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Review

Understanding and confronting snakebite envenoming: The harvest of cooperation

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ABSTRACT

During 45 years, the Instituto Clodomiro Picado (ICP, University of Costa Rica) has developed an ambitious scientific, technological, productive, and social program aimed at providing a better understanding of snakes and their venoms, contributing to the development, production and distribution of antivenoms, improving the prevention and management of snakebite envenomings, and strengthening human resources in science and technology. Among other topics, its research agenda has focused on the local tissue alterations induced by viperid snake venoms, i.e. myonecrosis, hemorrhage, dermonecrosis, extracellular matrix degradation, lymphatic vessel damage, and inflammation. In addition, the preclinical efficacy of antivenoms has been thoroughly investigated, together with the technological development of novel antivenoms. ICP's project has been based on a philosophical frame characterized by: (a) An integrated approach for confronting the problem of snakebites, involving research, production, extension activities, and teaching; (b) a cooperative and team work perspective in the pursuit of scientific, technological, productive, and social goals; (c) a search for excellence and continuous improvement in the quality of its activities; and (d) a vision of solidarity and compassion, based on the realization that snakebite envenomings mostly affect impoverished vulnerable populations in the rural settings of developing countries. A key aspect in this program has been the consolidation of international partnerships with groups of all continents, within a frame of academic and social cooperation, some of which are described in this review.

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1. The dawn of Costa Rican toxinology

The scientific study of venomous snakes and their venoms in Costa Rica started with the pioneering work of Clodomiro Picado Twight (1887–1944), during the first decades of the 20th century (Fig. 1). Picado, a talented person interested in natural history, was awarded a scholarship to perform advanced studies at La Sorbonne, from 1908 to 1913. During his last year in France, he also received training in Medical Microbiology at Institut Pasteur. Upon his return to Costa Rica, Picado was appointed Director of the Clinical Laboratory of Hospital San Juan de Dios, at that time the main health center in the country. In addition to introducing modern methods for laboratory diagnosis, which represented a leap forward in the medical practice in the country, he established an ambitious research program which covered a wide array of topics, including Medical Microbiology, Immunology, Experimental Pathophysiology, Clinical Chemistry, Hematology, Therapeutics, Phytopathology, and General Biology (Gutiérrez, 1986).

One of the subjects that captured the attention of Picado was the problem of snakebite envenoming, which was then, as it is now, a serious public health hazard in the country. Taking an integrated approach to the subject, he developed a serpentarium at the hospital, collected venoms, and studied a variety of aspects including the biology of snakes, the toxicology of venoms, and the therapy of envenomings. His contributions in the field of Toxinology are summarized in the book *Serpientes Venenosas de Costa Rica. Sus Venenos. Seroterapia Antiofídica* (Picado, 1931). It may be said that Picado introduced in Costa Rica what is currently known as 'translational research', since he went from the laboratory studies to the implementation of practical solutions to the problem. Being aware of the developments that had taken place since 1901 in Brazil, associated with the production of the first antivenoms in Latin America, at Instituto Butantan, under the leadership of Vital Brazil Mineiro da Campanha (Brazil, 1911), Picado hypothesized that the similarities between the venoms of Brazilian and Costa Rican viperid snakes could imply that antivenoms manufactured in Brazil would be effective in Costa Rica. Through an agreement with Instituto Butantan, Brazilian antivenoms were imported to Costa Rica, and used in the treatment of patients. The success of this approach initiated the modern era of snakebite envenoming therapy in Central America (Picado, 1931).

In addition to scientific research and the introduction of antivenoms, Picado also worked in the political front, promoting, in association with the Minister of Health of Costa Rica, Solón Núñez Frutos, a unique and highly progressive legislation that, among other issues, implemented the use of antivenoms in the country, the access of this therapy in rural settings, and the obligation of land owners to keep a stock of antivenom in case their workers suffered a snakebite (Picado, 1931; Gutiérrez, 2010).

The legacy of Clodomiro Picado germinated in the 1960s as an

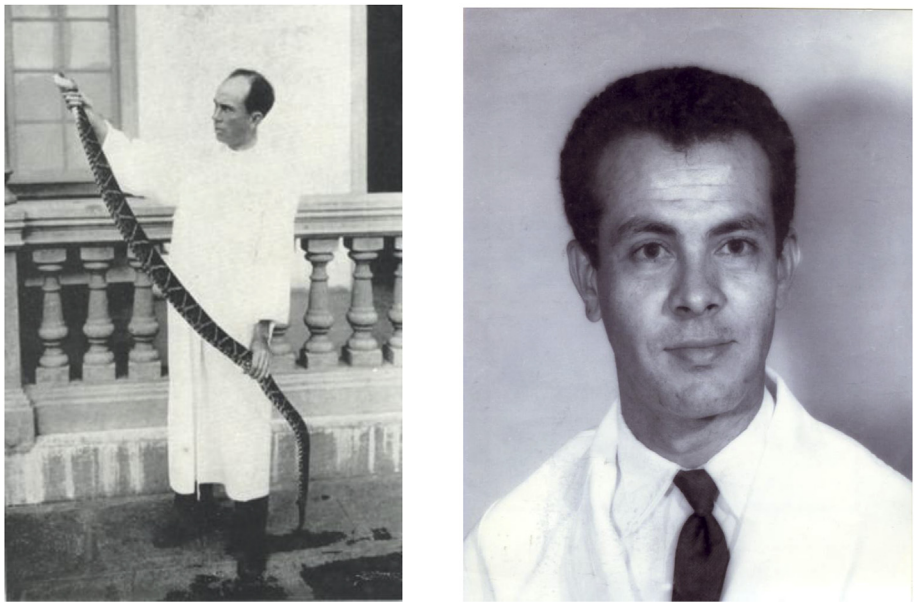

Fig. 1. Pioneers of Toxinology in Costa Rica. Left, Clodomiro Picado Twight (1887–1944). After completing advanced studies in France, Picado was appointed Director of the Clinical Laboratory of Hospital San Juan de Dios (San José, Costa Rica). In addition to his work on diagnostic laboratory aspects, he developed an ambitious research program which included, among other topics, the study of snakes, snake venoms, and snakebite envenomings. Right, Róger Bolaños Herrera (1931–2007), professor of Immunology at the School of Microbiology (University of Costa Rica). In the decade of 1960 he was involved in an inter-institutional program aimed at producing antivenoms in Costa Rica. Owing to the success of this project, the Instituto Clodomiro Picado was created in 1970, and Bolaños was its Director from 1970 to 1980. He performed studies on snake venoms and antivenoms, and promoted an innovative integrated approach for confronting snakebite envenomings, involving research, antivenom production, teaching and extension. Reprinted from Toxicon 50: 170–171, Gutiérrez, *Bothrops asper*: Beauty and peril in the Neotropics, copyright 2009, and Toxicon 54: 901–903, Gutiérrez, Obituary: Róger Bolaños, copyright 2007, with permission from Elsevier.

interinstitutional ambitious project known as *Programa de Sueros Antiofídicos* (Program for Antivenoms). This effort, jointly promoted by the Ministry of Health, the University of Costa Rica and the Embassy of the United States of America in Costa Rica, was aimed at initiating the local production of antivenoms (Gutiérrez, 2010). It was developed in the context of a profound transformation of the public health institutions in Costa Rica, as part of the social-democratic project that dominated the political landscape of the country in those years (Jaramillo, 1993). Under the leadership of Róger Bolaños (1931–2007) (Fig. 1) and Herschel H. Flowers, the program succeeded in producing the first batches of polyvalent and anticoral antivenoms in 1967 (see Gutiérrez (2010) for a detailed account of the origins and goals of the project).

The success of this initiative led to the creation of Instituto Clodomiro Picado (ICP), in 1970, as the institution responsible for the manufacture of antivenoms for the country, with Róger Bolaños appointed as its first Director. Bolaños, a professor of Immunology at the School of Microbiology of the University of Costa Rica, promoted an integrated approach to the issue of snakebite envenoming, one that inherited and expanded the philosophy implemented by Clodomiro Picado Twight (Gutiérrez, 2007, 2010). In 1972, through an agreement between the Ministry of Health and the University of Costa Rica, the administration of ICP was transferred to this university; this event has had significant implications for the strengthening of Toxinology and of antivenom production in the country.

2. The philosophical basis of an ambitious project

Róger Bolaños and his colleagues at ICP had a vision on how to study and confront snakebite envenoming which, since then, has been the philosophical basis for the development of ICP. This philosophy, which fits within the main goals of the University of Costa Rica, is based on four main pillars:

(a) The view that, for an effective approach to this complex public health problem, an integrated perspective had to be implemented, including (i) scientific and technological research on snakes, venoms and antivenoms; (ii) development, production and distribution of antivenoms; (iii) strengthening of human resources through graduate and undergraduate teaching programs at the university; and (iv) extension programs to convey the basic knowledge of prevention and management of snakebite envenomings to communities, vulnerable social groups, and health professionals.

(b) A permanent search for excellence in all aspects of ICP's activities, getting away from comfort zones, and searching for new scientific, technological, productive and social challenges.

(c) The promotion of a cooperative and team work approach in the fulfillment of objectives. The confrontation of the difficult tasks that ICP had to undertake was possible only through a collective agenda, characterized by a continued coordination between the various sections and workers. This cooperative perspective was implemented not only within ICP, but also in the collaborations that this institute has established with other groups in many countries along the years.

(d) A philosophy of solidarity and compassion, based on the fact that snakebite envenomings cause much suffering among vulnerable human groups living in impoverished rural settings. Thus, in the long term, the efforts carried out on various fronts by ICP have had the aim of reducing such suffering, through science, technology, and social entrepreneurship.

3. Scientific research at ICP: towards understanding the complex landscape of local tissue damage induced by viperid snake venoms

One of the most drastic consequences of viperid snakebite envenomings is the development of local pathological effects, characterized by edema, myonecrosis, dermonecrosis, blistering, and hemorrhage (Warrell, 2004; Cardoso et al., 2009). If not treated promptly and effectively, this may result in permanent tissue damage and sequelae, with long lasting physical and psychological effects in affected people. This subject has been of interest to researchers at ICP since the early years of the decade of 1980. After a general characterization of myotoxic and hemorrhagic activities of Costa Rican viperid venoms (Gutiérrez and Chaves, 1980), various research projects have contributed to understanding the complexity of these phenomena.

3.1. Myonecrosis

When describing the myotoxic activity induced by the venom of *Bothrops asper*, the most important venomous snake of Central America, it was proposed that the quantification of the plasma levels of the enzyme creatine kinase (CK) could be used as a marker to assess the extent of myonecrosis (Gutiérrez et al., 1980). The isolation of a myotoxic phospholipase A_2 (PLA_2), and the study of its mechanism of action, was performed in collaboration with Charlotte L. Ownby, the author's PhD supervisor, at Oklahoma State University (USA). It was proposed that this myotoxin induces acute muscle damage by first affecting the integrity of skeletal muscle plasma membrane, through phospholipid hydrolysis, thus promoting a prominent calcium influx which sets the stage for a series of intracellular degenerative events that lead to irreversible cell damage (Gutiérrez et al., 1984a, b).

Further studies in Costa Rica led to the isolation of several new myotoxins from various snake venoms (see Lomonte and Rangel (2012) for a review). Surprisingly, some of these myotoxins had PLA_2 structure, but were devoid of catalytic activity (Lomonte and Gutiérrez, 1989). Through a collaboration with the group of Ivan I. Kaiser, at the University of Wyoming (USA), it was found that one of these toxins, *B. asper* myotoxin II, is a Lys49 PLA_2 homologue, presenting modifications not only at position 49 but also in some residues of the so called 'calcium-binding loop' (Francis et al., 1991). This myotoxin has become a useful tool to investigate the mechanisms involved in skeletal muscle damage in the absence of phospholipid hydrolysis. Working with this toxin at the laboratory of Lars Å. Hanson, at the University of Göteborg (Sweden), Bruno Lomonte identified a stretch of hydrophobic and cationic residues in the C-terminal region of myotoxin II which was responsible for membrane damage (Lomonte et al., 1994a). Later on, the 3D structure of this myotoxin was described in collaboration with Rhaguvir R. Arni, of Universidade Estadual Paulista (Brazil) (Arni et al., 1995). The ability of catalytically-active and inactive myotoxins to disrupt bilayers was then explored using liposomes as models; it was observed that liposomes made of negatively-charged phospholipids were more susceptible to the action of these toxins (Díaz et al., 1991; Rufini et al., 1992).

The use of myoblast/myotube cell cultures for assessing the cytotoxic activity of snake venom myotoxins has been instrumental for deciphering various aspects of their mechanism of action (Lomonte et al., 1999), such as the role of toxin dimerization in the ability to induce cytotoxicity (Angulo et al., 2005), the effect of cholesterol and membrane fluidity in the susceptibility of myoblasts (Rangel et al., 2011), and the synergistic action of Asp49-and Lys49 PLA_2s (Mora-Obando et al., 2014). On the other hand, when exploring myotoxicity induced by elapid venom PLA_2s, and in

collaboration with the groups of Monica Thelestam and Hans Jörnvall, at Karolinska Institutet (Sweden), Alberto Alape-Girón proposed a hypothesis describing the evolutionary steps in the acquisition of myotoxicity in these PLA_2s, and identified key residues in these PLA_2s necessary to exert acute muscle damage (Alape-Girón et al., 1999).

More recently, through a collaboration with the group of Cesare Montecucco, at the University of Padova (Italy), new insights have been gained on the mechanism of action of viperid myotoxic PLA_2s. Using a mass spectrometric approach to quantify phospholipids and lysophospholipids in muscle tissue and myotubes in culture, it was found that a catalytically-active myotoxic PLA_2 induced predominantly hydrolysis of phosphatidylcholine, whereas a catalytically-inactive PLA_2 homologue did not, thus demonstrating that intracellular PLA_2s do not seem to play a key role in membrane damage in this model (Fernández et al., 2013). Moreover, using single cell fluorescent measurement with Fura-2, it was found that both types of myotoxins induced a rapid increment in cytosolic calcium, which was largely abrogated by eliminating extracellular calcium (Cintra-Francischinelli et al., 2009). A possible adaptive role for having catalytically-active and -inactive myotoxic PLA_2s in a single venom was inferred from studies showing a synergism between these two types of myotoxins in promoting damage to muscle cell plasma membrane (Mora-Obando et al., 2014).

On the basis of these experimental observations, a unified view of the mechanism of action of these myotoxins has been proposed (Gutiérrez and Ownby, 2003; Montecucco et al., 2008; Lomonte and Rangel, 2012) (Fig. 2). Myotoxic PLA_2s and PLA_2 homologues interact with as yet unidentified 'acceptors' in skeletal muscle cell plasma membrane, provoking rapid membrane disruption by catalytically-dependent and independent mechanisms. The main consequence of this membrane perturbation is a rapid influx of extracellular calcium which, in turn, results in: (a) Hypercontraction of myofilaments, (b) calcium uptake by mitochondria, eventually resulting in calcium overload and mitochondrial damage, and (c) activation of calcium-dependent intracellular proteinases (calpains) and probably PLA_2s.

3.2. Hemorrhage

Local and systemic bleeding are two frequent and serious manifestations of viperid snakebite envenomings (Warrell, 2004; Cardoso et al., 2009). The ability of these venoms to induce hemorrhage depends on the action of zinc-dependent metalloproteinases (SVMPs) on the microvasculature (Fox and Serrano, 2005; Gutiérrez et al., 2005a). The characterization of hemorrhagic SVMPs from the venom of *B. asper* was undertaken in a collaborative project between ICP and Michael Ovadia and Gadi Borkow, from the University of Tel Aviv (Israel) (Borkow et al., 1993; Gutiérrez et al., 1995). A P-I SVMP isolated from this venom, named BaP1, has become a useful tool for investigating the mechanism of microvessel damage and hemorrhage (Gutiérrez et al., 1995; Watanabe et al., 2003). Its primary and tertiary structures were described through collaborations with Raghuvir Arni (Universidade Estadual Paulista, Brazil) and Jay W. Fox (University of Virginia, USA), and later on with Torsten Lingott and Irmgard

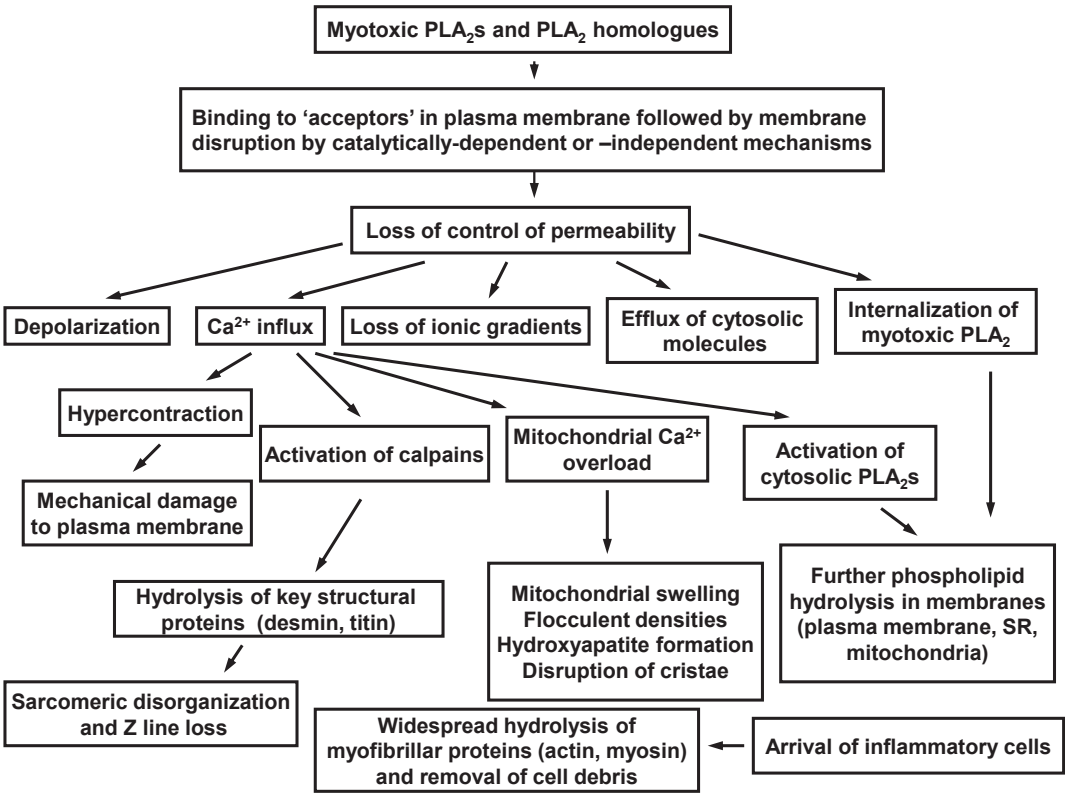

Fig. 2. Proposed sequence of cellular events occurring as a consequence of the action of myotoxic PLA_2s and PLA_2 homologues from viperid venoms in skeletal muscle cells. Myotoxins bind to as yet unidentified 'acceptor' sites at the plasma membrane, inducing a rapid and drastic membrane perturbation which is based on catalytically-dependent (in the case of Asp49 PLA_2s) and catalytically–independent (in the case of Lys49 PLA_2 homologues) events. Membrane damage results in the loss of the control of permeability to ions and macromolecules. The main consequence of this alteration is a prominent Ca^{2+} influx which triggers a series of intracellular degenerative events, such as myofilament hypercontraction, mitochondrial alterations, and activation of cytosolic Ca^{2+}-dependent proteinases and PLA_2s. Muscle cells rapidly pass the 'point of no return' and become irreversible damaged. Reprinted, with slight modifications, from Toxicon 42: 915–931, Gutiérrez and Ownby, Skeletal muscle degeneration induced by venom phospholipases A_2: Insights into the mechanisms of local and systemic myotoxicity, copyright 2003, with permission from Elsevier.

Merfort (University of Freiburg, Germany) (Watanabe et al., 2003; Lingott et al., 2009).

The mechanism of action of hemorrhagic SVMPs has been explored in depth at ICP, using a variety of experimental approaches that include light and electron microscopy, intravital microscopy, immunohistochemistry, endothelial cell cultures, Western blot analysis, and proteomic characterization of exudates collected in the vicinity of damaged tissue. Light and electron microscopic observations revealed a rapid disruption of capillary vessel structure after injection of SVMPs, characterized by a drop in the number of pinocytotic vesicles and a reduction in the width of endothelial cells, associated with a distention and eventual disruption of capillary wall (Moreira et al., 1994; Rucavado et al., 1995). Interestingly, SVMPs do not induce direct damage of endothelial cells in culture, but instead provoke the detachment of these cells, which eventually die by apoptosis (Lomonte et al., 1994b; Rucavado et al., 1995; Díaz et al., 2005).

The action of SVMPs on the basement membrane (BM) surrounding endothelial cells in capillaries has been investigated by Teresa Escalante, Alexandra Rucavado, Cristina Herrera, and the author, in collaboration with the groups of Jay W. Fox (University of Virginia, USA), Sussan Nourshargh and Mathieu-Benoit Voisin (Queen Mary University of London, UK), and Eladio F. Sánchez (Fundaçao Ezequiel Dias, Brazil). By a combination of an *in vitro* model, based on hydrolysis of Matrigel, a BM preparation from a sarcoma cell line, and *in vivo* and *ex vivo* assessment of BM hydrolysis, based on Western blotting and immunohistochemistry analyses, it was found that SVMPs are able to degrade laminin, type IV collagen, nidogen, and heparan sulphate proteoglycan (Escalante et al., 2006, 2011a; Herrera et al., 2015). When comparing the action of P-I hemorrhagic and non-hemorrhagic SVMPs, a striking difference was noticed regarding the hydrolysis of type IV collagen (Escalante et al., 2011a). It was thus suggested that enzymatic degradation of this BM component is a key step in the pathogenesis of hemorrhage, owing to the prominent role that this collagen plays in the mechanical stability of BM and the capillary wall. This hypothesis has been recently supported by further observations with hemorrhagic P-I, P-II, and P-III SVMPs (Herrera et al., 2015).

These studies have been complemented by a new approach to assess venom-induced tissue damage, based on the proteomic characterization of exudates collected in the vicinity of tissues injected with venoms or isolated toxins. These analyses, performed in collaboration with Jay W. Fox, provide a 'window' through which more subtle aspects of tissue damage can be explored (Escalante et al., 2009, 2011a; Rucavado et al., 2011). In particular, fragments of several extracellular matrix (ECM) proteins have been detected, thus revealing hitherto unknown aspects of SVMP action in the tissues.

Taken together, these experimental findings on the mechanism of action of hemorrhagic SVMPs have been unified in a 'two-step model' (Fig. 3). Initially, hemorrhagic SVMPs bind and hydrolyze components in the BM of capillary vessels, the degradation of type IV collagen being of particular relevance. P-II and P-III SVMPs bind more specifically to capillary BM than P-I SVMP, owing to the presence of exosites in the disintegrin, disintegrin-like and cysteine-rich domains of these SVMPs (Baldo et al., 2010; Herrera et al., 2015), thus explaining their generally higher hemorrhagic activity. BM degradation results in the weakening of the mechanical stability of the capillary wall; as a consequence, the hemodynamic forces normally operating in the vasculature *in vivo*, i.e. hydrostatic pressure and shear stress, provoke the distention and the eventual disruption of the capillary wall, leading to extravasation (Gutiérrez et al., 2005a; Escalante et al., 2011b).

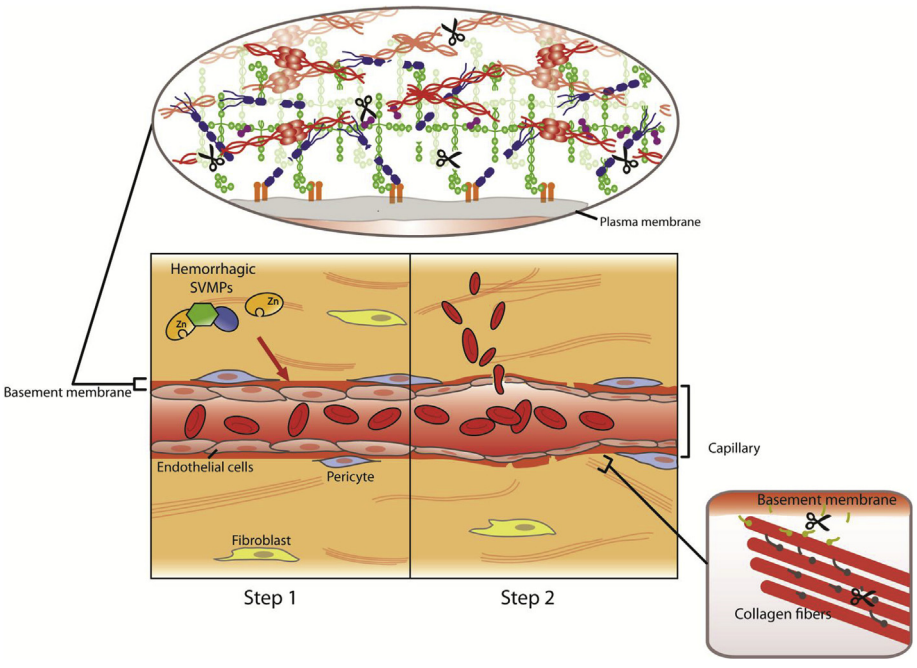

Fig. 3. Proposed 'two-step' mechanism of action of snake venom hemorrhagic zinc-dependent metalloproteinases (SVMPs) in capillary blood vessels. SVMPs hydrolyze key components of the basement membrane (BM) that surrounds endothelial cells in capillary blood vessels. P-III SVMPs in general have a higher hemorrhagic potential than P-I SVMPs due to the presence of exosites in the disintegrin-like and cysteine-rich domains, which enable them to bind to targets in the BM. The most important substrate for the proteolytic activity of hemorrhagic SVMPs is likely to be type IV collagen, since it plays a key role in the mechanical stability of the BM and hence of the capillary wall. After this first step, i.e. proteolysis of BM components, the capillary wall becomes weakened, and the action of hemodynamic biophysical forces operating in the circulation, i.e. hydrostatic pressure and shear stress, provokes the distention of endothelial cell and, eventually, the disruption in the integrity of the capillary wall, resulting in extravasation. Reprinted from Journal of Proteomics 74: 1781–1794, Escalante et al., Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases, copyright 2011, with permission from Elsevier.

3.3. Damage to skin and to lymphatic vessels

Two additional aspects of viperid venom-induced local pathology that have been investigated at ICP are the effects on skin and lymphatic vesicles. SVMPs induce blistering and dermonecrosis, as a consequence of the degradation of proteins of the dermal–epidermal junction (Jiménez et al., 2008; Escalante et al., 2009). On the other hand, *B. asper* venom and a Lys49 PLA_2 homologue from this venom induce a reduction in the lumen of collecting lymphatic vessels in the mesentery, followed by the halting of lymph flow (Mora et al., 2008). This effect is secondary to the action of this myotoxin on smooth muscle cells of the lymphatic vessel wall.

3.4. Exploring the causes of deficient skeletal muscle regeneration after venom-induced myonecrosis

Clinical observations indicate that regeneration of skeletal muscle after myonecrosis in viperid snakebite envenoming is often deficient, thus resulting in permanent tissue loss and disability. A successful muscle regenerative process depends on a number of factors, namely the removal of necrotic material, the presence of intact blood supply and innervation, and the permanence of the BM surrounding necrotic fibers, which serves a scaffolding role in the regenerative process (Ciciliot and Schiaffino, 2010). These requirements are largely hampered in muscle tissue affected by viperid snake venoms. In the case of the venom of *B. asper*, our studies in collaboration with Alexandra Rucavado (ICP) and Rosario Hernández and Patricia Saravia-Otten (Universidad de San Carlos, Guatemala) have shown that the density of microvessels and of axons within intramuscular nerves is greatly reduced, as a consequence of the action of tissue-damaging toxins (Gutiérrez et al., 1984c; Arce et al., 1991; Hernández et al., 2011). The observation of TUNEL-positive regenerative cells strongly supports the view that the tissue microenvironment does not provide the conditions necessary for regeneration (Hernández et al., 2011). Through a collaboration with Stefano Gastaldello (Karolinska Institutet, Sweden) and Patricia Saravia-Otten, it was found that homogenates of muscle tissue collected several days after injection of *B. asper* venom and toxins inhibit myoblast cell proliferation and fusion into myotubes in a cell culture model (Saravia-Otten et al., 2013). It is expected that a more thorough understanding of the basis of this poor regenerative process may lead the way to therapeutic interventions aimed at improving this outcome.

3.5. Inflammation in venom-affected tissues

The action of viperid snake venoms in the tissues is associated with a complex inflammatory reaction, characterized by edema, pain, and inflammatory cell infiltrate. Inflammation induced by the venom of *B. asper* and by purified SVMPs and myotoxic PLA_2s and PLA_2 homologues has been investigated through collaborations between ICP and the groups of Catarina F.P. Teixeira and Yara Cury (Instituto Butantan, Brazil) (see reviews by Teixeira et al., 2003a, 2009). Edema and pain occur as a consequence of the activation of several inflammatory pathways, with the release of histamine, and the generation of prostaglandins, leukotrienes, kinins, nitric oxide, complement anaphylatoxins, and cytokines, together with the synthesis of proteinases, mostly matrix metalloproteinases (MMPs) (Rucavado et al., 2002; Teixeira et al., 2009). These and other mediators act on venular endothelial cells and on a variety of nociceptors in afferent neurons, provoking edema and pain. PLA_2s and SVMPs also act directly on resident macrophages and other inflammatory cells, inducing their activation (Zamunér et al., 2001; Zuliani et al., 2005; Leiguez et al., 2011).

Whether inflammation contributes to further tissue damage in muscle injected with the venom of *B. asper* is an open question that has been explored by Fernando Chaves and colleagues at ICP, and by researchers at Instituto Butantan (Teixeira et al., 2003b; Chaves et al., 2005, 2006). Other studies have focused on the possible role in venom-induced pathology of tissue components, i.e. danger signals or DAMPs, released as a consequence of myonecrosis and ECM degradation. For example, the release of ATP from damaged muscle cells results in the expansion of myocyte damage through the action of this nucleotide on purinergic receptors, resulting in the increment of cytosolic calcium and the consequent cytotoxicity (Cintra-Francischinelli et al., 2010).

The large body of experimental observations gathered over the years in the study of venom-induced local tissue damage by researchers of ICP and other groups is summarized in Fig. 4. There is a direct toxic action of venom components, mostly SVMPs, PLA_2s and PLA_2 homologues. These early pathological events, in turn, generate an inflammatory reaction, in association with the direct effect of venom components on resident and inflammatory cells. The myriad of inflammatory mediators released in the tissue set the onset of reparative and regenerative events, but might also contribute to further tissue damage and to impaired regeneration. A more deep understanding of this complex scenario is necessary for the design of knowledge-based therapeutic interventions aimed at ameliorating the magnitude of tissue damage and at improving the regenerative outcome. These are urgent challenges that demand renewed collaborative research efforts.

4. Towards a better characterization of the preclincal efficacy of antivenoms

The traditional way to evaluate the capacity of antivenoms to neutralize snake venoms is based on the lethality neutralization test, which is the gold standard for assessing antivenom potency (WHO, 2010; Gutiérrez et al., 2013). This test involves the incubation of a fixed dose of venom with various dilutions of antivenom, using a control group in which venom is incubated with saline solution instead of antivenom. After an incubation interval, aliquots of the mixtures, containing a 'challenge dose' of venom (usually either 3, 4 or 5 Median Lethal Doses, LD_{50}s) are injected in mice, and lethality is observed. Antivenom efficacy is thus expressed as the Median Effective Dose (ED_{50}), i.e. the ratio venom/antivenom at which half of the injected animals survive (WHO, 2010; Gutiérrez et al., 2013). Using this experimental approach, Bolaños demonstrated that the polyvalent (viperid) antivenom manufactured at Instituto Clodomiro Picado (ICP) is effective in the neutralization of lethality of Costa Rican venoms of snakes from the family Viperidae (Bolaños, 1971).

Following early developments, especially by David Theakston and colleagues, at the Liverpool School of Tropical Medicine (UK) (Theakston and Reid, 1983), and by researchers at ICP, a new rationale of the assessment of the preclinical efficacy of antivenoms was developed. Since viperid, and some elapid, venoms induce a variety of pathologically- and pathophysiologically-relevant effects, in addition to lethality, it was considered that a more thorough characterization of antivenoms' preclinical efficacy should be based on the analysis of the neutralization of various toxic and enzymatic activities (Gutiérrez et al., 2013). In the early years of the 1980s, our group at ICP initiated a long lasting effort to characterize the efficacy of ICP antivenoms (Gutiérrez et al., 1981, 1985, 1996), and then applied these methodologies to assess other antivenoms against venoms from different regions of the world. Simple methods were adapted or developed for the study of hemorrhagic, myotoxic, dermonecrotic, edema-forming, coagulant, defibrinogenating, proteolytic, hyaluronidase, and PLA_2 activities of venoms (see

reviews by Gutiérrez et al., 1996, 2013).

Regarding the neutralization of *Bothrops* sp snake venoms, which inflict the highest number of snakebites in Latin America, these studies have revealed a high extent of cross-neutralizing ability of various antivenoms against several *Bothrops* sp venoms (see for example Bogarín et al., 2000; Rojas et al., 2005; Segura et al., 2010a). One of the most extensive studies performed in this subject involved the participation of ten Latin American public institutions from Brazil, Argentina, Peru, Bolivia, Colombia, Panama and Costa Rica, under the auspices of the Program CYTED (*Ciencia y Tecnología para el Desarrollo*) (Segura et al., 2010a). In contrast to the extensive cross-neutralization of *Bothrops* sp venoms by antivenoms, it was found that antivenoms produced using venoms of the rattlesnake *Crotalus simus* from Central America are ineffective in the neutralization of venoms of subspecies of *Crotalus durissus* from South America, and viceversa (Saravia et al., 2002). This methodological platform has been also used to investigate the preclinical efficacy of antivenoms used in sub-Saharan Africa (Segura et al., 2010b; Sánchez et al., 2015), and in Papua New Guinea (Vargas et al., 2011).

A significant step forward in our understanding of antivenom's ability to bind venom components came as a corollary of the developments in the field of snake venom proteomics, i.e. 'venomics', mostly through the work of the group of Juan J. Calvete, at the Instituto de Biomedicina de Valencia (Spain) (Calvete et al., 2007). Through an intensive collaboration with Calvete's group, scientists at ICP have been able to characterize the proteomes of almost all the venoms of Costa Rican snakes of the families Viperidae and Elapidae (see Lomonte et al. (2014) for a review). Using the information gathered from proteomics, the methodology known as 'antivenomics' was developed (Lomonte et al., 2008; Pla et al., 2012). The so called 'second generation' antivenomics protocol consists in performing affinity chromatography, by passing snake venoms through a Sepharose gel coupled with antivenom antibodies. In this way, venom components which are recognized, or not recognized, by antivenom antibodies can be identified and ascribed to the corresponding venom protein families (Pla et al., 2012; Gutiérrez et al., 2014). Using this approach, a detailed account of the immunoreactivity of the polyvalent (Viperidae) antivenom manufactured at ICP, when confronted with many snake venoms, has been obtained (see Table 1 in Gutiérrez et al., 2014). The combination of neutralization tests and antivenomics now provides a powerful methodological approach to analyze in detail the preclinical efficacy of antivenoms, and to make knowledge-based decisions on how to improve antivenoms' neutralizing coverage (Gutiérrez et al., 2013).

5. Confronting snakebite envenoming from a broad perspective: Central America and beyond

5.1. Improvements and innovation in antivenom manufacture

In addition to the described research, ICP has approached the problem of snakebite envenoming by an integrated perspective which includes the development, production and distribution of antivenoms, and the creation of extension programs and advocacy efforts at various levels. During its first decade, ICP manufactured the antivenoms that were needed in Costa Rica (Bolaños and Cerdas, 1980). On the basis of permanent innovation, the volume of antivenom production steadily increased over the years, and ICP started to cover the antivenom needs of other Central American countries. These improvements have been possible due to the commitment and dedication of the staff of the Industrial Division, the Administrative Section, and the Directors of ICP. This institute provides polyvalent (Viperidae) and anti-coral (Elapidae) antivenoms to Panama, Nicaragua, Honduras, El Salvador, Guatemala, and Belize. More recently, ICP polyvalent antivenom has been distributed to Ecuador, Peru, Colombia, Trinidad and Tobago, and

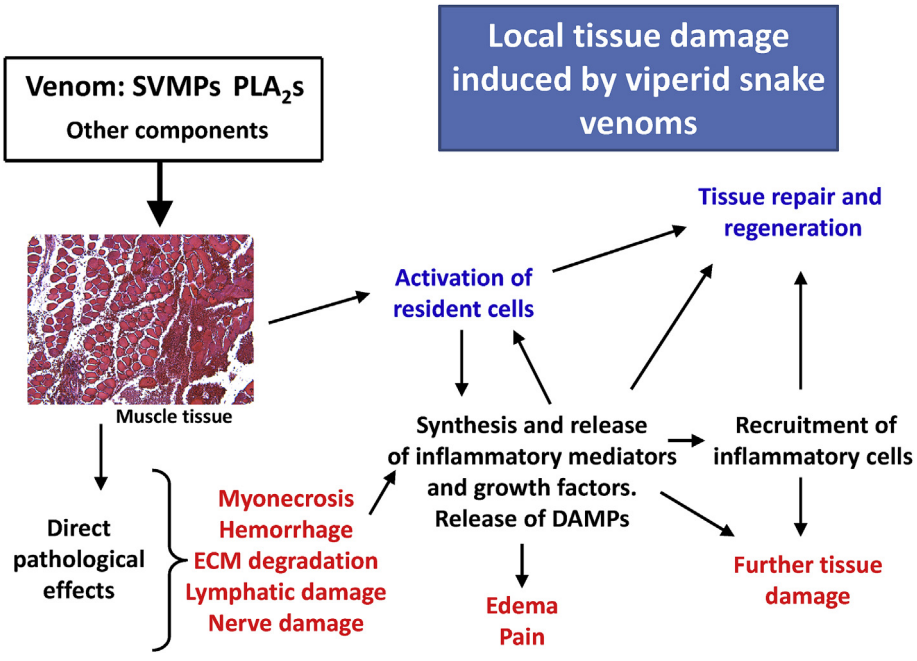

Fig. 4. Scheme that summarizes the main events in the pathogenesis of local tissue damage and inflammation induced by viperid snake venoms. Venom components, mostly myotoxic phospholipases A_2s (PLA_2s) and zinc-dependent metalloproteinases, induce direct pathological effects upon injection in muscle tissue, such as myonecrosis, hemorrhage, degradation of extracellular matrix (ECM), and damage to lymphatic vessels and nerves. Such widespread tissue pathology generates an inflammatory response which causes pain and edema. Resident cells in the tissue, such as macrophages and mast cells, become activated and contribute to the inflammatory scenario which might lead to resolution of tissue damage or may provoke further pathology. In turn, inflammation is followed by reparative and regenerative responses which, depending on the balance between the various processes in the tissue, may result in functional regeneration or in scarring and tissue loss. Modified from Gutiérrez et al. (2007b).

the island of Saint Lucia.

Initially, antivenoms were manufactured at ICP by a salting-out procedure involving ammonium sulphate precipitation of plasma proteins (Bolaños and Cerdas, 1980). A significant innovation was introduced in the early 1990s by the use of caprylic acid precipitation to separate non-immunoglobulin from immunoglobulin proteins in equine plasma (Rojas et al., 1994), following previous developments in Brazil (dos Santos et al., 1989). This protocol generates antivenoms of high purity and yield, and of good physicochemical quality, thus becoming an alternative for antivenom production; in fact, it is being currently used in laboratories of several countries in Latin America and Asia. The creation of the Technological Development Section (SEDETEC) at ICP provided the impetus for the improvement of the technologies for antivenom manufacture, and has generated new antivenoms for other regions. Among other achievements, Guillermo León, Álvaro Segura, Mariángela Vargas, María Herrera, and Mauren Villalta, at SEDETEC, have adapted a new 'two phase aqueous system' method for equine plasma fractionation (Vargas et al., 2015), and have introduced improvements in aspects such as freeze-drying (Herrera et al., 2014b) and stability (Segura et al., 2009) of antivenoms, in addition to investigating the mechanisms of adverse reactions to these immunobiologicals (León et al., 2013), among other contributions.

5.2. A better understanding of snakebite epidemiology: providing information for public health interventions

ICP has been interested in the study of the epidemiology of snakebites in Costa Rica (Bolaños, 1984; Arroyo et al., 1999; Sasa and Vázquez, 2003). In addition, the use of Geographical Information Systems (GIS) tools allowed a detailed analysis of the incidence of snakebites in the various regions of the country, and showed that the highest incidence occurs in the Central and South Pacific regions, in the Talamanca mountain range, in the Caribbean, and in some counties of the Northern region (Hansson et al., 2013). Therefore, although the general incidence of snakebites in the country is around 14 per 100,000 population per year, drastic differences in incidence occur at the county and district levels, some of which have incidences higher than 100 per 100,000 population per year (Hansson et al., 2013). The study also identified locations where transportation to health facilities is more delayed. Another study found that the incidence of snakebites is affected by weather fluctuations, and an association was observed between snakebites and the cold and hot phases of El Niño Southern Oscillation (ENSO) (Chaves et al., 2015). Moreover, it was described that snakebites affect predominantly poor areas, thus reinforcing the concept that this is a 'disease of poverty' (Harrison et al., 2009).

This epidemiological information is of high value for decision makers at the public health system of the country, since it allows the identification of the most vulnerable regions that require special attention. For instance, the deployment of antivenoms to primary health care centers, in addition to clinics and hospitals, can be decided on the basis of this knowledge, in order to ensure the availability of antivenoms where access to hospitals and clinics is delayed. These developments underscore the relevance of establishing channels of communication and coordination between research groups and public health authorities and practitioners.

5.3. Promoting prevention and appropriate management of snakebite cases through extension programs

Since the onset of ICP, an extension program was established for conveying the basic concepts of prevention and early management of snakebites in Costa Rica. It has been targeted especially at high risk communities and groups, such as rural settings, including those of indigenous ethnic groups, agricultural workers, institutions whose staff has to travel to rural areas, and primary and secondary education centers in areas of high incidence of snakebites. A wide array of teaching materials has been prepared, such as manuals in both Spanish and languages of indigenous groups, as in the case of Cabécar communities (Instituto Clodomiro Picado, 2009). These extension activities involve the participation of students from the University of Costa Rica. Additionally, there is a continuous education program on the treatment of snakebite envenomings, directed to professionals in the medical field, mostly physicians and nurses. It includes teaching in university courses, as well as seminars and workshops in clinics and hospitals throughout the country. The use of information and communication technologies has allowed the projection of these programs to rural hospitals in Costa Rica and in other Central American countries. These extension activities, over several decades, have improved the knowledge of the general population on how to prevent snakebites and what to do in the event of an accident. They have also contributed to a better clinical management of snakebite envenomings in health facilities.

5.4. Regional cooperation in Latin America

Latin America has had a long tradition in antivenom production, starting with the pioneering work of Vital Brazil at Instituto Butantan (Vital-Brazil, 1987). There are antivenom manufacturing laboratories in Mexico, Costa Rica, Colombia, Venezuela, Ecuador, Peru, Bolivia, Brazil, and Argentina (Gutiérrez et al., 2007a). In the last decade, regional integration has been promoted between public laboratories devoted to the production and quality control of antivenoms, and ICP has been directly involved in such projects. These initiatives have been supported by the Pan American Health Organization (PAHO), the Japanese International Cooperation Agency (JICA), the Program CYTED (*Ciencia y Tecnología para el Desarrollo*) and, more recently, the Organization of American States (OAS). Activities performed include workshops, technical consultations, training programs, and collaborative research projects (see Gutiérrez et al., 2007a, 2009). The involvement of Instituto Butantan, among other institutions, has been essential, especially through the leadership of Fan Hui Wen. These initiatives have strengthened the regional capacity to produce and control antivenoms, and have consolidated a regional network of institutions (Fig. 5).

Cooperative research projects between Latin American groups have been developed in subjects as varied as biochemistry of snake venoms, the mechanism of action of venoms and toxins, and preclinical assessment and clinical evaluation of efficacy and safety of antivenoms. For instance, through a long standing collaboration between ICP and Rafael Otero-Patiño and colleagues, in Colombia, a number of controlled, double-blind, randomized clinical trials have been performed in that country using the polyvalent antivenom produced at ICP (Otero et al., 1999, 2006; Otero-Patiño et al., 2012).

5.5. Antivenom development for other regions of the world

The experience gained by ICP in technological development, and in the manufacture and distribution of antivenoms in Latin America, paved the way, more than a decade ago, to new projects for the development of novel antivenoms for other regions of the world. Following a WHO workshop held in Potters Bar, London, in 2001 (Theakston et al., 2003), and in the light of a severe crisis in antivenom availability in sub-Saharan Africa (Theakston and Warrell, 2000), ICP joined a partnership with David G. Theakston and Robert Harrison (Liverpool School of Tropical Medicine, UK), David A. Warrell (University of Oxford, UK), Abdusalam Nasidi, Nandul

Fig. 5. Network of public institutions for the production and quality control of antivenoms in Latin America. This network, supported by organizations such as CYTED (Ciencia y Tecnología para el Desarrollo), OAS (Organization of American States), and PAHO (Pan American Health Organization), as well as by the institutions themselves, has promoted a dynamic partnership based on workshops, seminars, collaborative research projects, training of staff, and technical consultations. Through these cooperative initiatives, the regional capacity to produce and control antivenoms has been greatly improved in Latin America. ICP has played a leading role in these efforts. BIRMEX: Laboratorios de Biológicos y Reactivos de México; ICP: Instituto Clodomiro Picado (Costa Rica); INVIMA: Instituto Nacional de Vigilancia de Medicamentos y Alimentos (Colombia); INS: Instituto Nacional de Salud; U Panama: Universidad de Panamá; Biotecfar (Universidad Central de Venezuela); ENFARMA (Ecuador); U de San Marcos: Universidad Nacional Mayor de San Marcos (Perú); INLASA: Instituto Nacional de Laboratorios de Salud (Bolivia); FUNED: Fundação Ezequiel Dias (Brazil); Vital Brazil: Instituto Vital Brazil (Brazil); INCQS: Instituto Nacional de Controle de Qualidade em Saúde (Brazil); Butantan: Instituto Butantan (Brazil); CPPI: Centro de Produção e Pesquisa de Imunobiológicos (Brazil); ANLIS: Administración Nacional de Laboratorios e Institutos de Salud (Argentina).

Fig. 6. ICP has participated in an international partnership aimed at generating antivenoms, improving snakebite envenoming treatment, and performing toxinological research in Nigeria. This partnership, known as EchiTAb Study Group, has involved the Federal Ministry of Health of Nigeria, the Liverpool School of Tropical Medicine (UK), the University of Oxford (UK), and the company MicroPharm, together with ICP. Among the various outcomes of this cooperation, two antivenoms [EchiTAb G (Micropharm) and EchiTAb-Plus-ICP (ICP)] were developed and are regularly distributed to Nigeria. In addition, several research projects have contributed to a better understanding of venoms and envenomings induced by snakes in this country.

Durfa and other Nigerian colleagues (Federal Ministry of Health of Nigeria), and the company Micropharm (UK) (Fig. 6). This consortium, known as EchiTAb Study Group, developed several initiatives aimed at improving antivenom availability for Nigeria. On the basis of epidemiological, clinical, and immunological knowledge, a new antivenom was developed at ICP, using a mixture of the venoms of *Echis ocellatus*, *Bitis arietans* and *Naja nigricollis* for immunization, and the purification of antibodies from horse plasma by the caprylic acid fractionation method (Gutiérrez et al., 2005b). This polyspecific antivenom, initially named 'Pan-African antivenom', and then known as 'EchiTAb-Plus-ICP', proved to be effective at the preclinical level in the neutralization of the three venoms used for immunization, as well as of other venoms of medical relevance of the genera *Echis*, *Bitis* and *Naja* (Gutiérrez et al., 2005b; Segura et al., 2010b; Calvete et al., 2010). Then, this antivenom was evaluated in a large clinical trial performed in Nigeria, and showed satisfactory efficacy and safety profiles in envenomings by *E. ocellatus* (Abubakar et al., 2010). It has been registered in various countries and is now regularly distributed to sub-Saharan Africa.

Following this successful experience, a new partnership was established between ICP and the universities of Melbourne (Australia) and Papua New Guinea (PNG), with the participation of David Williams, Kenneth Winkel, Simon Jensen, Owen Paiva and Teatulohi Matainaho, together with the group of the Industrial Division of ICP. The goal was to develop a potent and safe monospecific antivenom to be used in PNG for the treatment of envenomings by the elapid snake Taipan (*Oxyuranus scutellatus*). In parallel with the proteomic characterization of the venom (Herrera et al., 2012), the new antivenom was shown to be effective at the preclinical level (Vargas et al., 2011; Herrera et al., 2014a). It is now being tested in a clinical trial in PNG and, depending on the results, the long term purpose is to manufacture this antivenom at ICP for distribution and use in PNG. The support of ICP's Directors, Gustavo Rojas, Yamileth Angulo and Alberto Alape, has played a key role in the success of these international initiatives.

Another project has started as a collaboration between ICP, the University of Peradeniya (Sri Lanka), and the non-governmental organization Animal Venom Research International (AVRI, USA), with the participation of Indika Gawarammana, Daniel Keyler, Kimberly McWhorter, Roy Malleappah, and Laki Wickremesinghe, and the group of the Industrial Division of ICP. As a first step, a new polyspecific antivenom effective against the venoms of the most important poisonous snakes of Sri Lanka will be developed. Following a clinical trial, scheduled to start in 2016, the project aims at implementing the transfer of the technology used at ICP for antivenom production to Sri Lanka, with the support of the Technology Transfer Unit (PROINNOVA) of the University of Costa Rica, in order to allow the local manufacture of the antivenom in this Asian country (Keyler et al., 2013). If successful, this new model of cooperation might be adapted, in the future, to other countries and regions.

5.6. Advocacy: raising global awareness of the impact of snakebite envenoming

The commitment of ICP in the search for solutions to reduce the impact of snakebite envenoming has also involved, together with people of other institutions and countries, a number of advocacy efforts. These include the participation in international workshops and congresses, and in the preparation of the *WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins* (WHO, 2010). Moreover, papers have been prepared to promote awareness on the problem of snakebites (Gutiérrez et al., 2006, 2010, 2013), and contacts with national and international health authorities have been established. ICP has been supportive of the Global Snakebite Initiative (GSI), which is currently implementing actions to confront the problem of snakebite envenoming

and its consequences (Williams et al., 2010; www.snakebiteinitative.org).

6. Concluding remarks

This overview highlights part of what ICP has achieved, in Costa Rica and in partnership with groups in many countries, for a better understanding of venomous snakes, venoms, and their effects at experimental and clinical levels. These efforts at the scientific realm have been linked to the development of novel technological platforms for the production and quality control of antivenoms, thus contributing to the improvement of antivenom availability and accessibility in Latin America, sub-Saharan Africa, and PNG. Extension programs at national and regional levels have provided a better understanding of the basic aspects of snakebite prevention and management to communities and health professionals. Finally, as a fourth component of this multifaceted strategy, ICP has strengthened human resources in Costa Rica and elsewhere, through teaching activities at graduate and undergraduate levels. The involvement of students in the research projects of ICP has been of paramount importance. The basic philosophy that has guided this institutional project has been based on the search for excellence and new challenges, a cooperative and collaborative view of scientific work, an integrated approach to understanding and solving a complex public health problem, and a vision of solidarity and compassion.

Ethical statement

The writing of this article followed national and international ethical guidelines in the preparation of scientific manuscripts.

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The achievements discussed in this review have been possible through the commitment and dedication of the people who have worked at ICP during 45 years. In addition, highly fruitful collaborative projects have been performed with colleagues of institutions in many countries; their cooperation has been essential in the fulfillment of the goals described, and is therefore deeply acknowledged. Thanks are due to Prof Alan Harvey for supporting the publication of this review. Finally, the support of the authorities of Universidad de Costa Rica over the years, and of several international agencies which have provided funding for our projects, is also recognized.

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