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IS THE OPISTOGLYPH *CLELIA CLELIA* DUVERNOY'S GLAND SECRETION HAEMORRHAGIC IN HUMANS?

¿ES HEMORRAGICA LA SECRECION DE LA GLANDULA DE DUVERNOY DE LA OPISTOGLIFA *CLELIA CLELIA* EN HUMANOS?

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RESUMEN

Se propone una alternativa a la utilización de roedores, para medir actividades hemorrágicas presentes en la secreción de la glándula de Duvernoy (DGS), utilizando embriones de pollo. Hemos usado un sistema viviente, sin sensibilidad nerviosa, que es la fase muy joven del embrión del pollo, como una opción para la prueba hemorrágica que normalmente ensayamos en mamíferos. La membrana alantoidea ofrece un sistema vascular, que permite una observación fácil. No se observó ninguna lesión hemorrágica en embriones de pollo, cuando fueron ensayados con la secreción de la glándula de Duvernoy de *Clelia clelia*. Control positivo, usando veneno de *Bothrops Lanceolatus* mostró un halo alrededor del cuadrado de papel de filtro impregnado con dicho veneno. Aunque, se han descrito casos clínicos mordidos por *Clelia clelia* con manifestaciones hemorrágicas, ésta no fue demostrada experimentalmente, haciendo sospechar que las equimosis y petequias son procesos de naturaleza alérgica, producidos por substancias proteicas, enzimas, hidratos de carbono y peptidos pequeños componentes de la DGS de la *Clelia clelia*.

ABSTRACT

A rodent alternative to the measurement of Duvernoy's gland secretion (DGS) haemorrhagic effects using hens' eggs is proposed. We have used a non-sentient living system, such as very young stage of the chick embryo, as an option to haemorrhagic testing in mammals. The yolk sac membrane offers an easy observation vascular system. None haemorrhagic lesion in hen's eggs was observed with Clelia clelia DGS. *Bothrops lanceolatus* positive control showed a halo distant the square and bleeding was also present around the sector below the square. Although in patients' clinical cases bitten by *Clelia clelia* haemorrhagic activities are evidenced, haemorrhage was not experimentally demonstrated, making to suspect that the echymosis and petechias presence are processes of allergic nature produced by substances such as proteins, enzymes, carbohydrates and short peptides components of DGS from *Clelia clelia* snake.

Palabras clave: Secreción de la glándula de Duvernoy, embriónes de pollo, *Clelia clelia*, veneno de serpiente, *Bothrops lanceolatus*.

Key words: Duvernoy's gland secretion, chicken embryos, *Clelia clelia*, snake venom, *Bothrops lanceolatus*.

INTRODUCTION

In the last thirty years the bite of postulated-to be harmless colubrids have been better studied (McAllister, 1963; Grogan, 1974; Nickerson and Henderson, 1976; Jaume, 1983; Ferlan *et al.*, 1984; Minton, 1990; Kuch and Jesberger, 1993; Assakura *et al.*, 1994: Rodriguez-Acosta *et al.*, 1997). It is now accepted that many colubrids snakes previously believed to be non venomous are, in fact, not (Poey, 1873). The expression of a Duvernoy's gland joined with opistoglyphous (grooved teeth) dentition is a signal for toxic potential in snakes. Remembering that this gland secretion as a digestive fluid it is composed of complex mixtures of enzymes, low molecular weight polypeptides, metal ions and glycoproteins necessary for the digestive process of the prey. Authors (Pinto *et al.*,

1991) have reported a *Clelia clelia* child bite which exhibited pronounced oedema and local haemorrhage without systemic symptoms. All local symptoms disappeared gradually over a period of 3 days. Based in our clinical experience and this case reported in the literature, we have tried to measure the haemorrhagic activity from *Clelia clelia* Duvernoy's gland secretion in a chicken embryo model (Dunn and Boone, 1976).

MATERIALS AND METHODS

Chicken embryos. Eggs were prepared using a modified method of Dunn and Boone (1976). Briefly, hatching eggs (Animal House of the Tropical Medicine Institute of the Universidad Central de Venezuela) were collected on day 1 and incubated until day 5 at 37°C in a humid incubator. On day 5, under sterile conditions, each egg was cleaned with 70% isopropanol, breaking out of its shell into a petri dish. Then the haemorrhagic tests were carried out for 5 hours.

Squares and their uses. Squares of 2mm were cut from Whatman no. 2 filter paper using a scissors. Duvernoy's gland secretion (DGS) concentrations in a total volume of 2.0 L were practised to each square. Each square was then applied to either the anterior or posterior vitelline vein on the yolk. Control test were carried out using saline solution as negative and *Bothrops lanceolatus* venom as positive (haemorrhagic) instead of DGS. Incubation at 37°C was continued for 5 hours and the embryos were observed at 1, 3, 5 hourly intervals and the haemorrhagic activity at 5 hours was photographed for the examination. A groups of experimental hen's eggs were maintained for 24 hours.

Duvernoy's gland secretion. Secretion was obtained with the same technique that we have used to obtain snake venom. Briefly, we made it to bite the rubber-covered rim of a glass so that the lower jaws were on the inside of the container. 100 L were extracted each time, making a pool, centrifuged at 5°C for 10 min at 5.000 rpm and the supernatant was maintained at – 80° C in dark bottles until use. Three different DGS were assayed for their haemorrhagic effect on the chick embryos. The secretions were tested at concentrations ranging from 0.3 to 2.0 g/2 L for haemorrhagic activity in the egg assay.

Determination of haemorrhage. We have tested in the hen's egg assay haemorrhagic activity surrounding square absorbed with *B. lanceolatus* venom (positive control) which could be visualised after 5 hours incubation at 37°C. The concentration necessary to cause a haemorrhagic halo 2 mm wide was accepted as a basis haemorrhagic dose (BHD) (Sells *et al.*, 1997). *Clelia clelia* DGS were tested under same conditions.

Estimation of protein. The protein determination method followed that of Bradford (1976).

RESULTS

In hen's eggs treated with *Clelia clelia* DGS non-haemorrhagic lesions were observed (Figure 1). DGS tended to be easily released from the paper square. For such hydrosoluble secretions, the egg assays always gave negative results.

In the hen's egg assay, a halo of haemorrhage surrounding square absorbed with *B. lanceolatus* venom (positive control) could be visualised after 5 hours incubation at 37°C (Figure 2). In this positive control haemorrhage was evident as a halo distant the square, bleeding was also present around the sector below the square. We found that a doorsill of *B. lanceolatus* concentration might be surpassed before there was any bleeding at all. As expected saline solution gave non-haemorrhagic (negative) results.

DISCUSSION

Today, it is accepted that many colubrids snakes previously suspected to be harmless are, in fact, not (Grenard, 1994). The phenomenon that bite of postulated-to be harmless colubrids at this time it has been demonstrated that it can be at least mildly toxic (Poey, 1873; Minton, 1990; Machado and Rodriguez-Acosta, 1997). Reports of accidents with colubrid snakes are caused injurious reactions in

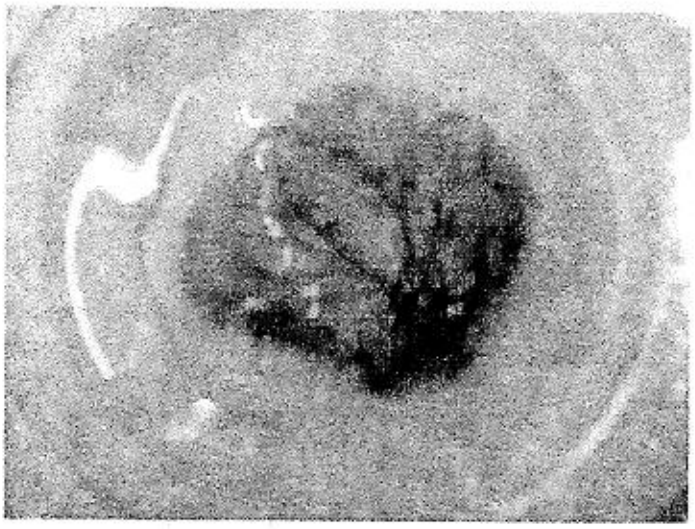

Figure 1. Chicken embryos treated with *Clelia clelia* DGS: non-haemorrhagic lesions were observed after 5 hours incubation at 37 °C.

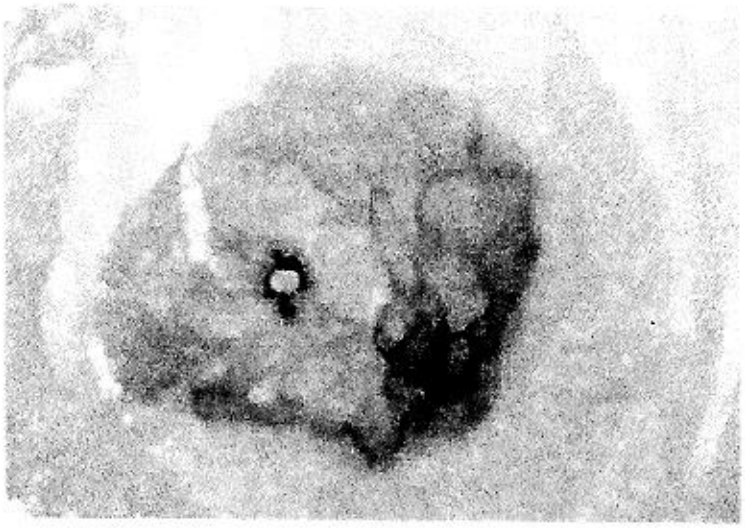

Figure 2. Chicken embryos treated with *Bothrops lanceolatus* (positive control): a halo of haemorrhage surrounding square absorbed with *B. lanceolatus* venom could be visualised after 5 hours incubation at 37 °C.

humans, among others: affects local blood coagulation and causes local haemorrhages (Nickerson and Henderson, 1976; Assakura *et al.*, 1994; Rodriguez-Acosta et al., 1997). The presence of Duvernoy's gland connected with opisthoglyphous dentition is identification for toxic potential in snakes. For instance, *Clelia clelia* has Duvernoy's glands and opisthoglyphous dentition. This snake lives in almost all the tropical and subtropical areas of our country, from the sea level until up 2.000 meters. The species reaches to measure until about 2.50 meters of longitude (Lancini, 1979).

Reports of human cases envenoming by *Clelia* genus colubrid snakes, considered as not venomous, showing evidence of symptoms of envenoming (Pinto *et al.*, 1991). This induced us to study the possible haemorrhagic activity present in this secretion. Among the deleterious components of DGS, some authors (Assakura *et al.*, 1994) proposed a haemorrhagic effect caused by a haemorrhaging that render the vasculature leaky and thus cause both local and systemic bleeding.

Sells *et al.* (1997), proposed the shell-less egg cultures for the assessment of the biological activities of venom and their antidotes. In this work, such a model provides and excellent alternative screen for secretion-induced haemorrhagic activity demonstration. Accurate measuring of the haemorrhagic lesion is complex in *in vivo* scheme. Nevertheless, using mice system it is possible to achieve adequately correct measurement (Kondo *et al.*, 1960; Sells *et al.*, 1997; Rodriguez-Acosta *et al.*, 1998). If we compare both *in vivo* mice and egg model, the haemorrhagic response is also dosage dependent. For instance, *B. Lanceolatus* venom at 0.2–0.5 g produced haemorrhagic halos increased from 1.0 mm to nearly 3.0 mm respectively. In the other hand, *Clelia* DGS concentrations from 0.2 to 0.5 g did not produce any haemorrhagic at all.

Our selection of an egg model, facing to the traditional rodent model is based on different reasons, humanitarian because the assay is a non-sentient one (Sells *et al.*, 1997), the common characteristics that avian and mammalian tissues seem to have (Ohsaka *et al.*, 1973; Sells *et al.*, 1997) and it is an excellent model to prove that the activity could be of allergic nature, since in the non previously sensitised chicken embryo a haemorrhagic allergic phenomenon should not be developed.

McAllister (1963) proposed the possibility of allergic responses to the secretions of DGS in colubrids (*Heterodon platyrhinos*). Grogan (1974) reported the presence of a haematoma in an accidental envenomation produced by eastern hognose

snake (*Heterodon platyrhinos*), the patient had history of numerous bites by other colubrid snakes of different species. Although an allergic reaction cannot be completely discarded, the inoculation of extraneous proteins, in some cases, unusual glucides, into the circulatory system of previously sensitised people could induce an allergic response. Haemorrhagic activity, such as the bleeding and haematoma are not typical allergic reactions in clinical medicine but they could be. The inoculation of allergens present in snake saliva could feasibly give rise to a localised allergic reaction evidenced by bleeding. Exist a high number of enigmatic bleeding disturbances that are non-symptomatic until something like snakebite injury occurs.

There is a group of genes in the human genome which respond to a single and usual polypeptide antigens, many of which occur in the secretions of supposed non-venomous snakes, as well as in arachnid, insects and other animals such as cats, dogs, reptiles and rats that have enzymes and other proteins and/or carbohydrates in the saliva with no established venomous effect on human beings.

Just if colubrid snakes with Duvernoy's gland do not secrete toxins compared with crotalids or elapids, its saliva could possibly introduce proteins, glucides and enzymes, susceptible of generate immediate immune reactions of hypersensibility. And still if a first inoculation produces insignificant or not reaction, a second one to such a previously sensitised patient has the potential of inducing a very severe hypersensitivity response (Grenard, 1994). In our experiments, the development of an allergic reaction in the chicken embryo would imply a previous relationship with the toxic substance, that is not the case.

Even, *Clelia clelia* DGS has not haemorrhagic activity, the controversy remains as to whether or not members of *Clelia* and other non-venomous colubrids should be reclassified from totally harmless to venomous opisthoglyph species or to species, which are just cautiously unpleasant. The truth that even the distress of their reported bites to date have resulted in no lethality indicates that this group is peculiar and should be reputable as such.

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