2010). *M. gibbosus* stings have been reported as been made by a yellow-color scorpion, but its symptoms cannot be separate according to regional records. *M. gibbosus* is a well-known widespread species of the Aegean and Central Anatolia regions. Clinical reports from Marmaris town of Muğla Province of Aegean region indicate that its venom causes erythema, swelling, local burning, and pain. Despite the fact that antivenom therapy was administrated,

at least two deaths (9- and 13-month-old babies) were recorded due to cardiac and respiratory effects of the yellow scorpion venom (Altinkaynak et al. 2002).

Iurus kinzelbachi and *Protoiurus kraepelini*

Recently, a more in-depth revision of genera *Iurus* and *Protoiurus* from the family Iuridae of Turkey reported four new species: *I. kinzelbachi* (from Aydin and Izmir provinces), *P. asiaticus* (from Adana, Adıyaman, Kahramanmaraş, Mersin, and Niğde provinces), *P. kadleci* (Antalya Province and its Alanya town), and *P. kraepelini* (Antalya, Isparta, Konya, Karaman, Mersin, and Muğla provinces) (Soleglad et al. 2012). For these reasons, two previous studies reporting electrophoretic profiles of *I. dufoureius asiaticus* soluble venom have been revised again based on the new systematic literatures. Keskin and Koc (2006) collected specimens of *I. kinzelbachi* from Aydın Province and has been misidentified as *I. d. asiaticus*. Additionally, Ozkan et al. (2007b) collected *P. kraepelini* from Muğla Province and misidentified it also as *I. d. asiaticus*. However, when the gels are compared concerning on molecular mass of the components, the differences can be easily realized. *I. kinzelbachi* gave at least 28 different protein bands between 6.5 and 205 kDa (Fig. 4a), whereas *P. kraepelini* gave only eight major bands between 29 and 116 kDa (35, 42, 48, 53, 68, 78, 92, and 110 kDa) by electrophoresis studies of the authors (Fig. 4b). These results also showed that *I. kinzelbachi* was enriched with low-molecular-weight peptide lower than 14.2 kDa as expected from scorpion venom neurotoxins. As given in Fig. 4b, lower than 36 kDa peptides were not observed on the *P. kraepelini* venom gel. These differences may explain different compositions of the two species. On the other hand, it is very difficult to observe small peptides by 7.5 % SDS-PAGE, the method chosen for *P. kraepelini* venom; Tris–tricine SDS-PAGE method is known to be sensitive for small protein as used by Keskin and Koc (2006).

Soluble venom of P . *kraepelini* was reported to be toxic to mice via intracerebroventricular injection, and LD_{50} was determined as 47.7 μg/20 g mouse body weight, but s.c. injection of the venom was not lethal to mice. Injection of the venom (range of the venom is 9.8–78.4 μg/20 g) causes several symptoms: squeaking, mouth rubbing, weakness, mastication, jumping, hyperactivity, trembling, humpback, salivation, and paralysis. The family Iuridae venom components are considered to be non-dangerous to human; lack of knowledge still awaits further molecular, biochemical, and pharmacological analysis.

Epidemiological and Clinical Studies of Turkish Scorpion

In Turkey, medical records of the clinical observations are not specific to the species of scorpions. Scorpions species are estimated by the authors concerning on their colors and geographic distributions.

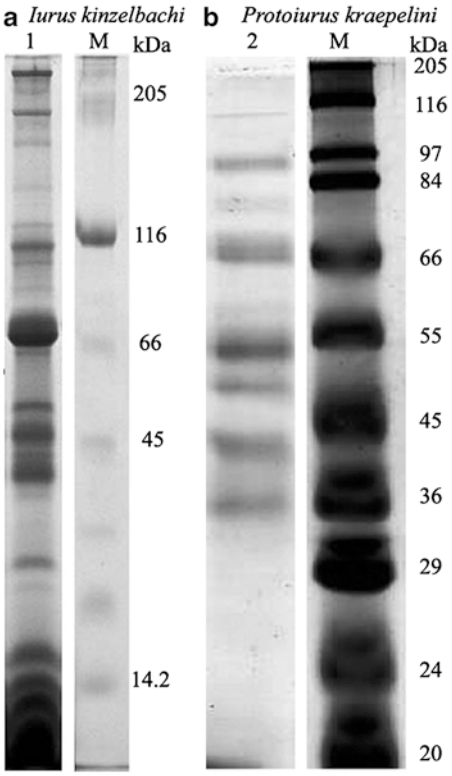

Fig. 4 Electrophoretic profiles of the crude venom of *Iurus kinzelbachi* and *Protoiurus kraepelini*. (**a**) Venom of *I. kinzelbachi* resolved using Tris–tricine SDS-PAGE (1) (Copyright 2006, with permission from Turkish Society for Parasitology); (**b**) *P. kraepelini* resolved using 7.5 % SDS-PAGE. M represents the molecular weight markers in kDa (Copyright 2007, with permission from Elsevier)

Geographic distributions of the most dangerous scorpions of Turkey are given in Fig. 5. Medically important scorpions, *L. abdullahbayrami, B. macrocentrus, M. eupeus*, and *M. gibbosus,* are known to be widespread "yellow-color" species of the Southeastern region of Turkey, usually named simply "yellow color." "Black scorpion" defines the species *A. crassicauda* for the Southeastern region, whereas black color also defines *P. kraepelini and I. kinzelbachi*, both not considered to be dangerous to humans, in Aegean and Mediterranean region of Turkey. Therefore, here, the findings are summarized mainly concerning on their regional records and colors.

A. crassicauda is a black scorpion responsible for the highest number of envenomation specifically in Şanlıurfa Province of Southeastern Anatolia region of Turkey. In 2003 (May to September), 598 cases of human accidents were reported, including 299 due to stings by black scorpion *A. crassicauda*, 152 by yellow scorpions, and 138 were unknown. Black scorpion *A. crassicauda* envenomation showed that local effects were in the ratio of 97.3 % pain, 86.0 % hyperemia, 66.6 % swelling, 8.0 % burning, 0.7 % numbness, and 0.7 % itching. Systemic effects were 11.4 % dry mouth, 11.4 % thirst, 8.4 % sweating, 7.7 % nausea, 2.3 % dyspnea, 2.0 % vomiting, 1.3 % lacrimation, 1.3 % restlessness, 0.3 % syanose,

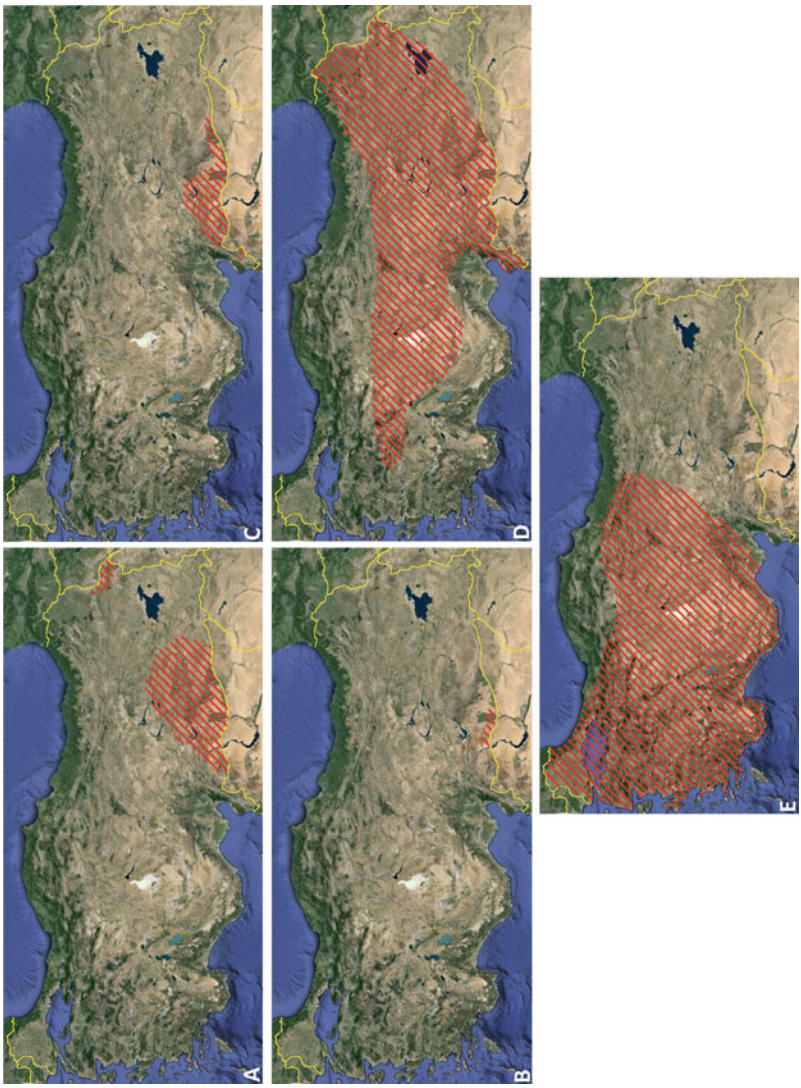

Fig. 5 Geographic distribution of the most dangerous scorpions of Turkey. *Androctonus crassicauda* (**a**), *Buthacus macrocentrus* (**b**), *Leiurus abdullahbayrami* (**c**), *Mesobuthus eupeus* (**d**), and *Mesobuthus gibbosus* (**e**)

0.3 % local spasm, 0.3 % increase of secretion, 4.1 % hypertension, 8.9 % hypotension, and 2.5 % tachycardia (Ozkan et al. 2006). In Diyarbakır Province of the region, in 2005 and 2006, 52 cases were reported: 24 stings (46.2 %) were caused by black scorpion (*A. crassicauda*), 1 sting (1.9 %) was caused by yellow scorpion identified as *L. quinquestriatus* (most likely *L. abdullahbayrami* or another yellow scorpions as described above), and 27 stings were unknown. Predominant stings produce the following symptoms: cold extremities (38.4 %), autonomic storm (38.4 %), tachycardia (36.5 %), dyspnea (23.0 %), and paleness (15.3 %). Hypertension (7.6 %) and hypotension (3.8 %) were uncommon. Moreover, there were also symptoms of cholinergic stimulation, including excessive sweating (32.6 %) and vomiting (3.8 %). All patients recovered after antivenom treatment except one child who died due to pulmonary edema (Bosnak et al. 2009). In Midyat town of the province of Mardin, in 2007 and 2008, from a total of 317 cases that were registered, 99 cases occurred with children. According to the color of scorpions, 61 were black (*A. crassicauda*), 17 were yellow (estimated *M. eupeus* by authors), and 21 (21.2 %) were unknown. The local effects were in the ratio of 97.9 % local pain, 54.5 % hyperemia, 26.3 % swelling, 19.2 % itching, 13.1 % burning, and 8.1 % numbness. Additionally, malaise, dry mouth, sweating, thirst, headache, nausea, pallor, tachycardia, vomiting, dizziness, restlessness, hypotension, tachypnea, local spasm, hypothermia, and convulsion were observed in the ratio of fewer than 9 %. One death was reported with cardiac and respiratory arrest after 6 h of envenomation (Uluğ et al. 2012). In the region of Batman Province, 120 envenomations were reported in 2007 (March to October). From this 86 accidents were attributed to black color scorpion and 34 by yellow ones. Like other records of the provinces, pain (97.5 %) was major symptom. Moreover, mark/redness (65 %), numbness (51.7 %), and edema (25.8 %) were also commonly observed (Al et al. 2009). In Adana Province of the Mediterranean region, a boy patient, 9 years old, was reported to be stung by yellow scorpion. He was admitted to the hospital with foaming in the mouth just after 10 min of scorpion sting and then presented heart failure and priapism (Karakus et al. 2012). In Hatay Province of Mediterranean region, a 4-year-old boy died due to pulmonary edema and blood acidosis following scorpion sting (Karakus et al. 2013). Several scorpion sting case accidents from Malatya, Bitlis, and Erzurum provinces of the Eastern Anatolia region were reported to have caused toxic myocarditis.

In Marmaris town of Muğla Province of the Aegean region, 24 children were hospitalized. Seven of them (~29 %) were stung by black (likely *I. kinzelbachi*), 3 (~12 %) by yellow (likely *M. gibbosus*), and 14 (~58 %) were unknown. Erythema, swelling, local burning, and pain were observed in all cases. Also hyperglycemia (54 %) and hyponatremia (12 %) were recorded. Two of them (girls 9 and 13 years old) died within 24 h after being stung by pulmonary edema and circulatory failure. *Euscorpius* species are considered as nonmedically important scorpions and widespread species of the Black Sea region. In Trabzon Province of the region, a h *emolytic*–uremic syndrome was recorded following a scorpion sting (Mocan et al. 1998).

Antivenom Studies and Administration

Due to the medically important scorpion species found in Turkey, people stung by scorpions need medical attention. Human envenomation requires serum therapy and symptomatic treatments. Polyvalent antivenom (PHI antivenom) has been prepared by maceration of telsons by the Public Health Institution of the Health Ministry of Turkey in Ankara (Caliskan et al. 2013a). The private company VSP also produces effective antivenom since 2011 named as "Acsera." Production of polyvalent antivenom in both laboratories has been performed by injection of the crude venom of *A. crassicauda* into horses. The antibodies of the polyvalent scorpion antivenom can neutralize the toxic effects of venoms of the black and yellow scorpion as well as that of the venoms of other scorpion species in the country.

On the other hand, previous studies show that the antivenom (PHI antivenom) which is prepared using *A. crassicauda* venom is more effective in neutralizing the venom of Algerian species *Androctonus australis* than antivenom which is prepared using *A. australis*. Furthermore, PHI antivenom is capable of neutralizing venoms from the scorpions *Buthus occitanus, Tityus serrulatus, and Tityus bahiensis* when compared to their homologous antivenoms (Whittemore et al. 1961). Ulug et al. (2012) reported that scorpionism is classified into three clinical categories in Midyat State Hospital of Mardin Province of Turkey: Class I indicated local signs including local pain, erythema, and paresthesia restricted to the sting area; Class II indicated shivering, fever, excessive sweating, nausea, vomiting, diarrhea, hypertension, and priapism; and Class III indicated cardiovascular, respiratory, or neurological symptoms (such as cardiogenic shock, pulmonary edema, altered consciousness, and convulsive crisis). Additionally, several studies indicated that biochemical analyses were performed such as measure of hemoglobin, total white blood cell count (WBC), platelet count, prothrombin time, serum glucose, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), sodium, potassium, chloride, and calcium levels (Karakus et al. 2013). Administration of the antivenom was reported to be dependent on the severity of toxicity, and one or two 5 ml PHI antivenom ampoules were needed to be injected to envenomated patients. Clinicians reported that PHI antivenom was diluted to a total volume of 100 ml and infused intravenously over 20 min. Furthermore, to avoid reverse reactions of antivenom, treatment included not only antivenom therapy but also intravenous hydration, analgesia, antihistamines, and steroids administration (Al et al. 2009; Uluğ et al. 2012).

Conclusion and Future Directions

Proteomic- and transcriptomic-based analyses show that single scorpion venom might contain more than 100 peptidic components. Up to date, an important number of toxins have been isolated from the venom of several scorpion species of the

world, most of which are relatively short peptides. These peptides modulate cellular communication by either blocking the ion channels or modifying their gating mechanisms, which cause abnormal cell depolarization and impair proper function. Due to their relatively small size, compacted by several disulfide bridges, high potency and selectivity, rich biodiversity, and medical importance, scorpion toxins are considered as valuable and almost untapped resource for pharmaceutical agents that still need more research. The new methodology known as venomics, allowed obtaining information by mass spectrometry and sequencing cDNA clones which facilitated the discovery of thousands of novel sequences. For Turkey, it is clear that the future work is huge and our knowledge in this regard is still limited. Much more scientific work is still required because the 23 species (Buthidae and non-buthid groups) have not been sufficiently studied yet. Numerous epidemiological and clinical studies indicate that scorpion envenomation mostly occurred during the summer period and moderate or severe cases have been all treated with the horse polyvalent antivenom (Adiguzel 2010). The studies also indicate that the PHI antivenom has been successfully used for scorpionism in the country. Otherwise, identification of structure and molecular targets of scorpion venom peptides will provide more information about medically important species and will provide to design and production of superior antivenom of the country.

Cross-References

► Scorpion Venoms: Pathogenesis and Biotherapies

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Scorpion Venom Research Around the World: *Heterometrus* Species

15

Antony Gomes and Aparna Gomes

Contents

Abstract

Scorpions are venomous, nocturnal, invertebrate (Arthropoda) arachnids and a living fossil. The scorpion genus *Heterometrus* belongs to the family Scorpionidae. There are 33 species of *Heterometrus* scorpion found throughout the world. Envenomation by *Heterometrus* species is frequent in Asian tropical countries during summer and monsoon time (May–October). Intense pain, burning sensation, redness, swelling, and hypotension are common clinical signs. The death reports regarding the scorpions sting are not available

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except for a few reports in children and infants. In India, no specific antiserum is available against *Heterometrus* venom, and symptomatic treatment is still provided to the victims. Pharmacological studies of venom showed that *Heterometrus bengalensis* venom caused hypotension, decreased respiratory rate, contracted several smooth muscle preparations, promoted the release of kinin and had indirect hemolytic effects. *Heterometrus* venom induced several metabolic changes (brain, liver enzyme) in animal studies. *Heterometrus* venom possesses several bioactive constituents, among which PLA2 enzyme, substance L, Toxin-HB, HsTX1, kappa-Hefutoxin 1, heteroscopin 1, Hp1090, HmTx, and HsAp have been reported. A rabbit anti-*H. bengalensis* venom has been reported to neutralize the lethal and neuromuscular actions of *H. bengalensis* venom. Certain herbal extracts showed neutralizing capacity against *H. bengalensis* venom in experimental models. Both *Pluchea indica* and *Hemidesmus indicus* root extract neutralized *H. bengalensis* venom's lethal, neurotoxic actions in animal models. A protein toxin (Bengalin, MW 72 kDa) having cytotoxic activity against leukemic cells and anti-osteoporosis activity in female albino rats has been identified from *H. bengalensis* venom and shows promise for further drug development clues.

Introduction

Scorpions, one of the deadly venomous arthropods distributed through the tropical countries, account for a huge number of envenomation accidents leading to mortality and morbidity, especially among young children. Out of the more than 2,000 named scorpion species, some 50 are dangerous to humans. In India, there are 99 species of scorpions, including a large number of the *Heterometrus* species. Information on scorpion venom is essential for the treatment of patients and for the development of a specific therapeutic antidote. Recently, it has been observed that scorpion venom constituents have therapeutic potential for certain diseases. This chapter reviews the available research information on scorpion venom of the *Heterometrus* species with special reference to venom pharmacodynamics, active constituents, and therapeutic potentials.

The Scorpion *Heterometrus* Species

Scorpions were on Earth long before humans. The exact evolutionary path is difficult to trace, probably from Eurypterids or the giant water scorpions, which existed more than 400 million years ago (MYA) during the Silurian period. Until the upper Devonian period 345 MYA, almost all scorpions were aquatic in habits. About 30 fossil scorpion species have been reported from Europe and North America, found in Carboniferous rocks from 325 MYA. All these fossil scorpions

Fig. 1 The Indian black scorpion *H. bengalensis* (Koch) (**a**) The Indian giant scorpion *Heterometrus swammerdami* (Simon) (**b**)

were air breathing and terrestrial in habits. There is very little difference in morphology of the extinct and present-day scorpions; their essential morphology has remained unchanged for hundred of millions of years. Thus, they are often termed as "living fossils."

Scorpions are found all over the world except in the Arctic region. They are most commonly found in tropical, sub-tropical, arid, and semi-arid zones. All scorpions are strictly nocturnal and shy animals that normally stay under the stones, barks, crevices, and dark corners of houses and avoid water. According to habitats, scorpions are divided into (1) burrowing (psammophilous or pelophilous), (2) rock dwelling (lithophilous), and (3) arboreal (Tikader and Bastawade 1983).

Scorpions are divided into some 24 extant families, among which the *Heterometrus* belongs to family Scorpionidae, subfamily Scorpioninae, and genus *Heterometrus* (Hemprich & Ehrenberg). *Heterometrus* species are distributed in India, Sri Lanka, Burma, Borneo, the Philippines, and Africa. About 33 species of *Heterometrus* are found mainly in tropical and sub-tropical regions of southeastern Asia, including Cambodia, Laos, Thailand, Vietnam, India, Sri Lanka, Nepal, and China. *H. scaber* and *H. fulvipes* are found in the southern part of India, and *H. bengalensis* and *H. swammerdami* are found in the eastern part of India (Fig. 1).

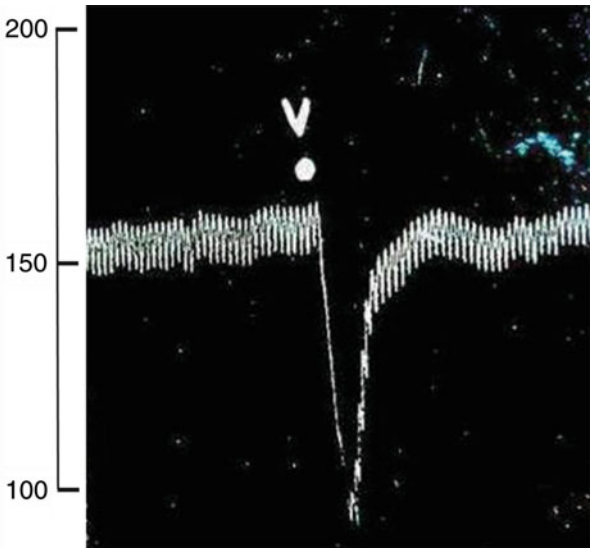

Fig. 2 Effect of *Heterometrus bengalensis* venom on cat blood pressure

Heterometrus Venom Pharmacodynamics

The clinical signs of *Heterometrus* envenomation include local burning pain, local swelling, redness or discoloration, hypotension, restlessness, respiratory distress, nausea and vomiting, abdominal pain, thirst, headache, pyrexia, shivering, and shocklike state (Nag Chaudhuri 1976). There is no specific treatment against *Heterometrus* envenomation in India due to the unavailability of an antiserum. Thus, only symptomatic treatments are provided to victims.

Caius and Mhaskar (1932) first reported that the venom of two *Palamnaeus* (outdated name for *Heterometrus*) species, *H. bengalensis* and *H. fulvipes*, produced dose-dependent prolonged bradycardia in dog heart, which was followed by tachycardia and increased force of myocardial contraction. The heart stopped at full systole condition. In decerebrated vagotomized animals, no bradycardia and tachycardia were observed immediately after venom injection. Babu et al. (1971) reported the toxic effects of *H. fulvipes* venom on cockroach heart, which stopped beating immediately after injection.

H. bengalensis venom (0.1–0.2 mg/kg, i.v) produced hypotension in cat blood pressure (Fig. 2). No tachycardia was observed due to repeated venom administration. The hypotensive response was not influenced by antagonists of acetylcholine, histamine, or serotonin, indicating that the hypotensive action was not influenced by cholinergic, histaminergic, or serotonergic pathways. The hypotensive effect of the venom was also present in the vagotomized cat, indicating that the effect was not due to central action. Thus, the mechanism of action of hypotensive response in cat induced by *H. bengalensis* venom remained unsolved. *H. bengalensis* venom also induced hypotension in rat and guinea pigs, an action which was also

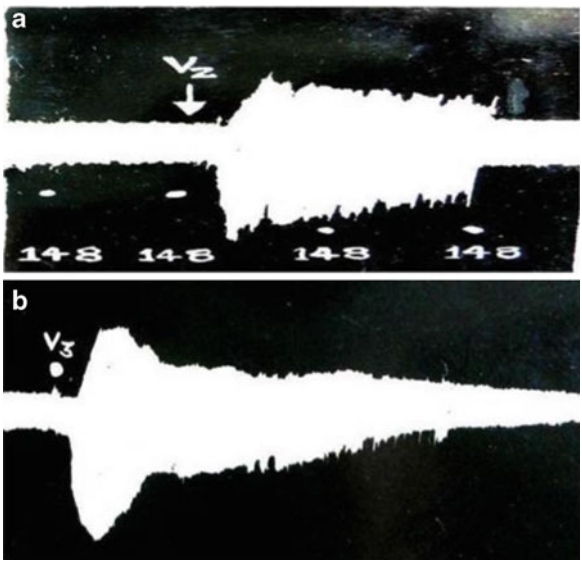

Fig. 3 Effect of *Heterometrus bengalensis* venom on isolated guinea pig auricle (a) and heart (b)

unaffected by cholinergic, histaminergic, and serotonergic blockers. Venom was found to increase capillary permeability but did not degranulate mast cells in in vitro experiments.

H. bengalensis venom (10–200 μg) on isolated guinea pig auricle and isolated guinea pig heart increased the force of contraction but not the rate (Fig. 3). Electrocardiographic changes produced by *H. bengalensis* venom (4 mg/kg, i.v) were bradycardia and complete dissociation of auricular ventricular complex. *H. bengalensis* venom produced significant changes in respiration in rat, guinea pig, and cat at dose level of 0.2–1 mg/kg, i.v. At lower dose it decreased respiratory rate and at higher dose produced temporary apnea. LD_{50} of *H. bengalensis* venom in male albino mice was found to be 11.52 mg/kg, i.v.

Lahiri and Nag Chaudhuri (1982) showed that the venom of *H. bengalensis* contracted several isolated smooth muscle preparations; the action was not mediated through cholinergic, histaminergic, serotonergic, and prostaglandin receptors. Later Kar et al. (1983) identified a smooth muscle contractile material designated as substance L (L = lipid) from *H. bengalensis* venom, which produced a contractile effect on isolated smooth muscles, an action which was unaffected by histamine, serotonin, acetylcholine, and prostaglandin inhibitors.

H. bengalensis venom was found to induce the release of considerable amounts of kinin from human blood kininogen (Lahiri and Nag Chaudhuri 1983). Aprotinin was found to inhibit kinin release. Plasma kinin level was also found to be elevated in vivo after intravenous injection of the venom into the guinea pig. This venom did not induce the release of acetylcholine, histamine, and serotonin. The released kinin could certainly contribute to hypotension and pain induced by the envenomation. Aprotinin-like compounds may antagonize venom-induced hypotension, pain, etc. The venom did not show any hemolytic activity on human RBC, but it produced

hyperglycemic response in rabbits. Venkaiah et al. (1983a) reported that *H. fulvipes* venom failed to produce any direct hemolytic activity on sheep RBC. However, sheep RBC sensitized with homologous hemolysin was lysed with the venom, and the factor was identified as a low molecular weight peptide containing a disulfide group. This factor present in *H. fulvipes* venom having a complement-like hemolytic effect was found to be thermolabile, dialyzable, and sensitive to the action of 2-mercaptoethanol. Fujimoto and Kaku (1982) reported the presence of both direct and indirect hemolyses (due to PLA2) in the venom of scorpion *H. gravimanus*. The direct hemolysins were presumably glycoproteins because they were positive in sugar reactions.

Gwee et al. (1993) documented the pharmacological activity of the venom of the black scorpion *H. longimanus*. The venom mimicked the agonist action of noradrenaline by acting directly on post-junctional alpha adrenoceptors in the anococcygeus muscle of rat. Noradrenaline and dopamine were present in the venom, whereas adrenaline was absent. The presence of noradrenaline in venom was deemed responsible for the post-junctional alpha agonist action of the anococcygeus muscle.

The involvement of black scorpion *H. spinifer* venom in cholinergic and adrenergic effects was established through isolated chick biventer cervicis muscle and isolated rat anococcygeus muscle in the presence/absence of agonists/antagonists. A high amount of acetylcholine and noradrenaline were detected by electrospray ionization mass spectrometry, which could explain the observed cholinergic and adrenergic effects. *H. spinifer* venom did not mediate any transmitter release (Nirthanan et al. 2002; Gwee et al. 2002).

Dasgupta et al. (1989a) established the immunological cross-reactivity and paraspecificity of the Indian black scorpion *H. bengalensis* venom with that of other scorpion venom available in Eastern India. It was found that the venom of *H. swammerdami*, the giant Indian scorpion, possessed cross-reactivity with the Indian black scorpion *H. bengalensis* venom but not with the Buthidae or Vaejovidae families of scorpions.

Dasgupta et al. (1989b) prepared monovalent rabbit antiserum against *H. bengalensis* venom. The antiserum showed positive precipitin bands in immunogel diffusion and immune electrophoresis and showed a high titer value. The rabbit antiserum gave protection against the lethal action of *H. bengalensis* venom in male albino mice. The serum also gave protection against venom-induced cardiac arrest, smooth muscle contractions, and neuromuscular paralysis in in vitro experiments.

Dasgupta et al. (1989c) reported the comparative detoxification and protection of the venom of *H. bengalensis* by toxoid antiserum. Among all the detoxifying agents used in this study, formaldehyde was the best detoxifying agent and showed 2.3 % loss of protein and sixfold detoxification. The formaldehyde-detoxified venom toxoid was immunogenic in the rabbit and developed anti-scorpion serum. The toxoid antiserum effectively neutralized *H. bengalensis* venom-induced lethal action and antagonized smooth muscle contraction, venom-induced cardiac arrest, and venom-induced neuromuscular blockade on isolated rat phrenic nerve-diaphragm and isolated chick biventer cervicis preparations. The possibility of using formaldehyde toxoid for antiserum production and immunization was recommended.

Dasgupta et al. (1990) for the first time prepared mice antiserum against Toxin-Hb, a heat-labile protein toxin (MW 10 Kda) isolated from the Indian black scorpion venom *H. bengalensis*. The anti-Toxin-Hb antiserum antagonized the Toxin-Hb-induced lethal actions and neuromuscular paralysis on isolated rat phrenic nerve-diaphragm and isolated chick biventer cervicis preparations. *H. bengalensis* venom tissue distribution was detected by enzyme-linked immunosorbent assay (ELISA) in rabbit tissues. The venom content was found in the order of liver > kidney > spleen > lung > heart > diaphragm > brain. The venom tissue distribution may provide a better understanding for antiserum treatment of victims (Dasgupta et al. 1991).

The venom yield of *H. bengalensis* was found to be 49 ± 10 mg/100 scorpion. The protein content of the venom was 71.6 ± 5 mg. The minimum lethal dose was 20 mg/kg, i.v.; 60 mg/kg, i.m.; and 65 mg/kg, s.c. in male albino mice. The minimum dose of venom for edema was 50 μg, sub planter, for apnoea was 16 mg/kg, i.v, for clotting of plasma was 30 μg and one unit of PLA2 10 μg. The minimum cardiotoxic dose was 4 mg and minimum neurotoxic dose was 2 mg/ml in in vitro studies.

Heterometrus envenomation induced several changes in the metabolic profile of victims that might aggravate the pathophysiological conditions of the patients. The metabolic effects are due to the direct/indirect actions of the venom constituents. Venom-induced release of mediators is also involved in this process. Babu et al. (1971) found that the venom of *H. fulvipes* could inhibit succinate dehydrogenase, lactic dehydrogenase, and cholinesterase activity in cockroach muscle and nerve homogenate. Prameelamma et al. (1975) observed that venom of *H. fulvipes* cause an increased level of sheep brain glutamate dehydrogenase and protease activity. They also observed that *H. fulvipes* venom inhibited sheep brain succinate dehydrogenase and cholinesterase activity. *H. bengalensis* venom induced hyperglycemia in rabbits. The hyperglycemic effects were maximum at 2 h of observation and lasted for 12 h. Venkaiah and Parthasarathy (1983b) showed that the venom of *H. fulvipes* caused mitochondrial swelling and inhibited mitochondrial succinate and glutamate dehydrogenase activity, thus uncoupling the state 4 respiration of rat liver mitochondria. *H. bengalensis* venom increased the liver glutathione, glutathione peroxidase, and glutathione transferase activity in male albino rats. *H. bengalensis* venom (0.1 mg/20 g mice, i.v.) significantly increased the urinary marker calcium/phosphate level, serum hepatic marker AST/ALT level, serum myotoxic marker CPK/LDH, and prooxidant markers after 4 h of injection. It has been proposed that the metabolic changes induced by scorpion venom may be helpful in the treatment protocol.

Natural Products Against *Heterometrus* Envenomation

The aqueous extract of 64 plants was tested on the venom *H. laoticus* scorpion against fibroblast cell lysis. Only two plant extracts of *Andrographis paniculata* and *Barringtonia acutangula* provided protection against this scorpion venom (Uawonggul et al. 2006).

Polysaccharide compounds identified from the root extracts of *Pluchea indica* and *Hemidesmus indicus* were found to neutralize the black scorpion *H. bengalensis* venom-induced lethal action, edema, urinary changes, hepatotoxic and myotoxic activity, PLA2 activity, and prooxidant and proinflammatory effects in in vivo/in vitro animal experiments. This study was expected to open a new area of development of herbal antidote against *H. bengalensis* venom (Das 2011).

Heterometrus Venom Active Components

The presence of PLA2 in the venom of the South Indian *H. scaber* was first reported by Kurup (1965). *H. scaber* venom PLA2 was also found to act on intact human RBC and RBC ghosts. Nair and Kurup (1973) showed the presence of glycosaminoglycan in *H. scaber* venom. The indole compounds present in the venom were identified as 5-hydroxytryptophan and histamine, along with two unidentified indole compounds. The presence of the enzyme acid phosphatase, ribonuclease, 5-nucleotidase, hyaluronidase, acetylcholine esterase, and phospholipase was also reported. A toxic protein present in the venom of *H. scaber* was identified by acetone precipitation, ammonium sulfate fractionation, and DEAE-Sephadex chromatography. The toxic protein was a glycoprotein (MW 15,000 D) containing 1.74 % glucosamine, 0.87 % galactosamine, 0.313 % sialic acid, 3.25 % fucose, and 0.45 % neutral sugar. The protein produced hyperglycemia (in sublethal doses) and LD_{50} were found to be 0.72 mg/kg, i.v., in rats (Nair and Kurup 1975).

The biochemical constituents of *H. scaber* venom were established by Nair (1980a). Nair (1981) also identified the chemical constituent and toxic protein present in *H. scaber* venom and their sublethal doses. Fujimoto and Kaku (1982) isolated hemolysin (direct and indirect) from the crude venom of *H. gravimanus* by Sephadex G-100 gel filtration followed by SD Sephadex C-25 column chromatography. The direct hemolysin was presumably a glycoprotein. Enzymes present in *Heterometrus* species venom were also reported by Achyuthan et al. (1982). Kar et al. (1983) identified Sub L from the venom of *H. bengalensis* by solvent extraction, gel filtration, and thin-layer chromatography. Substance L was a glycophospholipid in nature and contracted several smooth muscles. Kar et al. (1986) established the isoenzyme pattern of venom PLA2 from *H. bengalensis* venom, the molecular weights of which were 12,950 and 30,000 D. The enzyme PLA2 was purified from *H. fulvipes* venom (Ramanaiah et al. 1990a). The MW of the enzyme was 16,000 D with an active pH of 7.4 at 50 °C. The Km value was 1.8×10^{-3} M. Calcium, magnesium, and zinc ions stimulated enzyme activity, whereas mercury and EDTA inhibited the enzyme activity. Fluorescence emission maximum was found between 310 and 320 nm. They also isolated hyaluronidase (E.C.3.2.1.35) from *H. fulvipes* venom by gel filtration on Sephadex G-75 and DEAE ion exchange chromatography. The enzyme showed single band in PAGE and a MW of 82,000 D; optimum pH was found to be 4.0 and heparin inhibited the enzyme activity (Ramanaiah et al. 1990b).

Dasgupta et al. (1990) identified a lethal toxin (Toxin-Hb) from the venom of the scorpion *H. bengalensis* from Eastern India by CM-cellulose ion exchange chromatography. Toxin-HB was a heat-labile basic protein having a MW of 10 kD and LD_{50} value of 0.48 mg/kg, i.v., in male albino mice. It produced irreversible blockage of isolated rat phrenic nerve-diaphragm and chick biventer cervicis preparation. Anti-Toxin-Hb antiserum raised in mice gave fivefold protection against the lethal action and also effectively antagonized the neuromuscular blockade on isolated rat phrenic nerve-diaphragm and chick biventer cervicis preparation.

Ramanaiah and Venkaiah (1992) purified a manganese-containing enzyme superoxide dismutase (SOD) from scorpion *H. fulvipes* venom by ammonium sulfate, gel filtration on Sephadex G-100, and DEAE cellulose ion exchange chromatography. MW of the enzyme was 100,000 Da, optimum pH 8.5, and optimum temperature 45 °C. The enzyme activity was inhibited by metal chelation, EDTA, *O*-phenanthroline, and diethyl dithiocarbamate. An antiserum raised against *H. fulvipes* venom inhibited the SOD activity.

A new toxin (HsTx1) had been purified from the venom of *H. spinifer*; it is a 34-residue peptide having four disulfide bridges. HsTx1 shows 53 % and 59 % sequence identity with *Pandinus imperato*r toxin 1 (Pi1) and maurotoxin. It was only 32–47 % identical with other K^+ channel toxins. HsTX1 was a potent inhibitor of the rat Kv1.3 channels and competed efficiently with ^{125}I-kaliotoxin for binding to the voltage-gated K^+ channels (Lebrun et al. 1997).

The 3D structure of K^+ channel inhibitor HsTX1 was determined by NMR and molecular modeling (Savarin et al. 1999). This protein belonged to the scorpion short toxin family containing 29–39 amino acids and having 3–4 disulfide bridges. The fourth bridge had no influence on the global conformation of HsTX1. They also proposed that Tyr 21, Lys 23, Met 25, and Asn 26 were involved in the biological activity of HsTX1. Arg 33 was also important for the activity of the four disulfide-bridged toxin. Docking calculation confirmed that Arg 33 could contact Asp 386 on the K^+ channel and thus played the role of an additional positively charged residue of the toxin functional site.

The impact of the fourth disulfide bridge in scorpion venom of the alpha KTx6 subfamily was established by Carrega et al. (2005). It was shown that removal of the fourth disulfide bridge of HsTx1 did not affect its helical structure, whereas its two-stranded beta sheet was altered from a twisted to a non-twisted configuration. This structural change in the three disulfide-bridged HsTx 1 was accompanied by a marked decrease in Kv1.1 and Kv1.3 current blockage and by alteration in the toxin to channel molecular contacts. In contrast, a similar removal of the fourth disulfide bridge of Pi1 toxin had no impact on its structure-function.

Kappa-Hefutoxin 1, a novel class of weak potassium channel toxins from scorpion *H. fulvipes* venom, was identified by Srinivasan et al. (2002). A novel feature of the kappa-Hefutoxin 1, unlike other scorpion toxins, was that it not only blocked voltage-gated K^+ channels but also slowed the activation of kinetics of Kv1.3 currents. Alanine mutants failed to block the channels, indicating the importance of the functional diad composed by Tyr 5 and Lys 19. Zarrabi and

Naderi-Manesh (2008) established the interaction of kappa-Hefutoxin 1 with voltage-gated K^+ channels by docking and molecular dynamics. They indicated that a 3D model of the kappa-Hefutoxin1 complex could be helpful for drug design.

Nirthanan et al. (2005) reported that weak K^+ channel toxin kappa-KTx from *H. spinifer* venom showed 60 % identity with kappa-Hefutoxin 1 isolated from *H. fulvipes* venom. Despite the presence of equivalent functional diad (Y5 and K19), kappa-KTx1 failed to reproduce the K^+ channel blocking activity of kappa-Hefutoxin1 due to an additional positive charge (lysine K20) that could hinder the critical electrostatic interactions.

Heteroscorpine-1 (HS-1) toxin was purified from the Thai giant scorpion *H. laoticus* venom (Uawonggul et al. 2007). HS-1 showed 80 % similarity to Panscorpine and Opiscorpine and had inhibitory activity on several microorganisms. Hariprasad et al. (2007) reported the cloning sequence analysis and homology modeling of a novel PLA2 from *H. fulvipes* venom. The cDNA sequence codes for the mature portion of the group PLA2 consisted of 103 amino acids. This sequence was 80 % identical with the Indian red scorpion (*Hottentotta tamulus*) venom. Most of the essential features of group III PLA2 like the Ca^{2+} binding loop and catalytic residue were conserved. Heteromtoxin (HmTx), a novel group III heterodimeric PLA2 from the scorpion *H. laoticus* venom, was reported by Incamnoi et al. (2013). The PLA2 was an acidic protein (PI value 5.6), having MW of 14018.4 Da and 131 amino acids. The 3D structure of HmTx consisted of three conserved alpha helices that were highly similar to the conserved sequence of bee venom PLA2.

Proteomic bioinformatics analysis of African black scorpion *H. longimanus* venom confirmed that the venom was composed of potent mixture of toxin and antimicrobial and ionic channel inhibitors (Bringans et al. 2008). Ma et al. (2010) established the molecular diversity of toxic compounds from the scorpion *H. petersii* venom by proteomics-transcriptomics analysis. Ten known families of venom peptides and protein were identified, including two families of K^+ channel toxins, four families of antimicrobial and cytolytic peptides, and one family each of Ca^{2+} channel toxin, Lal-like peptide, PLA2, and serine protease. In addition, 12 atypical families, including acid phosphatases, diuretic peptide, and ten orphan families, were also identified in these studies.

A new natural alpha helical peptide (Hp1090) from the venom gland of the scorpion *H. petersii* was identified through cDNA technology. Hp1090 peptide inhibited hepatitis C infection (IC50 of 7.62 μg/ml) and it is viricidal for HCV in vitro, directly interacting with the viral membrane, thus decreasing the infectivity (Yan et al. 2011).

A novel class of antimicrobial peptides (HsAp, HsAp2, HsAp3 and HsAp4) were identified from the venom gland of *H. spinifer* (Nie et al. 2012). Each peptide was composed of 29 amino acid residues and was cationic and weakly amphipathic. No significant homology was found with other known peptides. HsAp was able to inhibit the growth of both gram-negative and gram-positive bacteria with MIC value of 11.8–51.2 μM. Genomic analysis indicated that the genes of all the four peptides were intron less.

Heterometrus Venom Therapeutic Potentials

In Ayurvedic, Unani, Chinese, and Homeopathic medicine, the use of venoms and toxins has been mentioned (Pal et al. 2002). In India, Africa, and Cuba, different scorpion venoms were used in various pathophysiological conditions (Gomes et al. 2010). The folk-traditional applications of venoms-toxins in health and diseases lead to the development of modern drug/drug leads against several pathophysiological conditions. Several lead compounds and antivirals against cancer, osteoporosis and arthritis have shown promising results, some of which are discussed below.

Anticancer/Antileukemic Activity

Das Gupta et al. (2007) established the antileukemic activity of *H. bengalensis* venom on leukemic cell lines U937 and K562. Venom inhibits cell growth, and the IC50 value of the venom was found to be 41.5 μg/ml (U937 cells) and 88.3 μg/ml (K562 cells). The scorpion-venom-treated cells showed signs of apoptosis, e.g., membrane blebbing, chromatin condensation, and DNA degradation as confirmed by confocal, fluorescence, and scanning electron microscopy (Figs. 4 and 5). Flow cytometry also revealed a significant amount of apoptotic cells (early and late stage) induced by *H. bengalensis* venom. A venom-induced cell cycle arrest was observed with maximum cell accumulation at the sub-G1 phase. Thus, the antiproliferative, cytotoxic, and apoptogenic activity of *H. bengalensis* venom was established against human leukemic cells.

H. bengalensis venom significantly inhibited EAC cell proliferation and increased the survival time of EAC BALB/c mice. It also significantly reduced the tumor (induced by 3-methyl chloranthene) weight and volume in BALB/c mice. Histopathological studies of the tumor revealed karyolysis and vacuolization in tumor tissue sections induced by the venom.

A high molecular weight protein (Bengalin) was purified from *H. bengalensis* venom that showed antileukemic activity on U937 and K562 cell lines in vivo (Gupta et al. 2010). The *N*-terminal 20 amino acid sequence of Bengalin was identified (G-P-L-T-I-L-H-I-N-D-V-H-A-A/R-F-E-Q/G-F/G-N-T) and showed no similarity with any other protein isolated from other scorpion venoms. Bengalin showed antiproliferative activity having IC50 value of 3.7 and 4.1 μgm/ml in U937 and K562 cells, respectively. Besides its apoptogenic activity, it also arrested cell cycle at sub-G1 Phase. It produced morphological changes in leukemic cells suggesting apoptogenic nature. It decreased Bcl-2/Bax ratio, HSP70 and HSP90 expression, and mitochondrial membrane potential; increased activity of caspase 9 and 3;, and induced PARP cleavage. These studies are the first hints on the molecular mechanism by which *Heterometrus* venom affects the leukemic cell and may hold clues for future anticancer drug development.

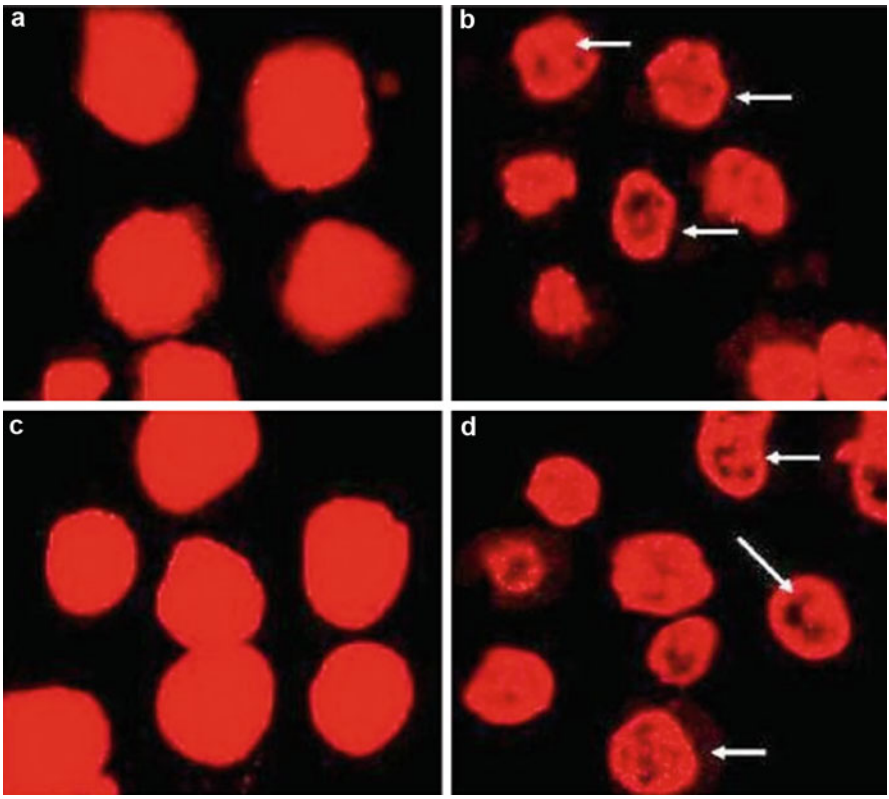

Fig. 4 Confocal microscopic images of control U937 cells (a), K562 cells(c) and scorpion (*H. bengalensis*) venom-treated U937 (b), K562 (d) cells using propidium iodide. The control cells showed intact nucleus and the venom-treated cells showed apoptotic bodies in both the cells indicated by arrows. Magnification (1000æ)

Anti-osteoporosis Activity

Gomes et al. (2009) established that the venom of the scorpion *H. bengalensis* possessed anti-osteoporosis activity in female albino rats as observed through urinary calcium, phosphate, creatinine, and hydroxyproline level. Venom also significantly regulated serum calcium, phosphate, tartrate-resistant acid phosphatase, interleukin 1, interleukin 6, TNF-alpha, and parathormone level in osteoporosis female albino rats. Bone minerals (calcium, phosphate, sodium, zinc, magnesium) were also influenced by this venom. This study confirmed that *H. bengalensis* venom may influence bone remodeling process by stimulating bone formation and reducing bone resorption process of osteogenesis.

Bengalin exhibited significant anti-osteoporosis activity in female albino osteoporosis rats, which was confirmed through urine, serum, and bone parameters. Bone mineral density (DEXA scan) of osteoporosis rats was improved due to Bengalin.

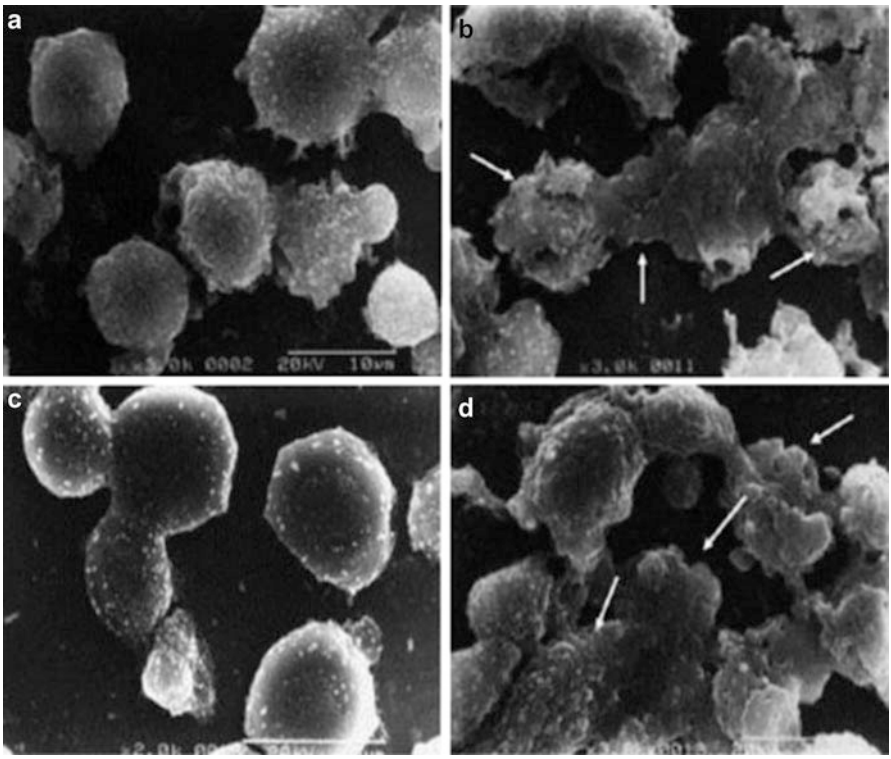

Fig. 5 Scanning electron microscopic images of control U937 (a), K562 (c) and *H. bengalensis* venom-treated U937 (b), K562 (d) cells. The control cells showed intact plasma membrane, but the treated cells clearly show deep ridges and furrows as well as severe membrane blebbing as indicated by arrowhead. Magnification (3000æ)

Bengalin showed cardiotoxic and neurotoxic effects through in vitro experiments. Bengalin was involved in the improvement of osteoporosis bone status through increased bone mineral deposit with the coordinated actions of hormones, cytokines, and bone cell activity. It also showed that Bengalin acted at the osteoclasts, which was confirmed through the involvement of interleukins and parathormone, and also inhibited the binding of RANKL with the RANK to decrease the osteoclastic activity (Haldar et al. 2010).

Therefore, Bengalin, a protein toxin from the scorpion *H. bengalensis* venom, shared antileukemic and anti-osteoporosis activity in experimental models. Further studies will confirm the future of Bengalin as a therapeutic agent in diseases.

Antibacterial and Antiviral Activity

Ahmed et al. (2012) reported that *H. xanthopus* venom contained antimicrobial peptides similar to hadrurin, scorpine, and Pandinin 1 and 2. *H. xanthopus* diluted

and crude venom could kill *B. subtilis* ATCC 6633, *S. typhimurium* ATCC 14028, *P. aeruginosa* ATCC 27853, and *E. faecalis* ATCC 14506 with highest activity against *B. subtilis*. From the venom of *H. spinifer*, several antimicrobial peptides have been identified. Among them, HsAp was effective against growth of gram-negative and gram-positive bacteria; it also inhibited growth of tested fungus (Nie et al. 2012). Crude venom of *H. laoticus* also showed antibacterial activity against *B. subtilis*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. Heteroscorpine 1 was purified from this venom, having a molecular weight of 8–93 KDa that was 300 times more potent to kill *B. subtilis*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* than the crude venom. Hp1090, an amphiphatic alpha helical peptide isolated from scorpion *H. petersii*, had viricidal activities against HCV particles in in vitro model and prevented the initiation of HCV infection. It acted directly on the phospholipid membrane and increased its permeability exerting direct effect on the viral membrane (Yan et al. 2011).

Conclusion and Future Directions

From the above review, it is evident that *Heterometrus* venoms contain several bioactive constituents that are directly and indirectly responsible for the envenomation pathophysiology. Some of these bioactive molecules have therapeutic potential and should be researched in more detail. Fundamental research on scorpion venom is essential for elucidating the actions, mechanism, and antagonism of venom toxic constituents for the alleviation of human suffering and death against scorpion sting.

Scorpion sting is a major health problem in underdeveloped tropical/subtropical countries. However, scorpion venom research is a neglected area in these countries, where the most deaths occur (though often not recorded systematically). There are several reasons for which scorpion venom research is still neglected: (1) availability of authenticated scorpion/scorpion venom is very difficult and there is no scorpion venom bank, (2) yield of scorpion venom is very low as compared with large venomous animals such as snakes, and (3) lack of awareness about the need for scorpion venom research in this part of the world.

Cross-References

▶ Scorpion Venom Research Around the World: Indian Red Scorpion

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Scorpion Venom Research Around the World: Indian Red Scorpion

16

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Contents

Abstract

The Indian red scorpion is the medically most important scorpion on the Indian subcontinent, and a sting is often lethal without treatment, especially to young children. The correct identification of this species is *Hottentotta tamulus*; the more commonly known names, *Buthus tamulus* and *Mesobuthus tamulus*, and the suggestions of subspecies (Pocock) are incorrect and should be discouraged. Mild cases of *H. tamulus* envenoming show vasoconstriction and hypertension resulting from a massive release of catecholamines. Severe cases result in hypotension, pulmonary edema and myocardial dysfunction. Animal studies indicate that catecholamine release is as a consequence of prolonged sodium channel activation and potassium channel inhibition. Pulmonary edema results

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from many subcellular events, including kinin activation and stimulation of central serotonin receptor subtypes. Treatment with a specific antibody (with or without the α-adrenoreceptor blocker, prazosin, dependent on symptoms and clinical severity) is presently the preferred therapy. The few venom constituents that have been characterized in detail include iberiotoxin, a specific blocker of high conductance, calcium-activated potassium channels; tamapin, a specific blocker of low conductance calcium activated potassium channels (K_{Ca} 2.2 subtype); and the short peptide insectotoxins, ButaIT and BtlTx3.

Introduction

Two predominant species of scorpion are found in India – the red scorpion and the black scorpion (Caius and Mhaskar 1932). The black scorpion (*Heterometrus*) is an impressive beast, growing up to 20 cm long with formidable and powerful pedipalps. However, from a venomous point of view, such scorpions are essentially harmless and are often kept as pets. In comparison, its physically less impressive cousin, the much smaller red scorpion (Fig. 1), growing between 5 and 9 cm long, is the most important scorpion of medical significance on the Indian subcontinent. It is found throughout India, in eastern Pakistan, and at lower elevations in Nepal and has recently been identified in Sri Lanka on the Jaffna Peninsula (Ranwana 2013). This widespread distribution indicates that the red scorpion can survive in a variety of habitats, from coastal tropical plains to semiarid inland plateaus.

Although originally named *Scorpio tamulus*, the scorpion was correctly identified as belonging to the *Hottentotta* genus (*Hottentotta tamulus*) as early as 1914 (Kovarik 2007). Nevertheless, it is almost exclusively (and mistakenly) referred to in the scientific and clinical literature as *Buthus tamulus* or *Mesobuthus tamulus*, names that are in widespread popular use today. Although Pocock originally distinguished various subspecies (*concanensis, sindicus, gujaratensis*, and *gangeticus*), according to coloration and distribution, terminologies that are often still used, these should be correctly regarded as color morphs rather than subspecies (Kovarik 2007).

The authors were unable to find a single paper in PubMed or Web of Science (December 2013) using the keywords *Hottentotta tamulus*. The authors suggest that both names are used concurrently for several years until the research community becomes familiar with the correct nomenclature.

Pathophysiology and Clinical Aspects

Given the diversity of proteins and peptides in scorpion venom, it is perhaps not surprising that the pathophysiological effects of venom are equally complex. One functional role of venom is to act as a deterrent to predators, and the initial injection of potassium ions in what is known as “prevenom” (Inceoglu et al. 2003) most probably accounts for the initial, excruciating pain felt when an individual is stung.

Fig. 1 The Indian Red Scorpion, *Hottentotta tamulus*. (Copyright P.N. Strong)

Red scorpion venom contains different toxins which either slow the inactivation of sodium channels or block potassium channels, thereby dramatically increasing the release of neurotransmitters at nerve endings (Narahashi et al. 1972; Rowan et al. 1992; Vatanpour et al. 1993). Both sympathetic and parasympathetic neurons and the adrenal medulla are stimulated, releasing acetylcholine, catecholamines, and angiotensin (Moss et al. 1973; Murthy and Vakil 1988). The release of catecholamines has been graphically described as an "autonomic storm."

The release of catecholamines through prolonged sympathetic stimulation, as a consequence of red scorpion envenomation, leads, in humans, to a variety a symptoms. Mild cases result in severe vasoconstriction and systemic hypertension, whereas severe cases result in hypotension, pulmonary edema, and myocardial dysfunction, the latter often leading to cardiogenic shock and death (Karnad 1998). Young children, the elderly, and those who are immunocompromised are particularly susceptible.

Animal studies, especially by Murthy, Deshpande, and their respective groups, have provided a wealth of experimental data to underpin clinical observations. Metabolic data from Murthy's group include cardiac sarcolemmal defects, reduced insulin secretion, and alterations in both carbohydrate and fat metabolism (see inter alia, Murthy 1982; Murthy and Ag 1986; Murthy and Medh 1986; Murthy et al. 1988). Deshpande's group has shown that venom-induced pulmonary edema and augmentation of cardiac reflexes (Deshpande et al. 1999) are produced through a variety of cellular processes, including the stimulation of central serotonin (5-HT_{1A}) subtypes (Bagchi and Deshpande 1999), kinin activation of a nitric oxide/cGMP pathway (Kanoo et al. 2009), and histamine release mediated by H_1 receptors on mast cells (Dutta and Deshpande 2011).

From animal studies, a number of different treatments have been proposed. In experimental animals, insulin has been shown to correct metabolic and cardiac abnormalities (Murthy et al. 1990), 2-deoxyglucose (increases insulin sensitivity) has been shown to reduce cardiopulmonary abnormalities (Choudhry et al. 2011), and aprotinin (a kinin inhibitor) has been shown to reverse cardiac abnormalities (Pandey and Deshpande 2004).

In envenomed child patients, insulin/glucose has been demonstrated to normalize blood pressure and reverse pulmonary edema (Murthy et al. 1991). Vasodilators, including captopril and nifedipine, have been used to correct both venom-induced hyper- and hypotension (Krishnan et al. 2007), although the use of captopril, an ACE inhibitor, has been questioned since it has been shown that captopril-induced cardiopulmonary changes are similar to venom (Bagchi and Deshpande 1998). However, the most successful drug treatment has been the use of prazosin, an α-adrenergic blocker, championed by Bawaskar (Bawaskar and Bawaskar 1996). As well as counteracting the effects of increased catecholamine production, prazosin also acts as a vasodilator to correct hemodynamic changes. Finally, the drug is cardioprotective, reversing metabolic changes associated with cardiac abnormalities brought on by the suppression of insulin secretion.

In comparison to its use in the treatment of snakebite, serotherapy for scorpion sting has been more controversial (see inter alia Freire-Maia et al. 1994; Gueron and Ovsyshcher 1987; Gueron and Sofer 1994; Ismail 1993; Ismail 1995). In the case of *H. tamulus*, the use of an antivenom serum $F(ab')_2$ fragment (Kankonkar et al. 1998) was shown to neutralize venom in the circulation and to be highly effective in treating symptoms of severe envenoming (Natu et al. 2006). This study was followed up with a prospective, open-labeled clinical trial, comparing the effects of serotherapy and/or prazosin (Natu 2010) in which it was found that as long as the antivenom dose given was dependent on clinical severity, the antivenom led to earlier clinical recovery than treatment with prazosin alone. It was further suggested (see also Natu 2012) that prazosin should be reserved for patients presenting with hypertension and pulmonary edema, although antivenom should also be given in these cases, to speed recovery. The results of these trials were subsequently independently confirmed in a randomized open-label clinical trial (Bawaskar and Bawaskar 2011), demonstrating that the antivenom, administered in conjunction with prazosin, hastened recovery when compared to the administration of prazosin alone.

Venom Constituents

In common with most other medically important scorpion venoms, *H. tamulus* venom is dominated by disulfide-rich peptides that interact with ion channel proteins. Enzymes are in low abundance compared to snake venoms. However, even after taking into consideration the identified peptides and a few other peptides and proteins that have been partially characterized, the total number investigated is less than 20. It is salutary to think how far we have to go to achieve complete venom characterization when one considers, using mass spectrometry techniques (MALDI-TOFMS and ESI-MS) in the range 2,000–10,000 Da, that over 600 distinct m/z values have been identified (70 % detected by both methods) after removing putative adducts from the final dataset (Newton et al. 2007).

Geographical differences in venom composition have been shown for snakes, spiders, cone snails, and scorpions. In Maharashtra state, the composition

Table 1 Sodium channel toxins in *H. tamulus* venom

	Reference	
***H. tamulus* α-toxin (basic)**	a	E D G Y L L N R D T G C K V S C G T C R Y C N D
AahIT4	b	E H G Y L L N K Y T G C K V W C V I N N E E C G Y L C N K R R G G Y Y G Y C Y F W K L A C Y C Q G A R K S E L W N Y K T N K C D L
***H. tamulus* α-toxin (acidic)**	c	G E D G Y I A D G D N C T Y I C T F N N Y C H A L C T D K K G D S G A C D W W V P Y G V V C W C E D L P T P V P I R G S G R C
BmK-M8	d	G R D A Y I A D S E N C T Y F C G S N P Y C N D V C T E N G A K S G Y C Q W A G R Y G N A C Y C I D L P A S E R I K E P G K C G

Toxins from *H. tamulus* are in bold. Reference Na^+ channel toxins are included for comparison. Yellow backgrounds: conserved Cys residue backbone. Blue backgrounds: other conserved residues.

[a] *Lala and Narayanan (1994)*
[b] *Loret et al (1991)*
[c] *Sharma et al (2006)*
[d] *Li et al (1996)*

of *H. tamulus* venom varies, dependent on whether it is collected from scorpions found in the tropical Konkan coastal region as opposed to those found on the semiarid Deccan plateau, up to 600 m (Newton et al. 2007). These differences may have clinical ramifications as it has been reported that within the state of Maharashtra, fatal human envenomations only occur within the Konkan region (Bawaskar and Bawaskar 1999). Intraspecific variations have also been seen using SDS-PAGE, between the venoms of scorpions collected in Western India (Ratnagiri, Chiplun, and Ahmednagar), as distinct from those collected in Chennai, in the south of the country (Badhe et al. 2006). The same authors have detected differences in the blood sodium levels of mice injected with these differently sourced venoms (Badhe et al. 2007).

Although sodium channel toxins (α-toxins) are among the most well characterized of scorpion venom toxins, none have been completely characterized from *H. tamulus* venom (Table 1). Habermann's group originally identified two toxic peptides (i.v. route) which had molecular masses (SDS-PAGE) of 7,500 and 8,000, suggesting the presence of α-toxin(s) (Chhatwal and Habermann 1981). Lala's group subsequently characterized a 7,800 Da protein with similar i.v. lethality and determined the sequence of the first 24 amino acid residues (Lala and Narayanan 1994). These residues have maximum sequence homology with the N-terminal residues of AahIT4, a sodium channel toxin from *A. australis* (Loret et al. 1991), although it would appear that Cys-19 in the *H. tamulus* sequence is probably mis-identified (see Table 1).

A classical α-like toxin with four disulfide bridges has been isolated from *H. tamulus* venom and its crystal structure determined at 2.2 Å resolution (Sharma et al. 2006). Unfortunately the assay used to follow purification is unclear, and no biological activity of the peptide has been described. The sequence shares 50 % identity with both classical α-toxins and α-like toxins, with greatest identity to BmK-M8, an acidic neurotoxin from *Mesobuthus martensii* Karsch (Li et al. 1996) (Table 1). Most α-toxins are basic peptides and unusually this peptide is highly acidic (pI = 4.3); although the three-dimensional structure is similar to other α-toxins, the N-terminal region has a remarkably high concentration of negatively charged residues. Other distinct features – positively charged residues at the C-terminus and several Pro residues within the third β-sheet – have led the authors (Sharma et al. 2006) to suggest that this toxin represents a new subclass. However, this issue will not be clarified until some substantial and detailed biological activity studies are undertaken.

Table 2 Potassium channel toxins in *H. tamulus* venom

	Reference	
Iberiotoxin	a	Q F T D V D C S V S K E C W S V C K D L F G V D R G K C M G K K C R C Y Q
Charybdotoxin	b	Q F T N V S C T T S K E C W S V C Q R L H N T S R G K C M N K K C R C Y S
BKT2	c	V G C A E C P M H C K G K M A K P T C E M E V C K C H I G
BmP02	d	V G C E E C P M H C K G N N A N P T C D D G V C N C N V
Tamulotoxin	e	D L I D V K C I S S Q E C W I A C K K V T G R F E G K C Q N R Q C R C Y
LqH 15-1	f	G L I D V R C Y D S R Q C W I A C K K V T G S T Q G K C U N K Q C R C Y
Noxiustoxin	g	T I I N V K C T S P K Q C S K P C K E L Y G S S A G A K C M N G K C K C Y N N
Tamapin	h	A F C N L R R C E L S C R S L G L L G K C I G E E C K C V P Y
Scyllatoxin	i	A F C N L R M C Q L S C R S L G L L G K C I G D K C E C V K H
Tamulustoxin	j	R C H F V V C T T D C R R N S P G T Y G E C V K K E K G K E C V C K S

Toxins from *H. tamulus* are in bold. Reference K^+ channel toxins are included for comparison. Yellow backgrounds: conserved Cys residue backbone. Blue backgrounds: other conserved residues.

[a] *Galvez et al (1990)*
[b] *Gimenez-Gallego et al (1988)*
[c] *Dhawan et al (2003)*
[d] *Romi-Lebrun et al (1997)*
[e] *Escobas et al (1997)*
[f] *Marshall et al (1994)*
[g] *Possani et al (1982)*
[h] *Pedarzani et al (2002)*
[i] *Chicchi et al (1988)*
[j] *Strong et al (2001)*

Iberiotoxin (M_r 4.3 kDa) (Table 2) was the first molecule characterized in *H. tamulus* venom, and it was originally identified through a screen of different scorpion venoms, to characterize molecules that could modulate charybdotoxin binding to smooth muscle membranes (Galvez et al. 1990). Iberiotoxin (Kα-1 toxin family, see (Rodriguez de la Vega and Possani 2004) for nomenclature) shares 68 % sequence identity with charybdotoxin (see Table 2) and also blocks high-conductance calcium-activated channels (BK channels) with picomolar affinity ($K_i = 250$pM, bovine aortic sarcolemmal membranes). Both peptides are N-terminally blocked with a pyroglutamate residue. Iberiotoxin is much less basic than charybdotoxin, with four additional aspartate residues, and this has a great influence on its channel binding. The former binds with a 10-fold higher affinity, due to the time that is spent bounding in the mouth of the ion channel (~200s vs. only ~10s for charybdotoxin) (Candia et al. 1992). In addition to having a higher affinity for BK channels than charybdotoxin, iberiotoxin also has a much higher specificity; charybdotoxin also blocks intermediate conductance calcium-activated potassium channels as well as voltage-gated K_v channels. This specificity has enabled iberiotoxin to be used as a tool to help dissect pharmacological mechanisms of great complexity – for example, endothelium-derived hyperpolarizing factor (EDHF) pathways (Edwards et al. 2010).

Using the same strategy, tamapin (M_r 3.4 kDa) (Table 2) was isolated from *H. tamulus* venom, using a ^{125}I-apamin-binding screen (Pedarzani et al. 2002). Apamin is a bee venom toxin, specific for low-conductance, calcium-activated potassium channels (SK channels or $K_{Ca}2.x$ family). In contrast to apamin, which does not distinguish between SK channel subtypes, tamapin is highly selective for $K_{Ca}2.2$ channels (see Table 3). Tamapin binds with extremely high affinity to rat

Table 3 IC_{50} values for tamapin block of SK channel subtypes stably expressed in HEK293 cells (Pedarzani et al. 2002)

Channel subtype	$K_{Ca}2.1$	$K_{Ca}2.2$	$K_{Ca}2.3$
Tamapin (IC_{50} nM)	42	0.024	1.7

brain synaptosomes ($K_i = 12$ pM); it belongs to the Kα-5 toxin family and is amidated at the C-terminus. An isomer, tamapin-2, was also isolated from the venom, having similar binding activity ($K_i = 8$ pM) but differing by a single residue (His/Tyr) at the C-terminus. A comparison of mass spectrometric and sequence data would suggest that tamapin-2 is also C-terminally amidated. Tamapin shares greatest identity with scyllatoxin (leiurotoxin), another peptide that blocks SK channels (Chicchi et al. 1988), isolated from *Leiurus quinquestriatus hebraeus* (Table 2).

Another toxin purified from *H. tamulus* venom, which on the basis of sequence comparisons may block calcium-activated potassium channels, is tamulotoxin (Escoubas et al. 1997). Unfortunately, apart from the original abstract with sequence information, no pharmacological data on tamulotoxin has been published. Tamulotoxin shares most similar to toxin Lqh15-1 (Marshall et al. 1994), isolated from *Leiurus quinquestriatus* venom, although frustratingly, pharmacological studies of the latter toxin are also limited. Tamulotoxin also shares limited similarity, not only with iberiotoxin/charybdotoxin but also noxiustoxin/margatoxin (K_v blockers); it has been catalogued as member of the Kα-16 toxin family (Rodriguez de la Vega and Possani 2004).

Several toxins have been characterized from *H. tamulus* venom that block voltage-gated K_v channels. Tamulustoxin (M_r 3.9 kDa) (Table 2) is another toxin that was isolated on the basis of a toxin screen, this time screening the venom for toxins that inhibited ^{125}I-toxin I binding to rat brain synaptic plasma membranes (Strong et al. 2001). Toxin I is a dendrotoxin homologue; dendrotoxins block certain voltage-gated potassium channels ($K_v1.1$, $K_v1.2$, and $K_v1.6$). Tamulustoxin is an "orphan" toxin in the current potassium channel scorpion toxin classification (Rodriguez de la Vega and Possani 2004); although it shares the common cysteine backbone with all other potassium channel toxins, there are twice as many amino acids (eight as opposed to four) between the fourth and fifth Cys residues, more reminiscent of scorpion hemolymph defensins (Cociancich et al. 1993). The C-terminal residue of tamulustoxin also appears to be amidated. The pharmacology of tamulustoxin is incomplete; the toxin blocks $K_v1.6$ channels, although it is not very potent (37 % inhibition at 500 nM). A single amino acid variant (Val-6 → Ile) of tamulustoxin was also isolated and confirmed by RT-PCR, confirming the presence of both mRNA species in the venom gland. Ile-6 tamulustoxin was inactive, and although it might be at first glance surprising that such a conservative substitution can affect biological activity, there are several examples of Ile/Val substitutions affecting various processes, such as transport, assembly, and protein – small molecule interactions. It was estimated that approx 90 % of all tamulustoxin genes encode the Val-6 isomer.

Table 4 Chloride channel toxins (insectotoxins) in *H. tamulus* venom

	Reference	
BtlTx3	a	R C P P C F T T N P N M E A D C R K C C G G R G Y C A S Y Q C I C P G
ButaIT	b	R C G P C F T T D P Q T Q A K C S E C C G R K G G V C K G P Q C I C G I Q
Chlorotoxin	c	M C M P C F T T D H Q M A R K C D D C C G G K G R G K C Y G P Q C L C R

Toxins from *H. tamulus* are in bold. Chlorotoxin is included for comparison. Yellow backgrounds: conserved Cys residue backbone. Blue backgrounds: other conserved residues.

[a] *Dhawan et al (2002)*
[b] *Wudayagiri et al (2001)*
[c] *DeBin et al (1993)*

BKT2 (M_r 3.4 kDa) is another potassium channel toxin isolated from *H. tamulus* (Dhawan et al. 2003). The amino acid sequence of BKT2 identifies it as a Kα-9 toxin (Table 2). BKT2 is a very weak inhibitor of Kv1.1 channels (IC_{50} = 5 μM), but since it has not been examined on any other channel, it is unclear at present whether the toxin is simply a low-affinity inhibitor or whether it binds more tightly to an as yet undetermined subtype. Unfortunately no functional clues can be obtained from examining other Kα-9 toxins; they are either of low affinity (>1 μM) or uncharacterized specificity (Rodriguez de la Vega and Possani 2004). The three-dimensional structure of recombinant BKT2 has recently been determined using an isotope-edited NMR method (Kumar et al. 2011). In comparison with high-affinity K_v peptides which have an extended C-terminal region, the equivalent residues of BKT2 fold back on themselves to form a loop, stabilized by the β-sheet. Another unusual feature of BKT2 with respect to other potassium channel toxins is the lack of aromatic residues in the former. BKT2 shows most structural similarity with BmP02, isolated from *Mesobuthus martensii* Karsch (Romi-Lebrun et al. 1997). Since BmP02 weakly competes with apamin for binding to rat brain synaptosomes (Romi-Lebrun et al. 1997), it would be interesting to see whether BKT2 has any effect on SK channels.

Many scorpion venoms contain short peptides (approx 35 amino acids) with four disulfide bridges. These peptides have significant sequence relatedness but diverse functional activities. They are variously known as short insect toxins ("insectotoxins") or chlorotoxin-like peptides after the prototype and most highly characterized member. Chlorotoxin itself, although originally characterized as blocking the cytoplasmic face of epithelial chloride channels (DeBin et al. 1993), remains a functional enigma, and its mechanism of action is controversial.

Two different peptides from *H. tamulus*, both belonging to this family, were characterized in a short space of time by different groups, using functional, insect-based assays. ButaIT (Mr 3.9 kDa, 37 amino acids, Table 4), was isolated using a tobacco budworm (*Heliothis virescens*) assay (Wudayagiri et al. 2001), whereas BtlTx3 (Mr 3.8 kDa, 35 amino acids) was isolated using a cotton bollworm (*Helicoverpa armigera*) assay. Both ButaIT and BtlTx3 are homologs of chlorotoxin (see Table 4) and other short insect toxins. Both peptides caused irreversible flaccid paralysis at similar concentrations (BtlTx3 LD_{50} = 5 μg/100 mg larva), and ButaIT was nontoxic to mice at a dose of 3 μg/g. Both peptides have four disulfide bridges, and modeling studies suggest, in common with other short insectotoxins, both

peptides possess a short α-helix and a β-sheet consisting of three antiparallel β-strands. Two cDNA clones (BTChl-1 and BTChl-2) have been characterized from the mRNA of *H. tamulus* venom glands (Newton et al. 2002; Newton 2004). BTChl-1 encodes BtITx3 and BTChl-2 encodes ButaIT.

With a view to developing insecticides for crop protection, recombinant forms of ButaIT have been developed by two groups. Hammock and colleagues (who originally isolated the peptide) constructed a synthetic ButaIT gene encoding a bombyxin signal sequence, which they then proceeded to express in the Baculovirus *Autographa californica* (Rajendra et al. 2006), whereas Gatehouse and colleagues (Trung et al. 2006) constructed a synthetic ButaIT gene fused to the N-terminus of snowdrop lectin, which was subsequently expressed in the yeast *Pichia pastoris*.

An early study of *H. tamulus* venom has suggested the presence of a range of enzymes, including acetylcholinesterase, alkaline phosphatase, acid phosphatase, 5′ nucleotidase, and deoxyribonuclease (Achyuthan et al. 1982). These data should be treated with a degree of circumspection, as the authors themselves indicate that different batches of venom vary broadly in their enzyme activity levels. Nevertheless, as with all scorpion venoms, enzyme levels are low when compared to the amounts found in snake venoms, and therefore, in light of earlier discussions (Newton et al. 2007), such variability should not be considered surprising.

One activity that has been definitely confirmed is phospholipase A_2. A group III secretory phospholipase A_2 has been cloned from an *H. tamulus* venom gland cDNA library using a primer from the nucleotide sequence from imperatoxin, a phospholipase A_2 enzyme from the scorpion *Pandinus imperator* (Hariprasad et al. 2009). In common with other scorpion phospholipases, the sequence of the transcript of *H. tamulus* phospholipase has a C-terminal extension which is predicted to be cleaved but to remain associated with the rest of the enzyme through a disulfide bridge, resulting in the formation of an active heterodimeric native enzyme. Recombinant His-tagged *H. tamulus* phospholipase A_2 was enzymatically active and showed a pH optimum and calcium dependency in common with other phospholipases. However, SDS-PAGE under reducing conditions produced only a single band suggesting that the predicted furin-type cleavage had not taken place and that the formation of a heterodimer is not essential for enzyme activity. The authors have subsequently modeled the enzyme, including the predicted large enzymatic subunit and the small nonenzymatic subunit, based on bee venom phospholipase A2 and the MU2 adaptin subunit of the AP2 clathrin adaptor, respectively (Hariprasad et al. 2011).

Following on from their physiological studies, Deshpande's group have partially characterized a pulmonary edema producing toxin from *H. tamulus* venom (Deshpande et al. 2005). The toxin (PoTx) was also identified on the basis of augmenting a phenyldiguanide-induced reflex response in rats. The toxin is basic (retained on a cation ion-exchange column), is not neurotoxic (neurotoxic activity removed after dialysis through an 8 kDa filter), and has a molecular weight of approx 100 kDa. The biological effects of PoTx were blocked by aprotinin, in a manner analogous to crude venom. Over 30 years ago, Chhatwal and Habermann

partially characterized a protease inhibitor from *H. tamulus* venom (Chhatwal and Habermann 1981), but this has not, to the authors' knowledge, been followed up. The same authors also demonstrated that the venom contained a histamine releaser.

Conclusions and Future Directions

Although not as well characterized, venom from the Indian red scorpion contains the same broad spectrum of ion channel toxins that predominate in the venoms of many other scorpion of medical importance (e.g., *Tityus, Leiurus,* and *Androctonus*). There is also a similar lack of enzyme activity. As with most species, there is evidence for novel activities, and in the case of *H. tamulus*, the presence of a high molecular weight, pulmonary edema -inducing toxin is particularly intriguing.

When working with pooled venom batches (as for nearly all of the papers discussed in this chapter), knowledge of the origins of the venom sample is increasingly recognized as being of great importance. For example, both tamapin and ButaIT were only detected in *H. tamulus* venom from a single region (Newton et al. 2007). This study has also highlighted another important aspect of discovering new toxins; tamapin, iberiotoxin, and ButaIT, three of the most important toxins characterized in *H. tamulus* venom, were identified through functional screens, although none appear in a table of the most abundant venom constituents from mass spectrometry data – low-abundance molecules may be easily overlooked in genomic studies or cDNA libraries generated from RNA transcripts.

Cross-References

► Scorpion Venom Research Around The World: *Heterometrus* Species

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Scorpion Venom Research Around the World: Chinese Scorpion *Mesobuthus martensii* Karsch

17

Zhi-Rui Liu and Yong-Hua Ji

Contents

Abstract

Scorpions, or "Quanxie" as named in traditional Chinese medical prescriptions, have long been exploited as remedy for expelling or relieving many disorders and syndromes, such as chronic pain, convulsions, cardiovascular diseases, or even tumors. In Chinese traditional medicine, the active part of scorpions is considered to be the tip of the tail containing a great reservoir of active components. However, it was not until recent decades that the pharmacological mechanisms of the venom and toxins of Chinese scorpion have been

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systematically studied. These toxins are known to be toxic polypeptides that specifically target membrane ion channels, through either enhancing the channel activity or suppressing the peak currents of channels, hereby drastically affecting the excitability of local neuronal circuits. Recent mapping of receptor sites of these toxins located on ion channels validates themselves as potential tools for probing and modulating the channel subtypes or gating properties involved in physiological/pathological conditions. While this chapter briefly covers the current knowledge regarding the traditional use of scorpions in traditional Chinese medicine, its main subject is the "state-of-the-art" view of physiological and pharmacological actions of neurotoxins found in the Chinese scorpion *Mesobuthus martensii* Karsch associated with ion channels, including nociceptive/antinociceptive effects, epileptic/antiepileptic functions, cardiovascular modulation, and antitumor effects. Meanwhile, the toxin-driven intracellular events parallel to behavioral phenotypes and the underlying linkage between structure and function of ion channels elucidated by molecular interactions with toxins will also be discussed.

Introduction

The "Combat poison with poison" principle has long been defined as one of the most spiritual essences in Chinese traditional medicine, which is known to employ chemically or biologically toxic drugs originated from plants, microorganisms, venomous animals, and heavy metal compounds to the prevention and treatment of many severe disorders. Among the medically important venomous species tailored to biotherapies since thousands of years ago, such as snake, centipede, toad, bee, etc., scorpions or "Quanxie" account as one of the most extensively documented remedies in Chinese herbalism.

In China, there exist 18 scorpion species which fall into five families, spreading most parts of China and surrounded countries (Table 1), namely, Buthidae, Chaerilidae, Ischnuridae, Scorpionidae, and Scorpiopidae (Zhu et al. 2004). Buthidae family comprises 10 species, in which *Mesobuthus martensii*, also named as *Buthus martensii* Karsch (BmK), widely distributed in north China, Mongolia, South Korea, and even in Japan, stands for the main scorpion species in China and receives the most intensive studies. The second largest family, Scorpiopidae, owns the largest number of scorpion species endemic to China, i.e., *Scorpiops jendeki*, *Scorpiops margerisonae*, *Scorpiops petersii*, and *Scorpiops tibetanus*, three of them are found in Tibet which is the richest source of most of scorpion species and other venomous animals in China. Although wide in geographical distribution and diverse in species classification, few epidemiologic data are reported, which suggest that the scorpionism, an actual public health problem in several parts of the world, is not a public health problem in China according to a global appraisal concerning the worldwide epidemiology of scorpionism based on the well-established Medline data bank search (Chippaux and Goyffon 2008).

Table 1 Distribution of scorpion species in China and around countries

Family	Genus	Species	Distribution
Buthidae	*Hottentotta Birula*	*Hottentotta alticola*	China, Afghanistan, Iran, Pakistan, Tajikistan
	Isometrus Ehrenberg	*Isometrus maculatus*	China (Taiwan, Hainan), Cambodia, India, Laos, Thailand, Vietnam etc.
	Lychas	*Lychas mucronatus*	China (Yunnan, Guangxi, Hainan), Cambodia, India, Japan, Laos, Thailand, Vietnam, etc.
		Lychas scutilus	China (Shanghai), South Andaman (India), Myanmar, Thailand, etc.
	Mesobuthus	*Mesobuthus eupeus mongolicus*	China (Inner Mongolia, Xinjiang), Mongolia
		Mesobuthus eupeus thersites	China (northwest), Iran (northeast), Kazakhstan, Tajikistan, Turkmenistan, Uzbekistan, etc.
		Mesobuthus martensii	China (Inner Mongolia, Beijing, Liaoning, Shanxi, Hebei, Henan, Shandong, Anhui, Jiangsu, Fujian, Hubei), South Korea, Mongolia, Japan
		Mesobuthus martensii hainanensis	China (Hainan)
	Olivierus Farzanpay	*Olivierus caucasicus intermedius*	China (northwest), Iran (northeast), Kazakhstan, Tajikistan, Turkmenistan, Uzbekistan, etc.
		Olivierus caucasicus przewalskii	China (northwest), Mongolia, Tajikistan, Uzbekistan
Chaerilidae	*Chaerilus*	*Chaerilus pictus*	China (Tibet), Bangladesh, India
Ischnuridae	*Liocheles*	*Liocheles australasiae*	China (Hainan), Bangladesh, India, Japan (south), South Korea, Myanmar, Philippines, Thailand, Vietnam, etc.
Scorpionidae	*Heterometrus*	*Heterometrus longimanus*	China (southwest), Indonesia, Malaysia, Philippines, Singapore
		Heterometrus petersii	China, Cambodia, Laos, Philippines, Vietnam
Scorpiopidae	*Scorpiops Peter*	*Scorpiops hardwickii*	China (Tibet), India, Nepal, Pakistan
		Scorpiops jendeki	China (Yunnan)
		Scorpiops margerisonae	China (Tibet)
		Scorpiops petersii	China (Tibet, Sichuan (west)), Bhutan, India, Pakistan
		Scorpiops tibetanus	China (Tibet)

This review systematically introduces the updated knowledge and ongoing progress of the investigations on the venom and toxins of Chinese scorpion, especially on BmK species. Through presenting the active components of BmK venom, molecular structure, classification, and physiological targets in organisms, the pharmacological essences of this species as used in Chinese herbalism will hopefully be clarified and evaluated. More meaningfully, this review will widen the view of potential applications to the understanding of the underlying mechanisms of neuronal disorders, such as pain and epilepsy. Also, by exploring the molecular interactions between BmK toxins and ion channels, the significant role of BmK toxins as probing tools for the study of structure-function relationship of ion channels will be highlighted, which validates themselves as molecular templates or medically leading compounds for developing novel drugs to channelopathies.

Toxic Constituents of BmK Scorpion Venom

The main function for Chinese scorpion venom is to defend and prey. The venom of BmK is considered to be only weakly toxic to mammals when compared to the venom of other famous scorpion species belonging to Buthidae family (Table 2). The low toxicity to mammals of BmK venom can fairly explain the reason for scare case of scorpion envenomation in China. Comparatively, the venom of BmK is highly toxic to insect species (Table 3), which might be because of the higher injection amount to the insect body than that to mammalian counterparts.

Table 2 LD_{50} values for partial scorpion species documented in the book contributed by Liu et al. (2007)

Scorpion	Distribution	LD_{50} (mouse)/ $mg \cdot kg^{-1}$	Administration method
Androctonus australis	From North Africa to West Pakistan	0.32	–
Androctonus mauretanicus mauretanicus	Morocco	0.31	i.v.[a]
Mesobuthus martensii Karsch	China, northeast of Asia	2.40	s.c.[b]
Buthus occitanus tunetanus	Sudan, Africa	0.90	–
Centruroides sculpturatus	From Mexico to Arizona state in the southwestern United States	1.12 ~ 1.46	s.c.
Leiurus quinquestriatus	Africa, Middle East, Israeli, Syria	0.25	s.c.
Tityus serrulatus	South America	0.66	i.v.

[a]Intravenous injection
[b]Subcutaneous injection

Table 3 Toxic activity and targeted ion channels of BmK venom or toxins

Classification	Toxin name	Length (in a.a)	LD_{50}	MLD[a] (mg/kg mouse)	CPU[b]	Targets	References
	Venom	–	2.4 (mg/kg mouse)[c]	0.074	1 μg/100 mg (blowfly larvae)	–	(Ji et al. 1996)
					2 μg/500 mg (cricket)		
α-like	BmK I	64	–	0.0018	0.29 μg/100 mg (blowfly larvae)	VGSC	(Ji et al. 1996)
					0.75 μg/500 mg (cricket)		
	BmK II	64	–	0.0111	0.88 μg/100 mg (blowfly larvae)	VGSC	(Ji et al. 1996)
					1.25 μg/500 mg (cricket)		
	BmK αIV	66	0.208 (μg/20 g mouse)[d]	–	–	VGSC	(Chai et al. 2006)
			0.47 (μg/100 mg cockroach)				
α-excitatory anti-insect	BmK IT	70		1 μg/500 mg (cricket)	7 ng/100 mg (blowfly larvae);	VGSC	(Ji et al. 1994)
β-depressant anti-insect	BmK IT2	61		ND	0.2 μg/500 mg (cricket)	VGSC	(Ji et al. 1994)
β-like	BmK AS	66		ND	ND	VGSC	(Ji et al. 1999)

(*continued*)

Table 3 (continued)

Classification	Toxin name	Length (in a.a)	LD_{50}	MLD[a] (mg/kg mouse)	CPU[b]	Targets	References
Weak β and α′	BmK abT	64		1.5	5 μg/cockroach	VGSC	(Ji et al. 2002)
KTx	Martentoxin	37		ND	ND	BK_{2+}	(Ji et al. 2003)
KTx	BmP01	29	>5 (μg/20 g mouse)	ND	ND	SK_{Ca2+}	(Romi-Lebrun et al. 1997)
KTx	BmP02	28	5 (μg/20 g mouse)	ND	ND	SK_{Ca2+}, Ito,Kv	(Romi-Lebrun et al. 1997; Tong et al. 2000)
KTx	BmP03	28	10 (μg/20 g mouse)	ND	ND	SK_{Ca2+}, Ito,Kv	(Romi-Lebrun et al. 1997; Tong et al. 2000)
KTx	BmP05	31	37.1 (ng/20 g mouse)	ND	ND	SK_{Ca2+}	(Romi-Lebrun et al. 1997)

[a]After intracerebroventricular (i.c.v.)
[b]Contraction paralysis unit
[c]After intraperitoneal injection (i.p.)
[d]Minimal lethal dose

Generally, the toxic mixture of scorpion venom mainly consists of pharmacologically active proteins, such as enzymes and neurotoxins. The BmK venom is composed of 45.58 % carbon, 5.83 % hydrogen, 15.21 % oxygen, and 28.8 ~ 29.2 % sulfur (Liu et al. 2007).

Usually, toxic enzymes are also present in scorpion venom, although vary with different species accounted. Among them are phospholipases A_2 (PLA_2), which can present hemolytic activity. In addition, hyaluronidase, known to facilitate the penetration of other toxins during envenomation, can also be found in the venom of BmK. This toxin class will not be discussed in this review.

Up till now, tens of different toxins or homologous peptides have been isolated from the BmK venom, only several representatives were pharmacologically or toxicologically characterized, which deemed to be specific modulators or blockers on voltage-gated ion channels. Traditionally, based on their size and functional characterizations, they are divided into two groups: long-chain and short-chain polypeptide toxins.

Long-Chain Polypeptide Toxins

This class of BmK toxins usually is composed of 60–70 amino acids organized by four disulfide bridges. Most long-chain BmK toxins have been characterized as voltage-gated sodium channels (VGSC)-specific modulators. Initially, they were classified into mammalian toxins (MTx), insect toxins (ITx), and crustacean toxins (CTx) based on their toxicity to mice, insect adult/larvae, and isopods, respectively. However, the concurrent investigations found this classification seemed not to be strict as there exist some toxins having distinct paralytic effects to both mammals and isopods. Comparatively, the classification of ITx was reasonable, showing high specificity to insect species. Consequently, Zlotkin et al. further defined the ITx into two major groups: contraction paralysis-type (CP-type) toxins which induced a rapidly reversible, fast contraction paralysis in blowfly larvae and flaccid paralysis-type (FP-type) toxins which induced a slow progressive onset of flaccid paralysis preceded by a short transient phase of contractility into larvae (Zlotkin et al. 1972). The toxicity on insects of each type of toxins can therefore be quantified by CPU and FPU (flaccid paralysis unit). Table 3 listed the partial CPU values of several representative long-chain toxins from BmK venom.

According to their mode of actions and binding affinities on sodium channels, scorpion toxins are divided into (1) α-toxins that include classical α toxins (highly specific to mammals), α-insect toxins (highly specific to insects), and α-like toxins (acting on both mammals and insects, but unable to bind to rat brain synaptosomes) and (2) β-toxins that include anti-mammalian β toxins, anti-insect excitatory/depressant β-toxins, and β-like toxins (acting on both mammalian and insect VGSCs) (Rodriguez de la Vega and Possani 2005). They are identified as specific gating modulators of sodium channels by affecting activation or inactivation kinetics. Even until now, this classification is continuously subjected to challenges

in dealing with some structural and functional transition toxins which blurred the boundary between different scorpion toxin subgroups.

By mapping these toxins to the receptor sites they target on sodium channels, they can be classified into (1) site-3 modulators, which bind to DI S5-S6 and DIV S3-S4 of the VGSCs, and (2) site-4 toxins, which bind to DII S1-S2, S3-S4, and DIII S5-S6 of the VGSCs (Catterall et al. 2007). The binding properties and molecular mechanism of site-3/site-4 modulators from BmK venom will be discussed in section "Molecular Interaction of BmK Neurotoxins with Membrane Ion Channels."

BmK I (UniProt number: P45697) is the most abundant neurotoxin in BmK venom, amounting to ~70 % in dry weight of all the neurotoxins in the whole venom (Liu et al. 2007). It is proved to be a site-3 sodium channel modulator and belongs to the α-like toxin group. BmK I could be toxic to both mammals and insects (see Table 3). The toxicity test showed that BmK I was six times more potent than BmK II (UniProt number: P59360), a toxin purified from the same venom. Only two amino acid replacements were found: at position 59, Val in BmK I was replaced by Ile in BmK II; and at position 62, a basic Lys residue in BmK I was substituted by a neutral Asn residue in BmK II. These features suggest that, apart from the conserved residue K58 critical for α toxins activity (Fig. 1), the positively charged residue (Lys or Arg) in the C-terminal position 62 (or 61 or 63) may also play an important role in facilitating the interaction between scorpion neurotoxins and the receptor on sodium channels, pioneering the paradigm of "point to face" of toxins (Ji et al. 1996).

BmK αIV (UniProt number: Q4TUA4), a 66-peptide toxin cloned from venomous glands of BmK, is unique to other known long-chain α toxins in structure and is considered to be a transitional toxin between classic α-toxins and α-like toxins, standing to be an independent clade in phylogenetic tree of α-toxins (Fig. 2). BmK αIV has potent toxicity in mice and cockroaches, with the LD_{50} of 0.21 μg/20 g of body weight to mice and 0.47 μg/100 mg of cockroach body, respectively (Table 2). It is well defined that the interaction surface of α-toxins and sodium channels contained at least two distinct domains. The core domain (residues 17, 18, 38, and 44) appeared in BmK αIV is fairly conserved as that of other α-toxins, which is responsible for recognition of receptor site-3 on sodium channels. The NC domain (residues 8–10 and 56–64) is responsible for high affinity and the specificity toward sodium channel subtypes (Bosmans and Tytgat 2007). In this domain, the N-terminus residues D8-D9-K10 in BmK αIV were similar to those of AaH II (D8-D9-V10), whereas it differed in Lqh αIT (K8-N9-Y10) and BmK I (K8-P9-H10) (Fig. 1). The C-terminus segment (position 56–64) of BmK αIV was similar to that of LqhαIT, a representative α-insect neurotoxin, indicating that this region might be involved in BmK αIV binding to insect sodium channel subtypes. Residue P17 in LqhαIT, which might interact directly with cockroach sodium channel receptor site, was replaced by glycine in most classical α-mammal toxins, as well as in BmK αIV. In addition, the extra sequence N64-G65-G66 of BmK αIV is rarely seen in other known scorpion neurotoxins (Chai et al. 2006). Thus, the diversities of functional surface formed by the above regions might be

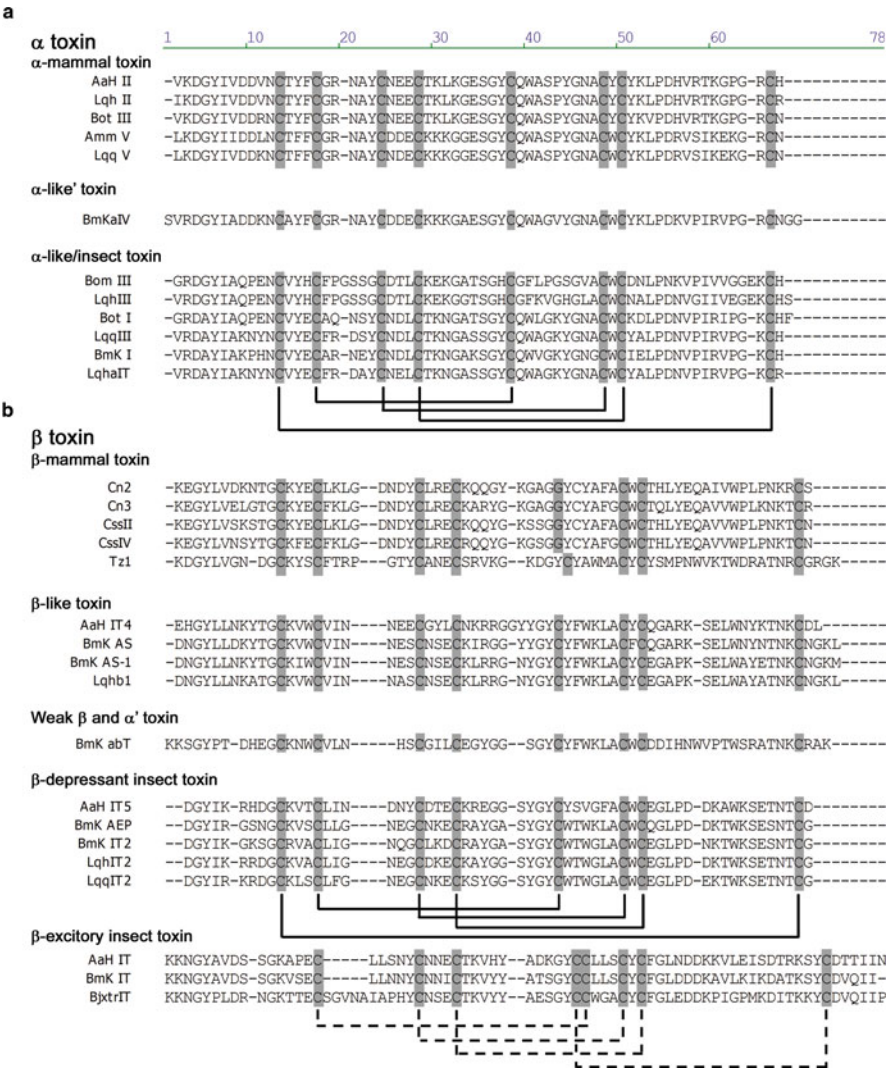

Fig. 1 Sequence alignment of long-chain scorpion toxin representatives of various pharmacological groups (see text). They were aligned taking as reference the Cys residues (shaded in *gray*). The length of toxin was indicated at the *top* of alignment. Dashes indicate gaps which were introduced to maximize similarities. The conserved (*solid line*) and the non-conserved (*dashed line*) disulfide bridges are depicted at the *bottom*

determinants for subtle binding and toxicity features of BmK αIV and indicated that BmK αIV may be a distinct structural and functional hybrid of α-toxins.

Similarly, BmK abT (UniProt number: Q9NBW2) showed poor structural identity (30 ~ 35 %) with most other known toxins purified from BmK venom (e.g., BmK I, BmK IT (O61668), and BmK IT2 (P68727)) and also very low

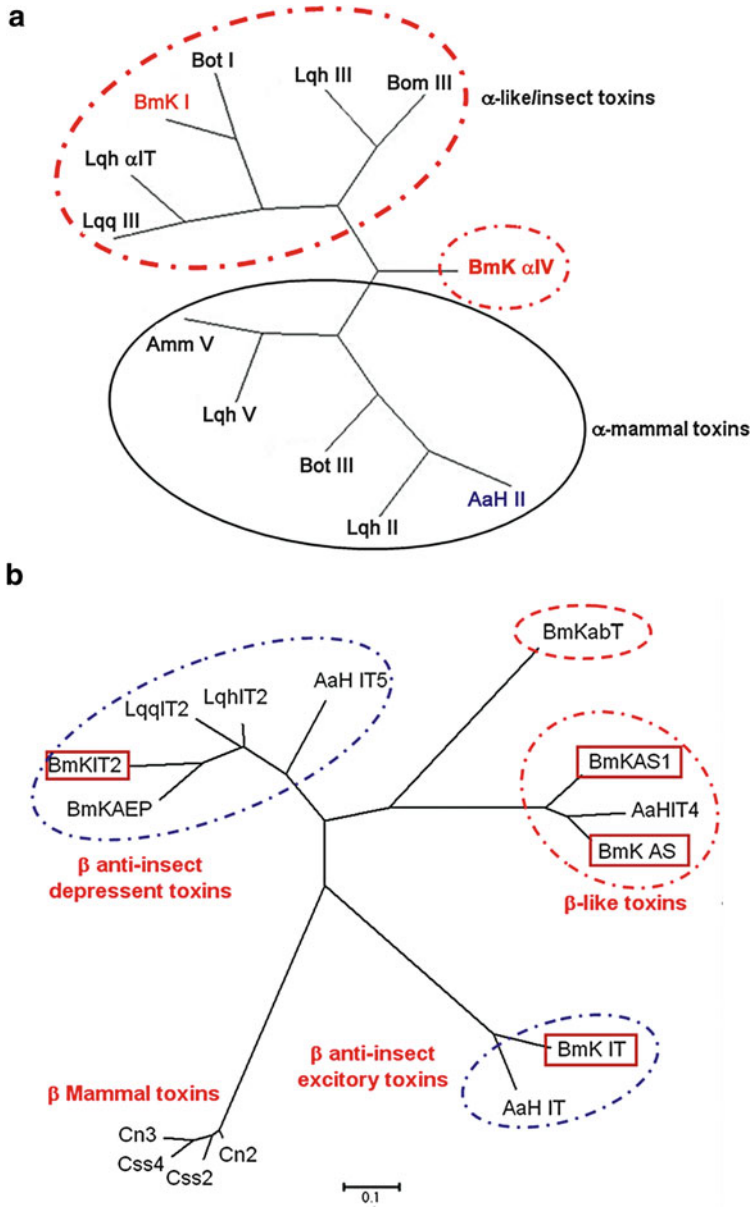

Fig. 2 Phylogenetic tree of representative long-chain scorpion toxins. (**a**) α-toxins, (**b**) β-toxins. Toxins from BmK were highlighted by red boxes. The genetic distances and relatedness of the toxins have been calculated by neighbor-joining method. This phylogenetic tree has been made using the programs CLUSTALW and MEGA 5.2

similarity (20 ~ 30 %) with many other scorpion toxins such as Css II, a typical β-mammal toxin, and Lqq V and AaH I, two α-mammal toxins. Toxicity assay showed that BmK abT could work on both mammals and insects, with the MLD of about 1.5 μg per mouse and the dose induced significant paralysis about 5 μg on cockroach (Table 3). The patch-clamp recording on dorsal root ganglion (DRG) neurons showed that this toxin could prolong the action potential and increase the amplitude of the peak Na^+ currents, which are the typical characters of α-toxin. Given its functional consistency with α toxins but structural homology with β toxins, BmK abT is regarded as novel transitional member between α- and β-toxins and stands for an independent clade of all the toxins considered (Fig. 2).

BmK IT2 is deemed to be the most potent insect-depressant toxin in BmK venom. Structural analysis indicated that BmK IT2 shares high identity, about 88 %, with Lqh IT2, the most intensively investigated β-depressant insect toxin so far. BmK IT2 was also found to be homologous to BmK AEP (UniProt number: P15228), a 61-residue toxin having eminent antiepilepsy effect in rats, which indicated the similar function of BmK IT2. The pathological properties and underlying mechanisms involved with BmK IT2 will be discussed in more detail later.

BmK AS and BmK AS-1 (UniProt number: Q9UAC9 and Q9UAC8) are two neurotoxin possessing a low sequence relatedness, identity lower than 30 % with other α- or β-toxins, but sharing high similarity in sequence only with the anti-insect toxin AaH IT4, a unique anti-insect toxin and a ligand of Na^+ channels obtained from Sahara scorpion *Androctonus australis*. Therefore, together with AaH IT4, BmK AS/AS-1 are clustered into an independent subgroup of scorpion toxins, named as β-like toxins. Toxicity tests in vivo showed that both BmK AS and BmK AS-1 have no toxic effect on mammals, and their toxicity on insects is weaker than other BmK neurotoxins mentioned above. It is worth to notice that BmK ASs seem to have multiple pharmacological effects, such as stimulating [^{3}H]-ryanodine binding to the skeletal-type ryanodine receptors similar to the imperatoxin activator and binding to sodium channels. Moreover, BmK AS was also found to enhance [^{3}H]-noradrenalin release from rat hippocampus slices (Kuniyasu et al. 1999) (it was named as BmK-PL in that paper). It has been demonstrated that the charged residues located in N-terminal and C-terminal regions (NC domain) of scorpion toxins play a multiple role in the interaction with their target receptors. The ionization properties either in NC domain between BmK ASs and AaH IT4 seem to be different, due to the His residue at position 2 in AaH IT4 being replaced by a neutral residue Asn-2 in BmK ASs, and a basic residue Lys-65 in BmK ASs is absent in AaH IT4 (Fig. 1). In addition, one Met residue at C-terminal of BmK AS-1, when oxidized, the resulted sulfoxide variant, called BmP09, brought about a dramatic switch from a Na^+ channel modulator to a BK channel blocker (Yao et al. 2005). These subtle but substantial alterations may render the structure of BmK ASs to be more flexible than AaH IT4, which might explain the multivalent functions as mentioned above.

BmK IT was the first excitatory insect toxin found in BmK venom and second in scorpion species of worldwide range (the first excitatory insect toxin found was AaH IT from *Androctonus australis* venom), which is indicated to have applications in pest control. The arrangement pattern of disulfide bridges in BmK IT is unique, when compared to other type of scorpion toxins, in that the first bridge-forming Cys residue is shifted to an inner location (at position 38) which is often seen at position 12 in mammal toxins structure (see dotted line in Fig. 1). This distinct disulfide-linking pattern may well explain the specificity to insect but no toxicity to mammals for BmK IT and AaH IT. Loret et al. have pointed out the important role of K28 and K51 in the activity of AaH IT and reported that the modification of H30, K34, and R60 had no significant impact on the biological activity (Loret et al. 1990). Since K28 and K51 are also conserved in the structure of BmK IT, while H30, K34, and R60 seen in AaH IT are substituted by Y30, S34, and T60 in BmK IT, it thus suggests that the positive charge K28 and K51 are important for CP-type insect toxins, while residues at position 30, 34, and 60 play minor roles. Moreover, the amino acid residue is Pro at position 14 in AaH IT, but it appears to be Ser in BmK IT. This change may also account for the activity difference between BmK IT and AaH IT (Fig. 1).

Short-Chain Polypeptide Toxins

BmK venom has been shown to have weak blockade of voltage-gated K^+ channels in NG108-15 cells, while a reversible blocking effect could also be seen on transient outward K^+ currents in neonatal rat ventricular cells. These observations indicated that there are components acting on Kv channels in the BmK venom. This group of toxins usually consists of 28–40 amino acid residues (2.8–4.5 kD), whose structures are rich in alkaline residues and tightly compacted by three to four disulfide bridges. By far, most of these toxins are identified as K^+ or Cl^- channel blockers (Rodriguez de la Vega and Possani 2004).

According to the commonly accepted classification forwarded by Tytgat et al. (1999), short-chained scorpion toxins can be divided into four major families (α-, β-, γ-, and κ-KTxs). According to the sequence updated from the latest UniProtKB/Swiss-Prot Tox-Prot database (http://www.uniprot.org/program/Toxins), 14 toxins have been found in the BmK venom evident in protein level, among which 13 of them are classified as α-KTxs comprising 10 different subgroups, one is considered being chlorotoxin-like peptide (Fig. 3). Briefly, BmTX1 and BmTx2 (UniProt number: Q9NII6 and Q9NII5) belong to the α-KTx 1.x subgroup, which are known to be potent blockers of both large-conductance calcium-activated potassium channels (BK channels) and voltage-gated potassium channels (K_v1.3/KCNA3); BmKTX (UniProt number: Q9NII7) is included in α-KTx 3.x subgroup, which is deemed to be voltage-gated K^+ channel blockers; BmP05 is an α-KTx 5 subgroup member toxin homologous to P05, which is found

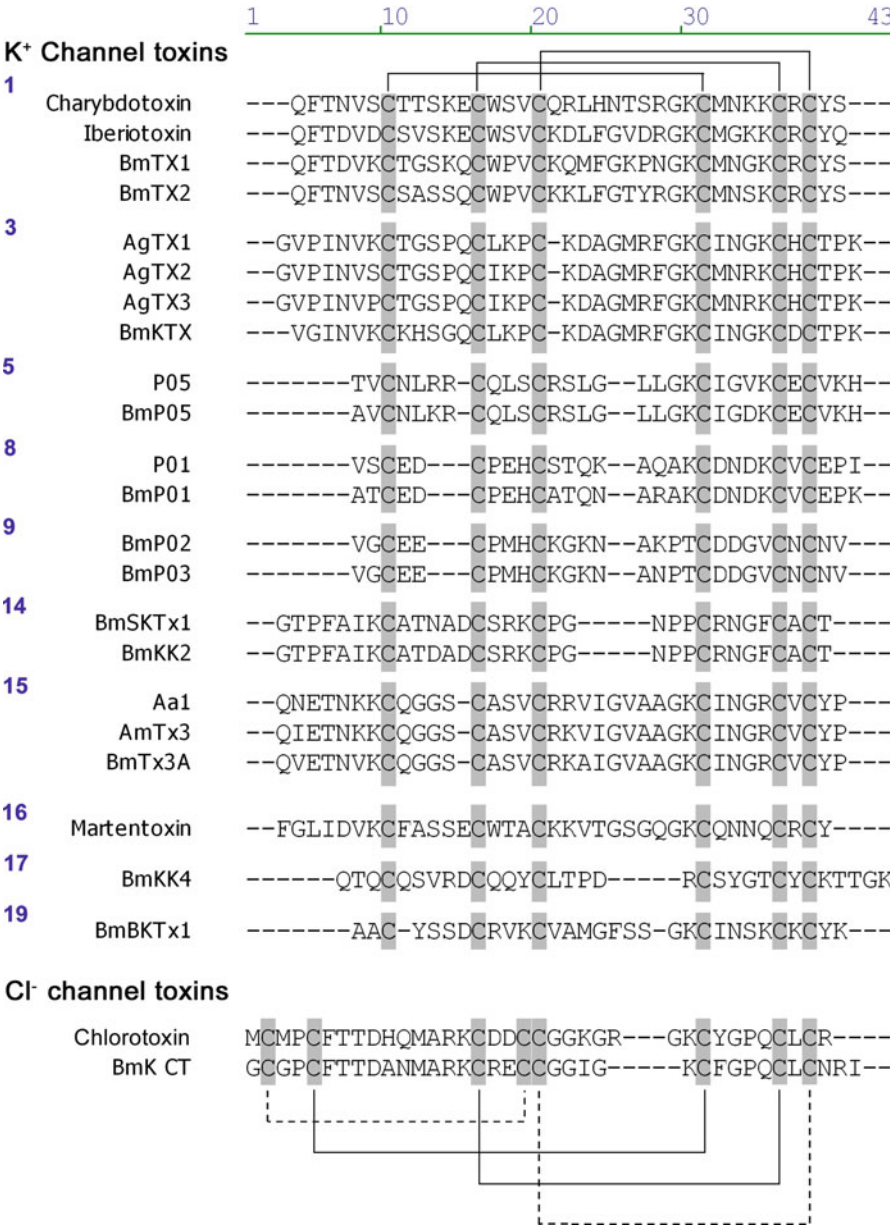

Fig. 3 Sequence alignment of BmK short-chain toxins and representatives of known homologous (see text). Numbers in blue indicate the subgroup of α-KTx. The layout descriptions were the same as in Fig.1

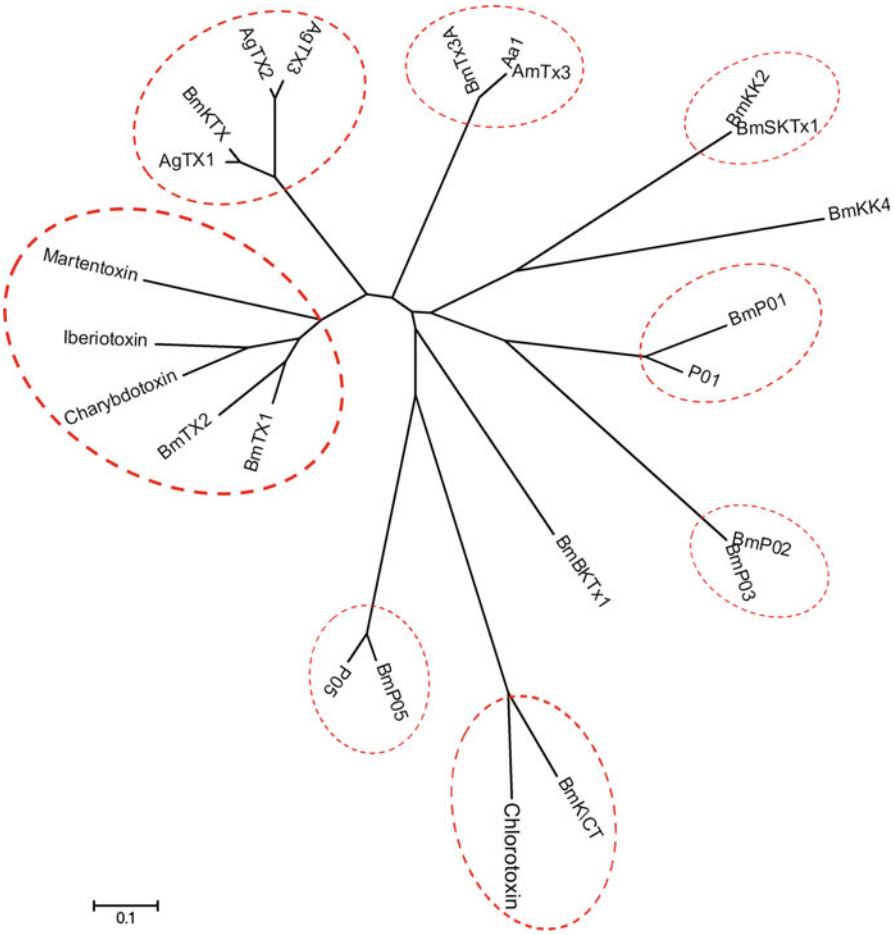

Fig. 4 Phylogenetic tree of representative short-chain scorpion toxins. Major clustering groups were highlighted by *dotted circles*. The construction method has been described in Fig. 2

to be specific to small-conductance K^+ (SK) channels (Fig. 4); and BmTx3A (UniProt number: Q8I0L5) is a toxin classified as α-KTx 15.2 because of its inhibitory activity on Ia currents of cerebellum cells and hKv11.1 expressed in oocytes. Interestingly, since the functioning sites of BmTx3A for the blockade of $hK_v11.1$ were spatially separated and independent from that of Kv on cerebellum cells, BmTx3A was also grouped into γ-KTx family.

Martentoxin (UniProt number: Q9NBG9), a 37-peptide of α-KTx16 toxin, is the most intensively studied short-chained toxin specific to BK channels in BmK venom. By sequence comparison with other K^+ channel toxin, Martentoxin showed a poor sequential similarity (35–50 %) with CTX (Charybdotoxin) and KTX (Kaliotoxin), even with other short-chained toxins from the same venom, including BmTX1-2 and BmKTX. The secondary structure of Martentoxin consists

of a triple-stranded β-sheet connected to an α-helical structure. This helix encompasses 10 residues from S11 to K20. The three strands of β-sheet comprise residues G2-D5, Q27-N30, E33-C36, and C30-N33 with a type I'β turn centered on N31-N32. In solution, this toxin adopts a typical CSαβ structure, with an α-helix connected to a triple-stranded β-sheet by three disulfide bridges, which is conserved among other toxins belonging to α-KTx family. The functional surface (β-face) of Martentoxin varies much with other classic BK channel toxins (such as ChTX and IbTX), which indicates a potentially unique interaction pattern with BK channels (see section "Molecular Interaction of BmK Neurotoxins with Membrane Ion Channels").

BmP02 and BmP03 (UniProt number: Q9NJP7 and Q9U8D1), belonging to α-KTx 9.x subgroup, are the smallest K^+ channel ligands identified in BmK venom. They are 28-residue peptide found to be capable of inhibiting the currents of transient outward K^+ channel in adult rat ventricular myocyte and had weak activity on SKCa channels. Like P01, BmP02 exhibited a low toxic activity in mice after intracerebroventricular injection (LD_{50} were 5 and 10 μg per mouse, respectively; Table 2) and competed only weakly ($K_{0.5} > 1$ μM) with iodinated apamin for binding to SKCa channels. NMR spectroscopy analysis revealed that BmP02 formed an α/β fold, the typical three-dimensional structure adopted by most short-chain scorpion toxins. This α/β fold was largely distorted in BmP02, whose α-helix was shortened to only one turn, and the loop connecting the helix to the first β-strand exhibited conformational heterogeneity. The instability of BmP02 structure in solution could be attributed to a proline at position 17, which is usually a glycine in other KTxs. This had a significant influence on the structure and function of BmP02. According to its structure, BmP02 was supposed to interact with its target via the side chains of K11 and K13 (Xu et al. 2000).

The Pharmacological and Pathological Modulation of BmK Neurotoxins

Both physiological and pharmacological evidence implicated a critical role of VGSCs in the development and maintenance of hyperexcitability observed in primary afferent neurons following nerve and tissue injury. In case of peripheral nerve injury, DRG neurons generate spontaneous action potentials or abnormal high-frequency activity that contributes to chronic pain. VGSCs are pivotal to the initiation and propagation of action potentials, and hence, the critical role of VGSCs in many types of chronic pain syndromes has been extensively demonstrated. Since scorpion venom is a rich source of bioactive modulators interacting specifically with various ionic channels in excitable membranes, especially those long-chain polypeptides that selectively modulate the functional features of sodium channel subtypes, BmK sodium channel modulators and venom are deemed to be outstanding dissectors of the pain mechanism and a valuable source to identify novel analgesic agents.

Epilepsy is an episodic disorder of the nervous system arising from the excessive synchronous and sustained discharge of a group of neurons. Abnormal focal or

generalized synchronized electrical discharges within the central nervous system (CNS) induce epileptic behavioral seizures. As VGSCs play a significant role in neuronal excitability, the abnormal synchronized electrical discharges may be evoked by the alteration of sodium channels gating or expression. On the other hand, it is also clarified that antiepileptic drugs may paradoxically worsen seizure frequency or induce new seizure types in some patients with epilepsy. Therefore, learning about the molecular and cellular mechanism of sodium channel involvement in epileptogenesis and identification of new potential antiepileptic drugs may form two sides of an organic whole. Long-chain BmK neurotoxic polypeptides specifically targeting on sodium channels have been reported to possess inductive or suppressive effects on epileptic seizures.

This section will also cover the application of BmK toxins for the research on glioma and cardiovascular cells, which may hopefully provide clues for understanding the pathogenesis and development of treatment strategies for the associated disorders.

Nociceptive/Antinociception of BmK Venom/Toxins

Long-chain BmK neurotoxins possess nociceptive/antinociceptive effects on inflammatory pain in terms of their diverse functions to the Na^+ channels. For example, BmK I is considered to be one of the major contributors of the nociceptive effect induced by the BmK venom, and the antinociceptive activities of BmK ASs (BmK AS and BmK AS-1) and BmK IT2 have been identified as well. In the present review, we summarized the nociceptive/antinociceptive effects of BmK toxins in rats and cellular mechanisms involved in central or peripheral sensation induced by these toxins.

Nociception and Inflammation of BmK Venom/Toxins

Experimental Scorpion Sting Studies

BmK venom injection induces a tonic, monophasic nociceptive response characterized by continuously flinching, licking, and lifting of the paw ipsilateral to injection, but not of the contralateral paw. The mean pain scores induced by BmK venom dose-dependently reached peak intensity within 20 min, then decreased slowly, and lasted for more than 1 h after the injection of BmK venom. With the relative amount of BmK I, similar patterns of nociceptive spontaneous behaviors evoked by the crude BmK venom were also produced. Differently, the mean pain scores induced by BmK I were much lower than those induced by BmK venom. It thus suggests that BmK I is a major contributor to the nociceptive responses elicited by scorpion BmK sting (Bai et al. 2010). Moreover, BmK abT has been verified to be another contributor of pain induced by BmK venom. Compared with other well-characterized pain stimuli such as bee venom, formalin, and carrageenan, the "nature tonic pain" induced by BmK venom/BmK I possesses distinct features in induction, development, and maintenance.

Peripheral Hypersensitivity of Nociception and Inflammation Induced by BmK Venom/Toxins

BmK I could induce several kinds of inflammatory pain-related behaviors including spontaneous pain companied with unique episodic paroxysms, primary thermal hypersensitivity, and unique mirror-image mechanical hypersensitivity with different time course of development, which could be suppressed by morphine, indomethacin, or bupivacaine to different extents. The dramatic attenuation by pretreatment with resiniferatoxin (RTX), an ultrapotent analog of capsaicin, on BmK I-induced pain-related behaviors, paw edema, and spinal L4–L5 c-Fos expression demonstrated that capsaicin-sensitive primary afferent neurons played important roles in pain induced by BmK I.

In periphery, plasma extravasation, recruitment of immune cells, degranulation of mast cells, and histamine release at injury site had been demonstrated to contribute to BmK venom-induced peripheral sensitization and inflammatory responses in rats (Chen et al. 2002). Similar with that of BmK I, the functional depletion of capsaicin-sensitive primary afferent fibers by systemic administration of resiniferatoxin (RTX) also attenuates rat pain-related behaviors and paw edema induced by BmK venom.

Using the single-fiber recording technique, it was found that BmK I could induce dramatic increase in excitability of rapidly adapting (RA) and type I slowly adapting (SAI) low-threshold mechanical A fibers of rat by increasing the response to stimulation at higher frequencies and altering the firing pattern to burst model (Liu et al. 2009). Thus, the gigantic abnormal activity in low-threshold mechanical A fibers is involved in BmK scorpion sting pain.

Central Sensation of Nociception and Inflammation Induced by BmK Venom/Toxins

While it has been assumed that a long-chain sodium channel modulator such as BmK I could not go through BBB (brain-blood barrier) by either adsorptive- or receptor-mediated endocytosis mechanisms, it was concluded that the pharmacological effects of these toxins might be mainly resulted from direct modulation on peripheral nervous system absolutely (Li et al. 2000). Recent investigations suggested that BmK I/venom may induce nociception and inflammation on central nervous system through an indirect pathway.

In central, spinal astrocyte and microglia in bilateral spinal cord are activated with different time courses following BmK venom administration (Jiang et al. 2009). Dynamic release of excitatory amino acids from dorsal horn could be also triggered by peripheral administration of BmK I/venom (Zhang et al. 2002). The increase of c-Fos expression, which is a functional marker of neurons activation, could be evoked spatially and temporally in rat spinal cord by intraplantar injection of BmK I/venom. Additionally, bilateral upregulation of neuronal nitric oxide synthase (nNOS) expression in spinal cord was involved in BmK venom-induced pain (Liu et al. 2008). Intracellular phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in spinal cord dorsal horn was found to be activated by peripheral administration of BmK I/venom as well. The distribution of the

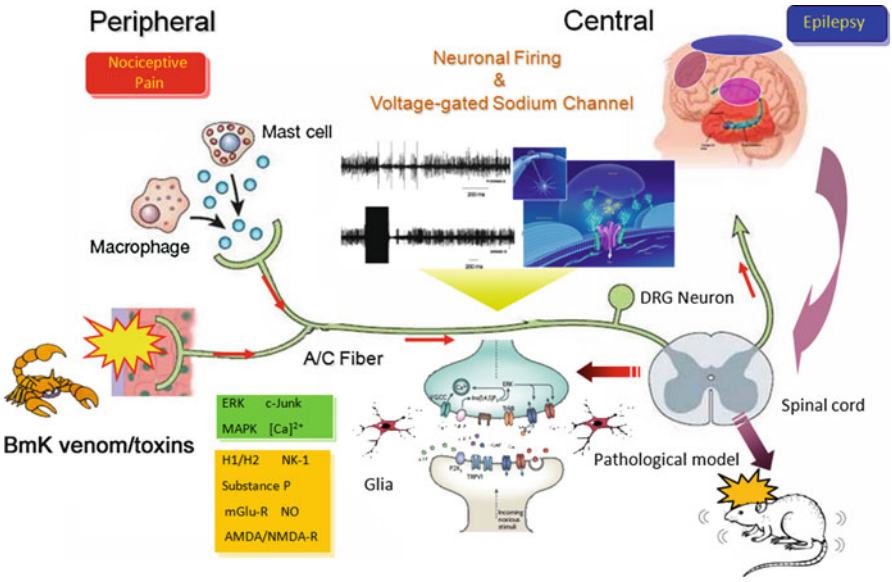

Fig. 5 Schematic illustration of central and peripheral mechanisms of BmK I-induced excitability

neurons kindled by BmK I in related forebrain areas is distinct from those in classic formalin-induced pain model (unpublished data).

Taken together, nociceptive responses induced by BmK I/venom may contribute to the activation of multiple signaling pathways associated with peripheral and central sensitization (Fig. 5).

Antinociception and Anti-inflammation of Sodium Channel-Specific Site-4 Modulators

Nociception induced by inflammatory chemicals or electrical stimulation could be reduced significantly by peripheral administration of BmK AS or BmK AS-1 in rats (Chen and Ji 2002). Likewise, formalin-induced spontaneous pain, carrageenan-evoked thermal hyperalgesia, and the C component of the nociceptive reflex flexion evoked by electrical stimulation could be suppressed by peripheral local administration of BmK AS or BmK AS-1 (Liu et al. 2008). Intrathecal injection of BmK IT2 could suppress formalin-induced spontaneous pain behaviors and spinal c-Fos expression (Bai et al. 2007). In the peripheral system, a similar biphasic inhibition of Na^+ currents by BmK IT2 was found in rat small DRG neurons. The inhibition of TTX-R Na^+ currents by BmK IT2 was much more evident than that of TTX-S Na^+ currents, but the TTX-S Na^+ currents were inhibited more rapidly. The different action kinetics of BmK IT2 on the TTX-S and TTX-R Na^+ currents suggested the production of the biphasic inhibition of BmK IT2 on the C component of the nociceptive flexion reflex. The first inhibitory phase on the C component might be caused by inhibition of the TTX-S Na^+ channels, whereas the second phase might result mainly from decrease of the TTX-R Na^+ currents (Tan et al. 2001).

Similar with the case of BmK IT2, BmK AS could also cause a dose-dependent inhibitory effect on C component of nociceptive responses and depress the peak TTX-S and TTX-R Na^+ currents. Moreover, on small DRG neurons, BmK AS could efficaciously and directly suppress inflammation-induced hyperexcitability of primary sensory neurons and in turn decreased central sensitization (Chen et al. 2006).

Epileptogenesis/Anticonvulsion of BmK Toxins

The Epileptic Seizures Induced by BmK Toxins

Minor partial/limbic seizures could be induced by the lower dose of BmK I (0.1 μg), whereas strong generalized motor seizures and high lethality could be evoked by the higher dose of BmK I (0.5 and 1 μg).

Similarly, BmK αIV could also induce epileptic behavioral and electroencephalographic seizures through the unilateral intracerebroventricular injection. In contrast to BmK I, BmK αIV beared distinct specificity for induction of epileptic seizures. The dose of BmK αIV used for the seizure occurrence was higher but the stages of seizures were lower, and the latent period of seizures evoked by BmK αIV was longer.

Though other proteins might be involved in the epileptogenesis induced by the neurotoxins, the sodium channels expressed in the central nervous system such as hippocampal region were the vital targets. The inactivation of Na^+ currents in rat-cultured hippocampal neurons was prolonged significantly by BmK I while the inactivation time and the peak amplitude of the currents from $Na_v1.2$ channels expressed in *Xenopus laevis* oocytes were both increased by BmK αIV (Chai et al. 2006). $Na_v1.6$, one of the major contributors to neuronal firing in central nerve system, was found to be significantly modulated by BmK I through amplifying the persistent currents and prolonging the inactivation time course (He et al. 2010).

BmK αIV-evoked epileptogenesis may be related to the glutamate release from synaptosomes associated with an increase in Ca^{2+} and Na^+ influx. The similar increase in $[Ca]^{2+}$ and $[Na]^+$ could also be found in rat brain synaptosomes after BmK I administration. The glutamate release and ion influx were completely blocked by TTX, suggesting that the induction of BmK I-evoked epileptic seizures may be solely attributed to TTX-S sodium channels located on the nerve terminal, which subsequently enhances the Ca^{2+} influx to cause an increase of glutamate release.

Anticonvulsion of BmK Toxins

Pentylenetetrazol (PTZ)-induced generalized seizures in behavior and electroencephalograph (EEG) could be significantly inhibited by unilateral injection of BmK IT2 into CA1 region of hippocampus. The hippocampal c-Fos expression during status epilepticus was suppressed by pretreatment of BmK IT2. Concurrently, the latency to status epilepticus onset induced by pilocarpine was also prolonged. Since BmK IT2 could inhibit the peak Na^+ currents of TTX-S channels in the cultured hippocampus neurons as seen in DRG neurons, it was assumed that VGSCs were involved in the decrease of neural excitability modulated by BmK IT2 in the central neural system (Zhao et al. 2008).

Likewise, BmK AS could suppress seizure-associated behavior and reduce both the number and duration of high-amplitude, high-frequency discharges (HAFDs) in the PTZ model. This anticonvulsant activity was attributed to the inhibition of I_{Na} in rat hippocampal neurons. In contrast, BmK AS did not affect the epileptiform EEG in the pilocarpine model over the same dose range, although it did increase the latency to status epilepticus onset and slightly, but significantly, reduced the seizure score.

Cardiovascular Modulation of BmK Toxins

In addition to the modulation of BmK toxins on neuronal systems, BmK toxins are capable of producing complex mechanical and electrical activity changes in heart muscle which could be explained by the induction of neurotransmitter release from postganglionic nerve fiber and the modulation on ion channels.

Cardiac Excitation-Contraction

The blood pressure in conscious rats could be significantly increased by BmK venom. The increase in blood pressure occurred very quickly and was relatively permanent. Notably, BmK venom could not modify the contraction frequency but increase the force of contraction of isolated atrial strips or isolated arterial strips from the aorta and renal and vertebral arteries. The latter contraction occurred very slowly, suggesting a direct effect of BmK venom on cardiac myocytes. This was in accordance with the accumulative evidence of the increased metabolism of IP3 in dispersed cardiac myocytes and the unchanged $[Ca^{2+}]_i$ in cultured vascular smooth muscle cells and endothelial cells after the administration of BmK venom. Contrarily, heart rate was not changed by the BmK venom in conscious rats.

BmK I was capable of evoking complex modulation on rat cardiovascular activity by increasing the maximal left ventricular-developed pressure ($LVDP_{max}$) and dp/dtmax, positive chronotropic effect accompanied with varied coronary flow rates, and negative inotropic action and bradycardia. In addition, tachycardia and complex cardiac arrhythmias were induced by BmK I as well (Sun et al. 2005).

Electrophysiological Basis

Of course, the mechanism for the cardiovascular effects of BmK scorpion venom still awaited extensive investigation because the components of the venom are diverse. Except for cardiac sodium channel that may account for the most part in cardiovascular modulation by BmK toxins, other ion channels or intracellular receptors targeted by BmK toxins may also play indispensable roles.

Voltage-Gated Sodium Channels

The mechanism underlying the increase in rat heart contractility modulated by BmK I is considered to be the accumulation of intracellular Na^+ through voltage-gated sodium channels. In single ventricular myocyte, by administration of BmK I, the inactivation process of Na^+ current was significantly delayed without any changes of its amplitude. Additionally, it was unexpected to find that the overall

intracellular Na^+ and Ca^{2+} concentration in rat cardiac myocytes could be augmented through Na^+-Ca^{2+} exchanger. Given the fact that this augmentation of Na^+ and Ca^{2+} could be completely removed by administration of TTX, it may allow us to safely infer that the cardiac VGSCs are indeed the targets of BmK I. Thus, the Ca^{2+} release from sarcoplasmic reticulum (SR) may account for the positive isotropic effects induced by BmK I (Sun et al. 2005).

More direct evidences of VGSC involvement in cardiovascular modulation of BmK I come from the in vitro electrophysiological characterization of BmK I on cardiac sodium channel isoform $Na_v1.5$ expressed in HEK293t cells, which confirmed that BmK I could target cardiac VGSC by impairing the inactivation processes and induce substantial persistent sodium currents. Moreover, BmK I slowed down the recovery from inactivation and increased peak currents (the EC_{50} for increasing peak current by BmK I was 99.4 ± 20.1 nM). The steady-state activation of $Na_v1.5$ was also found to be shifted to more hyperpolarized potentials by BmK I (Feng et al. 2008).

Potassium Channels

Unlike the structural and functional diversity of VGSCs relying large upon the variable isoforms of α subunit, different BK channels were discriminated by the subtypes of β subunits coexpressed with α subunits in tissues. Among them, BK channel (α + β1) is considered to be a key player in balancing excessive vasoconstriction in the cardiovascular system.

In the presence of $[Ca^{2+}]_i$, Martentoxin could enhance the activities of BK channel (α + β1) subtypes in dose-dependent manner with EC_{50} of 495 nM while not shift the steady-state activation of BK channels. Interestingly, this enhancement was independent on the concentration of $[Ca^{2+}]$ applied with, in contrast to the absolute acceleration in binding between toxin and BK channels under higher $[Ca^{2+}]_i$ (Tao et al. 2011). The remarkable enhancement of BK channel activity by Martentoxin in cardiac cell may therefore suggest a potential perspective to utilize Martentoxin as a scaffold for designing novel drug candidates to relieve cardiovascular dysfunctions.

Antitumor and Cell Apoptosis

Tumor growth on severe combined immunodeficiency bearing malignant glioma U251-MG tumor xenografts could be significantly inhibited by BmK venom. After the administration of BmK venom, tumor growth was slowed obviously. Correspondingly, cell death, especially apoptosis, was induced by BmK venom.

Various Na^+, K^+, and Cl^- channels have been detected in glioma cells. Though the correlation between ion channels and proliferation of neuroblastoma cells or glioma cells remains to be investigated, it is speculated that neurotoxins from BmK may exert the antitumor effects via the modulation of ion channel activity. For example, proliferation and migration of human glioma cells were significantly affected by some blocker or openers of BK channels. Some ligands specific for BK channels such as Martentoxin have been shown to have antitumor effects

(Tao et al. 2011). In addition, BmK AS could modulate the gating mechanism of the voltage-gated Na^+ channels and alter the intracellular Na^+ concentration of the neuroblastoma cells (Tan et al. 2003). More direct evidences came from a Cl^- channel toxin, BmK CT (UniProt number: Q9UAD0), which effectively inhibit the proliferation and metastasis of glioma cells.

Molecular Interaction of BmK Neurotoxins with Membrane Ion Channels

The structure of different VGSC subtypes identified to date is highly conserved, all of which contain four homologous domains (DI-DIV), each with six putative transmembrane segments (S1–S6). Due to the high sequence similarity, characterizing or pharmacologically targeting specific members of the large family of VGSCs has proven to be challenging. Long-chain neurotoxins (60–76 amino acid residues) from BmK venom have previously been characterized as specific modulators of VGSCs. It was found that these toxins specifically bind to distinct regions on different VGSCs in either overlapped or separated forms, which suggests that their binding sites on VGSCs are diverse (review in Liu et al. 2011). Also, key residues involved in toxin-channel inter-recognition or subtype-selective patterns were uncovered by site-directed mutagenesis. These findings make BmK neurotoxins good candidates for probing the pharmacological and structural diversity of VGSCs in mammals or insects.

Similarly, for the case of BK channels, Martentoxin has been investigated with a comparatively higher preference for these channels over other voltage-gated potassium (Kv) channels. Since the specific drug tool probing for clarifying structure-function of BK channel subtypes and related pathology remain scarce, it is of importance to illuminate the underlying mechanism of molecular interaction between Martentoxin and BK channels.

Toxins found to target specific ion channels may have potential applications in therapeutics against pathological conditions or in bio-pesticide development. As for it, this section will address the current knowledge based on the studies of pharmacological characterizations and molecular determinants of BmK toxins.

Pharmacological and Molecular Mechanisms of BmK Toxins Interaction with Sodium Channel Subtypes

The gating properties of the VGSCs are correlated to the shape of action potential as well as the firing pattern. It has been well established that α-scorpion toxins associate the VGSCs at receptor site-3 and inhibit the inactivation, while β-toxins bind receptor site-4 and facilitate the activation. However, the modulation patterns vary among the toxins acting on the same receptor site. The modulatory profiles of three representative BmK toxins, i.e., BmK I, BmK IT2, and BmK AS, have been extensively investigated on VGSC subtypes.

BmK I could discriminate well for the three neuronal VGSCs that were independently expressed in *Xenopus* oocytes with the auxiliary β1 subunit: $Na_v1.6α/β1$ responded with a large increase of both transient and persistent currents, which correlated to a prominent reduction in the fast components of inactivating current in a dose dependency. Moreover, BmK I could also accelerate the slow inactivation development and delayed recovery through binding to $Na_v1.6/β1$ in the open state; $Na_v1.2/β1$ was less affected with only increased fast time constants of inactivating current upon the application of BmK I at high concentration (500nM); and $Na_v1.3α/β1$ was nearly insensitive (He et al. 2010). Residue-swap analysis verified that an acidic residue (e.g., Asp1602 in $mNa_v1.6$) within the domain IV S3–S4 extracellular loop of VGSCs was crucial for the selectivity and modulation pattern of BmK I, which was in agreement with other α-scorpion toxins binding to sodium channels (Zuo and Ji 2004; Bosmans and Tytgat 2007).

For the insect toxin BmK IT2, the functional characterization demonstrated that BmK IT2 could strongly hyperpolarize the activation of the insect VGSC $DmNa_v1$ from *Drosophila*, but hardly act on $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$, three mammalian central neuronal sodium channel isoforms, which was consistent with the insect-specific toxicity and higher binding affinity of BmK IT2 to insect nerve cords. By replacing each domain (DI-DIV) of $Na_v1.2$ with that of $DmNa_v1$, BmK IT2 specificity for $DmNa_v1$ could be conferred by DIII while the interaction with channel voltage sensor was mediated through binding to DII. Analysis of subsequent $DmNa_v1$ mutants further highlighted that, in addition to the contribution of E896, L899, and G904 in DII S4 for functional action of BmK IT2, residues in DIII pore loop, esp. I1529 and R1530, were critical for recognition and binding of BmK IT2 (He et al. 2011). Hence, DIII SS2-S6 is considered to be the recognition site with species selectivity for BmK IT2 and DII S3b-S4 is considered the functional voltage-trapping site of VGSCs.

BmK AS could modulate the VGSCs in a unique U-shaped dose-dependent manner. In the *Xenopus* oocytes, BmK AS depolarized the voltage dependence of activation and inactivation of $Na_v1.2$ at 0.1 and 500 nM whereas hyperpolarized them at 1 nM. In the ND7–23 cells, BmK AS hyperpolarized the voltage dependence of activation and inactivation at 0.1, 1 and 100 nM but not 10 nM (Zhu et al. 2009). This unique U-shaped dose-dependent modulation pattern of BmK AS could also be observed for $Na_v1.3$ (Liu et al. 2012). Like AaH IT4, BmK AS/AS-1 were capable of competing with anti-insect scorpion toxins for binding to the sodium channel of insects with a high affinity but low capacity site; it also competed the binding of α-type scorpion toxins to the mammal sodium channel with low affinity but high capacity binding site (Li et al. 2000). Hence, it is possible to assume that BmK AS possess the ability to bind both site-3 and site-4, exerting modulation on both voltage-dependent activation and inactivation of VGSCs. At medium concentrations, BmK AS tend to bind with site-3 by modulating inactivation more pronouncedly at medium concentration, and meanwhile binding with site-4 at low or high concentrations by negative-shifting the activation more significantly. These multifaceted features resemble another β-like toxin Lqhβ1,

which could induce a shift in the voltage dependent of activation to more negative membrane potentials and a reduction in sodium peak currents, while having a weak effect on inactivation for cardiac VGSC and a marked effect on rat brain and skeletal muscle VGSCs as well.

Novel Interaction Model of Martentoxin with BK Channels

As a specific ligand of BK channels, Martentoxin was identified to have potentials discriminating most BK channel subtypes efficiently in a different way compared to other toxins.

Martentoxin (10 μmol/L) nonspecifically inhibits voltage-dependent Na^+ current (I_{Na}) and voltage-dependent delayed rectifier K^+ current (I_K) but without any effect on transient K^+ current (I_A). Both interactions with Na^+ and K^+ channels were irreversible. On the contrary, 100 nmol/L Martentoxin potently blocked BK channel currents in adrenal medulla chromaffin cells in which β2 subunit coexpressed with α subunit (Ji et al. 2003). Martentoxin has a remarkable preference (about 10^3-fold) for BK (α + β2) channel subtype over Kv channels and Nav channels and strongly blocked IbTX-insensitive neuronal BK channels (α + β4) ($IC_{50} = 78$ nmol/L). The BK channel (one α subunit only) was almost insensitive to Martentoxin (Shi et al. 2008). These results fully demonstrated that Martentoxin could potently discriminate α and α + β4 BK subtypes. Furthermore, Martentoxin exhibits a higher preference in outward K^+ current increase on the BK channel subtype expressed in glioma cells (gBK) over BK (α + β1) channel by about 10-fold (Tao et al. 2011). Herein, the subtype selectivity of Martentoxin was possibly attributed to the integrative modulation of different β subunits or alternative-splicing forms of α subunit for BK channels.

One interesting character for the blockade mode of Martentoxin is that it could modulate the activities of neuronal BK channel subtype (α + β4) in a Ca^{2+}-dependent manner. The neuronal BK channel (α + β4) currents could be reduced by Martentoxin in the presence of low cytoplasmic Ca^{2+} concentration, but increased in high Ca^{2+} concentration. This was corresponding to the notion that β4 subunit reduces BK channel openings at low cytoplasmic Ca^{2+} concentration, while increased channel openings at high cytoplasmic Ca^{2+} concentration (Tao et al. 2011). It thus indicated that the pharmacological features of Martentoxin would be reversed when subjecting to the conformational change of BK channels.

One the other hand, the activities of gBK and BK channel (α + β1) subtypes were both enhanced by Martentoxin only when there was free Ca^{2+} in the external environment. When Ca^{2+} was completely removed from the pipette solution, the activity of gBK channel was inhibited. By contrast, this situation was not occurred in case of BK channel (α + β1) whose activity was unaltered by Martentoxin (Tao et al. 2011). Therefore, the modulation of Martentoxin on various BK cannels was Ca^{2+}-dependent. The Ca^{2+} binding sites of BK channels may be potential targets interacting with Martentoxin. However, this hypothesis seemed to be challenged in that Martentoxin may not be able to reach the cytoplasmic face of the cell where

most Ca^{2+} binding sites locate. Another possible mechanism is that Martentoxin may indirectly interact with Ca^{2+} binding sites through binding with β subunits as they are spatially approximate to each other.

Conclusions and Future Directions

The pros and cons of scorpion venom utilized for drug purposes have always been the great interests for toxinologists and pharmacologist since the ancient China. This field is severely lacking research intended to bridge the understanding that BmK venom is both acutely pro-nociceptive and pro-convulsant, but also antinociceptive and anticonvulsant. The current and ongoing researches on BmK toxins will enable us to fully realize the biological essences of BmK venom, which, in turn, provide more comprehensive and broader space to reexamine the possible pharmacological effects and structural optimization on natural BmK toxins that will ultimately determine any potential clinical applications for the widespread use.

Even so, there are apparently a lot more challenging questions for taking advantages of such valuable resources. Moreover, since structural diversity and complex biological effects induced by BmK toxins, it is worth to carry out more parallel investigations both in vitro and in vivo regarding the pharmacological and pathological responses. What are the factors linking seemingly conflict two sides, such as pro-nociception and antinociception, for the effectiveness of BmK toxins? Are there any downstreaming molecules that possibly facilitate the modulation of BmK toxins? Additional BmK toxin-induced pharmacological effects may exist, but how should their causes be identified? Establishing a mechanistic link between toxins to diseases is urgently needed for a beneficial and reasonable strategy in the future studies.

Like the philosophical metaphor of the spear and the shield documented in traditional Chinese fables, which goes that an impenetrable shield is impossible to coexist with a spear that finds nothing impenetrable, the study of BmK toxins cannot be treated exclusively without considering the interactions with their natural targets. Meanwhile, advances in pharmacogenomics and genetic information have suggested that individual sensitivity to toxins might be diverse and case by case, which illuminate the significant and complicated role of various receptors in actual physiological environment. This and other several important open questions remain to be answered. For example, what is the basis for the temporal or spatial expression pattern of ion channels when toxins manifest in the channelopathies? A growing number of partner molecules and modulatory factors are being shown to interact with ion channels, whose receptor sites and pharmacological effects may be mutually overlapped or allosteric with BmK toxins. Also, some indirect factors or "multi-hit" mechanisms must be also seriously taken into account in dealing with toxin-induced neuropathies. Finally, how to explain the distinct question why excitatory ITxs such as BmK IT and AaH IT were extremely lethal to insects but have no toxicity on mammals? Why BmK I, distinct from well-studied AaH II, a classic anti-mammal α-toxin, was toxic to both insect and mammals, could it be

attributed to the subtle divergence of toxins or the diversity of targets (selectivity, sensitivity, affinity, specificity, etc.), or both, and what are the underlying reasons why BmK scorpion was insensitive to its own toxins? Only with the advent of more evidences from structural characterization, revolutionary view, and exquisite work on dynamic toxin-targets interactions these mysteries will possibly be unveiled.

With the increasing development of advances in analytical techniques, the researches of BmK toxin are turning to be easier and deepening than ever before. These and other questions about BmK toxins mentioned above will hopefully be uncovered and continue to help us expand understanding the nature of channelopathies, which will certainly help to bring out new changes in therapeutic approaches.

Cross-References

► Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
► Potassium Channel Blocking Peptide Toxins from Scorpion Venom

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Scorpion Venom Research Around the World: *Tityus serrulatus*

18

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Contents

Abstract

Tityus serrulatus is considered the most dangerous scorpion in Brazil. It is widely distributed, especially in the Southeast region, and is responsible for the highest number and most severe accidents. This chapter focuses on

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Tityus serrulatus scorpion venom (Tsv) and aspires to unravel its complex composition with emphases on its isolated proteins, their targets, structures, and functions. It takes a closer look at the peptides related to the Na^+ and K^+ channel toxin families, NaTx and KTx, respectively, including their toxin precursors. Additionally, a hyaluronidase, a serine proteinase, metalloproteinases, and many other proteins/peptides, such as a nontoxic protein (Ts4), PAPE peptides, bradykinin-potentiating peptides (BPP), antimicrobial peptides (AMPs), anionic peptides, and venom peptides with undetermined functions, were reported.

Introduction

Only 34 among the more than 1,500 known scorpion species in the world produce venom lethal to humans and all but one belonging to the Buthidae family (for review, see Cologna et al. 2009). *Tityus serrulatus* (Fig. 1) is considered to be the most dangerous scorpion in Brazil, where it is widely distributed. It is vulgarly known as Brazilian yellow scorpion and causes serious accidents which can lead to death, especially in children.

Scorpion venoms are extremely versatile, being effective offensively against insect preys and defensively against vertebrate enemies. It has been suggested that their outstanding and dramatic effects on mammals are due to strong conservation of receptors and ion channels across diverse taxa.

The effects of *Tityus serrulatus* venom (Tsv) in various animal species were reported by Magalhães in 1946 (Almeida et al. 2002). Just after 20 years, the first separation of its components was reported by Gomez and Diniz in 1966. Ever since, the progression of knowledge about the Tsv composition, its effects, and its biotechnological application has risen. Scorpion toxins have been used as molecular probes to study the structure and function of Na^+ and K^+ channels, as well as to study the gating mechanisms of these channels, to isolate them from native tissues, and to understand the physiological role of specific channel ligands. In addition, scorpion toxins may be used as tools in physiopathological studies for the full comprehension of several channels related to diseases such as cancer, Alzheimer, and Parkinson. Since some of these toxins victimize insects in a specific way, they also contributed to the development of new pesticides. Lastly, the full characterization of *T. serrulatus* components and their effects will lead for more efficient envenoming treatments (for review, see Cologna et al. 2009).

Toxicity of Tsv

The sting of *T. serrulatus* causes local pain and, depending on the envenoming severity, paresthesia, sweating, vomiting, hypertension, neurological manifestations, alternation between agitation and exhaustion, cardiorespiratory alterations, pulmonary edema, and circulatory failure triggering death are also observed (for review, see Cologna et al. 2009). This plethora of symptoms and signs are attributed

Fig. 1 *Tityus serrulatus* (Lutz and Mello 1922), also known as yellow scorpion. This scorpion's body is 5–7 cm long

to the effects of toxins interacting with voltage-gated sodium channels of excitable cell membranes, causing them to release a large amount of neurotransmitters (Possani et al. 1999).

In healthy adults, most scorpion stings are less severe and may not need medical treatment. However, children and elderly people with pre-existing heart disease are at risk of death. All scorpion victims should always be kept under observation during 4–6 h after the sting and in case of moderate to severe accidents be monitored for at least 24 h.

The lethal dose of Tsv required to kill 50 % (LD_{50}) of rats or mice has been experimentally attested by several researcher groups. The LD_{50} of Tsv is 25 μg/mouse (20 g) after intraperitoneal injection of soluble venom (Possani et al. 1977), 24.6 μg/mouse (20 g) after subcutaneous injection (Pucca et al. 2011), 7.5 μg/mouse (20 g) after intravenous (i.v.) injection, and 0.098 μg/mouse (20 g) after intracerebroventricular (i.c.v.) injection (Arantes et al. 1989). On the other hand, the i.v. and i.c.v. LD_{50} of toxins Ts2, Ts6, Ts3, and Ts1 were, respectively, 6.42 and 0.074, 16.52 and 0.22, 2.04 and 0.034, 1.52 and 0.022 μg/mouse (20 g) (Arantes et al. 1989).

The LD_{50} is 10.8 ± 0.6 mg/kg for adult male rats after subcutaneous injection of a saline extract of crude Tsv, while adult female rats and weanling (male and female) rats are, respectively, around 2 and 3.5 times more sensitive to the venom (Nunan et al. 2001). Thus, the effect of the scorpion envenoming in children is probably related to body maturation factors, such as number of receptor sites and pharmacokinetic parameters when compared with adults (Nunan et al. 2001). Besides that, children's blood–brain barrier is more permeable to small peptides, such as toxins able to act on voltage-gated Na^+ or K^+ channels (Nunan et al. 2003).

The LD_{50} values for manually extracted venom are approximately twice or three times lower than those for venom extracted by electrical stimulation. The contraction of the venom gland can be controlled by the scorpion during manual extraction

of the venom, being closer to reality than electrical stimulation. Manual extraction releases only toxins, while electrical stimulation releases also nontoxic components (Kalapothakis and Chávez-Olórtegui 1997 *apud* Pucca et al. 2011).

An adequate assessment of scorpion LD_{50} is an important step for accurate evaluation of antivenom sera potencies and the optimization of serotherapy (Krifi et al. 1988 *apud* Pucca et al. 2011). In retrospect to comparing LD_{50} values, several parameters have to be taken into account as there are: the route of venom administration, the animal model (strain, species, body weight, sex and age), the venom batch and the venom extraction procedure.

Isolation of Toxins

In order to extract the venom from *Tityus serrulatus* scorpions kept in captivity, an electric charge equivalent to 12 V is applied on the tail segment with the hazardous telson, stimulating the release of the venom. Hundreds of small droplets of venom from several specimens of Ts are accumulated in a glass tube or plate and desiccated on a vacuum pump or freeze-dried. The collected dried venom is a complex mixture as mentioned before and should be dispersed in a suitable buffer solution and centrifuged to separate the mucus from the protein solution.

Ts crude soluble venom has been fractionated by size exclusion chromatography, followed by ion exchange chromatography and rechromatography. Ts toxins have also been isolated through preparative reversed phase HPLC and rechromatography on a C18 column. Another reliable purification procedure consists in applying Ts venom on a carboxymethyl-cellulose-52 column (Arantes et al. 1989). This protocol assures a swift recuperation of pure Ts1, the major toxin from Ts venom, in just one purification step. After this first fractionation step, the desired fraction is rechromatographed on an ion exchange column or on a reversed phase C18 column (Pessini et al. 2001; Cologna et al. 2011).

Composition of the Venom

Tsv is composed of insoluble mucus, many neurotoxic proteins composed of 60–70 or 30–42 amino acid residues that affect, respectively, Na^+ or K^+ channels, bioactive amines, hypotensins, proteinases, a hyaluronidase, a bradykinin-potentiating peptide, a kallikrein inhibitor, allergenic proteins, and other peptides whose biological functions are still not clarified.

Estimates indicate that Tsv comprises over 300 toxins, most of which are small molecules (molecular mass <10 kDa) (Pimenta et al. 2001). This diversity of molecular masses from Tsv has been investigated using MS-based proteomic approaches, which allowed to detect posttranslational modifications, such as phosphorylation and N-glycosylation in toxins from Tsv. Proteomics led to a huge amount of new information considering the absence of information on the complete genome sequence of *Tityus* sp. (Verano-Braga et al. 2013).

Some bioactive amines, including histamine and 5-hydroxytryptamine, were found at low concentrations in Tsv. The most common function of histamine in venom is to produce pain at the site of injection, an advantage when the venom is used for defense. Tsv also contains compounds that induce release of histamine from mast cells, causing an increase in vascular permeability which facilitates the access of its toxic components into the blood circulation and contributes to profound hypotension.

Despite the description of phospholipases A_2 in scorpion venoms, no activity had been detected in Tsv (Possani et al. 1977; Venancio et al. 2013) till its transcript was identified in the *T. serrulatus* cDNA library (Alvarenga et al. 2012).

The absence of L-amino acid oxidase and phosphodiesterase activities has been noted in scorpion venoms. In addition to the absence of phosphodiesterase activity (Possani et al. 1977), Tsv contains neither catecholamines nor acetylcholine. However, after intravenous injection of Tsv, a great increase of epinephrine and norepinephrine release was reported with concomitant increase of the mean arterial pressure in rats previously catheterized (Vasconcelos et al. 2005).

Because of their interactions with ionic channels in excitable membranes and their role in the envenoming, neurotoxins with low molecular mass became the most studied components of Tsv.

Tsv neurotoxins are represented by long-chain Na^+ channel toxins (NaTx), such as α-toxins which inhibit the inactivation and β-toxins responsible for decreasing the excitation threshold of this channel. This triggers depolarization and mediators' release. The venom also contains short-chain K^+ channel toxins (KTx) with low toxicity acting on K^+ channels, which might be used as potential medicines (for review, see Cologna et al. 2009).

In 2009 a nomenclature for TsV components was proposed where the isolated proteins of which the amino acid sequences are completely determined are named consecutively, to avoid confusion and systematize the nomenclatures adopted previously by Dr. Giglio's and Dr. Possani's groups (for review, see Cologna et al. 2009). Following this systematized nomenclature, here it is proposed to appoint not completely mature toxins using the toxin name followed by the single-letter code of each amino acid residue that appear after the C-terminal of the mature protein, aiming to distinguish the immature toxin (e.g., Ts1-G) from its precursor (containing the signal peptide).

The primary sequences, molar masses, and the names used by different authors to describe the several components isolated from Tsv or identified by peptidomic or transcriptomic analyses are shown in Table 1.

A phylogenetic tree was constructed with Tsv components whose amino acid sequences were completely determined (Fig. 2).

It is interesting to note that all the toxins belonging to the same toxin group are closely related to each other (Fig. 2), such as NaTxs (in red and orange) and KTxs (in violet and pink). **Ts4** (black), a nontoxic protein, is closely related to **Ts1** and shares 65.57 % of identity with it. The **β-KTxs** (in pink in Fig. 2) were joined together in the same branch and are near the **α-KTxs** (in violet). An outstanding observation is that **Ts10** shares 92.3 % of identity with Ts5 and Ts17, **α-NaTxs**;

Table 1 Proteins and peptides from *Tityus serrulatus* venom

Name[1]	Other names	Sequences[2]	UniProt or GenBank /PDB	Molecular mass[3]	Reference*
Ts1 β-NaTx	TsTX-I; toxin γ; Toxin T_2IV; Toxin Ts7; Ts VII; Toxin VII; TsTX-VII; Tityustoxin VII; Toxin III-10; Toxin II-11; Toxin T_1VIII	KEGYLMDHEGCKLSCFIRPSGYCGRECGIKKGSSGYCAWP ACYCYGLPNWVKVWDRATNKC	1NPI 1B7D	6878	*
Ts1-G β-NaTx	Isoform of Ts1	KEGYLMDHEGCKLSCFIRPSGYCGRECGIKKGSSGYCAWP ACYCYGLPNWVKVWDRATNKCG	---	---	(Coelho et al., 2014)
Ts1 precursor β-NaTx	Beta-neurotoxin precursor Toxin Ts7 precursor Toxin VII precursor	*MKGMILFISCLLLIGIVVEC*KEGYLMDHEGCKLSCFIRPS GYCGRECGIKKGSSGYCAWPACYCYGLPNWVKVWDRATNK CGKK	P15226	7191	*
Ts2 α-NaTx	TsTX-III; III-8; Tst2; Toxin II; TsTX-II; Tityustoxin II; Toxin T1-IV; beta toxin TsII	KEGYAMDHEGCKFSCFIRPAGFCDGYCKTHLKASSGYCAW PACYCYGVPDHIKVWDYATNKC	P68410 PM0077533 (PMDB model)	6985	*, (Cologna et al., 2012)
Ts3 α-NaTx	TsTX; Tityustoxin; Toxin IV-5 TsIV-5; Ts IV alpha-toxin; Ts IV; Toxin-4; Tityustoxin IV; Toxin IV; Ts III; Toxin-3; Toxin T_2III_I	KKDGYPVEYDNCAYICWNYDNAYCDKLCKDKKADSGYCYW VHILCYCYGLPDSEPTKTNGKC	---	7227	*
	Toxin-3	KKDGYPVEYDNCAYICWNYDNAYCDKLCKDKKADSGYCYW VHILCYCYGLPDSEPTKTNGKCKS	---	7442	
Ts3 precursor α-NaTx	Toxin-4	KKDGYPVEYDNCAYICWNYDNAYCDKLCKDKKADSGYCYW VHILCYCYGLPDSEPTKTNGKCKSGK	---	7627	*
	Toxin-5, Toxin IV-5 precursor Precursor Tityustoxin	*MVVVCLLTAGTEG*KKDGYPVEYDNCAYICWNYDNAYCDKL CKDKKADSGYCYWVHILCYCYGLPDSEPTKTNGKCKSGKK	P01496	7755	
Ts4	TsTX-VI; Tityustoxin-6; Tityustoxin VI; TsTXVI; Toxin VI; Ts VI	GREGYPADSKGCKITCFLTAAGYCNTECTLKKGSSGYCAW PACYCYGLPESVKIWTSETNKC	P45669	6704	*
Ts4-G	Ts4 with Gly in the C-terminal	GREGYPADSKGCKITCFLTAAGYCNTECTLKKGSSGYCAW PACYCYGLPESVKIWTSETNKCG	---	6747	*
Ts4 precursor	Non-toxic protein NTxP precursor; TsNTxP; TsnTxp; NTxp	*MKRMILFISCLLLIDIVVG*GREGYPADSKGCKITCFLTAA GYCNTECTLKKGSSGYCAWPACYCYGLPESVKIWTSETNK CGKK	AAC25688 AAC25689 O77463	7003	(Guatimosim et al., 1999)
Ts5 α-NaTx	TsTX-V; Tityustoxin-5; Tityustoxin V; alpha toxin TsTX-V; Ts V; Toxin V	KKDGYPVEGDNCAFACFGYDNAYCDKLCKDKKADDGYCVW SPDCYCYGLPEHILKEPTKTSGRC	P46115	7187	*
Ts6 α-KTx	TsTX-IV; α-$KTx_{12.1}$; Potassium channel toxin alpha-$KTx_{12.1}$; Butantoxin; BuTX	WCSTCLDLACGASRECYDFCFKAFGRAHGKCMNNKCRCYT	P59936 1C55 1C56	4503	*
Ts7 α-KTx	TstX-Kα; Tityustoxin K-alpha; Potassium channel toxin alpha-KTx 4.1; TsII-9; TsTx-K-alpha; TSK4; Toxin II-9	VFINAKCRGSPECLPKCKEAIGKAAGKCMNGKCKCYP	P46114 1HP2	3939	*
Ts8 β-KTx	Tityustoxin K-beta; TSK2 TsTX-K beta; TsTx-Kβ;	KLVALIPNDQLRSILKAVVHKVAKTQFGCPAYEGYCNDHC NDIERKDGECHGFKCKCAKD	P69940	6712	*
Ts8 propeptide β-KTx	Potassium channel toxin beta-KTx 1 precursor	*MERKLALLLILGMVTLASC*GLREKHVQKLVALIPNDQLRS ILKAVVHKVAKTQFGCPAYEGYCNDHCNDIERKDGECHGF KCKCAKD	P69940	7660	*
Ts9 α-KTx	Ts κ; Ts Kappa; Neurotoxin Ts-kappa; TsKapa	VVIGQRCYRSPDCYSACKKLVGKATGKCTNGRCDC	1TSK	3776	*
Ts9 propeptide α-KTx	Potassium channel toxin alpha-KTx 4.2 precursor	*MKVLYGILIIFILCSMFYLS*QEVVIGQRCYRSPDCYSACK KLVGKATGKCTNGRCDC	P56219	4033	*
Ts10	Peptide T; Bradykinin-potentiating peptide	KKDGYPVEYDRAY	Q9TWR4	1603	*
Ts11	TsPep1; Peptide TsPep1	KPKCGLCRYRCCSGGCSSGKCVNGACDCS	P0C174	2936	
Ts12	TsPep2; Peptide TsPep2	TVKCGGCNRKCCAGGCRSGKCINGKCQCY	P0C175	2991	*
Ts12 precursor	TsPep2; Peptide TsPep2 precursor	*MKFSCGFLLIFLVLSAMIATFSEVEA*TVKCGGCNRKCCAG GCRSGKCINGKCQCYGRSDLNEEFENYQ	P0C175	4573	
Ts13	TsPep3; Peptide TsPep3	TVKCGGCNRKCCPGGCRSGKCINGKCQCY	P0C176	3017	
Ts14	Hypotensin-1 (TsHpt-I)	AEIDFSGIPEDIIKQIKETNAKPPA	P84189	2723	*
	Hypotensin-2 (TsHpt-II)	AEIDFSGIPEDIIKEIKETNAKPPA	P84190	2724	
	Hypotensin-3 (TsHpt-III)	AEIDFSGIPEDIIKQIKETNAKPP	P84191	2652	
	Hypotensin-4 (TsHpt-IV)	AEIDFSGIPEDIIKEIKETNAKPP	P84192	2653	
Ts15 α-KTx	Potassium channel toxin alpha-KTx 21.1	GKFGKCKPNICAKTCQTEKGKGMGYCNKTECVCSEW	P86270	3953	(Cologna et al., 2011)
Ts16 α-KTx	Tityustoxin-16	GCMKEYCAGQCRGKVSQDYCLKHCKCIPR	P86271 2LO7	3300	(Bordon et al., 2011; Saucedo et al., 2012)
Ts17 α-NaTx	U-BUTX-Ts1a	*MNYFIFLVVACLLTAGTEG*KKDGYPVEGDNCAFACFGYDN AYCDKLCKDKKADSGYCYWVHILCYCYGLPDKEPTKTSGR CKPGKK	---	7547	(Alvarenga et al., 2012)

(*continued*)

Table 1 (continued)

Name[1]	Other names	Sequences[2]	UniProt or GenBank /PDB	Molecular mass[3]	Reference*
Ts18 α-NaTx	U-BUTX-Ts1b	*MNFRFPFLLMITISKIGAVLT*FGNDVRPRNQYGNEIFCPH QGFNQKCNAICKRKKYLLGFCDQKTCLCKVRP	---	5971	(Alvarenga *et al.*, 2012)
Ts19 β-KTx	U-BUTX-Ts1c	*MVATNRCCVFALLFALLLVHSLTEA*GKGKEILGKIKEKII EAKDKMKAGWERLTSQSEYACPAIDKFCEDHCAAKKAVGK CDDFKCNCIKL	P86822	7354	(Alvarenga *et al.*, 2012)
Ts19 frag I β-KTx	Ts19 fragment I	KIKEKIIEAKDKMKAGWERLTSQSEYACPAIDKFCEDHCA AKKAVGKCDDFKCNCIK	P86822	6458	(Carmanhan *et al.*, 2013)
Ts19 frag II β-KTx	Ts19 fragment II	KDKMKAGWERLTSQSEYACPAIDKFCEDHCAAKKAVGKCD DFKCNCIKL	P86822	5519	(Cerni *et al.*, 2013)
KTx or CaTx	TsTx-VII	GHZGYGS…	---	6700-6800[4]	(Sampaio *et al.*, 1997)
Hypotensin -like	Hypotensin-like peptide (Fragment)	ADVDFTGIADSIIK	P86824	---	(Rates *et al.*, 2008)
Kallikrein inhibitor	Kallikrein Inhibitor	KNSTCLDLAxGASRECYDPCF…	---	4489[4,5]	(Ferreira *et al.*, 1998)
Enzymes	Hyaluronidase (Fragment)	KFKVYWEVPSFLCSKRFKICVTEVLTSHEILVNQ…	P85841	51kDa[6]	(Pessini *et al.*, 2001; Richardson *et al.*, 2008b)
	Venom metalloproteinase, VMP (Fragments)	SPCIIIDYLCVTETCFCERFKTNKELLEYITVMFTGVQNL LDTLNLGIVGVAQDYSDYNERVDTVAHETAHLIGAPHDEE GPCQDT	P85842 (Non-adjacent residues)	---	(Richardson v 2008c)
	Venom metalloproteinase antarease, VMPA (Fragments)	DDDCIVVEYYIVTDSAFTKRFKSNSALTNYVTVMFTGxxx xxxxxELGIGVRLLGVTTFTEKTEPSFIKDNLIPGPPAAF DPDVLISAMSKYYCNHQTGLAKDTDLIFLITARGMGDPRE DGTVDINTAGIANSAGVCKPCFKSGIATDDSDYNERVDTL AHESVHLLGSPHDGEGPNLVSLGSPGAANCPAxxxxxxxx xKAGYIMGNRNDKVNKYKFSN/PCTKKCVEYLLSKPTASC IFQQCSD	P86392 (Sequence uncertainty)	25,500 ± 100 Da[5]	(Fletcher *et al.*, 2010)
Other components	TsII-10-2	TFIDVKCGSSKECLP…	---	---	(Possani *et al.*, 1982)
	Toxin T_1V_I	GHFGK… (72 amino acid residues)	---	7549[4]	(Sampaio *et al.*, 1983)
	Toxin To46, Toxin Tc46	KEGYLFGSRG	P84686	---	(Verano-Braga *et al.*, 2013)
	Pape peptide precursor	*MNRKTLLVIFFVTLLIAEEVNS*FRLGGFLKKIWRSKLVKR LRSKGKQLLKEALAPEPAPEPAPEPAPEAAPEAAPEPAAA APERRRRR	---	7300	(Alvarenga *et al.*, 2012)
	Pape peptide fragment	KQLLKEALAPEPAPEPAPEPAAPEAAPEPAAAAPE	P86821	---	(Rates *et al.*, 2008)
	Venom peptide 1	KHFGKDSNFPFGT	P86825	---	(Rates *et al.*, 2008)
	Venom peptide 2	KHFGKDSNFPF	P86825	---	
	Venom peptide 3	YDRYEVVYR	P86826	---	
	Venom peptide 4	YDRYEVVY	P86826	---	
	Venom peptide 5	YDRYEVV	P86826	---	
	Venom peptide 6	FGVLNFF	P86827	---	
	Venom peptide 7	RLRSKGKK	P86828	---	
	Venom allergen 5, Antigen 5, Cysteine-rich venom protein, CRVP (Fragments)	ECPALYRRYSKEHTFCKTKNQKCNIKRWGVSQDDRNTIIN LHNKVRNNIALGQDQSGRLPAAGDMLEMEWDDELAQIAQK LADQCVFKHDCDDCRKVENFDVGQNIFTRGITAVEIPDPF KSWTQLYVCNYGPAGNLDDSELYKVDKPCEKCPSNTCCGS HCKKHNKSTSYLGLCDVLNGSGPDFDETDFSNYIFNCDFK PESDCNNKVEGS	P85840 (Non-adjacent residues)	---	(Richardson *et al.*, 2008a)
	Putative antimicrobial peptides, AMP	*MQMKYLIPIFFLVLIVADHCHA*FLGMIPGLIGGLISAFKG RRKREITSQIEQYRNLQKREAELENLLANLPVY	JK732086	5842	(Alvarenga *et al.*, 2012)
		*MQMKYLIPIFFLVLIVADHCHA*FLGMIPGLIGGLISAFKG RRKREITSQIEPYRNLQKREAELENLLANLPVY	JK732409	5811	
		*MQIKHLITLFFLVLIVADQCSA*FLSLIPSLVGGLISAFKG RRKREISAQIEQYKDLQKREAELEELLDRLPMY	JK732667	5946	
	Ponericin-like	*MQFKKQLLVIFFAYFLMVHESEA*FLGSLLSLGSKLLPKVV GLFTRKRERSLKKRDLEKFYDPYQGNLEMERLLKQLPIY	JK732079	6623	
		*MQFKKQLLVIFFAYFLMVHESEA*FLGSLLSLGSKLLPKVV GLFTRKRERSLKKRDLEKFYDPYQRNLEMERLLKQLPIY	JK732889	6722	
	Anionic peptide	*MVSKSLIVLLLVSVLVSTFFTTEA*YPASFDDDFDALDDLD DLDLDDLLDLEPADLVLLDMWANMLDSQDFEDFE	JK31735	5798	

[1]Nomenclature suggested by Cologna et al. (2009). NaTx, Na^+-channel toxin; KTx, K^+-channel toxin
[2]Signal peptide is highlighted in *gray*;
[3]Molecular mass of the oxidized monoisotopic toxin (S-S) calculated by the **Sequence Editor** 3.2 program – the signal peptide was not considered
[4]Determined by amino acid analysis;
[5]Determined by mass spectrometry
[6]Estimated by SDS-PAGE
—, no data
…, the primary sequence was not completely determined
*Reported by Cologna et al. (2009)
Cys residues are in *blue*. Distinct amino acid residues determined at transcriptome and mature protein are highlighted in *green*

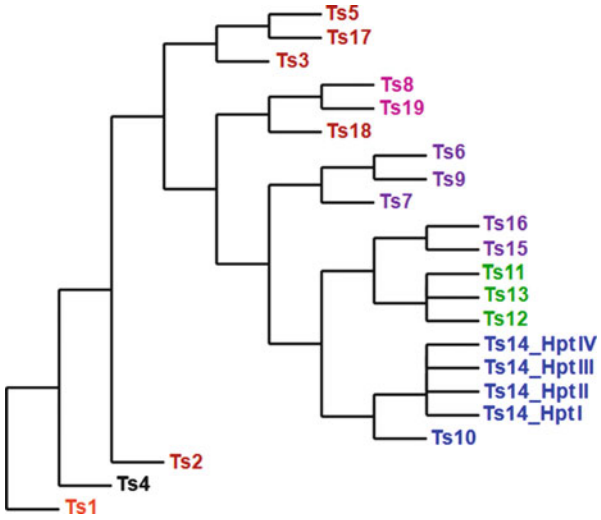

Fig. 2 Phylogenetic tree of peptides and toxins from Tsv using the maximum parsimony method. Each branch of the tree ends with the name of the peptide. The tree was constructed using the Phylogeny analysis program (http://www.phylogeny.fr/). The "A la Carte" mode was used with ClustalW for multiple alignment, parsimony method using TNT (Tree analysis using New Technology) for tree building, and TreeDyn for tree rendering. α- and β-NaTx are in *red* and *orange*, respectively. Ts4, a nontoxic protein, is shown in *black*. α- and β-KTx are colored in *violet* and *pink*, respectively. The bradykinin-potentiating peptide and hypotensins are in *blue*. The other peptides whose sequence was completely determined are represented in *green*

however, it is a bradykinin-potentiating peptide and it is in a branch close to **hypotensins** (in blue in Fig. 2). The components of the latter belong to a new structural family of peptides able to potentiate the hypotensive effects of bradykinin (Verano-Braga et al. 2008).

Tityus serrulatus venom (Tsv) has been studied for many years, and several toxins have already been described. The classification of the different components of Tsv presented in this chapter is based upon their pharmacological action. However, some peptides identified in the venom have not been functionally characterized yet, and they have been classified according to the primary sequence similarity with other well-characterized venom components.

Na^+ Channel Toxins (NaTx)

Voltage-gated sodium, calcium, and potassium channels play a key role in electrical signaling of excitable cells. They are critical elements required for action potential generation and conduction. Since ion channels play important roles in many physiological processes, they are molecular targets for a broad range of potent neurotoxins. Scorpion neurotoxins have been shown to affect the ion permeability of excitable cells and have been intensively studied because they represent

excellent models for investigating structure/function relationships and they are also fine probes for studying ionic channel functions.

Voltage-gated sodium (Na_V) channels are a large superfamily of transmembrane proteins that play a key role in the initiation and propagation of action potential in excitable cells and are responsible for the initial depolarization of the membrane. Nine mammalian Na_V channels isoforms ($Na_V1.1$–1.9) have been identified, and they are targets of neurotoxins which strongly alter their basic functions: conductance, voltage-dependent activation, and voltage-dependent inactivation. Na_V channels consist of a pore-forming α-subunit and auxiliary β-subunits. The α-subunit contains four homologous domains (DI-DIV), each made out of six hydrophobic transmembrane segments (S1–S6), connected by both intracellular and extracellular loops. The membrane reentrant loop between S5 and S6 forms the narrow extracellular end of the pore, and the intracellular loop between DIII and DIV, in particular the IFM sequence, is believed to act as an inactivation gate. The voltage sensor domain consists of the S1–S4 segments. The S4 segments, which contain repeated motifs of a positively charged amino acid residues followed by two hydrophobic residues, move outward in response to depolarization to initiate a conformational change that opens the pore (for review, see Catterall et al. 2007).

Scorpion toxins targeting voltage-gated sodium (NaTx) channels are widely used as powerful tools to study the molecular structure and function of these channels. They are known as long-chain toxins (60–76 amino acid residues), cross-linked by four disulfide bridges. NaTx can be divided into two groups (α- and β-toxins), according to their mechanism of action and binding to different sites on the extracellular surface of the Na_V channel.

α-Scorpion toxins bind to the extracellular receptor site-3 on the Na_V channel and slow or block its fast inactivation by preventing outward movement of the S4 segment from domain IV of these channels. Basic amino acids of the α-toxins and amino acids located on the S3–S4 loop of domain IV (highlighting hydrophobic residues and Glu residues in S3b and Arg residues in S4), as well as on the loops S5–S6 (domains I and IV) of the Na_V channel pore are involved in toxin binding (Rogers et al. 1996; Bosmans et al. 2008). **(a) 'α-classic'**, highly active in mammal, their binding affinity to sodium channels is reduced by membrane depolarization; **(b) 'anti-insect'**, that show high toxicity towards Na_V channels of insects, their binding to neuronal membranes is independent of membrane potential, and **(c) 'α-like'**, that are active in both mammals and insects Na_V channels, with a preference for insects (Gordon et al. 2007).

β-Scorpion toxins bind to the extracellular loop of segments S3–S4 of domain II, the receptor site-4 of Na_V channels, and induce both a shift in the voltage dependence of Na_V channel activation in the hyperpolarizing direction and a reduction of the peak sodium current amplitude. These toxins trap segment S4, keeping it in the activated position, producing spontaneous and repetitive action potentials. β-toxins are classified according to their pharmacological preference for insect and mammalian Na_V channels into four groups: **(a) βm**, active on mammalian Na_V channels; **(b) βi**, which selectively act on insect Na_V channels; **(c) β-like**, for those without

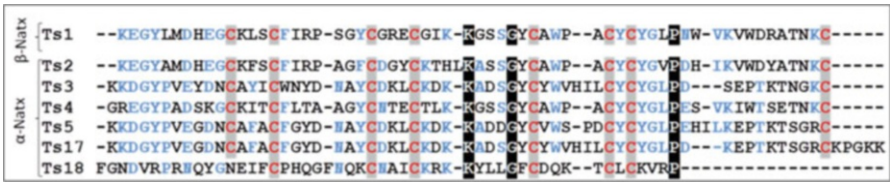

Fig. 3 Sequence alignments of NaTxs from Tsv. The conserved residues are in *blue*, and those highly conserved are highlighted in *black*. The amino acid residues in *black* indicate low consensus. Cys residues are in *red* and shaded in *gray*. Alignment was generated by MultAlin

preference between mammalian and insect Na_V; and **(d) β_α**, for toxins that present primary structure of β-toxins, but with a functional α-effect (Bosmans et al. 2007).

So far, seven neurotoxins from the Brazilian scorpion *T. serrulatus* active in Na_V channels have been described and characterized (Fig. 3). Indeed, NaTxs, the main agents responsible for the toxic effects of *T. serrulatus* envenoming, present high expression (about 6 % of the total transcripts and 18 % of the venom transcripts); in particular, this was observed for Ts1 and Ts2 (Alvarenga et al. 2012).

Ts1

Ts1, the major toxin of Tsv, corresponds to 16 % of the crude soluble venom and contributes significantly to venom toxicity (Vasconcelos et al. 2005). It is also the best-studied toxin from Tsv and was initially named Toxin γ by Possani et al. (1977) and Ts VII by Bechis et al. (1984), who first established its amino acid sequence.

Ts1 is classified as a β-like toxin, binds at site 4 of the Na_V channels, and is active on both mammalian and insect cells. This toxin has been reported to have high affinity for Na_V channels using binding studies on membrane synaptosomes (Barhanin et al. 1982). Electrophysiological studies performed with Ts1 on peripheral nerve membrane of *Xenopus laevis*, under current- and voltage-clamp conditions, demonstrated that it depolarizes the membrane, induces spontaneous activity, reduces the amplitude of the action potential, and increases its duration. Ts1 (440 nmol/L) induces inward Na^+ current flow at resting potential, shifts the voltage dependence of activation toward more negative potential values (~10 mV), and reduces the maximum Na^+ permeability to about 20 % (Jonas et al. 1986).

Ts1 can interact with other domains in addition to the canonical interaction with domain II. Bosmans et al. (2008) showed that the transfer of the paddle motifs from domains II, III, or IV from $rNa_V1.2a$ renders the $K_V2.1$ channel sensitive to Ts1 (TsVII), revealing that this toxin can interact with multiple paddle motifs from $rNa_V1.2a$ channel. Similar effects were observed with the transfer of the domain II, III, or IV paddle motifs from hNav1.9, with the domain II chimera exhibiting the largest inhibition in the presence of Ts1 (Bosmans et al. 2011). In contrast, Ts1 selectively binds to the voltage sensor in domain II from $rNa_V1.4$ channel, showing that its interactions can differ between Na_V channel subtypes (Bosmans et al. 2008).

Additionally, Ts1 (100 nM) produces a dramatic facilitation of rNav1.9 currents with little or no effect on rNav1.8, showing that this toxin can discriminate between these two TTX-r Na_V channels (Bosmans et al. 2011).

Study performed by Campos et al. (2007) showed that the binding of the Ts1 to skeletal muscle sodium channels, Na_V 1.4, not only immobilizes domain II voltage sensor in an activated state, but also causes large hyperpolarizing shifts in the activation of other voltage sensors. These findings reveal the cooperative or coupled behavior of voltage sensors in Na_V channels and explain how Ts1 has such profound effects on the voltage-dependent gating process.

Ts1 also exhibits strong toxicity to insects. It binds with high affinity to the same site as AaH IT on the insect sodium channel (De Lima et al. 1986). Therefore, in addition to its important application in studies of structure/function of Na_V channels it can also be used to design new bioinsecticides.

In vivo studies performed with Ts1 showed that it is able to induce an increase in plasma levels of aminotransferase, amylase, creatine kinase, and lactate dehydrogenase, liver congestion, pulmonary and renal hemorrhage, hypertrophy, and degeneration of cardiac areas. Ts1 (30 μg/kg) induces pronounced hypertension, with the maximal pressor effect at 2.0–3.5 min after toxin administration, with concomitant increase in plasma catecholamines. The hypertensive effect is considered the major aetiological factor responsible for the development of cardiac failure and pulmonary edema (Vasconcelos et al. 2005).

In vitro assays show that macrophages exposed to Ts1 increase the production of proinflammatory cytokines IL-1α and IL-1β after 12 h; IL-6 and TNF after 24 h; as well as IFN-γ and NO after 72 h. In contrast, increase of IL-10, an anti-inflammatory cytokine, was observed after 120 h, indicating that Ts1 has an important immunomodulatory effect on macrophages (Petricevich et al. 2007, apud Zoccal et al. 2011). Similar results were obtained by Zoccal et al. (2011) using J774.1 murine macrophage cell line.

Ts1 is a basic protein composed of 61 amino acids cross-linked by four disulfide bridges (Table 1 and Fig. 3). Ten of its amino acid residues are positively charged (Arg, Lys, His); five are negatively charged (Asp, Glu) and there are nine aromatic residues (Trp,Tyr, Phe). Its amino acid sequence deduced from the cDNA nucleotide sequence shows that the mature toxin is the product of a precursor (Table 1) containing a signal peptide of 20 residues, the mature toxin and an extra Gly-Lys-Lys tail at the C-terminal region. Thus, the posttranslational modifications of Ts1 involve the removal of the signal peptide by a signal peptidase, cleavage of C-terminal Lys residues by a carboxypeptidase, and the conversion of the remaining Gly-extended toxin into a des-Gly protein by an α-amidating enzyme (Martin-Eauclaire et al. 1992).

Polikarpov et al. 1999 (*apud* Cologna et al. 2009) showed that positively charged Lys at positions 1 and 12 as well as a negatively charged Glu at position 2 are likely determinants of the specificity of β-toxins. They observed that all residues identified as functionally important are located at one side of the molecule (Face A), which is therefore considered as the Na_V channel recognition site. Face A is also characterized by a number of aromatic interactions.

Ts2

The α-toxin Ts2 was previously described as a β-neurotoxin based on receptor binding assays (Mansuelle et al. 1992 *apud* Cologna et al. 2012) and on the high sequence identity (72 %) shared with Ts1 (Table 1 and Fig. 3). However, despite sharing a very similar primary structure, Ts1 and Ts2 present opposite effects regarding to NO, TNF-a, IL-6, and IL-10 production (Zoccal et al. 2011). Ts2 stimulated the production of IL-10, suggesting an anti-inflammatory activity for this toxin. Additionally, Sampaio et al. 1991 (*apud* Cologna et al. 2009) demonstrated that Ts2 induces prolongation of the action potential of myelinated fibers of rabbit vagus nerve, a classic effect of α-neurotoxins. More recently, Ts2 was assayed against eight subtypes of Na_V channels (rNa_V1.2, rNa_V1.3, rNa_V1.4, hNa_V1.5, mNa_V1.6, rNa_V1.7, rNa_V1.8, and the insect channel DmNa_V1) and showed to be able to inhibit rapid inactivation of Na_V1.2, Na_V1.3, Na_V1.5, Na_V1.6, and Na_V1.7, although not affecting Na_V1.4, Na_V1.8, or DmNa_V1 (Cologna et al. 2012). These results confirm that it is able to discriminate between mammal and insect channels, with no activity for DmNa_V1. De Lima et al. 1986 (*apud* Cologna et al. 2012) had already found that Ts2 is nontoxic for insects when injected in blowfly larvae (*Sarcophaga argyrostoma*).

Interestingly, Ts2 affects the inactivation and shifts the voltage dependence of activation of Na_V1.3 channels (an effect that is characteristic of β-toxins), with no reduction in the sodium peak amplitude. Its dual behavior (α-type/β-type effect) for this channel shows that the pharmacological sensitivity of Na_V channel subtypes to different modulators is very complex (Cologna et al. 2012).

The 3D structure to Ts2 was modeled using the Ts1 structure as a template, based on their high sequence identity (72 %). Its fold presents three antiparallel β-strands and one α-helix interlinked by four disulfide bridges (cysteine-stabilized α-helix/β-sheet motif), forming two opposite faces (A and B). Comparison between Ts1 and Ts2 structures shows that four substitutions are located in face A: Gly24Asp (comparable to Ts3 and Ts5 α-toxins), Asn50Asp (an acidic residue at position 50 is frequent in α-toxins, while β-like toxins contain a highly conserved Asn at this position), Trp51His (this Trp residue is important for Ts1 activity), and Arg57Tyr. Together, these substitutions induce important changes to the face A surface that may contribute to recognition of target sites in Na_V channels. Ts2 represents the newest member of a small group of toxins with the structural features of β-toxins but displaying α-like activity (Cologna et al. 2012).

Ts3

The effects of the α-neurotoxin Ts3 (previously named TsTX, Tityustoxin, or TsIV-5) on Na_V channel inactivation have been studied by Kirsch et al. (1989). A model (stop model) proposed to explain the effect of Ts3 on Na_V channel considers that the binding of site three toxins acts as a stop that prevents the complete movement of the segment S4 of domain IV (IVS4), slowing the inactivation, but not interfering with the channel activation (Campos et al. 2004).

Campos et al. (2008) analyzed the effects of Ts3 on the muscle Na_V channels ($Na_V1.4$) expressed in *Xenopus* oocytes using fluorescence–voltage relationship. Ts3 (200 nM) specifically impairs the conformational change that leads to fast inactivation of $Na_V1.4$ channels; consequently, the decay of the currents became slower. Ts3 acts by partially blocking the movement of the S4 segment of domain IV, which would be enough to inhibit the inactivation but still allowing a normal activation to occur. The toxin significantly shifted the fluorescence–voltage relationship of domain I to more positive potentials, showing a strong coupling between domains I and IV. Additionally, Ts3 increased the amplitude of the sodium current compared to control conditions, but did not change significantly the voltage dependence of the activation.

Ts3 contains 62 amino acid residues, has a molecular mass of 7.2 kDa, and forms four disulfide bridges (Possani et al. 1999). Many studies have been conducted to elucidate Ts3 function and structure. This toxin induces the release of several mediators, such as catecholamines, acetylcholine, NO, GABA, aspartate, and glutamate, due to their primary action on the Na_V channels (for review, see Cologna et al. 2009).

The precursors of Ts3 (Table 1), containing the C-terminal sequence Gly-Lys-Lys, are processed by caboxypeptidases. The Lys residues are removed and the remaining Gly-extended peptide is converted into a des-Gly peptide amine by an α-amidating enzyme to give a serine amide as C-terminal end (Martin-Eauclaire et al. 1994). Although the biological relevance of this posttranslational modification remains unclear, it can provide possible distinct molecular targets and effects.

Ts5

Ts5 is an α-neurotoxin able to increase the duration of the composite action potential of rabbit vagus B fibber, an effect abolished by tetrodotoxin. The α-type effect of this toxin was confirmed by a study performed using the rate of $^{86}Rb^+$ release from depolarized rat pancreatic β-cells as a measure of K^+ permeability changes. Ts5 increases the rate of the marker outflow in the presence of 8.3 mM glucose. This effect was persistent and slowly reversible, showing similarity to that induced by veratridine, a toxin that delays Na_V channels inactivation. By extending the depolarized period, Ts5 indirectly affects β-cell K_V channels, thus increasing K^+ permeability (Marangoni et al. 1995).

Ts5 represents 2 % of the soluble venom and shows high toxicity ($LD_{50} = 94 \pm 7$ μg/kg, i.v.) in mice. In vivo assays shows that Ts5 is able to induce an increase in plasma levels of catecholamines, with concomitant increase in arterial pressure on conscious unrestrained rats (Vasconcelos et al. 2005). It is also able to induce the release of NO in isolated retractor penis muscle. Additionally, Ts5 inhibited both ^{3}H-GABA (γ-aminobutyric acid) and ^{3}H-DA (dopamine) uptake from rat synaptosomes, in a Ca^{2+}-dependent manner, as consequence of depolarization, involving Na_V channels, but it is not able to affect the ^{3}H-Glu (glutamate) uptake (Cecchini et al. 2006).

Most of the effects of scorpion toxins are indirect and due to the release of adrenergic and cholinergic neurotransmitters. However, Ts5 is also able to interact directly with Na_V channel in vascular smooth muscle cells, inducing an increase in Ca^{2+} cytosolic concentration (Neto et al. 2012).

Ts17

Ts17 (U-BUTX-Ts1a) is a probable new NaTx since it presents 86 % identity with Ts5 (Fig. 2). It was found in the transcriptome profiling of the *T. serrulatus* venom gland (Alvarenga et al. 2012). Figure 3 shows the alignment of the Ts17 sequence with the NaTx from Tsv already described.

Ts18

Ts18 (U-BUTX-Ts1b) was identified in the *T. serrulatus* cDNA library and was considered a NaTx, because its sequence matched significantly with the U1-buthitoxin-Hj1a (identities = 63 %), a predicted NaTx derived from *Hottentotta judaicus* (Alvarenga et al. 2012).

K^+ Channel Toxins (KTx)

Potassium channels (K^+ channels) play a crucial role in numerous biological processes such as blood pressure regulation, immunity, neurotransmitters release, heart rate, insulin secretion, smooth muscle contraction, cell volume regulation, and cell proliferation (to review see Mouhat et al. 2008). They are responsible for keeping the equilibrium of the membrane potential of excitable cells, contributing to membrane repolarization during the action potential. K^+ channels represent the largest and most diverse family of ion channels in terms of subtypes, structure, and function (Mouhat et al. 2008) and have been implicated in a number of human pathologies such as asthma, cardiac arrhythmia, T-cell-mediated autoimmune disease, immune response to infection and inflammation, and hypertension (Bergeron and Bingham 2012). Due to their importance in many biological processes, K^+ channels became a significant target for several compounds including animal venoms and toxins (Catterall et al. 2007).

Scorpion toxins acting on K^+ channels (KTx) generally are compact peptides composed of 23–42 amino acid residues, reticulated by 3–4 disulfide bonds (Rodríguez De La Vega and Possani 2004). These toxins act in synergism with those active on Na^+ channels resulting in abnormal nerve functioning. The combination of both types of toxins causes changes in the neurotransmitters' release resulting in paralysis and death of the prey (Mouhat et al. 2008). Based on their molecular target, sequence similarities, and cysteine distribution, the scorpions' toxins active on K^+ channels (KTx) were classified in four families named α, β, γ, and κ-KTx. In addition, a systematic nomenclature was also proposed (Rodríguez De La Vega and Possani 2004). The α-KTx, the largest subfamily, comprises peptides of 23–43 amino acids stabilized by 3–4 disulfide bonds and folded within the CSα/β scaffold, the most common for scorpion toxins (Mouhat et al. 2008). The unusual β-KTx group is characterized by long peptides (~60 amino acid residues) linked by three disulfide bridges with the same CSα/β architecture of α-KTx. γ-KTx's are *hERG* (ether-à-go-go) channel blockers with 36–47 amino acid residues connected by three or four disulfide bridges. This subfamily has the same CSα/β framework of the previous subfamilies (Rodríguez De La Vega and Possani 2004). The representatives of the last family, the κ-KTx, are relatively

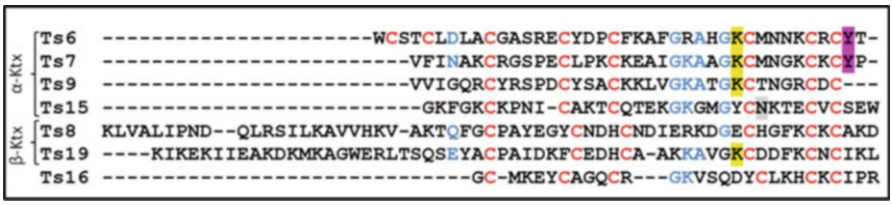

Fig. 4 Sequence alignments of KTxs from Tsv. The conserved residues are in *blue*. Cys residues are highlighted in *red*. Residue Lys highlighted in *yellow* is responsible for disrupting potassium conduction in the selectivity filter (Banerjee et al. 2013) and residue Tyr highlighted in *pink* is part of functional dyad. Asn residue highlighted in *gray* represents a putative N-glycosylation site (Verano-Braga et al. 2013). Alignment was generated by MultAlin

poor K^+ blockers and display a different tertiary arrangement with two α-helices stabilized by two disulfide bonds also known as CSα/α scaffold (Mouhat et al. 2008).

The main mechanism of action of scorpion toxins on K^+ channels is pore occlusion via adequate positioning of the toxin β-sheet onto the channel pore. A strategically positioned lysine on the β-sheet side and an aromatic residue separated by 6.6 Å form the "functional dyad" (Fig. 4), which plays a critical role in the interaction with voltage-gated K^+ channels. Such structure was reported in toxins from several animals' species and, generally, is present despite the type of peptide scaffold and half-cystine pairing pattern. The side chain of the basic residue of the dyad places itself into the K^+ channel pore, whereas the hydrophobic residues (aromatic and aliphatic) stabilize the complex toxin/receptor, leading to a physical occlusion of the channel. Although the importance of this pharmacophore is widely recognized, toxins lacking the functional dyad and still presenting significant effect on potassium channels have also been described. These findings highlight the existence of other important regions of the toxin that mediate their interaction with Kv channels (Mouhat et al. 2008; Banerjee et al. 2013).

This structural diversity of scorpion toxins represents a valuable research tool in different areas of knowledge. Scorpion toxins active on K^+ channels have been essential to the investigation and understanding of the physiological role of these channels. The persevering research on this field resulted in the identification, localization, and classification of novel K^+ channel subtypes as well as its structural and pharmacological elucidation (Bergeron and Bingham 2012). In addition, considering the role of K^+ channels in numerous diseases, scorpion toxins active on this channel family may assist in the bioengineering of therapeutic scaffolds and development of novel drugs.

Among the wide variety of compounds predicted to compose Tsv, up to this date, only seven were described to act on Kv channels (Fig. 4).

Ts6

Ts6, previously named as TsTx-IV, was first isolated and described in 1989 by Arantes and coworkers (Arantes et al. 1989) as a 41-amino-acid-residue peptide. Later on, the absence of the last residue Asn41 was verified, confirming that Ts6 and the 40-amino-acid-residue-long peptide butantoxin were actually the same

toxin (Oyama et al. 2005). Ts6 was shown to block the high-conductance Ca^{2+}-activated K^+ channels in Leydig cells (Novello et al. 1999) and to inhibit the binding of 125 I-kaliotoxin into its receptors in rat brain synaptosomes, suggesting an additional affinity for $K_v1.3$ channels with an IC_{50} of 46 nM. This effect was reduced about 100-fold when the first six N-terminal residues, including the unusual disulfide bridge which forms an N-terminus ring, were cleaved (Pimenta et al. 2003a). In addition, the blocking effect of Ts6 on *Shaker* B K^+ channels with Kd of 660 nM was described, highlighting the ability of this toxin to interact with diverse types of K^+ channels with different affinities (Coronas et al. 2003).

Structural studies reveal the presence of the global CSαβ scaffold on the tertiary arrangement of Ts6. Interestingly, Ts6 structure is linked by four disulfide bridges, a characteristic first elucidated for short peptides from *T. serrulatus* for this striking toxin (Novello et al. 1999; Oyama et al. 2005). This novel feature, the presence of an extra disulfide bridge in the N-terminal of the toxin (Fig. 4), seems not to be related with the structural stability, as observed for other disulfide bonds, but it may play an important role in receptor specificity. Moreover, Ts6 has the ability to preserve its active conformation even when submitted to drastic pH variation, thus expanding the range of cellular conditions in which the toxin may exhibit its function (Oyama et al. 2005).

The immunomodulatory effects of Ts6 were also investigated and the results show that Ts6 can stimulate the production of NO, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in J774.1 cell, which are enhanced under LPS co-stimulation. Those effects observed were suggested to be independent of toxin/ion channel interaction, since Ts6 has shown similar inflammatory effects to Ts1, a specific Na^+ channel modulator (Zoccal et al. 2011).

Ts7

Formerly known as TsTx-Kα, Tityustoxin K-alpha, TsII-9, and TSK4, Ts7 was first described as a selective blocker of the ^{86}Rb efflux through non-inactivating delayed-rectifier-type K^+ channels present in synaptosome preparations, with an IC_{50} of 8 nM. Its action on voltage-gated non-inactivating K^+ current was also confirmed in hippocampal and cerebellar neurons. In contrast, Ts7 was reported to block low-voltage-activated, partially inactivating K^+ currents in neurons of dorsal root ganglion (DRG) (Matteson and Blaustein 1997). In addition, Ts7 was assayed on fibroblast cells transformed to express $K_v1.2$ subtype channel resulting in a block effect with very high affinity. This toxin has already been used as a pharmacological tool to evaluate the involvement of $K_v1.2$ subunits in the generation of total K^+ currents in native cells (Dodson et al. 2002). The action of Ts7 on $K_v1.3$ channels expressed both in mammalian cell lines (L929) and in *Xenopus laevis* oocytes were also investigated and revealed high affinity for both expression systems (Kd = 19.8 at pH 7.4; Kd = 3.9 at pH 7.5, respectively). The authors also reported a pH dependence of the blocking activity of Ts7. These results indicated that Ts7, or α-KTx4.1, may also represent a useful tool for probing the physiological role of Kv1.3 channels (for review, see Cologna et al. 2009).

The interaction of Ts7 with a cloned K^+ channel from Squid (sqKv1A) was also evaluated. To further study the interaction between this toxin and the mentioned

channel, the 3D structure of Ts7 was determined by NMR spectroscopy and a model Ts7/sqKv1A complex was generated. The importance of lysine at position 27 (K27) (Fig. 4) was confirmed, and this residue seems to be inserted into the ion conducting pathway, causing the block of the channel pore. The model also corroborates to the hypothesis that the pH-dependent block observed is related to a histidine residue (H351) present in the outer vestibule of the channel, which seems to repels the positively charged residues of the toxin at low pH (Ellis et al. 2001 *apud* Cologna et al. 2009).

Ts8

Ts8 or TsTx-K beta is a 60-amino-acid-residue peptide, linked by three disulfide bridges with an experimental molecular mass of 6,716 Da (Legros et al. 1998). Despite the unusual long chain, Ts8 was characterized as a selective blocker of voltage-gated non-inactivation K^+ channels in synaptosome preparations (Rogowski et al. 1994). The complete sequence was very different from those of the 60–70-residue toxins active on Na^+ channels or those of the 23–42-residue toxins active on K^+ channels and therefore was the first member of the β-KTx subfamily. Its amino acid sequence was also confirmed by cDNA which encodes a precursor consisted of a signal peptide (19 amino acid residues), a propeptide (8 amino acid residues), and the mature chain with 60 amino acid residues (Legros et al. 1998).

Ts9

Ts9, Ts kappa, 1TSK, Neurotoxin Ts kappa, or α-KTx4.2 is a short peptide (3784.4 Da) purified from Tsv, considered as a very high potent ligand for small-conductance apamin-sensitive calcium-activated K^+ channels (SK). Ts9 is able to efficiently compete with apamin for binding on these channels. The solution structure of Ts9 has been determined by NMR techniques, which led to the full description of its 3D conformation: a short alpha helix and a three-stranded antiparallel beta sheet (Blanc et al. 1997).

Ts15

Ts15 or α-KTx21.1 is described as a short peptide with 36 amino acid residues, cross-linked by three disulfide bridges, with a molecular mass of 3,961 Da. This toxin was assayed on a wide range of K^+ channel subtypes and has shown a blocking effect on $K_v1.2$, $K_v1.3$, $K_v1.6$, and Shaker IR in a nanomolar range, while it does not block the other K_V isoforms tested ($K_v1.1$, $K_v1.4$, $K_v1.5$, $K_v2.1$, $K_v3.1$, $K_v4.2$, $K_v4.3$, and *hERG*). It was demonstrated that Ts15 preferentially blocks $K_v1.2$ and $K_v1.3$ channels with an IC_{50} value of 196 ± 25 and 508 ± 67 nM, respectively (Cologna et al. 2011). The preference of Ts15 for $K_v1.2$ was related with the quantity of basic amino acid residues in the C-terminal region of the toxin (Fig. 4), which was proposed to be less in Kv1.2 high-affinity toxins when compared with the ones with high affinity for Kv1.3. Ts15 did not share high similarity with any of the 14 Ts toxins previously described, neither with KTxs deposited in the data bank, and therefore was considered a bona fide novel type of toxin (Cologna et al. 2011). Recently, a posttranslational modification in the structure of Ts15 was reported, which became the first N-glycosylated toxin ever described in Tsv (Verano-Braga et al. 2013).

Ts16

Ts16, as well as Ts15, was screened on a wide variety of K^+ channels such as $K_v1.1$–$K_v1.6$, $K_v2.1$, $K_v3.1$, $K_v4.2$, $K_v4.3$, and $K_v7.1$ and Shaker IR and *hERG* using the two-electrode voltage-clamp technique. Interestingly this toxin selectively blocks $K_v1.2$ channels, without any effect on the other K^+ subtypes (Bordon et al. 2011). Since this toxin shares 62 % of identity with Tt28 or α-KTx20.1 from *T. trivittatus* venom, it would be reasonable to classify it at the same α-KTx20 subfamily and therefore name it as α-KTx20.2 (Saucedo et al. 2012). However, Saucedo and collaborators (2012) have stated an unexpected new cysteine pattern accompanied by a different arrangement of the secondary structure topology into a CSα/α scaffold, the characteristic scaffold of the κ-KTx. The unconventional structure, which consists of an antiparallel helix–loop–helix topology stabilized by three disulfide bonds, was determined for the recombinant version of Ts16 (rTs16) and provides new insights on the structural versatility of scorpion peptides. These findings could suggest the classification of Ts16 as a member of the κ-KTx subfamily, the only group of scorpion toxins which adopt the same structural pattern. However, by structural analyses, the authors hesitated to classify it since the helix–loop–helix topology of rTs16 seems to be an elaboration of CSα/β scaffold, rather than a variation of the κ-KTx α-hairpin (Saucedo et al. 2012).

Ts19

The partial sequence of Ts19 was first detected by peptidomic analysis as two overlapping peptide fragments of 12 and 9 amino acid residues and was suggested to be fragments from the propeptide region of a β-KTx-like toxin (Rates et al. 2008). In a newly transcriptomic study, Ts19 precursor was fully determined and consists of a signal peptide of 25 amino acid residues followed by the mature toxin composed of 66 amino acid residues (Alvarenga et al. 2012). Just recently, two mature fragments of Ts19 were sequenced by Edman degradation and deposited in the UniProt data bank as Ts19 fragment I (Carmanhan et al. 2013) and fragment II (Cerni et al. 2013). The determined sequences suggest a presence of a propeptide region in the precursor described by Alvarenga and coauthors (2012) composed of eight amino acid residues. The toxin Ts19 fragment I has nine additional residues in the N-terminal region, starting with residues KIK, when compared with Ts19 fragment II (Table 1). The predicted Ts19, Ts19 fragment I, and Ts19 fragment II all share high identity with TsTKMK and TtrKIK, both β-KTx-like toxins (98 % and 94 % of identity, respectively) from *T. stigmurus* and *T. trivittatus* (Alvarenga et al. 2012). Up to this date, no experimental data were published to confirm the activity of this toxin and to evaluate the functional differences among the two described fragments.

Proteinases

Proteinases, such as exopeptidases, are linked with the posttranslational processing of peptides and proteins from the venom and their precursors. Besides that, they are involved in the inhibition of platelet aggregation, activation of the complement

system, modulation of cytokine production, and diffusion of toxic components, since they degrade proteins of interstitial matrix (Cologna et al. 2009).

Proteolytic activity has been detected in some scorpion genera. In 1946, some effects observed in various animal species after a Ts sting, such as necrosis, hemolysis, and gangrene, were attributed to proteolytic enzymes in the venom. Probably these enzymes process and activate the toxins, facilitate the diffusion of toxic components, and activate trypsinogen, contributing to pancreatitis often observed in the victims. However, their functions in the venom are still unknown (Almeida et al. 2002). Concerning the pancreatitis, study performed with alpha (Ts2 and Ts3) and beta (Ts1) toxins from *T. serrulatus* venom shows that the acinar cells are stimulated by these peptides to discharge their zymogen granules. Additionally, the appearance of large vacuoles and some loss of morphological integrity was observed. These results indicate that probably a synergistic action between the venom components is responsible by the pancreatitis (Possani et al. 1991).

There are few studies about serine proteinases from Tsv. Gelatinolytic activity was detected in the first fraction of Tsv filtered on Sephadex G-50, whose activity was inhibited by PMSF, suggesting this fraction contains a serine proteinase; however, its isolation was unsuccessful (Almeida et al. 2002). In another study, the gelatinase activity was not found in Tsv, leading to speculation that these discrepancies could be due to the sensitivity of the methods of measurement or intraspecific/interspecific variations in venom composition (Venancio et al. 2013).

A metalloproteinase from Tsv, known as antarease, whose name is derived from the principal star Antares in the constellation Scorpio, was isolated. Its primary structure was partially determined by the sequencing of its N-terminal and internal peptides cleaved with trypsin, Asp-N, Arg-C, CNBr, Glu-C, and Lys-C. Antarease cleaves SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which are involved in the essential step that leads to fusion of vesicles with cellular membranes and are responsible for selective transport between cellular compartments. Therefore, antarease could be used for treating muscle spasms and other disorders, e.g., diabetes (Fletcher et al. 2010).

Another metalloproteinase had its N-terminal partially determined (Richardson et al. 2008c) and a dynorphin-cleaving metalloproteinase similar to antarease VAMP2 was detected in Tsv (Venancio et al. 2013).

The proteinases MQ-5 and MQ-7, isolated from Tsv, were able to activate the complement system, playing an important role during the inflammatory process after scorpion sting (Bertazzi 2007).

Hyaluronidase

Hyaluronidases have been detected in the venoms of snakes, bees, wasps, spiders, lizards, fishes, and scorpions. These enzymes possess high antiedematogenic activity and cleave hyaluronan, the major glycosaminoglycan of the extracellular matrix, being responsible for spreading toxic components through the tissues of the victim/prey. Since hyaluronidase inhibition would retard toxins' diffusion, improving

antivenom therapy and reducing its side effects, several classes of chemical compounds have been studied as potential hyaluronidase inhibitors, such as flavonoids, alkaloids, antioxidants, terpenoids, lanostanoids, antibiotics, glycosaminoglycans, polysaccharides, proteins, polyphenols, fatty acids, anti-inflammatory drugs, or synthetic organic compounds.

A 51 kDa hyaluronidase from Tsv was isolated (Pessini et al. 2001) and its 34 first amino acid residues from its N-terminal were determined (Richardson et al. 2008b). Ts hyaluronidase potentiated the action of Ts1, increasing the levels of serum enzymes, and its enzymatic activity was inhibited by some flavonoid compounds with determined structural characteristics (Pessini et al. 2001). It was also reported that Ts hyaluronidase activity is similar to that determined for some bothropic venoms (Venancio et al. 2013).

Hyaluronidase isolated from Tsv induced mononuclear increase in the bronchoalveolar space after intranasal inoculation of 16 U in C57Bl/6 mice (Bitencourt et al. 2011). It decreases the concentration of hyaluronan and thus prevents the bleomycin-induced pulmonary fibrosis development. Besides that, it ameliorated pneumofibrosis by mesenchymal-like cell recruitment and did not cause edema formation and neither induced the increase in lung vascular permeability. Considering those findings, hyaluronidase became a promising tool in the treatment of pulmonary fibrosis.

Other Components

Other components include non-neurotoxic venom components previously described for *T. serrulatus* venom.

Ts4

Ts4 (TsTX-VI, TsNTxP) presents a primary structure similar to Ts1 (Fig. 2, Table 1), but its hydropathic index indicates that it is more hydrophobic than Ts1. These characteristics can be useful in clarifying the relationship between primary structure and biological function of scorpion toxins (Marangoni et al. 1990; Chávez-Olórtegui et al. 1997).

Ts4 was considered nontoxic to mice since it was unable to induce the characteristic symptoms of toxicity produced by Tsv and the Na_V scorpion toxins. However, it induced an allergic reaction, lacrimation, and spasm of the hind legs of mice and produced dose dependent GABA and Glu liberation of synaptosomes that were blocked by tetrodotoxin (Marangoni et al. 1990). Polyclonal antibodies elicited by Ts4 are cross-reactive with several toxins from Tsv. Antibodies raised against peptides corresponding to residues 1–15 and 47–61 were able to neutralize the venom toxic fraction, indicating that these residues might be involved in the toxic action of the scorpion neurotoxins. These results indicate that this protein may be of interest in the production of antivenoms for clinical use (Chávez-Olórtegui et al. 2002).

Ts11, Ts12, and Ts13

Ts11 (TsPep1, 2,936 Da), Ts12 (TsPep2, 2,991 Da), and Ts13 (TsPep3, 3,017 Da) are 29 amino acid residues long, highly reticulated by a new pattern of four disulfide bridges, which make them the most constrained structures of scorpion venom-derived peptides known up to date. They present high sequence similarities with some KTx, as can be observed in the phylogenetic tree of peptides and toxins from Tsv (Fig. 2), but their biological function has not been determined yet. They are devoid of toxicity in mice. Ts13 was found to be a close isoform of Ts12 and the difference between them is just a change in position 13 (Ala/Pro). Ts11 showed 58.6 % of sequence homology with Ts12. Interestingly, the sequence of Ts12 corresponds only partially to that of the precursor (Table 1), indicating a specific posttranslational maturation process with the cleavage of a non-negligible C-terminal portion of 13 amino acid residues (Pimenta et al. 2003b).

Ts10 and Ts14 (Hypotensins)

Bradykinin-potentiating peptides (BPPs), which are able to inhibit the angiotensin-converting enzyme (ACE) activity, are random-coiled linear peptides characterized by the BPP amino acid signature (an N-terminal pyroglutamic acid and the sequence Ile-Pro-Pro at the C-terminal). The BPP first isolated from *Bothrops jararaca* venom was essential to develop the commercial antihypertensive drug, captopril. Since then, many other BPPs were found in venom of other snakes, spiders, and scorpions.

Surprisingly, Ts10 (peptide T) has not a typical BPP amino acid signature, but it was able to potentiate bradykinin, by inhibiting ACE activity (Ferreira et al. 1993). It presents 13 amino acid residues, 1603.7 Da (Table 1), and is very different from the other components of the venom with hypotensive action (hypotensins), as shown in the phylogenetic tree presented in Fig. 2.

Ts14, also known as hypotensins (TsHpt-I, II, III, and IV), are random-coiled linear peptides. Their primary structure (Table 1) presents a similar BPP amino acid signature, with a noncanonical Lys residue prior to the conservative Pro-Pro doublet. TsHpt-I (2,732 Da) induces hypotension and potentiates the bradykinin hypotensive effects when administrated to normotensive rats. It is able to induce endothelium-dependent vasorelaxation dependent on NO release, but could not inhibit ACE activity (Verano-Braga et al. 2008). Differently from other BPPs, TsHpt-I acts as an agonist of the B2 receptor, a subtype of kinin receptor, inducing NO synthesis and consequent vasodilatation. Synthetic peptides containing the Lys-Pro-Pro at C-terminal kept the ability to activate this receptor and could induce a transient hypotension (Verano-Braga et al. 2010).

The complete sequence of hypotensin-like and hypotensin-1 precursors was identified in *T. serrulatus* venom gland transcriptome (Alvarenga et al. 2012). TsHpt-I and TsHpt-II are phosphorylated (Verano-Braga et al. 2013).

PAPE Peptides

Second only to the neurotoxins, the PAPE peptide (8.5 % of the total transcripts) was the most highly expressed transcript in the *T. serrulatus* venom gland transcriptome (Alvarenga et al. 2012). In this protein rich in proline, the PAPE tetrapeptide (Pro-Ala-Pro-Glu) iteratively appears in the sequence. Additionally, it presents three proline-containing motifs (PEPAP, AAPE, and PEPAAAAPE). Rates et al. (2008) report the identification/sequencing of 28 peptides that could be identified as fragments from PAPE protein. While the signal peptide is highly conserved among these peptides, the N-terminal region is moderately conserved and the C-terminal region shows very little conservation. Despite its higher expression level, the function of the PAPE peptides in *T. serrulatus* venom remains unknown.

Antimicrobial, Ponericin-Like, and Anionic Peptides

Sequences of antimicrobial peptides (AMPs, 17 %) and anionic peptides (3 %) were abundant in the venom gland transcriptome of *T. serrulatus*. Although the precise function of these components has not been elucidated yet, their abundance suggests an important role in the biological function in the venom gland (Alvarenga et al. 2012). Three different sequences coded an **AMP** highly similar to the putative AMP of *T. costatus*. These AMPs may act as protectors against bacterial infection or potentiators of neurotoxin action. One anionic peptide sequence was identified in the transcriptome of *T. serulatus*. This class of peptides has been reported as highly expressed and conserved among the Buthidae scorpion species. They might play antimicrobial activity or an important role in pH balance, since neurotoxins are basic peptides. Additionally, two ponericin-like sequences were found and they, probably, present antimicrobial function (Alvarenga et al. 2012).

Conclusion and Future Directions

Tityus serrulatus venom presents several underexplored compounds with promising pharmacological effect. Further characterization of these peptides will help to clarify their role in the envenoming and will propitiate its use as potential drugs or tools for biological systems' studies.

Scorpion neurotoxins, due to their high affinity and specificity, have provided important information about the structure and the function of Na_V and K_V channels, affecting both permeation and gating properties. Moreover, neurotoxins turn out to be invaluable tools to distinguish and to reveal unique properties of different Na_V channel isoforms. The demonstrated antimicrobial activities of AMPs have indicated their potential for use as anti-infective drugs.

Some bioactive proteins/peptides (such as KTxs, AMPs, and hypotensins) present in *Tityus serrulatus* venom are an important resource for the investigation

and characterization of molecules applicable in pharmaceutical research and biotechnology. Such richness can be useful to biotechnology in many ways, with the prospection of new drug candidates being the most promising.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ Potassium Channel Blocking Peptide Toxins from Scorpion Venom
- ▶ Scorpion Venom Gland Transcriptomics
- ▶ Scorpion Venom Interactions with the Immune System
- ▶ Scorpion Venoms: Pathogenesis and Biotherapies
- ▶ Scorpionism and Dangerous Species of Brazil

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Part VI

Scorpion Toxins

Androctonus Toxins Targeting Voltage-Gated Sodium Channels

19

Marie-France Martin-Eauclaire, Najwa Abbas, Brigitte Céard, Jean-Pierre Rosso, and Pierre E. Bougis

Contents

Abstract

The *Androctonus* genus constitutes a serious threat to human health in Northern Africa and Southwest Asia because some of the *Androctonus* subspecies produce the most dangerous venoms for mammals. These venoms have provided several high selective affinity ligands, which specifically interact with sodium, potassium, chloride, and calcium channels. However, the vast majority of lethal toxins, even present in the venom at few percent, is active on voltage-gated sodium (Na_v) channels and is responsible of almost the whole venom toxicity in mice by subcutaneous injection. During the last four decades, an increasing amount of data was published on the isolation, chemical, pharmacological, and immunological characterization of several structurally distinct families of these extremely active *Androctonus* toxins, which induce different biological answers when applied to Na_v channels. These toxins have been further extensively used to study the functioning and decipher the topology of Na_v channels. This chapter

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reviews the current knowledge on *Androctonus* toxins active on Na_v channels only, at the structural, biological, and immunological level. The organization of their gene and mRNA precursor will also be approached.

Introduction

The *Androctonus* Scorpion Genus

Scorpions constitute an ancient and ecologically successful group of animals being exemplified in the fossil's trace for some 400 million years and currently comprising for around 2,000 different species. Indeed, as most of their morphological features have remained largely unaltered, they are considered by some authors as true living fossils. These arthropods are a serious threat to human health in many countries throughout the world, particularly in South and Central America and in North Africa. During decades, they have been classified into six families: Bothriuridae, Scorpionidae, Buthidae, Vejovidae, Chlaerilidae, and Chactidae (Bücherl 1971). However, it has now been described in 18–20 families (see ▶ Chap. 1, "Scorpion Diversity and Distribution: Past and Present Patterns," in this book). The Buthidae family is widespread around the world. With 86 genera and 990 species, and this number is still growing, it is the largest one among the scorpion families. The most dangerous scorpions for human being, because they produce venoms lethal for mammals, belong to this family. Only about 20 Buthidae species can be lethal and therefore cause a major public health problem in the countries where they are found. They include the genera *Androctonus* (Northern Africa to Southwest Asia), *Buthus* (Mediterranean), *Leiurus* (Northern Africa and Middle East), *Mesobuthus* (Asia), *Parabuthus* (Western and Southern Africa), *Centruroides* (Southwest USA, Mexico, Central America, Caribbean region), and *Tityus* (Central and South America, Caribbean region).

Scorpions from the genus *Androctonus* are very big-sized animals, which can inject up to 500 μg of venom especially rich in lethal toxins (Fig. 1). In the Maghreb, *Androctonus australis* in Algeria (hector morph) and in Tunisia (subspecies garzonii) and *Androctonus mauretanicus* in Morocco are responsible of about 100,000 stings per year. Among them 1–7 % lead to death according to the geographical zone and the patient age, the children being more vulnerable to scorpion stings. Their median lethal dose (LD_{50}) by subcutaneous injection is between 1 and 6 μg per mouse of 20 g; thus they are considered as among the most lethal scorpion species in the world. Their venoms have been widely studied, and the most toxic components are now well characterized.

As example, three *Androctonus* species have been reported from Tunisia: *Androctonus amoreuxi*, *Androctonus aeneas*, and *Androctonus australis*. The latest has been divided in two subspecies: *Androctonus australis garzonii* Goyffon & Lamy and *Androctonus australis* hector Koch. Variation of ribosomal DNA sequences of the ITS1, 5.8S, and ITS2 regions of 14 representatives of the four

Fig. 1 The scorpion *Androctonus mauretanicus mauretanicus from Morocco*

taxa in Tunisia was reported (Ben Ali et al. 2000) and described the high polymorphism of the ITS regions. In some instances in both intra- and interspecific comparisons, it was difficult to unambiguously align the sequences, and it appeared difficult to recognize the species on the basis of these sequences. The conducted phylogenetic analysis inquires the validity of the subspecies status of *Androctonus australis garzonii* versus hector but grouped together *Androctonus amoreuxi* and *aeneas*. Little information is available in the literature concerning the venoms of other *Androctonus* species (see Table 1: Different *Androctonus* species identified; in red, scorpions with some toxins described in the database Uniprot).

These other *Androctonus* species venoms are not really studied so far. Why? Different reasons could be considered. Or their venom toxicity is not really dangerous for humans? Or they live on a dispersed area and stings and fatal cases are scarce? Or the countries concerned never achieved systematic studies on these venoms. In Maghreb, the three Pasteur Institutes (Casablanca in Morocco, Alger in Algeria, and Tunis in Tunisia) have largely contributed to *Androctonus mauretanicus* and *Androctonus australis* venoms studies, their primary goal being the production of specific and efficient antivenoms for serotherapy purpose.

In Fig. 2 (localization of *Androctonus* species) is shown the large area where *Androctonus* species are found. In Table 2 is given the medium LD_{50} of different *Androctonus* venoms.

The Scorpion Venom Constituents

Scorpion venoms are highly complex mixtures of peptides, nucleotides, lipids, mucoproteins, biogenic amines, some rare enzymes, and other unknown substances. As a consequence, each secretion possesses a wide spectrum of bioactivities. The complex cocktail of bioactive molecules contained in scorpion venom appears to have evolved primarily for subduing prey and also plays a highly effective role in chemical defense. Polypeptide toxins from scorpion venom have

Table 1 Different *Androctonus* species identified. According to Dupré Gérard, 2004, World bibliography of scorpions (Internet site The Scorpion Fauna)

Species	Subspecies
Androctonus afghanus	
Androctonus aleksandrplotkini	
Androctonus amoreuxi	
Androctonus australis	
	A. australis (Aah)
	A. australis garzonii (Aag)
	A. australis libycus
Androctonus baluchicus	
Androctonus bicolor	
	A. bicolor aeneas
Androctonus crassicauda	
Androctonus dekeyseri	
Androctonus finitimus	
Androctonus gonneti	
Androctonus hoggarensis	
Androctonus liouvillei	
Androctonus maelfaiti	
Androctonus mauretanicus	
	A. mauretanicus mauretanicus (Amm)
Androctonus sergenti	
Androctonus eburneus[a]	
Androctonus maroccanus[a]	
Androctonus togolensis[a]	

[a]Rein, J. O. 2009 (internet site The Scorpion Files)
For more details, see in this book the contribution from Lourenço, ▶ Chap. 1, "Scorpion Diversity and Distribution: Past and Present Patterns"
In red: scorpions with toxins described in Uniprot database

high degrees of specific actions on and interactions with the ion channels and receptors in excitable membranes. Predictions suggest that close to 100,000 distinct polypeptides are present in all identified scorpion species, and from this total, only 1 % is currently known (Possani et al. 1999). Furthermore, for several of these peptides, no function is described and has yet to be assigned. In fact, four different families of scorpion toxins, which specifically interact on ionic channels, have been characterized so far. They are active on sodium, potassium, chloride, and calcium channels. In Buthidae venoms, even present in the venom as few percent of the dried secretion, the vast majority of lethal toxins is active on voltage-gated sodium (Na_v) channels and is responsible of almost the whole venom toxicity by subcutaneous injection (Martin-Eauclaire and Couraud 1995; Pedraza-Escalona and Possani 2013; Possani et al. 1999).

The scorpion venom compounds are highly stable and resistant to denaturation. The high number of disulfide bonds that reticulated these small (20–70 amino acid residues) peptides explains this stability. Glycine and proline residues are relatively

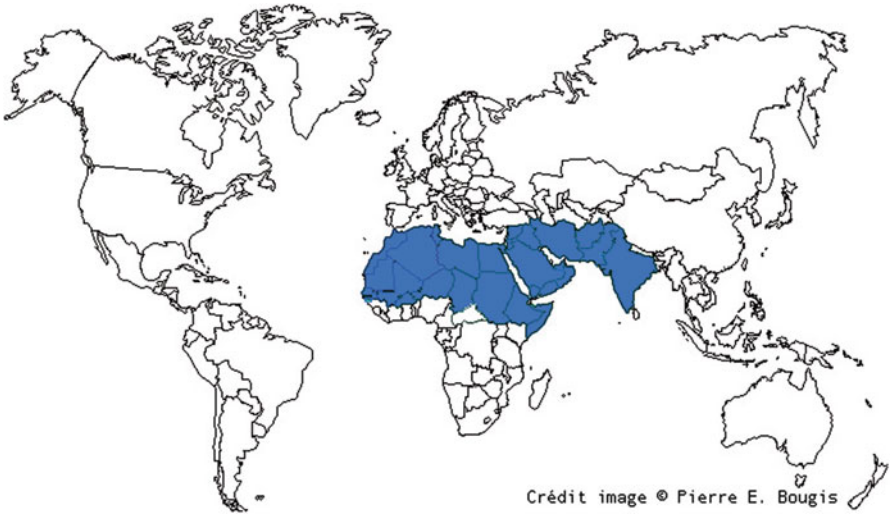

Fig. 2 World localization of the *Androctonus* species

Table 2 LD_{50} in mice (C57Bl/6) of some *Androctonus* venoms

Species	LD 50 mg/kg	LD 50 µg/ 20gr mouse	Injection route	References
Androctonus amoreuxi	0.75	15	s.c	Abbas et al. (2009)
***Androctonus australis* (hector)**	0.32	6.4	s.c	Martin and Rochat (1986)
Androctonus bicolor	1.21	24.2	i.v	Martin-Eauclaire (Unpublished data)
	0.31	6.2	s.c	
Androctonus crassicauda	0.32	6.4	i.v	Martin-Eauclaire (Unpublished data)
	0.64	12.8	s.c	
Androctonus mauretanicus	0.31	6.2	sc	Alami et al. (2003)

s.c subcutaneous, *i.v* intravenous

abundant in toxin amino acid sequences and may provide the balance of the polypeptide chain flexibility necessary for recognition of multiple targets and for survival in a hostile environment. Scorpion toxins display a high degree of relatedness at the level of their three-dimensional (3D) structure, despite having more limited sequence homology, and they have the ability to interact with a wide variety of channel targets in a sequence-specific fashion. Thus, because of these tightly folded and stable structures, scorpion toxins provide ideal molecular scaffolds in basic research, probing the structure of the ion channels and studying their activation and inactivation processes, as well as in drug development. Another important

application of scorpion toxins is based on their ability to discriminate between vertebrate and invertebrate channels. Insect-selective scorpion toxins could serve as templates for further development of novel pesticides (Arnon et al. 2005; Housset et al. 1994).

Structure and Function of the Buthidae Scorpion α- and β-Toxins

Pharmacological Properties

As already mentioned above, the whole toxicity of scorpion venom for human being is mainly attributed to the activity of the long-chain toxins, which bind with high affinity to Na_v channels of excitable cells acting as gating modifiers. Na_v channels by opening rapidly are crucial for generating the rising phase of the action potentials. Toxins increase or decrease ion flux by affecting either activation or inactivation kinetics and consequently disturb the action potentials (Catterall 2012; Martin-Eauclaire and Couraud 1995; Possani et al. 1999).

The scorpion toxins specific for Na_v channel can be divided into two classes: α-toxins, which bind to site 3, and β-toxins, which bind to site 4.

The α-scorpion toxins bind to the paddle sensor of domain IV and by holding it in its inward position, slow down the fast inactivation process of the channel, provoke a transient increase in sodium permeability, and induce a persistent depolarization of cell membrane. Their binding is dependent on membrane potential and decreases at depolarized potentials (Bosmans et al. 2008; Catterall 2012).

The β-type toxins are the major components in the New-World scorpion venoms, contrary to the Old-World scorpion venoms, which contain a majority of α-type toxins. They were isolated mainly from the North-American *Centruroides* scorpions. They trap and stabilize the paddle sensor of domain II in its outward, activated position, thereby enhancing channel activation in response to subsequent depolarization and negatively shifting the voltage dependence of activation (Bosmans et al. 2007; Catterall 2012; Cestèle et al. 2006; Pedraza-Escalona and Possani 2013; Possani et al. 1999; Zhang et al. 2012).

Gene Organization and Primary Structure

The Na_v channel-specific scorpion toxins are single-chain peptides composed of 60–76 amino acid residues highly reticulated by four disulfide bridges. So far, hundreds of distinct scorpion peptides have been purified and characterized from about 20–30 different species of scorpions (Billen et al. 2008). The amino acid sequences of many others have been also obtained by cDNA sequencing. However, in that case, there is always some uncertainty about the C-terminal sequence and amidation of mature peptides. Thus, the reader has to be extremely careful when describing the proposed sequence in the data bank.

In particular, the first scorpion toxin cDNAs cloned were described from a cDNA library made from telsons of *Androctonus australis* (Bougis et al. 1989). Using oligonucleotide probes, the precursors of scorpion toxins active on mammals or on insects have been decrypted from full-length cDNAs of about 370 nucleotides. The precursors of the toxins I (AaH I) and II (AaH II) from *Androctonus australis*

contain signal peptides of about 20 amino acid residues and extensions of basic amino acids at their COOH-terminal ends. Thus, processing steps are required to generate mature toxins. Monkey kidney COS-7 cells transfected with the AaH II cDNA were used to successfully express AaH II, which was the first recombinant scorpion toxin report (Bougis et al. 1989).

The promoter structure and the intron-exon organization of the AaH I gene was further investigated. The gene transcriptional unit was 793 base pairs long, with a single intron of 425 base pairs located near the end of the signal peptide. The transcription initiation site AACAA was determined. Upstream, a promoter region composed of a CCAAT box and a TATA box has been also identified (Fig. 3). Moreover, putative elements for binding the transcriptional factors MAT-alpha 2, Pit-1, and IEF1 were also present. A strong DNA bending (bending angle of 61°) centered on the transcription initiation site of the gene was revealed by computer modeling. Other minor deflections of the helix axis gave an overall curvature of nearly 90°, which was significantly stronger than similar structures already reported in eukaryotic cells (Delabre et al. 1995).

Secondary and Tertiary Structures

Na_v channel-specific scorpion toxins have an α/β scaffold (βαββ), which contains one α-helix and a three-stranded antiparallel β-sheet, connected by three loops of different sizes and highly variable compositions (Housset et al. 1994; Possani et al. 1999).

The C^1 of the N-terminal part of the toxin forms a disulfide bridge with the C^8 in the C-terminal part. The three other disulfide bridges tightly fold the α-helix and β-sheets. This structural motif is called cystine-stabilized helix (CSH). The cysteine pair of the α-helix motif, spaced by a tripeptide ($C^3 \times C^4$), binds with the pair of cysteine residues of the β3 strand ($C^6 \times C^7$). C^5 residue located at the β2 strand is connected to the C^2. These structural elements are conserved in almost all the scorpion toxins targeting Na_v channel reported so far. These three conserved disulfide bonds stabilize the core of scorpion toxins. One exception is found for the excitatory toxins specific to insect. The fourth disulfide bond in excitatory toxins is established in an atypical way between the contiguous C residue in their β2 strand and the C^8 at the C-terminal end of the toxin (Fig. 4). Injected into fly larvae, these toxins induce an immediate reversible fast contraction paralysis caused by repetitive activity of motor nerves resulting from the activation of sodium currents at more negative membrane potentials (Darbon et al. 1982).

All Na_v channel-specific toxins show a large solvent-exposed cluster of aromatic and hydrophobic residues known as conserved hydrophobic surface (Housset et al. 1994; Krimm et al. 1999). Finally, the 3-D structure studies on scorpion toxins have revealed subtle differences in the spatial arrangement of the turn preceding C^1 (N region), the C-terminal region, and the region between C^5 and C^6 (β2-β3 loop). Few general features can be assigned to the different groups of toxins. For Old-World scorpion toxins, like *Androctonus* toxins, a β-loop of maximal length is linked to an α-type activity and blocks the fast inactivation process of the channel. The rare and most active α-type toxins described in New-World

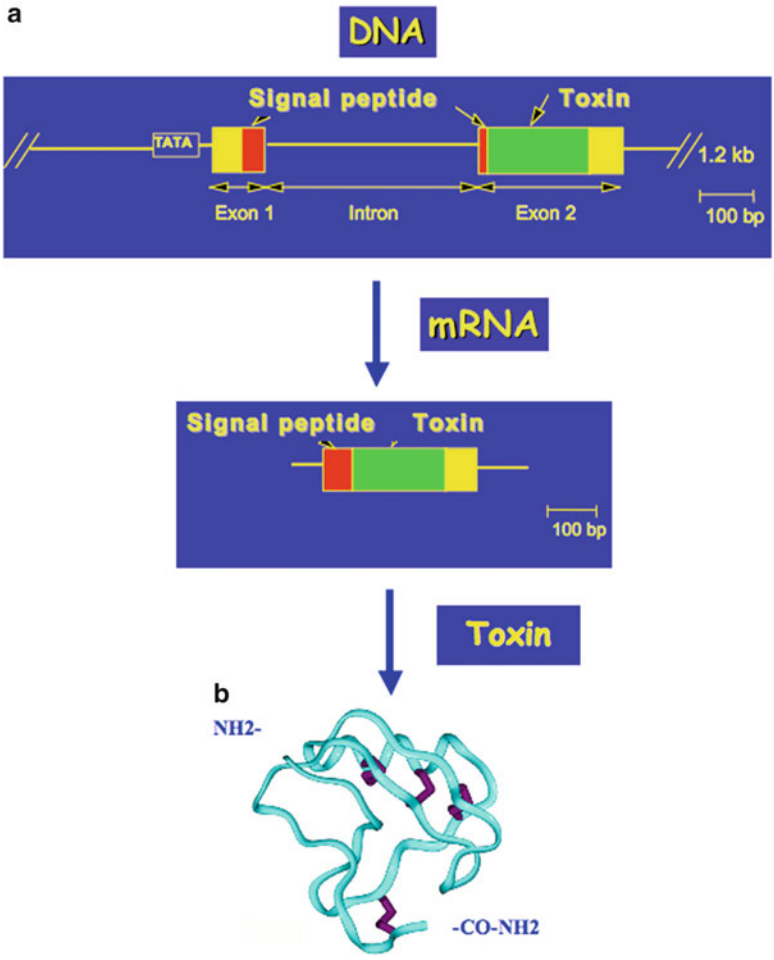

Fig. 3 Gene and precursor organization of the classical alpha-toxin AaH II. *Top*, Organization of the DNA gene: two exons are split by a single intron. In *yellow*, untranslated region; in *red*, signal peptide for exportation; in *green*, oligonucleotides to be traduced in the native toxin. *Middle*, Precursor organization of AaH II: In *red*, signal peptide for exportation; in *green*, oligonucleotides to be traduced in the native toxin; in *yellow*, posttranslational processed amino acids, in order to achieve a C-terminal amidation. *Bottom*, 3D structure of native AaH II, after processing and folding. The N-terminal is free and the C-terminal histidine is amidated

Buthidae (as *Centruroides* and *Tityus*) venoms have a β-loop of intermediate size, sharing this characteristic with Old-World depressant anti-insect toxins which, on the contrary, exhibit a β-type activity and a different pharmacological effect (Pedraza-Escalona and Possani 2013; Possani et al. 1999). These depressant anti-insect toxins suppress evoked action potentials due to a strong depolarization of the axonal membrane (Gurevitz et al. 2007; Nakagawa et al. 1997; Possani et al. 1999). However, if they are largely found in several Buthidae venoms from Old-World

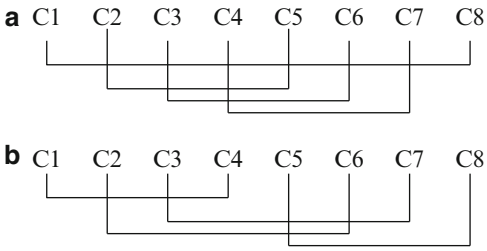

Fig. 4 (**a**) Disulfide bridges organization from scorpion α-toxins specific to mammals and (**b**) from contracturant anti-insect toxins

(as *Buthus* and *Leiurus*), only one of these depressant anti-insect toxins has been described in *Androctonus* venom so far (Nakagawa et al. 1997).

At last, a new substructural group of long-chain toxins acting on Na_v channels was recently characterized. The Birtoxin-like toxins (in reference to the first member identified) are shorter than other long-chain scorpion toxins with only three disulfide bridges instead of the four usually found in α- and β-toxins (Inceoglu et al. 2002). The majority of the sequences described were deduced from PCR-amplified cDNAs from *Androctonus australis* and *Androctonus crassicauda* venom glands. These molecules are partially related to β-scorpion toxins in primary structure (Abbas et al. 2011; Caliskan et al. 2006; Inceoglu et al. 2002). At the pharmacological level, this new protein family is still poorly studied because of the weak activity of its members. The electrophysiological characterization of their effects on various Na_v channels revealed that they preferentially act as β-toxins by shifting the voltage dependence of activation to more negative membrane potentials.

The *Androctonus* Toxins Active on Na_v Channels

The present chapter is only dedicated to the *Androctonus* toxins able to modify the Na_v channel functioning. The properties of the shorter toxins blocking different K^+ channel subtypes and their interactions with their specific receptors have been recently described elsewhere (Martin-Eauclaire and Bougis 2012).

The toxin variability in *Androctonus* venoms was assessed first by low-pressure chromatography (Martin and Rochat 1986), then by high-performance liquid chromatography (HPLC), by ELISA or radioimmunoassay, and recently by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analyses. Important variations were observed in venom contents according to the mode of extraction (electrical or manual stimulation of the scorpion postabdomen), to the precise geographic area of the animal collect, from a single specimen to another, and even in the venom of a single specimen extracted at different times (Martin-Eauclaire et al. 2013).

After four decennia of research on the *Androctonus* venoms, what is finally characterized without ambiguity in these secretions? As example, besides the α-toxins, which are particularly lethal for mammals and constitute the major toxicity for human being, *Androctonus australis* venom contains a small amount of β-type peptides with no or weak toxic activity when inoculated to the mice by

subcutaneous injection. In particular, the most potent insect-selective β-toxins with an excitatory function ever described, AaHIT, is found in *Androctonus australis* venom (Darbon et al. 1982).

The *Androctonus* α-Toxins Which Bind the Site 3 in Na_v Channel Domain IV

First based on primary structure analogies, cross-neutralization, and immunoprecipitation, α-scorpion toxins from Buthidae were classified into four groups (Granier et al. 1989; Martin-Eauclaire and Couraud 1995). Sequence comparison among the various groups reveals less than 30 % similarity, whereas the toxins within each group may differ up to 50 %. This classification was then confirmed by radioimmunoassay and was in concordance with the structural groups (Granier et al. 1989). An antibody raised against a member of a structural-antigenic group is able to recognize and neutralize only the toxins of the same group. However, the preference of scorpion α-toxins for Na_v channels varies greatly, and some of them are also highly active on insects. This implies that receptor site 3, despite its commonality, varies on insect versus mammalian channels. Thus, latter on, based on their biological activity on mammals and/or insects, toxicity symptoms, electrophysiological properties, and binding assays, α-toxins have been classified into three subgroups: (I) classical α-toxins, (II) α-like toxins, and (III) α-toxins specific for insects (Gordon et al. 1996).

The sequences of at least the 25 primary structures of α-toxins have been depicted so far from *Androctonus* venoms, either by Edman degradation or by cDNAs cloning, and are found in the data bank (Fig. 5; sequences from Expasy Bioinformatics Resource Portal).

The Classical α-Toxins

Androctonus Australis Classical α-Toxins

In the *Androctonus australis* hector venom, the classical α-toxins are exclusively lethal to mammal (e.g., AaH II, taken as reference for the classical α-toxins) and are particularly highly active on mammalian brain Na_v channel. The $LD_{50}$by intracerebroventricular (i.c.v.) injection range is only 0.5 ng for AaH II per 20 g mouse body weight. They also bind with high affinity to rat brain synaptosomes ($K_D = 0.2$ nM for AaH II). The use of ^{125}I-AaH II in binding and displacement experiments in rat brain synaptosomal preparations is one of the main criteria for classification of unknown peptides into classical α-toxin (Martin-Eauclaire and Couraud 1995).

These classical α-toxins are the most dangerous for mammals as they are highly lethal by subcutaneous injection. By this route, 90 % of *Androctonus australis* venom lethal activity in mice is constituted by its four classical α-toxins (AaH I to IV), which represent only a low amount in weight from a crude venom. AaH II alone, which accounts for 50 % of lethality by subcutaneous injection in mice, is described as the most active classical α-toxin and represents the archetype of anti-mammalian α-toxin (Martin and Rochat 1986). AaH II exhibits the highest affinity described for Na_vchannel site 3. Voltage-clamp experiments using neuronal

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P01479    6803  KRDGYIVYPNNCVYHCVPP---CDGLCKKNGGSSGSCSFLVPSGLACWCKDLPDNVPIKDTSRKCT    Andau I
D5HR50    6875  VRDGYIVYPNNCVYHCIPA---CDGLCKKNGGTSGSCSFLIGSGIACWCKDLPDNVPIKDPSQKCTR*  Andcr ntx 1
Q86SE1    7047  VRDGYIVYPHNCVYHCIPS---CDGLCKENGATSGSCGYIIKVGIACWCKDLPENVPIYDRSYKCY    Andam H1
JQ975126        VRDGYIVDDKNCVYHCIPP---CDGLCKKNGGKSGSCSFLVPSGLACWCKALPDNVPIKDPSYKCHKR* Andcr Acra4
P01480    6822  VRDGYIVDSKNCVYHCVPP---CDGLCKKNGAKSGSCGFLIPSGLACWCVALPDNVPIKDPSYKCHS   Andau III
P45658    6883  GRDGYIVDSKNCVYHCYPP---CDGLCKKNGAKSGSCGFLVPSGLACWCNDLPENVPIKDPSDDCHK   Andau IV
D5HR53    7020  VRDGYIVDFKNCVYRCVPP---CDGLCKKNGGKGGSCSFLIGSGLACWCNALPDNVPIKDPLHKCPKR  Andcr ntx 4
P0C910    7001  GRDGYIVDTKNCVYHCYPP---CDGLCKKNQAKSGSCGFLYPSGLACWCVALPENVPIKDPNDDCHK   Andma tox III
Q86SD9    6935  ARDGYIAQPNNCVYHCIPLSPGCDKLCRENGATSGKCSFLAGSGLACWCVALPDNVPIKIIGQKCT    Andam H3
D5HR54    7162  ARDGYIAQPHNCVYPCIPLSPGCDNLCKENGATSGKCSFLLESGLACWCVALPDNVPIKIEGQKCTR*  Andma α-ntox10
JQ975129        VRDGYIVKPTNCVIHCIPFSPGCDKDCKEKGAASGYCQAFGKHGNGCWCIDLPDKVPIKDPNQDCTR*  Andcr Acra7

P01484    7244  VKDGYIVDDVNCTYFCGRNA-YCNEECTKLKGESGYCQWASPYGNACYCYKLPDHVRTKGP-GRCH#   Andau II
Q9blm4    7187  KKDGYIVDDKNCTFFCGRNA-YCNDECKKKGAESGYCQWASPYGNACYCYKLPDRVSTKKK-GGCN#   Andau P1005
D5HR51    7422  IKDGYIVDDRNCTYFCGTKS-YCNGECTKLKGESGYCQWASPYGNACYCYKLPDHVRTKGP-GRCKGR* Andcr ntx 2
D5HR52    7491  IKDGYIVDDRNCTYFCGRNA-YCNEECTKLKGESGYCQWASPYGNACYCYKLPDHVRTKAP-GKCNGR* Andcr ntx 3
JQ975130        VRDGYIVDDKNCTFFCGRNAY-CNDECKKKGGESGYCQWASPYGNACWCYKLPDRVPIKEKG-RCNGR* Andcr Acra8
D5HR48    7260  LKDGYIVNDINCTYFCGRNA-YCNEECIKLKGESGYCQWASPYGNACYCYKLPDHVRTKGP-GRCNDR* Andbi ntx8
Q7YXD3    7375  LKDGYIVNDINCTYFCGRNA-YCNELCIKLKGESGYCQWASPYGNSCYCYKLPDHVRTKGP-GRCND   Andma VIII
P01482    7293  LKDGYIIDDLNCTFFCGRNA-YCDDECKKKGGESGYCQWASPYGNACWCYKLPDRVSIKEK-GRCN#   Andma toxin V
Q2YHM1    7308 GVRDAYIADNKNCIFTCYRDS-YCKTECIKNGAETGYCIWIGEYGNACWCIKLPNKVPIKVP-GKCN*  AndmaVIIIrgp1
Q2YHM1    7280 GVRDAYIADNKNCIFTCYKDS-YCKTECIKNGAETGYCIWIGEYGNACWCIKLPNKVPIKVP-GKCN*  AndmaVIIIrgp2
Q2YHM1    7102 GVRDAYIADNKNCIFTCYKDS--CKTECIKNGAETGYCVWIGKYGNACWCIKLPNKVPIKVP-GKCN*  AndmaVIIIrgp3
D5HR49    7158  VRDAYIADDKNCIFTCFRNS-YCNTECTKNGAEAGYCIWIGKYGNACYCIKLPDKVPIKVP-GKCNGR* Andbi ntx9
Q86SE0    6998  VRDGYIADAGNCGYTCVAND-YCNTECTKNGAESGYCQWFGRYGNACWCIKLPDKVPIKVP-GKCN    Andam H2
Q9blm3    7170  ARDAYIAKNDNCVYECFQDS-YCNDLCTKNGAKSGTCDWIGTYGDACLCYALPDNVPIKLS-GECHR*  AndauP985

Q9BLM0    5538  VRDGYFVEPDNCVVHCMPSSEMCDRGCKHNGATSGSCKAFSKGGNACWCKGL*                  Andau Ntx P993
Q9BLM1    5904  VRDGYFVEPDNCLVYCMPSPEICDRGCKRYGATSGFCKEFSKGENFCWCKGL*                  Andau Ntx P996
Q9BLM2    5656  VRDGYFVEPDNCVIYCMPSSEVCDRGCKHNGATSGTCKEFSKGGNVCWCKGL*                  Andau Ntx P1008
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Fig. 5 Sequences of the α-toxins decrypted so far from different *Androctonus* subspecies. These sequences found in the data bank (Expasy Bioinformatics Resource Portal) were obtained either by Edman degradation or by cDNAs cloning. They are aligned here according to the *Androctonus australis* hector toxin I (AaH I) for the first structural group and to *Androctonus australis* hector toxin II (AaH II) for the second structural group, AaH I and AaH II being the first *Androctonus* toxins purified and sequenced (see Martin-Eauclaire and Couraud 1995). *Andau Androctonus australis*, *Andma Androctonus mauretanicus*, *Andam Androctonus amoreuxi*, *Andcr Androctonus crassicauda*, *Andbi Androctonus bicolor*. #, C-terminal amidated; *Amino acid sequence deduced from cDNA. Masses are both in bold and underlined

$rNa_v1.2$ and skeletal muscle $rNa_v1.4$ channels expressed in *Xenopus* oocytes showed that the EC_{50} values of the toxin-induced slowing of the channel inactivation are 2.6 ± 0.3 nM and 2.2 ± 0.2 nM, respectively (Alami et al. 2003).

The *Androctonus australis* venom contains in addition two extremely potent classical α-toxins, AaH I and AaH III, which are almost as toxic as AaH II when injected by subcutaneous route, but also the less toxic AaH IV (Martin and Rochat 1986). These three toxins belong to the same structural and immunologic group (group I), which differs from that of AaH II (group II), and share high sequence homologies (Granier et al. 1989). AaH I was the first scorpion toxin described in the international literature and the first sequenced. Later, closer AaH I analogues were purified from different subspecies of *Androctonus australis*: AaH I' was in the venom of *Androctonus australis* garzonii from Tunisia, with an Ile in position 17 instead of a Val for AaH I; AaH I" was found in the venom of *Androctonus australis* hector from Algeria, and its sequence differs from that of AaH I only by the presence of an extra Arg at the C-terminal end. AaH I, AaH I', and AaH I" have exactly the same lethal activity and the same biological properties. When the AaH I precursor has been depicted, it was demonstrated that its C-terminal residue was an extra Arg, further removed by post-traductional processing (Bougis et al. 1989). Thus, it is tempting to argue that AaH I" is only a non-processed AaH I.

No X-ray structure from the group I toxins is available. ^{1}H-nuclear magnetic resonance assignment strategy has been applied to AaH III. Major structural features common to α-toxins were found: i.e., a helix of 2 and half turns linked by two disulfide bridges to the central strand of a triple-stranded antiparallel beta-sheet and, as in the AaH II X-ray structure (Housset et al. 1994), a hydrophobic core including aromatic rings, which lie orthogonal to one another (termed the "herring-bone" arrangement).

Androctonus mauretanicus mauretanicus Classical α-toxins

Concerning the *Androctonus mauretanicus mauretanicus* venom, obtained by manual handling of the animal, about only 70 components were found in its toxic fractions using LC-MALDI-TOF-MS. Among them Amm III, Amm IV, and Amm V were sequenced and biologically characterized as classical α-toxins. They are responsible of about 73 % of the venom lethality by subcutaneous injection. The Amm V itself is responsible of 47 % of the venom lethality and belongs to the same structural and immunological group as the archetypal α-toxin AaH II. Unexpectedly, using competition experiments against the radioiodinated β-toxin Css II bound to its binding site on rat brain synaptosomes, a β-toxin active on mammals was also found, the first one identified in the venom from a North African scorpion (Zerrouk et al. 1991).

Later, the *Androctonus mauretanicus* venom was screened using a specific polyclonal antiserum directed against AaH II. This led to the isolation of Amm VIII, which gave a highly positive response in ELISA tests but was totally devoid of toxicity when subcutaneously injected (Alami et al. 2003). In voltage-clamp experiments on rNav1.2 and rNav1.4, expressed in *Xenopus* oocytes, the EC_{50} values of the toxin-induced slowing of inactivation were 29 ± 5 and 416 ± 14 nM, respectively. Because of its very low affinity for the skeletal muscle Nav1.4 channel and its total lack of toxicity when injected by peripheric route, the Amm VIII was first used as a natural anatoxin to induce successfully neutralizing antibodies against the most potent scorpion α-toxins of the structural and immunological group 2. Better, the anti-Amm VIII serum was able to prevent the association of ^{125}I-AaH II with its receptor and was also able to remove the toxin already bound to its binding site on rat brain synaptosomes (the half-life of the complex ^{125}I-AaH II-receptor site was 12 min in the absence of anti-Amm VIII serum but decreased to only 2 min in the presence of anti-Amm VIII serum). In vivo, this serum had also a protective effect in mice: it was able to neutralize 42 LD_{50} of AaH II (measured by subcutaneous injection) by milliliter of serum. It was finally demonstrated that the antiserum against Amm VIII protected mice from the AaH II lethal action because Amm VIII elicited antibodies able to only recognize AaH II discontinuous-type epitopes (Alvarenga et al. 2010).

Also, because of its special nontoxic properties, the analgesic effects of Amm VIII were further evaluated in mice by intraperitoneal route. The toxin increased hot plate and tail flick latencies in a dose-dependent manner and induced increased c-fos mRNA expression in spinal cord (Martin-Eauclaire et al. 2010). However, as the pain relief induced by the toxin was reversed par naloxone (a μ2 opioid receptor antagonist), it might implicate the activation of an endogenous opioid system and be partly the result of the "diffuse noxious inhibitor control" (DNIC) activation and

of a counter irritation phenomenon. Then, the Amm VIII electrophysiological response was studied by patch clamp on both tetrodotoxin TTX-sensitive (TTXs) and TTX-resistant (TTXr) Na^+ currents in nociceptive dorsal root ganglion (DRG) neurons and in immortalized DRG neuron-derived F11 cells. Amm VIII at 1 μM and AaH II at 50–100 nM strongly enhanced voltage-gated Na^+ currents by selectively modulating TTXs Na_vchannels (probably $Na_v1.7$). This result was in perfect accordance with a previous study showing that AaH II could modulate the cloned $Na_v1.7$ channel from peripheral sensory and sympathetic neurons, as described for the toxins BmKM1 from *Mesobuthus martensii* Karsch and OD1 from *Odonthobuthus doriae* (Maertens et al. 2006). On the contrary, Amm VIII or AaH II affected neither $Na_v1.8$ nor $Na_v1.9$ at concentrations up to 1 μM (Abbas et al. 2013). This was also confirmed by the work of Bosmans et al. (2011), who used a protein engineering approach to dissect the contributions of the four voltage sensors from $Na_v1.8$ or $Na_v1.9$ channels to the channel function and pharmacology. They show that the individual structural paddle motifs (corresponding to the S3b–S4 segment of each $Na_v1.8$ and $Na_v1.9$ voltage sensor domain), when transplanted into K_v channels, can be exploited for screening toxins from venomous organisms. In this study, AaH II was totally unable to act on the $Na_v1.8$ and $Na_v1.9$ chimeras, even at 1 μM.

Finally, in the *Androctonus mauretanicus* venom, Amm III is closely related to AaH IV (90 % of sequence identity) and is classified as a member of the antigenic group I, with AaH I, III, and IV. However, in RIA experiments, unlabeled Amm III inhibits the binding of ^{125}I-AaH I with a weak affinity (EC_{50} of 54 nM, as compared with 0.12nM for AaH I).

Other *Androctonus* (*crassicauda*, *bicolor*, and *amoreuxi*) Classical α-Toxins

Androctonus crassicauda is a dangerous neglected scorpion and one of the most toxic species in the world, which produces venom with an intravenous LD_{50}in mice of 0.32 mg/kg. When looking at data banks, four oligonucleotide sequences encoding putative classical α-toxins are depicted from its genomic DNA using specific primers for genome walking (Weinberger et al. 2010): Acra ntx1 (clone D5HR0) and Acra ntx4 (clone D5HR53) show amino acid sequence close to those of the structural and immunological group I toxins (respectively, 87 % and 78 % similarity with AaH I); Acra ntx2 (D5HR51) and Acra ntx3 (D5HR52) show amino acid sequence close to those of the structural and immunological group II toxins (respectively, 89 % and 92 % similarity with AaH II). The four sequences bear additional basic residues at their C-terminal. These residues would be probably processed and not present in the native toxin. Also, Acra ntx2 (D5HR51) and Acra ntx3 (D5HR52) bear a penultimate Gly residue for C-terminal amidation. More recently, a new toxic peptide, named Acra4, because it is the fourth peptide completely characterized from this scorpion venom, was also reported (Caliskan et al. 2013). The toxin has a weak LD_{50}in mouse by i.c.v. injection (50.5 ng/20 g mouse body weight, compared to 0.5 ng for AaH II). Its affinity, obtained using patch-clamp recordings toward Na_vchannels expressed in F11 cell line, is in the order of 1 μM. Additionally, four cDNAs were cloned from total RNA of the

venomous glands and reported as potentially toxic. Their deduced amino acid sequences displayed high similarities with those of other *Androctonus* classical α-toxins, in particular of the structural and immunological group 1.

Also identifed from a genomic library of *Androctonus bicolor*, the clone Andbi ntx8 (D5HR49) quite similar to structural group II toxins at the amino acid level (97 % of similarity with Amm VIII and 92 % with AaH II) and the clone D5HR49, which encodes the putative sequence of a α-like toxin, Andbi ntx9, were described (Weinberger et al. 2010). However, no information on the immunological properties of all these gene products is given. At the level of their biological properties, a few representatives of the new sequences were produced in recombinant form. Their activities were assayed by injection to blowfly larvae, for their ability to inhibit sodium current inactivation of $DmNa_v1$ (*Drosophila*), $rNa_v1.2$ (rat brain), and $rNa_v1.4$ (rat skeletal muscle) sodium channels expressed in *Xenopus oocytes* (Weinberger et al. 2010). Andbi ntx8 was active on $rNa_v1.2$ and almost inactive on the other channel subtypes, like its close homologue AmmVIII. Andbi ntx8, Andbi ntx9, and Acra ntx4 were weakly active ($ED_{50} > 2$ μg per 100 mg larvae). Acra ntx4 was inactive at the three channel types, whereas Andbi ntx9 was weakly active only on $DmNa_v1$. Thus, the clones are encoding toxins, which, in fact, turn to be α-like toxins, according to their biological activity and the definition given previously (Gordon et al. 1996).

Concerning the chemical, pharmacological, and immunological characterization of the toxic components isolated from the *Androctonus amoreuxi* venom, it was shown that the major "long-chain" toxins described are not from the classical α-toxin group, but belong to the α-like toxins group (Abbas et al. 2009), which will be treated in the next paragraph. However, beside the purification of the AamH1, AamH2, and AamH3 major toxins, ELISA were used to test all the HPLC fractions with different serums previously raised against the most lethal or original toxins representing the major structural and immunological groups from Buthidae venoms. Several cross-reactivities with the anti-AaH II serum were observed and suggested the presence of minor toxins belonging to the AaH II immunological group II in the *Androctonus amoreuxi* venom. These toxins remain to be purified and characterized (Abbas and Martin-Eauclaire, personal communications).

The Anti-insect α-Toxins and the α-Like Toxins

The anti-insect α-toxins as described are selectively toxic for insects (e.g., LqhαIT from *Leiurus quinquestriatus hebraeus*). They cause contractile paralysis of the insect, after a time lag due to extreme prolongation of the action potential by slowing the inactivation of sodium current. Their binding affinity to insect neuronal preparations is high ($K_D = 0.06-1$ nM). In fact, the α-like toxins and the α-anti-insect toxins are distinct from each other in that α-insect toxins are nontoxic to mammal brain even at high concentration (Cohen et al. 2006; Gordon et al. 1996). The α-like toxins are highly toxic for both mammals and insects by i.c.v. and subcutaneous injections (e.g., Lqh III from *Leiurus quinquestriatus hebraeus* and BmK M1 from *Mesobuthus martensii*) (Goudet et al. 2001; Krimm et al. 1999).

They have a rather good LD_{50}(23–50 ng/20 g per mouse) by i.c.v. injection but do not bind to rat brain synaptosomes (which contain mostly $Na_v1.2$) and barely compete for ^{125}I-AaH II binding to rat brain synaptosomes (Gordon et al. 1996; Gurevitz et al. 2007; Krimm et al. 1999).

None of these toxins was purified and clearly characterized so far in *Androctonus australis* and *Androctonus mauretanicus* venoms, but this does not mean that they are lacking. Some clones encoding new amino acid sequences close to those of anti-insect α-toxins and α-like toxins were described, but the deduced proteins were not isolated yet (Martin-Eauclaire, Céard, Alami, and Bougis; personal communication). It was shown that the major lethality for mice recorded in these two venoms is mainly associated to classical α-toxins and, thus, we can suspect that these gene products are just weakly toxic to mice.

Recently, the immunological and pharmacological properties of the three major α-type toxins from the scorpion *Androctonus amoreuxi*, AamH1, AamH2, and AamH3 have been characterized (Abbas et al. 2009). The immunological tests (ELISA, RIA) have demonstrated that they belong to the immunological groups 3 and 4 of α-like toxins, i.e., that they are closer to *Buthus occitanus* and *Leiurus quinquestriatus* main toxins than to *Androctonus* toxins. In accordance, the *Androctonus amoreuxi* venom was better neutralized by the antiserum raised against the venom of *Buthus occitanus tunetanus* than by the antisera raised against scorpion venoms from the same genus *Androctonus*. From a pharmacological point of view, AamH1, AamH2, and AamH3 from *Androctonus amoreuxi* were lethal to mice by i.c.v. injection, but they were poorly active by subcutaneous injection. Electrophysiological tests showed a significant effect of the three toxins on currents through sodium channels expressed in *Xenopus* oocytes ($rNa_v1.2$ from rat brain, $rNa_v1.4$ from rat skeletal muscle and *Drosophila*, $DmNa_v1$) arguing that they are active on both mammal and insect. While AamH1 removes fast inactivation only in neuronal $rNa_v1.2$ channel and has no effect on skeletal muscle $rNa_v1.4$ channel, AamH2 affects both neuronal $rNa_v1.2$ and skeletal muscle $rNa_v1.4$ channels. AamH3 was lethal to mice by i.c.v. injection despite its lack of activity on the neuronal $rNa_v1.2$ channel (Abbas et al. 2009).

As AamH1 and AamH2, but not AamH3, have anti-insect and anti-mammal activities, they can be classified as α-like toxins. This is probably the reason why the venom is not as toxic for the mouse (LD_{50} around 20 μg) as those of *Androctonus australis* or *mauretanicus* ($LD_{50} < 5$ μg), which contain only highly lethal classical α-toxins constituting between 75 % and 90 % of the total crude venom lethality (Martin and Rochat 1986; Zerrouk et al. 1991).

Androctonus bicolor genomic clone D5HR49 encodes the putative sequence of Andbi ntx9 (Weinberger et al. 2010). This sequence is very close to that of AamH2 and, thus, Andbi ntx9 could be considered as an α-like toxins. The toxin was shown weakly active on blowfly larvae and on $DmNa_v1$ only (Weinberger et al. 2010). Also, from genomic DNA of *Androctonus mauretanicus mauretanicus*, several sequences were depicted: one encoding an already described putative toxin, AmmVIII rgp variant 3, and a second one encoding a molecule relatively distant from all known toxins. This putative toxin was numbered Amm10 (considering that

AmmVIII rgp variant 3 was encoding "Amm9"). They were expressed in *E. coli* for their biological characterization. Amm10, as Andbi ntx9 and Acra ntx4, was found weakly active on insects ($ED_{50} > 2$ μg per 100 mg larvae). Amm9 was inactive for the three channels tested ($rNa_v1.2$, $rNa_v1.4$, and $DmNa_v1$), whereas Amm10 was weakly active only at $DmNa_v1$ (Weinberger et al. 2010).

Key Amino Acid Residues Involved in the Interaction Between *Androctonus* α-Toxins and Na_v Channels

The amino acid residues involved in the interaction between α-toxins and the S3b–S4 loop in domain DIV of Na_vchannels were determined by direct mutagenesis in both toxins and channels. In general, acidic and hydrophobic amino acid residues in the DIV S3b–S4 loop and basic and hydrophobic residues in the toxins are implicated in toxin-channel interaction and toxin action (Bosmans et al. 2008; Rogers et al. 1996).

However, other parts of the channel may also be involved. Topology of Na_vchannel model suggests that domains DI and DIV are adjacent in the tertiary structure of the channel protein. Thus, it is not surprising that, previously, the extracellular loops between segments S5 and S6 of domains DI and DIV were first said involved, at least in part, in the scorpion α-toxin receptor site 3. This result was obtained using different techniques consisting of photoaffinity labeling, cleavage with proteases or cyanogen bromide, and antigen mapping with sequence-specific antibodies and by blocking the toxin binding with sequence-specific antibodies (Catterall 2012). Now, it is demonstrated that scorpion toxins bind to the outer end of the S3–S4 loop of the voltage sensors in both resting and activated states (Catterall 2012). Diverse classes of toxins interact in a different manner with this channel region, depending on the bioactive surface of each toxin (Leipold et al. 2006). Furthermore, structural flexibility issues of scorpion α-toxins are also likely to contribute to Na_vchannel subtype selectivity (Krimm et al. 1999).

Key Amino Acids Identified in Na_v Channels and Shown Crucial for the Binding of *Androctonus* Classical α-Toxins

The E^{1613} in the DIV S3b–S4 loop was first identified as a major determinant for α-classical toxin- Na_vchannel interaction because the conversion of E^{1613} into R or H in the neuronal $Na_v1.2$ channel inhibits the classical α-toxin binding to the channel (Rogers et al. 1996). Also, the affinity of AaH II to the $Na_v1.2$ DIV paddle motif (which was shifted into the Kv2.1 channel) decreases greatly by $F^{1610}A$, $E^{1613}A$, $L^{1614}A$, and $Y^{1918}A$ and to less extant by $M^{1599}A$, $I^{1615}A$, $E^{1616}A$, $V^{1627}A$, $I^{1628}A$, and $I^{1633}A$ substitution (Fig. 6). $R^{1629}A$ and $L^{1630}A$ substitution, on the contrary, increase the AaH II affinity (Bosmans et al. 2008).

However, E^{1613} is important for classical α-toxins (as AaH II) activity on $Na_v1.2$, but less necessary on $Na_v1.4$ where other residues could be implicated in the interaction toxin-channel (Bosmans et al. 2008; Leipold et al. 2006).

AaH II slows current inactivation of $Na_v1.2$, $Na_v1.4$, and $Na_v1.7$ channels (Alami et al. 2003; Bosmans et al. 2008; Maertens et al. 2006) and of

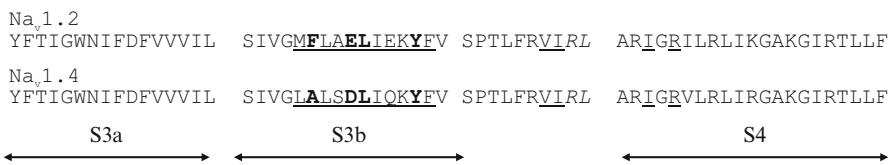

Fig. 6 Amino acids of the neuronal $Na_v1.2$ and skeletal muscle $Na_v1.4$ channels involved in the binding site of AaH II on the S3–S4 segment of the domain IV voltage sensor. The data are according to Bosmans et al. 2008. They have been obtained by point mutation of each amino acid of the DIV paddle motif using Alanine scanning. Arrows correspond to part of the S3a, S3b, and S4 transmembrane segments from neuronal $rNa_v1.2$ and skeletal muscle $rNa_v1.4$ channel domain IV. All underlined amino acids are involved in the AaH II interaction, and those in bold are highly crucial for the binding. Modification of the two amino acids in italic (RL) leads to an increase of AaH II affinity

TTX-sensitive Na_v currents of DRG (mainly $Na_v1.7$) but has no effect on their TTX-resistant currents expressed by $Na_v1.8$ and $Na_v1.9$ channels (Abbas et al. 2013). In fact, sequence analysis reveals that the DIV S3–S4 linker is longer in $Na_v1.8$ than in $Na_v1.4$ by four amino acids: SLEN. A constructed chimera, $Na_v1.4$-SLEN, carrying SLEN at the analogous position in the DIV S3–S4 linker, was finally found resistant to classical α-toxin (Saab et al. 2002).

Key Amino Acids on *Androctonus* Classical α-Toxins

The role of the disulfide bridges was shown crucial for the toxin activity itself. Reduction and methylation of only one disulfide bridge (C1-C8) totally destroy the AaH II lethality and bioactivity. Chemical modifications and punctual mutations of amino acid residues in the classical α-toxins from *Androctonus* have also pointed out some residues important for their activity.

The 3-D structure of AaH II showed that all residues important for the toxin-channel interaction were clustered on one face of the toxin and suggested a multipoint interaction with the Na_vchannel. Those residues involved in the "toxic region" appeared to belong to the C-terminal (CT domain, residues 56–64) and N-terminal regions, which form together the NC domain. Special importance was also referred to the five residues reverse turn between β1 and α helices (RT domain).

The interaction of AaH II with its receptor site on the Na_vchannel was probed using five antibody populations selected for their specificity toward various regions of AaH II (Granier et al. 1989). These studies indicated that two antigenic sites were involved in the molecular mechanisms of toxicity neutralization. One is located around the disulfide bridge including the cysteine residues 12 and 63, which links the N- and C-terminal parts of the toxin (called NC domain), and one encompassing residues 50–59 (CT domain), Fab fragments specific to the region around the disulfide bridge 12–63, inhibited the binding of the ^{125}I-labeled AaH II to its receptor site. These two antigenic regions were inaccessible to their antibodies when the toxin was bound to its receptor site.

The residue K^{58} in the C-terminal was found to be imperative for the pharmacological function of α-toxin family and confirmed the critical role of the C-terminal region

for its interaction with Na_v channel (Kharrat et al. 1989). For example, in AaH II, the replacement by punctual mutagenesis of K^{58} by a hydrophobic (V and I) or acidic (E) amino acid residue immediately led to an inactive analogue (Legros et al. 2005). Chemical modifications of R^2 and R^{60} in AaH I and R^{56} in AaH II (modified by phenylglyoxal or p-hydroxyl-phenylglyoxal), W^{38} in AaH II, and W^{45}, Y^5, Y^{14}, and Y^{60} in AaH III showed that the charged residues in the N- and C-terminal, as well as the aromatic residues belonging to the CSH motif, to the C-terminal, and to the β2–β3 loop (residues 37–44), also played an important role in the molecular mechanisms of α-toxin bioactivity and their interaction with Na_v channel (Kharrat et al. 1989).

Natural mutations in close analogues of AaH II led to better understand the participation of some amino acid residues in its toxicity. As example, Bot III from *Buthus occitanus tunetanus* is a classical α-toxin, which differed from AaH II by only three residues ($R^{10}V$, $V^{51}L$, $N^{64}H$). However, Bot III exhibits a much weaker affinity for Na_vchannels than AaH II. Mutation studies on cloned and expressed α-toxin Bot III and AaH II show that (a) the affinity of recombinant Bot III-OH and recombinant AaH II-OH for the Nav channel is reduced compared to the native toxins, (b) the single mutation $N^{64}H$ is responsible for the difference of toxicity and affinity between rAahII-OH and rBotIII-OH, and (c) the addition of the sequence GR to rBot III-OH leads to the loss of biological activity (Benkhadir et al. 2004). Conversely, others authors show that, in AaH II, the lack of amidation on the C-terminal residue H^{64} does not affect the pharmacological activity but addition of a G^{65} in C-terminal decreases the affinity only by a factor two (Legros et al. 2005).

Also, as said above, the anatoxin Amm VIII shows 87 % sequence identity with AaH II. However, on the contrary to AaH II, it is not active on the skeletal muscle channel $rNa_v1.4$ and weakly on the neuronal channel $rNa_v1.2$. As it carries an unusual extension at its C-terminal end, consisting of an additional D due to a point mutation in the cDNA penultimate codon, it was first supposed that this extra amino acid residue could induce a steric hindrance, which dramatically reduces the target recognition by Amm VIII. The molecular modeling showed that this C-terminal extension does not lead to an overall conformational change in Amm VIII, but drastically modifies the charge repartition. Consequently, it was proposed that the electrostatic dipole moment of the molecule could explain the loss of activity (Alami et al. 2003). Lqh2 from *Leiurus quinquestriatus hebraeus*, another close AaH II analogue, shows as AaH II, a high affinity for a variety of mammalian Na_v channels such as $Na_v1.2a$, $Na_v1.4$, $Na_v1.5$, $Na_v1.6$, and $Na_v1.7$, and exhibits 90 % sequence identity with Amm VIII, which shows a clear preference for $rNa_v1.2a$ over $rNa_v1.4$. To identify the amino acid residues that determine this differential effect, Lqh2 was mutated in a sequential manner focusing on residues that vary in Amm VIII, particularly at the NC domain. Then, the mutant activities against $rNa_v1.2a$ versus $rNa_v1.4$ were investigated (Weinberger et al. 2010). Interestingly, substitutions D^8/A/K/N (where A, K, or N replaced D^8), V^{10}/A/Y/I (where A, Y, or I replaced V^{10}), or R^{64} by N followed by a D addition (R^{64}N-D, a single substitution where two residues replaced one), as appears in Amm VIII, did not alter the activity for $rNa_v1.4$. Therefore, guided by the idea that the general shape of the NC domain was important, complete exchange of the NC domain in Lqh2 by its Amm VIII counterpart, achieved

through substitutions $D^{8}N$, $V^{10}I$, and $R^{64}N$-D, resulted in a toxin mutant with an activity similar to that of Amm VIII. Finally, the C-tail and the five-residue turn were mutated separately. The activity of mutant Lqh2-$R^{64}N$-D was similar to that of Lqh2. However, mutant Lqh2 $D^{8}N$, $V^{10}I$ in concentrations up to 5 μm hardly slowed the decay of the sodium current at $rNa_v1.4$, $hNa_v1.5$, and $rNa_v1.6$, although the sodium peak current increased especially at $hNa_v1.5$ (Weinberger et al. 2010).

The *Androctonus* β-Toxins Which Bind the Site 4 in Na_v Channel

The binding of β-toxins to receptor site 4 induces both a shift in the voltage dependence of Na_vchannel activation in the hyperpolarizing direction and a reduction of the peak sodium current amplitude. The S1–S2 and S3–S4 linkers of the domain II voltage sensor are part of site 4 (Cestele et al. 2006; Campos et al. 2007; Bosmans et al. 2008; Pedraza Escalona and Possani 2013). However, the S3–S4 segment of the domain III voltage sensor was also shown involved in the binding of some β-toxin (Campos et al. 2007; Bosmans et al. 2008). At last, it was proposed that both effects of β-toxins on Na_v channels (left shift on the activation curve and reduction of peak conductance) are due to distinct interacting sites, the peak current reduction requiring probably a more complex domain organization (Pedraza Escalona and Possani 2013).

The β-type toxins are the major components in the New-World (North and South America) scorpion venoms, on the contrary to the Old-World scorpion venoms, which contain a majority of α-type toxins. According to their functional properties, β-toxins have be clustered into (i) classical β-toxins only active on mammalian Na_v channels, (ii) insect-selective β-toxins with an excitatory or depressant function, and (iii) β-like toxins that are active both on mammals and insects (Bosmans et al. 2007, 2008; Gurevitz et al. 2007; Pedraza Escalona and Possani 2013; Possani et al. 1999; Zhang et al. 2012). Even if classical β-toxins highly toxic for mammals are not described in Old-World Buthidae venom, the majority of specific anti-insect β-toxins were, in contrast, isolated from these venoms, and they evolved principally into depressant and excitatory toxins with a high selectivity for insects. *Androctonus* venoms contain some of these β-type peptides mainly active on insects and without toxic activity when inoculated to mammals by subcutaneous injection.

Now, about 23 β-toxins, including already 17 Birtoxin-like peptides, are characterized from the venoms of different subspecies of *Androctonus*. Figure 7 shows the amino acid sequences of some β-toxins, all mainly active on insects, purified from *Androctonus* venoms.

Anti-insect Excitatory β-Toxins

Upon injection of excitatory toxins into fly larvae, they induce an immediate reversible fast contraction paralysis caused by repetitive activity of motor nerves resulting from the activation of sodium currents at more negative membrane potentials (Arnon et al. 2005; Darbon et al. 1982).

AaH IT1 from *Androctonus australis*, archetype of the excitatory β-toxin, was the first anti-insect toxin described and, 40 years after its purification, stays the most

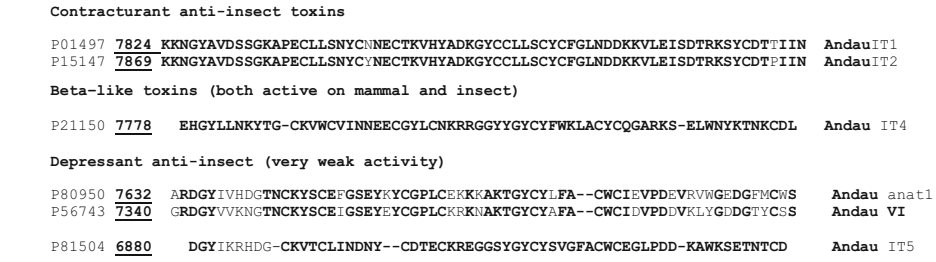

Fig. 7 Sequences of the β-toxins characterized from the venoms of different *Androctonus* subspecies. Except AaH IT4, which is also toxic for mammals, these β-toxins are all active on insects. Homologous amino acids are in bold. Masses are both in bold and underlined. *Andau Androctonus australis*

specific and the most active against insect (Zlotkin et al. 1971). Excitatory insect toxins (composed of 70–76 amino acid residues) are devoid of any activity in mammals, even when injected in milligram amounts by intracerebroventricular injection to mice (De Dianous et al. 1987). These toxins bind specifically only to insect neuronal membranes independently of voltage and do not interact with the mammalian sodium channel (De Lima et al. 1986; Gurevitz et al. 2007). ^{125}I-AaHIT and other excitatory toxins define a high-affinity (K_d ~ 0.2–3 nM) binding site in insect neuronal membranes. AaH IT1 only affected DmNav1/tipE but not rNa_v1.2, indicating its perfect insect selectivity (Bosmans et al. 2007). So, it was used as a probe to study the insect nervous system excitability and as a reference model for designing new biopesticides.

The AaH IT1 gene (Bougis et al. 1989) was cloned into the baculovirus and synthesized in the hemolymph of tobacco budworms (*Heliothis virescens*). The insect's death was increased, thus limiting feeding damage to the plant (Stewart et al. 1991).

The 3-D structure of AaH IT1 and AaH IT2, a second anti-insect toxin closely related, was determined by ^{1}H-nuclear magnetic resonance (NMR), which has confirmed that one of their disulfide bonds is shifted compared to the other long-chain toxins (Darbon et al. 1982). In excitatory toxins, this bridge connects the β2 strand to the C-terminal end, resulting in a β-loop of minimal length. This specific structure decreases their molecular flexibility, as shown by circular dichroism experiments, and may be responsible of their high specificity for the insect Na_vchannels. Furthermore, circular dichroism (CD) data show that this class of toxin should possess an additional α-helical region and a β-sheet, not found in toxins active on mammals. This proposed additional α-helical region was confirmed by X-ray crystal structure analysis of Bj-xtrIT, an excitatory insect toxin from *Hottentota judaicus* (Oren et al. 1998).

Anti-insect Depressant β-toxins

They consist of about only 61 amino acid residues and share the same fold as classical β-toxins. Structurally, an intermediate β-loop is related to depressant

insect toxins. They induce a slow progressive onset of flaccid paralysis preceded by a short transient phase of contractility upon injection into insects. They suppress evoked action potentials due to a strong depolarization of the axonal membrane. Under voltage-clamp conditions, depressant toxins cause a decrease in sodium peak current and affect the inactivation process of the insect Na_v channel by inducing a constant inward current at negative membrane potentials (Gurevitz et al. 2007).

At the level of their pharmacology, the depressant toxins binds on two noninteracting sites on cockroach neuronal membranes: the first site is of high affinity (K_D1–4 nM) and the second is of low affinity (K_D ~ 200–500 nM). Competition binding experiments between excitatory and depressant toxins using locust and cockroach neuronal membranes revealed that depressant toxins inhibit competitively the binding of the excitatory toxin ^{125}I-AaH IT, whereas excitatory toxins compete only for the high-affinity site of depressant toxins. However, in neuronal membranes of lepidopteran and blowfly heads, the excitatory toxin AaH IT had only a minor effect on depressant toxin binding, suggesting differences in the binding sites of anti-insect β-toxins in various insect species (De Lima et al. 1986; Gurevitz et al. 2007).

No highly potent toxin similar in amino acid sequence or pharmacology to those characterized in the *Leiurus quinquestriatus*, *Buthus occitanus*, or *Mesobuthus martensii* venoms was identified so far in *Androctonus* venoms. However, some molecules giving a weak progressive onset of flaccid paralysis in insects were found. AaH IT5, from the *Androctonus australis* hector venom, is very potent against the tobacco budworm, *Heliothis virescens*, and shows distinct insect specificity (Nakagawa et al. 1997). AaH VI, also purified from *Androctonus australis*, causes a very slow, gradual paralysis in *Blattella germanica* cockroaches (LD_{50} = 8.5 μg for animal) and is not toxic to mice. AaH VI was the first characterized glycosylated toxin described (Hassani et al. 1999). Its sequence (66 amino acid residues) shares little homology with the other depressant toxins and is heterogeneously N-glycosylated on a single site, N^9. Also, a nontoxic polypeptide from the same venom, AaH STR1, shares 88 % of identity with AaH VI. Its 3-D structure, determined by two-dimensional NMR techniques, revealed the existence of a short β-loop (typical structural features of New-World β-type toxins) (Blanc et al. 1997).

The β-Like Toxins

They are highly active on both insect and mammalian sodium channels. They induce typical depressant effects upon injection to blowfly larvae. In the toxins of this group are described several toxins from Old- or New-World scorpion venoms. The archetype of these β-like toxins is Ts1 (named also TsVII or Tsγ), from the Brazilian scorpion *Tityus serrulatus* (Bosmans et al. 2007; Campos et al. 2007; De Lima et al. 1986; Possani et al. 1999). AaHIT4 and Lqhβ1, far less toxic than Ts1, were later described in Old-World scorpion venoms (Gordon et al. 2003; Loret et al. 1991). These toxins compete for the excitatory (AaHIT1) and depressant toxin (LqhIT2) binding sites on sodium channels in insect neuronal preparations (Cohen et al. 2006; Gordon et al. 2003), as well as for the binding site 4 of the classical β-toxins CssII or CssIV on rat brain synaptosomes (Gordon et al. 2003).

AaHIT4 seems to be related to these β-like toxins because it is also recognized by antibodies raised against the reference β-toxin, CssII, but not by antibodies raised against the α-toxin, AaH II, or the excitatory toxin, AaHIT. However, AaHIT4 competes with both α-(AaH II) and β-(CssII) classical anti-mammalian toxins for their binding site to rat brain synaptosomes, but with moderate affinity (Loret et al. 1991). In *Androctonus mauretanicus* and *Androctonus amoreuxi*, three new molecules, with very close amino acid sequences to AaH IT4, have been also identified by screening the displacement of ^{125}I-Css IV bound to its binding site on rat brain synaptosomes (unpublished data).

The Birtoxin-Like (BTX-L) Peptides

The Birtoxin-like peptides are slightly shorter than other long-chain scorpion toxins. They are around 56–58 amino acids long and have only three disulfide bridges instead of the four usually found in long-chain peptides. Probably, the Birtoxins have an alternative system for keeping the polypeptide in a 3-D conformation retaining biological activity without the fourth disulfide bridge. The first characterized Birtoxin was isolated from the venom of the South African scorpion *Parabuthus transvaalicus* (58 amino acid residues, 6543.6 Da). From the same venom, other Birtoxin-like were further isolated: Dortoxin is a lethal peptide, Bestoxin causes writhing in mice but is not lethal, Altitoxin is a highly depressant-lethal peptide, and Ikitoxin, which differs from Birtoxin by a single amino acid residue change, from G to E at the position 23, lowering the activity of the toxin (Inceoglu et al. 2002).

This new protein family is still scarce. Aside the five analogues described in *Parabuthus transvaalicus*, the majority of the known sequences were deduced from PCR-amplified cDNAs from *Androctonus australis* and *crassicauda* venom glands (Abbas et al. 2011; Caliskan et al. 2006) and are, thus, putative (Fig. 8). Multiple sequence alignment implies that they are more related to β-scorpion toxins in primary structure. Therefore, these peptides should be included in a new substructural group of scorpion toxins called "the Birtoxin-like" toxins, by reference to the first member identified (Inceoglu et al. 2002). Indeed, the electrophysiological characterization of the effects of some Birtoxin-like toxins, achieved by different international groups on various Na_v channels, revealed that they act as β-toxins by shifting the voltage dependence of activation to more negative membrane potentials.

However, their biological activities on Na_v channels are highly heterogeneous, some protein being lethal or toxic for mammals and others not. They also exhibit an insecticidal activity and, moreover, a blocking effect on K_v channels (K_v1.1 and K_v1.3) has also been described for two of these toxins, KAaH1 (clone AaF1CA8) and KAaH2 (clone AaF1CA22), from the venom of *Androctonus australis*. KAaH1 blocks K_v1.1 and K_v1.3 channels expressed in *Xenopus* oocytes with IC_{50} values of 5 and 50 nM, respectively, whereas KAaH2 blocks only 20 % of the current on K_v1.1. KAaH2 is not active on K_v1.3 channels at 100 nM concentration. In the KAaH1 model, the functionally proposed important residues F^{26} and K^{29} are close to each other and are located in the α-helix. These residues may constitute the

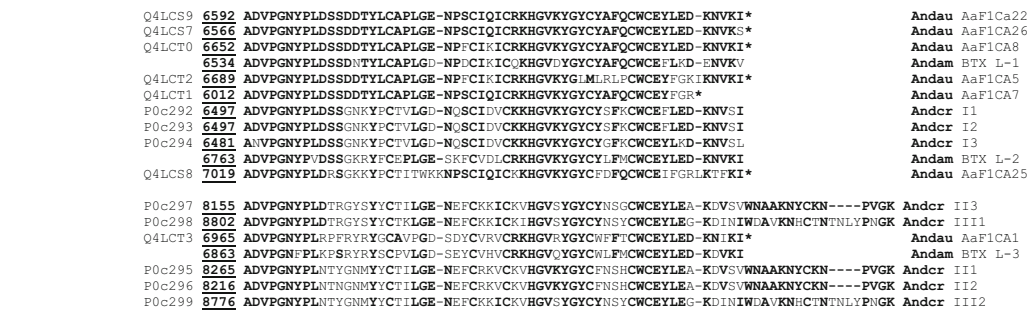

Fig. 8 Amino acid sequences of the Birtoxin-like peptides decrypted from different *Androctonus* subspecies. These sequences were obtained both by Edman degradation and by cDNAs amplification. *Amino acid sequence deduced from cDNA. Homologous amino acids are in bold. Masses are both in bold and underlined. Andau *Androctonus australis*, Andam *Androctonus amoreuxi*, Andcr *Androctonus crassicauda*

so-called functional dyad observed for short α-KTx scorpion toxins in the β-sheet. KAaH1 or KAaH2, as all other members of the family, show important sequence similarity with anti-mammal β-toxins specific for Na_vchannels, but only weak β-like effects were observed when they were tested (1 μM) on expressed brain $Na_v1.2$ channels (Srairi-Abid et al. 2005).

Another Birtoxin-like, AaBTX-L1, was also isolated from the *Androctonus australis* venom. It was not able to displace of the ^{125}I-labeled Css IV bound to its receptor site, even at 0.1 μM. Similarly, AaBTX-L1 (clone AaF1CA25) was also totally unable to compete on rat brain synaptosomes with ^{125}I-labeled KTX (high-affinity ligand of $K_v1.1$ and $K_v1.3$) and^{125}I-labeled Apamin (ligand of the SK_{Ca}channel) up to 0.1 μM. Thus, AaBTX-L1 was definitively not a pore blocker of K^+ channels, even if it is structurally related to the K1.3 blockers KAaH 1 and KAaH 2. Further electrophysiological study on the insect *Drosophila* para/tipE channel shows that AaBTX-L1 was able to shift slightly the activation ($V_{1/2}$) and the reversal potential toward more hyperpolarized potentials, which was fully consistent with the β-type activity of the scorpion anti-insect-specific toxins (Abbas et al. 2011).

It cannot be excluded that KAaH1 binds to the $K_v1.1$ or $K_v1.3$ voltage sensor, as tarantula toxins, to inhibit the normal channel opening. Thus, it could be hypothesized that the lack of the fourth disulfide bridge present in α- and β-toxins (bridge C1-C8) led to an unusual mobility of these molecules. It is possible that the high flexibility of the BTX-like toxins allows them to adopt a transient conformation not only adapted to the $K_v1.1$ or $K_v1.3$ voltage sensors but also able to recognize any $Na_v1.2a$ binding site which induces an additive β-effect.

From *Androctonus crassicauda* venom, two Birtoxin-like, Acra1 (toxic to mice and constituting 2.8 % of the total protein in the soluble venom) and Acra2 (lethal to mice and only 1 % of the soluble venom), were isolated (Caliskan et al. 2006). Acra1 was fully sequenced and Acra2 only partially. Additionally, several clones were obtained from a cDNA library of the venomous gland of one specimen.

Their gene sequences suggested the presence in this venom of three distinct groups of peptides. In particular, a new type of peptide was identified with odd number of cysteines (seven), allowing the formation of heterodimers with molecular masses in the range of 16,000 atomic mass units.

More recently, three novel peptides, AamBTX-L1, AamBTX-L2 and AamBTX-L3, were further isolated and characterized in the *Androctonus amoreuxi* venom (Abbas et al. 2011). Of the three peptides, only one, AamBTX-L3, was highly toxic and able to recognize the same binding site as a classical β-toxin specific for mammal on neuronal Na_vchannels. This work was the first report claiming that a Birtoxin-like toxin was able to displace the radioiodinated β- toxin Css IV bound on rat brain membranes. The fact that Birtoxin-like toxins were highly conserved at the structural level, but produce distinct in vivo effects, led to speculate that each peptide may bind to a different channel subtype, thus displaying a range of activities. Considering AamBTX-L1 and AamBTX-L2, both the absence of toxicity in mice and total lack of competition with the anti-mammal β-toxin^{125}I-labeled Css IV might be consistent with the β-type activity of the scorpion anti-insect-specific toxins. Electrophysiological experiments on the *Drosophila* Na_vchannel expressed in oocytes indicated that AamBTX-L1 and AamBTX-L2 from *Androctonus amoreuxi* were able to induce a strong shift of the voltage dependence of activation characteristically produced by scorpion β-toxins (Christian Legros, personal communication). Thus, it was proposed that, as AaBTX-L1 from *Androctonus australis*, these two new toxins from *Androctonus amoreuxi* were β-toxins active on insects. This increasing number of β-toxins active on insects characterized from scorpion venoms illustrates their insecticidal potential and an efficient means for scorpions to capture their prey.

Conclusion and Future Direction

In *Androctonus* venoms, in particular in well-studied *Androctonus australis* and *Androctonus mauretanicus* venoms, the lethality for mammals is almost entirely due to α-toxins. It was shown that the neutralization of the classical α-toxins by specific antisera inhibits completely the venom lethal activity, because these α-toxins are not only the most abundant but also the most lethal in the venom. Unfortunately, the large antigenic polymorphism of this group of scorpion toxins, due to their wide structural polymorphism, complicates the serotherapy (Granier et al. 1989).

Now, it is perfectly admitted by the toxinologists community that the toxins specific for insects, which induce contractive or depressant effects in the animal, are also β-toxins according to their electrophysiological effects and binding competitions with radiolabeled toxins on insect synaptosomes. The preliminary description of the anti-insect toxin binding site is in favor of the domain II voltage sensor (Karbat et al. 2007). In *Androctonus australis* and *Androctonus mauretanicus* venoms, the whole lethality for insect (blowfly larvae as experimental model) is due to the contracturant anti-insect β-toxin, used as bioinsecticide. Some other

β-toxins active both on mammals and insects were also characterized in the venoms from *Androctonus australis*, *Androctonus mauretanicus*, and *Androctonus amoreuxi*. However, their contribution to the overall venom toxicity to model mammals and insects counts for almost nothing (Martin-Eauclaire et al. unpublished data).

The recent work on the Birtoxin-like family described in at least three *Androctonus* venoms now asks a question without any clear answer: what are these new components, which are found in a quite large amount in the venoms studied, used for? Their pharmacological diversity is intriguing, and the role of these molecules in the *Androctonus* venoms obscure. In fact, the idea that the binding site (localized on the channel thanks to labeled molecules) and the pharmacological response are not necessarily tied emerges (Cohen et al. 2006). It was proposed that some apparently inactive molecules could bind the channel without inducing any effect. So, although α-toxins and β-toxins bind to two different sites on the Na_v channel, synergic effects were reported when they were both co-injected into insects and a mutual allosteric interaction between their receptor sites on the locust Na_vchannel was found. This fact suggests a new functional role for weakly toxic polypeptides in enhancing the effect of other active neurotoxins in arthropod venom, because of allosteric interactions between toxin and Na_v channel receptor sites (Cohen et al. 2006). Thus, the binding of a nontoxic ligand to receptor site 4 ("silent binding") could induce a conformational change that does not alter channel gating, but influences toxin binding at receptor site 3, and finally leading to enhanced toxicity.

In fact, synergistic lethal effects in mice (up to 50 % increase) were already observed by mixing together toxic and not toxic fractions isolated during the purification of *Leiurus quinquestriatus quinquestriatus* or *Buthus occitanus tunetanus* venoms and are in favor of this hypothesis. However, no synergy at all was so far observed during the purification of the *Androctonus australis* venom, in which several Birtoxin-like toxins are characterized (Martin-Eauclaire, personal communication).

As a final conclusion, it can be said that the study of *Androctonus* venoms has always been focused, not only on the serotherapy improvement (*Androctonus* stings being the most lethal for human being in the whole world) but also on the search for new venom peptides and their functional characterization. A pool of physiologically obtained *Androctonus* venom may possess about one hundred different peptides, as already shown by MALDI-TOF mass spectrometry (Martin-Eauclaire et al. 2013). Most of these peptides are highly reticulated by disulfide bridges, in order to stabilize and protect a biologically active conformation, which, usually, interact specifically with ionic channels. These large transmembrane proteins play crucial roles in multiple physiological processes such as nerve conduction, signal transduction, gene transcription, cell proliferation, etc. So far, *Androctonus* toxins have already provided powerful tools for the identification, purification, structure determination, and functional characterization of ion channels, as well as analysis of channel kinetics and gating properties, and for understanding their physiological contributions. Thus, these investigations still open novel valuable perspectives in drug development.

Cross-References

- ▶ Modern Venom Profiling: Mining into Scorpion Venom Biodiversity
- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ Scorpion Diversity and Distribution: Past and Present Patterns

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Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels

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Abstract

Scorpion alpha and beta toxins interact with voltage-gated sodium channels (Na_vs) at two pharmacologically distinct sites. Alpha toxins bind at receptor site 3 and inhibit channel inactivation, whereas beta toxins bind at receptor site 4 and shift the voltage-dependent activation toward more hyperpolarizing potentials. The two toxin classes are subdivided to distinct pharmacological groups according to their binding preferences and competition for receptor sites at Na_v subtypes. To elucidate the surface of interaction of the two toxin classes with Na_vs and clarify the molecular basis of varying toxin preferences, an efficient expression system was established. Mutagenesis accompanied by toxicity, binding, and electrophysiological assays, in parallel to determination of the three-dimensional structure using NMR and X-ray crystallography, uncovered the bioactive surfaces of toxin representatives of all pharmacological groups. Exchange of external loops between channels that exhibit marked differences in sensitivity to various toxins accompanied by point mutagenesis highlighted channel determinants that play a role in toxin selectivity. These data were used in further mapping of the brain channel $rNa_v1.2a$ receptor sites for the beta-toxin Css4 (from *Centruroides suffusus suffusus*) and the alpha-toxin Lqh2 (from *Leiurus quinquestriatus hebraeus*). On the basis of channel mutations that affected Css4 activity, the known structure of the toxin and its bioactive surface, and using the structure of a potassium channel as template, a structural model of Css4 interaction with the gating module of domain II was constructed. This initial model was the first step in the identification of part of receptor site 4. In parallel, a swapping and a mutagenesis approach employing the $rNa_v1.2a$ mammalian and $DmNa_v1$ insect Na_vs and the toxin Lqh2 as a probe were used to search for receptor site 3. The channel mapping along with toxin dissociation assays and double-mutant cycle analyses using toxin and channel mutants identified the gating module of domain IV as the site of interaction with the toxin core domain, thus describing the docking orientation of an alpha toxin at the channel surface.

An enticing phrase often used in describing new scorpion neurotoxins affecting voltage-gated sodium channels (Na_vs) praises their potential use as leads in future design of insecticides or therapeutics. Apart of an evident need to attract the reader, has this claim ever come near to realization? During more than three decades of extensive research, the pharmacology of a large arsenal of scorpion toxins and the way they modulate sodium channel activation or inactivation have been described. Residues with a role in bioactivity have been identified for numerous scorpion toxins representing various pharmacological groups, and initial details as to channel regions involved in toxin recognition (receptor sites) were highlighted. Still, a

comprehensive molecular description of how these toxins interact with Na_vs and modulate their function has not yet been achieved, and only an initial perspective has recently emerged. This gap in knowledge is attributed in large to the fact that the structure of the eukaryotic sodium channel has not yet been determined and is probably further complicated by conformational rearrangements of the toxin-channel complex during gating. Since a channel receptor site, composed of amino acid residues brought together by its fold, may alter as the channel opens and closes, identification of these residues may require determination of the structure of the toxin-channel complex at various channel states. These limitations delay realization of the great potential of scorpion peptide toxins as leads in rational design of selective, industrially significant peptide mimetics. This review focuses on recent attainments leading to better characterization of the receptor sites of scorpion alpha and beta toxins and manipulation of toxins selectivity, as well as raises the main obstacles still on way for gaining complete understanding of the interactions between Na_vs and their toxin modulators.

Scorpion Toxins That Modulate Na_v Activation

Scorpion toxins typified by the ability to shift the voltage dependence of Na_v activation toward more negative membrane potentials are classified as β-toxins (Fig. 1; Catterall 1992; Martin-Eauclaire and Couraud 1995; Catterall et al. 2007; Gurevitz et al. 2007). Despite their common effect on channel activation, they vary greatly in sequence, in selectivity, and in the symptoms they induce when injected to animals. On the basis of the pharmacological effect and binding studies, the receptor of scorpion β-toxins at Na_vs was named neurotoxin receptor site 4 (Jover et al. 1980; Barhanin et al. 1982). Site 4 was characterized in insect Na_vs by the high-affinity (0.1–2 nM) voltage-independent binding of the anti-insect-selective excitatory (e.g., AahIT and Bj-xtrIT) and depressant (e.g., LqhIT2, Lqh-dprIT$_3$) toxins (reviewed in Gurevitz et al. 2007). For mammalian Na_vs this site was characterized in binding assays using the radiolabeled β-toxin Css2 and rat brain synaptosomes (Jover et al. 1980).

On the basis of binding competition assays, scorpion β-toxins were divided into four pharmacological groups (Fig. 1; Zlotkin 1999; Possani et al. 1999; Gurevitz et al. 2007): (1) anti-mammalian β-toxins found in scorpions of the New World (e.g., Css4 from *Centruroides suffusus suffusus*, Cn2 from *Centruroides noxious*) that bind with high affinity in a voltage-independent manner to receptor site 4 at rat brain synaptosomes; (2) anti-insect-selective excitatory β-toxins (e.g., AahIT from *Androctonus australis*, Bj-xtrIT from *Hottentotta judaicus*) that when injected into blowfly larvae induce spastic paralysis, resulting from repetitive activity of motor nerves consequent to increased currents and slowed inactivation of Na_vs (Froy et al. 1999); (3) anti-insect depressant toxins (e.g., LqhIT2 and Lqh-dprIT3 from *L. q. hebraeus*, BotIT2 from *Buthus occitanus tunetanus*) which exert high affinity for insect Na_vs and are practically harmless when injected to mice (Ben-Khalifa et al. 1997; Strugatsky et al. 2005). These toxins induce flaccid paralysis in injected

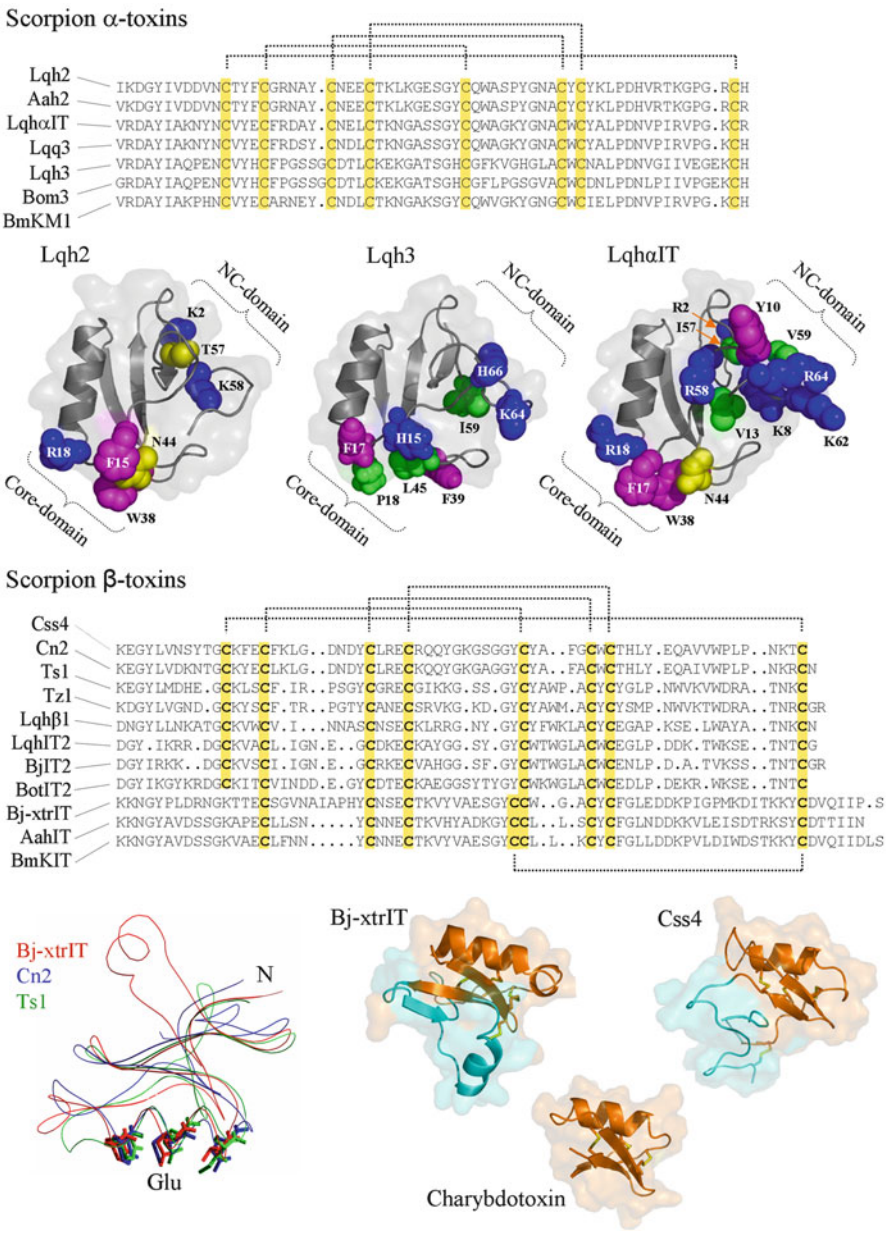

Fig. 1 Sequence and structure alignments of scorpion alpha and beta toxin representatives. Sequences were aligned according to the conserved cysteine residues (*yellow* background), and the disulfide bonds formed between cysteine pairs are shown. *Dots* indicate gaps for best alignment. *Lqh Leiurus quinquestriatus hebraeus, Aah Androctonus australis, Lqq L. q. quinquestriatus, Bom Buthus occitanus mardochei, BmK Mesobuthus martensii* Karsch, *Css Centruroides suffusus suffusus, Cn Centruroides noxius, Ts Tityus serrulatus, Tz Tityus zulianus, Bj Buthotus judaicus*

blowfly larvae, which are opposite to the contraction paralysis symptoms induced by excitatory toxins. Study of their effect on a cockroach axon under current or voltage-clamp conditions revealed depolarization and block of evoked action potentials as well as strong decrease in the inward transient peak current (Ben-Khalifa et al. 1997); (4) β-toxins that bind with high affinity to both mammalian and insect Na_vs, such as Ts1 from *Tityus serrulatus* (New World; Barhanin et al. 1982) and Lqhβ1 from *L. q. hebraeus* (Old World; Gordon et al. 2003). Most β-toxins that affect mammals modify the activation of $rNa_v1.2$ rat brain and $rNa_v1.4$ skeletal muscle Na_vs in a similar manner, but have no effect on $hNa_v1.5$ cardiac channel (Cestèle et al. 1998). These observations have shown that despite the general similarity among Na_v subtypes, their receptor-binding sites for these toxins vary.

Of note are several tarantula toxins that differ considerably in structure from scorpion β-toxins and still bind at receptor site 4, such as ProTx-II (30 amino acid long) from *Thrixopelma pruriens* and CcoTx2 (33 amino acid long) from *Ceratogyrus cornuatus* (Middleton et al. 2002; Bosmans et al. 2006; Smith et al. 2007), which compete with scorpion β-toxins for binding, or HWTX-IV, a 35-residue toxin from *Ornithoctonus huwena*, which shows preference for neuronal channels (Xiao et al. 2008). These peptides, bearing an ICK fold (inhibitory cystine knot), induce a shift in voltage dependence of channel activation to more depolarizing potentials (stronger depolarization is required to activate the channel), in contrast to the hyperpolarizing shift in voltage dependence of channel activation induced by scorpion β-toxins. Still, the ability of these spider toxins to compete for

Fig. 1 (continued) (now called *Hottentotta judaicus*), *Bot Buthus occitanus tunetanus* (Gordon et al. 2007; Gurevitz et al. 2007). *Upper part*: Lqh2 and Aah2 are classical anti-mammalian α-toxins, LqhαIT and Lqq3 are named anti-insect α-toxins despite their effect on mammalian skeletal muscle channels, and Lqh3, Bom3, and BmKM1 are α-like toxins. The presented structures of Lqh3 and LqhαIT have been determined (PDB codes 1FH3 and 2ASC, respectively). Lqh2 structure was modeled on the basis of the known structure of Aah2 (PDB code 1AHO) using the SWISS-MODEL protein homology-modeling server (EXPASY). Ribbons indicate the backbone structures covered by a semitransparent molecular surface of the toxins. Residues of the bioactive surfaces (Karbat et al. 2004a, 2007; Kahn et al. 2009) are space filled and colored according to their chemical nature (aliphatic, *green*; aromatic, *magenta*; polar, *yellow*; and positive, *blue*). The bioactive surface in the three toxins is divided into a core domain and an NC domain. *Lower part*: Css4, Cn2, Ts1, and Tz1 are classical β-toxins; Lqhβ1 was found and characterized in the "New World"; LqhIT2, BjIT2, and BotIT2 are depressant toxins; Bj-xtrIT, AahIT, and BmKIT are excitatory toxins. The structures of Bj-xtrIT, Cn2, and Ts1 were determined (PDB codes 1BCG, 1Cn2, and 1NPI, respectively) (note the common "hot spot" at the "pharmacophore" of the three toxins, which differ markedly in selectivity toward insects and mammals (Cohen et al. 2004). The structural models of Css4 (based on the NMR structure of Cn2; PDB code 1CN2), Bj-xtrIT (PDB code 1BCG), and charybdotoxin (potassium channel blocker; PDB code 2CRD) at the lower right-hand side are covered by semitransparent molecular surfaces and are spatially aligned. The moieties representing ΔΔBj-xtrIT and ΔΔCss4 are colored in orange on the ribbon structure of the parent toxins, and the cyan ribbons represent deleted N- and C-termini (note the resemblance of the core in the three toxins suggesting common ancestry (Froy and Gurevitz 1998; Froy et al. 1999; Cohen et al. 2008). The models were prepared using PyMOL

the receptor of scorpion β-toxins suggests that the boundaries of site 4 are wider than those pictured for the scorpion toxins. Thus, receptor site 4 at various channels should be determined in detail for each toxin separately to explain the variations in potency and efficacy. In conclusion, scorpion β-toxins are divided into four groups according to their binding features at receptor site 4 of various Na_vs. They all affect channel activation, and two groups show selectivity to insects.

Scorpion Toxins That Modulate Na_v Inactivation

Scorpion toxins that inhibit sodium current inactivation under voltage-clamp conditions have been classified as α-toxins (reviewed in Catterall 1992 and Gordon et al. 2007). These toxins are similar in structure and function (Fig. 1), and they bind in a voltage-dependent manner at a channel site determined pharmacologically as Na_v receptor site 3. The inhibition of channel inactivation has been attributed to perturbation of the outward movement of DIV/S4 voltage sensor upon channel activation, which is coupled to the conformational change leading to channel fast inactivation (Yang and Kuo 2003; Cestèle et al. 2006; Campos et al. 2008). Interestingly, certain peptide toxins from sea anemones {e.g., Av2 (ATX II) from *Anemonia viridis* (named previously *Anemonia sulcata*) or Av3 (Moran et al. 2009)} and from spiders (e.g., δ-atracotoxin-Hv1 from *Hadronyche versuta*, Hexathelidae: Atracinae), that share no sequence homology or structure similarity with scorpion α-toxins, compete in binding for receptor site 3, and their inhibitory effect on the inactivation process is similar (reviewed in Catterall 1992; Gordon 1997; Rash and Hodgson 2002; King et al. 2008). This heterogeneity in parallel to the resemblance in effect suggest that the boundaries of receptor site 3 from a structural viewpoint are apparently broader than those involved in the binding of scorpion α-toxins (Gordon et al. 1996) and might also overlap partially at DIV/S3-S4 external loop with site 6 of δ-conotoxins, which induce a similar effect (Heinemann and Leipold 2007). Along with the vast differences in preference of scorpion α-toxins for Na_v subtypes, a detailed description of this receptor site in various channels is required for each toxin derivative.

Despite the similar mode of action and toxicity to mice when administered subcutaneously, scorpion α-toxins differ prominently in respect to their preference for insect versus mammalian Na_vs as well as in potency when examined in binding competition assays using various neuronal membrane preparations. Accordingly, they were divided into three major groups (reviewed in Gordon and Gurevitz 2003; Gordon et al. 2007): (1) α-Toxins that bind with high affinity to rat brain synaptosomes (K_d in the range of 0.2–0.3 nM) and are highly active in mammalian brain (LD_{50} = 0.5–20 ng/20 g mouse by intracerebroventricular injection). These toxins are very weak when injected to insects or when tested on sodium current inactivation in insect neuronal preparations. Toxins of this group have been first identified in the venom of *Androctonus australis* (e.g., Aah1, Aah2, Aah3) due to their high toxicity for humans and then in other scorpion species (e.g., Lqq5 in *Leiurus quinquestriatus quinquestriatus* and Lqh2 in *Leiurus quinquestriatus hebraeus*;

Kahn et al. 2009). They are found in "Old world" (Africa, Asia, Europe) scorpions (Martin-Eauclaire and Couraud 1995; Froy and Gurevitz 2003), but were also identified in scorpion venom of the "New world" (Americas), e.g., toxin V of *Centruroides sculpturatus* (Possani et al. 1999). The affinity of these toxins for mammalian Na_vs is markedly reduced upon membrane depolarization due to conformational alterations at the binding site (reviewed by Catterall 1992; Gordon 1997). Aah2, the most potent α-toxin of this group (LD_{50} = 0.5–1.0 ng/20 g mouse by i.c.v. injection) serves as a prototype in binding assays for classification of unknown anti-mammalian α-toxins (Gordon et al. 2007). Another feature that typifies these toxins is the allosteric increase in their affinity for receptor site 3 by binding of alkaloid toxins such as veratridine and batrachotoxin at receptor site 2 (Catterall 1992; Gordon et al. 2007). (2) α-Toxins highly active on insects and very weak in mice by i.c.v. injection (LD_{50} = ~40 mg/20 g mouse; e.g., LqhαIT, Lqq3, and BotIT1). They bind with high affinity to insect neuronal preparations (0.061–1.0 nM) and hardly compete (at the μM range) with iodinated Aah2 on binding at rat brain synaptosomes. Moreover, these toxins hardly affect the rat brain channel $rNa_v1.2a$ expressed in *Xenopus* oocytes (at the μM range). In contrast to the anti-mammalian α-toxins, the binding of LqhαIT to insect neuronal membranes is independent of membrane potential but, like Aah2, is allosterically enhanced by veratridine binding at receptor site 2 implying a similar mode of binding. LqhαIT is the most potent and best characterized toxin in this group and is used as a marker of receptor site 3 at insect Na_vs. Two additional anti-insect α-toxins have been reported in recent years, BjαIT from the black scorpion *Hottentotta judaicus* (previously *Buthotus judaicus*) and phaiodotoxin from the Mexican scorpion *Anuroctonus phaiodactylus* of the Iuridae family (reviewed in Gordon et al. 2007). Yet, the selectivity of these two toxins and definite classification have not been fully determined and require further evaluation on various Na_v subtypes. (3) α-Like toxins, such as Lqh3 and Lqh6 (from *L. q. hebraeus*), Bom3 and Bom4 (from *Buthus occitanus mardochei*), and BmK M1 (from *Mesobuthus martensii* Karsch), are toxic to insects by injection and exert high apparent affinity for insect neuronal membranes (Gordon et al. 1996) and $DmNa_v1$ (*Drosophila para*) channels expressed in *Xenopus* oocytes (Karbat et al. 2007). These toxins are also active on mice by i.c.v. injection (LD_{50} = 23–50 ng/20 g mouse) although they do not affect the rat brain channel $rNa_v1.2a$ and hardly compete with iodinated Aah2 on binding at rat brain synaptosomes (Gordon et al. 1996; Gilles et al. 2000). These unique features distinguish α-like toxins from the anti-insect α-toxin group, while their distinction from anti-mammalian α-toxins relies on lack of activity at $rNa_v1.2a$ or binding to rat brain synaptosomes.

The differences among scorpion α-toxins imply that classification of new members on the basis of the criteria established requires thorough analysis of their toxicity to insects and mice by i.c.v. and peripheral injections; electrophysiological effects on insect and mammalian brain and skeletal muscle ($Na_v1.2a$, $Na_v1.4$) channels; and binding affinity for site 3 on insect and rat brain synaptosomes. In conclusion, scorpion α-toxins are divided into three groups on the basis of their preference for Na_v subtypes. They bind at receptor site 3 and affect channel inactivation.

Emerging View of the Receptor Sites for Scorpion Alpha and Beta Toxins

The interaction of scorpion toxins with their Na_v receptor sites has been the focus of extensive research for several decades (reviewed in Rodriguez de la Vega and Possani 2007), yet molecular and structural details about these interactions were limited and have emerged only with the establishment of cloning procedures for the toxins and of an efficient bacterial expression system (LqhαIT anti-insect α-toxin, Gurevitz and Zilberberg 1994; Zilberberg et al. 1997; LqhIT2 and Lqh-dprIT$_3$ depressant β-toxins, Turkov et al. 1997; Strugatsky et al. 2005; Bj-xtrIT anti-insect excitatory toxin, Froy et al. 1999; Lqhβ1 and Css4 anti-mammalian β-toxins, Gordon et al. 2003; Cohen et al. 2005; Lqh3 α-like toxin, Karbat et al. 2007; Lqq5 and Aah2/Lqh2 anti-mammalian α-toxins, Banerjee et al. 2006; Kahn et al. 2009) and later in yeast (e.g., BmK M1 α-like toxin: Shao et al. 1999). From this point on the study of the toxins did not depend any longer on tedious purifications from crude venoms or chemical modifications that were limited to reactive residues (reviewed in Gurevitz 2012), but rather enabled substitution of any desired single or combination of amino acid residues and massive production of unmodified and mutant toxins for functional and structural analyses. Ever since, the bioactive surfaces of toxin representatives of all pharmacological groups of the α- and β-classes were elucidated. Comparison of bioactive surfaces highlighted commonalities associated with the similar mode of action, and differences that likely dictate deviation in selectivity toward channel subtypes (Zilberberg et al. 1997; Oren et al. 1998; Froy et al. 1999; Karbat et al. 2004a, 2006, 2007; Cohen et al. 2004, 2005, 2007; Ye et al. 2005; Kahn et al. 2009; Weinberger et al. 2010).

Na_v Receptor for Scorpion β-Toxins: Determination of the receptor site for Na_v modifiers, such as scorpion toxins, is complicated in the lack of channel structure and the fact that ion channels are membrane proteins dipped in a lipid bilayer that modifies their electrostatic environment as well as conformational rearrangements during gating. Since the channel receptor is composed of amino acid residues brought together by the three-dimensional protein fold, identification of residue participants requires structural determination of the toxin-channel complex. As this requirement has not been met so far, the experimental approach undertaken was to identify channel residues whose substitution affected toxin binding and/or activity (Fig. 2a). Another approach was to first identify by mutagenesis channel regions involved in toxin selectivity and then search for specific residues with the utmost impact. By analyzing toxin activity on chimeras made between cardiac, skeletal muscle, and brain Na_vs, channel domain II has been shown to be important for binding of Ts1 from the scorpion *Tityus serrulatus* (Marcotte et al. 1997). This conclusion was corroborated by a decrease in Css4 activity upon substitution of Glu779 at S1-S2 and Glu837, Leu840, and Gly845 at S3-S4 extracellular loops at DII of the brain channel rNa_v1.2a (Cestèle et al. 1998).

Exchange of DII in rNa_v1.2a, which is insensitive to scorpion excitatory toxins, with its *Drosophila* channel DmNa_v1 equivalent, resulted in a functional channel chimera that was sensitive to the excitatory toxin AahIT. This has implied that at

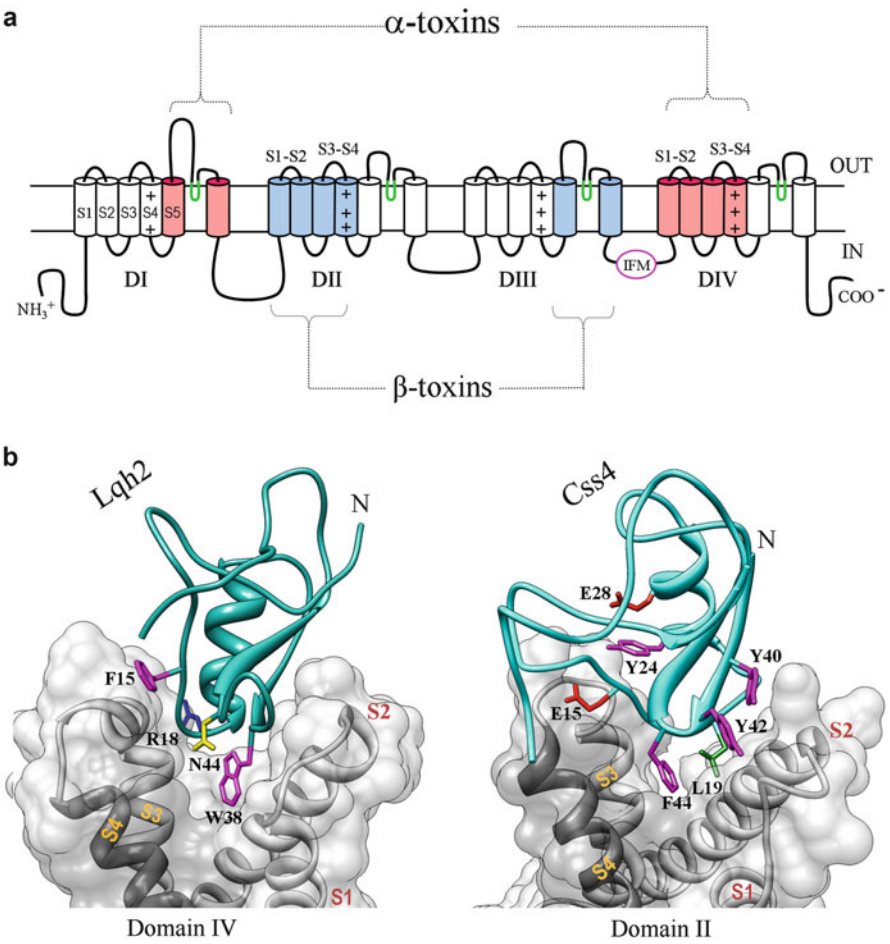

Fig. 2 Binding site for scorpion α- and β-toxins at Na_vs. (**a**) Scheme of a sodium channel pore-forming α-subunit and regions assigned to receptor sites 3 (*red*) and 4 (*blue*). DI, DII, DIII, and DIV assemble in the membrane around the channel pore (*green*). The S1-S2 and S3-S4 linkers at the gating module of DIV and S5-S6 at the pore module of DI form receptor site 3 of scorpion α-toxins (Gur et al. 2011; Wang et al. 2011). The equivalent regions in DII and DIII form receptor site 4 of scorpion β-toxins (Zhang et al. 2012). (**b**), Face of interaction between Lqh2 α-toxin and Css4 β-toxins with the rat brain channel $rNa_v1.2a$. The toxins (in *blue*) intrude at a cleft between S1-S2 and S3-S4 of domains IV (Lqh2) and II (Css4). Both toxins are believed to also interact with the external linkers of the juxtaposed pore domain (DI for Lqh2 and DIII for Css4), which are not shown. The toxin residues with utmost bioactive role are indicated. *N* indicates N-terminus

least part of receptor site 4 is associated with domain II (reviewed in Gurevitz et al. 2007; Rodriguez de la Vega and Possani 2007). Based on these results as well as on substitutions at $rNa_v1.2$ that changed the sensitivity to the toxin Css4 (Cohen et al. 2005), and by using the published structure of a closely related β-toxin, Cn2 (PDB code 1CN2), as well as the X-ray structure of a potassium channel

(Long et al. 2005) as template, an initial putative model of Css4 docking at the gating module of $rNa_v1.2a$ was constructed (Cestèle et al. 2006). Although the structural model portrayed for the first time a putative face of interaction between a scorpion β-toxin and a sodium channel at a cleft in the gating module of DII, further experimental validation was still required. Furthermore, the molecular details at the face of interaction that dictate selectivity remained obscure, although it was proposed to be associated with the pore module. Additional substitutions at the pore module of domain III (DIII/SS2-S6) of $rNa_v1.4$ have shown the role of Glu1251 and His1257 in the interaction with the β-toxin Tz1 (Leipold et al. 2006), which led Cohen and his colleagues to examine these residues in $rNa_v1.4$ by employing Css4 mutants in double-mutant cycle analysis (Cohen et al. 2007). The ΔΔG values obtained suggested close proximity between Phe14 of the toxin and Glu592 at DII/S1-S2 of the channel, Arg27 of the toxin and Glu1251 at DIII/SS2-S6 of the channel, and Glu28 of the toxin with both Glu650 at DII/S3-S4 and Glu1251 at DIII/SS2-S6 of the channel. These results have corroborated the suggestion that receptor site 4 spans channel domains II and III and also demonstrated that despite the differences in interactions with the rat brain and skeletal muscle Na_vs, Css4 recognizes a similar region at both channel subtypes.

Further mapping of Css4 interaction with $rNa_v1.2$, focusing on extracellular loops S1-S2 and S3-S4 at DII, highlighted the critical role of Glu779 and Pro782 at the S1-S2 loop and five positions surrounding a previously identified key-binding determinant, G845, at the S3-S4 loop, which constitutes a hotspot of high-impact residues. Two of these substitutions (A841N and L846A) reduced the voltage-sensor trapping effect of Css4, while the other three, N842R, V843A, and E844N, increased the voltage-sensor trapping (Zhang et al. 2012). A molecular model constructed with the Rosetta membrane modeling and ligand-docking algorithms suggested direct interactions between toxin and channel amino acid pairs (Fig. 2b). In light of the view that scorpion β-toxins interact with Na_vs at two surfaces, one comprising S1-S2 and S3-S4 in the voltage sensor of DII and the other in the external linkers of the pore module of DIII, the results of analysis of the insecticidal depressant β-toxin Lqh-dprIT$_3$ on $BgNa_v$ (the sodium channel of the cockroach *Blattella germanica*) were puzzling as substitutions at the voltage sensor of DIII were found to increase the toxin effect on channel activation (Song et al. 2011). Rather than offering the voltage sensor at DIII as a possible binding site for Lqh-dprIT$_3$, these results were rationalized in that the channel hypersensitivity was due to coupling between neighboring voltage sensors during channel activation (Chanda et al. 2004), rendering an allosteric effect that improved the ability of the toxin to trap DIIS4 in its outward position (Song et al. 2011; see also Cestèle et al. 1998, 2001, 2006).

To date, a structural model that pictures in detail the docking orientation and entire face of interaction between the sodium channel and scorpion β-toxins (receptor site 4) has not yet been established. Still, accumulating data suggest that this receptor encompasses domains II and III of the Na_v, and the toxin's effect on activation is due to trapping of S4 movement at DII upon membrane depolarization. Such a description may be more challenging than anticipated given the

conformational rearrangements of the channel during gating, which might reflect on induced conformational fit of the ligand and its receptor (e.g., Tsushima et al. 1999).

Na_v Receptor for Scorpion α-Toxins: Mapping the receptor sites for a variety of Na_v ligands has classified the binding site recognized by scorpion α-toxins affecting the inactivation process as neurotoxin receptor site 3 (reviewed in Catterall et al. 2007). This classification chronologically followed the previous designation of neurotoxin receptor sites 1 for tetrodotoxin and 2 for batrachotoxin (reviewed in Gordon 1997). Interestingly, despite of functional and structural resemblance, Na_v subtypes in various species or tissues of the same organism (e.g., insect channel $DmNa_v1$ and mammalian brain and heart channels $Na_v1.2$ and $Na_v1.5$) often show great differences in sensitivity to α-toxins of different pharmacological subgroups (Gordon et al. 2007), whereas other channels (e.g., the mammalian skeletal muscle channel $Na_v1.4$) are almost equally sensitive to these α-toxins. This feature along with the ability of structurally different toxins from spiders and sea anemones to compete for the same site in binding studies (Gordon et al. 2007; Moran et al. 2009) suggest that receptor site 3 is a macrosite with broader boundaries than those initially assumed. Minute variations among residues that compose this site have been shown to account for large differences in sensitivity to and accessibility of scorpion α-toxins (Gordon et al. 2007; Gurevitz 2012).

Initial studies to identify receptor site 3 in the rat brain channel $rNa_v1.2a$ pointed to external linkers DI/S5-S6, DIV/S5-S6, and DIV/S3-S4 as putative participants (Fig. 2a; reviewed in Catterall et al. 2007). Mutagenic dissection of these linkers in mammalian and insect channels highlighted a conserved negatively charged residues at DIV/S3-S4 (Glu1613 in $rNa_v1.2a$, Glu1428 in $rNa_v1.4$, and Asp1701 in $DmNa_v1$), whose substitution with arginine decreased prominently the activity of α-toxins (Rogers et al. 1996; Leipold et al. 2004; Karbat et al. 2007). Ever since, further description of receptor site 3 has been delayed for more than a decade before two combined approaches recently shed more light on this binding site (Gur et al. 2011; Wang et al. 2011). One approach was to exchange channel regions that determine toxin selectivity. The DIV/S1-S2, DIV/S3-S4, DI/S5-SS1, and DI/SS2-S6 external linkers in the *Drosophila* channel $DmNa_v1$, which is highly sensitive to LqhαIT and insensitive to Lqh2, were exchanged with their equivalents of the rat brain channel $rNa_v1.2a$, which is highly sensitive to Lqh2 and insensitive to LqhαIT. The channel chimera was highly sensitive to Lqh2 and insensitive to LqhαIT. These results correlated these linkers with toxin selectivity and with receptor site 3. Additional substitutions of linkers and individual residues and analysis of gain or loss of Lqh2 and LqhαIT functions exposed specific channel residues involved in toxin binding. Depolarization-induced dissociation experiments revealed that substitution of core domain residues at the toxin surface facilitated dissociation from the channel and from channel mutants modified at DIV/S3 (Phe1610) and at DIV/S3-S4 (Glu1613). This has indicated that the toxin core domain interacts with the gating module of DIV, a conclusion corroborated by double-mutant cycle analysis suggesting close proximity between Phe15 and Asn44 of the toxin and Phe1610 and Glu1613 of the channel at the gating module of DIV. The mutagenic approach has also shown the importance of DI/S5-S6 (pore module)

and the external part of DIV/S3 (gating module) of rNa$_v$1.2a for Lqh2 action (Fig. 2b). Collectively, these results enabled to construct structural models of the putative interaction of the toxin core domain with gating module DIV of the channel, while the NC domain of the toxin has been proposed to interact with the external loops at the pore module of DI (Gur et al. 2011; Wang et al. 2011). In addition to the identification of channel determinants that dictate toxins specificity, the results of Gur and her colleagues (2011) and Wang and her colleagues (2011) provided an initial view of the docking orientation of a scorpion α-toxin at a voltage-gated sodium channel. Despite this achievement, a detailed description of the toxin interaction with the channel at the pore module awaits further investigation. As the binding of scorpion α-toxins to Na$_v$s is voltage dependent, which closely correlates with the voltage dependence of channel activation, the specific effect of α-toxins on inactivation suggests that changes in membrane potential affect the structure of receptor site 3. Therefore, the channel region that includes receptor site 3 is involved in coupling channel activation and inactivation, and binding of scorpion α-toxins likely prevents the conformational change required for fast inactivation (Catterall et al. 2007).

The interaction of Lqh2 α-toxin with rNa$_v$1.2a is reminiscent of the interaction of Css4 β-toxin with the channel at domain II (Cestèle et al. 2006). The two types of scorpion toxins bind at a cleft in the gating module of domains IV and II, respectively (Fig. 2b) and affect the movement of the S4 voltage sensor at either domain IV (α-toxins) or domain II (β-toxins). The interaction of the toxins with the external less mobile region of the pore module (α-toxins with the pore module of DI and β-toxins with the pore module of DIII) probably provides an anchor that enables the core domain of the toxin to adjust the binding at the cleft between S1-S2 and S3-S4. This bipartite interaction likely increases the binding affinity and avoids toxin falloff upon slight changes in membrane potential.

Although structural details that would enable fine comparison of receptor site 3 on insect and mammalian sodium channels are not yet available, various pharmacological studies have indicated differences in (i) the binding affinity of toxins to insect and rat brain Na$_v$s, (ii) the sensitivity to membrane potential in binding of various site 3 toxins to insect and rat brain Na$_v$s, (iii) the allosteric interactions between receptor sites for scorpion α-toxins and those for brevetoxins (site 5) or pyrethroids (site 7) on insect and rat brain Na$_v$s, and (iv) the allosteric interactions between receptor sites for scorpion α- and β-toxins (Cohen et al. 2006; reviewed in Gordon et al. 2007). Despite these differences, a single E1613D substitution at DIV/S3-S4 unexpectedly converted rNa$_v$1.2a to high sensitivity toward the toxin LqhαIT, which otherwise could hardly affect this channel (Gur et al. 2011). This has clearly indicated that Glu1613 at DIV/S3-S4 in rNa$_v$1.2a is a key factor that hinders LqhαIT interaction with receptor site 3, although Asp1428, its equivalent at DIV/S3-S4 in rNa$_v$1.4, does not hinder LqhαIT binding and effect. The gain of rNa$_v$1.2a sensitivity to LqhαIT upon a single substitution demonstrates that the brain channel bears a receptor site for LqhαIT, and the primary reason for lack of LqhαIT activity is the hindrance caused by Glu1613. Collectively, these results suggest that receptor sites 3 in the brain and insect channels are localized similarly but are not identical.

Early studies of the effects of toxins on neuronal membrane preparations revealed an increase in the binding affinity of the scorpion toxin LqhαIT for locust Na_vs in the presence of brevetoxin-1 that binds at receptor site 5 or in the presence of pyrethroids that bind at receptor site 7. A similar experiment using rat brain synaptosomes revealed that in the presence of brevetoxin-1 or pyrethroids, the binding of Aah2 or Lqh2 anti-mammalian toxins either decreased or did not change, respectively. On the other hand, the binding of pyrethroids to rat brain or to insect Na_vs has improved in the presence of the anti-mammalian α-toxin Lqq5 or the anti-insect α-toxin LqhαIT, respectively. These results pointed to differences in receptor site 3 between insect and mammalian Na_vs. However, differences in receptor site 3 were also detected in Na_vs of different insect species as indicated, for example, by the variations in binding affinity of LqhαIT for cockroach over locust neuronal membrane preparations, or the differences in effect of the scorpion toxin BmK M1 on the *Drosophila* channel $DmNa_v1$ expressed in frog oocytes versus DUM neurons isolated from locusts (reviewed in Gordon et al. 2007).

Concomitant application of the pyrethroid deltamethrin and site 3 toxins, such as the scorpion toxin LqhαIT or the sea anemone toxin ATX II (now called Av2), at insect Na_vs resulted in synergic effects (reviewed in Gordon et al. 2007). Co-injection of the anti-insect-selective excitatory (e.g., AahIT and Bj-xtrIT) and depressant (e.g., LqhIT2) scorpion β-toxins with the α-toxin LqhαIT to blowfly larvae also increased the toxicity in a cooperative manner (Herrmann et al. 1995). More peculiar was the increased toxicity of α-toxins active on insects when injected either with a nontoxic mutant of the excitatory β-toxin, Bj-xtrIT^{E15R} (a competitive antagonist of Bj-xtrIT; Karbat et al. 2004b), or with weakly active scorpion depressant β-toxins, likely resulting from positive allosteric interactions between the corresponding receptor sites (Cohen et al. 2006). In conclusion, receptor site 3 for scorpion α-toxins has been assigned to domains IV and I, and their effect on inactivation follows trapping of the movement of S4 voltage sensor at domain IV.

Manipulation of Scorpion Toxin Selectivity

The vast diversification in sequence and function of scorpion toxins and their remarkable tolerance to mutagenesis implies structural flexibility and raises challenges of rational design toward novel therapeutics (e.g., for inherited diseases that involve mutated Na_vs; Ryan and Ptacek 2010) or highly selective insecticides (Zlotkin 1999; Gurevitz et al. 2007; Gordon et al. 2007). Herein are a few examples that demonstrate the potential of such an approach:

Manipulation of Toxin Selectivity: Mutagenic dissection of α-toxins and comparison of the activity of mutant derivatives at Na_v subtypes identified variations in sequence and structure likely determining the differences in selectivity (Gordon and Gurevitz 2003). To validate these findings Karbat and his colleagues (2004a) and later Kahn and his colleagues (2009) exploited two α-toxins from *Leiurus quinquestriatus hebraeus*, LqhαIT and Lqh2, which vary greatly in preference for the insect channel $DmNa_v1$ and the mammalian brain channel $rNa_v1.2a$, and

following identification of their functional surfaces exchanged them between the two toxins. The resulting swap in toxin selectivity has shown that rational design of these polypeptides is possible. This led Kahn and his colleagues to examine whether activity of Lqh2, which affects to a similar extent skeletal muscle ($Na_v1.4$), cardiac muscle ($Na_v1.5$), and neuronal ($Na_v1.6$) and brain ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$) sodium channels, could be restricted to only brain channels. The experimental design was based on differences in sequence between Lqh2 and the α-toxin Amm8 from *Androctonus mauretanicus mauretanicus*, a considerably less active toxin than Lqh2 at the $Na_v1.4$ channel. Of all substitutions examined, those at positions 8 and 10 of the five-residue turn resulted in a toxin mutant $Lqh2^{D8N,V10I}$ that hardly affected any of the Na_vs but those of the brain. Thus, a subtle alteration in toxin structure resulted in a toxin mutant capable of differentiating between receptor site 3 of $Na_v1.4$–1.6 channels and those of the $Na_v1.1$–1.3 (Kahn et al. 2009).

Design of a β-Toxin Antagonist: In an attempt to identify scorpion β-toxin residues involved during channel activation in voltage-sensor trapping, Karbat and colleagues substituted negatively charged residues of the excitatory toxin Bj-xtrIT and found Glu15 and Glu30 as key players in toxin action. However, while substitution of Glu30 affected both the toxicity and binding, substitution of Glu15 with Arg abolished the activity but hardly affected toxin binding. This uncoupling of activity from binding has unexpectedly provided an efficient antagonist of Bj-xtrIT, as indeed was shown in toxicity assays where the antagonist protected fly larvae from the unmodified toxin (Karbat et al. 2004b). A similar result was later obtained with the anti-mammalian β-toxin Css4, where E15R (note the same position) provided an efficient antagonist of Css4, as was also shown by its ability to protect mice injected with the unmodified toxin (Karbat et al. 2010). Since Glu15 is not conserved in all β-toxins, the mechanism of trapping in terms of structural changes and the role of residue 15 in scorpion β-toxins remain to be described.

Design of a Selective Activator of the Skeletal Muscle Sodium Channel: Although Na_vs are conserved, their varying sensitivities to scorpion toxins indicate differences in the receptor sites that could be used for manipulation of toxin selectivity. By thorough mutagenesis of the scorpion β-toxin Css4 and analysis of the binding of the toxin mutants to the rat brain channel $rNa_v1.2a$ and the skeletal muscle channel $rNa_v1.4$, Cohen and his colleagues (2007) discovered prominent differences. By combining three of the substitutions, they obtained a mutant, $Css4^{F14A\text{-}E15A\text{-}E28R}$, which was highly active at the skeletal muscle channel and practically inactive at the brain channel. Since Css4 enhances channel activation, the elimination of its activity at brain Na_vs has provided a specific activator of the skeletal muscle Na_v. This selectivity prompted the examination of the therapeutic potential of $Css4^{F14A\text{-}E15A\text{-}E28R}$ by applying it onto an $rNa_v1.4$ channel mutant bearing the same mutation found in the genetic disorder hypokalemic periodic paralysis (hypoPP). The mutant toxin restored the impaired gating properties of the modified channel expressed in *Xenopus laevis* oocytes, demonstrating a tentative new means for treatment of neuromuscular disorders with reduced muscle excitability (Cohen et al. 2007).

Truncation of Scorpion β-Toxins Exposes an Ancestral Bioactive Surface: Residues involved in the interaction of scorpion β-toxins with receptor site 4 at Na_vs have been assigned to the conserved βαββ core and the C-tail (Cohen et al. 2004, 2005; Karbat et al. 2006). This prompted Cohen and his colleagues to assess the contribution of residues of the toxin core to the binding by removing large segments of the N- and C-termini of the anti-insect and anti-mammalian β-toxins Bj-xtrIT and Css4 and analyzing the bioactivity of the residual truncated toxins. The truncated β-toxins (ΔΔBj-xtrIT and ΔΔCss4) were nontoxic upon injection and did not compete with the parental toxins on binding at receptor site 4, but were capable of modulating in an allosteric manner the binding and effects of site 3 scorpion α-toxins. While reducing the binding and effect of the scorpion α-toxin Lqh2 at mammalian Na_vs, they enhanced the binding and effect of LqhαIT at insect sodium channels. These results implied a novel bioactive surface at ΔΔBj-xtrIT and ΔΔCss4 that prior to these manipulations was hidden underneath the toxin exteriors. These results corroborated the suggestion that the toxins presently found in scorpion venom might have developed from smaller ancestor molecules (Froy and Gurevitz 1998; Cohen et al. 2008).

Putative Potential of Scorpion Toxins as Leads of Insecticides and Therapeutics

The ever-growing need to increase agricultural outputs and inevitable use of insecticides and herbicides and the low doses of scorpion toxins that affect insects with high selectivity (e.g., Froy et al. 1999; Strugatsky et al. 2005) have raised the potential of these toxins in insect pest control (Gordon 1997; Zlotkin 1999; Gurevitz et al. 2007). Utilization of a scorpion toxin or an engineered derivative as a drug or insecticide requires high efficiency, structural stability, amenability for genetic manipulation, and most importantly, specificity for a target Na_v. Fractionation of scorpion venom has illuminated toxins that show specificity for insect Na_vs (e.g., depressant and excitatory β-toxins), raising their potential as leads for design of insecticides (Gordon 1997; Zlotkin 1999; Gurevitz et al. 2007). Yet, realization of this potential depends on means developed for delivery of recombinant toxins to their receptor sites at Na_vs of insect pests, which in nature is simply mediated by stinging. A plant engineered to express the toxin not necessarily would be toxic to a chewing larva due to the extreme degrading environment in the guts and inability to penetrate through the peritrophic membrane into the hemolymph. These limitations require design that would, on the one hand, preserve activity and, on the other hand, acquire penetration capabilities upon ingestion. It seems, though, that simple formulation might not satisfy this requirement due to the harsh proteolytic conditions in insect guts. Evidently, the toxins should be modified so as to improve their bioavailability. Despite these difficulties there are a few examples of scorpion toxins used to acquire resistance to insect pests: ButaIT, a toxin from the scorpion *Mesobuthus tamulus,* was fused to the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), and the fusion was expressed in the yeast *Pichia pastoris*.

The insecticidal potential of this fusion had improved over just GNA upon feeding of larvae of the tomato moth *Lacanobia oleracea* or the rice brown planthopper *Nilaparvata lugens* (Trung et al. 2006). BmK IT, from *Mesobuthus martensii* Karsch, introduced into *Brassica napus* plants expressing in parallel a chitinase enzyme for digestion of the insect gut membrane, provided some resistance of the transgenic plants to insect pests (Wang et al. 2005).

A totally different approach was to exploit the natural ability of existing insect-selective vectors (e.g. baculoviruses) to penetrate into insects upon infection and engineer them with genes encoding a variety of different neurotoxins so that they would be expressed within the infected host (reviewed in Gurevitz et al. 2007). However, the use of engineered baculoviruses raises public concern over the risks to humans and livestock (Cory 2000), which led to a situation where peptide mimetics based on natural insect killers are preferred objectives sought by the industry. This approach requires, however, extensive study of the toxins and their face of interaction with the target Na_v. In the lack of target channel structure, a complete molecular description of toxin-channel interaction has been delayed and so was the potential use of scorpion toxins as therapeutics. However, there are examples where the therapeutic potential of a toxin has been realized prior to resolution of the structure of the target ion channel, such as Prialt (ziconotide), a naturally occurring peptide conotoxin from the cone snail *Conus magus*. The high selectivity of this toxin in blocking N-type calcium channels involved in excitatory neurotransmitter release from primary afferent nerve terminals enabled registration as a drug for treatment of pain (McIntosh et al. 1982; Miljanich 2004). Another heralded achievement was the engineering of ShK 192, a highly active analogue of the naturally occurring toxin peptide ShK-L5 from the sea anemone *Stichodactyla helianthus*, to blocking of $K_v1.3$ channels and raising a therapeutic potential in autoimmune diseases mediated by effector memory T cells (Pennington et al. 2009).

The recent reports on the structure of bacterial voltage-gated sodium channels (Payandeh et al. 2011; Zhang et al. 2012) give hope that it remains a matter of technological breakthrough before the structure of a heterotetrameric Na_v is solved, which would enable determination of fine details regarding toxin-channel interactions and design of antagonists to various channelopathies. The structure of the bacterial homotetrameric Na_v has shown great resemblance with the spatial organization of the potassium channel and revealed new molecular details about the channel pore domain and the mechanism that conducts selectively Na^+ ions. However, mostly due to the substantial differences between the external linkers in the bacterial over their much longer equivalents in eukaryotic Na_vs, the resolved structure of the bacterial channel not necessarily contributed to a better molecular description of receptor sites 3 and 4. Perhaps crystallization of chimeras comprising the membranous part of the bacterial channel carrying the external linkers of a eukaryotic sodium channel and able to bind toxins would illuminate the neurotoxin receptor sites at atomic resolution. Still, considering putative changes in toxin-channel interactions during gating, determination of the receptor sites of toxin modifiers should be performed with channels at various intermediary states

(e.g., Lacroix et al. 2012). In an alternative approach to structure determination, a few initiatives have been documented by measuring structural alterations in the channel utilizing bound fluorescent tags (e.g., Bezanilla 2000; Asamoah et al. 2003; Villalba-Galea et al. 2009) or monitoring changes in the voltage sensor and in the entire channel during gating using the accessibility of avidin to different-length tethered biotin reagents attached to the prokaryotic voltage-dependent potassium channel K_vAP (Ruta et al. 2005). In another experiment, the kinetics of voltage-sensor movements and gating was measured by immobilizing the voltage sensors using a bifunctional photo-cross-linking reagent attached to an introduced cysteine (Horn et al. 2000). Such measurements in the absence or in the presence of a toxin might illuminate its effects on channel dynamics during gating.

Cross-References

- ▶ Androctonus Toxins Targeting Voltage-Gated Sodium Channels
- ▶ Scorpion Venom Research Around the World: Chinese Scorpion *Mesobuthus martensii* Karsch

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Potassium Channel Blocking Peptide Toxins from Scorpion Venom

21

Adam Bartok, Gyorgy Panyi, and Zoltan Varga

Contents

Abstract

In the last three decades, numerous peptides isolated from scorpion venom have been identified as members of the KTx, or potassium channel-blocking group of toxins. This chapter provides an overview of the four families of KTx, named α, β, γ, and κ, discussing characteristic structural features and K^+ channel selectivity of these peptides. Methods of KTx peptide identification and isolation, as well as techniques for the assessment of the efficacy of potassium channel blockade, are described. With the advancement of molecular biology, molecular dynamics simulations, and nuclear magnetic resonance (NMR) techniques, many details of the toxin-channel interaction have been clarified and models of different modes of toxin binding have emerged. A table summarizing all currently known 133 members of the KTx group of peptides is presented, including their systematic and common names, along with their affinities for the major target K^+ channels, which may be in the low picomolar range.

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These peptides have provided vital information about the topology of the external pore region of K^+ channels highlighting similarities and even minute differences. In addition to being valuable exploratory molecular tools, peptide blockers of K^+ channels with high affinity and selectivity offer great potential for therapeutic use in a wide variety of diseases as was illustrated by several successful trials in animal models.

Introduction

The venom of scorpions contains a rich mixture of various compounds including many peptide components with a wide range of molecular weights. The biologically active constituents are often small peptide toxins that modulate the ion channels in the plasma membrane of a variety of cells. Some of these toxins alter the operation of Na^+-, Cl^--, or ryanodine-sensitive Ca^{2+} channels, but the largest and best-studied group consists of toxins that block K^+ channels (KTx).

Potassium channels represent the largest and most diverse ion channel type in the human organism with very wide tissue distribution and functional roles (Shieh et al. 2000). Peptide toxins that bind to specific K^+ channels have proven to be very valuable for two reasons:

1. They can be used as efficient molecular tools to learn about ion channel structure and function. The ability to examine how mutations in the toxin and/or channel affect the interaction offers great flexibility in the use of these peptides. Docking simulations with toxins of known structure make it possible to pinpoint minor structural differences in the topology of closely related channels, which may then explain observed functional differences between them. Blocking a certain subset of K^+ channels by high selectivity toxins can distinguish between the functions of similar channels expressed by the same cell.
2. Considering the enormous variety of the physiological and pathophysiological roles of K^+ channels and their cell-/tissue-specific expression distribution, they are attractive pharmacological targets in the therapy of several diseases. Successful experiments in animal disease models with potassium channel-blocking toxins have provided proof of concept for the feasibility of these efforts.

Primary Structure of KTxs

A large number of scorpion toxins have been identified by the isolation of mRNA from the venom gland. The reverse-transcribed cDNA sequences can be used to predict the amino acid sequences of the peptides expressed in the venom gland. These sequences are generally 50–60 amino acid-long precursor sequences containing signal peptides and other residues that are removed after translation. Many of such KTx peptides have not been tested on K^+ channels yet; however, all

long-chain peptides have putative or confirmed mature, short-chain derivatives in the α-KTx group. Other toxins were directly isolated from scorpion venoms and purified with high-performance liquid chromatography (HPLC) method, and their amino acid sequences were obtained by Edman degradation or MS/MS. In most of the cases, the amount of peptide isolated from the venom is not sufficient to determine the sequence, disulfide pairing, 3D structure, and the receptor specificity of the toxin. Knowing the amino acid sequences, toxins can be synthesized with recombinant techniques or chemical synthesis methods which allow the production of the peptides in large amount.

Secondary and Tertiary Structures and Classification of KTxs

The group of scorpion toxins targeting K^+ channels comprises short-chain (23–43 residues) and long-chain (42–84 residues) peptides, whose structure is stabilized by three or four disulfide bridges. At present, KTx toxins are classified into four families, α, β, γ, and κ, based on structural similarities and their specificities for various K^+ channels. Except for the κ-KTxs, all members of the other three families share a characteristic structural motif, called cysteine-stabilized α/β motif (CS-αβ), in which the α-helix is connected to a strand of the β-sheet (consisting of at least two strands, i.e., an αββ topology) by two disulfide bridges in C_i–C_j and C_{i+4}–C_{j+2} configuration. Although the CS-αβ-fold is a dominant structural feature among KTxs, it is not exclusive for this class of molecules, as peptides with different functions also share this motif (Dimarcq et al. 1998; Thomma et al. 2002; Caldwell et al. 1998; Zhao et al. 2002). Thus, the K^+ channel-blocking property should not be assumed solely based on the presence of this fold.

A highly conserved pair of residues, dubbed "the functional dyad" in many KTxs, was found to be important for high-affinity block of various K^+ channels. It consists of a lysine, whose positively charged side chain protrudes into the negatively charged environment of the selectivity filter of the potassium-conducting pore, and a hydrophobic (often aromatic) residue often situated nine positions downstream in the sequence, sterically separated by about 7 Å from the lysine. The dyad is found on the β-sheet side of the toxins. The dyad performs the same function even in toxins from sea anemone that have folds different from the signature CS-αβ. Besides the dyad, other residues also play crucial roles in forming the contact surface with the channel, thus determining selectivity (see below). Moreover, KTxs without the dyad that still block K^+ channels with high affinity have also been described, suggesting that the dyad is not an essential element for blockade (Batista et al. 2002).

Members of the KTx group of scorpion toxins that were discovered the earliest and shared high sequence and structural homology were classified into the α family, and the nomenclature α-KTxm.n was introduced to denote the *n*th member of the *m*th subfamily among the α-KTx toxins. The toxins included in the original classification were short (<40 residues) and contained six conserved cysteines. Since then the α-KTx family has vastly expanded, now including 133 members, and

ranging from 23 to 43 residues in size. Most of them have 3 disulfide bonds; however, all members of families 6, 12 (except 12.5 and 12.7), and 23 are stabilized by 4 disulfide bonds. α-KTx toxins are generally known to block *Shaker*-type Kv channels and Ca^{2+}-activated potassium channels.

Toxins of the β-KTx family are longer than the α-KTx toxins (47–84 residues stabilized by 3 disulfide bonds) and originate from the Buthidae, Caraboctonidae, and Scorpionidae families. These toxins contain two functionally different domains: a freely moving, possibly α-helical N-terminal segment, and a more compact cysteine-rich C-terminal segment that contains the signature CS-αβ structural motif. The N-terminal segment confers cytolytic activity to the toxin, while the C-terminal domain is responsible for K^+ channel-blocking ability. These toxins are further divided into three classes based on sequence similarity.

A separate family, γ-KTx, has been devoted to KTx toxins interacting with K^+ channels of the *ether-á-go-go*-related gene (ERG) family. γ-KTxs are all stabilized by four disulfide bonds except members 2.1 and 2.2. Their length ranges from 36 to 47 amino acids. The topography of the outer pore/turret region of the ERG family of channels is quite different from that of most other Kv channels; therefore, it is not surprising that a particular toxin is not likely to block both groups of channels. However, there has been one toxin, BmTx3, found, which belongs to subfamily α-KTx15 and yet blocks hERG channels (Huys et al. 2004a). A more detailed study revealed that two basic residues on the α-helix side of the toxin interact with the hERG channel, most likely with residues in the turret region, distant from the selectivity filter. On the other hand, BmTx3 also possesses the K_i-Y_{i+9} functional dyad on the β-sheet side, via which it was shown to block A-type K^+ currents, and thus was suggested to have two interaction surfaces, each acting on different channels. Interestingly, despite the presence of the dyad, the toxin does not block *Shaker*-type channels. Some of the γ-KTxs were found to be selective among human and rat ERG1, ERG2, and ERG3 channels, such as γ-KTx1.1, γ-KTx1.7, γ-KTx1.8, and γ-KTx2.1, which blocked these channel subtypes with varying affinities and therefore can be employed for the discrimination of these channels (Restano-Cassulini et al. 2006, 2008). There are currently 29 toxins that belong in the γ-KTx family.

The newest family of K^+ channel-blocking toxins from scorpion venom is the κ-KTx family of peptides consisting of 22–28 residues. They originate from scorpions in the Scorpionidae and Liochelidae families. Unlike members of the other KTx families, which are based on the CS-αβ scaffold, κ-KTx toxins adopt a structure that is formed by two parallel α-helices linked by two disulfide bridges. Although the presence of the functional dyad in hefutoxins, the first κ-KTxs to be described, and Om-toxins suggested that their targets would be K^+ channels, their affinities were found to be very low on the assayed channels. At present 18 κ-KTxs are listed in UniProt.

The systemic and common names of all currently known KTx toxins are listed in Table 1.

Table 1 List of KTx toxins isolated from scorpion venoms (main reference: http://www.uniprot.org/docs/scorpktx). The columns contain the Swiss-Prot accession number, the systemic and other names of the toxins, and the number of amino acids and disulfide bridges. The biological activity if determined is represented by the inhibition of a given channel (IC_{50} or Kd in brackets) if not stated otherwise (activation, cytotoxic effect, etc.). The abbreviated terms in the Method column indicate the principle of the measurement of the toxin-channel interaction (electrophysiology: voltage-clamp on insect or vertebrate cells; radio ligand: radioactive ligand binding assay; Xenopus: voltage-clamped Xenopus laevis oocytes; Rb^+ flux: measurement of radioactive Rb^+ efflux through K^+ channels).

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
P13487	α-KTx 1.1	Charybdotoxin, ChTX, ChTX-Lq1, ChTx-a	37	3	Kv1.2 (14 nM), Kv1.3 (2.6 nM) (Grissmer et al. 1994), Shaker (120 nM) (Goldstein et al. 1994), KCa1.1 (3 nM) (Meera et al. 2000), KCa3.1 (5 nM) (Wulff and Castle 2010)	electrophysiology
P45628	α-KTx 1.2	Charybdotoxin-2, ChTX-Lq2, ChTx-d	37	3	KCa (Lucchesi et al. 1989)	radio ligand
P24663	α-KTx 1.3	Iberiotoxin, IbTx	37	3	KCa1.1 (1 nM) (Meera et al. 2000)	*Xenopus*
P0C167	α-KTx 1.4	Limbatotoxin, LbTx, Limbatustoxin	37	3		
Q9NII6	α-KTx 1.5	Neurotoxin TX1, BmTX1	37	3	Kv1.3 (0.6–1.6 nM) (Romi-Lebrun et al. 1997a)	
Q9NII5	α-KTx 1.6	Neurotoxin TX2, BmTX2	37	3	Kv1.3 (0.6–1.6 nM) (Romi-Lebrun et al. 1997a)	
P45660	α-KTx 1.7	Toxin 15–1, Lqh 15-1	36	3		
	α-KTx 1.8	Reclassified as alpha-KTx 16.1				
P59848	α-KTx 1.9	Hongotoxin-2, HgTX2	36	3	Kv1.1 (30 pM) Kv1.2 (170 pM) Kv1.3 (86 pM) (Koschak et al. 1998)	Rb^+ flux

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
P83112	α-KTx 1.10	Parabutoxin-3, PBTx3	37	3	Kv1.1 (79 μM) Kv1.2 (547 nM) Kv1.3 (492 nM) (Huys et al. 2002)	*Xenopus*
P0C182	α-KTx 1.11	Slotoxin, SloTx	37	3	KCa1.1 (1.5 nM) (Garcia-Valdes et al. 2001)	*Xenopus*
P59943	α-KTx 1.12	Charybdotoxin b, ChTx-b	37	3		
P59944	α-KTx 1.13	Charybdotoxin c, ChTx-c	37	3		
H2ETQ6	α-KTx 1.14	Kcug1	37	3		
H2ER23	α-KTx 1.15	Kcug2	37	3		
P08815	α-KTx 2.1	Noxiustoxin, NTx, toxin II.11	39	3	Kv1.2 (2 nM), Kv1.3 (1 nM) (Grissmer et al. 1994)	electrophysiology
P40755	α-KTx 2.2	Margatoxin, MgTX	39	3	Kv1.1 (1.7–4.7 nM), Kv1.2 (6.4 pM), Kv1.3 (11.7 pM) (Bartok et al. 2014)	electrophysiology
P45629	α-KTx 2.3	Toxin-1, toxin I, toxin II.10.9.1, CllTx1	38	3	Rat brain K^+ channels (Martin et al. 1994)	radio ligand
Q9TXD1	α-KTx 2.4	Noxiustoxin-2, NTx2, NTx-2	38	3	Rat brain K^+ channels, bovine endothelial KCa (Nieto et al. 1996)	radio ligand, electrophysiology (KCa)
P59847	α-KTx 2.5	Hongotoxin-1, HgTX1	39	3	Kv1.1 (31 pM), Kv1.2 (170 pM), Kv1.3 (86 pM) (Koschak et al. 1998)	radio ligand
P59849	α-KTx 2.6	Hongotoxin-3, HgTX3	34 (partial sequence)			
P45630	α-KTx 2.7	Toxin II, toxin II.10.9.2, CllTx2	36	3	Rat brain K^+ channels (Martin et al. 1994)	radio ligand

P0C161	α-KTx 2.8	Toxin Ce1	39	3	Kv1.3 (0.71 nM) (Olamendi-Portugal et al. 2005)	electrophysiology
P0C162	α-KTx 2.9	Toxin Ce2	39	3	Kv1.3 (0.25 nM) (Olamendi-Portugal et al. 2005)	electrophysiology
P0C163	α-KTx 2.10	Toxin Ce3	38	3	Kv1.3 (366 nM) (Olamendi-Portugal et al. 2005)	electrophysiology
P0C164	α-KTx 2.11	Toxin Ce4	39	3	Kv1.3 (0.98 nM) (Olamendi-Portugal et al. 2005)	electrophysiology
P0C165	α-KTx 2.12	Toxin Ce5	39	3	Kv1.3 (69 nM) (Olamendi-Portugal et al. 2005)	electrophysiology
P85529	α-KTx 2.13	Toxin Css20	38	3	Kv1.2 (1.26 nM), Kv1.3 (7.1 nM) (Corzo et al. 2008)	electrophysiology
	α-KTx 2.14		37	3		
P24662	α-KTx 3.1	Kaliotoxin-1, KTX-1	38	3	Kv1.1 (0.1 nM), Kv1.2 (25 nM), Kv1.3 (1.5 nM) (Mourre et al. 1999), KCa (*H. pomatia* 2.5 nM) (Romi et al. 1993)	*Xenopus*/*Helix pomatia*
P46111	α-KTx 3.2	Agitoxin-2, AGTX-2, AgTx2	38	3	Kv1.1 (44 pM), Kv1.3 (4 pM), Kv1.6 (37 pM), Shaker (640 pM) (Garcia et al. 1994)	electrophysiology
P46112	α-KTx 3.3	Agitoxin-3, AGTX-3, AgTx3	38	3	Shaker 0.64 nM (Garcia et al. 1994)	*Xenopus*
P46110	α-KTx 3.4	Agitoxin-1, AGTX-1, AgTx1, leiurotoxin-2, leiurotoxin II, LeTx II	38	3	Kv1.1 (136 nM), Kv1.3 (41.7 nM), Kv1.6 (149 nM), Shaker (0.16 nM) (Garcia et al. 1994)	electrophysiology
P45696	α-KTx 3.5	Kaliotoxin-2, KTX-2	37	3	Rat brain K^+ channels, KCa (*H. pomatia*) (Laraba-Djebari et al. 1994)	radio ligand, electrophysiology

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
Q NII79	α-KTx 3.6	Kaliotoxin-1, KTX-1	38	3	Kv1.1 (203 pM), Kv1.2 (8.9 nM), Kv1.3 (171 pM) (Gao et al. 2010)	electrophysiology
P55896	α-KTx 3.7	Toxin OsK1, OsK-1	38	3	Kv1.1 (0.6 nM), Kv1.2 (5.4 nM), Kv1.3 (14 pM), KCa3.1 (225 nM) (Mouhat et al. 2005)	electrophysiology
P59886	α-KTx 3.8	Charybdotoxin-like peptide Bs 6, Bs6	38	3		
P59290	α-KTx 3.9	Kaliotoxin-3, KTX-3	37	3	Rat brain K^+ channels (Meki et al. 2000)	radio ligand
P0C908	α-KTx 3.10	BoiTx1	37	3	Shaker (3.5 nM) (Kozminsky-Atias et al. 2007)	electrophysiology
P0C909	α-KTx 3.11	OdK2	38	3	Kv1.3 (7.2 nM) (Abdel-Mottaleb et al. 2008)	*Xenopus*
P0C8R1	α-KTx 3.12	Kaliotoxin analog, Aam-KTX	38	3	Kv1.2 (10.4 nM), Kv1.3 (1.1 nM) (Abbas et al. 2008)	*Xenopus*
P46114	α-KTx 4.1	Tityustoxin K-alpha, TsTX-K-alpha, TSK4, toxin II-9, Ts7	37	3	Rat brain K^+ channels (Rogowski et al. 1994)	Rb^+ flux
P56219	α-KTx 4.2	Neurotoxin Ts-kappa, TsKapa, TsK	35	3	Rat brain K^+ channels (Legros et al. 1996)	radio ligand
P59925	α-KTx 4.3	Toxin TdK1	37	3	Shaker (280 nM) (D'Suze et al. 1999)	electrophysiology
P60210	α-KTx 4.4	Toxin Tc30	37	3	Kv1.3 (16 nM), Shaker (4.7 μM) (Batista et al. 2002)	electrophysiology
Q5G8B6	α-KTx 4.5		37	3		

P0CB56	α-KTx 4.6	Tst23	37	3	Kv1.2 (19 nM), Kv1.3 (10.7 nM) (Papp et al. 2009)	electrophysiology
P16341	α-KTx 5.1	Leiurotoxin-1, leiurotoxin I, LeTx I, scyllatoxin, ScyTx	31	3	Rat brain K^+ channels (Auguste et al. 1990)	radio ligand
P31719	α-KTx 5.2	Leiurotoxin I-like toxin P05, AmP05	31	3	Rat brain K^+ channels (Sabatier et al. 1993)	radio ligand
Q9TVX3	α-KTx 5.3	Neurotoxin BmP05, potassium ion channel blocker P05	31	3	Rat brain K^+ channels (Romi-Lebrun et al. 1997b)	radio ligand
P59869	α-KTx 5.4	Tamapin	31	3	Rat brain K^+ channels (Pedarzani et al. 2002)	radio ligand
P59870	α-KTx 5.5	Tamapin-2	31	3	Rat brain K^+ channels (Pedarzani et al. 2002)	radio ligand
Q10726	α-KTx 6.1	Potassium channel-blocking toxin 1, Pi1, Pi-1, PiTX-K-gamma	35	4	Rat brain K^+ channels, Kv1.2 (1.3 nM) (Mouhat et al. 2004), Kv1.3 (11.7 nM) (Peter et al. 2000), Shaker (32 nM) (Gomez-Lagunas et al. 1997)	radio ligand, electrophysiology
P80719	α-KTx 6.2	Maurotoxin, MTX	34	4	Kv1.1 (45 nM), Kv1.2 (0.8 nM), Kv1.3 (180 nM) (Kharrat et al. 1997), KCa3.1 (14 nM) (Castle et al. 2003)	*Xenopus*/Rb^+ flux
P59867	α-KTx 6.3	Neurotoxin HsTX1	34	4	Kv1.1 (7 nM) (Regaya et al. 2004), Kv1.1 (12.5 pM) (Lebrun et al. 1997)	electrophysiology
P58498	α-KTx 6.4	Potassium channel-blocking toxin 4, Pi4, Pi-4	38	4	Kv1.2 (8 pM) (M'Barek et al. 2003), Shaker (8 nM) (Olamendi-Portugal et al. 1998)	electrophysiology
P58490	α-KTx 6.5	Pi7, Pi-7, toxin-7	38	4		
Q6XLL9	α-KTx 6.6	OcKTx1	37	4		
Q6XLL8	α-KTx 6.7	OcKTx2	37	4		

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
Q6XLL7	α-KTx 6.8	OcKTx3	37	4		
Q6XLL6	α-KTx 6.9	OcKTx4	38	4		
Q6XLL5	α-KTx 6.10	OcKTx5	37	4		
P0C194	α-KTx 6.11	Male-specific potassium channel inhibitor IsTX	41	4		
P0C166	α-KTx 6.12	Anuroctoxin	35	4	Kv1.2 (6.14 nM), Kv1.3 (0.73 nM) (Bagdany et al. 2005)	electrophysiology
P84094	α-KTx 6.13	Spinoxin	34	4		
P84864	α-KTx 6.14	HgeTx1	36	4	Shaker (52 nM) (Schwartz et al. 2006)	electrophysiology
P85528	α-KTx 6.15	Hemitoxin	35	4	Kv1.1 (13 nM), Kv1.2 (16 nM), Kv1.3 (2 nM) (Srairi-Abid et al. 2008)	electrophysiology
C5J896	α-KTx 6.16	OcyC12	43	4		
P86116	α-KTx 6.17	Toxin OcyKTx2	34	4	Kv1.3 (18 nM), Shaker (52 nM) (Schwartz et al. 2013)	electrophysiology
	α-KTx 6.21	Urotoxin	37	4	Kv1.1 (253 nM), Kv1.2 (160 pM), Kv1.3 (91 nM), KCa3.1 (70 nM) (Luna-Ramirez et al. 2014)	electrophysiology
P55927	α-KTx 7.1	Toxin PiTX-K-alpha, pandinotoxin-alpha, potassium channel-blocking toxin 2, Pi2, Pi-2	35	3	Kv1.2 (32 pM) (Rogowski et al. 1996), Kv1.3 (44 pM) (Peter et al. 2001), Shaker (8.2 nM) (Gomez-Lagunas et al. 1996)	electrophysiology

P55928	α-KTx 7.2	Toxin PiTX-K-beta, pandinotoxin-beta, potassium channel-blocking toxin 3, Pi3, Pi-3	35	3	Kv1.3 (795 pM) (Peter et al. 2001), Shaker (140 nM) (Gomez-Lagunas et al. 1996)	electrophysiology
P56215	α-KTx 8.1	Neurotoxin P01, AmP01	29	3	Rat brain K^+ channels (Zerrouk et al. 1996)	radio ligand
Q9U8D2	α-KTx 8.2	Neurotoxin BmP01, potassium ion channel blocker P01	29	3	Rat brain K^+ channels (Romi-Lebrun et al. 1997b)	radio ligand
P80670	α-KTx 8.3	Toxin GaTx2, gating modifier of anion channels 2, leiuropeptide II, LpII, leiuropeptide-2	29	3	CIC-2 Cl^- channels (20 pM) (Thompson et al. 2009)	electrophysiology
P80671	α-KTx 8.4	Leiuropeptide-3, leiuropeptide III, LpIII	29	3		
P0CC12	α-KTx 8.5	OdK1	29	3	Kv1.2 (183 nM) (Abdel-Mottaleb et al. 2006)	electrophysiology
Q9NJP7	α-KTx 9.1	Neurotoxin BmP02, potassium ion channel blocker P02, toxin Kk6, BmKK6	28	3	Rat brain K^+ channels (Romi-Lebrun et al. 1997b)	radio ligand
Q9U8D1	α-KTx 9.2	Neurotoxin BmP03, potassium ion channel blocker P03	28	3	Rat brain K^+ channels (Romi-Lebrun et al. 1997b)	radio ligand
P80669	α-KTx 9.3	Leiuropeptide-1, leiuropeptide I, LpI	28	3		
P60209	α-KTx 9.4	Toxin BTK-2, Bt BTK-2	32	3	Kv1.1 (4.6 μM) (Dhawan et al. 2003)	electrophysiology
P84744	α-KTx 9.5	Neurotoxin Kbot1	28	3	Kv1.1 (145 nM), Kv1.2 (2.5 nM), Kv1.3 (15 nM), rat brain K^+ channels (Mahjoubi-Boubaker et al. 2004)	*Xenopus*/radio ligand

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
O46028	α-KTx 10.1	Cobatoxin-1, CoTx1, gtIX	32	3	Kv1.1 (24 μM), Kv1.2 (27 nM), Kv1.3 (5.3 μM), Shaker (1 μM), KCa3.1 (7.5 μM) (Jouirou et al. 2004), rat brain K^+ channels (Selisko et al. 1998)	electrophysiology
P58504	α-KTx 10.2	Cobatoxin-2, CoTx2	32	3	Kv1.1 (1 μM), Shaker (4.1 μM), rat brain K^+ channels (Selisko et al. 1998)	electrophysiology
P60164	α-KTx 11.1	Parabutoxin-1, PBTx1	37	3	Kv1.1 (150 nM) (Huys et al. 2004b)	electrophysiology
P60165	α-KTx 11.2	Parabutoxin-2, PBTx2	37	3	Kv1.1 (1 μM) (Huys et al. 2004b)	electrophysiology
Q6WGI9	α-KTx 11.3	Parabutoxin-10, PBTx10	36	3	Kv1.1 (1 μM) (Huys et al. 2004b)	electrophysiology
P59936	α-KTx 12.1	Butantoxin, BuTX, TsTX-IV, Ts6	40	4	KCa (mouse) (Novello et al. 1999)	electrophysiology
P0C168	α-KTx 12.2	Butantoxin, BuTX, TtBut	40	4	Shaker (660 nM) (Coronas et al. 2003)	electrophysiology
P0C185	α-KTx 12.3	Butantoxin-like peptide, Tco30	40	4		
P0C8L1	α-KTx 12.4	Butantoxin, BuTX, TstBut	40	4		
P0CH12	α-KTx 12.5	Neurotoxin KTx10	38	3	Kv1.1 (1.7 μM), Kv1.2 (12.6 μM), Kv1.3 (28 nM) (Liu et al. 2009)	electrophysiology
P0CI47	α-KTx 12.6		43	4		
P0CI48	α-KTx 12.7		38	3		
P83243	α-KTx 13.1	Toxin Tc1	23	3	Shaker (65 nM), mouse brain K^+ channels (Batista et al. 2000)	electrophysiology/ radio ligand

P83244	α-KTx 13.2	Toxin OsK2, OsK-2	28	3	Kv1.2 (97 nM) (Dudina et al. 2001)	electrophysiology
P84630	α-KTx 13.3	Toxin Tpa1	23	3	Shaker (200 nM) (Barona et al. 2006)	electrophysiology
P0C8L2	α-KTx 13.4	Toxin Tst-17	23	3	Shaker (3 μM) (Batista et al. 2007)	electrophysiology
Q967F9	α-KTx 14.1	Toxin Kk1, BmKK1	31	3		
Q95NK7	α-KTx 14.2	Toxin Kk2, BmKK2, BmTXKS3, neurotoxin BmP07, potassium ion channel blocker P07, KK1	31	3		
Q9BJX2	α-KTx 14.3	Toxin Kk3, BmKK3, neurotoxin SKTx2	31	3		
Q9BKB4	α-KTx 14.4	Neurotoxin SKTx1, BmSKTx1	31	3		
P60233	α-KTx 15.1	Peptide Aa1	37	3	Rat brain K^+ channels (Pisciotta et al. 2000)	electrophysiology
Q8I0L5	α-KTx 15.2	Toxin BmTX3, neurotoxin TX3, BmTX3A	37	3	hERG (1.9 μM) (Huys et al. 2004a), rat brain K^+ channels (Vacher et al. 2001)	electrophysiology
P60208	α-KTx 15.3	Toxin AmmTX3	37	3	Rat brain K^+ channels (Vacher et al. 2002)	electrophysiology
Q867F4	α-KTx 15.4	Toxin AaTX1, toxin Aa1	37	3	Rat brain K^+ channels (Pisciotta et al. 2000)	electrophysiology
Q86SD8	α-KTx 15.5	Toxin AaTX2	37	3		
P84777	α-KTx 15.6	Discrepin	38	3	Rat brain K^+ channels (D'Suze et al. 2004)	electrophysiology
Q5K0E0	α-KTx 15.7	Neurotoxin AamTX	37	3		
Q86BX0	α-KTx 15.8	Neurotoxin Kk4, BmKKx1	38	3		

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
D9U2A8	α-KTx 15.9	Neurotoxin KTx9	38	3		
P0C173	α-KTx 16.1	Tamulotoxin, TmTX	36	3		
Q9NBG9	α-KTx 16.2	Martentoxin, BmK 622, BmTx3B	37	3	KCa1.1 (80 nM) (Shi et al. 2008)	electrophysiology
Q8MQL0	α-KTx 16.3	Charybdotoxin-like toxin 1, Kchtx1, KTX1	37	3		
Q95NJ8	α-KTx 17.1	Toxin Kk4, BmKK4, toxin TXKs4	30	3	Rat brain K^+ channels (Li et al. 2003)	electrophysiology
P0CI46	α-KTx 17.2		31	3		
P60211	α-KTx 18.1	Toxin Tc32	35	3	Kv1.3 (10 nM), Shaker (74 nM) (Batista et al. 2002)	electrophysiology
P0C1X5	α-KTx 18.2	Toxin TdK2	34	3		
P0C1X6	α-KTx 18.3	Toxin TdK3	36	3		
P83407	α-KTx 19.1	Neurotoxin BmBKTx1, BmK37	31	3	Insect BK channels (Xu et al. 2004)	electrophysiology
P0C183	α-KTx 20.1	Toxin Tt28	29	3		
P86270	α-KTx 21.1	Tityustoxin-15, TS15	36	3	Kv1.2 (196 nM), Kv1.3 (508 nM) (Cologna et al. 2011)	electrophysiology
Q8MUB1	α-KTx 22.1	Toxin Kcugx, neurotoxin BmK38	40	3		
P0DJ31	α-KTx 23.1	Toxin Vm24, toxin alpha-KTx 21.1	36	4	Kv1.1 (30–40 nM), Kv1.2 (5–10 nM), Kv1.3 (2.9 pM), KCa3.1 (14–30 nM) (Varga et al. 2012)	electrophysiology
P0DJ32	α-KTx 23.2	Toxin Vm23, toxin alpha-KTx 21.2	35	4		

A7KJJ7	α-KTx 26.1	Neurotoxin BmK86	35	3	Kv1.3 (150 nM) (Mao et al. 2007)	electrophysiology
D9U2B2	α-KTx 26.2		39	3		
R4GUQ3	α-KTx 28.1	Toxin ImKTx104	27	3	Kv7.1 (11.7 μM) (Chen et al. 2012)	electrophysiology
D9U2A6	α-KTx 29.1	Neurotoxin-F, toxin LmKTx2	32	3		
R4GUQ1	α-KTx 29.2	Neurotoxin KTx3, toxin LmKTx71	32	3		
P0CI86	α-KTx 29.3	Neurotoxin-E, neurotoxin LmKTx95	32	3		
P0DL33	α-KTx 30.1	Toxin StKTx23	42	3	Kv1.3 (>1 μM) (Chen et al. 2012)	electrophysiology
P0DL34	α-KTx 30.2	Toxin SjKTx32	42	3		
P0DL35	α-KTx 30.3	Toxin SjKTx51	42	3		
β-KTx class 1 subfamily						
P69939		AaTXK-beta, AaTXKbeta, beta-KTx 2	64	3	Activation of Kv7.x channels (Landoulsi et al. 2013)	electrophysiology
B8XH40		BuTXK-beta, BuTXKbeta, Tx690	64	3		
Q9N661		BmTXK-beta-2, BmTX K-beta2, BmTXKbeta2, BmTX K beta2′, beta-KTx 4	64	3		
Q5G8A6		Tco-beta-KTx, TcobetaKTx, Tco 41.46-2, Tco 42.14	60	3		
Q0GY44		Tdi-beta-KTx, TdibetaKTx	60	3		
P69940		TsTXK-beta, tityustoxin K-beta, TsTX-K beta, TsTX K beta, TsTXKbeta, TSK2, beta-KTx 1	60	3	Rat brain K^+ channels (Rogowski et al. 1994)	Rb^+ flux

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
P0C2F3		Tst-beta-KTx, TstbetaKTx	60	3	Kv1.1 (96 nM) (Diego-Garcia et al. 2008)	electrophysiology
Q0GY46		Ttr-beta-KTx, TtrbetaKTx	60	3		
P0CI49		Neurotoxin beta-KTx 31.1	67	3		
A9XE60		MeuTXK-beta-1, MeuTXKbeta1	72	3		
A9XE59		MeuTXK-beta-2, MeuTXKbeta2	72	3		
D9U2A7		Neurotoxin beta-KTx 7	67	3		
β-KTx class 2 subfamily						
Q0GY41		Hge-beta-KTx, HgebetaKTx	58	3	Cytolytic (Diego-Garcia et al. 2008)	electrophysiology
P0CH57		MeuTXKbeta3, BeL-5, BeL-69, Meucin-24, MeuTXKbeta3	47	3		
Q9NJC6		BmTXK-beta, BmTX K-beta, BmTXKbeta, BmKLK, beta-KTx 3	61	3	Rabbit myocyte K^+ channels (Cao et al. 2003)	electrophysiology
Q0GY42		TcoKIK	47	3		
Q0GY43		TdiKIK, TdKIK	47	3		
P0C8W4		TstKMK, toxin 5536	47	3		
P86822		Beta-Ktx 2	64	3		
Q0GY45		TtrKIK	47	3		
P0CJ45		Neurotoxin beta-KTx 52.1	50	3		
D9U2B1		Neurotoxin beta-KTx 12	50	3		
P0CI42		Neurotoxin beta-KTx 14.3	48	3		
C6ZH27		Neurotoxin beta-KTx 17	48	3		

β-KTx class 3 subfamily						
Q0GY40		Hge-scorpine, Hg-scorpine-like 1, Hgscplike1, HgeScplp1, Hge36	48	3	HGE36 fragment on Kv1.1 (158 nM), cytolytic (Diego-Garcia et al. 2008)	electrophysiology
P0C2F4		Heteroscorpine-1, HS-1	76	3	Antibacterial (Uawonggul et al. 2007)	
C5J891		Scorpine-like, OcyC7	53	3		
P56972		Scorpine, scorpin, panscorpine	75	3	Antimicrobial (*Plasmodium*) (Conde et al. 2000)	
P86121		Scorpine-like	15	0		
Q5WR03		Opiscorpine-1	76	3	Antimicrobial (Zhu and Tytgat 2004)	
P0C8W5		Hg-scorpine-like 2, Hgscplike2, HgeScplp2	84	3		
Q5WR01		Opiscorpine-2	76	3		
Q5WQZ7		Opiscorpine-3	76	3		
Q5WQZ9		Opiscorpine-4	76	3		
Q86QT3	γ-KTx 1.1	Ergtoxin, ErgTx, ergtoxin-like protein 1, ErgTx1, CnErg1, CnErgTx1	42	4	hERG (8.5 nM) (Torres et al. 2003)	electrophysiology
Q86QV6	γ-KTx 1.2	Ergtoxin-like protein 1, ErgTx1, CeErg1, CeErgTx1	42	4		
Q86QV3	γ-KTx 1.3	Ergtoxin-like protein 1, ErgTx1, CgErg1, CgErgTx1	42	4		
Q86QU6	γ-KTx 1.4	Ergtoxin-like protein 1, ErgTx1, CsErg1, CsErgTx1	42	4		
Q86QV0	γ-KTx 1.5	Ergtoxin-like protein 1, ErgTx1, CllErg1, CllErgTx1	42	4		

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
Q86QU1	γ-KTx 1.6	Ergtoxin-like protein 1, ErgTx1, CexErg1, CexErgTx1	42	4		
P0C892	γ-KTx 1.7	CeErg4	42	4	hERG1 (12.8 nM), hERG3 (0.88 nM) (Restano-Cassulini et al. 2008)	electrophysiology
P0C893	γ-KTx 1.8	CeErg5	42	4	Similar effect to gamma-KTx 1.7 (Restano-Cassulini et al. 2008)	electrophysiology
Q9BKB7	γ-KTx 2.1	Neurotoxin BeKm-1	36	3	hERG (3.3 nM) (Korolkova et al. 2001)	electrophysiology
P59938	γ-KTx 2.2	Neurotoxin Kk7, BmKK7, BmKKx2	36	3		
P59939	γ-KTx 3.1	Ergtoxin-like protein 2, ErgTx2, CnErg2, CnErgTx2	43	4		
Q86QV5	γ-KTx 3.2	Ergtoxin-like protein 2, ErgTx2, CeErg2, CeErgTx2	43	4		
Q86QU5	γ-KTx 3.3	Ergtoxin-like protein 2, ErgTx2, CsErg2, CsErgTx2	43	4		
Q86QV2	γ-KTx 3.4	Ergtoxin-like protein 2, ErgTx2, CgErg2, CgErgTx2	42	4		
Q86QU9	γ-KTx 4.1	Ergtoxin-like protein 2, ErgTx2, ClIErg2, ClIErgTx2	43	4		
Q86QV7	γ-KTx 4.2	Ergtoxin-like protein 5, ErgTx5, CnErg5, CnErgTx5	43	4		

Q86QU0	γ-KTx 4.3	Ergtoxin-like protein 2, ErgTx2, CexErg2, CexErgTx2	43	4		
Q86QT9	γ-KTx 4.4	Ergtoxin-like protein 3, ErgTx3, CexErg3, CexErgTx3	43	4		
Q86QT8	γ-KTx 4.5	Ergtoxin-like protein 4, ErgTx4, CexErg4, CexErgTx4	43	4		
Q86QU8	γ-KTx 4.6	Ergtoxin-like protein 3, ErgTx3, CllErg3, CllErgTx3	43	4		
Q86QU7	γ-KTx 4.7	Ergtoxin-like protein 4, ErgTx4, CllErg4, CllErgTx4	43	4		
Q86QV4	γ-KTx 4.8	Ergtoxin-like protein 3, ErgTx3, CeErg3, CeErgTx3	43	4		
Q86QU4	γ-KTx 4.9	Ergtoxin-like protein 3, ErgTx3, CsErg3, CsErgTx3	43	4		
Q86QU3	γ-KTx 4.10	Ergtoxin-like protein 4, ErgTx4, CsErg4, CsErgTx4	43	4		
Q86QV8	γ-KTx 4.11	Ergtoxin-like protein 4, ErgTx4, CnErg4, CnErgTx4	43	4		
P59940	γ-KTx 4.12	Neurotoxin CsEKerg1	43	4	hERG (232 nM) (Nastainczyk et al. 2002)	electrophysiology
Q86QV9	γ-KTx 4.13	Ergtoxin-like protein 3, ErgTx3, CnErg3, CnErgTx3	43	4		
Q86QU2	γ-KTx 5.1	Ergtoxin-like protein 5, ErgTx5, CsErg5, CsErgTx5	47	4		
Q86QV1	γ-KTx 5.2	Ergtoxin-like protein 3, ErgTx3, CgErg3, CgErgTx3	47	4		

(*continued*)

Table 1 (continued)

Swiss-Prot accession	Systematic name	Other names	Length (amino acids)	Disulfide bonds	Biological effect	Method
P82850	κ-KTx 1.1	Kappa-hefutoxin-1, Kappa-HfTx1	22	2	Kv1.2 (150 μM), Kv1.3 (40 μM) (Srinivasan et al. 2002)	electrophysiology
P82851	κ-KTx 1.2	Kappa-hefutoxin-2, Kappa-HfTx2	23	2		
P83655	κ-KTx 1.3		23	2		
P0DJ33	κ-KTx 1.4	HSP009C	23	2		
P0C1Z3	κ-KTx 2.1	Toxin OmTx1	26	2		
P0C1Z3	κ-KTx 2.2	Toxin OmTx2	27	2		
P0C1Z4	κ-KTx 2.3	Toxin OmTx3	23	2		
P0C1Z3	κ-KTx 2.4	Toxin OmTx4	25	2		
P86110	κ-KTx 2.5	OcyC8, OcyKTx6	28	2	Kv1.1 (217 μM), Kv1.4 (71 μM) (Camargos et al. 2011)	electrophysiology
C5J893	κ-KTx 2.6	OcyC9	24	2		
P0DJ34	κ-KTx 2.7	HSP053C.1, toxin HeTx203, toxin kappa-KTx 2.6	24	2		
P0DJ35	κ-KTx 2.8	HSP053C.2, toxin HeTx204, toxin kappa-KTx 2.7	24	2		
P0DJ36	κ-KTx 3.1	HSP040C.1	27	2		
P0DJ37	κ-KTx 3.2	HSP040C.3	25	2		
P0DJ38	κ-KTx 3.3	HSP040C.4	25	2		
P0DJ39	κ-KTx 3.4	HSP040C.5	27	2		
P0DJ40	κ-KTx 4.1	HSP040C.2	27	2		
P0DJ41	κ-KTx 5.1	HelaTx1	27	2	Kv1.1 (9.9 μM), Kv1.6 (approx. 10 μM) (Vandendriessche et al. 2012)	electrophysiology

Characterization of Toxin-Channel Interactions, Mechanism of Block

To test the affinity of a given peptide to its receptors, several methods are available. At the time of the isolation of the first scorpion toxins (noxiustoxin in 1982 (Carbone et al. 1982)), the availability of cloned ion channel genes was limited. The first ion channel gene cloned in 1982 was the nicotinic acetylcholine receptor (nAChR) of the torpedo ray (Noda et al. 1982) followed by the voltage-gated sodium channel of electric eel in 1984 (Noda et al. 1984). Therefore, the general way to test the efficiency of a peptide in inhibiting K^+ channels was to isolate excitable cells generally from rat nervous system and measure the effect of the test substance on the endogenously expressed channels. Toxin-K^+ channel interactions can be tested on the fast-inactivating A-type current of these cells, which is generated by Kv1.4, Kv3.4, Kv4.1, Kv4.2, and Kv4.3 α-subunits (Vacher et al. 2004; Song et al. 1998; Song 2002) or on delayed-rectifier currents of Kv1.1, Kv1.2, Kv1.5, Kv1.6, Kv2.1, Kv3.1, and Kv3.2 channels (Song 2002). These cells also express Ca^{2+}-activated K^+ channels which makes them suitable to test the inhibitory effect of the toxins on small conductance (SK) channels (Legros et al. 1996; Jouirou et al. 2004). Other primary cell cultures were also used for testing, such as bovine aortic endothelial cells (Nieto et al. 1996) or neurons from snail (Laraba-Djebari et al. 1994) or rabbit (Crest et al. 1992).

After the cloning of individual ion channel genes and the application of heterologous expression systems in *Xenopus* oocytes, insect, or mammalian cells, more precise methods became available to determine the receptors of the toxins (Schwartz et al. 2013; Varga et al. 2012; Lebrun et al. 1997). Measurements can be done by radiography or with electrophysiological methods. Radiography methods can be direct or indirect. Direct measurements require the radioactive labeling (in most of the cases, ^{125}I) of the toxin which may alter the receptor specificity of the labeled toxin compared to the unlabeled form (Koch et al. 1997). Indirect assays are based on the competition of the test substance with a well-characterized radioactive labeled ligand (such as ^{125}I apamin or ^{125}I noxiustoxin) for the binding site (Legros et al. 1996; Pedarzani et al. 2002). The disadvantage of such measurements is that they measure the association and dissociation of the peptides to the targeted receptors at any contact surface. Kd (dissociation constant) values in such measurements do not necessarily represent the pore-blocking ability or the dose dependence of the inhibition of the ionic flux through the channels (IC_{50}). Determination of the radioactive $^{86}Rb^+$ flux is another general tool to test the K^+ channel inhibiting ability. Cells expressing voltage-gated K^+ channels are loaded with $^{86}Rb^+$ and then depolarized by high K^+-containing extracellular solution. $^{86}Rb^+$ flows through open K^+ channels and amount of extracellular $^{86}Rb^+$ can be determined with scintillation counter. Inhibitors of potassium channels decrease the $^{86}Rb^+$ flux in a dose-dependent manner; therefore, the half-inhibiting concentration (IC_{50}) with such method can be determined (Bartschat and Blaustein 1985; Koschak et al. 1998). Electrophysiological methods permit the direct measurement of ionic currents through voltage-clamped

membranes. For these measurements a variety of different cells or membrane preparations can be used. Primary cell lines (neurons, lymphocytes, etc.) expressing specific ion channels endogenously are widely used for the measurements (Schwartz et al. 2013; Varga et al. 2012; Vacher et al. 2001). Recombinant techniques allow the expression of specific ion channels in various cell types (*Xenopus* oocytes, mammalian cells, etc.) which has the advantage of measuring specific inhibitory effect of a toxin on a given ion channel with very low probability of aspecific effect due to the absence of endogenously expressed channels (Schwartz et al. 2013; Bagdany et al. 2005; Romi-Lebrun et al. 1997a).

The receptor site for KTxs is the K^+ channel pore; competition experiments confirmed that the toxins bind to a region that overlaps with the tetraethylammonium (TEA) binding site at the external entrance of the pore and that only a single peptide molecule is able to occupy the binding site at a given time (Varga et al. 2012; Miller 1988).

The relatively small size of KTxs enables them to deeply enter the vestibule of the channels allowing for multiple contact points and also exposes the majority of their residues, which results in highly variable interaction surfaces even due to minor changes in the sequence. These features enable the toxins to bind to channel surfaces in various orientations. There have been three major modes of interaction described between K^+ channels and KTxs. The most frequently identified and best-characterized interaction is via the functional dyad described above (Fig. 1a). In these cases the β-sheet side of the toxin faces the entrance of the channel pore and the lysine side chain in the selectivity filter, and the hydrophobic interaction of the other dyad residue mostly accounts for the high-affinity binding.

A different mode of interaction was described between KCa2.x channels and α-KTx4.2 and members of the α-KTx5 subfamily (Rodriguez de la Vega et al. 2003) (Fig. 1b). In these instances influential residues were localized on the α-helix side of the toxins. Two arginines (for TSκ (α-KTx4.2)) and three arginines (for P05 (α-KTx5.3)) were identified as critical for binding to small conductance calcium-activated potassium channels that made contacts with channel residues on the bottom of the vestibule and the turret region. Thus, compared to typical α-KTx-Kv channel interactions, the contact region is on the opposite side of the toxins and farther away from the selectivity filter.

Members of the γ-KTx family seem to employ yet another way to bind to hERG channels. As described above for BmTx3, γ-KTxs most likely bind to extracellular segments of the extended S5–S6 linker in ERG channels, which may form an extra amphipathic α-helix (Fig. 1c). As exemplified by ErgTx (Pardo-Lopez et al. 2002), this mode of block differs in several respects from the typical α-KTx mode of block, whose characteristics are mainly defined by the critical lysine's interaction with the selectivity filter. Due to the deep penetration of the lysine side chain, it not only interacts with potassium ions in the pore but also senses the electric field, which makes this mode of block by α-KTX sensitive to external K^+ concentration and to the applied membrane voltage. Since γ-KTxs lack the equivalent of the lysine and thus do not interact directly with the pore, the block is insensitive to K^+_{ext}, but not the membrane potential. This was explained by structural rearrangements in the

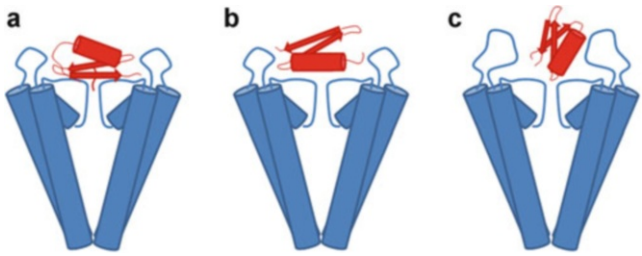

Fig. 1 (**a**) Typical blocking scheme of an α-KTx in the pore of a *Shaker*-related Kv channel. The interaction surface is on the β-sheet side of the toxin forming several close contacts with the bottom of the vestibule, and the side chain of the critical lysine protrudes deeply into the selectivity filter. (**b**) Block of the KCa2.2 (SK) channel by α-KTx5.3 occurs by an inverted orientation of the toxin compared to the typical α-KTx mechanism; the main interaction surface is on the α-helical side of the toxin. Channel residues involved in the interaction are localized in the turret region and the bottom of the vestibule. Brownian dynamics of the recognition of the scorpion toxin P05 with small-conductance calcium-activated potassium channels. (**c**) Interaction of γ-KTx2.1 with the HERG channel occurs mainly between the α-helix of the toxin and the large turret region of the channel. The toxin does not enter very deeply and does not fully block permeation

S5-P linker brought on by strong depolarization, which destabilizes ErgTx binding. Although the overlap of the ErgTx binding site with that of TEA places it at the outer mouth of hERG, the inability of ErgTx to produce total current block suggests an off-center binding position rather than a complete plugging of the pore as known for α-KTxs. γ-KTxs are assumed to bind with their α-helix side in an orientation different from the two previous modes and interact with residues even farther from the selectivity filter (Rodriguez de la Vega et al. 2003; Pardo-Lopez et al. 2002).

The block mechanism of the KTxs has been studied by several methods, the earliest ones involving a large number of mutations both in the toxin and channel sequences. The structure of the toxins was fairly well known from NMR studies (Bontems et al. 1991, 1992), and based on geometric constraints, useful conclusions could be drawn about the topology of the outer pore region of the channels. Using conservative and nonconservative mutations and measuring the binding affinities, the most influential residues were identified (Goldstein et al. 1994). Most KTxs carry a high net positive charge and thus are likely to be attracted toward the negatively charged environment of the selectivity filter by long-range interactions. This involvement of electrostatic interactions is supported by the ionic strength dependence of toxin binding (MacKinnon et al. 1989). However, even charge-neutralizing mutations of toxin residues that drastically affected binding affinity had little effect on association rates, implying that toxin affinity is mostly determined by pairwise close contact interactions with channel residues. Residues forming "close contact" were defined as those whose conservative mutations resulted in great changes in binding affinity and in which the affinity change mostly arose from the dissociation rate of the toxin.

A very influential residue, the mutation of which changed binding affinity by several orders of magnitude, was identified in the "wall" of the vestibule or turret

region of the *Shaker* channel (F425G mutation), whose role was confirmed for the corresponding residue in Kv1.3 as well (position 380) (Aiyar et al. 1995). Strikingly, this residue is very far from the cluster of other critical residues surrounding the entryway of the pore. It was shown that this residue does not contribute to normal binding of the toxin, but can greatly reduce accessibility to the pore by steric hindrance if a bulky residue is situated here. This finding underlines the fact that even residues that are not located on the typical interaction site of the toxin or the channel can have an effect on the formation of a specific channel-toxin complex, which may be a determining factor in the channel selectivity of a toxin.

Applying thermodynamic mutant cycle analysis, the closely interacting residue pairs could be pinpointed with even higher accuracy (Ranganathan et al. 1996). In this technique residues of the toxin and the channel are mutated individually and then simultaneously, and based on the binding affinities of the various combinations, a pairwise coupling energy is calculated, which characterizes the tightness of the interaction of the pair. These early studies established the critical role of the central (dyad) lysine and recognized that it must interact with residues forming K^+ binding sites in the pore based on the external K^+ concentration dependence of the binding. In contrast to the pore-blocking mechanism discussed above, some spider toxins bind to the voltage sensors and modify channel gating instead of plugging the conduction pore (hanatoxin). Chimeric toxins constructed of two other toxins active on different channels were also used to learn about the relevance of various peptide regions in the binding to different K^+ channel subtypes (Regaya et al. 2004). Then, the calculated and hypothesized interaction topology can be further refined by docking simulations that use homology models of the target channel based on known X-ray crystallographic structures of a related channel and typically NMR-derived structures of the toxins. Comparison of the results of docking calculations with different channels can provide clues about which channel residues may allow or prevent high-affinity binding of the toxin.

The identified receptors of KTx toxins can be found in Table 1.

Binding and Selectivity of α-KTxs at the Molecular Level: Docking Simulations and NMR Structure Determinations of the Complexes

From the results obtained using a variety of techniques listed above, the picture of a general blocking mechanism has emerged that is employed by the majority of confirmed high-affinity K^+ channel-blocking toxins. Most toxins carry a high net positive charge and thus are likely to be attracted toward the negatively charged environment of the selectivity filter by long-range interactions. This involvement of electrostatic interactions is supported by the ionic strength dependence of toxin binding (MacKinnon et al. 1989). As described above, many toxins feature the conserved functional dyad that superimposes spatially even in toxins of various lengths and structures (Menez 1998; Dauplais et al.1997) and is a good indicator of high-affinity K^+ channel blockade. However, as sequence comparisons and docking

simulations reveal, the hydrophobic residue of the dyad may have a major influence on the selectivity of a toxin such that the often present tyrosine shows preference for Kv1.2 channels over Kv1.3, while a threonine at that position directs toxin preference toward Kv1.3. Recent studies confirmed these expectations with toxins in which the hydrophobic dyad residue was mutated (Bartok et al. 2013).

A similar strategy was used to convert charybdotoxin (ChTx), which blocks several Kv channels and KCa3.1 into a more selective toxin (Rauer et al. 2000). Docking simulations aided by thermodynamic mutant cycle analyses revealed minor structural differences in the otherwise very similar topology of the external vestibules of Kv and KCa channels. A cluster of negatively charged residues was found in the turret of Kv1.3, not present in KCa3.1. A lysine residue of ChTx, which lies close to this cluster in the bound state, was mutated to negatively charged residues, which significantly reduced the affinity for Kv1.3 and therefore improved selectivity for KCa3.1.

Most models of toxin binding assume rigid topological structures for both the channel and toxin surfaces that must be complementary to a certain extent for the formation of the contact points that establish tight binding. However, recent NMR studies challenged this view and suggested that both structures are capable of flexible rearrangements during the formation of the channel-toxin complex (Lange et al. 2006). Using solid-state NMR spectroscopy (ssNMR), which is performed in a medium with limited mobility compared to the classical liquid-state NMR, the docking of kaliotoxin (KTX, α-KTx3.1) to a KcsA-Kv1.3 chimeric channel was studied. The pore region of Kv1.3, which contains the binding site for KTX, was inserted into KcsA, a bacterial K^+ channel with known crystal structure at the time, and structural changes were investigated upon KTX binding.

The authors observed significant ssNMR chemical shift changes for several KTX residues that are found on one side of the KTX three-dimensional structure bound to the channel and confirmed the general layout of the interaction surface from previous models describing KTx-Kv channel complexes. The results indicated that the structure of the outer and inner helices of KcsA-Kv1.3 was mostly unaffected by KTX binding, but changes were detected in both the pore helix and the selectivity filter, which were quite significant for the GYG signature selectivity filter residues. Their data suggests that the critical lysine side chain is inserted more deeply into the selectivity filter than previous models had assumed and that its methylene groups replace water molecules in the entry region of the pore. This insertion induces a new conformational state of the filter with characteristics of both the conducting and collapsed conformation that was described for KcsA. This reorientation, along with small changes in the toxin itself, is thought to strengthen the binding by allowing a more intimate contact between the toxin and the pore.

A follow-up study by the same group further investigated this phenomenon and found similarities between the structural changes associated with toxin binding and C-type inactivation, a process which makes Kv channels nonconducting during prolonged depolarizations via rearrangement of the external pore region (Zachariae et al. 2008). Using molecular dynamics simulations, ssNMR, and electrophysiological measurements, they showed that upon toxin binding, rotation of external

pore residues widens the pore and increases the number of contacts with the toxin, which both contribute to increased affinity. Thus, the original "lock and key" model of toxin binding was modified to a "hand and glove" or "induced fit" model to account for the mutual flexibility and adaptation of the two partners.

Therapeutic Applications

Many of the toxins of various venomous species are known to exert their harmful effects through interactions with the ion channels expressed by the cells of the prey. With detailed knowledge of the role of an ion channel in a cell's functions and the effects of peptide toxins on the channel, the behavior of cells or even organs can be manipulated in a desired way to achieve therapeutic goals. The high number of potassium channel genes expressed in the human body and the variety of cellular functions that they perform present many potential targets for such medical goals. Although the pharmacological properties of small molecule channel modulators are generally better suited for therapeutic applications, peptide toxins still have some advantages that make them attractive as drug candidates. One important aspect of these is that the greater contact area of the peptides compared to small molecules with the target channel allows a higher-affinity binding; thus, a lower concentration of the blocker is required. The other aspect again arises as a result of the higher number of contact points with the channel, which enables the toxin to differentiate among channels with similar, but still slightly differing structures. As described in previous sections, even minute differences in the topology of the interaction surfaces can lead to great changes in binding affinity. The resulting selectivity is a critical characteristic of drug molecules as this prevents unwanted side effects by avoiding interactions with off-target channels.

Several in vivo experiments in animal disease models have proven the efficacy and applicability of small K^+ channel-blocking peptides (Varga et al. 2012; Koshy et al. 2014). Although some of these experiments were performed with toxins originating from other species (ShK toxins from *Stichodactyla helianthus*), the similar size, structure, and mechanism of action assure that KTxs from scorpions would be just as effective in these applications (Dauplais et al. 1997).

The best-studied target of therapeutic application is the voltage-gated Kv1.3 channel expressed by lymphocytes. In patients with autoimmune diseases, the disease-associated autoantigen-specific T cells were identified as co-stimulation-independent effector-memory T cells, which express a high number of Kv1.3 channels. This was confirmed in multiple sclerosis, type 1 diabetes mellitus, and rheumatoid arthritis patients (Markovic-Plese et al. 2001; Wulff et al. 2003). As the activation and proliferation of the effector-memory T cells responsible for most of the tissue damage can be suppressed by selective Kv1.3 blockers, major improvements can be achieved by the use of such peptides. This concept has been elegantly proven in experiments, in which disease development or progression was prevented in rat models of multiple sclerosis, type 1 diabetes mellitus, rheumatoid arthritis, contact dermatitis, and delayed-type hypersensitivity. An advantage of this approach is that it specifically

suppresses effector-memory T cell activation without compromising the protective immune response. Experiments have shown that at therapeutically relevant concentrations, the toxins did not cause toxicity in the animals (Beeton et al. 2001, 2006) and did not suppress the protective immune response to acute viral and bacterial infections.

Several naturally highly Kv1.3-selective KTxs have been identified, for example, the recently characterized Vm24 (α-KTx21.1) from the venom of *Vaejovis mexicanus smithi*, with very high affinity (K_d = 2.9 pM) and exceptionally high (>1,500-fold) selectivity over several other ion channels assayed, including the closest relatives of Kv1.3. It was also shown to reduce delayed-type hypersensitivity in rats; thus, it promises to be a valuable tool for applications requiring selective Kv1.3 blockade (Varga et al. 2012).

A Kv1.3-specific peptide was also found effective in counteracting the negative effects of elevated caloric intake by mice that were fed a diet rich in fat and fructose. It produced effects similar to the effects of Kv1.3 gene deletion, which included a reduction of blood levels of cholesterol, sugar, and insulin and enhanced insulin sensitivity. Overall toxin application resulted in decreased weight gain, adiposity, and fatty liver (Upadhyay et al. 2013).

Another disease where selective KTxs have potential therapeutic value is myotonic dystrophy type 1 (DM1), because voltage-gated K^+ channels are responsible for myoblast proliferation and differentiation.

Comparison of the functional potassium channel expression in myoblasts from healthy individuals to myoblasts from patients with DM1 revealed a switch from KCa1.1 to Kv1 channels. Specifically, Kv1.2 and Kv1.5 channel expression increased, along with a decrease in KCa1.1 expression in DM1 myoblasts. Pharmacological block of Kv1 channels in DM1 myoblasts was found to normalize proliferation and improve other factors of myotube production. In contrast, wound healing and myotube formation were impaired by selective inhibition of KCa1.1 channels in normal myoblasts. Thus, detrimental effects of the switch in K^+ channel expression associated with the early stage of myogenesis in DM1 may be counteracted by selective KTxs (Tajhya et al. 2014).

Besides effector-memory T cells in the synovial fluid, resident joint cells known as fibroblast-like synoviocytes (FLS) are also responsible for many of the pathogenic features of rheumatoid arthritis (RA). FLS in RA (RA-FLS) become invasive and cause joint damage by releasing proteases and proangiogenic and proinflammatory growth factors. RA-FLS were shown to upregulate KCa1.1 channels, which localize on the leading edge of the plasma membrane. Blockade of KCa1.1 inhibited cellular migration and invasion, along with the production of pathogenic factors by interfering with cytoskeletal rearrangements. Pharmacological inhibition of KCa1.1 also improved the clinical symptoms in rat models of RA (Tanner et al. 2014). As in the cases above, the use of a selective KTx inhibitor may render general immunosuppression unnecessary during RA treatment in the future.

Recent results indicate that K^+ channel inhibition may also be a beneficial tool in enhancing antitumor immunity (Koshy et al. 2013). Blockade of KCa3.1 channels was found to increase the degranulation and cytotoxicity of adherent natural killer cells and to increase the ability of these cells to reduce in vivo tumor growth.

Conclusion and Future Directions

The examples above illustrate the wide spectrum of potential applications, in which K^+ channel-specific scorpion toxins of high affinity and selectivity may be used to accomplish therapeutic goals. With the number of identified KTxs growing by the day and the expansion of the body of knowledge on K^+ channel distributions and functions along with details of the toxin-channel interactions, this spectrum is likely to broaden even more, and routine clinical use of these peptides may soon become reality.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ Scorpion Venom Interactions with the Immune System
- ▶ Scorpionism and Dangerous Species of Mexico

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Part VII

Venomics

Scorpion Venom Gland Transcriptomics

22

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Contents

Abstract

For decades, the study of venomous animals has focused on the isolation and biochemical characterization of specific venom components that have medical or biotechnological importance. Indeed, scorpions have been extensively studied under this optics, which has led to the identification of hundreds of different transcripts encoding toxic peptides. However, scorpions are interesting organisms not only because of their toxin diversity but also because they represent the most ancient terrestrial animals that fossil records have identified. About 2,000

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species have been described around the world, which also implies that scorpions are extremely well-adapted arthropods that have managed to survive in different environmental conditions. Even though the divergence timing of scorpions places them as interesting model organisms for evolutionary inferences, little is known about the genomic organization, speciation events, and population dynamics of these arthropods.

Different "omic" approaches have become a very powerful strategy for understanding the complexity of venomous animals. Transcriptomics, in particular, has been widely used to explore the transcriptional diversity of venom glands of several scorpion species. Recently, high-throughput sequencing platforms have substantially improved our capacity to describe biological features of scorpions but, most importantly, have outlined new directions toward a more complete understanding of the evolution of these arthropods.

In this chapter, those transcriptomic strategies followed in the last two decades that went from cDNA cloning to next-generation sequencing methods will be described. Some biological and evolutionary questions about scorpion speciation and venom diversification will also be addressed. Finally, an attempt to raise some future directions in the field will be made.

Introduction

The transcriptome is the complete set of RNAs that is present in a cell, a tissue, or an organ at any given time. This includes the protein-coding messenger RNAs (mRNAs), ribosomal (rRNAs), transfer (tRNAs), and other noncoding or small RNAs. Since the transcriptome is specific for a particular cell type and is affected by the specific conditions where those cells live, including the external environment, it can be considered a snapshot of the genes that are actively expressed in those conditions at any given time. The study of particular transcriptomes therefore allows focusing on the subset of genes expressed in the relevant cell types or tissues without having to study the complete set of gene products that the organism's genome encodes. This is particularly important for venomous species, such as scorpions, since they are relevant not only for their biology or ecology but mostly for the venom they produce, which is restricted to a specific organ: the venom gland.

Several tools and techniques have been developed in order to have a comprehensive profile of the transcripts present in a given cell population. Historically, the possibility of cloning individual DNA molecules that are complementary (cDNAs) to mRNAs coding for proteins of interest led to the development of cDNA libraries. These libraries constitute collections of cDNA sequences cloned into vectors. The first scorpion venom gland cDNA library was reported in 1989 (Bougis et al. 1989) for the North African scorpion *Androctonus australis*. From then on, for more than two decades, the construction of cDNA libraries has been the main source of contribution to the discovery of new protein precursor sequences in many other scorpion species. The cDNA library sequencing has usually been performed by the traditional and expensive chain-termination DNA sequencing method, which limits

the number of sequenced individual clones and therefore the amount of information mined from the libraries. With the advent of high-throughput sequencing technologies, known as RNA-seq, the amount of information that can be gathered from the cDNA libraries has grown exponentially. Scorpions are not an exception, and by now, reports have emerged describing the whole transcriptome sequencing of two species using next-generation methods: the African scorpion *Pandinus imperator* (Roeding et al. 2009) and the Mexican *Centruroides noxius* (Rendón-Anaya et al. 2012).

The cDNA library construction generalities and the specific points will be considered, focusing on both, the traditional and the high-throughput methodologies for transcriptome analysis, their advantages and disadvantages. The most relevant examples of applications in the study of scorpion transcriptomes will be referred. The future of the field of transcriptomics for scorpions will also be addressed.

Scorpion cDNA Library Construction

A cDNA library consists of a collection of DNA sequences that are complementary to the RNAs that are present in a group of cells in a particular environment, individually cloned into vectors. The nature of the study to be performed determines the conditions in which the specimens are kept before the cells are harvested, the election of the tissue or organ from where they are dissected, and the cDNA library construction protocols employed. Scorpions are undoubtedly relevant members of the ecosystems they inhabit, but the interest they attract is mainly due to their capacity to produce a very complex venom, with a variety of bioactive components. Among them, there is a diverse group of toxins and other peptides with a great potential as therapeutics and tools for studying molecular interactions, with a special emphasis on ion channels. The transcriptome analysis in these organisms is therefore usually directed to unraveling the expressed peptide components of the venoms. In scorpions, the venom is produced exclusively in the venom glands, two very well-delimited structures within the last segment of their metasome (tail), the telson. Hence, it is the tissue that is usually processed to produce the cDNA libraries (Fig. 1). The telson is easily dissected and homogenized to release the cell contents, including the RNAs, into an RNase-inactivating buffer. It is best to select healthy individuals from their natural environment for this procedure, and it is advisable to use freshly collected specimens whenever possible. Though the collection of specimens is unavoidable, the unlimited availability of cloned sequences after the library is constructed eliminates the need for further recollection, outweighing by far the problem of extracting a few individuals from the environment. It should also be noticed that differences in the venom profiles between the two genders have been found (De Sousa et al. 2010). Thus, it could be reasonable to generate independent cDNA libraries for males and females. In order to stimulate the mRNA production, the specimens are usually milked to depletion 2–5 days before the telson is dissected.

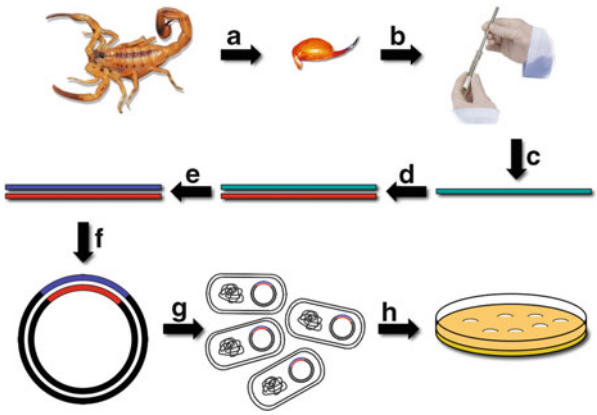

Fig. 1 Scorpion cDNA library construction steps: (**a**) Telson dissection. (**b**) Tissue homogenization. (**c**) Total RNA purification. (**d**) First-strand cDNA synthesis by reverse transcription. (**e**) Double-stranded cDNA synthesis by PCR. (**f**) Ligation into vector. (**g**) Bacterial transformation. (**h**) Growth and selection of colonies

The first cDNA strand is generated by an RNA-dependent DNA polymerase, the reverse transcriptase (RT) that will create a complementary copy of the RNAs. All known DNA polymerases need a primer to function, and the reverse transcriptase is not an exception. The choice of primers is another important consideration. In most of the times, the interest resides in the protein-coding mRNAs, but in others, a complete study, including the other noncoding RNAs, might be the objective. In the first case, the goal is to make copies of the mRNA exclusively. Like most eukaryotic mRNAs, the majority of scorpion mRNAs are polyadenylated at the 3′ end, so the use of a poly-dT primer will selectively amplify mainly the mRNAs. For the second case, a mix of random hexameric primers is the choice, and all RNAs will be reverse-transcribed.

For the generation of the second cDNA strand, there are a large number of choices available, including different commercial kits with different strategies. The simplest way is to treat the RNA/DNA hybrid product from the previous step with a combination of RNase H and a DNA polymerase. The RNase H will randomly cut the RNA in the hybrid, providing small RNA primers for the DNA polymerase to synthesize the second strand. Several kits provide ways to generate cDNAs flanked by adaptors that facilitate the cloning of the cDNAs into vectors, either by ligation (providing sites for restriction endonucleases) or by recombination (providing the specific recombinase target sequences).

Once the double-stranded cDNA is produced, it is cloned into the chosen vector (either plasmidic or phage derived), and individual clones (transformed bacteria or lysis plaques, respectively) can be isolated if desired. The library can then be screened by traditional methods or be used for massive sequencing.

There are several intrinsic advantages in generating scorpion cDNA libraries that are independent of the screening and characterization methods. One was mentioned earlier: once the library is obtained, there is no further need to extract living specimens from their environments in order to fulfill the need for DNA sequences for a wide range of projects. Large enough cDNA libraries made from scorpion venom glands should contain all the sequence information related to all

the protein components of the venom. This remains true for underrepresented transcripts corresponding to poorly expressed components (though their isolation and identification pose some challenges; see below), even those that are basically undetectable by standard proteomic methods. Since cDNA is copied from mature RNAs, there is no intron sequence present nor any information on regulatory sequences (for that, a genomic DNA library should be created instead). On the other side, the lack of introns is quite an advantage since the sequence of the encoded protein may be unambiguously assigned. Cloned cDNA sequences constitute a major source of DNA for protein expression in different heterologous systems, including bacteria, thanks again to the lack of introns (Quintero-Hernández et al. 2011). Many scorpion peptides are translated from mRNA as precursors: pro-peptides (endogenous peptides, not secreted) and pre-pro-peptides (those with a secretion signal peptide). Posttranslational modifications generate the mature peptides. That information is lost for proteomic analysis, but transcriptomes include it. Some other posttranslational modifications can be predicted from the cDNA sequence, including putative phosphorylation or glycosylation sites, disulfide bridges (though the specific connectivity cannot be assigned sometimes), or the amidation of the C-terminus, which is a very relevant modification for scorpion toxins, as some amidated toxins have been shown to have a higher affinity for their target than the non-amidated variants (Benkhadir et al. 2004). The availability of the derived complete amino acid sequence allows to design synthetic peptides and from them to generate antibodies that will aid in the localization and purification of the parental proteins. The comparison of the predicted peptide sequences could aid in designing immunogens for the generation of antivenom sera with cross-reactivity against the main toxic components of geographically related species (Becerril et al. 1996) (see ► Chap. 6, "Recombinant Neutralizing Antibodies, A New Generation of Antivenoms").

Scorpion cDNA Library Screening by Traditional Methods

When the cDNA library construction methodologies became widely available, different methods for library screening were also developed. As mentioned earlier, the first reported scorpion cDNA library was screened by means of colony hybridization with specific oligonucleotide probes designed from the known peptide sequence of the mature toxins. The authors were able to isolate the clones containing the precursors for AaHII and several other toxins specific for mammals and insects from some 400,000 different clones (Bougis et al. 1989).

A similar approach based on the reverse translation of known mature peptide sequences to design specific oligonucleotides, but relaying on the polymerase chain reaction (PCR), was first employed to amplify the cDNA precursor sequence of the BjIT2 toxin from the Asian scorpion *Buthotus judaicus* (Gurevitz et al. 1990). Since the primers are designed from the sequence of the mature toxin, the amplified sequence is only a fragment or a subsequence of the complete cDNA. The first report employing this strategy allowed the cloning of the cDNA of Na^+

channel-blocking toxins from the Mexican scorpion *C. noxius* Hoffmann (Becerril et al. 1993). The amplified partial cDNA can be used to determine the complete cDNA sequence by means of the RACE (Rapid Amplification of cDNA Ends) protocols. After the first strand cDNA is amplified by reverse transcription, an adaptor with known sequence is ligated to its 5′ end. A direct primer specific for the 5′ adaptor sequence is used with a reverse gene-specific primer (derived form the cloned fragment) to amplify the 5′ region of the cDNA (5′-RACE), and a direct gene-specific primer (also from the fragment) is used together with a poly(dT) to amplify the 3′ region (3′-RACE). The complete cDNAs for the precursors of the neurotoxins from the Chinese scorpion *Mesobuthus martensii* Karsch, BmK AS and BmK AS-1, BmP01, BmP03, BmP05, and the insect-specific BmK IT-AP were the first to be cloned using the 5′-RACE and 3′-RACE techniques (Lan et al. 1999; Wu et al. 1999; Xiong et al. 1999). To date more than a hundred different scorpion cDNAs have been isolated and sequenced with these tools (for a comprehensive review see Quintero-Hernández et al. 2011).

A different strategy for cDNA library exploration is the sequencing from more or less randomly selected clones, which is called expressed sequence tags (ESTs) (Adams et al. 1991 and Adams et al. 1993). Bacteria from the library are plated in a dilution that allows individual clones to be isolated and analyzed by colony PCR (a PCR technique where the template DNA is not purified: total DNA from lysed cells is added to the reaction). Vector-specific primers flanking the cloning region are used for amplification. Gel electrophoresis is then used to analyze the PCR products and to select the clones that will be sequenced. The selection is based on the colony PCR product size, so that a heterogeneous group of colonies is then chosen to purify the vector and sequence the cloned cDNA. Since no gene-specific primer is used, the results are in correspondence with the cDNA size range selected for sequencing. Sometimes, no such selection is applied and random clones are directly sequenced. This holistic approach will give a partial snapshot of the specimen's whole transcriptome. Otherwise, the focus can be put on large cDNAs (including enzymes as phospholipases, proteases, etc.) or smaller ones (coding for toxins, antimicrobial peptides, etc.).

The application of EST sequencing to uncover the global gene expression of a venom gland, as well as the description of the toxins potentially represented in a venom composition by transcriptomic studies, was first described for snake venom (Junqueira-de-Azevedo et al. 2002). The value of this strategy for scorpion venom study was then demonstrated with a cDNA library from the Mexican scorpion *Hadrurus gertschi*. The authors were able to isolate and sequence 147 ESTs, some even coding for undescribed putative toxins and other proteins (Schwartz et al. 2007). The number of sequences made available per study has been increasing since the first report. For example, the random sequencing of the venom gland cDNA libraries from *Lychas mucronatus*, *Isometrus maculatus*, and *Scorpiops margerisonae* resulted in 551, 743, and 730 ESTs, respectively (Ma et al. 2012), illustrating the power of this technique.

To date, the random sequencing of ESTs has been conducted with the following buthid species: *Buthus occitanus israelis* (Kozminsky-Atias et al. 2008), *Tityus*

discrepans (D'Suze et al. 2009), *Lychas mucronatus* (Ruiming et al. 2010 and Ma et al. 2012), *Hottentotta judaicus* (Morgenstern et al. 2011), *Isometrus maculatus* (Ma et al. 2012), *Tityus serrulatus* (Alvarenga et al. 2012), *Tityus stigmurus* (Almeida et al. 2012), and *Centruroides tecomanus* (Valdez-Velázquez et al. 2013). Within the non-buthids, reports are available for *Hadrurus gertschi* (Caraboctonidae, Schwartz et al. 2007), *Opisthacanthus cayaporum* (Liochelidae, Silva et al. 2009), *Scorpiops jendeki* (Euscorpiidae, Ma et al. 2009), *Heterometrus petersii* (Scorpionidae, Ma et al. 2010), *Pandinus cavimanus* (Scorpionidae, Diego-García et al. 2012), *Scorpiops margerisonae* (Euscorpiidae, Ma et al. 2012), and *Urodacus yaschenkoi* (Urodacidae, Luna-Ramírez et al. 2013).

As more transcriptome analyses of this kind have become available in the literature, a striking pattern of expression for toxins and other peptides has begun to appear. For example, it is well known that the large majority of the scorpion species that are of medical relevance belong to the Buthidae family. They are (in) famous for the Na^+ channel-modulating toxins present in their venoms that are capable of depolarizing the axonal membranes, with dire consequences for the stung victims, including death (see ▶ Chap. 20, "Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels"). Transcriptomes from the milked venom gland of members of the Buthidae family show that their most abundant transcripts are precisely those for the Na^+ channel-modulating toxins, with relative abundances as high as 54.2 % for *B. occitanus* (Kozminsky-Atias et al. 2008), while they are basically absent in non-buthid species. In the non-buthid scorpions, on the other hand, transcripts coding for K^+ channel-blocking ß-toxins (see chapter "▶ Potassium Channel Blocking Peptide Toxins from Scorpion Venom"), antimicrobial and cytolytic peptides, are more abundant (Diego-García et al. 2007). A somewhat surprising finding is that, within the Buthidae family, the level of those Na^+ channel toxin's expression seems to be very dependent on the physiological state of the venom gland. Two transcriptomics analyses that used the resting (not milked) venom gland as starting material reported relatively lower levels of transcripts for Na^+ channel toxins: 1.3 % for *T. stigmurus* (Almeida et al. 2012) and 6.7 % for *H. judaicus* (Morgenstern et al. 2011). There is still the possibility that these results could reflect the particularities of these two scorpions, since their transcriptomes from milked glands are not available for comparison.

Although the need for the isolation of individual clones to be sequenced can be seen as painstaking, its rewarding counterpart is that the experimenter has the clones perfectly identified and matched with the determined sequence, which facilitates further experiments to be performed with those sequences. They can be subcloned into expression vectors to heterologously produce the encoded proteins. The cDNA sequences can be used as templates to generate probes (e.g., by primer extension) for the screening of scorpion genomic DNA libraries, for genetic analyses or Southern blots, for the screening for homologous genes in other scorpion species, for studying gene expression, for interference experiments, and for a large etcetera.

The disadvantage of these methods resides in their cost. Since the sequencing is performed by the expensive Sanger method, they are not easy, nor cheap, to

upscale. This implies that only a relatively small subgroup of all clones gets sequenced; thus, they are only partially quantitative and focused on abundant transcripts. A relevant component of the scorpion transcriptomes is therefore missed: the transcription levels of low expressed transcripts. Tag-based methods have been developed to overcome this limitation, including serial analysis of gene expression (SAGE, Velculescu et al. 1995), massively parallel signature sequencing (MPSS, Brenner et al. 2000), and cap analysis of gene expression (CAGE, Kodzius et al. 2006). None of them has been applied to scorpion transcriptomes, mainly due to the need for reference genome to work properly.

Scorpion Transcriptome Analysis with High-Throughput Technologies

With the advent of so-called next-generation or high-throughput sequence technologies, the possibility of massively sequencing cDNA libraries (RNA-seq or whole transcriptome shotgun sequencing, WTSS) has become a reality (Morin et al. 2008). For RNA-seq, a library of cDNA fragments with flanking adaptors at one or both ends is created from RNA. Only small amounts of RNA are required, since the cDNAs are not cloned. The fragments are then subjected to direct high-throughput shotgun sequencing from the adaptors to generate a large collection of short reads (30–700 nucleotides, depending on the DNA sequencing technology used). Following sequencing, the reads are aligned to a reference genome (when one is available) or assembled de novo to produce a detailed transcription map with both the sequences of all transcripts and their expression levels (Wang et al. 2009).

Two different reports have taken advantage of massive sequencing, both of them using the 454 pyrosequencing platform, to explore the transcriptional universe of the scorpion venom glands. Pyrosequencing relies on the "sequencing by synthesis" principle: a complementary strand is synthesized enzymatically from the single-stranded cDNA template. The four nucleotides are added separately, and in a given order, to the reaction. The unreacted nucleotides are degraded before the next one is added. Every time a new complementary nucleotide is incorporated by the DNA polymerase, pyrophosphate (PPi) (hence the name of the method) is stoichiometrically released and detected by a chemiluminescent reaction with another enzyme, revealing the letter in the sequence. The intensity of the light is proportional to the number of times this same nucleotide is present in a row. The process is repeated until the sequencing is completed (Ronaghi et al. 1998; Margulies et al. 2005). With current capacities, an average of 700 nucleotides are determined per read, but array-based instruments are capable of generating a million reads per run, so that in a single run, 700 Mb can be read with a single machine (http://454.com/products/gs-flx-system/).

The African scorpion *P. imperator* was the first species to be examined under this approach (Roeding et al. 2009). In contrast to the abovementioned reports, this study did not focus on the discovery of new toxins, but rather aimed at making a comprehensive multigene-based phylogenetic analysis of arthropods. *C. noxius* was

the second Buthid species to be analyzed with this platform (Rendón-Anaya et al. 2012). In this report, the transcriptomes of resting and active (milked) venom glands were compared and contrasted to a third cDNA library obtained from RNA extracted from the body after telson removal. Altogether, around 19,000 different potential transcripts were identified. The functional annotation revealed that the use of microRNAs (miRNAs) as a posttranscriptional control mechanism is widely distributed among eukaryotes as the main components of the small RNA machinery are conserved in *C. noxius*. Additionally, a phylogenomic analysis of concatenated coding genes uncovered important differences in evolution rates of specific sets of genes. By means of a quantitative analysis of the transcriptional profiles of two different telson conditions, several regulatory and metabolic responses were detected, such as high representation of carbohydrate, lipid and amino acid metabolism, proteasome activity, membrane transport, and signal transduction pathways differentially expressed.

Although pyrosequencing was the methodology of choice for the two reported massive transcriptome analyses made with scorpions to date, several other methods of high-throughput sequencing are available to researchers nowadays, each with its advantages and disadvantages (Liu et al. 2012). These technologies have dramatically reduced sequencing cost while significantly increasing the throughput. This is a field under rapid development, and new improved and cheaper technologies are under development (Schadt et al. 2010). There is no doubt that these technologies will drive the field of scorpion transcriptomics in the near future, replacing the Sanger-based sequencing methods.

Future Directions for Scorpion Transcriptomics

Even though these analyses have placed us in the middle of a large amount of molecular data, it is easy to see how far we are from really understanding the evolution of the scorpion venom. So far, we have some evidence that important toxin genes might be overexpressed right after the electric milking of the venom glands (Morgenstern et al. 2011; Rendón-Anaya et al. 2012), but, which other factors alter these toxin profiles? It has been suggested that the length and location of introns in toxin genes could alter to some extent their expression, as observed for K^+ channel toxins in *M. martensii* (Nie et al. 2012; Zeng et al. 2012). It is also possible that environmental conditions determine the venom composition. Indeed, the transcriptome of the venom glands from *L. mucronatus* (Ruiming et al. 2010) revealed important differences of the transcriptional profile of two geographically distinct populations (Yunnan and Hainan-sourced). Other factors such as sex and age might affect the transcriptional and proteomic profiles of the venom glands as well, which should necessarily imply differences at the regulatory level that need to be explored. In a recent report, it was observed that miRNAs could be involved in the control of the venom phenotype in the rattlesnake *Crotalus simus simus* (Durban et al. 2013). In this particular case, the comparison of the transcriptional activity of the venom glands of neonate and adult specimens suggested that age-dependent

changes in the concentration of miRNA modulating the transition from a crotoxin-rich to a metalloproteinase-rich venom from birth through adulthood could potentially explain the proteomic differences in the venom composition of *C. s. simus*. Given this evidence, the natural question to ask would be what is the potential role of the scorpion miRNA machinery in the venom gland during toxin production (Rendón-Anaya et al. 2012)?

Another unsolved question that researchers have evaluated for a long time is how do scorpions achieve such a wide diversity of toxin peptides. A parsimonious explanation would be gene duplication and functional diversification accompanied by strong positive selection. An intriguing example, for which this alternative has been examined, is the venomous mammal *Ornithorhynchus anatinus* (Wong et al. 2012). The combination of transcriptomic data with the reference genome revealed that only 16 of 107 platypus genes with high similarity to known toxins evolved through gene duplication, suggesting that gene duplications alone do not explain the "venome" of the platypus. This leads to the possibility that other mechanisms, such as alternative splicing and mutation, may be important in venom innovation. This has also been proposed for some scorpion toxins from *M. martensii* (Zeng et al. 2012), in which the BmKbpp toxin was proposed to be the result of a recombination event at the transcript level, opening the possibility of trans-splicing driving the functional diversification of venom peptides.

From this discussion, it becomes clear that, in order to understand the venom complexity of scorpions as well as other significant biological insights, a comparative genomic approach should become the next needed step in the field (Fig. 2a–c). Scorpions represent an excellent evolutionary model and unexplored subjects for population genetic studies as they are the most ancient terrestrial animals that fossil records have identified. Cladistic and phylogenetic analysis (Pisani et al. 2004; Jeyaprakash and Hoy 2009) suggested that they arose ~350 Ma ago, and, after a physical separation upon the partition of the African and South American continents (~150 My ago), several speciation events gave rise to different genera such as *Buthus*, *Mesobuthus*, *Parabuthus*, *Hottentotta*, *Leiurus*, and *Androctonus* in Africa and Asia and *Tityus* and *Centruroides* in South and North America, respectively (Fet et al. 2003). Speciation of Asian scorpions in particular was recently associated to climate changes (aridifications and glaciations) during the Mid-Miocene and Pleistocene periods. Using one mitochondrial and three nuclear genes, although limited in the number of sequences, it was possible to describe how intensified aridifications from Mid-Miocene onward drove the diversification of Mesobuthid scorpions and that a switch to a more humid habitat that occurred close to the most common ancestor of *M. martensii* and the lineage of *M. caucasicus* led to the adaptation of *M. martensii* to a humid environment (Shi et al. 2013). Such results outline the importance of phylogeographic studies in our interpretation of scorpion history and venom evolution (Fig. 2d, e). Furthermore, considering the geographic overlap of scorpion species in particular areas, it becomes easy to imagine how outcrossing and successive admixtures before reproductive barriers are established are feasible events after speciation took place, opening the possibility that the genome organization might be a mosaic of genomic fragments from parental

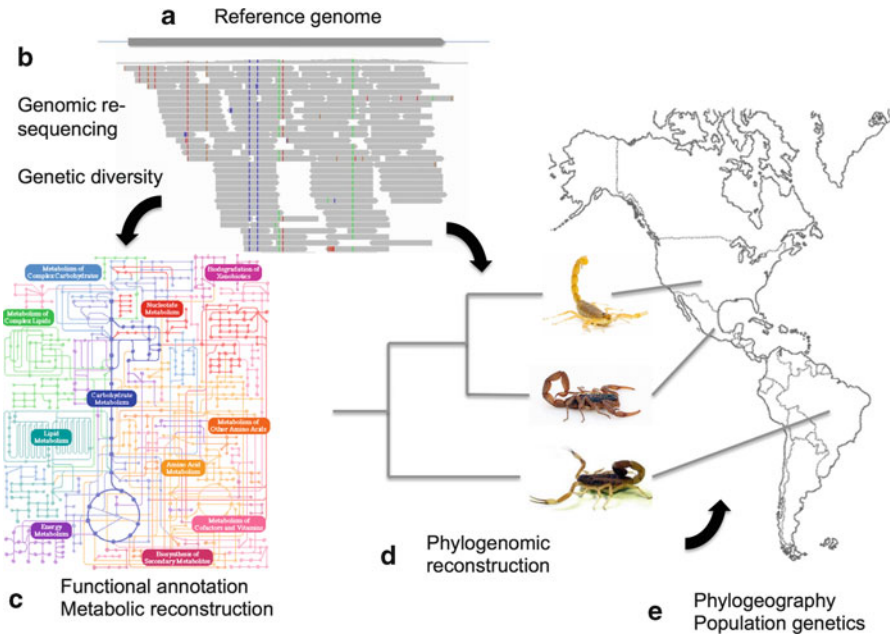

Fig. 2 Future directions in scorpion genomic studies. (**a**) The generation of a reference genome is the first step toward deeper comparative analysis. (**b**) Genome re-sequencing, mapping, and SNPs identification will depict the genetic diversity of scorpion species. (**c**) Functional annotation and metabolic reconstruction can be achieved after genome assembly. (**d**) Detailed phylogenomic reconstructions using orthologous and paralogous genes identified after whole genome comparison will illustrate the real tree of scorpion species. (**e**) Phylogenomic observations can ultimately be related to geographic conditions, climate changes, and population dynamics

scorpion species. In spite of these interesting evolutionary aspects, a small amount of reports have tried to elucidate genomic features of scorpions, leading to contrasting chromosome numbers and genome size estimations. Karyotype determination has concluded that scorpion chromosomes vary in number and morphology, ranging from a diploid number of <10 up to >100 chromosomes (Schneider et al. 2009; Schneider and Cella 2010). Additionally, flow cytometry experiments indicate that the genome size of buthid scorpions might be comprised between 600 (*M. martensii*, Li et al. 2009) and 880 Mbp (*Centruroides vittatus*, Hanrahan and Johnston 2011).

The most important limitation for the maturation of a genomic strategy that would naturally lead to population genomics and phylogeographic studies has been the cost of obtaining true genome-scale data. Nevertheless, new sequencing tools such as restriction site-associated DNA (RAD) sequencing, a method that simultaneously types and scores thousands of sequence variants (such as single nucleotide polymorphisms (SNP)), open the possibility of gathering genomic information across multiple individuals at a genome-wide scale in natural populations (Hohenlohe et al. 2012). These technologies should become increasingly important for evolutionary genetics, even in organisms with few genomic resources like scorpions.

Existing model organisms are limited when it comes to answering evolutionary and ecological questions. Advances in sequencing have radically expanded the reach of genetic studies to non-model organisms and thus should allow us to exploit the potential of these fascinating arthropods in the short term.

Cross-References

- ▶ Molecular Description of Scorpion Toxin Interaction with Voltage-Gated Sodium Channels
- ▶ Potassium Channel Blocking Peptide Toxins from Scorpion Venom
- ▶ Recombinant Neutralizing Antibodies, A New Generation of Antivenoms

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Modern Venom Profiling: Mining into Scorpion Venom Biodiversity

23

Jennifer J. Smith and Paul F. Alewood

Contents

Abstract

Scorpions and their sting are infamous for causing pain, morbidity, and, in some cases, death. However, research into scorpion venoms has revealed the presence of components that potentially have beneficial properties for humans. Such components may be developed into therapeutics or bioinsecticides. In order to assess the biodiversity of components present in scorpion venoms, proteomic and transcriptomic approaches have been applied to numerous scorpion species. This chapter presents our current knowledge in the field of venom-wide studies of scorpions. Discussions on the pros and cons of several proteomic and transcriptomic techniques used to investigate scorpion venoms are also included.

Introduction

For millennia, scorpions and the ability of their venom to cause morbidity and death have inspired fear, awe, and superstition across many human cultures. Such was the influence of scorpions in ancient civilizations that their image was lent to

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mythological deities such as the Egyptian goddess Serket and Scorpio of Greek mythology (Cloudsley-Thompson 1990). In contemporary times, scorpion stings are still a significant source of human fear, illness, and injury, with an estimated 1.2 million human envenomations per year resulting in approximately 3,000 deaths (Chippaux and Goyffon 2008). Modern science has revealed scorpion venoms are complex mixtures of components including salts, lipids, small molecules, and proteins (Eauclaire-Martin and Couraud 1995). The constituents primarily responsible for physiological effects and, hence, of medical interest are the venom peptides (Sofer 1995). In the 1970s and 1980s, advancements in chromatographic techniques, such as the development of high-performance liquid chromatography (Martin and Rochat 1986; Michaelis et al. 1973), enabled the separation and purification of venom peptides to homogeneity, thus allowing for the first time the study of individual peptides to determine their function. The first scorpion toxin peptides characterized were found to affect action potentials through either modulation of voltage-gated sodium channel activity or blockade of voltage-gated potassium channels (Rodriguez de la Vega and Possani 2004). Toxins that affected potassium channels were found to be around 35 residues in length, while those that targeted sodium channels were about 65 residues long. Consequently, potassium channel toxins were termed "short chain" and sodium channel toxins called "long chain" (Rodriguez de la Vega and Possani 2004, 2005). Crystallographic and spectroscopic analyses of both short- and long-chain toxins revealed their peptide backbone adopts a fold consisting of an alpha helix connected to three antiparallel beta sheets via three or four disulfide bonds, named the cysteine-stabilized alpha/beta (CSαβ) motif (Bontems et al. 1991; Cornet et al. 1995). Currently, about 600 of the ~750 identified scorpion venom peptide sequences are classified as CSαβ folded sodium or potassium channel toxins (Jungo et al. 2012). Although CSαβ toxins dominate the literature on scorpion venoms, scorpion venom peptides are known to adopt numerous other folds, including linear alpha helices, the inhibitor cystine knot motif, and the cysteine-stabilized alpha/alpha (CSαα) motif (Dai et al. 2001; Mosbah et al. 2000; Srinivasan et al. 2002). Moreover, their molecular targets are not restricted to sodium or potassium channels, as toxins have been found that affect other channels such as intracellular ryanodine receptors and voltage-gated calcium channels (see Fig. 1) (Lee et al. 2012; Valdivia et al. 1992).

Of the 1,900 scorpion species worldwide, the venom from only ~100 species has been researched, with about 30 species containing venom that is harmful to humans. Nearly all of these dangerous species belong to the Buthidae family (Isbister et al. 2003). Due to the historical partiality of studying medically important species, it is perhaps not surprising that current literature is biased toward the research of buthid venoms, with over 85 % of known scorpion venom peptides originating from less than 50 Buthidae species (Jungo et al. 2012). The remaining 14 scorpion families encompassing approximately 900 species are likely to contain a plethora of peptides with novel function and structure. The sheer enormity of this virtually untapped resource of venoms makes assembling a picture of the diversity of peptides and proteins in scorpion venoms a significant challenge. However, recent advancements in technology now enable the compilation of accurate and comprehensive profiles of venom components, namely, through a combination of modern proteomic and transcriptomic approaches.

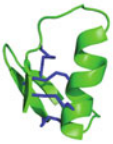
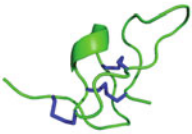
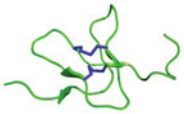
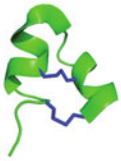
Structure	Fold; Molecular Targets	Size range (nearest 0.5 kDa)
	Cystine-stabilised α/β (short-chain); voltage-gated and calcium-activated potassium channels	2.5 to 5
	Cystine-stabilised α/β (long-chain); voltage-gated sodium channels	6.5 to 8.5
	Inhibitor cystine knot (containing antiparallel β sheets and α helix); small conductance chloride channels, matrix metalloprotease-2, annexin A2, voltage-gated potassium channels	3.5 to 5
	Inhibitor cystine knot (mainly random coil); ryanodine receptors	4
	Disulfide-directed hairpin; ryanodine receptors	4
	Cystine-stabilised α/α; voltage-gated potasisum channels	2.5 to 3
	Non-disulfide bonded α helical; antibacterial and cytolytic	1 to 5

Fig. 1 Structure, fold, and molecular targets of known scorpion toxins

Transcriptomic Profiling

In the past 12 years, the price of sequencing DNA has dropped drastically, from over USD$5,000 per megabase in 2001 to USD$0.06 in 2013 (Wetterstrand 2013). The cost breakthrough occurred in 2008 with the development of "next-generation" sequencing methods such as Illumina and SOLiD technologies. Since 2007, venom

gland transcriptomes of 16 scorpion species spanning 6 families have been published. However, all but one of these studies employed the traditional method of cDNA plasmid library generation followed by random selection of clones for Sanger sequencing of expressed sequence tags (ESTs) (Almeida et al. 2012; D'Suze et al. 2009; Diego-Garcia et al. 2012; Kozminsky-Atias et al. 2008; Luna-Ramirez et al. 2013; Ma et al. 2009, 2010, 2012; Morgenstern et al. 2011; Rendon-Anaya et al. 2012; Ruiming et al. 2010; Schwartz et al. 2007; Silva et al. 2009; Valdez-Velazquez et al. 2013; Abdel-Rahman et al. 2013). Only one study to date has used the newer 454 pyrosequencing method in which approximately one million reads were obtained per sample analyzed (Rendon-Anaya et al. 2012). Even though Illumina and SOLiD technologies are less expensive, Sanger and 454 sequencing (USD$2,400 and USD$10 per megabase, respectively) are still the preferred method of choice for transcriptomic analyses of venom glands. This is mainly due to the considerably longer read lengths achievable using Sanger (400–900 bp with the AB3730xl) and 454 (700 bp with GS FLX) methods compared to Illumina (90 bp with the Hiseq 2000) and SOLiD (85 bp with the 5500xl) (Liu et al. 2012). The time-consuming, challenging, and costly process of de novo transcriptome assembly is assisted by longer reads, which allows for better identification of overlapping sequences and improved accuracy of contig construction.

Although the depth of coverage varied considerably between the 16 studies using traditional Sanger methods, with the number of high-quality readable clones ranging from ~100 to over 800, a wide variety of toxin families were still detected in the studies with smaller cDNA libraries. These families include toxins that affect sodium, potassium, and calcium channels, as well as metalloproteases, phospholipases, and antimicrobial peptides (Figs. 1 and 2) (Rodriguez de la Vega et al. 2010). Notably, transcripts that encode sodium channel toxins are of low abundance or entirely absent in non-Buthidae scorpions, while they are the most prevalent toxin type in Buthidae transcriptomes (Ma et al. 2012). This evolutionary divergence between Buthid and non-Buthid scorpions may be helpful to genetic studies of phylogenetic relationships between different scorpion species.

Most scorpion transcriptomes have been obtained using RNA from venom glands harvested 2–5 days after venom milking. This time period is when the glands are regenerating their venom and mRNA levels are at a maximum, which is referred to as the "active" or "replenishing" state (Alami et al. 2001; Zeng et al. 2002). In active venom glands, transcripts that encode homologues of known toxins and venom peptides account for between 24 % and 78 % of ESTs with a discernable open reading frame (Diego-Garcia et al. 2012; Kozminsky-Atias et al. 2008). Currently, there are only two transcriptomic investigations of venom glands that had not been milked in the days before harvesting, i.e., glands in their "resting" or "replete" state (Morgenstern et al. 2011; Rendon-Anaya et al. 2012). Both resting gland studies were of Buthidae scorpions, and it appears transcripts encoding toxins, especially sodium channel toxins, are not as highly expressed in the resting gland compared to the active gland. Lower transcript levels of certain toxins may indicate the encoded

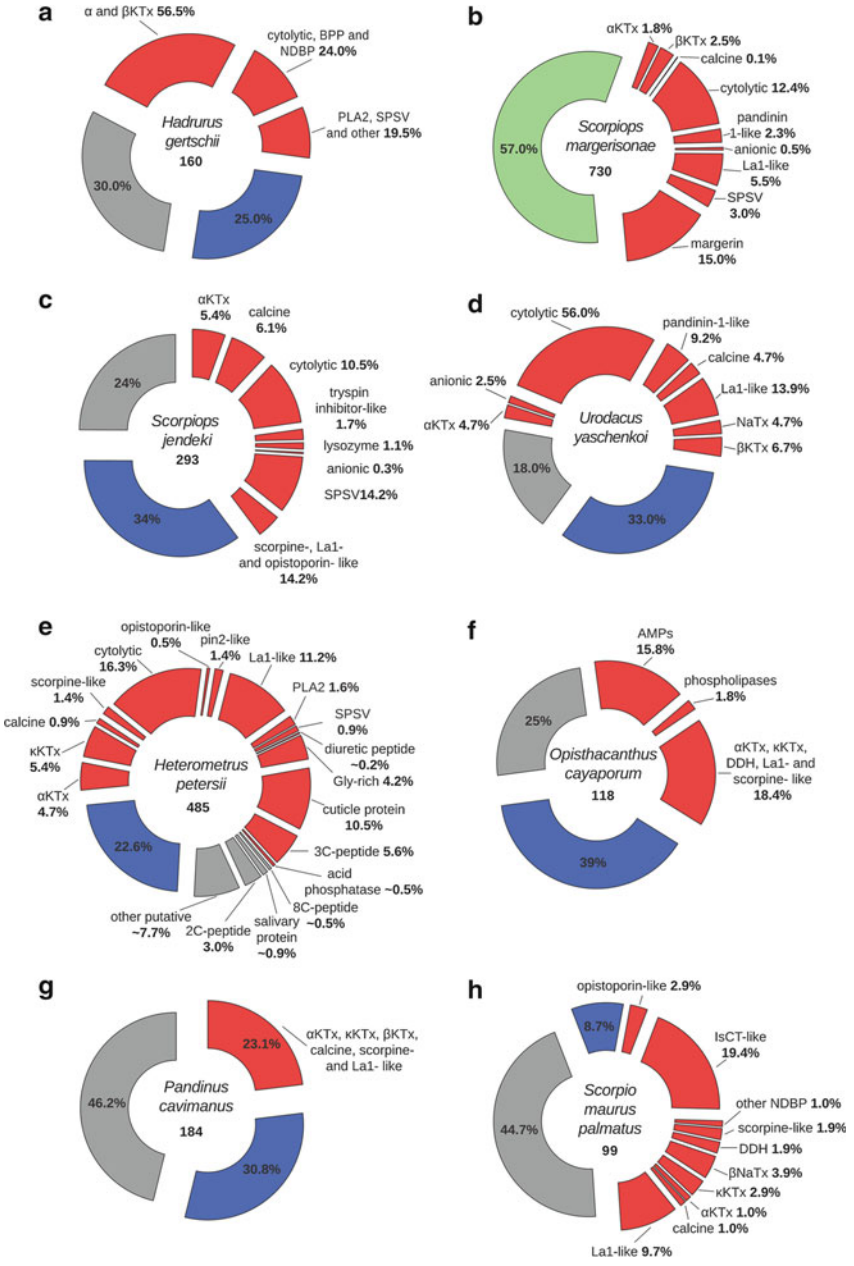


Fig. 2 (continued)

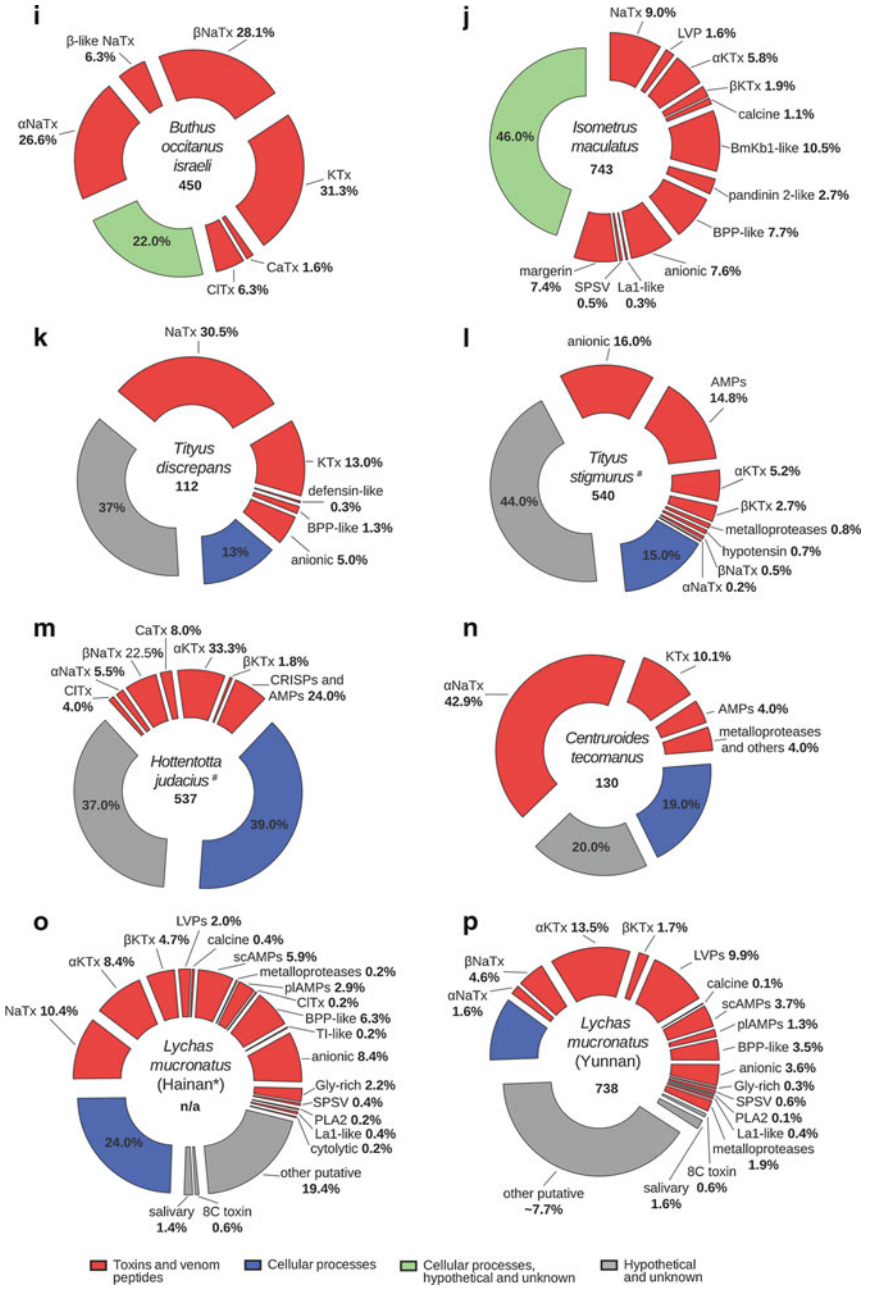


Fig. 2 Relative proportion of transcripts in each category from transcriptomic analyses of scorpion venom glands. Sequences without an ORF excluded. The number in the center of each chart in bold indicates the number of readable sequences or clones in each study. Abbreviations are as follows: *αKTx* alpha potassium channel toxin, *αNaTx* alpha sodium channel toxin, *βKTx* beta

peptides are still in adequate quantities in the venom, as they may possess high chemical stability and resistance to degradation; thus the metabolically demanding process of transcription and translation of those peptides are unnecessary (Morgenstern et al. 2011; Rendon-Anaya et al. 2012). Moreover, resting glands have a higher representation of transcripts corresponding to cellular and environmental information processing, such as membrane transport and signal transduction pathways (Rendon-Anaya et al. 2012). It is important, therefore, to analyze actively transcribing venom glands in order to capture maximal toxin transcript diversity.

Transcriptomic analysis has proved to be a valuable tool in providing an overview of the variety of peptides and toxins that can be found in scorpion venoms. Nevertheless, there is not necessarily a correlation between levels of transcription and venom peptide expression. Furthermore, a large percentage of ESTs in all scorpion venom transcriptomic studies to date encode putative venom peptides with no homology to existing toxins and no known function. Next-generation sequencing methods can also introduce erroneous variation in transcriptomic libraries through sequencing errors and insertions and deletions (indels). Although sequencing errors are very low (usually <0.1 %) (Liu et al. 2012) and indels usually occur in homopolymer regions, which, except for the polyA tail, do not occur as frequently in mRNA as in genomic regions, these errors could be incorrectly assigned as sequence variations. Conversely, overstringent library assembly could lead to exclusion of low abundance transcripts, resulting in an underrepresentation of venom diversity. Therefore, additional methods such as proteomic, genomic, and biochemical characterization of venom peptides are required to accurately assign and assess the range of diversity in the venom peptidome. The first genome study of a scorpion species, *Mesobuthus martensii* (Cao et al. 2013), was recently reported, and future publications of reference scorpion genomes will help to deconvolute transcriptomic data.

Fig. 2 (continued) potassium channel toxin, *βNaTx* beta sodium channel toxin, *SPSV* serine proteases from scorpion venom, *CaTx* calcium channel toxin, *ClTx* chloride channel toxin, *LVP* lipolysis-activating peptide, *CRISPs* cysteine-rich secretory proteins, *AMPs* antimicrobial peptides, *PLA2* phospholipase A2, *TI-like* trypsin-inhibitor-like, *NDBP* non-disulfide-bonded peptides, *8C* eight cysteine peptides, *BPP-like* bradykinin-potentiation-peptide-like, # transcriptome of gland in resting state, * percentages estimated. (**a**) *Hadrurus gertschi* (Schwartz et al. 2007), (**b**) *Scorpiops margerisonae* (Ma et al. 2012), (**c**) *Scorpiops jendeki* (Ma et al. 2009), (**d**) *Urodacus yaschenkoi* (Luna-Ramirez et al. 2013), (**e**) *Heterometrus petersii* (Ma et al. 2010), (**f**) *Opisthacanthus cayaporum* (Silva et al. 2009), (**g**) *Pandinus cavimanus* (Diego-Garcia et al. 2012), (**h**) *Scorpio maurus palmatus* (Abdel-Rahman et al. 2013), (**i**) *Buthus occitanus israelis* (Kozminsky-Atias et al. 2008), (**j**) *Isometrus maculatus* (Ma et al. 2012), (**k**) *Tityus discrepans* (D'Suze et al. 2012), (**l**) *Tityus stigmurus* (Almeida et al. 2012), (**m**) *Hottentotta judaicus* (Morgenstern et al. 2011), (**n**) *Centruroides tecomanus* (Valdez-Velazquez et al. 2013), (**o**) *Lychas mucronatus* (From Hainan province, China) (Ruiming et al. 2010), (**p**) *Lychas mucronatus* (From Yunnan province, China) (Ruiming et al. 2010)

Proteomic Profiling

While transcriptomic analyses can deduce all the peptide sequences that may potentially be expressed in the venom of a particular species, it cannot provide information on posttranslational modifications (PTMs) that may be present or whether the venom peptides are even expressed at all. In order to validate the expression of sequences obtained through transcriptomes, mass spectrometric and proteomic analyses are required. Currently, there are only five studies on scorpion venoms that use a combined transcriptomic and peptidomic approach (Diego-Garcia et al. 2012; Luna-Ramirez et al. 2013; Ma et al. 2010; Valdez-Velazquez et al. 2013; Abdel-Rahman et al. 2013). In four of these studies, the peptide masses of chromatographically separated venom components were compared to the theoretical masses of predicted peptide sequences obtained from the cDNA library. Surprisingly, only a handful of masses predicted from the venom gland transcriptomes matched the masses of actual components found in the venom. In one report, the masses of just two predicted peptides corresponded to experimentally determined masses (Diego-Garcia et al. 2012). Since mass alone is a poor predictor of the presence of a corresponding peptide, sequence tags obtained by MS/MS are needed to correlate mass and transcriptome data. Indeed, experimental validation of predicted sequences on a transcriptome-wide level has only been demonstrated in one study, where crude venom was digested with trypsin prior to nano-LC-ESI-MS/MS of the resulting peptide fragments (Ma et al. 2010). The MS/MS fingerprints of the peptide fragments were then matched to databases composed of the translated transcriptome. Using this approach, fragments corresponding to the majority of predicted venom peptide families were found. Venom peptides that were highly transcribed but not found in the venom typically possessed features that made mass spectrometric detection difficult. These characteristics include either a lack of positively charged residues (Arg or Lys) or an abundance at every second or third residue resulting in tryptic fragments too small to be detected by the MS acquisition method used (Ma et al. 2010). It may also be possible that contributing factors to the poor correlation seen between the peptidomes of undigested scorpion venoms and their corresponding venom gland transcriptomes are posttranslational modifications such as phosphorylation and N-glycosylation (Hassani et al. 1999; Verano-Braga et al. 2013). Also, proteolysis of peptides occurs within some scorpion venoms, producing successions of serially truncated peptides that would only appear as one sequence in a transcriptome (Verano-Braga et al. 2013; Rates et al. 2008; Smith et al. 2012). Another contributing factor to the deficiency of observable masses consistent with full-length predicted peptides may be that current methods might not be able to ascertain the correct processing of scorpion venom peptide mRNA transcripts, such as cleavage or retainment of proposed “pro regions”; thus accurate predictions of some mature toxin sequences and their masses may not be possible (Diego-Garcia et al. 2005). Future studies of transcript processing and better methodologies for predicting cleavage and posttranslationally modified sites may enable consolidation between the transcriptome and peptidome of scorpion venom glands.

A factor to be mindful of when trying to associate the peptidome with the transcriptome is ensuring the search engine used to correlate the MS/MS peptide fingerprints to the transcriptome library is set up to accommodate PTMs such as amidation and phosphorylation, which are frequently present in scorpion peptides. Common search engines and software used include MASCOT, Spectrum Mill, ProteinPilot, and X! Tandem. The majority of these programs can be modified or instructed, with varying degrees of success, to account for PTMs as well as limited degrees of sequence variation; however, differences in the identification and assignment of peptides can result from the use of different software.

Besides combined transcriptome and proteome studies, venoms from more than another 20 scorpion species, 14 of which are buthids, have been profiled using only proteomic techniques (Rodriguez de la Vega et al. 2010; Smith et al. 2012; Caliskan et al. 2012; Newton et al. 2007; Rodriguez-Ravelo et al. 2013) (Table 1). Over 100 peptides were present in the venom of each species. From current proteomic studies, it is apparent that there is a difference in mass profiles between buthid and non-buthid species, with buthid venoms containing more masses between 6 and 9 kDa than non-buthids (Rodriguez de la Vega and Possani 2005). Scorpion peptides in this mass range are typically sodium channel toxins, and their scarcity in non-buthid venoms agrees with the lack of sodium channel transcripts in non-buthid transcriptomic studies (Ma et al. 2009, 2010; Schwartz et al. 2007). Peptides in this mass range that are present in non-buthid venoms are usually long-chain potassium channel toxins or long antimicrobial peptides that include homologues of scorpine (Conde et al. 2000; Diego-Garcia et al. 2007). The dominant components of both buthid and non-buthid venoms are linear alpha helical or randomly coiled peptides under 2 kDa that are typically antimicrobial or cytolytic, such as the opistoporins and IsCT homologues, or hormonelike, such as bradykinin-potentiating peptides and hypotensins (Rodriguez de la Vega et al. 2010). Another large group of venom components are between masses of 2 and 5 kDa, which usually correspond to short-chain potassium channel toxins, although a number of scorpion peptides that target other receptors are also found in this mass range. These include peptides that adopt a three-dimensional fold called the inhibitor cystine knot and whose targets include small conductance chloride channels, intracellular calcium release channels known as ryanodine receptors, and potassium channels (Mosbah et al. 2000; DeBin et al. 1993; Gao et al. 2013). Ryanodine receptor toxins that adopt the two-disulfide fold called the disulfide-directed hairpin also fall in this mass range (Smith et al. 2011). Proteins above 9 kDa comprise a small proportion of scorpion venom components and have not been well characterized, with only a few proteins found to have homology to phospholipase A2, lysozyme, or hyaluronidase enzymes (Batista et al. 2007; Cologna et al. 2009; Schwartz et al. 2008) (Fig. 3).

Currently, the most common workflow when analyzing the proteome of scorpion venoms involves fractionation using one or more chromatographic steps, such as ion exchange, size exclusion, reversed-phase, and the more recently developed zwitterionic-hydrophilic interaction chromatography, followed by mass spectrometric analysis of fractions (Xu et al. 2012). The two primary methods of mass

Table 1 Scorpion venom species and analyses performed on their venom and/or venom glands

	Analyses performed	
Species name	Transcriptomic	Mass spectrometric
Centruroides noxius	Yes	No
Scorpiops jendeki	Yes	No
Tityus discrepans	Yes	Yes
Opisthacanthus cayaporum	Yes	Yes
Heterometrus petersii	Yes	Yes
Pandinus cavimanus	Yes	Yes
Lychas mucronatus	Yes	No
Isometrus maculatus	Yes	No
Scorpiops margerisonae	Yes	No
Tityus stigmurus	Yes	Yes
Hottentotta judaicus	Yes	No
Urodacus yaschenkoi	Yes	Yes
Hadrurus gertschi	Yes	No
Buthus occitanus israelis	Yes	No
Centruroides tecomanus	Yes	Yes
Tityus serrulatus	No	Yes
Tityus cambridgei	No	Yes
Tityus costatus	No	Yes
Tityus pachyurus	No	Yes
Androctonus crassicauda	No	Yes
Androctonus mauretanicus mauretanicus	No	Yes
Liocheles australasiae	No	Yes
Parabuthus transvaalicus	No	Yes
Tityus bahiensis	No	Yes
Leiurus quinquestriatus quinquestriatus	No	Yes
Leiurus quinquestriatus hebraeus	No	Yes
Heterometrus longimanus	No	Yes
Scorpio maurus	Yes	Yes
Urodacus armatus	No	Yes
Urodacus elongatus	No	Yes
Lychas marmoreus obscurus	No	Yes
Vaejovis spinigerus	No	Yes
Opistophthalmus glabrifrons	No	Yes
Pandinus imperator	No	Yes
Buthacus macrocentrus	No	Yes
Rhopalurus junceus	No	Yes
Mesobuthus tamulus	No	Yes

spectrometry in use are electrospray ionization (ESI) and matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) (Ashton et al. 1994; Hillenkamp et al. 1991). The abundance of low molecular weight scorpion toxins makes them amenable to top-down peptidomic analyses. Partial sequences can be obtained with

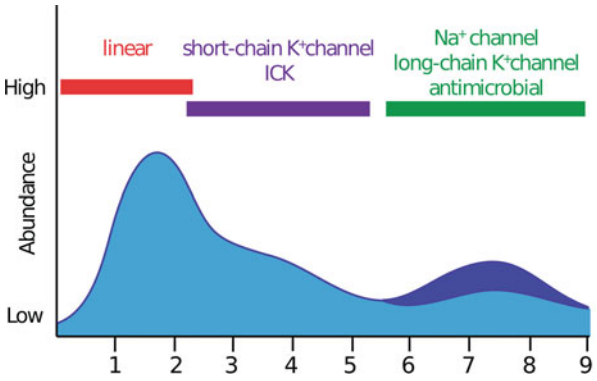


Fig. 3 Stylized representation of scorpion venom mass landscapes. *Light blue* represents non-buthid venoms; *dark blue* represents buthid venoms

de novo sequencing using tandem mass spectrometry or through the use of MALDI-ToF matrices such as 1,5-DAN and 5,1-ANL that enable in-source decay (ISD) fragmentation of peptides (Fukuyama et al. 2006; Osaka et al. 2013). 1,5-DAN also causes partial reduction of cystines in the MALDI-ToF laser plume, thus providing important information on the number of disulfide bonds that are present in peptides (Fukuyama et al. 2006). The use of ISD fragmentation for analysis of venom peptides is relatively new, and further development of the technique is required to overcome its limitations, such as the undetectability of many peptides with current ISD matrices.

In MS/MS, the most commonly used method of generating peptide fragments is by collision-induced dissociation (CID). However, CID often yields selective backbone fragmentation, resulting in incomplete sequence information, and PTMs are frequently not retained. Newer techniques of generating fragment ions, namely, electron transfer dissociation (ETD) and electron capture dissociation (ECD), circumvent these problems associated with CID. However, the primary limitation of these newer techniques is the requirement for precursor ions with a high charge, i.e., a low m/z ratio, in order to produce useful fragmentation. Although low m/z ratios are achieved with ESI, ETD and ECD analysis of peptides with MALDI-ToF requires strategies to increase the charge state, e.g., through chemical modification of cysteine residues to convert them to a dimethyl lysine analogue (Ueberheide et al. 2009).

In order to collect a comprehensive mass list, it is important to analyze venom fractions using both ESI and MALDI-ToF methods. This is because the masses obtained using MALDI-ToF and ESI are vastly different, with studies showing common masses between both methods can be as low as 12 % of the total observable masses (Smith et al. 2012). Most studies have also checked their mass lists for potential modifications caused by user handling of venoms, such as oxidation (+16 Da) and acid-driven glutamine to pyroglutamic acid formation (−18 Da), which may result in overestimation of peptide components. Overall, the proteomic approach of analyzing scorpion venoms has successfully yielded insights into the diversity of components found within.

Conclusion and Future Directions

The combined approach of transcriptomic and proteomic profiling is essential to garner the range of peptides and proteins found in the venom of each scorpion species. Innovations in mass spectrometric techniques such as the development of novel MALDI-ToF matrices facilitate detailed analysis of venom components and improvements in detection limits of mass spectrometers enable mass profiling of venoms from scorpions that yield low venom volumes. With ever-lowering prices and rapidity, transcriptomic sequencing of venom glands will become more routine and their integration with accompanying proteomic data essential for a full interpretation of the peptidic venom components. The main obstacle in the pursuit of exploring the diversity of scorpion venom peptides is the pharmacological characterization of novel peptides. Since computer modeling and simulations of toxin-receptor interactions are not yet able to predict the target of a novel peptide, the molecular target for new peptides still needs to be delineated through screening techniques such as organ bath preparations, cell-based assays, and oocyte-expressed receptor testing. This blind screening approach is the bottleneck in exploring the biodiversity of scorpion venom components as it can take months, if not years, to ascertain the target of peptides with no homology to existing toxins.

Future proteomic, genomic, and transcriptomic studies promise to reveal peptides with new folds and receptor targets, which may be useful as drug leads, pharmacological tools, and bioinsecticides (Smith et al. 2013).

Cross-References

► Scorpion Venom Gland Transcriptomics

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