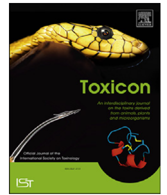
Toxicon 139 (2017) 87–93

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon



An improved technique for the assessment of venom-induced haemorrhage in a murine model

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A R T I C L E I N F O

Article history:
Received 18 August 2017
Received in revised form
23 September 2017
Accepted 8 October 2017
Available online 9 October 2017

Keywords:
Antivenom
Haemorrhage
Minimum haemorrhagic dose
Neutralisation
Snake venom

A B S T R A C T

Haemorrhage is a common clinical manifestation in envenomings caused by bites from snakes of the family Viperidae. Therefore, knowing the haemorrhagic potential of venoms and the capacity of antivenoms to neutralise this effect are of paramount relevance in toxinology. The most widely used method for quantifying haemorrhage involves the intradermal injection of venom (or a mixture of venom/antivenom) in mice, and the assessment of the resulting haemorrhagic area in the inner side of the skin. Although this method allows a straightforward assessment of the haemorrhagic activity of a venom, it does not account for haemorrhagic lesions having a similar area but differing in the depth and intensity of haemorrhage. We have developed an approach that allows the assessment of both area and intensity of a venom-induced haemorrhagic lesion using computational tools and propose a unit to represent the combination of these two factors as a measure of haemorrhage intensity, namely haemorrhagic unit (HaU). A strong correlation was observed between haemoglobin extracted from a haemorrhagic lesion and the associated HaUs. The method was used to determine the haemorrhagic activity of the venoms of *Bothrops asper*, *Echis ocellatus* and *Crotalus basiliscus* and the haemorrhage neutralising capabilities of the three associated antivenoms. Overall, the ease of use, as well as the time involved in this new method, makes its implementation very feasible in the determination of haemorrhagic activity of venoms and its neutralisation by antivenoms in the murine model.

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1. Introduction

Snakebite envenomings pose a significant risk to public health globally, especially in tropical and subtropical regions (Chippaux, 1998; Gutiérrez et al., 2006; Kasturiratne et al., 2008; Warrell, 2010). It is estimated that five million snakebites per annum occur worldwide, leading to 125,000 deaths (Chippaux, 1998). Despite its substantial public health threat, snakebite has not received the attention it deserves by the global scientific community and by public health authorities. These aspects, together with the fact that it largely affects impoverished populations in rural communities (Harrison et al., 2009), makes it a 'neglected tropical disease' (Brown, 2012; Gutiérrez et al., 2006).

Currently, the only specific treatment for systemic envenoming is the intravenous administration of antivenom (Brown, 2012; Gutiérrez et al., 2006; Lalloo and Theakston, 2003; Theakston et al., 2003). Antivenoms are manufactured by immunizing large animals, usually horses, with snake venoms. Owing to the considerable variation in snake venom composition (Calvete, 2011; Casewell et al., 2014; Chippaux et al., 1991), the ability of antivenoms to neutralise different snake venoms has to be carefully assessed at the preclinical level (Gutiérrez et al., 2017). This is particularly relevant when antivenoms raised against the venoms of some snake species are tested against venoms from different species, i.e. heterologous neutralisation.

The gold standard for assessing the preclinical efficacy of antivenoms is based on the neutralisation of lethal effect of venoms in mice (WHO, 2010; Gutiérrez et al., 2013). In addition, and owing to

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https://doi.org/10.1016/j.toxicon.2017.10.005
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the complex pathophysiology of snakebite envenomings, other relevant effects should be tested as well. One such effect is haemorrhage, since many snake venoms, predominantly viperid, induce local and systemic haemorrhage, which is responsible for prominent local tissue damage and cardiovascular disturbances (Warrell, 2010; Escalante et al., 2011). Thus, the experimental assessment of the haemorrhagic activity of venoms, and its neutralisation by antivenoms, is an important tool in toxinological research.

The most widely used method for analysing haemorrhage is the skin test originally developed in rabbits by Kondo et al. (1960), and later on adapted for use in rats (Theakston and Reid, 1983) and mice (Gutiérrez et al., 1985). In the adaptation of this method for mice, a range of different venom concentrations are injected intradermally in the abdominal region. After 2 h, mice are humanely sacrificed and carefully dissected in order to allow the assessment of the inner surface of the skin. Subsequently, the area of the haemorrhagic lesion is measured, and the diameter of the haemorrhagic lesion is estimated. The Minimum Haemorrhagic Dose (MHD) is then determined as the amount of venom that results in a haemorrhagic lesion of 10 mm diameter (Gutiérrez et al., 1985). Although this method allows a straightforward assessment of the haemorrhagic activity of venoms, it has limitations. The main drawback of this procedure is that haemorrhagic lesions having a similar diameter might vary in their depth and in the intensity of haemorrhage (De Roodt et al., 2000). The current methodology does not allow an accurate assessment of these aspects of the haemorrhagic lesions owing to single-variable approach.

The aim of this study was to improve and expand the rodent skin methodology through the application of computational and image analysis tools, allowing a more efficient and accurate analysis of venom induced haemorrhage.

2. Materials and methods

2.1. Snake venoms

Venom of *Bothrops asper* (batch number 03–06 Bap P) was collected from adult specimens captured in Costa Rica and maintained in captivity at the Serpentarium of Instituto Clodomiro Picado, Universidad de Costa Rica. Venom of *Crotalus basiliscus* (batch number Cbb-000301) was collected from 15 adult specimens captured in Mexico and maintained in captivity at the Serpentarium of Laboratorios de Biológicos y Reactivos de México S. A. de C. V. (BIRMEX). Venom of *Echis ocellatus* (batch number 326.051) from Ghana was obtained from Latoxan. Samples of venom correspond to pools obtained from many specimens, and were stabilized by lyophilisation and stored at −20 °C. Solutions of venoms in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 buffer (PBS) were prepared immediately before use.

2.2. Antivenoms

The following antivenoms were used: (a) polyvalent antivenom, manufactured by Instituto Clodomiro Picado (batch number 5720416POLQ); it is prepared from the plasma of horses immunized with a mixture of venoms of *B. asper, Crotalus simus* and *Lachesis stenophrys*. (b) Polyvalent antivenom, manufactured by BIRMEX, batch number SV-189, using the venoms of *B. asper* and *C. basiliscus* for immunization of horses. (c) EchiTab-plus-ICP polyvalent antivenom, manufactured by Instituto Clodomiro Picado (batch number 5750416PALQ), using the venoms of *Echis ocellatus, Bitis arietans* and *Naja nigricollis* for immunization of horses.

2.3. Description of the new method to quantify haemorrhagic activity

The method describes the use of a free image analysis software (Inkscape 0.91) to determine area and intensity of haemorrhagic lesion. The equipment required for this measurement consists of a camera, a standard colour sheet (Pantone with RGB values with the colours 1895 C, 1905 C, 1915 C, 1925 C and 1935 C), a ruler and a light box.

Once skin samples are ready to be analysed, the dissected skin of mice is placed over a white A4 paper sheet. Then, the sheet with the skin is introduced in the light box, and the Pantone and ruler are placed next to the samples. Afterwards the light box is sealed, leaving only an opening for the camera (Supplementary file S1). A photograph is taken while ensuring minimal/no surface reflections or varying lighting across the samples.

A detailed description of the methodology described together with an example to illustrate the procedure is shown in Supplementary file S2. An Excel file is included in Supplementary file S3 to facilitate the processing of the data and the estimation of haemorrhagic activity and its neutralisation by this method.

2.3.1. Luminance conversion factor

This step will establish a correction factor using the true R, G and B values shown in the Pantone. Using the software Inkscape 0.91 (https://inkscape.org/download/), the R, G and B values from the standard Pantone sheet (colours 1895 C, 1905 C, 1915 C, 1925 C and 1935 C) are determined. The software shows the values of each colour in terms of red, green and blue (R, G and B values), which should be converted to luminance according to the following equations. The first step involves adjusting the RGB values for the gamma correction and thus transforming these values to sRGB (scaled RGB) values (Pierre et al., 2015; IEC, 2006). However, since the RGB values are in a value range from 0 to 255, and are required to be in the range from 0 to 1 for further calculations, they need to be divided by 255 (Eq. (1)), where C value is R, G or B values.

$$C\ scaled = \frac{C_{value}}{255} \tag{1}$$

If the R, G or B scaled values obtained using equation (1) are higher or equal than 0.04045, the following formula to adjust the R, G or B scaled value should be used (Eq. (2)), where C scaled corresponds to R, G or B scaled values.

$$C\ adjusted = \left(\frac{C_{scaled} + 0.055}{1 + 0.055}\right)^{2.4} \tag{2}$$

On the contrary, if the value is lower than 0.04045, each of the R, G or B scaled values are divided by 12.92 (Eq. (3)) (Pierre et al., 2015; IEC, 2006), where C scaled is R, G or B scaled values.

$$C\ adjusted = \frac{C_{scaled}}{12.92} \tag{3}$$

Each R, G or B adjusted value is then multiplied by the corresponding conversion factors, according to the luminosity function, which describes the average spectral sensitivity of human visual perception of brightness and states that green light contributes the most to the intensity perceived by humans, and blue light the least. This multiplication will then provide a luminance value for the colour of interest (Eq. (4)) (Pierre et al., 2015; IEC, 2006).

$$Luminance(LU) = 0.2126R_{adjusted} + 0.7152G_{adjusted} + 0.0722B_{adjusted} \tag{4}$$

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