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Inhibition of VEGF-Flk-1 binding induced profound biochemical alteration in the hippocampus of a rat model of BBB breakdown by spider venom. A preliminary assessment using FT-IR spectroscopy

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ABSTRACT

Phoneutria nigriventer spider venom (PNV) contains ion channels-acting neuropeptides that in rat induces transitory blood-brain barrier breakdown (BBBb) in hippocampus in parallel with VEGF upregulation. We investigated whether VEGF has a neuroprotective role by inhibiting its binding to receptor Flk-1 by itraconazole (ITZ). FT-IR spectroscopy examined the biochemical status of hippocampus and evaluated BBBb in rats administered PNV or ITZ/PNV at periods with greatest toxicity (1-2h), recovery (5h) and visual absence of symptoms (24h), and compared to saline and ITZ controls. The antifungal treatment before venom intoxication aggravated the venom effects and increased BBB damage. FT-IR spectra of venom, hippocampi of controls, PNV and ITZ-PNV showed a 1400 cm^{-1} band linked to symmetric stretch of carboxylate and 1467 cm^{-1} band (CH_2 bending: mainly lipids) that were considered biomarker and reference bands, respectively. Inhibition of VEGF/Flk-1 binding produced marked changes in lipid/protein stability at 1-2h. The largest differences were observed in spectra regions assigned to lipids, both symmetric (2852 cm^{-1}) and asymmetric (2924 and 2968 cm^{-1}). Quantitative analyses showed greatest increases in the 1400 cm^{-1}/1467 cm^{-1} ratio also at 1h. Such changes at period of rats' severe intoxication referred to wavenumber region from 3106 cm^{-1} to 687 cm^{-1} assigning for C-H and N-H stretching of protein, Amide I, C = N cytosine, N-H adenine, Amide II, CH_2 bending: mainly lipids, C-O stretch: glycogen, polysaccharides, glycolipids, z-type DNA, C-C, C-O and CH out-of-plane bending vibrations. We conclude that VEGF has a neuroprotective role and can be a therapeutic target in PNV envenomation. FT-IR spectroscopy showed to be instrumental for monitoring biochemical changes in this model of *P. nigriventer* venom-induced BBB disruption.

1. Introduction

The *Phoneutria nigriventer* spider venom (PNV) is a depository of large molecular diversity, which action involves the nervous and muscular system and interferes in the hemodynamics. Fractionation of venom reveals a plethora of toxins with broad spectrum of pharmacological action in insects and mammals which results mainly from interference on the physiology of sodium, potassium and calcium channels and disturbance of glutamate and acetylcholine release (see De Lima et al., 2016 for review). Crude PNV has been considered a potential source of molecules for the design of new drugs to be used in pharmaceutics and agrochemical products. In Brazil, more than 90% of human accidents with *P. nigriventer* are of low level and causes just profuse sweating in the victims and local pain, edema and erythema; accidents scored as severe cause besides nausea and vomiting, blurred vision, agitation, priapism, hypertension, tachycardia, arrhythmias, respiratory distress and convulsion. Less than 0.5% of accidents are graded as severe (Bucaretchi et al., 2017). In rats, the crude PNV reproduces most of the toxic signs observed in humans (Le Sueur et al., 2003, 2004; Rapôso et al., 2007; Mendonça et al., 2013). All these effects are self-limiting thus making the researches with *P. nigriventer* venom components even more attractive.

Our group has shown that the crude venom of *P. nigriventer* promotes temporary opening of the blood-brain barrier (BBB) in the

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hippocampus and cerebellum of rats, which courses with a likewise toxic state and transitory flaccid and spastic paralysis of the hind limbs and intense salivation (Le Sueur et al., 2003, 2004). Moreover, the crude PNV activates populations of neuronal nitric oxide synthase (nNOS)-positive neurons in brain regions of different stereotaxic coordinate as shown by the expression of cFOS protein (Cruz-Höfling et al., 2007; see also, Cruz-Höfling et al., 2016 for review).

The BBB main component is the endothelium of capillaries whose structure is unique due to the lack of pores, high electrical resistance and adhesion and tight junctional proteins covering completely the cell-cell contacts. This apparatus prevents the paracellular transport of hydrophilic molecules but did not avoid the lipophilic molecules as O_2 and CO_2 that diffuses freely through the cell membrane alongside a concentration gradient (Grieb et al., 1985; Abbott et al., 2018). Thus, the bidirectional trafficking of molecules is obligatorily through the transcellular route via endocytosis, formation of caveolae, and transport of vesicles, all tightly regulated by membrane receptors and carriers; other BBB structures comprise the basement membrane, the end-feet processes of perivascular astrocytes and pericytes all-encompassing and working in concert for the selectivity of the barrier (Pardridge, 2003). The homeostasis of the central nervous system (CNS) relies on a healthy BBB able to prevent the ions and metabolites fluctuations occurring in the peripheral blood; certain medical conditions, such as neurodegenerative diseases or toxic substances, as PNV, can break down the functioning of the BBB and its role as guardian of brain homeostasis. It is worth mention that a steady-state BBB remains a major obstacle for drug delivery and therapeutic intervention in pathologies of the CNS (Pardridge, 2012). Studies dealing with natural substances that open transitorily the BBB with minimal side effects are of interest.

Our previous studies have shown that a mechanism underlying the opening of the BBB by the *P. nigriventer* spider venom could likely involve the vascular endothelial growth factor (VEGF) and receptors (Mendonça et al., 2012, 2013, 2014). We have found time-dependent increases of VEGF and receptors in rats systemically injected with PNV. VEGF has a major regulatory role on endothelial cell proliferation, angiogenesis and vascular permeability, including at the BBB (Ferrara, 2000). Growing number of evidences have now been referring to a neuroprotective role to VEGF following brain insult (Rosenstein and Krum, 2004; Yasuhara et al., 2004; Góra-Kupilas and Josco, 2005; Sun and Guo, 2005), particularly in hippocampus (Cammalleri et al., 2011). VEGF ligand regulation is evoked mainly by the VEGFR-2 signaling, a transmembrane tyrosine kinase receptor also named Flk-1 (Ferrara et al., 2003). In fact, the inhibition of Flk-1 leads to neuronal deterioration following traumatic brain/hippocampus's injury (Sköld et al., 2006; Lee and Agoston, 2009), and worsening of illness condition. We do not know whether the increased expression of VEGF in the hippocampus of rats administered crude PNV earlier reported (Mendonça et al., 2012, 2013, 2014) would be a triggering of a causal protective factor aimed at modulating the venom's homeostatic perturbations. One way to clarify this is by blocking VEGF ligand through the inhibition of Flk-1. Itraconazole is a Food and Drug Administration (FDA)-approved agent belonging to the family of azole antifungal drugs. Reports describe itraconazole as having antiangiogenic properties due to the inhibition of VEGF/Flk-1 binding (Chong et al., 2007; Aftab et al., 2011; Nacev et al., 2011).

Hence, the aim of the current work is to examine how the inhibition of Flk-1/VEGF binding by itraconazole echoes on both the neurotoxic condition of the animals and BBB integrity, as well as to analyze possible changes in functional groups in the hippocampus molecular content of rats administered PNV.

To achieve this purpose we followed the evolution of animals during the experimental period, evaluate BBB status by infusing Evans blue vital dye and used vibrational spectroscopy to assessing the biochemistry of the hippocampus of rats that received systemic administration of *Phoneutria nigriventer* crude venom before and after itraconazole treatment.

2. Materials and methods

2.1. Ethics statement

The institution's Committee for Ethics in Animal Use (CEUA/IB/UNICAMP, protocol n. 32223-1) approved this study and the experiments were carried out according to the Brazilian Society of Laboratory Animal Science (SBCAL) guidelines.

2.2. Animals, venom and itraconazole

Male Wistar rats (*Rattus norvegicus*), aged 6 weeks (190 ± 50 g) were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of UNICAMP and housed under standard laboratory conditions, and receiving food and water *ad libitum*. The lyophilized *P. nigriventer* spider venom was kindly donated by Dr. Evanguedes Kalapothakis (Department of Biology, IBS, UFMG, Belo Horizonte, MG, Brazil) and stored at −20 °C until its use. Itraconazole was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Exposure to Phoneutria nigriventer venom and itraconazole and their controls

Since our previous studies showed that both the clinical condition of animals and BBB-disruption were self-limited, and the VEGF upregulation was time-related, the analyses were taken at a time-window from 1 to 24 h, corresponding to clinical evolution of envenomed animals.

PNV - Rats received an intravenous (i.v.) injection of PNV (0.85 mg/kg in 0.5 mg/ml of 0.9% sterile saline) through the tail vein (Le Sueur et al., 2003). After 1 and 2 h (intervals with greatest signs of envenomation), 5 h (interval with signs of animals recovery) and 24 h (interval with no detectable envenomation sign), the animals (n = 3/interval, total of 12 animals in this group) were euthanized by decapitation after CO_2 inhalation and the brains were immediately removed.

ITZ - The animals received i. v. injection of itraconazole (10 mg/kg dissolved in a mixture of 15% dimethylsulfoxide (DMSO) plus 85% of 0.9% sterile saline solution (Carrer et al., 2013), 30 min prior to the injection of PNV. Itraconazole + PNV-treated rats (ITZ-PNV group) were euthanized at same intervals and as for PNV alone group: 1, 2, 5 and 24 h (n = 3/time, total of 12 animals in this group). The effectiveness in inhibiting the VEGF-Flk-1 binding by itraconazole was assessed by immunoprecipitation; the data showed a significant decrease of the VEGF-Flk-1 immunecomplex in the hippocampus of ITZ-PNV rats in comparison to the found in the PNV group (unpublished results).

Control - Two groups of controls were used: One received the same volume (0.5 mg/ml) of 0.9% sterile solution (i.v.) and the other control group received itraconazole; the animals of both groups were euthanized after 5 h (n = 3/group, total of 6 animals in control groups) We used just one interval because a previous pilot experiment had indicated the normality of animals' behavior of these two groups irrespective of the survival period. Our goal was to keep the number of animals used in the experiments to a minimum if unneeded.

After immediate brains excision from the scalp, the hippocampi from saline and ITZ controls, PNV- and ITZ-PNV-treated rats were carefully dissected, according to Paxinos and Watson (1986) rat brain atlas and frozen in liquid nitrogen (−195.79 °C; −320 °F) until its use. Fresh brain has the advantage to allow identifying visually different regions and anatomical boundaries because they show different colors and shapes. For hippocampi dissection, the brains were placed in Petri dish containing physiologic solution and a cross-section was made with a scalpel. The hippocampus was paler than the cortex and then carefully dissected with surgical material (tweezers, spatula) and removed from the left and right brain hemispheres. Then, the hippocampi (n = 3 animals per group were frozen immediately in liquid nitrogen (−195.79 °C; −320 °F) until use.