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MECANISMOS ENVOLVIDOS NA QUEBRA DA BARREIRA HEMATO-ENCEFÁLICA INDUZIDA PELO VENENO DA ARANHA *Phoneutria nigriventer*. ESTUDOS *in vivo E in vitro*.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Histologia.

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Dedico esta tese

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RESUMO

O veneno da aranha *Phoneutria nigriventer* (PNV) é sabidamente neurotóxico, edematogênico e pode causar hipertensão e convulsões nos casos graves de envenenamento. Nosso objetivo foi investigar a habilidade do PNV em quebrar a barreira hemato-encefálica (BHE) de ratos, bem como estudar, *in vivo* e *in vitro*, os mecanismos envolvidos nesse processo. A ação do PNV sobre a permeabilidade da BHE foi avaliada por métodos ultra-estruturais utilizando-se o traçador extracelular nitrato de lantânio. Nossos resultados mostraram que o veneno, injetado por via endovenosa, provoca significativa ($p < 0,05$) quebra da BHE 1 e 9 dias após o envenenamento, particularmente no hipocampo. A quebra foi caracterizada pela presença de grande quantidade de vesículas pinocíticas contendo o traçador no citoplasma das células endoteliais, além da presença do traçador no espaço intercelular. No tecido neural circunjacente, foi observado edema vasogênico, porém, sem infiltrado inflamatório ou indícios morfológicos evidentes de sofrimento neuronal. Em seguida, investigando alguns mecanismos envolvidos nesse processo, constatamos que a barreira paracelular parece não ter sido afetada pelo veneno, uma vez que não foram detectadas diminuição nos níveis de expressão ou aumento de fosforilação das proteínas ZO-1, ocludina e β-catenina, associadas às junções intercelulares. Contrariamente, uma tendência de aumento na expressão de ocludina foi observada após o tratamento com o PNV. A quebra da BHE *in vivo* parece ter ocorrido primariamente através do aumento do transporte vesicular transendotelial mediado por microtúbulos, uma vez que o pré-tratamento com colchicina bloqueou completamente as alterações da permeabilidade provocadas pelo veneno. Com o objetivo de esclarecermos se o veneno possuía uma ação direta sobre os elementos da BHE, ou se a quebra era decorrente de distúrbios hemodinâmicos, utilizamos modelos *in vitro,* com três linhagens celulares: endotelial ECV304, glioma astrocitário C6 e epitelial MDCK. Nessas condições, o efeito direto do PNV sobre a viabilidade e vias de transporte das células em cultura pode ser estudado, enquanto os possíveis efeitos hemodinâmicos são excluídos. Nossos resultados mostraram que o PNV não possui efeito citotóxico sobre as linhagens ECV304 e MDCK. Entretanto, um efeito citotóxico foi observado na linhagem C6 somente com alta dose do veneno. A ação do PNV sobre as vias de transporte celular foi testada nas células MDCK, onde o PNV estimulou a endocitose apical. Já a barreira paracelular parece não ter sido afetada, uma vez que não foram observadas diminuições da resistência elétricas transepitelial (R_T), diminuição na expressão ou alteração na distribuição das proteínas juncionais ZO-1, ocludina e E-caderina e nos filamentos de actina. Contrariamente, um aumento significativo da R_T foi observado nas células MDCK expostas a alta dose de veneno. Em conclusão, o PNV parece induzir a quebra da barreira hemato-encefálica, estimulando primariamente o transporte transcelular mediado por vesículas pinocíticas.

ABSTRACT

Phoneutria nigriventer spider venom (PNV) is neurotoxic, edematogenic and can cause hypertension and convulsions in severe envenoming accident. Our objective was to investigate the ability of PNV in breaking the blood-brain barrier (BBB) of rats, as well as to study, *in vivo* and *in vitro* experimental conditions, the mechanisms involved in this process. The action of PNV on the BBB permeability was evaluated by ultrastructural methods by using the lantanum nitrate as an extracellular tracer. Our results showed that the venom, when endovenously injected, evokes significant ($p < 0.05$) BBB breakdown at 1 and 9 days after envenoming, particularly in the hippocampus. This process was evidenced by the presence of numerous pinocytotic vesicles containing the tracer within the endothelial cell cytoplasm, besides the presence of the tracer in the intercellular space. In the surrounding neural tissue was observed vasogenic edema, without inflammatory infiltrate or evident morphologic indications of neuronal suffering. The paracellular endothelial barrier seemed do not affected by the venom, since there was no decrease in the expression or changes in the phosphorylation state of the ZO-1, ocludin and β-catenin proteins, which are associated with the intercellular junctions. Contrarily, a tendency of increase in the ocludin expression was observed after PNV treatment. The BBB breakdown may have occurred through an increase in the microtubules-mediated transendotelial vesicular transport, since pretreatment with colchicine completely blocked the alterations in brain vascular permeability evoked by the venom. In order to study whether the venom possesses a direct action on the BBB elements, we used *in vitro* models employing three distinct cellular lineages: the ECV304 endothelial, the C6 astrocytic glioma and the MDCK epithelial cells. In *in vitro* condition, the direct effect of PNV on the viability and cellular transport routes could be studied, while the haemodynamic effects were excluded. Our results showed that PNV does not possess cytotoxic effect on the ECV304 and MDCK cell lineages. However, a cytotoxic effect was observed in the C6 lineage at the highest concentration of PVN used. The action of PNV on the cellular transport pathways was evaluated in MDCK cells, where PNV stimulated the apical endocitosis. The paracellular barrier seemed not be affected, since no decrease was observed in the transepithelial electrical resistance (TER) neither changes in the expression and cellular distribution of junctional and cytoskeletal proteins. Contrarily, a significant increase in TER was observed in MDCK cells exposed to high concentration of PNV. In conclusion, PNV seems to induce the BBB breakdown by disturbing primarily the transcellular endothelial transport mediated by pinocytotic vesicles.

LISTA DE ABREVIATURAS

Abreviaturas em português:

BHE - barreira hemato-encefálica

HRP – peroxidase de rabanete

JA – Junção de aderência

JO – Junção de oclusão

PNV - veneno de *Phoneutria nigriventer*

R_T - resistência elétrica transepitelial

Abreviaturas em inglês:

BBB - blood-brain barrier

BBBb - blood-brain barrier breakdown

HRP - horseradish peroxidase

PNV - *Phoneutria nigriventer* venom

TER - transepithelial electrical resistance

TJ - tight junction

SUMÁRIO

I - INTRODUÇÃO

1. Epidemiologia e história natural da aranha *Phoneutria nigriventer*

Os acidentes causados por araneísmo constituem no Brasil um problema de Saúde Pública. Dentre estes, os acidentes causados pelas aranhas da espécie *Phoneutria nigriventer* constituem cerca de 42% dos casos registrados no Estado de São Paulo (Barraviera, 1992; Bucaretchi *et al.,* 2000).

As aranhas do gênero *Phoneutria* são popularmente conhecidas como aranhas armadeiras, devido à posição que assumem quando se sentem ameaçadas, apoiando-se nas patas traseiras e erguendo as dianteiras procurando picar a vítima (Figura 1) (Vellard, 1936; Lucas, 1988). São aranhas agressivas, que enfrentam seus oponentes. Possuem pêlos curtos e acinzentados, com comprimento do corpo de até 17 cm quando em posição de ataque. Não constróem teias e possuem hábitos crepusculares e noturnos (Lucas, 1988). Habitam as proximidades de árvores de folhas grandes como coqueiros, bananeiras e palmeiras, ou ficam sob pedras, dentro de tocas, cupinzeiros e casas, onde penetram freqüentemente na época do acasalamento, ou deslocadas pela chuva. Dentro das casas, elas freqüentemente se escondem dentro dos calçados, e a maioria dos acidentes ocorre pela manhã, ao calçá-los (Vellard, 1936; Lucas, 1988). Os acidentes podem também acontecer durante a limpeza doméstica ou, ainda, no trabalho de profissionais como agricultores, comerciantes de frutas e verduras ou da construção civil (Lucas, 1988).

Figura 1 - *Phoneutria nigriventer.* Foto cortesia Prof. Dr. Augusto Abe / IB, Unesp - Rio Claro (SP)

Indivíduos picados pela aranha apresentam manifestações clínicas, cuja gravidade permite a classificação do acidente em leve, moderado e grave. São classificados como acidentes leves, aqueles em que as vítimas apresentam os seguintes sintomas: dor irradiada no sítio da picada, edema local, parestesia

e sudorese generalizada. Nos acidentes moderados, são observados tremores, paralisia espástica, sialorréia, taquicardia, hipertensão arterial, agitação psicomotora e distúrbios visuais (Brazil e Vellard, 1925, 1926; Lucas, 1988). Nos casos graves, observam-se vômitos freqüentes, hipotensão arterial, bradicardia, dispnéia, insuficiência e arritmias cardíacas, convulsões, choque, coma, priapismo, edema pulmonar agudo, parada cardiorespiratória (Schenberg e Pereira Lima, 1978; Bucaretchi, 1992). No entanto, a maioria dos acidentes é do tipo leve ou moderado, sendo raros os casos graves, os quais ocorrem principalmente em crianças e idosos. No caso das crianças, a gravidade deve-se, provavelmente, à quantidade de veneno inoculada em relação ao peso corporal e, no caso dos idosos, à fragilidade do organismo. Não tem sido registrada necrose local, coagulação sangüínea anormal ou hemólise após o envenenamento por este gênero de aranha (Bucaretchi, 1992).

O tratamento dos acidentes leves é sintomático, com infiltração de um anestésico local, sem vasodilatador. Este procedimento, repetido por mais duas ou três vezes, tem sido suficiente na maioria dos casos mas, nos casos de dor persistente, indica-se o uso de analgésicos sistêmicos mais potentes. Nos casos de manifestações sistêmicas (acidentes moderados e graves) e/ou com envolvimento do sistema nervoso central, é aplicada a soroterapia específica, que consiste de 5 a 10 ampolas de soro anti-aracnídico, por via intravenosa ou subcutânea, com objetivo de neutralizar os efeitos do veneno circulante o mais rapidamente possível. Na maioria dos casos o prognóstico é bom, estando o paciente recuperado em no máximo 24 horas (Lucas, 1988; Bucaretchi, 1992).

2. Composição química e atividade farmacológica do veneno

Do ponto de vista bioquímico, o veneno da aranha *Phoneutria nigriventer* (PNV) é composto por uma mistura de polipeptídeos básicos de baixa massa molecular (3.500-9.000 Da) (Gomez *et al.,* 2002), de conhecida ação neurotóxica, e por pequena quantidade de histamina (0,06-1 %) e serotonina (0,03-0,25 %) (Diniz, 1963; Schenberg and Pereira-Lima, 1978). A partir do PNV dialisado, ou seja, livre das aminas vasoativas histamina e serotonina, várias frações foram isoladas. As técnicas de filtração em gel e cromatografia de fase reversa revelaram a existência de três frações neurotóxicas e letais em camundongos, denominadas PhTx1, PhTx2 e PhTx3 (Rezende *et al.,* 1991) e uma fração de ação inseticida, denominada PhTx4 (Entwistle *et al.,* 1982). As frações diferem entre si pela massa molecular, composição de aminoácidos, seqüência do N-terminal e ação biológica. A partir dessas frações, várias neurotoxinas puras foram caracterizadas (Entwistle *et al.,* 1982; Diniz *et al.,* 1990; Rezende *et al.,* 1991; Cordeiro *et al.,* 1992; 1993; Figueiredo *et al.,* 1995; Romano-Silva *et al*., 1996) e clonadas (Kalapothakis *et al*., 1998 a,b).

O modo de ação do PNV parece envolver, freqüentemente, a ativação ou atraso na inativação de canais iônicos (para revisão ver Gomez *et al.,* 2002; Rash and Hodgson, 2002; Antunes and Málaque*,* 2003). Modelos *in vivo* e *in vitro* têm contribuído para o avanço do conhecimento sobre os mecanismos de ação deste veneno. Alterações eletrofisiológicas e morfológicas induzidas pelo PNV bruto e frações foram observadas em fibras musculares *in vivo* e *in vitro*, decorrentes da ativação e/ou atraso na inativação de canais de Na^+ (Fontana e Vital Brazil, 1985; Vital Brazil, 1987; Vital Brazil e Fontana, 1993; Mattiello-Sverzut *et al.,* 1998; Mattiello-Sverzut e Cruz-Höfling, 2000). Em fibras nervosas periféricas, este efeito também foi observado. Neste caso, a ativação e/ou atraso na inativação dos canais de Na^+ promoveram um influxo excessivo do íon nos nodos de Ranvier, responsável pelas respostas repetitivas que levam à paralisia espástica (seguida pela flácida) em camundongos envenenados (Cruz-Höfling *et al.,* 1985; Love *et al.,* 1986; Love and Cruz-Höfling, 1986). Estudos com preparações *in vitro* de sinaptosomas isolados do córtex cerebral de ratos mostraram que a fração PhTx2 ativa canais de Na^+, os quais induzem um rápido aumento na concentração intracelular de Ca^{+2} com liberação de glutamato (Romano-Silva *et al.,* 1993) e liberação de acetilcolina por mecanismos dependentes do Ca^{+2} extracelular (Moura *et al.,* 1998). Toxinas purificadas a partir das frações PhTx3 e PhTx4, por sua vez, bloqueiam canais de Ca^{+2}, os quais inibem a liberação de glutamato, [^{3}H]-dopamina e [^{3}H]-acetilcolina, respectivamente em preparações de sinaptosomas corticais de ratos (Prado *et al.,* 1996; Mafra *et al.,* 1999), preparações de corpo estriado de rato (Troncone *et al.,* 1995) e córtex de rato e plexo mioentérico de porcos da índia (Gomez *et al.,* 1995).

No sistema cardiovascular, Costa *et al.* (1996) demonstraram que altas doses de PNV dialisado, inoculado por via sistêmica, causavam hipertensão em ratos anestesiados, efeito este atribuído à abertura de canais de Ca^+, do tipo L, voltagem-dependentes. Em coelhos anestesiados, o PNV também produziu resposta hipertensora prolongada, tanto após a injeção endovenosa do veneno quanto após a injeção intracerebroventricular, sugerindo que o PNV induz alterações hemodinâmicas de origem periférica e central (Estato *et al.,* 2000).

O PNV tem sido utilizado, ainda, como ferramenta para o entendimento dos mecanismos relacionados à dor e à inflamação de causa nervosa, denominada de inflamação neurogênica. O PNV total ou dialisado, quando injetado na derme dorsal de ratos, provoca aumento da permeabilidade vascular local, com formação de edema (Antunes *et al.,* 1992). Este efeito é parcialmente causado pela ativação de receptores de histamina e serotonina (Antunes *et al.,* 1992; Costa *et al.,* 2001) e também pela ativação de receptores vanilóides (Costa *et al.,* 2000) e NK1 de taquicininas (Palframan *et al.,* 1996). Os receptores vanilóides e de taquicininas são encontrados em uma classe de fibras nervosas sensoriais, as fibras tipo C, sensíveis à capsaicina, existentes na pele. A ativação desses receptores leva à liberação de neuropeptídeos pró-inflamatórios pelos terminais nervosos, como a substância P, com conseqüente aumento da

permeabilidade local. Recentemente, Costa *et al.* (2003) demonstraram, em preparações de nervo vago, que o PNV ativa receptores de serotonina do tipo 5-HT_4, existentes em fibras nervosas nociceptivas, o que pode explicar o desenvolvimento da dor e inflamação observados após o envenenamento.

O aumento da permeabilidade vascular com formação de edema foi também observado na derme dorsal de coelhos (Marangoni *et al.,* 1993). Nesta espécie, no entanto, o mecanismo de ação do veneno mostrou-se independente da ativação de fibras nervosas sensoriais. Os autores mostraram que o PNV dialisado provoca a ativação do sistema de calicreínas teciduais, com posterior formação de calidina, a qual induz o aumento da permeabilidade vascular.

Em função dos efeitos neurotóxicos e edematogênicos do veneno e dos sintomas clínicos de convulsões, a ação do veneno de *P. nigriventer* no SNC tem despertado particular interesse. Souza e Cruz-Höfling (1997) demonstraram que o PNV, quando inoculado diretamente na ponte cerebral de ratos adultos, provocava lesão espongiótica extensa, caracterizada por morte celular no centro da lesão e áreas de desmielinização na periferia. Em tempos de sobrevida mais avançados, os autores observaram acúmulos de células gliais em torno das áreas afetadas, tornando-as císticas, enquanto que na periferia, oligodendrócitos promoviam a remielinização, embora de forma incompleta, dos axônios afetados. Em fases posteriores, a remielinização total das fibras nervosas do SNC ocorria por conta das células de Schwann, células gliais encontradas no SNP.

Recentemente, nós demonstramos que o PNV bruto, injetado por via endovenosa em ratos, provoca alterações vasculares no SNC, resultando na quebra da barreira hemato-encefálica (BHE), particularmente na região do hipocampo (Le Sueur*,* 1998). Através de métodos ultra-estruturais, utilizando o traçador extracelular nitrato de lantânio, foi demonstrado um aumento significativo da permeabilidade dos vasos sanguíneos hipocampais, desde 1 até 9 dias após a inoculação da solução de veneno. A quebra da BHE foi evidenciada pela presença de grande quantidade de vesículas de transporte contendo o traçador no citoplasma das células endoteliais, e pela presença do mesmo no espaço intercelular. Além disso, no parênquima cerebral circunjacente aos vasos afetados, foi observado edema vasogênico, caracterizado pelo intumescimento dos prolongamentos astrócitários, além do aumento na expressão da proteína acídica fibrilar glial (GFAP) astrocitária perivascular. Por outro lado, não observamos infiltrados inflamatórios ou indícios morfológicos evidentes de sofrimento neuronal. No entanto, os mecanismos envolvidos nesta quebra da BHE ainda não estão esclarecidos, bem como quais os constituintes da BHE e as vias de transporte afetadas pelo veneno.

3. Barreira hemato-encefálica e seus constituintes

A BHE constitui a interface física e metabólica entre o sangue e o cérebro. Tem sede nos capilares do SNC, que possuem características morfo-funcionais particulares que visam a preservação da integridade do tecido neural, propiciando um meio adequado ao desempenho de suas funções e manutenção da homeostase e, de certa forma, "isolado" das flutuações da composição sanguínea.

Os capilares do tecido nervoso central são formados por uma ou duas células endoteliais, unidas por complexas junções intercelulares, formando um epitélio simples, contínuo, de 2 a 5 μm de espessura (Rowland *et al.,* 1991). O citoplasma dessas células apresenta baixo índice de transporte vesicular transcitótico, traduzido por reduzido conteúdo de vesículas intracitoplasmáticas, além de grande quantidade de mitocôndrias, que refletem a alta atividade metabólica do endotélio cerebral (Reese e Karnovsky, 1967; Brightman e Reese, 1969; Johansson, 1992). Em conjunto, as junções intercelulares e o reduzido número de vesículas citoplasmáticas são responsáveis pelo trânsito altamente restritivo e seletivo de substâncias pelas vias paracelular e transcelular, respectivamente. Contribuem ainda para a estrutura da barreira, a presença de uma lâmina basal espessa na face abluminal das células endoteliais, a qual é contínua à lâmina basal de pericitos que, eventualmente, envolvem os capilares (Rowland *et al.,* 1991). Os pericitos são células mesenquimais quiescentes, que podem dar origem a outras células mesenquimais ou a células musculares lisas. Os pericitos contribuem para a regulação do fluxo sanguíneo e o tônus vascular, além de constituírem uma importante linha de defesa da BHE, por conta de sua atividade fagocítica (Balabanov e Dore-Duffy, 1998; Allt e Lawrenson, 2000).

Outra particularidade dos vasos sanguíneos cerebrais, que os distinguem dos capilares do restante do organismo, é que estes são envolvidos em toda a extensão por expansões citoplasmáticas de um tipo de célula glial, o astrócito (Figura 2) (Rowland *et al.,* 1991). Essas expansões astrocitárias são conhecidas como "pés vasculares" e, além de formarem uma barreira física, liberam fatores solúveis que constituem os principais responsáveis pelos processos de indução, manutenção e regulação das características de barreira da microvasculatura cerebral (Janzer, 1993; Igarashi *et al.,* 1999; Abbott, 2002). A natureza química dos fatores liberados pelos astrócitos ainda não é totalmente conhecida, no entanto, várias moléculas já foram identificadas, como fator de crescimento tumoral β (TGF-β), fator neurotrófico derivado de glia (GDNF), fator de crescimento de fibroblasto básico (FGFb), interleucina 6 (IL-6) e a hidrocortisona (Abbott, 2002). Estudos utilizando modelos de BHE *in vitro* mostraram que os fatores liberados pelos astrócitos têm papel decisivo na formação de junções de oclusão entre as células endoteliais e, conseqüentemente, no aumento da resistência elétrica transendotelial e na polaridade do endotélio. Além disso, esses fatores induzem a expressão funcional de enzimas e transportadores reconhecidos como marcadores do fenótipo da BHE, como a γ-glutamil transpeptidase, a fosfatase

alcalina, o receptor de transferrina, o transportador de glicose tipo 1 (GLUT-1) e a glicoproteína-P (Janzer, 1993; Allt e Lawrenson, 2000). Estudos *in vitro* demonstraram, também, que os fatores astrocitários são capazes de induzir a expressão de marcadores fenotípicos da BHE até mesmo em células endoteliais de origem não cerebral, como no endotélio da aorta bovina e da veia umbilical humana (Yamagata *et al.,* 1997; Kuchler-Bopp *et al.,* 1999). Além disso, a indução astrocitária é dependente da matriz extracelular sobre a qual as células endoteliais são cultivadas (Yamagata *et al.,* 1997; Hurst, 2000), denotando o papel fundamental dos astrócitos e da membrana basal endotelial no fenótipo da BHE.

Além dos fatores astrocitários, a presença de terminais nervosos próximos aos capilares, de pericitos, micróglias e mastócitos também contribuem para a manutenção, regulação da permeabilidade e reparo da BHE (Janzer, 1993; Abbott, 2002).

Além da eficiente barreira física, a BHE conta ainda com uma barreira metabólica, constituída por enzimas e pelo glicocálix que recobre a superfície luminal do endotélio cerebral. O sistema enzimático da célula endotelial é representado por enzimas citoplasmáticas, como as enzimas hidrolíticas lisossomais e extra-lisossomais (monoamina oxidase), e por enzimas associadas à superfície externa da membrana plasmática, das quais destacam-se as peptidases, responsáveis pelo metabolismo de substâncias potencialmente neuroativas, como a bradicinina, encefalinas e a substância P (Allt e Lawrenson, 2000). Já o glicocálix constitui uma camada de glicoconjugados (glicoproteínas + carboidratos) de superfície celular, ricos em resíduos de ácido siálico, o qual confere uma propriedade aniônica ao endotélio. O glicocálix age como uma peneira molecular, selecionando moléculas com base em sua massa molecular e carga iônica (Allt e Lawrenson, 2000).

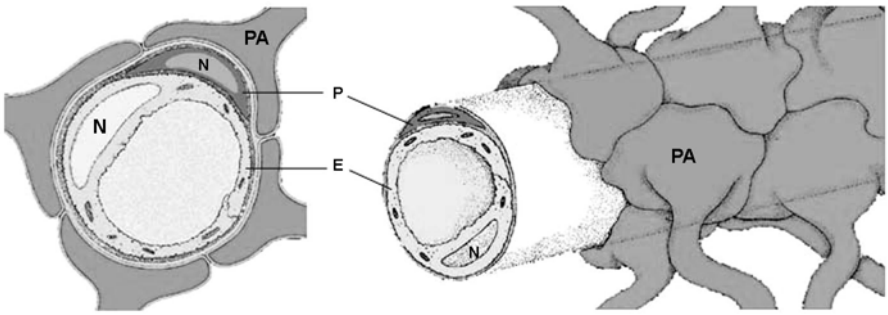

Figura 2- Representação esquemática dos constituintes da BHE: vista frontal (esquerda) e vista laretal (direita). Esquema modificado de Rowland *et al.* (1991). Legenda: E, endotélio; P, pericito; PA, pés astrocitários; N, núcleo.

3.1. Transporte transcelular

Devido à complexidade estrutural das junções intercelulares, que limitam a passagem de substâncias pela via paracelular, o transporte através da BHE ocorre preferencialmente através da membrana endotelial. A via transcelular é responsável pela transferência seletiva e bidirecional de substâncias entre o sangue e o cérebro. Pequenas moléculas lipofílicas, como o O_2, o CO_2 e o etanol, difundem-se livremente através da bicamada lipídica (Grant *et al.,* 1998). Já alguns componentes essenciais, como por exemplo a glicose e alguns aminoácidos, por serem altamente polares, possuem pobre capacidade de penetração através da membrana plasmática endotelial. Assim, com o intuito de atender à alta demanda energética e metabólica do tecido neural, essas substâncias são transportadas através de sistemas de carreadores específicos (Egleton and Davis, 1997), como os sistemas L, ASC e A, que transportam aminoácidos, e o sistema de carreadores GLUT, que transporta glicose de forma independente de insulina, dos quais o mais importante é o GLUT-1 (Janzer, 1993; Egleton and Davis, 1997; Janigro, 1999).

O transporte mediado por carreador ocorre através da interação entre a substância e o transportador, localizado na superfície da membrana plasmática. Este é um transporte saturável, onde o índice de transferência depende da taxa de ocupação do transportador. É um processo que ocorre sem que a substância transportada seja envolvida por membrana, e, sendo assim, não há a formação de vesículas. O transporte pode ser dependente ou não de energia e de íons Na^+, ou ainda, dependente do co-transporte com outra substância na mesma direção (simporte) ou em direção oposta (antiporte) (Egleton e Davis, 1997). Dentre os carreadores da BHE que promovem o efluxo de substâncias do cérebro, o mais abundante no endotélio cerebral é a glicoproteína-P. Este constitui um eficiente sistema de proteção do SNC contra xenobióticos, mas por outro lado, causa resistência ao tratamento de doenças neurológicas, por impedir a entrada de complexos imunes ou agentes terapêuticos no SNC (Sun *et al.,* 2003).

O transporte de peptídeos e proteínas para o tecido neural é restrito e seletivo e ocorre, basicamente, através dos processos de difusão e das vias de endocitose. O processo de difusão, livre ou facilitada, é bidirecional, ocorre de forma independente de energia e pode ser saturável ou insaturável. Este tipo de transporte é dependente das caracteríticas fisico-químicas da molécula, ou seja, do grau de lipofilicidade, massa molecular e estrutura química (Broadwell and Banks, 1993; Egleton and Davis, 1997).

Embora a taxa de endocitose na BHE seja muito reduzida quando comparada à existente em vasos periféricos, esta tem papel essencial na transferência de peptídeos e proteínas para o cérebro (Broadwell and Banks, 1993). Este processo envolve a invaginação da membrana plasmática e formação de vesículas,

com conseqüente internalização de micro e macromoléculas. Três mecanismos de endocitose são descritos na BHE (sumariados na figura 3):

- Endocitose de fase fluida: onde a célula capta parte da fase fluida do meio externo, ou seja, moléculas de água e pequenas proteínas solúveis. Neste caso, as partículas aderem, mas não se ligam à superfície da membrana. Através desse processo, a célula também recicla a sua membrana plasmática ao longo do ciclo celular;
- Endocitose adsortiva: processo pelo qual uma classe especial de moléculas é internalizada, como por exemplo as chamadas lectinas e moléculas carregadas positivamente ou catiônicas. Estas moléculas ligam-se seletivamente aos resíduos de carboidrato do glicocálix, que tem carga negativa, desencadeando assim, o processo de endocitose;
- Endocitose mediada por receptor: caracterizada pela interação entre um ligante disponível na circulação (como a insulina, vasopressina, interleucinas, a transferrina ligada ao íon ferro e lipoproteínas) e um receptor específico na superfície celular. A interação receptor-ligante aciona a internalização do complexo.

As vesículas internalizadas podem, em seguida, ter quatro destinos: a) via de reciclagem, onde molécula e/ou receptor retornam ao domínio da membrana plasmática de onde se originaram; b) via de degradação, após seguirem para os lisossomos; c) via de estocagem, para posterior exocitose ou utilização pela própria célula; e d) via transcitótica, processo mediado por microtúbulos (Nag, 1995), no qual a molécula e/ou o receptor segue para domínios diferentes da membrana plasmática, com ou sem passagem pelo aparelho de Golgi. Na transcitose, a molécula é liberada por exocitose em um domínio da membrana diferente daquele onde se originou (Broadwell and Banks, 1993; Friden, 1993).

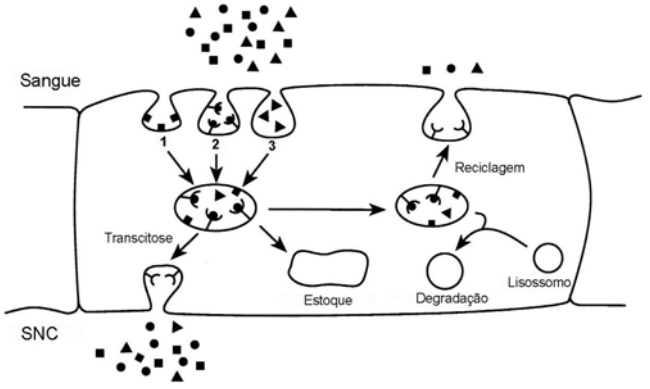

Figura 3 - Representação esquemática das vias de transporte transcelular na BHE, modificado de Friden (1993). Legenda: 1 - Endocitose adsortiva; 2 - Endocitose mediada por receptor; 3 - Endocitose de fase fluida.

3.2. Constituição e função da barreira paracelular

A função de barreira paracelular é determinada pelo complexo juncional intercelular (Huber *et al.,* 2001). Na BHE, as junções intercelulares formam uma rede contínua de proteínas enfileiradas e arranjadas em série de múltiplas barreiras, ocupando toda a extensão do contato interendotelial (Schulze e Firth, 1993; Hirase *et al*., 1997). As junções intercelulares são especializações da membrana plasmática que interconectam as células (Farquhar e Palade, 1963; Collares-Buzato, 2001). No endotélio cerebral, assim como em outros epitélios, dois tipos de junções intercelulares são particularmente relevantes: a junção de oclusão (JO) ou *tight junction* e a junção aderente (JA) ou *zonula adherens*. A JO constitui uma região de extrema proximidade das membranas plasmáticas das células adjacentes, com aparente fusão entre os folhetos externos dessas membranas. Localizada na porção apical do complexo juncional, a JO forma um anel contínuo ou zônula ao redor das células epiteliais, constituindo uma barreira que limita a difusão de íons e solutos pela via paracelular (Collares-Buzato, 2001; Farquhar e Palade, 1963). Na BHE, as JO localizam-se em uma região da membrana plasmática rica em colesterol e associada a caveolas (Nusrat *et al.,* 2000). A JA constitui um dispositivo eficaz de adesão intercelular e está envolvida na regulação da permeabilidade da JO (Brown e Davis, 2002).

Quanto à composição e ao arranjo molecular das junções, varias proteínas têm sido identificadas (figura 4). A JO é constituída por três classes de proteínas integrais transmembrana: as claudinas, a ocludina e a molécula de adesão juncional. As claudinas pertencem a uma família de proteínas multigene com mais de 20 isoformas já identificadas, das quais, na BHE, foram descritas as claudinas-1 e 5 (Lippoldt *et al.,* 2000). Essas proteínas formam dímeros que se ligam a claudinas da célula adjacente de forma homotípica ou heterotípica, formando o primeiro elo de contato intercelular (Furuse *et al.,* 1999). As claudinas associam-se a ocludina, a primeira proteína integral identificada na JO (Furuse *et al.,* 1993). Estudos recentes demonstraram que, diferentemente das claudinas, a ocludina não é requirida na formação da JO (Saitou *et al.,* 2000), mas, por outro lado, é uma importante proteína regulatória da função de barreira paracelular. Hirase *et al.* (1997) demonstraram que a presença da ocludina na JO da BHE está relacionada ao aumento da resistência elétrica e à diminuição da permeabilidade paracelular. A outra proteína integral identificada no complexo juncional é a molécula de adesão juncional (JAM), membro de uma superfamília de imunoglobulinas, cuja função está relacionada à regulação da migração de leucócitos pela via paracelular (Martin-Padura *et al.,* 1998).

Além das proteínas integrais, a JO conta ainda com várias proteínas citoplasmáticas, responsáveis, principalmente, pelo suporte estrutural da junção. Dentre elas estão as proteínas conhecidas como zonula ocludens (ZO-1, ZO-2 e ZO-3), que se associam diretamente às proteínas integrais da JO (Stevenson and Goodenough, 1984; Gumbiner *et al.,* 1991; Haskins *et al.,* 1998). Juntamente com a ocludina, as ZOs são

alvos de proteínas regulatórias, envolvidas no controle da organização e permeabilidade da junção (Staddon *et al.,* 1997). Outras proteínas citoplasmáticas têm sido identificadas, como a AF6, o antígeno 7H6 e a cingulina (Satoh *et al.,* 1996; Yamamoto *et al.,* 1997; Cordenonsi *et al.,* 1999), que promovem a ligação entre as ZOs e o citoesqueleto da célula endotelial. O citoesqueleto é constituído pelos filamentos de actina e miosina, que formam uma densa banda que envolve toda a célula, e interagem com a JO diretamente pela ligação com as claudinas e ocludina, ou através das proteínas citoplasmáticas associadas a esta junção.

A JA contém, como um de seus principais componentes a caderina, uma molécula de adesão dependente de Ca^{2+} (Geiger and Ayalon, 1992). As caderinas são glicoproteínas integrais que promovem a coesão entre células homotípicas adjacentes e interagem intracelularmente com o sistema de microfilamentos (constituído principalmente de F-actina) por intermédio de várias proteínas intracelulares tais como as cateninas (α, β, γ) (Ozawa *et al.,* 1989), α-actinina e vinculina (Knudsen *et al.,* 1995).

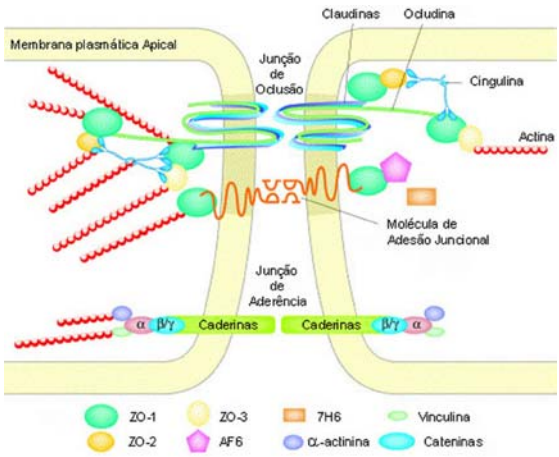

Figura 4 - Representação esquemática da organização molecular das junções intercelulares na BHE, modificado de Huber *et al.* (2001).

O arranjo e a complexidade das JO são atributos diretamente voltados ao controle da passagem de íons e solutos por entre as células e, conseqüentemente, envolvidos na fisiologia da barreira paracelular. A relativa baixa permeabilidade da barreira paracelular na BHE *in vivo* é comprovada pelos seus altos índices de resistência elétrica transepitelial (R_T), que são de aproximadamente 1500 - 2000 $\Omega.cm^2$. Estes valores se assemelham mais aos encontrados nos epitélios do tipo "tight" do que nos endotélios de vasos periféricos, os quais apresentam valores de R_T entre 3 e 33 $\Omega.cm^2$ (Huber *et al.*, 2001).

Apesar da complexa organização juncional, a permeabilidade paracelular dos microvasos cerebrais pode ser modulada, em situações especiais, por importantes proteínas regulatórias localizadas

próximo à região das junções. Entre elas destacam-se as proteínas kinases citoplasmáticas, que, uma vez ativadas, promovem a fosforilação de algumas proteínas juncionais como a β-catenina, ZO-1 e ocludina, principalmente nos resíduos de tirosina. Já as fosfatases, de forma antagônica, promovem a de-fosforilação das mesmas (Staddon *et al.,* 1997; Collares-Buzato *et al.,* 1998). O processo de fosforilação é extremamente dinâmico e culmina na abertura temporária das JO, com passagem de substâncias do lúmem do vaso em direção ao parênquima cerebral. No entanto, embora se conheçam várias substâncias ou situações fisiopatológicas que promovem a fosforilação das proteínas, os mecanismos que desencadeiam a ativação das proteínas kinases e fosfatases juncionais ainda permanecem obscuros.

3.3. Métodos de avaliação da integridade da BHE

A integridade morfológica dos componentes da BHE vem de longe sendo investigada através de métodos ultra-estruturais. Do ponto de vista funcional, grande avanço tem sido obtido utilizando-se traçadores ou marcadores extracelulares, tanto em estudos *in vivo* como *in vitro*. Devido às suas características fisico-químicas, os traçadores normalmente não atravessam a BHE. No entanto, em situações patológicas em que a permeabilidade da BHE está comprometida, estes podem penetrar no tecido neural e serem facilmente rastreados através de métodos morfológicos e/ou bioquímicos. Exemplos de marcadores extracelulares são: a peroxidase (HRP), a microperoxidase, o nitrato de lantânio, o lucifer yelow e compostos marcados com partículas radioativas. Adicionalmente, métodos de citoquímica enzimática e a utilização de anticorpos contra proteínas estruturais e regulatórias conhecidas representam importantes ferramentas de avaliação da integridade morfo-funcional da BHE em situações normais e patológicas.

A integridade funcional da barreira paracelular pode também ser avaliada experimentalmente por métodos biofísicos, através da medida eletrofisiológica da resistência elétrica transepitelial (R_T). Através desta metodologia, mede-se a resistência da camada de células à passagem da corrente elétrica. Uma corrente elétrica conhecida é gerada por dois eletrodos (Ag/AgCl), dispostos nas superfícies apical e basal do endotélio, e a resistência à passagem da corrente é medida por um voltímetro interno (Watlington *et al*., 1970). Desta forma, altos valores de R_T traduzem a alta complexidade das junções e o caráter altamente restritivo ao transporte paracelular e, portanto, a baixa permeabilidade da camada de células à passagem de íons e solutos. Por outro lado, baixos valores de R_T indicam a alta permeabilidade das junções intercelulares a estes componentes. No entanto, a medida da R_T da BHE é principalmente empregada em experimentos *in vitro* ou, então, pode ser feita *in vivo* a partir dos capilares da pia mater, devido ao seu acesso facilitado na superfície cerebral.

Vários modelos de BHE *in vitro* têm sido descritos na literatura, embora nenhum deles tenha conseguido preservar e/ou reproduzir totalmente as características observadas no endotélio cerebral *in vivo*. A cultura primária de células endoteliais obtidas de cérebro de boi, porco ou rato tem-se mostrado eficiente em preservar a capacidade de formação de JO e de efetuar processos de endocitose (Bowman *et al.,* 1981; Audus e Borchardt, 1987; Janzer e Raff, 1987; Franke *et al.,* 2000). A co-cultura com astrócitos, obtidos de cultura primária ou linhagens tumorais astrocitárias, ou ainda, o uso de meio condicionado de astrócitos, têm sido utilizados como estratégias para induzir um aumento na quantidade e complexidade das JO endoteliais e a reorganização do citoesqueleto de actina (Rubin *et al.,* 1991; Gaillard *et al.,* 2001). No entanto, o processo de isolamento do endotélio cerebral e o seu cultivo são muito exaustivos e dispendiosos economicamente, além de apresentarem dificuldades técnicas na prevenção à contaminação por outros tipos celulares como pericitos, células da leptomeninge e células musculares lisas. Além disso, a viabilidade das células endoteliais em cultura primária é muito limitada (Grant *et al.,* 1998).

Desta forma, o estabelecimento de uma linhagem imortalizada que preserve estável o fenótipo da BHE, tem sido alvo de grande interesse, tanto por parte da comunidade científica da área, como da indústria farmacêutica. Duas principais linhagens de células endoteliais têm sido empregadas como modelo da BHE: a linhagem RBE4, obtida de capilares do córtex de rato, e a linhagem ECV304, obtida de veia umbilical humana (Roux *et al.,* 1994; Hurst and Fritz, 1996). O co-cultivo com astrócitos, glioma astrocitário (linhagem C6), ou meio condicionado de astrócitos, tem otimizado a formação de monocamadas com propriedades eletrofisiológicas e funcionais semelhantes ao endotélio cerebral. No entanto, ambas apresentam algumas desvantagens como modelo de BHE. As células da linhagem RBE4, após várias passagens começam a se desdiferenciar e não mais estabelecer JO (F. Roux, comunicação pessoal), tornando-se assim, inviáveis para o estudo dos mecanismos de transporte através da BHE. A linhagem ECV304, por sua vez, dependendo da cepa utilizada, mesmo quando co-cultivada com astrócitos, não formam JO, e conseqüentemente, não apresentam valores significativos de R_T (Hurst, 2000).

Já a utilização de modelos *in vivo* no estudo da BHE apresenta algumas vantagens, como a preservação do arranjo anatômico de suas estruturas, a preservação das diferenças regionais do sistema nervoso e das respostas hemodinâmicas presentes em condições fisiológicas, patológicas e experimentais. No entanto, existem algumas dificuldades técnicas associadas ao estudo *in vivo*, como a fina espessura das células endoteliais cerebrais e a impossibilidade de acesso ao compartimento cerebral e plasmático simultaneamente, sem danos à BHE.

II – OBJETIVOS E JUSTIFICATIVAS

O objetivo do presente trabalho foi avançar no conhecimento dos possíveis mecanismos envolvidos no aumento da permeabilidade da BHE provocado pelo veneno bruto de *P. nigriventer* (PNV). Para isso, foram utilizados modelos *in vivo* e *in vitro*, com o intuito de investigarmos os possíveis constituintes da BHE e vias de transporte afetadas pelo PNV.

Estudo *in vivo*:

- ✓ O efeito do PNV sobre a permeabilidade paracelular da BHE de ratos foi avaliado através da expressão e fosforilação de algumas proteínas juncionais, pelos métodos bioquímicos de *Western Blotting* e imunoprecipitação;
- ✓ A ação do PNV sobre o transporte transcelular da BHE de ratos foi investigada bloqueando-se o transporte transcitótico mediado por microtúbulos, através da droga colchicina, anteriormente ao evenenamento dos animais. Em seguida, as alterações da permeabilidade da BHE foram avaliadas por método ultra-estrutural, utilizando-se o traçador nitrato de lantânio;

Estudo *in vitro*:

- ✓ O efeito citotóxico direto do PNV foi avaliado através de ensaio de viabilidade em três linhagens celulares (linhagem endotelial ECV304, glioma astrocitário C6 e linhagem epitelial MDCK);
- ✓ O efeito do PNV sobre a função da barreira paracelular foi avaliado através da medida da resistência elétrica transepitelial e da localização e expressão de algumas proteínas juncionais, utilizando como modelo a linhagem MDCK. Para isso, utilizamos métodos biofísicos, morfológicos (imunocitoquímica) e bioquímicos (*Western Blotting*). Além disso, a expressão e distribuição dos filamentos de actina, associados às junções, foram investigadas através de método citoquímico;
- ✓ A ação do veneno sobre a via de transporte transcelular foi investigada utilizando-se a peroxidase (HRP) como marcador de endocitose. A taxa de endocitose e transcitose de HRP através da monocamada de células MDCK foi avaliada quantitativamente, por métodos bioquímicos, enquanto que a HRP acumulada intracelularmente foi detectada pelo método de citoquímica ultra-estrutural.

No nosso estudo, a cepa de células endoteliais ECV304 utilizada, mesmo quando co-cultivada com células de glioma C6, não desenvolveu monocamada com junções de oclusão, crescendo de forma

empilhada, fatos comprovados por métodos morfológicos e eletrofisiológicos. Por conta disso, tornou-se inviável o estudo da ação do PNV sobre as vias de transporte endotelial nessa linhagem de células. Apenas o efeito citotóxico direto do veneno foi testado nas linhagens ECV304 e C6. O efeito do PNV sobre as rotas de transporte trans- e paracelular foi então avaliado utilizando-se apenas a linhagem MDCK. As células MDCK constituem uma linhagem epitelial estabelecida, obtida de rim de cão da raça Cocker Spaniel, largamente utilizada como modelo no estudo de citotoxicidade de drogas e na investigação das funções de barreira trans- e paracelular (van Deurs *et al.,* 1990; Collares-Buzato *et al.,* 1998; 2002). Estas células estabelecem junções intercelulares e formam monocamadas que apresentam altos valores de R_T. Adicionalmente, a linhagem MDCK possui organização dos filamentos de actina e expressão de alguns dos marcadores enzimáticos e antigênicos similares aos encontrados no endotélio cerebral (Cereijido *et al.,* 1980; Anderson and van Itallie, 1995; Veronesi, 1996).

III - ESTRUTURA DA TESE

A presente tese de doutorado contém 4 capítulos (incluindo o presente capítulo, que introduz o tema e objetivos desta tese). Os resultados estão organizados na forma de artigos científicos, apresentados no capítulo 2. O capítulo 3 apresenta as conclusões gerais do trabalho e o capítulo 4, as referências bibliográficas referentes ao capítulo 1.

O primeiro artigo (Artigo I) relata os efeitos do PNV, injetado por via endovenosa, sobre a BHE de ratos. A permeabilidade da BHE frente ao envenenamento foi avaliada através de métodos morfológicos e morfométricos, utilizando-se o traçador extracelular nitrato de lantânio aliado à microscopia eletrônica de transmissão. Nossos resultados demonstraram que o PNV provoca a quebra da BHE de ratos, desde 1 até 9 dias após o envenenamento, particularmente na região do hipocampo. A quebra da BHE foi caracterizada por grande quantidade de vesículas de transporte contendo o traçador no citoplasma das células endoteliais, além da presença do mesmo no espaço intercelular. No tecido neural circunjacente aos vasos afetados, foi observado edema vasogênico. No entanto, não observamos infiltrados inflamatórios ou quaisquer indícios morfológicos evidentes de sofrimento neuronal. Os dados morfológicos que compõem este artigo foram realizados durante o período do mestrado. No entanto, a morfometria, a redação do artigo científico, a submissão para publicação e as correções sugeridas pelos *referees* foram realizadas durante o período do doutorado. O artigo completo encontra-se publicado em periódico internacional: Le Sueur LP, Kalapothakis E, Cruz-Höfling MA (2003) Breakdown of the blood-brain barrier and neuropathological changes induced by *Phoneutria nigriventer* spider venom. *Acta Neuropathologica*, 105: 125-134.

No segundo artigo (Artigo II) foram investigados alguns mecanismos pelos quais o PNV pode ter induzido a quebra da BHE nos ratos. Através dos métodos bioquímicos de *Western Blotting* e imunoprecipitação, constatamos que a barreira paracelular, assegurada pelas junções intercelulares, parece não ter sido afetada durante o envenenamento pelo PNV, visto que não foram detectadas alterações nos níveis de expressão e fosforilação de algumas proteínas juncionais, nos tempos de sobrevida analisados. O aumento da permeabilidade da BHE parece ter ocorrido através do aumento do transporte vesicular transendotelial mediado por microtúbulos, uma vez que o pré-tratamento com colchicina, uma droga que despolimeriza microtúbulos, bloqueou completamente as alterações da permeabilidade provocadas pelo veneno. O artigo completo foi submetido a um periódico internacional. Este estudo também recebeu o prêmio de melhor trabalho científico apresentado no "V Congreso de Anatomía del Cono Sur, XXIV Congreso Chileno de Anatomía y XL Congreso Angentino de Anatomía", realizado no período de 12 a 16 de novembro de 2003 em Temuco, no Chile.

No terceiro artigo (Artigo III) investigamos o possível efeito direto do PNV sobre a viabilidade celular e a permeabilidade trans- e paracelular, utilizando como modelos *in vitro,* três linhagens celulares: a linhagem endotelial ECV304, o glioma astrocitário C6 e a linhagem epitelial MDCK. Nossos resultados sugerem que o PNV não possui efeito citotóxico direto sobre as células endoteliais ECV304 e epiteliais MDCK. Apenas um pequeno, mas significativo, efeito citotóxico foi observado sobre a linhagem tumoral astrocitária C6 submetida a altas doses de veneno. Nossos resultados mostraram, também, que o PNV possui ação direta sobre os mecanismos de endocitose apical das células epiteliais MDCK em cultura, como demonstrado por métodos bioquímicos e morfológicos. Já a permeabilidade da barreira paracelular, assegurada pelas junções intercelulares, parece não ter sido afetada. Este trabalho encontra-se em vias de submissão para publicação.

Breakdown of the Blood-Brain Barrier and Neuropathological Changes Induced by *Phoneutria nigriventer* Spider Venom

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ABSTRACT

The blood-brain barrier (BBB) is responsible for selective flux of substances between blood and brain. The selective permeability of the BBB is crucial for the maintenance of the brain microenvironment homeostasis and alterations in the barrier may be involved in many pathophysiological processes. *Phoneutria nigriventer* armed spider venom produces excitatory signals and symptoms in humans, and its recognized neurotoxic action suggests a potential ability to alter BBB permeability. The aim of the present study was to investigate the capacity of *P. nigriventer* venom (PNV) in promoting BBB breakdown in adult rats. After endovenous injection of 850 μg/kg of the whole venom, BBB lesions were evaluated from 18 h to 9 days by ultrastructural methods using the extracellular tracer lanthanum nitrate. Clinical signs and symptoms of rats showed acute neurotoxicity, with some of the animals presenting convulsions, but which were clinically resolved by 12 h post-envenoming.The results showed that PNV is able to increase BBB permeability, particularly in the hippocampus. Changes were first detected in arterioles and post-capillary venules 18 h to 5 days after venom inoculation. The increased permeation of the extracellular tracer peaked on the 1st day, representing about 42% of the examined vessels ($p < 0.01$). This appeared to occur by both transendothelial and intercellular routes, i.e. by pinocytic transport and through interendothelial junctions. Concomitantly, the surrounding tissue showed vasogenic edema and swollen astrocytic processes, without inflammatory infiltrate. The peak of the edema occurrence was observed on the 3rd day, in about 60% of the vessels ($p < 0.001$). Only by the 9th day enhanced capillary permeability was observed, and achieved 36% of the total capillaries ($p < 0.05$). The affected capillaries were characterized by increased number of pinocytotic vesicles, which, in addition, were filled with the extracellular tracer, but without visible transport through the interendothelial pathway. This work demonstrates that systemic PNV inoculation induces BBB breakdown through trans- and paracellular routes. It is concluded that BBB breakdown is an event not associated with the acute neurotoxicity exhibited by the rats.

Running Title: Blood-brain barrier breakdown by *Phoneutria nigriventer*

Key words: Blood-brain barrier - brain vasogenic edema - extracellular tracer - permeability - *Phoneutria nigriventer* venom

INTRODUCTION

The blood-brain barrier (BBB) constitutes the regulatory interface that mediates the restricted movement of substances between the bloodstream and the cerebral parenchyma. The structural basis of the BBB resides in the continuous-type brain capillaries whose endothelial cells (ECs) display well-developed intercellular junctions. Two properties of brain capillary ECs are responsible for the functional characteristics of the BBB: their very low rate of transcytotic vesicular transport and highly electrically resistant tight junctions [for review see 57]. Physiologically, paracellular transport is prevented while the transcellular pathway is highly selective. The EC phenotype is influenced and maintained by several factors including growth factors released by astrocytic end-feet that form a continuous sheath around the cerebral capillaries [7, 25, 29]. The low permeability of the BBB usually allows entry into the brain of only small hydrophobic molecules, a limited set of specifically transported nutrients as glucose and certain amino acids, and a few macromolecules such as transferrin, through receptor-mediated transport [29]. In addition, small and large hydrophilic molecules can penetrate the brain by active transport [56]. Alterations in the functioning of BBB as a highly selective barrier may be involved in many pathophysiological processes [5, 8, 14, 47, 48, 61].

Accidents by venomous animals may constitute an important public health problem. The South American wandering spider *Phoneutria nigriventer* (Ctenidae, Labidognatha), popularly known as armed-spider, is responsible for about 42% of human accidents caused by araneism in Brazil [13, 44]. Extremely aggressive, these spiders wander into rural dwellings and attack inhabitants in their sleep or work. Epidemiologically, *Phoneutria nigriventer* is one of the most important spiders in the country and its venom one of the most toxic.

The venom is composed of a mixture of polypeptides and biologically active molecules, most of them neurotoxic [9]. Various fractions have been isolated, characterized [15, 16, 21, 22, 23, 54, 55] and cloned [31, 32]. PNV inoculation in animals leads to a variety of pharmacological effects such as activation of voltage-dependent sodium channels leading to neuromuscular blockade of the phrenic nerve-diaphragm muscle preparation [24] and/or delayed inactivation of sodium channels *in vivo* [18], release of acetylcholine and norepinephrine by autonomic nerve endings in guinea pig auricles [62], contraction of rabbit vascular smooth muscle [2, 40], increased vascular permeability in rat and rabbit skin with local edema formation [3, 4, 6, 39], relaxation of rabbit *corpus cavernosum* tissue [35, 53], and a biphasic response on arterial blood pressure of rats characterized by a short-lasting hypotension followed by sustained and prolonged hypertension [17].

Based on clinical symptoms as convulsions, hypertension and other neurotoxic effects induced by the venom in the victims, the brain vessels and the central nervous system itself have been considered one of its possible targets. The aim of this work was to investigate the capacity of the PNV in promoting BBB breakdown in adult rats.

MATERIAL AND METHODS

Male Wistar rats (200-280 g) were obtained from an established colony maintained by the University's Central Animal House. Two lots of lyophilized *Phoneutria nigriventer* venom (PNV) from two pools of samples harvested from numerous spiders was supplied by Dr. Evanguedes Kalapothakis. The venom was stored at -20ºC and dissolved in 0.9% sterile saline solution immediately before use.

The animals were anesthetized by ether inhalation and divided into two groups. The treated group was given intravenously (*i.v.*) 850 μg/kg PNV (0.5 ml) in the tail vein, and the control group received *i.v.* injection of saline. The dose of 850 μg/kg was selected based on the clinical manifestations in rats which mimicked the neurotoxic manifestations of severe envenomation in humans in which there is pain, tachycardia, restlessness, sweating, hypertonia, priaprism, neurogenic shock and/or tonic convulsions (and death) (Schenberg & Pereira-Lima, 1966; Lucas, 1988; Bucaretchi *et al.,* 2000). The toxin- and saline-injected rats were killed at 18 h, 1, 3, 5 and 9 days (n = 5 rats/period; total n = 30) for transmission electron microscopic (TEM) studies.

After anesthesia with sodium pentobarbital (Sagatal 30-40 mg/kg, *i.p.*), the rats were perfused through the left ventricle with a cannula adjusted tightly into the ascending aorta with a 100 ml prefixative solution followed by 250 ml of the fixative using a peristaltic pump. The pressure of perfusion was monitored by mercury manometer and it never exceeded 70 mmHg. The prefixative solution contained tri-hydroxy-methyl aminomethane, NaCl, KCl, $MgCl_2$, $CaCl_2$, glucose and procaine [26] and the fixative 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 containing 2% lanthanum nitrate. Lanthanum ions added to a fixative serve as anionic tracer of small size (433 Da) and are easily visible in the electron microscope due to their very high electron opacity [49, 63]. This allows ultrastructural visualization and tracing of extracellular channels and/or some preferential routes for transfer of substances from the lumen of vessels to the surrounding parenchyma. After perfusion, the animals were maintained untouched overnight at 4ºC and, on the next day (18 h later), the brains were removed. This procedure was used to avoid the appearance of artifactual "dark" cells [13]. Samples of cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum were obtained under stereomicroscope and kept in the same fixative without lanthanum nitrate for 1 h. Then the samples were

rinsed in washer solution (0.15 M NaCl and 0.2 M sucrose), post-fixed in 1% OsO_4 diluted in the same solution, dehydrated in graded ethanol series and embedded in Epon 812. Semithin sections (1 μm thick) were cut in an ultramicrotome (Reichert S, Leica), stained with 1% toluidine blue and examined in a light microscope Eclipse E800 (Nikon). Relevant areas were selected for obtaining ultrathin sections (60 nm thick), which were mounted in copper grids (200 mesh) and double-stained with uranyl acetate and lead citrate for examination in a LEO 906 (Zeiss) TEM operated at 60 kV.

For a semiquantitative analyses of the tracer leakage and perivascular swelling frequencies, 30 vessels per animal were randomly selected and counted. The proportion of each of the two types of vascular injury was calculated by dividing the number of affected vessels by the total number of vessels examined. The data were tabulated and multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by Bonferroni test to compare the treated group and its respective control. Values were presented as mean $\pm$ SEM. $P< 0.05$ was considered statistically significant.

RESULTS

General observations

Prior to administration of venom all the rats were active; but 10 min after injection of 850 μg/kg *i.v.* of PNV the rats appeared motionless and excitatory signs of salivation, lachrymation, tremors, priapism, flaccidity followed by spastic paralysis of the posterior legs. From the five rats used in each period of envenoming only three rats showed tonic convulsion. The latter rats received PNV from a lot of venom different from that administered to the other two. Most of the animals, independent of the lot of venom they received showed signs and symptoms for about 12 h, at the end of which a clinical recovery occurred and the animals appeared again normal and active.

Light microscopy findings

Plastic sections of the control group, stained with toluidine blue, showed the neural parenchyma normal and well preserved (Fig. 1A). The occurrence of perivascular edema did not exceeded 10% of the examined vessels. In the PNV-treated group, time-dependent abnormalities were seen particularly in hippocampus (Fig. 1B-D). In the other CNS areas examined (cerebral cortex, thalamus, hypothalamus and cerebellum) changes in permeability were not detected, but these areas have not been exhaustively explored. The disturbances seemed more marked in white than in grey matter of the hippocampus, presenting the neuropil around the affected vessels with a spongiotic appearance (Fig. 1B). However,

changes in the cells morphology or necrosis were absent. The peak of the vasogenic edema was on the 3rd day, representing about 60% of the examined vessels (Fig. 2) ($p < 0.001$).

TEM examination

The control brains showed no ultrastructural abnormality (Fig. 3A). The extracellular tracer was confined into the vessel as a very electron-dense product adhering to the luminal surface of the endothelial cells (Fig 3B). At the junctional complex, the tracer occupied only the luminal aspect of the cleft, internal to the interendothelial tight junctions. As usual, rare tracer-emptied pinocytotic vesicles permeating the endothelial cytoplasm. The occurrence of one or two lanthanum-filled vesicles adhered to the luminal surface was found in 6% of the total number of vessels examined. Neither transendothelial lanthanum-filled transport vesicles detached from the adluminal surface nor tracer-impregnated basal lamina, which could be interpreted as the existence of vesicles trafficking, were found.

In the PNV-treated group, extravasation of lanthanum nitrate was observed particularly in the hippocampus at all time intervals, but the pattern of BBB permeability changed depending on the type of blood vessel and the duration of the experiment.

At 18 h extravasation of tracer was observed in some small-sized hippocampal arterioles and venules, but not in capillaries. While paracellular transport remained unaffected, the transcellular route was increased. Tracer-filled pinocytotic vesicles appeared to be heading toward the abluminal surface of the endothelial cells, but no tracer was observed penetrating cells in the neuropil. The rate of vessels displaying tracer leakage was about 31% of the total number of vessels examined, which corresponded to an increase of 460% in vessel with lesion as compared to the control group. Normal looking neural parenchyma containing intact myelinated nerve fibers and mitochondria was seen among swollen astrocytes.

One day after PNV inoculation, larger numbers of venules and arterioles were affected (Fig. 3C,D), but the capillaries continued to show absence of tracer transport. However, numerous rows of vesicles packed with tracer were aligned along the ab- and adluminal surfaces of endothelial cells, and tracer-filled pinocytotic vesicles fusing to the sarcolemma of the muscle cells of venules and arterioles could also be seen. Lanthanum also impregnated the basal lamina (intercellular space) of neighboring vascular smooth muscle cells. In addition, lanthanum deposits were found between adjacent endothelial cells either as a discontinuous or sometimes as a continuous line, suggesting that the tracer could be passing through open tight junctions (Fig. 3C). Pericytes with phagocytic activity also showed vesicles, vacuoles and phagosomes, some of them with extracellular tracer. These findings show that increased transport of substances occurs both in arterioles and venules after *i.v.* PNV from 24 h on. This time

showed the highest frequency in vessel damaged (42%); PNV treatment induced an increase of 630% in frequency of vessels with tracer leakage as compared to the control group ($p < 0.01$).(Fig. 4). However, it is not possible to state whether this takes place through the transcellular or the intercellular pathways, or both. In some regions, the lanthanum marker gained access to the perivascular neuropil, without being captured by neurons or glia.

Figure 4 summarizes the semiquantitative evaluation of vessels presenting tracer leakage in control and PNV-treated group.

Three and five days after PNV inoculation although the pattern of abnormalities was maintained (Fig. 5A-D), the frequency of vessels with tracer extravasation decreased for about 23% of the total number of vessels examined. On the other hand, the vasogenic edema reached the peak on the 3rd day after envenoming (Fig. 2 and 5A) and further diminished. Markedly widened astrocytic processes were seen around arterioles and venules, containing proteinaceous material, bundles of intermediate filaments, swollen mitochondria, and dilated endoplasmic reticulum.

Lanthanum leakage was observed in some hippocampal capillaries only on the 9th day p.i. and appeared as tracer-containing pinocytotic vesicles within endothelial cells, and free tracer in the adjacent basal lamina (Fig. 5E). The tracer was not observed permeating capillary intercellular junctions, indicating an increment of only transcellular transport. The occurrence of injured capillaries once again increased the frequency of tracer leaked vessels for about 36% ($p < 0.05$).

Neuropathological changes in the brain parenchyma were more severe at 9 days than in previous survival periods and there was a direct correlation between the extension of lanthanum extravasation and the intensity of neuropil alterations. Corroborating the results obtained on light microscope, the disturbances seemed more marked in white than in gray matter. In the former, the tracer was deposited among bundles of apparently intact nerve fibers and in the latter between edematous cellular processes, which gave the neuropil spongiotic appearance. As observed in earlier survival periods, the tracer in gray matter was not taken by cells even on the 9th day post-injection of PNV.

DISCUSSION

P. nigriventer crude venom is basically composed of relatively low molecular mass basic polypeptides (5,000-6,000 Da) [58] mainly with neurotoxic and excitatory action [9], in addition to a small content of histamine (0.06-1%) and serotonin (0.03-0.25%) [20, 60]. Signs and symptoms in human accidents are intense and include irradiated local pain, cramps, tremors, sudoresis, diarrhea, paralysis, arrhythmias, tachycardia, elevated arterial pressure, visual disturbances, tonic convulsions, priapism and

cardiorespiratory failure [9, 10, 11, 59]. Three major fractions were isolated from PNV (PhTx1, PhTx2 and PhTx3), all of them neurotoxic in mice [54]. Intracerebroventricular injection of PhTx1 toxin produces neurotoxic signs, as tail elevation, excitation and spastic paralysis of the hindlimbs. PhTx3 was lethal in mice and induced flaccid paralysis. PhTx2 comprises four sub-fractions (Tx2-1, Tx2-5, Tx2-6 and Tx2-9). Tx2-6 causes scratching, lachrymation, hypersalivation, sweating and agitation followed by spastic paralysis of the anterior and posterior extremities and death. PhTx2 therefore produces the majority of excitatory signs of the whole venom and both activate sodium channels responsible for neurotransmission. Tx2-6 is considered the principal component with significant role in the activation of sodium channels and has toxic activity in the central and autonomic nervous systems [15].

The present study indicates that intravenous administration of *Phoneutria nigriventer* whole venom is able to alter the permeability of the BBB, particularly in the hippocampus of rats. However, not all brain regions responded equally, nor the different types of microvessels were synchronous in relation to barrier breakdown. The way in which the barrier was broken was also different in pre-capillary and capillary vessels.

It was clear from the present experiments that microvessels of the hippocampus were more vulnerable to venom effects than vessels elsewhere in the CNS. The reason for such selectivity remains unknown. Although it is well-known that hippocampal neurons are more easily damaged by noxious stimuli than those of other brain regions, we found no evidence of neuronal lesions or necrosis in our preparations. It may be that venom toxins are not directly active on nerve cells. However, it has been shown that PhTx3 from PNV abolishes Ca^{2+}-dependent glutamate release in rat synaptosomes, being considered a potent calcium-channel antagonist that blocks glutamate exocytosis [27, 51]. This might counteract a possible damaging effect of venom on neurons through excitotoxicity. Our findings demonstrated that the BBB breakdown and vasogenic edema were significant in hippocampus from the 1st day p.i. on.

Another question refers to the type of blood vessel first affected by venom. Permeability increase was initially demonstrated in small-sized arterioles and venules, which were the main route of tracer passage up to the 5th day. Only by the 9th day were capillaries lanthanum-labeled. A greater susceptibility of venules and arterioles as compared to capillaries has also been demonstrated in experimental models of hypertension [45, 46], in bicuculline- [48] and metrazole-induced seizures [28]. A clear understanding of the phenomenon remains unknown. Possible explanations could be uneven occurrence of enzymes, transporters or receptors in distinct vascular segments [1] and differences in the structure or regulating mechanisms of endothelial tight junctions.

Venom induced increase in BBB permeability could depend on enhanced transcellular transport, leakage through tight junctions or both. The first route is supported by the observation of numerous tracer-filled vesicles attached to both sides of the endothelial cells and of tracer diffusely impregnating the basal lamina of microvessels, which was seen from 18 h to 9 days after PNV. Transcytotic vesicles are not a common feature of normal brain capillary endothelium [52] and this was also the case in our control rats in which the tracer remained restricted to vessel lumina. Enhanced transport through transcytotic vesicles has been evidenced in experimental models of hypercapnia [19], acute hypertension [8, 45], epileptic seizures [48], spinal cord lesions [36, 49] and in multiple sclerosis [14]. Our findings suggest that *Phoneutria* venom is also able to activate the transcytotic pathway in brain venules, arterioles and later also capillaries.

We also found extracellular tracer between two neighbouring endothelial cells suggesting that PNV could be able to open interendothelial tight junctions in hippocampal arterioles and venules. Capillaries did not exhibit this kind of transport even by the 9th day. Paracellular transport in brain arterioles and venules (but not in capillaries) was also described in rats during hypertension [46], in rabbit hypothalamus after bicuculline-induced seizures [48] and in multiple sclerosis [14]. However, Nitsch *et al.* [48] claimed that the presence of tracer between neighboring endothelial cells does not necessarily imply junctional opening, as tracer-filled pinocytotic vesicles may eject their content into the interendothelial cleft. Our results therefore are not conclusive as to whether the breakdown of the BBB occurs also through opening of junctions.

When circulating *P. nigriventer* venom eventually gains access to the brain it is conceivable that sodium channel acting neurotoxins may cause swelling of astroglial feet around the hippocampal vessels. These were present not only in the vicinity of lanthanum-marked vessels, but also around unaffected segments of vessels, mainly on the 3rd day p.i., suggesting that the venom may induce ionic disturbances and/or release of cytokines, which may produce effects at some distance from lanthanum permeable vessels. The astrocytes respond rapidly to CNS injury and swelling of foot process is one of the earliest responses. Swollen astrocytes are less capable of maintaining their homeostatic functions, such as uptake of ions and neurotransmitters [33]. One of the mechanisms likely to be involved in glial swelling is the inhibition of membrane Na^+-K^+-ATPase activity and the subsequent accumulation of cytoplasmic Na^+ and loss of K^+ [34]. Blockade of the Na^+-K^+-ATPase or sarcoplasmic reticulum-Ca^{2+} pump by myotoxic peptides that activate sodium channels has been suggested by Ownby et al. [50] in muscle cells. A similar mechanism could be involved in brain tissue.

The PNV-induced disturbances were more frequent and drastic in white than in gray matter. Likewise, white matter is preferentially affected in vasogenic edema, where the accumulation of water

occurs easily and diffusely, due to the greater freedom with which fluids can move through the parallel fiber bundles [43].

Increase of vascular permeability and subsequent edema has been reported in other biological systems after the PNV inoculation. Dialysed PNV (free of histamine and serotonin) injected intradermally leads to local edema in rats and rabbits [3], associated with activation of tissue kallikrein-kinin system and kallidin formation [39]. Swelling of nodal regions of myelinated axons has been described after PNV injection in sciatic nerve [18, 37, 38], and after incubation of phrenic nerve-diaphragm preparations with PhTx2 [41]. These findings have been attributed to the electrolytic disturbance caused by activation and/or delayed inactivation of voltage-dependent sodium channels inducing a higher influx of sodium and subsequent osmotic influx of water. Since pre-treatment with tetrodotoxin, a specific sodium channel blocker, completely prevented the changes [37], they were attributed to the altered physiology of ion channels.

It should be emphasized that the extracellular tracer was never seen inside neurons or glia demonstrating that there was no cell death, which could be attributed to the venom.

It is noteworthy that although BBB enhanced permeability progress from the 18th day p.i., it gained the maximum of permeability on the 1st day, and eventually achieved the most resistant segment of the cerebral microcirculation, the capillaries, by the 9th day p.i.. It is remarkable that more severe neuropathological changes in the neuropil were at 9 days just when capillaries became affected what also run in parallel with the extension of lanthanum extravasation throughout the surrounding parenchyma. Nevertheless, the marked signs and symptoms of acute neurotoxicity of the rats seemed to be completely resolved around 12 h after venom i.v. administration. These findings suggest that the BBB breakdown probably occurs concomitantly with the other systemic effects of PNV, but that such damage did not contribute to the severity of the clinical signs, i.e. the two events are not necessarily related. The BBB damage could just be an epiphenomenon aside the other systemic effects. This interpretation was taken from the observation that BBB damage was consistently observed in all rats treated with PNV injection, whereas convulsions were not. Thus, the acute toxicity of PNV appears likely to be associated with other (unknown) phenomena rather than with BBB breakdown. This situation is similar to that observed for convulxin, a toxin from the venom of the South American rattlesnake *Crotalus durissus terrificus*. This toxin causes convulsions when injected intravenously in cats and mice, but no such effect is seen when injected into the dorsal hippocampus. The convulsions seen with this toxin may be the result of vasoconstriction and reduced cerebral blood flow following intravascular platelet aggregation caused by the toxin (42). Finally, of the several anatomical areas examined, only the hippocampus showed convincing BBB breakdown with vasogenic edema and tracer leakage significant from the 1st day after

injection onwards. The susceptibility of the BBB in this region contrasts with the resistance of other regions of the brain. Although damage to the BBB in the hippocampus was time-dependent, it was not sufficient to explain the marked effects of PNV in the rats. Since the hippocampus accounts for only a small area of the cerebral parenchyma, it is possible for damage to the BBB in this region to be overlooked during gross clinical examination in PNV-treated rats. This fact could partly explain the lack of correlation between the clinical signs of experimental envenomation and progressive BBB damage.

In conclusion, this investigation demonstrated that the venom of *P. nigriventer* was able to increase the permeability of the BBB of rats, but the molecular mechanisms involved remain to be determined. Even in clinical signs graded as severe with *Phoneutria nigriventer* venom in rats, the disruption of BBB may likely not contribute to the acute signs of neurotoxicity. An extrapolation of these results to humans should be taken with caution. New experiments have been planned in our laboratory with the goal of investigating the (poly)peptide(s) responsible(s) for this effect and the underlying mechanisms by which they exert their *in vivo* effects.

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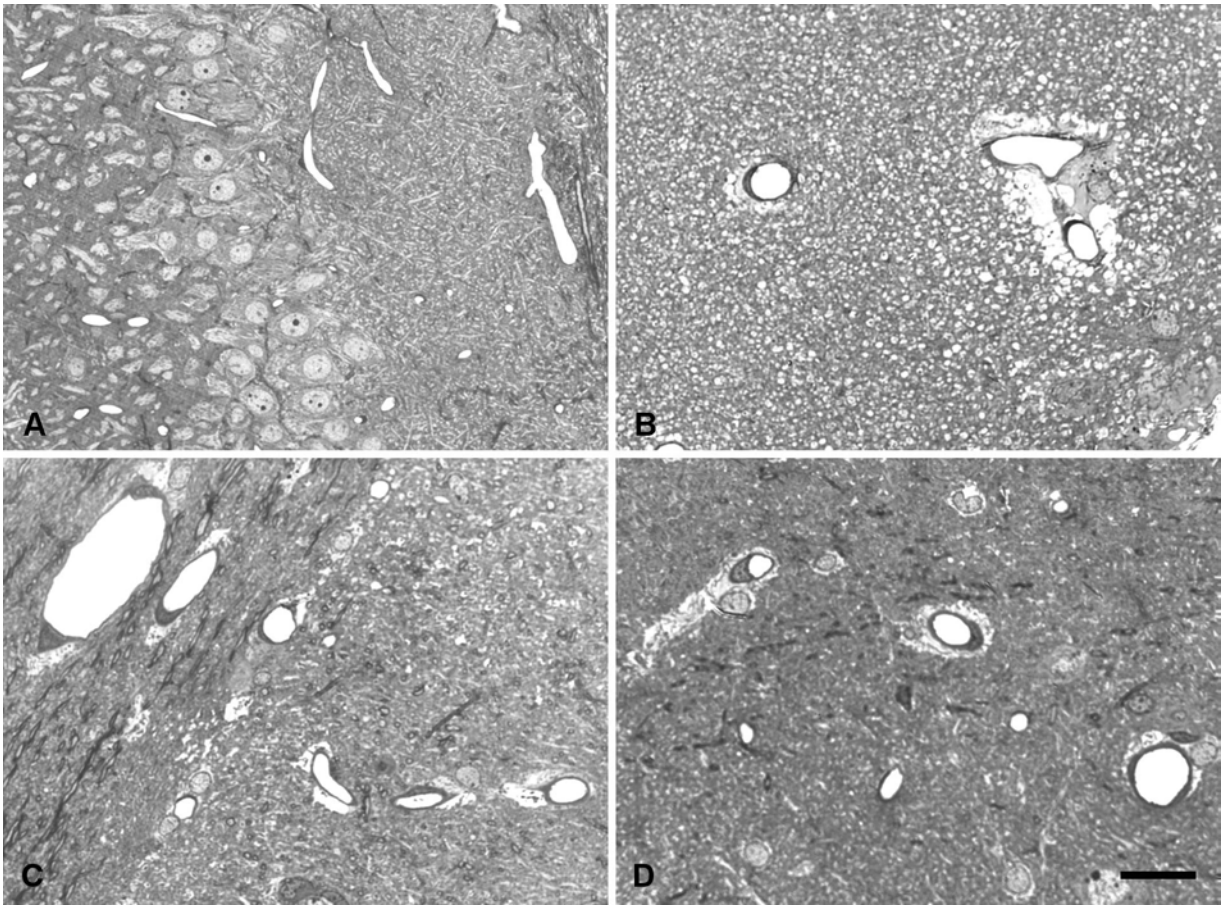

Figure 1- Toluidine-blue-stained sections of rats' hippocampus. **A**, control group showing intact appearance of the neural parenchyma. Note the absence of perivascular spaces in the various types of vessels. **B**, 1st day after PNV-envenoming. White matter region with vessels presenting perivascular swelling and vacuolated surrounding parenchyma. **C**, 3rd day after PNV-envenoming, note vessels of several calibres, all showing vasogenic edema. **D**, five days of the PNV injection. Note some vessels presenting edema beside other apparently intact; [Bars: 26 μm (A) and 13 μm (B-D)].

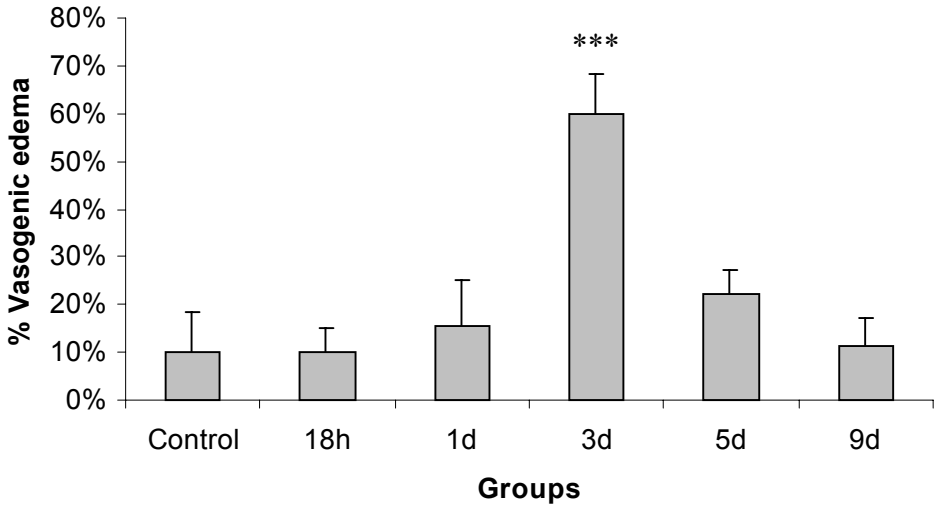

Figure 2 - Percentage of hipocampal vessels with vasogenic edema after PNV injection. Each bar represents the mean number of affected vessels (± S.E.) per period (30 vessels counted by animal; n=5/group). *** $P< 0.001$ compared with control group.

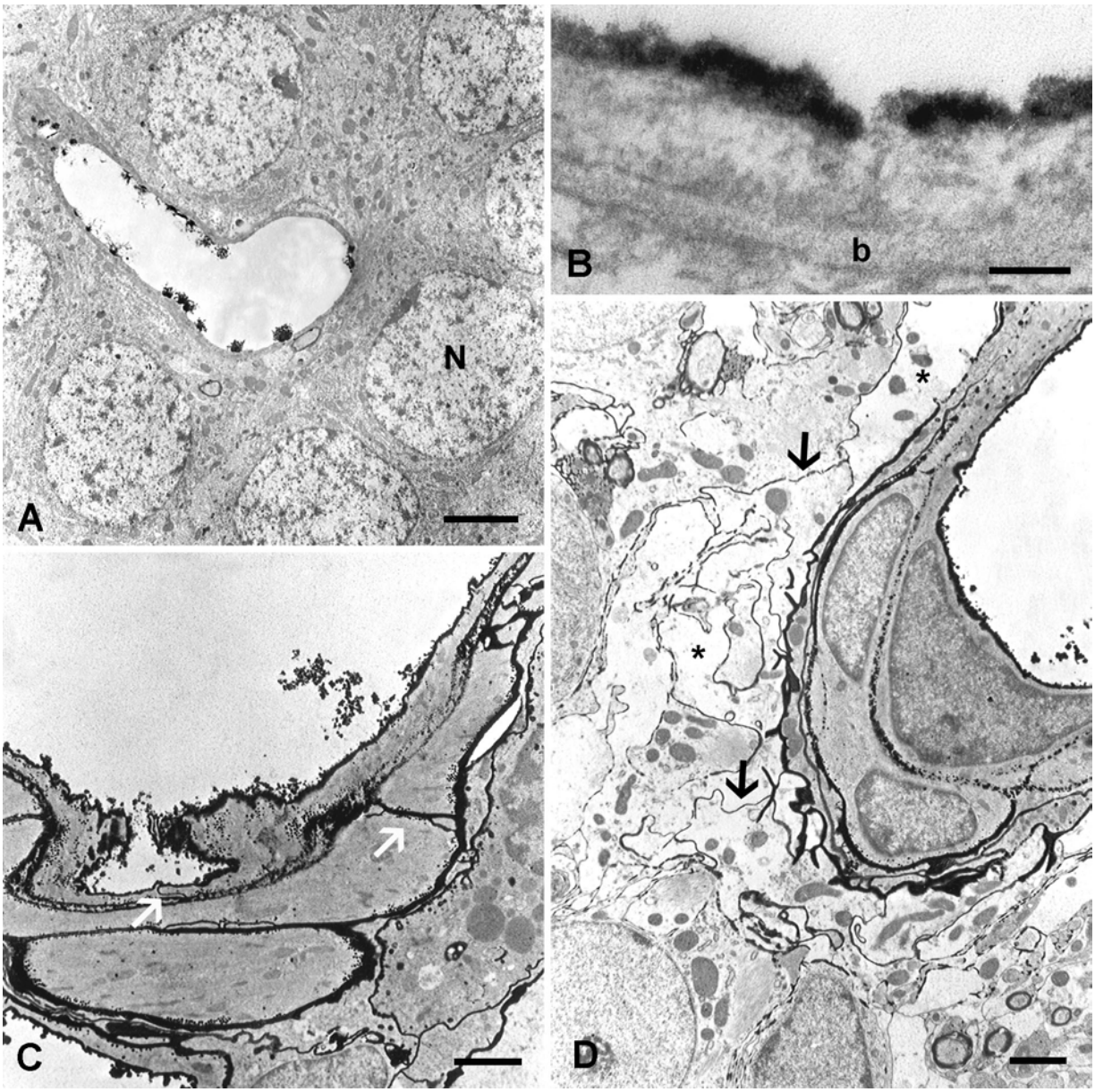

Figure 3 – **A** and **B**, hippocampal vessel of saline-treated rats (controls). **A**, Lanthanum tracer (arrow) is confined to the luminal surface of the endothelium. Neurons (N) are structurally intact. B, high magnification of part of an endothelial cell showing absence of transcytotic vesicles. **C** and **D**, electronmicrographs of hippocampus 1 day after envenomation. Extravasation of tracer was observed in arterioles (**C**) and venules (**D**) with perivascular extracellular spaces containing abundant tracer both among muscle cells (arrowhead), in the intercellular cleft (white arrow) and in the neuropil (black arrow). b = basal lamina; * = electronlucent astroglial feet; (Bars: 4 µm; 0.1 µm; 2 µm; 2 µm, respectively).

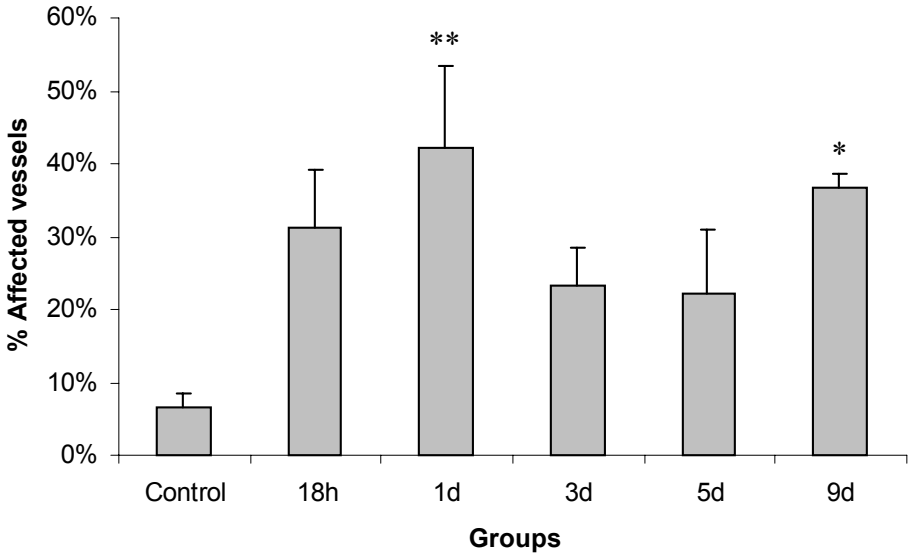

Figure 4 – Percentage of hipocampal vessels with extravasation of lanthanum nitrate after PNV injection. Each bar represents the mean number of affected vessels (± S.E.) per period (30 vessels counted by animal; n=5/group). * $P< 0.05$, ** $P< 0.01$ compared with control group.

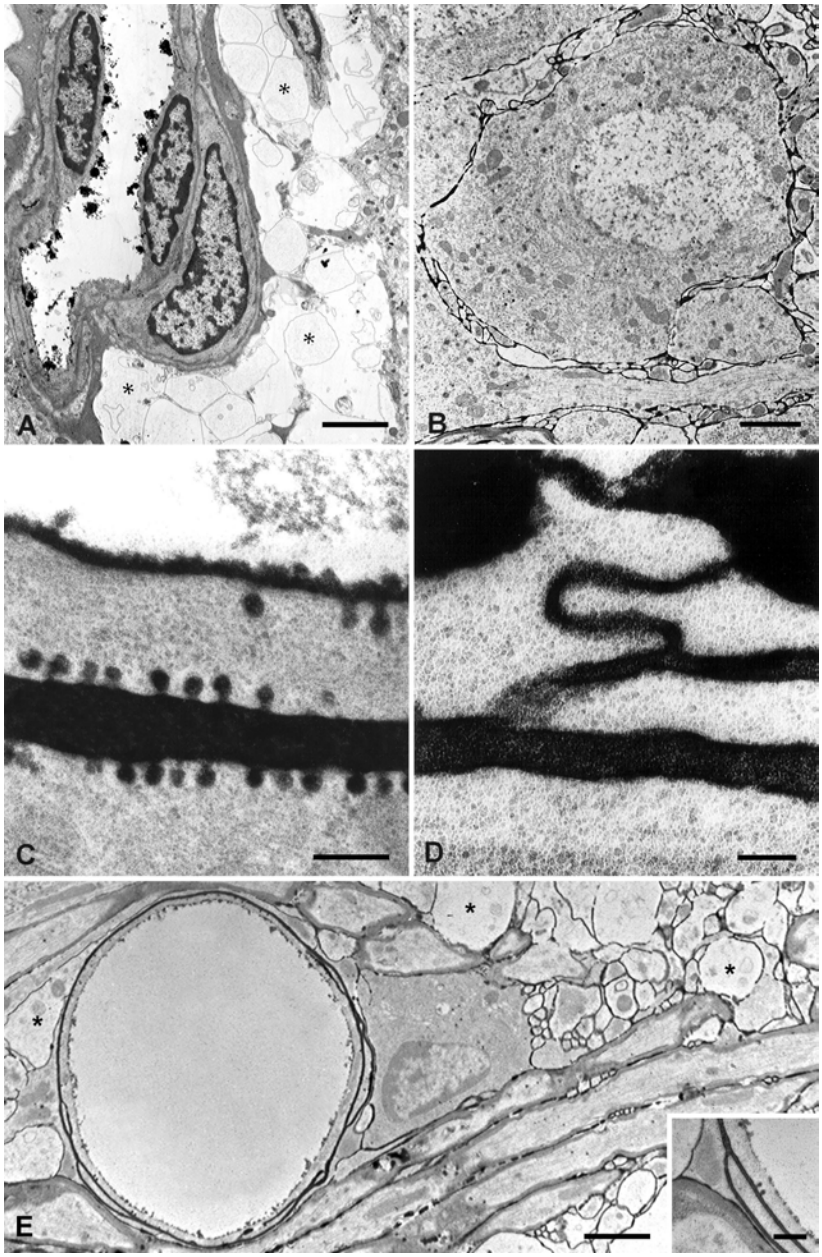

Figure 5 – **A**, three days of the PNV injection show marked vasogenic edema and lucent astrocytic feet around venules, indicating disruption of the BBB. The cytoplasm of these cells shows membranous profiles and flocculent protein precipitate (asterisks). **B**, **C** and **D**, five days after PNV i.v. injection. **B**, neurons near an affected vessel with lanthanum in the intercellular space but not within the nerve cell cytoplasm. **C**, detail of a venular segment shows vesicles containing the lanthanum on both sides of endothelial and smooth muscle cells. **D**, high magnification of an interendothelial junction showing tracer completely filling the cleft between two cells. In **E**, nine days after the PNV injection, a capillary where there was leakage of lanthanum, associated with numerous edematous cell profiles (*), suggesting tissue damage. Insert shows high magnification of **E** where some vesicles containing the tracer are contacting the basal lamina; (Bars: 3 µm; 2 µm; 0.2 µm; 1 µm; 1.5 µm; 0.5 µm, respectively).

Mechanisms Involved in the Blood-Brain Barrier Breakdown Induced by *Phoneutria nigriventer* Spider Venom in Rats

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ABSTRACT

We have recently demonstrated by electron microscopy, using lanthanum nitrate as an extracellular tracer, that the intravenous injection of *Phoneutria nigriventer* spider venom (PNV) induces blood-brain barrier (BBB) breakdown in rat hippocampus. One and nine days after PNV injection, tracer was found in pinocytic vesicles crossing the endothelium and in the interendothelial cleft, suggesting that BBB breakdown had occurred through enhanced transendothelial transport and/or tight-junction opening. In the present work, we investigated the mechanisms by which PNV (850 μg/kg, *i.v.*) increased the hippocampal microvascular permeability in rats. The expression and phosphorylation of some tight- and adherens junctions-associated proteins in hippocampal and hippocampal microvessel homogenates was assessed by western blotting and immunoprecipitation. The microtubule-dependent transcellular transport was also evaluated by quantitative ultrastructural methods in pretreated rats with colchicine (0.5 mg/kg, *i.p.*), prior to PNV injection. Western blots showed no significant increase in the expression of the tight junction-associated proteins ZO-1 and occludin or in the adherens junction-associated β-catenin after PNV administration. In addition, no changes were observed in phosphotyrosine content of occludin and β-catenin in PNV-treated rats compared with control animals. However, the disruption of microtubule-dependent transcellular transport by colchicine completely prevented ($p < 0.001$) PNV-induced leakage of the BBB tracer. These findings indicate that the increased BBB permeability evoked by PNV in rats probably resulted from enhanced microtubule-dependent transendothelial vesicular transport, with no substantial involvement of the paracellular barrier.

Running Title: BBB breakdown by *P. nigriventer* venom

Key words: Blood-brain barrier breakdown - hippocampus - microtubule-transcellular transport - *Phoneutria nigriventer* venom - tight junction

INTRODUCTION

Phoneutria nigriventer (Ctenidae, Labidognatha), popularly known as the "armed" spider, is an aggressive venomous spider found in South America (Lucas, 1988), responsible for about 40% of the spider bites in humans in Brazil (Ministério da Saúde, 1998; Bucaretchi *et al.*, 2000). In severe accidents, cardiovascular and neurological alterations such as arterial hypertension, tachycardia, arrhythmia, visual disturbances and tonic convulsions, have been described (Brazil & Vellard, 1925, 1926).

Experimentally, *P. nigriventer* venom (PNV) elicits a variety of pharmacological effects such as increased release of acetylcholine and norepinephrine by autonomic nerve endings in guinea pig atria (Vital-Brazil *et al.,* 1988), contraction of rabbit vascular smooth muscle (Antunes *et al*., 1990; Marangoni *et al*., 1993b), increased vascular permeability and local edema formation in rat and rabbit skin (Antunes *et al.,* 1992; 1993; Marangoni *et al*., 1993a; Bento *et al*., 1995) and a biphasic response in the arterial blood pressure of anesthetized rats which is characterized by short-lasting hypotension followed by sustained hypertension (Costa *et al.,* 1996). The α_1-adrenoreceptor-mediated convulsions and central haemodynamic alterations observed in rabbits suggest a central action for PNV (Estato *et al.,* 2000).

We have recently demonstrated through transmission electron microscopy using lanthanum nitrate as an extracellular tracer that the systemic injection of PNV causes blood-brain barrier (BBB) breakdown in adult rats, particularly in the hippocampus (Le Sueur *et al.,* 2003). Hippocampal arteriole and post-capillary venule disruption was observed one day after envenoming, whereas capillary was seen disrupted only at the ninth day after envenoming. Tracer extravasation occurred via the interendotelial cleft and by pinocytotic vesicles crossing the endothelium. The molecular mechanisms by which PNV increased the trans- and paracellular permeabilities in the hippocampal microsvasculature remain unknown.

The structural basis of the BBB resides in the continuous-type brain capillaries whose endothelial cells (ECs) display extremely tight intercellular junctions. The two pivotal properties of brain capillary ECs that account for the functional characteristics of the BBB are the highly electrical resistant tight junctions, that limit paracellular transport, and the very low rate of transcytotic vesicular transport, that yields a highly selective transcellular transport (Rubin & Staddon, 1999).

Proper functioning of the paracellular barrier depends on specialized proteins which form the tight- and adherens-junctions (Huber *et al.,* 2001). Specifically at the brain microvessels, these two types of cell membrane junctions occupy the entire length of the interendothelial contact zone (Schulze and Firth, 1993; Hirase *et al.,* 1997) and are responsible for the strong intercellular tightness and adhesion, which produces high transendothelial electrical resistance and decreased paracellular permeability in the CNS (Butt *et al.,* 1990). This junctional tightness is ontogenetically inducible and mantained throughout

life by diffusible factors released by astrocytic end-feet that form a continuous sheath around the cerebral capillaries (Igarashi *et al.,* 1999; Abbott, 2002). The state of phosphorylation of some tight junction-associated proteins, such as ZO-1 and occludin, and the adherens junction-associated protein β-catenin, plays an important role in establishing and regulating paracellular permeability (Staddon *et al.,* 1997; Huber *et al.,* 2001). Exogenous or endogenous agents may affect the stability of this system.

The very restrictive and selective transcellular transport in the BBB makes this route accessible only to small hydrophobic molecules such as CO_2 and O_2, and a limited set of carrier-transported nutrients (glucose and certain amino acids) and a few macromolecules such as transferrin, whose transport is mediated by receptors (Igarashi *et al.,* 1999). Small and large hydrophilic molecules can penetrate the brain by active transport (Rowland *et al*., 1991). The transcellular transport across the BBB and through peripheral endothelial cells is dependent on the subcellular organization of a microtubule network and an actin filament-based cytoskeleton (Liu *et al.,* 1993; Nag, 1995).

In this work, we investigated the mechanisms involved in the PNV-induced breakdown of the BBB in rats. Specifically, the effect of PNV on the expression and phosphorylation of some proteins associated with the interendothelial junctional complex was investigated. In addition, the action of the circulating PNV in enhancing microtubule-dependent transcellular transport was evaluated quantitatively by transmission electron microscopy using rats pretreated with the microtubule-disrupting drug, colchicine, prior to venom injection.

MATERIALS AND METHODS

Animals and venom

Male Wistar rats (250-300 g) were obtained from an established colony maintained by the Central Animal House Service at UNICAMP. Two lots of lyophilized *Phoneutria nigriventer* crude venom (PNV) from two pools of venom milked from numerous spiders was supplied by Dr. Evanguedes Kalapothakis (Federal University of Minas Gerais – UFMG, Belo Horizonte, MG, Brazil). The venom was stored at -20ºC and dissolved in 0.9% sterile saline solution immediately before use.

PNV envenomation

Male Wistar rats 8-10 weeks old were divided into two groups. One of the groups received a single intravenous (*i.v.*) injection of PNV (850 μg/kg in 0.5 ml) in the tail vein, while the other (control group) was given the same volume of saline solution. One and nine days post-injection, the venom- and

saline-injected rats were anesthetised with ketamine + xylazine (185 mg/kg and 14 mg/kg, respectively, *i.p.*) before killing. These periods were chosen based on morphological and morphometrical evidence of BBB breakdown induced by PNV in rats (Le Sueur *et al.,* 2003). Fourty-seven rats were used in this study: 27 rats for biochemical analysis of the expression and phosphorylation of junctional proteins, and 20 rats for ultrastructural evaluation of the effect of PNV on transcellular and paracellular transport. The experiments were done according to the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Isolation of the hippocampus and hippocampal microvessels

Control (n = 5) and PNV-treated rats (n = 10) were anesthetised and killed by decapitation one (n = 5) and nine (n = 5) days after saline or venom injection. The brains were quickly dissected and the hippocampus isolated and homogenized in an extraction cocktail (10 mM EDTA, 2 mM PMSF, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 μg of aprotinin/ml and 100 mM Tris, pH 7.4). The homogenates were centrifuged at 3,000 g for 10 min and the supernatants collected and stored at -70ºC until used for immunoblotting.

Hippocampal microvessels were isolated as described by Huber *et al.* (2002). Briefly, after decapitation (n = 4 for control; n = 4 for 1d PNV; n = 4 for 9d PNV), the brains were quickly removed and the hippocampus dissected and homogenized in a microvessel isolation buffer [103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO4, 1.2 Mm $MgSO_4$, 15 mM HEPES, 2.5 mM $NaHCO_3$, 10 mM D-glucose, 1 mM sodium pyruvate, and 10 g dextran/l (mol wt 70,000), pH 7.4], containing protease inhibitors (2 mM PMSF, 1 mM benzamide, 1 mM $NaVO_4$, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μg of aprotinin/ml and 10 μg of leupeptin/ml). One milliliter of homogenate and equal volume of ice-cold 26% dextran were then vortexed together. The mixtures were centrifuged at 5,600 g for 10 min and the pellets then resuspended in the above microvessel isolation buffer with anti-proteases and passed through a 125 μm mesh filter. For morphological evaluation of the isolation procedure, a drop of filtrate was dried on glass slide, stained with 1% toluidine blue and examined using an Eclipse E800 light microscope (Nikon). The remaining filtrate was centrifuged at 3,000 g for 10 min and the proteins were extracted from the pellets using 6 M urea lysis buffer (6 M urea, 0.1% triton X-100, 10 mM Tris, pH 8.0, 1 mM DTT, 5 mM $MgCl_2$, 5 mM EGTA and 5 mM NaCl) containing protease inhibitors at the same concentrations as described above.

Immunoblotting

Protein concentrations were determined with a Bio-Rad protein assay kit. Aliquots of hippocampus (50 μg) and hippocampal microvessels (40 μg) were applied to 6.5% or 8% polyacrylamide gels. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane by electroblotting, and the membrane then blocked overnight at 4ºC in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 with 5% non-fat milk. The blots were incubated at room temperature (RT) for 1 h with the following primary antibodies (Zymed; San Francisco, CA): polyclonal anti-ZO-1 (1:500 dilution), polyclonal anti-occludin (1:3000 dilution) or monoclonal anti-β-catenin (1:2000 dilution) diluted in buffer solution (TBS plus 0.1% Tween 20) containing 3% non-fat milk. The membranes were subsequently rinsed six times (10 min each) in buffer solution and then incubated with the respective HRP-conjugated secondary antibody (Sigma or Zymed, 1:1000 dilution) diluted in buffer with 1% non-fat milk for 1 h. After rinsing in buffer, the blots were developed on X-Ray film (Fuji Medical) using an enhanced chemiluminescence kit (Super Signal, Pierce). Densitometric analyses were done using Scion image software.

Immunoprecipitation

Samples of hippocampus and hippocampal microvessels were immunoprecipitated as described by Velloso *et al.* (1995), with some modifications. This method allowed determination of the state of phosphorylation of the tyrosine residues of some junctional proteins. Briefly, homogenates were incubated with 10% Triton X-100 and maintained in ice for 2 h. An aliquot containing 400 μg of total protein was diluted with 400 μl of extraction cocktail (containing 30 μg of aprotinin/ml) plus 0.5 μg of anti-occludin or 1 μg of anti-β-catenin antibodies, and incubated overnight at 4ºC. Protein A-Sepharose 6 MB (Pharmacia; Uppsala, Sweden) was added then to the sample to a concentration of 10% (w/v) and incubated at 4ºC for 4 h. The immunoprecipitate was centrifuged at 1,000 g (4ºC) for 15 min and the pellet then resuspended in washing buffer (2 mM sodium orthovanadate, 100 mM Tris pH 7.4, 1 mM EDTA, 0.5% Triton X-100) and centrifuged again at 1,000 g for 5 min. This procedure was repeated three times. After the last centrifugation, the pellet was dried, resuspended in 50 μl of milli-Q water plus 100 μl of Laemmli sample buffer (50 mM Tris, 2% SDS, 10% β-mercaptoethanol, 2% bromophenol blue) containing 100 mg of DTT/ml, and boiled for 5 min before electrophoresis.

Immunoblotting was done as described above, using a monoclonal anti-phosphotyrosine as primary antibody (Sigma, 1:500 dilution). To control for the immunoprecipitation procedure, after investigation of the phosphorylation state of occludin and β-catenin, membranes were stripped (0.1M β-

mercaptoethanol, 2% SDS in phosphate buffer**,** pH 7.4) for 10 min and reblotted with anti-occludin and anti-β-catenin antibodies, respectively.

Immunocytochemistry

In order to determine whether ZO-1, occludin and β-catenin were proteins exclusively located in the brain microvasculature the immunolabeling was assessed in saline-injected rats using a standard indirect immunofluorescence technique (Collares-Buzato *et al.*, 1998b). Rats were anesthetised and perfused transcardiacally with ice-cold PBS (0.1 M**,** pH 7.4) containing 10% sucrose. The brains were quickly dissected, included in OCT-Tissue Tek and frozen in n-hexane with liquid nitrogen. Cryostat sections (10 μm thick) were collected on poly-L-lysine-coated glass slides, air dried and fixed in 2% paraformaldehyde (in 0.1 M TBS**,** pH 7.4) at RT for 10 min. After washing in TBS, the sections were permeabilized with 0.1% Triton X-100 for 10 min and incubated with 0.1% Tween 20 in TBS containing 5% non-fat milk at RT for 1 h. The sections were then incubated at 4ºC overnight with primary anti-ZO-1 (1:200 dilution), anti-occludin (1:50 dilution) or anti-β-catenin (1:50 dilution) antibodies diluted in 1% non-fat milk in TBS. After this step, the sections were washed in TBS and incubated with the second antibody consisting of FITC-labelled anti-rabbit or anti-mouse IgG (Sigma, 1:100 dilution in 1% non-fat milk in TBS) at RT in the dark for 2 h. After washings, the sections were mounted in a commercial anti-fading agent (Vectashield, Vector Labs, Burlingame, CA) and examined by confocal laser scanning microscopy (CLSM; Bio-Rad MRC-1024).

Ultrastructural study

To investigate whether the PNV-induced BBB breakdown was microtubule-mediated, rats were divided into four groups (n = 5 rats/group; total n = 20). In the PNV-treated group (n = 5), the venom was injected as previously described (850 μg PNV/kg, *i.v.*) and the rats then killed one day after injection (Sal/PNV). Control rats (n = 5) were injected with saline *i.v.* instead of PNV (Sal/Sal). The other two groups of PNV- and saline-treated rats were injected with colchicine (0.5 mg/kg, *i.p.*; Sigma) 4 h prior to PNV (Cch/PNV, n = 5) or saline (Cch/Sal, n = 5) administration. The colchicine dose was selected based on a pilot assay (range of 0.5 to 1 mg/kg) and produced no signs of toxicity *in vivo* nor any morphological abnormalities in brain tissue as visualised in thin sections.

One day after the injection of PNV or saline, anaesthetised rats were perfused transcardiacally with 100 ml of prefixative solution (100 mM Tris, pH 7.2, 150 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 3.7 mM glucose and 3.6 mM procaine) followed by 250 ml of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, containing 2% lanthanum nitrate)

using a peristaltic pump (Le Sueur *et al.*, 2003). The perfusion pressure was monitored with a mercury manometer and never exceeded 70 mmHg. After perfusion, the rats were maintained at 4ºC overnight (18 h) before dissection of the brains. Samples of hippocampus were dissected under a stereomicroscope and kept in the same fixative without lanthanum nitrate for 1 h. The samples were then rinsed in washing solution (0.15 M NaCl plus 0.2 M sucrose), post-fixed in 1% OsO_4 diluted in the same solution, dehydrated in a graded ethanol series and embedded in Epon 812. Semithin sections (1 μm thick) were cut on a ultramicrotome (Reichert S, Leica), stained with 1% toluidine blue and examined by light microscopy. Ultrathin sections (60 nm thick) were cut, mounted on copper grids (200 mesh) and double-stained with uranyl acetate and lead citrate for examination in a LEO 906 (Zeiss) transmission electron microscope (TEM).

For quantitative evaluation of the tracer leakage, 30 microvessels (arterioles, venules and capillaries) per animal (total of 150 vessels/group) were randomly selected and counted. The extent of vascular leakage was calculated by dividing the number of affected vessels by the total number of vessels examined. Affected vessels were those containing three or more intracytoplasmic tracer-filled vesicles, or filled the interendothelial clefts and basal membrane, or both. Vessels with two or less vesicles were considered unaffected.

Statistical analysis

The results were expressed as means + SE, where appropriate. The densitometric values of the immunoreactive bands (biochemical studies) and the number of permeable vessels (ultrastructural/quantitative studies) were analysed using the Graphpad Prism software package. One-way analysis of variance (ANOVA) followed by the Bonferroni test was used to compare the treated and control groups. A value of $P< 0.05$ indicated statistical significance.

RESULTS

Effect of PNV on paracellular transport in the BBB

The effect of PNV on the paracellular barrier of the hippocampal endothelium was assessed based on the expression of some proteins associated with tight and adherens junctions and their degree of phosphorylation. The location of the junctional proteins ZO-1, occludin and β-catenin was demonstrated immunocytochemically. Bright staining was observed in the cerebral vessels, compared to the lack of labelling in the surrounding cerebral parenchyma (Fig. 1). Because of its specificity for the

microvasculature, the expression of these proteins was examined in samples of total hippocampus, as well as isolated hippocampal microvessels from saline- and PNV-treated rats.

Western blotting (n = 5 rats for each of the proteins) indicated no significant alterations in the hippocampal and hippocampal microvessels expression of ZO-1 (Fig. 2) or occludin (Fig. 3 A,B) after PNV injection, when compared with the controls. Although a tendency of increase in occludin expression had been observed, the lack of significance between the control and treated rats reflected the high variability in the responses to venom. Similarly, no changes were observed in β-catenin expression after PNV administration (Fig. 4 A,B). There were no changes in the phosphotyrosine content of occludin, or in the phosphorylation of the tyrosine residues of β-catenin in PNV-treated rats when compared with the controls (Fig. 5 A).

Effect of PNV on transcellular transport in the BBB

Microtubule-mediated vesicular transport as mechanism for the increased BBB permeability seen in PNV-injected rats was investigated ultrastructually by pretreating the rats with colchicine.

Saline-injected rats pretreated or not with colchicine maintained their BBB integrity, as shown by the absence of vascular permeability to lanthanum (Fig. 6A,C). In about 88% of the microvessels examined by TEM, the extracellular tracer was confined within the vessel lumen and was seen as a highly electron-dense material adhering to the luminal surface of the endothelium. The remaining 12% of the vessels, which contained 3-5 lanthanum-filled vesicles in the vicinity of the luminal membrane, were considered as leaky vessels (Fig. 7). However, no tracer-filled vesicles were observed attached to the basal surface of the endothelial cells, nor was there any tracer in the intercellular clefts or impregnating the basal lamina in the saline-saline and colchicine-saline treated rats (Fig. 6A,C). In addition, there were no morphological abnormalities in the perivascular parenchyma.

In contrast, in PNV-treated rats, the extravasation of lanthanum from the vessel lumen to the vascular wall was frequently observed (Fig. 6B). The main PNV target was small arterioles and venules, where numerous tracer-filled vesicles were seen at the cytoplasmic surface of the luminal and abluminal endothelial membrane and surrounding smooth muscle cells. Less frequently, tracer was seen impregnating the basal lamina and permeating the clefts between adjacent cells. In some cases, lanthanum was observed outside the vessels, among the structures of the neural parenchyma. About 40% of the vessels showed tracer leakage (Fig. 7).

The injection of colchicine (0.5 mg/kg, *i.p.*) prior to PNV significantly prevented the leakage of lanthanum from the hippocampal vessels lumen (compare Fig. 6A and 6D). In agreement with the

ultrastructural observations, quantification of the vessels with or without tracer extravasation showed that the percentage of leaky vessels in rats pre-treated with colchicine prior to PNV injection was significantly lower ($p < 0.001$) than that observed in rats treated with PNV alone (Fig. 7).

DISCUSSION

In this study, we investigated the cellular mechanisms involved in the PNV-induced BBB breakdown in rat hippocampus. Our findings indicate that the increased BBB permeability evoked by PNV probably occurred through enhanced transendothelial transport**,** while the paracellular barrier was unaffected 1 d and 9 d after envenoming. This conclusion was supported by 1) the lack of change in the expression and phosphorylation of junctional proteins, and 2) the complete inhibition of the PNV-induced BBB breakdown after pretreatment with colchicine.

Increased BBB permeability associated with the disruption of tight junctions has been demonstrated in several CNS pathologies, and has been related mainly to neuro-inflammatory events, such as multiple sclerosis (Plumb *et al.,* 2002), HIV encephalitis and Alzheimer´s disease (Fiala *et al.,* 2002). In these cases, disruption of the tight junctions allowed the paracellular flow of inflammatory cells from blood to brain; the exact molecular mechanisms underlying this phenomenon are still not fully understood. Experimentally, peripheral inflammation models (such as those produced by the subcutaneous injection of formalin, λ-carrageenan or complete Freund's adjuvant into the hind paw of rats) and the use of pro-inflammatory mediators have been shown to impair the functioning of BBB tight junctions and increase the paracellular permeability (Bolton *et al.,* 1998; Huber *et al.,* 2001b; 2002). These authors reported decreased occludin expression, whose presence at tight junctions has been related to increased electrical resistance across the BBB and decreased paracellular permeability (Hirase *et al.,* 1997). Huber *et al.* (2001b) suggested that the decreased expression of occludin and the increased BBB permeability could be the result of excessive phosphorylation of this protein.

The phosphorylation of some junctional proteins, such as ZO-1, occludin and β-catenin, has been indicated as one of the regulatory mechanisms involved in junctional permeability. Phosphorylation is an extremely fast process that culminates in temporary junctional opening, that allows the passage of cells and/or plasma constituents from blood to the brain (Rubin and Staddon, 1999). Phosphorylation occurs especially at tyrosine residues, through regulatory kinases and phosphatases located at the junctional site (Staddon *et al.,* 1995). Tyrosine phosphatase inhibitors or the overexpression of tyrosine kinases leads to a state of continuous phosphorylation, which decrease the transcellular electrical resistance and rapidly increases the ionic permeability of the tight junctions in epithelium and endothelium *in vivo* and *in vitro*

(Staddon *et al.,* 1995; 1997; Collares-Buzato *et al.,* 1998a). On the other hand, the decreased tyrosine phosphorylation of proteins involved in cell-cell contacts may correlate with the acquisition of paracellular barrier function during the embryonic development of chicks (Maher and Pasquale, 1998).

Our results showed that PNV-induced BBB breakdown was not associated with a decrease in the expression of tight and adherens junctional proteins or with an increase in the tyrosine phosphorylation of these proteins. In the case of occludin, there was a tendency for its expression to increase at 1 and 9 d after PNV injection, and could represent a compensatory mechanism in response to the venom-induced BBB breakdown that occurred mainly through the transcellular pathway. This increased occludin expression would be associated with a tightening of the endothelial tight junction, restricting even more the transit of molecules through the paracellular pathway. A similar explanation was also proposed by Song *et al.* (2002) who observed a slight increase in occludin expression during reoxygenation after hypoxia-induced BBB breakdown *in vitro*, and associated it with a decrease in paracellular permeability. In previous work, we detected the presence of lanthanum tracer between adjacent endothelial cells, suggesting that BBB leakage involved the paracellular route (Le Sueur *et al.,* 2003). However, based on the present data, we propose that the presence of extracellular tracer in interendothelial spaces is not indicative of junctional opening, but rather results from tracer-filled pinocytotic vesicles which release their contents into the interendothelial clefts.

Although our findings favour the hypothesis that PNV induced-BBB breakdown in rat hippocampus probably involves little or no contribution from the paracellular route, we cannot completely discard the involvement of tight junctions in this phenomenon since phosphorylation is a very fast, dynamic process (Rubin and Staddon, 1999) and junctional opening could be a transient episode during BBB breakdown.

The other mechanism involved in BBB breakdown relates to the transendothelial route. Stewart (2000) suggested that the restricted transcellular transport of proteins through BBB vessels could reflect low expression of specific protein-binding receptors that define the functions of the vesicles. In addition, the low rate of transcytotic transport in the BBB would be partly dependent on a network of microtubules and on the actin filament-based cytoskeleton (Liu *et al.,* 1993; Nag, 1995). In the BBB, actin filaments associated with junctional proteins and the plasma membrane would function as a powerful anchor to restrict the membrane flexibility, needed for pinosome formation, while the microtubule network would serve as rails for vesicle trafficking (Nag, 1995). BBB breakdown resulting from enhanced transport through transcytotic vesicles has been reported in experimental models of hypercapnia (Cutler and Barlow, 1966), acute hypertension (Bolwig *et al.,* 1977; Nag and Harik, 1987), epileptic seizures (Nitsch *et al.,* 1986), and in brain biopsy specimens from patients with multiple sclerosis (Claudio *et al.,* 1995)

and Alzheimer´s disease (Claudio *et al.,* 1996). Enhanced permeability associated with an increased number of pinocytic vesicles has been demonstrated by ultrastructural methods, with or without extracellular tracer.

The increased permeability to lanthanum after PNV injection were completely inhibited by disruption of the microtubular network. This result explains the significant increase in vesicular transport and the impregnation of the basal lamina with this tracer following envenoming (Le Sueur *et al.,* 2003), and suggests that PNV can activate the microtubule dependent-transcytotic transport system in rat hippocampal vessels. A similar mechanism appears to be involved in the BBB permeability to proteins in hypertensive rats, which was also blocked by pre-treating the rats with colchicine (Nag, 1995). This author also showed that rats infused with the actin filament-disrupting drug, cytochalasin B, had increased cerebrovascular permeability to HRP, indicating that the integrity of endothelial actin filaments was also important for maintaining the BBB impermeability to proteins during normal conditions.

The BBB can be modulated by a range of inflammatory mediators, including serotonin, histamine and bradkinin (Abbott, 2000). *P. nigriventer* venom consists mainly of relatively low molecular mass (3,500-9,000 Da) basic polypeptides (Gomez *et al.,* 2002) with neurotoxic and excitatory actions (Brazil and Vellard, 1925), and a small amount of histamine (0.06-1%) and serotonin (0.03-0.25%) (Diniz, 1963; Schenberg and Pereira-Lima, 1978). Thus, the presence of inflammatory mediators in the venom could explain the ability of PNV to increase the BBB permeability. Antunes *et al.* (1992) showed that the PNV injected intradermally increased the vascular permeability and edema formation in rat dorsal skin, partly through the activation of histamine H_1 and serotonin 5-HT receptors. In addition, Palframan *et al.* (1996) demonstrated the dialysed PNV (free of histamine and serotonin) increased of the vascular permeability in rat skin by stimulating capsaicin-sensitive sensory nerve fibers, leading to the local release of pro-inflammatory neuropeptides, such as P substance, independent of histamine and serotonin receptor activation. The stimulation of these fibers involves the tachykinin NK_1 (Palframan *et al*., 1996) and vanilloid (Costa *et al*., 2000) receptors activation. Recently, Costa *et al.* (2003) showed that the development of pain and inflammation after *P. nigriventer* spider bites could involve the activation of serotonin 5-HT_4 receptors in nociceptive sensory nerve fibers. Dialysed PNV also increases vascular permeability in rabbit skin (Antunes *et al.,* 1992), in a mechanism mediated by activation of the tissue kallikrein-kinin system and kallidin formation (Marangoni *et al.,* 1993). A polypeptide responsible for this effect has been isolated and characterized (Bento *et al.,* 1995).

In conclusion, the increase in BBB permeability caused by PNV involves primarily transcellular vesicular transport. It remains to be determined whether this increase involves a direct action of PNV on the brain endothelium and/or an haemodynamic disturbances caused by the venom.

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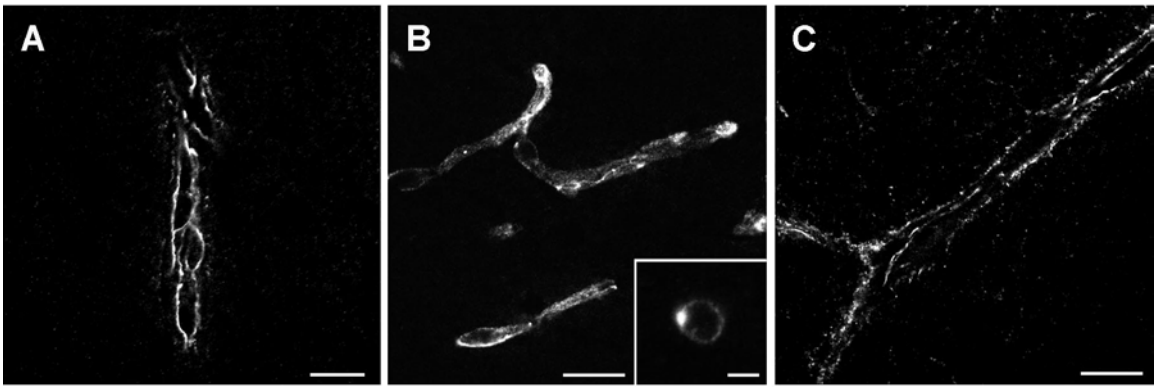

Figure 1 - Immunolabelling for ZO-1 (A), occludin (B) and β-catenin (C) in saline-injected rat hippocampus. Note the specific localization of these junctional proteins in the microvasculature. Inset shows a capillary with brighter points in the region of cell-cell contact. Bars = 20 μm (A-C) and 4 μm (inset).

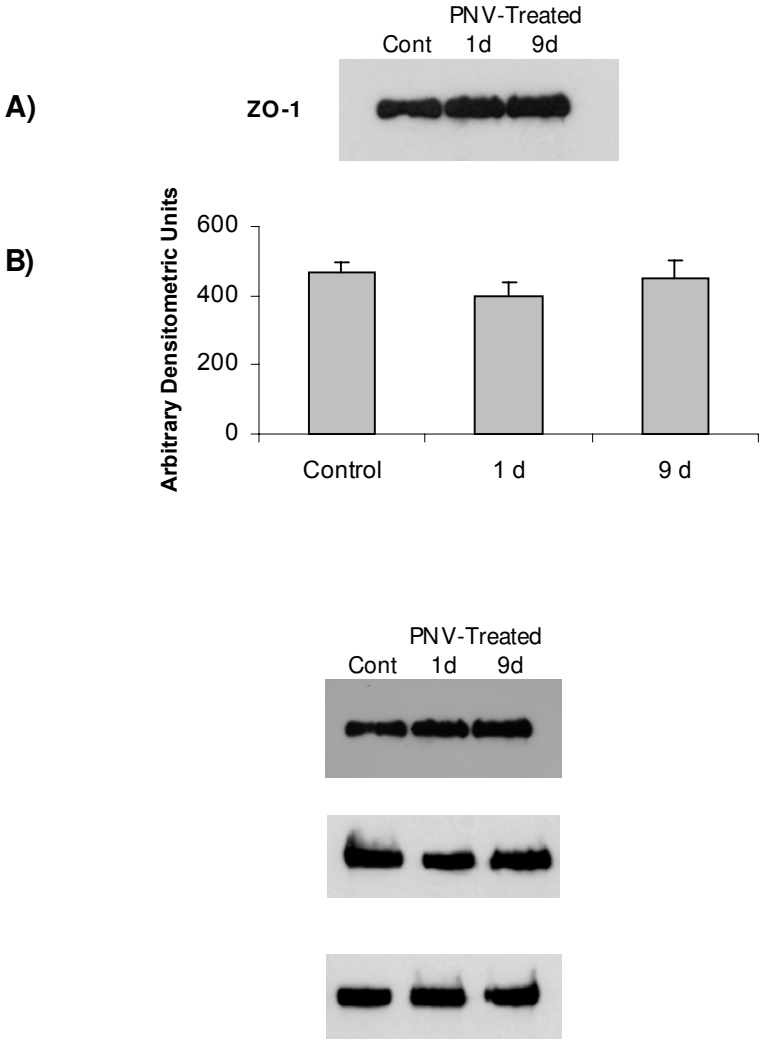

Figure 2 – Expression of the tight junction associated protein ZO-1 after PNV injection. ZO-1 expression was evaluated in samples of isolated hippocampal vessels from saline- and PNV-treated (1 d and 9 d) rats. **A**, a representative blot showing the tight junction-associated ZO-1 migrating at 225 kDa. **B**, the means (+ SE) densitometric units of the immunoreactive bands from four rats/group. PNV did not significantly alter ZO-1 expression compared with control.

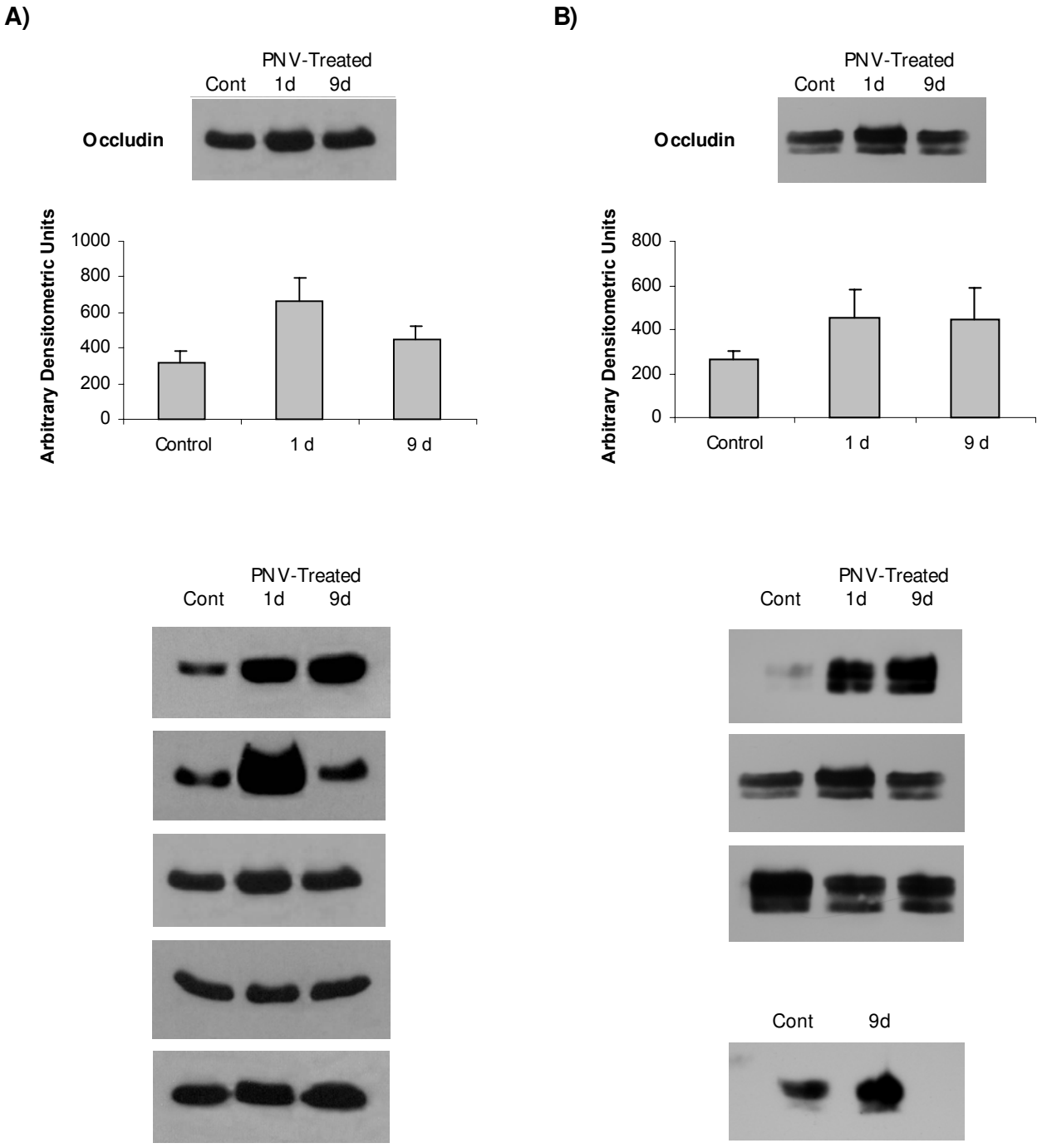

Figure 3 – Expression of the tight-junction associated protein occludin in rat hippocampus (A) and isolated hippocampal vessels (B) after PNV injection. The density of the immunoreactive bands was determined for five (**A**) and four (**B**) rats. Note the tendency of occludin expression to increase in PNV-treated rats at 1 d and 9 d, although this was not significant ($p > 0.05$). Representative blots images showing occludin migrating at 65 kDa are shown. The columns are the mean + SE.

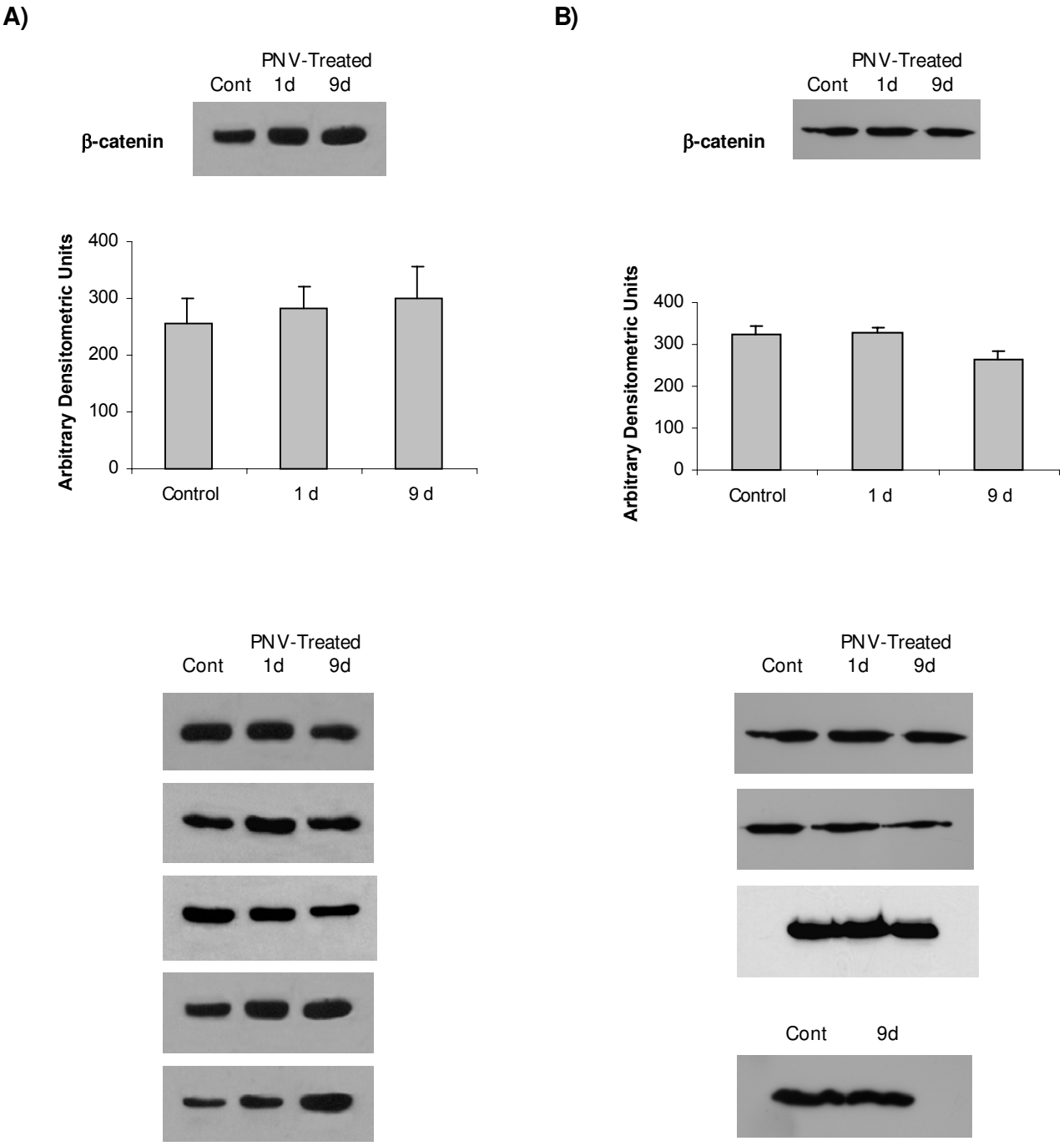

Figure 4 – Expression of the adherens junction associated protein β-catenin after PNV injection. The expression of β-catenin was investigated in rat hippocampus (A) and isolated hippocampal vessels (B). There was no significant change in β-catenin expression 1 d or 9 d after PNV injection when compared with the saline control. Representative blots of β-catenin migrating at 92 kDa are shown. The columns are the mean + SE of 5 (A) and 4 (B) rats.

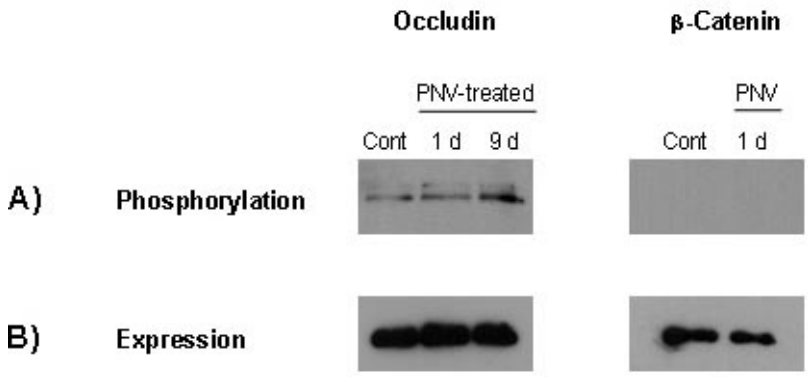

Figure 5 - PNV has no effect on the phosphorylation of junction-associated proteins in hippocampal vessels. Phosphorylation of junction-associated proteins was assessed in hippocampal microvessels isolated from control- and PNV-treated rats (1 d and 9 d). **A** - immunoblots for the detection of phosphotyrosine residues in occludin and β-catenin. There was no detectable change in phosphotyrosine content of occludin after treatment with PNV and no phosphorylation in β-catenin was seen either the control or PNV (1d) group. **B** - immunoblots for occludin and β-catenin after stripping the membranes.

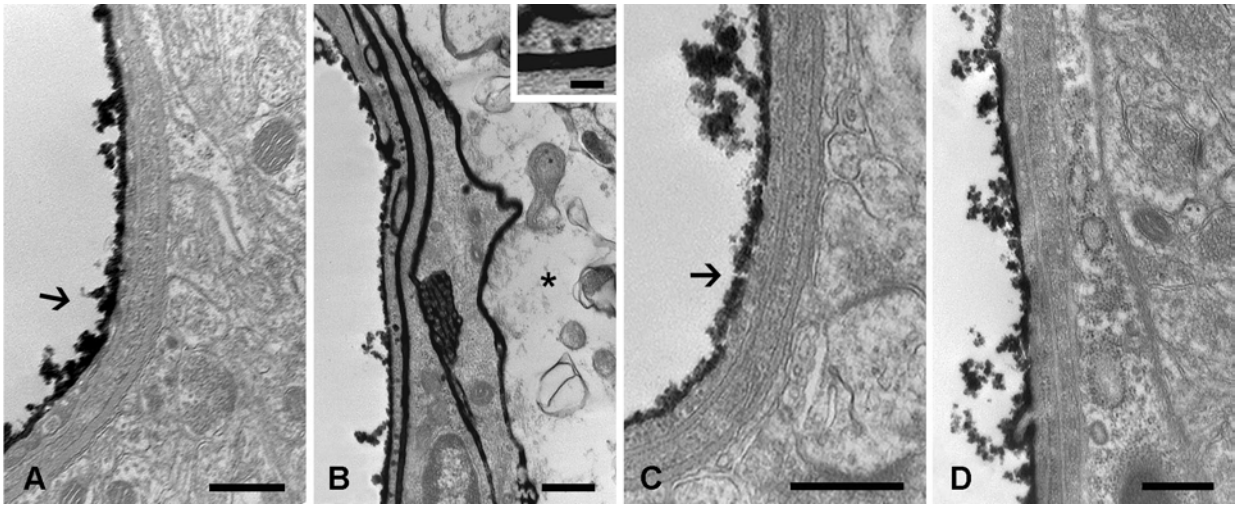

Figure 6 – Inhibition of PNV-induced BBB breakdown by colchicine. Electron micrographs of representative hippocampal microvessels from rats treated with PNV before perfusion with fixative containing the extracellular tracer lanthanum nitrate. **A** and **C** show capillaries from rats treated either with saline *i.p.* and *i.v.* (Sal/sal group) or 0.5 mg colchicine/kg *i.p.* before saline *i.v.* (Cch/sal group), respectively. Note the integrity of the vessels and the surrounding parenchyma. The extracellular tracer (arrows) was confined to the lumen of the vessels and no extravasation was observed beneath the vessel and surrounding tissue. In **B**, the venule of a rat that received 850 μg of PNV/kg *i.v.* 4 h after the injection of saline *i.p.* (Sal/PNV group). Note the leakage of lanthanum out of the lumen to the basal lamina of the endothelium and its presence in transcytotic vesicles (inset), indicating BBB breakdown. In addition, swelling of the astroglial end-feet was seen around the affected vessel (asterisk). In **D**, the disturbances of the BBB permeability were completely prevented when rats were treated with colchicine prior to PNV injection (Cch/PNV group). Bars = 0.5 μm.

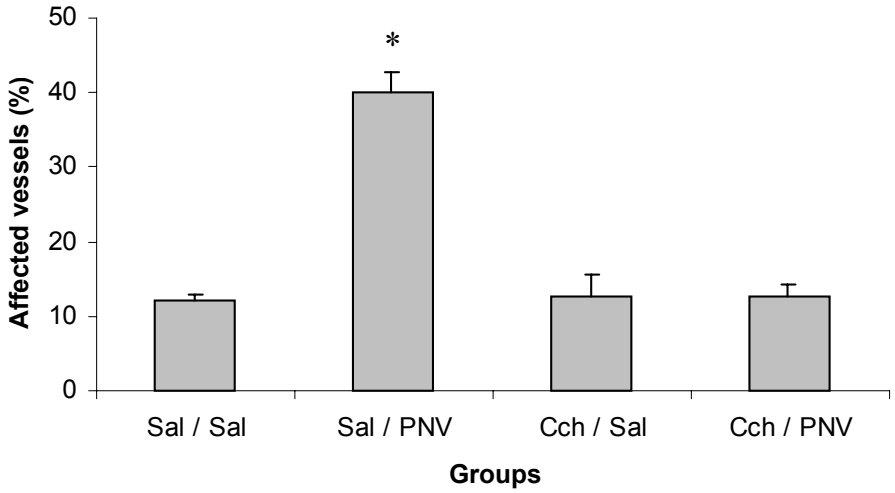

Figure 7 - Percentage of leaky hippocampal vessels in saline- and PNV-treated-rats. Each bar represents the mean number of affected vessels (+ SE) per treatment (30 vessels counted by rat; n = 5 rats/group). Note that treatment with colchicine (0.5 mg/kg, *i.p.*) prior to PNV injection (850 μg/kg, *i.v.*) completely prevented the BBB breakdown induced by the venom.

* $P < 0.001$ compared with all other groups.

In vitro effect of the *Phoneutria nigriventer* spider venom on cell viability, paracellular barrier function and transcellular transport in cultured cell lines

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ABSTRACT

We have recently demonstrated that endovenously injected *Phoneutria nigriventer* spider venom (PNV) is able to cause blood-brain barrier breakdown in rat hippocampus, which was mostly determined by an enhanced vesicular transendothelial transport. In this work we investigated the direct effect of PNV on cell viability of MDCK epithelial-, ECV304 endothelial- and C6 glioma-cell lines, and on cellular transport routes of MDCK monolayers. Our findings showed that the PNV (290 μg PNV/ml) has a direct cytotoxic effect ($p < 0.05$) on C6 glioma cells, but not on ECV304 endothelial and MDCK cells in culture. Also, the PNV did not cause any disturbance on the paracellular barrier function in cultured MDCK cells, as shown by the lack of significant change in the distribution and expression of the junctional proteins, ZO-1, occludin, E-cadherin and the cytoskeletal F-actin. In contrast, there was an enhancement in the transepithelial electrical resistance and a tendency of increased occludin expression. Conversely, the PNV significantly increased the apical endocytosis of HRP, which though was not followed by an equivalent exocytosis at the basal side. We conclude that the venom of *P. nigriventer* activates the transcellular transport pathway in MDCK cells whereas it reinforces the tightening of the paracellular route, as a compensatory mechanism. We also suggest that these effects were not due to cytotoxicity of the venom, since MDCK cells viability remained intact, but instead to a direct activating effect of *P. nigriventer* venom on a given via of the cellular transport.

Running title: Effect of *P. nigriventer* venom on cultured cells

Key words: cytotoxicity - ECV304 - glioma C6 - MDCK - paracellular permeability - *Phoneutria nigriventer* spider venom - transcytosis

INTRODUCTION

Phoneutria nigriventer (Ctenidae, Labidognatha) is an aggressive and wandering spider responsible for about 42% of human accidents caused by araneism in Brazil (Bucaretchi *et al.,* 2000). *Phoneutria nigriventer* venom (PNV) constitutes a rich source of biologically active substances, for this reason its biochemical and pharmacological properties have been widely studied. The PNV is mostly a mixture of low molecular mass basic polypeptides (3,500-9,000 Da) (Gomez *et al.,* 2002), and small amount of histamine (0.06-1%) and serotonin (0.03-0.25%) (Diniz, 1963; Schenberg and Pereira-Lima, 1978). Pharmacologycally, the venom acts on several classes of ion channels largely distributed in synaptosomes, nerve and muscle fibers (for review see Gomez *et al.,* 2002; Rash and Hodgson, 2002; Antunes and Málaque*,* 2003). When injected intradermally, PNV increases vascular permeability and consequently induces edema in rat skin, through mechanisms dependent both on activation of histamine and serotonin receptors (Antunes *et al.,* 1992), as well as the stimulation of capsaicin-sensitive sensory nerve fibers, that leads to locally releasing of pro-inflammatory neuropeptides, such as P substance (Palframan *et al.,* 1996). Dialysed PNV (histamine- and serotonin-free) also increases vascular permeability in rabbit skin (Antunes *et al.,* 1992), with involvement of the tissue kallikrein-kinin system and kallidin formation (Marangoni *et al.,* 1993), and elicits a biphasic response in the arterial blood pressure of anesthetized rats which is characterized by short-lasting hypotension followed by sustained hypertension (Costa *et al.*, 1996).

Recently, we observed using lanthanum nitrate as an extracellular tracer, that endovenous injection of PNV induces blood-brain barrier breakdown (BBBb) in hippocampus of rats (Le Sueur *et al.,* 2003). The increase of the BBB permeability occurred from one to nine days after PNV injection and the tracer was found within pinocytic vesicles crossing the brain endothelium and throughout the interendothelial cleft, suggesting that BBBb may have occurred through enhanced transendothelial transport and/or tight junction opening. Thereafter, investigating the cellular mechanisms of this process we found that the increase of the BBB permeability caused by PNV in rats probably occurred through enhanced transendothelial vesicular transport**,** while the paracellular barrier function remained unaffected at the period intervals studied (Le Sueur *et al.,* unpublished). This conclusion was supported by two observations: the venom did not induce any change in the expression and phosphorylation state of junctional proteins, and the PNV-induced BBBb was completely inhibited when the cytoskeleton-mediated pinocytotic vesicular traffic was blocked in rats pretreated with colchicine. However, it remained unclear whether the BBBb involved a direct cytotoxic effect of PNV on brain endothelium, and as such affecting it as a barrier, or if the venom had a direct effect on endothelial transport processes, and/or affected indirectly the BBB by causing a local haemodynamic disturbance.

In vitro models fit well to discriminate the above mentioned alternatives with the advantage of eliminating the possible haemodynamic effect of the venom as well as permitting a better control of the experimental conditions. The aim of this work was to investigate a possible direct cytotoxic effects of the PNV on the ECV304 endothelial cells, the C6 glioma cells and the MDCK epithelial cells. In addition a direct action of PNV on the paracellular barrier and transcellular transport system was evaluated in MDCK cultured cells. As for BBB endothelium, MDCK cell lineage is able to form a tight cell monolayer with similar organization of proteins associated with the junctional complex besides to form a belt-like distribution of actin filaments (Cereijido *et al.,* 1980; Rubbin *et al.,* 1991; Anderson and van Itallie, 1995; Huber *et al.,* 2001). These cells develop high transepithelial electrical resistance and display polarized distribution of ion pumps, channels and carriers, all of which participate actively in the vectorial transport of substances (Simons and Fuller, 1985; Cereijido *et al.,* 1989).

MATHERIALS AND METHODS

Reagents

Sterile plastic material for cell culture was supplied by Nunc (Roskilde, Denmark), Millipore (Bedford, USA), Falcon (Franklin Lakes, USA) or Corning (Corning, USA). Cell culture media and supplements were purchased from Cultilab or Nutricell (Campinas, Brazil). Primary and secondary antibodies were supplied by Zymed (San Francisco, USA) or Sigma (St. Louis, USA). All other chemicals and reagents were supplied by Sigma, Merck (Darmstadt, Germany) or Amresco (Solon, USA).

Cell culture

MDCK renal epithelial cells were obtained from the Adolf Lutz Institute (São Paulo, Brazil). ECV304 and C6 glioma cells were provided by Dr. H. B. Nader (UNIFESP, São Paulo, Brazil) and Dr. E. S. Ferro (USP, São Paulo, Brazil), respectively. ECV304 cells, C6 glioma cells and MDCK cells were cultured in Ham F-12 medium, Dulbecco´s modified Eagle´s medium (DMEM) and Eagle´s minimum essential medium (EMEM), respectively. All the culture media were supplemented with 10% fetal calf serum, 100 IU of penicillin/ml and 100 μg of streptomycin/ml and cells were grown at 37ºC in a humidified 5% CO_2 atmosphere. Cell culture medium was changed every 48 h and, at confluency, cells were passaged weekly with a solution containing 2.5 mg/ml of trypsin and 100 μg/ml of EDTA for MDCK and C6 cells or 3.5 mg/ml of pancreatin for ECV304 cells.

Exposure to *Phoneutria nigriventer* venom

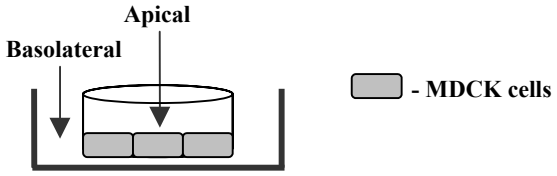
Two lots of lyophilized *Phoneutria nigriventer* crude venom (PNV) from two pools of crude venom milked from spiders were used. The venom was stored at -20ºC and diluted in culture medium immediately before use. After cell confluence, the culture medium was carefully removed by aspiration and replaced by fresh medium containing *P. nigriventer* crude venom at concentrations of 14.6 μg of PNV/ml or 292 μg of PNV/ml. The control group received the same volume of culture media without venom. After PNV-treatment the cells were maintained in a 5% CO_2 humidified atmosphere at 37ºC up to 24 h. The *in vitro* venom dose (14.6 μg of PNV/ml) was selected for further experiments based on the concentration of 0.85 mg of PNV/kg rat used before in *in vivo* experiments (Le Sueur *et al.,* 2003), and considering (i) rats with 250 g weight average and (ii) that a 250 g rat has a plasma volume average of 14.5 ml (Filep et al., 1997; Lo and Kaufman, 2001). Thus, the 14.6 μg of PNV/ml dose is obtained by applying the *in vivo* concentration (0.85 mg/kg = 0.21 mg/0.25 kg) to the plasma volume (14.5 ml) of a 250 g rat. To test whether the PNV effect was concentration-dependent or not we used the dose of 292 μg of PNV/ml, which was 20-fold larger than 14.6 μg of PNV/ml.

Cell viability (MDCK, ECV304 and C6 glioma cells)

Cell viability was analysed by using the neutral red viability assay (Borenfreund and Puerner, 1985). Cells were seeded in 96 well-tissue culture microtiter plates at density of 1.96 x 10^5 cells/well for MDCK, 0.64 x 10^5 cells/well for ECV304 and 0.32 x 10^5 cells/well for C6 glioma cells. The venom exposure started as soon as cell confluence was reached, i.e., three days for MDCK cells, four days for ECV304 cells and three days for C6 cells. Following 24 h PNV venom exposure, the media were replaced by the respective fresh medium containing 5 μg of neutral red dye/ml. The cells were incubated under 5% CO_2 humidified atmosphere at 37ºC for 3 h. The media containing the dye were then removed and cells washed twice with formol-calcium solution (40% formaldehyde, 1% anhydrous calcium chloride, v/w) for removing non-incorporated dye. Subsequently, 200 μl of a solution containing glacial acetic acid and ethanol (1:100, v/v) was added to each well and after 15 min of incubation at room temperature (RT), the plates were measured by reading at 540 nm in a microplate reader (Multiskan MS, Labsystems). The cell viability was determined by comparing the absorbance absolute values from PNV-treated cells with the absorbance mean value obtained from the control cells (without PNV treatment), which were taken as 100% cell viability.

Measurement of transepithelial electrical resistance (MDCK cells)

The PNV effect on the epithelial barrier function was assessed by measuring the transepithelial electrical resistance (TER) across MDCK monolayers, using two Ag/AgCl "chopstick" electrodes coupled to a combined voltmeter and constant current source (Evom-G, World Precision Instruments). For this purpose, MDCK cells were seeded at high density (5 x 10^5 cell/cm^2) into tissue culture inserts incorporated with permeable inorganic (Anocell, Nunc), nitrocellulose (Millicell HA, Millipore) or flexible track-etched (PET, Falcon) membrane filters. The inserts were placed into the 24 well-plate. For PNV treatment, the culture medium with or without venom was applied to both the apical and basolateral monolayer surfaces, as represented below. TER values were corrected for the effective growth area of the filter after subtracting the resistance of the filter with bathing solution and without cells. An increase in TER represents increased tight junction formation and a corresponding decrease in its permeability. The standard average of the TER displayed by MDCK monolayers at confluence (before PNV exposure) were ~ 180 Ω cm^2.



Immunostaining of junctional-associated proteins (MDCK cells)

The expression and distribution of some proteins associated with the tight- and adherens-junctions were evaluated using a standard indirect immunofluorescence protocol (Collares-Buzato *et al*, 1998). Briefly, after PNV exposure, MDCK monolayers grown on Anocell filter support were both fixed and permeabilized overnight with methanol at –20ºC. The filters were then rehydrated in 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 3% horse serum for 30 min and incubated at 4ºC overnight with the following primary antibodies: polyclonal anti-ZO-1 (1: 200; Zymed), polyclonal anti-occludin (1: 100; Zymed) or monoclonal anti-uvomorulin (1: 200; Sigma) diluted in PBS containing 3% horse serum. Each of the antibodies solution was applied to both apical and basolateral surfaces (80 µl each face). After this step, the filters were washed in PBS and 2 h-incubated with the secondary antibody consisting of FITC-labelled anti-rabbit or anti-mouse IgG (1:50; Sigma) diluted in the same solution, at RT in the dark. After washing with PBS, the monolayers were detached from the Anocell inserts and mounted in a commercial anti-fading agent (Vectashield, Vector Laboratories, Burlingame, USA). Cell staining was examined by confocal laser scanning microscopy (CLSM; Bio-Rad, MRC-1024). To compare the treated and untreated

control cell groups, the microscopic examination of both was done in the same session. The observation was confirmed in a minimum of two sets of experiment. Control of the reaction have the primary antibody incubation omitted.

Cytochemistry for F-actin (MDCK cells)

After 24 h of PNV treatment, MDCK cells grown on Anocell filters were fixed in 3.7% formalin (in 10 mM PBS, pH 7.4) for 30 min, permeabilized with 0.1% Triton-100 (in PBS) for 10 min and then incubated with TRITC-conjugated phalloidin (1 μg/ml in PBS) at RT in the dark for 2 h. Afterwards the monolayers were washed several times with PBS, detached from Anocell inserts and mounted with Vectashield (Vector Laboratories, Burlingame, USA). Cell staining was analysed by CLSM. To permit comparison between the treated and control groups, the microscopic examination was done along the same session and reassured in three different sets of experiments.

Immunoblotting (MDCK cells)

Control and 24 h PNV-treated MDCK cells, grown on Anocell membranes were scraped, added to 500 μl of ice-cold PBS (0.1 M**,** pH 7.4) and centrifuged at 1,000 g at 4ºC for 1 min. The pellets were sonicated with an anti-protease cocktail (100 mM TRIS, 10 mM EDTA, 2 mM PMSF, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium ortovanadate, 0.03 mg of aprotinin /ml) for 15 s and stored at -70ºC until used. Protein concentration was determined with the Bio-Rad DC protein assay. Aliquots containing 30 μg of total protein plus 30% of the total volume of Laemmli sample buffer (50 mM sodium phosphate, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, 2% β-mercaptoethanol) were incubated at 37ºC for 60 min and applied to 8% polyacrylamide gels. Following electrophoresis, separated proteins were transferred to nitrocellulose membranes (Bio Rad) by electroblotting. Membranes checking with Ponceau S dye were then blocked at 4ºC overnight with Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 plus 5% non-fat milk. The blots were then incubated at RT for 1 h with polyclonal rabbit anti-occludin primary antibody (1: 2000; Zymed) diluted in buffer solution (TBS plus 0.1% Tween 20) containing 3% non-fat milk. After rinsing six times (10 min each) with buffer solution the membranes were incubated with the goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) (1: 1000; Zymed) diluted in buffer solution with 1% non-fat milk for 1 h. After washings the blots were developed on X-Ray film (Fuji Medical) using an enhanced chemiluminescence kit (Super Signal West Pico Chemiluminescent Substrate, Pierce). Densitometric analyses were done using the Scion image software.

Evaluation of HRP Endocytosis and Transcytosis (MDCK cells)

The transcellular flux of HRP was made in MDCK monolayers grown on Millicell - HA filters and evaluated by biochemical assay as described by Bomsel *et al.* (1989), with some modifications. Briefly, following 24 h PNV exposure, the EMEM was replaced by fresh medium without phenol red containing 1 mg of HRP/ml (Type II; Sigma) and 0.05 % BSA (fraction V; Sigma), and added to either the apical or the basolateral monolayer side. The HRP uptake by MDCK cultured cells was carried out at 37ºC for 60 min. During this time, the treated cells remained exposed to PNV. Aliquots of the contralateral supernatant were then sampled and cells cooled to 4ºC and washed (4 x 10 min each, under agitation) with ice-cold PBS (10 mM, pH 7.4) containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.2 % BSA. The cells were then lysed with 1% Triton X-100 and 0.5% SDS in PBS at 4ºC for 45 min, and the sample lysates were collected. TER was measured at the beginning and before cell lysis for checking the epithelial barrier integrity. HRP activity either in the lysate (endocytosed HRP) or in the contralateral medium (transcytosed HRP) was revealed after mixture 1:10 samples and substrate solution, containing 0.1 ml of 0.3% H_2O_2 and 0.8 ml of 1g % o-dianisidine (dissolved in absolute methanol) to each 10 ml of 50 mM phosphate buffer, pH 5.0 (Steinman and Cohn, 1972). The absorbance values were obtained at 462 nm by kinetic assays in a microplate reader (Molecular Devices, Spectra Max 340).

HRP cell trafficking

The fate of HRP particles uptaken was traced by cytochemistry according Balda *et al.* (1996), with some modifications. Briefly, MDCK cells grown on rat-tail collagen-coated PET filters were incubated with 5 mg of HRP/ml of sterile saline added to the apical surface. HRP uptaking was allowed at 37ºC for 10 min and then stopped by cooling the filters on ice. The filters were washed six times for 3 min with cold saline and the cells re-incubated at 37ºC for 90 min in Hanks sterile solution (136 mM NaCl, 5.4 mM KCl, 0.95 mM $CaCl_2$, 0.39 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 4.2 mM $NaHCO_3$). During this incubation additional period, the monolayers were again exposed to the venom (90 min). The cell monolayers were then fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) for 30 min. To visualize the internalized HRP, after washing in buffer, the monolayers were incubated with 0.1% diamino-benzidine (DAB) diluted in the same buffer for 1 min, and then H_2O_2 (30% volume) was added to a final concentration of 0.01% to trigger the reaction. After 2 min incubation in the dark, the reaction was stopped by washing the filters with buffer. After removing from the inserts, the cell monolayers were post-fixed in 2% OsO_4 at 4^{o}C for 1 h and block-stained with 1% uranyl acetate in maleate buffer (50 mM, pH 5.2) in the dark for 1 h (Parton *et al.,* 1989). Monolayers were then dehydrated through graded ethanol series and embedded in resin Epon 812. Sections of 80-90 nm were cut

in an ultramicrotome (Leica, Reichert S) and transferred to copper grids (200 mesh). After double-staining with uranyl acetate and lead citrate, the sections were examined in a transmission electron microscope (TEM; Zeiss, Leo 906) operated at 60 kV.

Statistical Analysis

All numerical results were analysed using the Graphpad Prism software package and expressed as the mean $\pm$ standard error (SE). The statistical significance among the control and PNV-treated groups was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni test. A value of $P< 0.05$ indicated statistical significance.

RESULTS

The screening for PNV toxicity effect over the three cultured cell lines tested was assessed by measuring neutral red cell uptake (Fig. 1). The amount of dye taken by the population of cells in culture is directly proportional to the number of viable cells. Our results showed a 8% significant ($p < 0.05$) decrease in cell viability of the C6 glioma cells only when they were exposed to 292 μg of PNV/ml for 24 h. This venom concentration is 20 folds higher than the 14.6 μg/ml (corresponding to the 0.85 mg/kg used before in *in vivo* studies, Le Sueur *et al.,* 2003). These data suggest a subtle cytotoxic effect of the PNV in this cell type. In contrast, PNV had no effect on cell viability of ECV304 and MDCK, even at the highest dose tested. In addition, the dose of 14.6 μg of PNV/ml was not cytotoxic in the three cell lines.

To assess whether the PNV alters the paracellular barrier function of MDCK cultured cells, parameters such as TER, expression/distribution of some junctional proteins and F-actin filaments were analysed. TER was measured across MDCK cells monolayer grown on permeable filters and exposed either to 14.6 or 292 μg of PNV/ml of venom. As shown in Fig. 2, PNV induced a significant increase of about 140% in TER after 24 h exposure to 292 μg of PNV/ml in comparison with control group. However, PNV had no significant effect on this parameter when the venom concentration was 14.6 μg/ml in comparison with the control.

The expression and distribution of junctional proteins following PNV exposure seen by indirect immunofluorescence (Fig. 3) showed that tight junction-associated proteins, occludin and ZO-1, were detected mainly at the apical regions of the cell-cell contacts, while the E-cadherin protein, associated with the adherens junction, was found along the whole lateral membrane at the intercellular contact. Our results suggest that both the expression and distribution of ZO-1 and E-cadherin were not affected by PNV

treatment (14.6 μg/ml not shown and 292 μg/ml shown in Fig. 3). In contrast, immunostaining for occludin revealed that the PNV-treated MDCK monolayers, at concentration of 292 μg/ml, displayed higher and well-defined occludin staining at the membrane sites of cell-to-cell contact in comparison with the control cultured cells. By western blotting, it was observed a tendency, although not significant, towards an increase in the expression of occludin following PNV exposure when compared to the control MDCK cells (Fig. 4). Monolayers exposed to 14.6 μg/ml of PNV showed the same pattern of intensity and distribution of occludin staining observed in control group.

TRITC-phalloidin staining revealed no changes in the distribution of F-actin filaments in MDCK cells following PNV exposure (Fig. 5). Both PNV-treated and control monolayers showed F-actin filaments throughout the cytoplasm at the sites where the microvilli were inserted apically. F-actin filaments were also detected at the cell periphery forming the perijunctional actin ring and at the basal cell surface, forming the stress fibers.

The effect of PNV on the transcellular transport was assessed by biochemical and ultrastructural methods using HRP as a marker. For the biochemical assay, the HRP was added either to the apical or basolateral surface of the MDCK monolayers. After 60 min of continuous marker internalisation at 37ºC, the biochemical quantification of the captured HRP by the MDCK cells revealed a significant increase in the HRP endocytosis when exposed to 14.6 μg of PNV/ml (Fig. 6 A). The increased endocytosis occurred when HRP was applied apically and was about 50% higher than in the control cells. However, the quantification of HRP in the opposite medium of the administration showed a non significant increase in HRP transcytosis in PNV-treated cells as compared to the control (Fig. 6 B). When HRP was added basolaterally, no changes in HRP endocytosis or transcytosis were detected after PNV treatment in comparison to control MDCK monolayers (Fig. 6 C,D). The results showed that the TER remained constant during the whole period of experimentation (data not shown), so validating the findings on endocytosis and transcytosis of HRP across the monolayer.

Corroborating the biochemical findings, ultrastructural analysis revealed that the PNV-treated MDCK monolayers display higher number of intracellular structures containing the marker HRP only when this was added apically (Fig. 7). Moreover, the images obtained by TEM also confirmed that no such labeling was present along the intercellular clefts and spaces. Cytochemically, after 10 min of HRP incubation, the marker was seen impregnating spherical and differently-sized membranous structures in MDCK monolayers. Upon the venom exposure (either with 14.6 or 292 μg of PNV/ml concentration) there was a marked increase in the number of HRP-labeled small vacuoles (Fig. 7 D, E, G, H). Interestingly, in PNV-treated cells, a relatively high amount of HRP-unlabeled small vesicles was seen budding from the apical cell membrane, or located just underneath this membrane site (Fig. 7 G). These

electron lucent small vacuoles, free of HRP, were interpreted as being early (or primary) endosomes. Some of the electrondense small vacuoles (or large vesicles), may represent secondary lysosomes in process of marker degradation (Fig. 7 E). In all groups analysed, the integrity of the cell-to-cell and cell-to-matrix junctions was always maintained (Fig. 7 F).

DISCUSSION

Accidents caused by *Phoneutria nigriventer* spider bites has long been known to cause in humans clinical signs indicative of neurotoxic and cardiovascular actions, such as hypertension, tachycardia, arrhythmia, visual disturbances and tonic convulsions (Brazil and Vellard, 1925; 1926). Experimentally, we have demonstrated that when endovenously injected, the PNV induced BBB breakdown in rat hippocampus, primarily through an enhanced transcellular vesicular transport (Le Sueur *et al.,* 2003; Le Sueur *et al.,* unpublished). However, the mechanisms underlying this phenomenon were still poorly understood. In this work we used three cultured cell lines in order to better understand the biological effects of the venom in controlled experimental *in vitro* models, as a strategy to comprehend the mechanisms which could be underneath the enhanced permeabilization of the BBB in rats. To assess this, we investigated an until unexplored possible direct action of the PNV upon both the structural elements of the BBB *in vitro*, *i.e.* ECV304 endothelial and C6 glioma cell lines, and transport routes of the epithelial MDCK cultured cells.

Our findings revealed that the PNV exibited a direct subtle cytotoxic effect that was only observed in the C6 glioma cells and when exposed to 292 μg of PNV/ml for 24 h. The other cell lines (endothelial ECV304 and epithelial MDCK cells) did not show any change in viability after PNV exposure. An *in vivo* cytotoxic effect on astroglial cells was also seen during PNV-induced BBBb in hippocampus of Wistar rat (Le Sueur *et al.,* 2003), or when PNV is injected *in situ* in the pons of rats (Souza and Cruz-Höfling, 1997). Swelling of astrocytes end-feet that unsheath the hippocampal vessels was revealed ultrastructurally and suggested that PNV may induce ionic disturbances in these cells. Swollen astrocytes are less capable of maintaining their homeostatic functions, which may have contributed for the impairment of the BBB functioning caused by PNV (Le Sueur *et al.,* 2003). Recently, Brillault *et al.* (2002), using an *in vitro* BBB model, have shown that glial cells co-cultured with endothelial cells and exposed to hypoxic and oxygen plus glucose deprivation (OGD) conditions, induced an increase in the endothelial transcellular transport while paracellular barrier was unaffected, suggesting that stressed glial cells may induce deleterious effect on endothelia. ECV304 endothelial cell line when co-cultured with C6 glioma cells have been demonstrated to develop strong BBB phenotype, including junctional tightening

and up-regulation of BBB markers (Hurst and Fritz, 1996; Abbott, 2002). In this work, the ECV304 cell strain did not display well-developed tight junctions and increased transendothelial electrical resistance even when co-cultured with C6 cell line. For this reason, we could not test the PNV effect on transcellular transport pathways in this BBB *in vitro* model.

The direct effect of PNV on the paracellular barrier function and transcellular transport was evaluated on cultured MDCK cell line. These cells has long been widely used for studies in cellular transport, including BBB experimental model (Rubbin *et al.,* 1991; Veronesi, 1996), since the MDCK monolayer diplays complex tight junction and increased TER, as well as expresses some enzymatic and antigenic cell markers which were also found in cerebral endothelial cells (Veronesi, 1996). Tight junction (TJ) constitutes the most apical component of the junctional complex in epithelium and endothelium. The complexity of TJ strains defines the paracellular barrier function, that limits the movement of water, ions and solutes between cells (Anderson, 2001). TJ is composed by two major transmembrane proteins, claudins and occludin, that not only interact mutually but also bind homotypically to others localized in neighbouring cells to form the primary seal of the tight junction (Furuse *et al.*, 1993; 1999; Anderson, 2001). Besides, the cytoplasmatic tail of these proteins binds up zonula occludens proteins (ZO-1) (Stevenson and Goodenough, 1984), which in turn, associates with several cytoplasmic peripheral proteins (ZO-2, ZO-3, cingulin, 7H6 antigen and symplekin) and actin-based cytoskeleton (Gumbiner *et al.,* 1991; Citi *et al.,* 1991; Zhong *et al.,* 1993). The TJ integrity can be modulated by adherens junction-associated proteins that are responsible for cell-cell adhesion. Adherens junction is composed by Ca^{2+}-dependent transmembrane glycoproteins termed cadherins (Geiger and Ayalon, 1992) that associate via their cytoplasmatic tail to a group of proteins such as the catenins (α-, β-, γ-), α-actinin, vinculin and actin-based cytoskeleton (Ozawa *et al.,* 1989; Reynolds *et al.,* 1994; Gumbiner, 1996). Changes in the expression and/or distribution of these proteins alter the junctional functioning, leading to an increase of the paracellular permeability, as reported in several experimental conditions (Collares-Buzato *et al.,* 1994; 1998; Rubbin and Staddon, 1999; Lippoldt *et al.,* 2000).

In our work, the PNV treatment did not seem to disturb the paracellular barrier function of MDCK cells. This conclusion was supported by the lack of change in the distribution and expression of the junctional proteins, ZO-1, occludin and E-cadherin, and cytoskeletal F-actin. Regarding the occludin expression, there was a tendency for increasing, as demonstrated by western blotting. Similar finding was observed in 1 day- and 9 day-sample from rat hippocampus, after endovenous injection of PNV (Le Sueur *et al.*, unpublished). Instead of the expected decrease in TER of MDCK cells after PNV exposure, surprisingly an increase in TER was seen after treatment with 292 μg of PNV/ml for 24 h. Although the reason for this phenomenon remains obscure, its is likely that both the increase of TER and occludin

expression, observed *in vivo* and *in vitro* experiments, could represent a compensatory mechanism by which the strengthen of the paracellular tightening is a reactive response of the cultured cells to the weakening of the restrictive transcellular transport mechanisms evoked by PNV exposure. This kind of interpretation is plausible from a physiological point of view and does not exclude a putative existence o toxins in the venom that could account by themselves for the increased expression of all or some of the junctional proteins. Such an interpretation could be also applied to the *in vivo* studies done before to which an increase in occludin expression was also observed (Le Sueur *et al.,* unpublished). These findings support strongly the view that the mechanisms controlling both routes, paracellular and transcellular, work in concert, in such a way that the impairment of one route may lead to the strength of the other.

Transcellular transport is bidirectional, usually energy dependent, and governed by the cell-specific profile of transporters and channels located on the apical and basolateral cell membranes (Bomsel *et al.,* 1989; Egleton and Davis, 1997; Allt and Lawrenson, 2000). In this work, the effect of PNV on endocytosis and intracellular traffic was evaluated in MDCK cells using HRP and biochemical and ultrastructural methods. We found that PNV increased the endocytosis of HRP selectively from the apical surface, whereas basolateral endocytosis was unaffected. This result supports the existence of important functional differences in the apical and basolateral endocytic pathway in MDCK cells, although the mechanism underlying the stimulation of apical endocytosis by PNV in this cell line remains unknown. Increased apical endocytosis of HRP was also observed in MDCK cells after treatment with the fungal metabolite brefeldin A, which induces blockade of protein secretion (Prydz *et al.,* 1992). Gottlieb *et al.* (1993) have shown that the adsorptive and fluid phase endocytic events occurring at the apical membrane of MDCK cells were distinct from those at the basolateral surface, being the former dependent on the integrity of the cytoskeletal microfilaments. In addition, in our experimental model, the increased endocytosis was not accompanied by an enhancement in the HRP transcytosis from apical to basolateral direction. One possible explanation for this result is that part of the endocytosed HRP would be destined for recycling or was degraded. Supporting this view, the presence of a marked number of HRP-unloaded vesicles and secondary lysosomes in the cytoplasm of MDCK cells, as seen by electron microscopy. The expressive population of unlabeled vesicles (in addition to the labeled ones) after PNV exposure could be a strong evidence that the venom of *P. nigriventer* activates the epithelial cell transport through the transcellular via. This is a very attractive hypothesis for explaining the massive presence of unlabeled vesicles in the MDCK monolayers, and it is supported by the well established biochemical and pharmacological characteristics of this venom, which is non-cytolytic, but which alters the membrane permeability to sodium ions. Neurotoxins containing small basic peptides (as the ones found in the *P. nigriventer* venom) are well-known to activate (Fontana and Vital Brazil, 1985) or delay the inactivation (Cruz-Höfling *et al*, 1985; Love and Cruz-Höfling, 1986) of Na^+ channels and have been also shown to

affect the Na^+-K^+-ATPase of the cell membrane (Mebs and Ownby, 1990). The pharmacological action of the neurotoxins from the *P. nigriventer* spider venom has been extensively studied by a group of researchers to which belong one of the authors of this work (E. K.). The group has demonstrated that these neurotoxins act as potent activators and blockers of ionic channels, including Ca^{2+} and K^+(Gomez *et al.,* 2002). The importance of ions, particularly the calcium as an universal signaling agent for the biological processes, is unequivocal and well established. Then, the interference in the physiology of ionic channels would certainly interfere with the physiology of the biological events, among which the transcellular transport dependent on these ions. In addition, the venom induces hyperalgesia mediated by IL-1β and IL-6 (Zanchet, 2004). IL-6 has been shown to promote increased fluid-phase endocytosis and transcytosis in CNS-derived endothelial cells (Duchini *et al*., 1996). Subsequent studies need to be done to investigate the possible involvement of these interleukins on the PNV induced-BBBb.

In accordance with our *in vitro* findings, we also observed an enhanced transcellular vesicular transport of an extracellular tracer ($LaNO_3$) in hippocampal vessels of rats after endovenous injection of PNV (Le Sueur *et al.,* 2003). This increased transport probably occurred through a microtubule-dependent mechanism, since a complete inhibition of the PNV-induced BBB breakdown was seen in rats pretreated with colchicine prior to PNV injection (Le Sueur *et al.*, unpublished).

Our *in vitro* studies indicate that PNV possesses a direct action on transcellular transport pathway in MDCK cells. However, in *in vivo* conditions (Le Sueur *et al.*, 2003) we cannot discard an additional hemodynamic effect of the PNV contributing to enhanced BBB permeability in rats. Costa *et al.* (1996) demonstrated that endovenously injected PNV induces hypertension in anesthetised rats, due to activation of L-type voltage-dependent Ca^{2+} channels. An increase in the BBB permeability, through enhanced transendothelial vesicular transport, caused by hypertension has been reported by Nag (1995), Zumkeller and Dietz (1996) and Al-Sarraf and Philip (2003). In addition, to a direct effect the circulating PNV can be inducing the release of endogenous factors from the neural tissue and/or endothelium, which, in turn, would contribute to modulate the BBB permeability.

In conclusion, neutral red cytotoxicity assay revealed a subtle direct cytotoxic effect of PNV on C6 glioma cells, while the viability of ECV304 and MDCK cells was unaffected under PNV exposure. We demonstrated that the venom increase the endocytosis of HRP at the same time that reinforces the tightening of the paracellular route. It was suggested that such effects result from a cross-talk between the mechanisms involved in the two pathways in such a way that the weakening of one route promotes the strengthening of the other. We also suggest that these effects were not due to the cytotoxicity of the venom, since MDCK cells viability remained intact, but instead to a direct activating effect of *P. nigriventer* venom on a given via of cell transport probably mediated by Na^+, Ca^{2+} and K^+ channels

affected by the venom. Although this work does show a direct *in vitro* effect of the PNV on epithelial transport, we cannot discard at moment the possibility of an additional local haemodynamic disturbance caused by PNV upon the increase of the transcellular endothelial permeability observed in *in vivo* conditions. Further studies are also need to determine which of the PNV´neurotoxins was (were) responsible for altering cellular transport in MDCK monolayers, or in BBB of rats.

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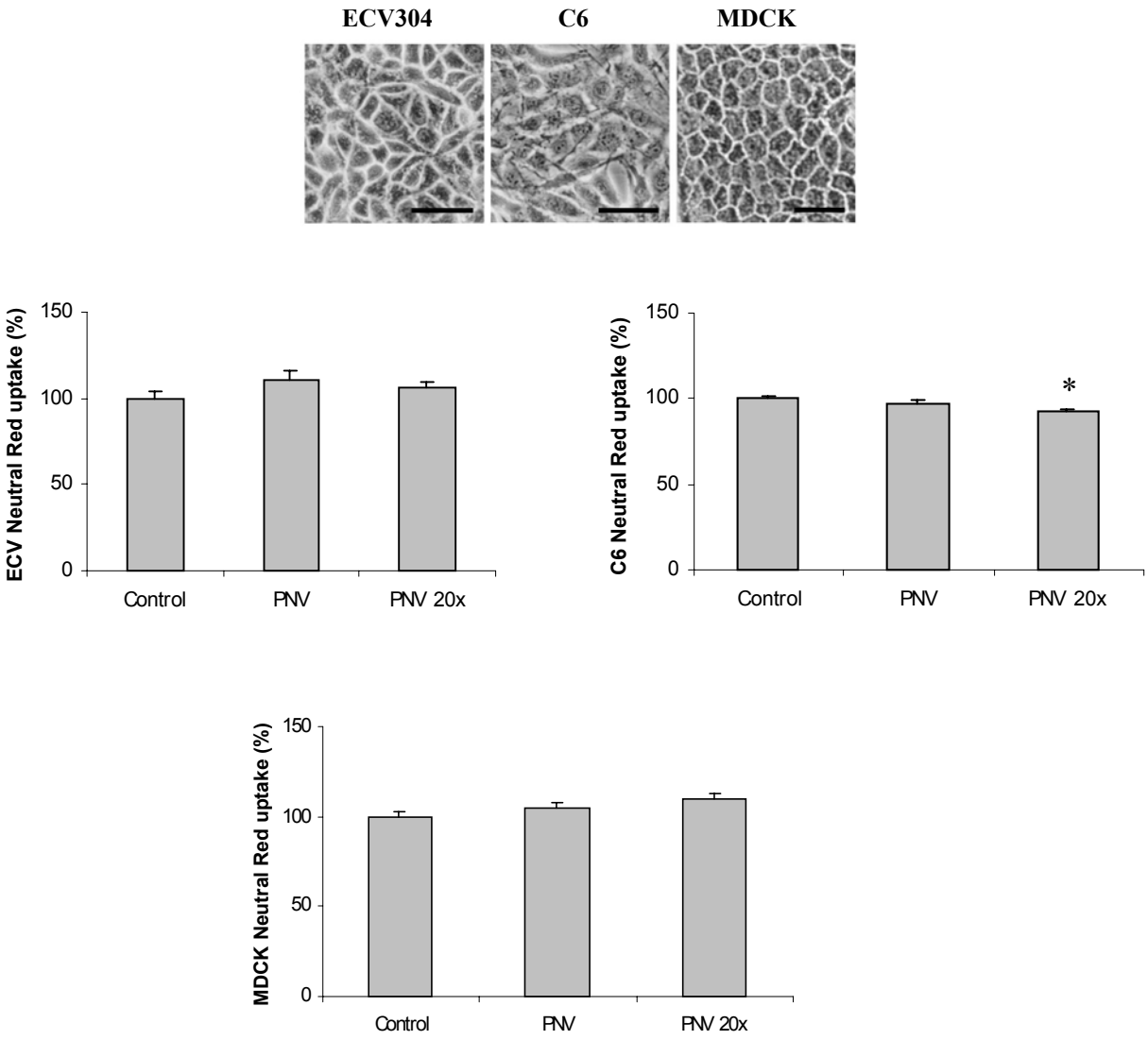

Figure 1 – Cell viability of ECV304 endothelial cells, C6 glioma and MDCK epithelial cells following PNV treatment. Cell viability was assessed by measuring neutral red cell uptake. Cells were exposed for 24 h either to 14.6 μg of PNV/ml (PNV) or 292 μg of PNV/ml (PNV 20x). The control cells were cultured in medium without venom. The C6 glioma cells submitted to 292 μg of PNV/ml for 24 h showed a decrease in cell viability (n = 18 monolayers for each group). No significant effect on cell viability was observed after PNV treatment of two other cellular lineages, ECV304 (n = 19 monolayers/group) or MDCK (n = 12 filters/group). Insets show contrast phase photomicrographs of cells at confluency, grown on plastic flasks. * $P < 0.05$ compared with the corresponding control. Bars: 8 μm (ECV304 and C6); 1 μm (MDCK).

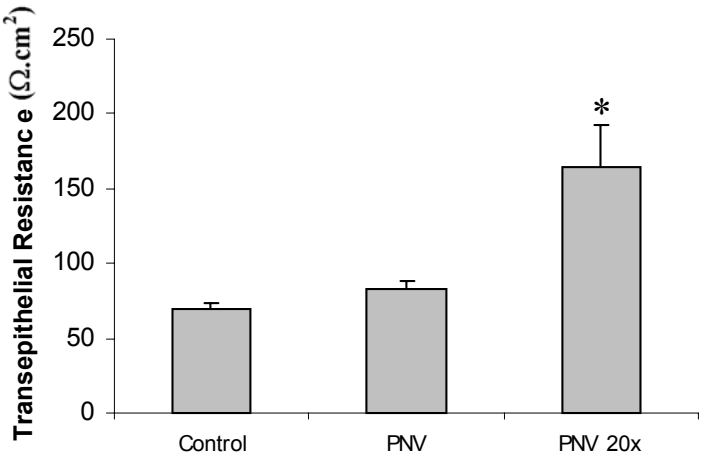

Figure 2 - Transepithelial electrical resistance (TER) across MDCK monolayers following PNV exposure for 24 h. PNV at concentration of 14.6 μg/ml (PNV) or 292 μg/ml (PNV 20x) was applied to the apical and basolateral surfaces of the monolayers, while the control group received culture medium without venom. Note the significant increase in TER following 292 μg/ml PNV-exposure for 24 h. The columns are the mean + SE of 23 to 28 monolayers per group from eight independent experiments. * $P < 0.001$ compared with the corresponding control and with MDCK cells exposed to 14.6 μg of PNV/ml.

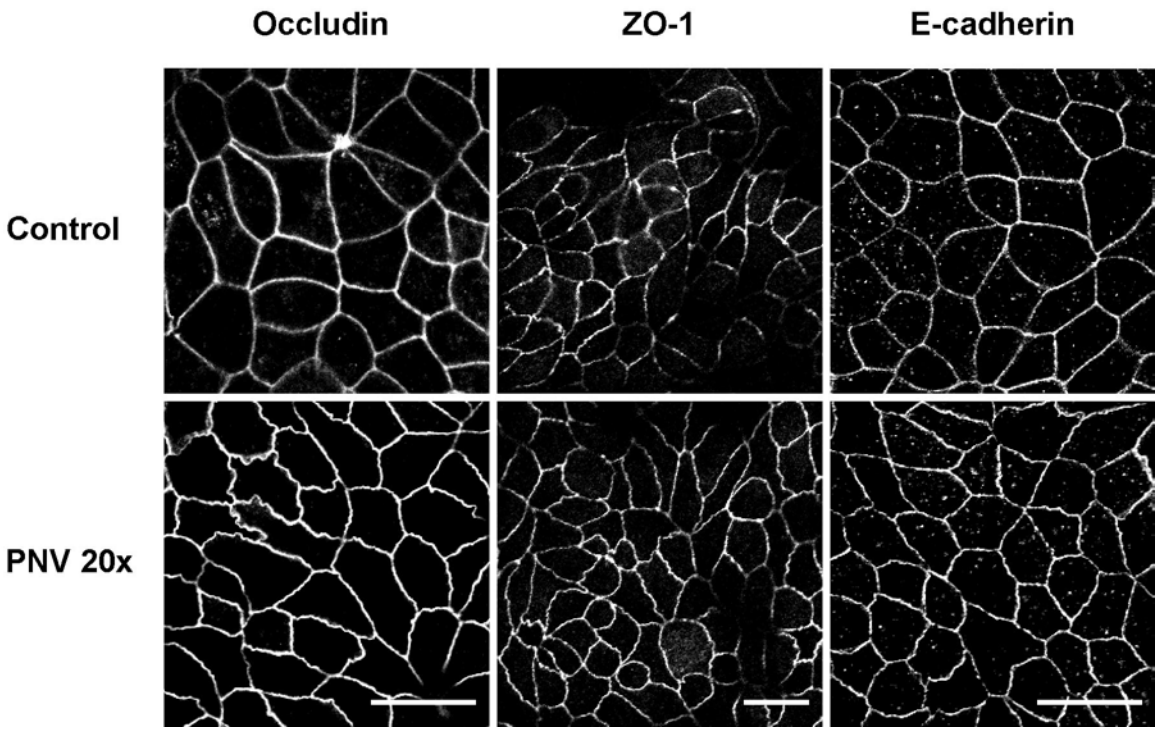

Figure 3 - Cellular expression and distribution of the tight junction-associated proteins, occludin and ZO-1, and the adherens junction-associated E-cadherin, following PNV treatment. The monolayers were fixed and immunostained using indirect immunofluorescence protocol. The photomicrographs are representative confocal "en face"(X-Y) images obtained from MDCK monolayers using the same level of CLSM sensitivity for both experimental groups. Occludin showed subtle higher and well-defined staining at apical portion of the cell-cell contact in PNV-treated cells (292 μg of PNV/ml) when compared with control cells. The distribution and intensity of staining for ZO-1 and E-cadherin did not change after PNV treatment when compared with control group. Bar = 20 μm.

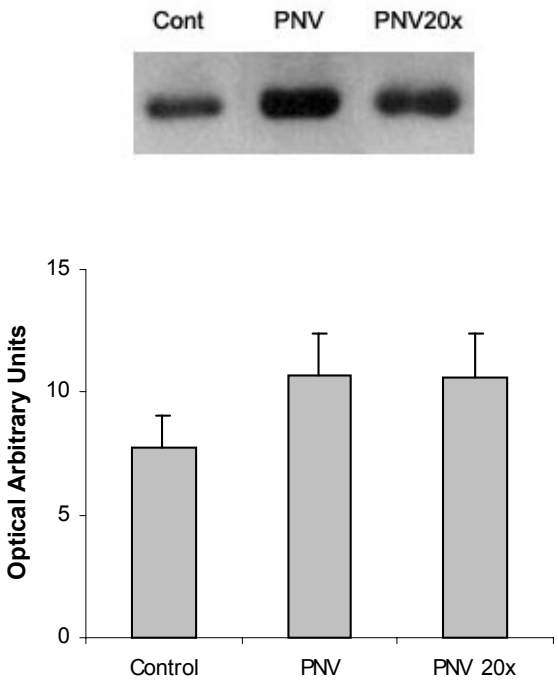

Figure 4 - Expression of the tight junction-associated occludin after PNV exposure, assessed by immunoblotting. Occludin expression was evaluated in samples from MDCK cells treated with either 14.6 μg of PNV/ml (PNV) or 292 μg of PNV/ml (PNV 20x). The graph shows the means (+ SE) of densitometric values of the immunoreactive bands from three monolayers/group (b). In a, representative blot image of the occludin, migrating at 65 kDa, is shown. Note that following PNV treatment there was a tendency towards an increase in occludin expression although this change was below the statistical significance level.

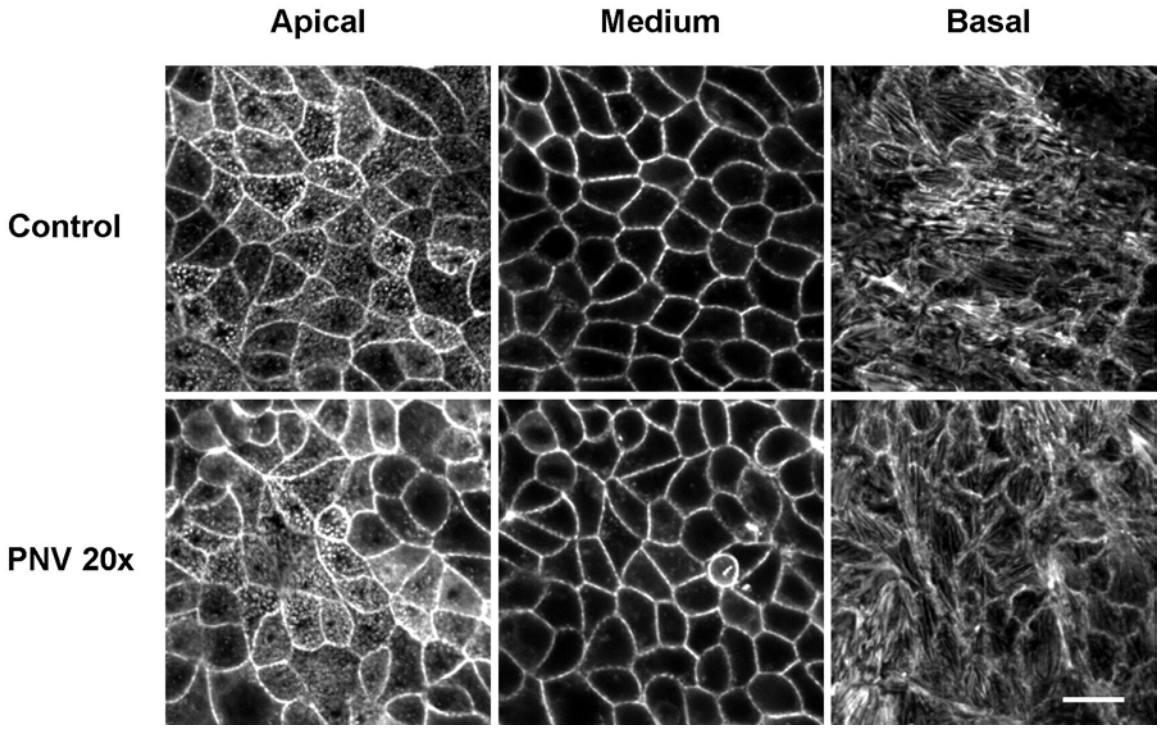

Figure 5 - Distribution of F-actin in MDCK monolayers following PNV treatment (292 μg PNV/ml) revealed by TRITC-phalloidin staining. Panels are X-Y confocal images representing optical sections taken at the apical, middle or basal portions of the monolayers, using the same level of CLSM sensitivity for both experimental groups. No changes in the F-actin distribution were observed after the PNV exposure (292 μg/ml) when compared with control cells. Bar = 30 μm.

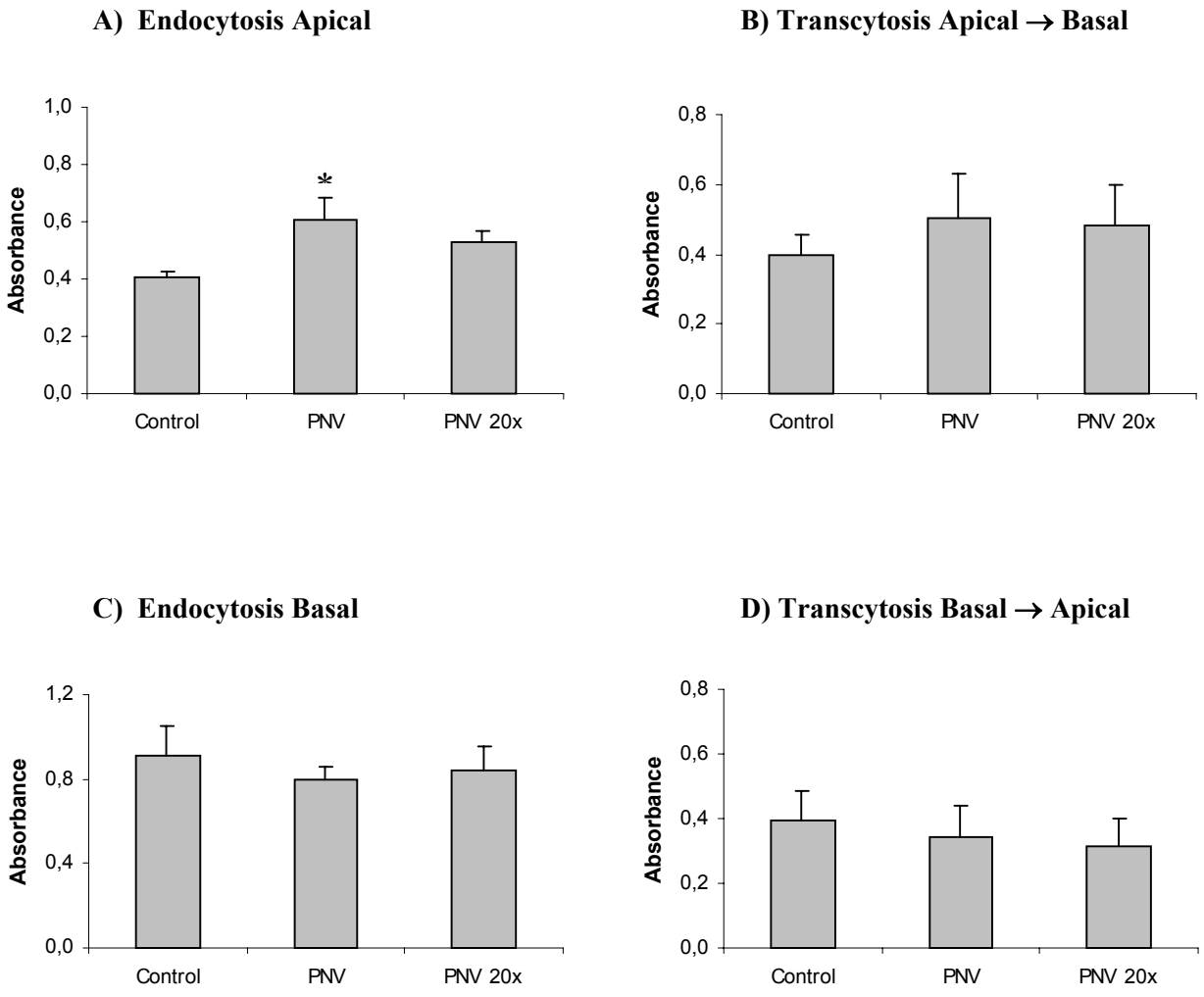

Figura 6 - Endocytosis and transcytosis of HRP following PNV treatment. Each column represents the mean + SE of 4 to 8 filters from four independent experiments. Amount of endocytosed HRP was assessed by HRP quantification in the lysate after apical (A) or basolateral (C) uptake. The amount of HRP trancytosed after apical and basolateral uptake was assessed by HRP quantification in the basolateral (B) and apical (D) culture medium, respectively. Note the significant increased endocytosis of HRP following 24 h exposure to PNV at concentration of 14.6 μg/ml (n = 8 for each group). However, no significant differences were observed in the basolateral endocytosis and transcytosis when compared the control and treated groups. * $P < 0.05$ compared with the corresponding control.

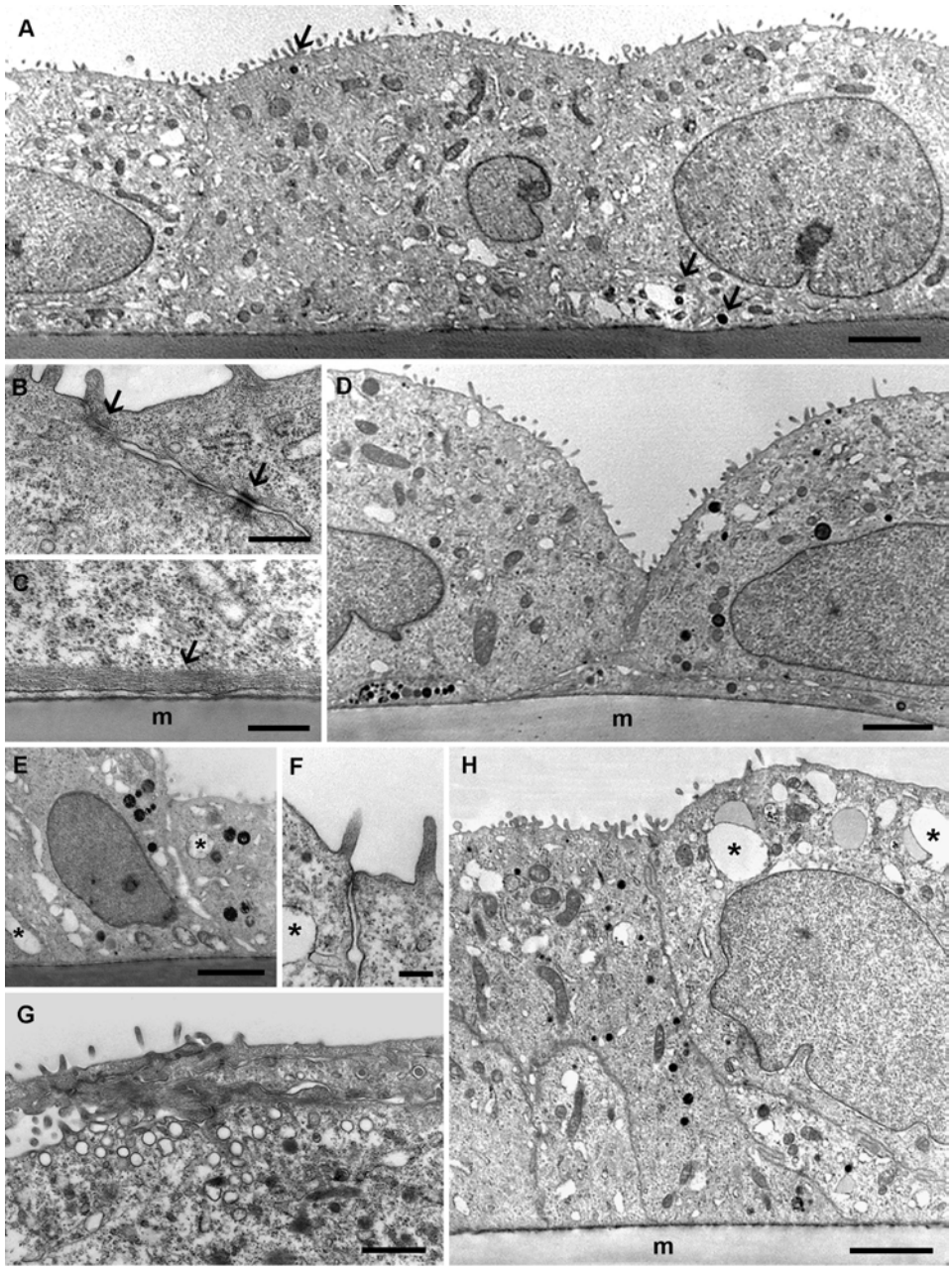

Figure 7 - Intracellular content of HRP as revealed by ultrastructural method following 24 h-PNV exposure. A - C, panoramic view and details of control MDCK cells (exposed to culture medium without venom for 24 h) showing the integrity of the monolayer and a few number of intracellular HRP-labeled vesicles (A, arrowheads). Typical intercellular junctions (B, arrowheads) as well as well-developed focal adhesion junction with associated stress fibers (C, arrowhead) could be observed. Increased number of spherical vesicles containing HRP was found in the MDCK cells exposed to 14.6 μg of PNV/ml (D and E) and 292 μg of PNV/ml (H). PNV-treated cells also showed higher number of non-HRP labeled vacuoles (asterisks) and vesicles without tracer (probably early endosomes) close to the apical membrane (G), despites an intact junctional complex at the intercellular contact region (F). Legend: Insert membrane (m). Bars = 2 μm (A, D, E, H); 0.4 μm (B); 0.3 μm (C); 0.2 μm (F); 1 μm (G).

CONCLUSÕES GERAIS

***In vivo*:**

- O veneno bruto da aranha *Phoneutria nigriventer* injetado por via endovenosa, na dose de 0,85 mg/kg, provoca significativa quebra da barreira hemato-encefálica de ratos, particularmente na região do hipocampo cerebral, 1 e 9 dias após o envenenamento;
- A conseqüência mais evidente da quebra da BHE provocada pelo PNV é a ocorrência de edema vasogênico no tecido neural, caracterizado pelo intumescimento dos pés astrócitários perivasculares. Por outro lado, o PNV não induz resposta inflamatória, recrutamento de micróglias para o sítio da lesão ou quaisquer indícios morfológicos evidentes de sofrimento neuronal;
- A barreira paracelular, assegurada pelo complexo juncional, parece não ser o alvo primário do PNV em ratos. Esta conclusão foi baseada na ausência de alterações nos níveis de expressão das proteínas juncionais ocludina, ZO-1 e β-catenina, bem como no estado de fosforilação das proteínas ocludina e β-catenina. Contrariamente, a barreira paracelular parece fortalecida 1 e 9 dias após o envenenamento, fato interpretado como decorrente de um mecanismo compensatório à quebra da BHE ocorrida pela via transcelular, ou ainda, como conseqüência do aumento da permeabilidade pela via paracelular, ocorrido no intervalo entre os tempos de sobrevida analisados, e portanto não detectados neste estudo;
- Fortes indícios sugerem que o aumento da permeabilidade da BHE provocada pelo PNV 1 e 9 dias após a injeção nos ratos tenha ocorrido através do aumento do transporte vesicular transendotelial mediado por microtúbulos, uma vez que o pré-tratamento com colchicina bloqueou completamente as alterações da permeabilidade induzidas pelo veneno;
- A quebra da BHE no hipocampo de ratos pode ser um epifenômeno, isto é, pode não ter relação com os sinais clínicos de convulsões, uma vez que as alterações da permeabilidade da BHE foram observadas independentemente da ocorrência deste sinal clínico em alguns dos animais experimentais. Além disso, enquanto os sinais clínicos duravam cerca de 18 horas, as alterações da permeabilidade foram evidenciadas até 9 dias após a injeção do PNV, sugerindo que alterações sub-clínicas continuam ocorrendo e que contribuem para a quebra da BHE;

***In vitro*:**

- A viabilidade da linhagem endotelial ECV304 não foi afetada pelo PNV;
- Alta dose de PNV induz pequeno, porém significativo ($p < 0,05$), efeito citotóxico sobre a linhagem tumoral astrocitária C6;
- O PNV não é citotóxico para as células MDCK, porém afeta a sua propriedade de barreira epitelial;
- Alta dose de PNV provoca aumento da resistência à passagem de íons pela via paracelular da monocamada de células MDCK em cultura, através de mecanismos não conhecidos, mas que não estão relacionados a um aumento significativo na expressão das proteínas associadas à junção de oclusão, ocludina e ZO-1;
- O PNV possui ação direta sobre os mecanismos de endocitose apical das células epiteliais MDCK em cultura. Esta conclusão foi baseada no aumento significativo da captação apical do marcador HRP pelas células expostas ao PNV, quando comparadas ao controle (células não envenenadas);
- O aumento da endocitose do marcador HRP provocado pelo veneno nas células MDCK em cultura não está associado a alterações morfológicas detectáveis na distribuição dos filamentos de actina neste tipo celular;
- A quebra da BHE em ratos, pode não estar associada a um efeito citotóxico direto do veneno sobre as células endoteliais cerebrais, porém pode ter o envolvimento dos astrócitos. Estas hipóteses basearam-se nos resultados dos modelos *in vitro*;
- Embora os resultados da ação do PNV sobre as células MDCK não possam ser transportos para a situação da BHE *in vivo,* eles indicam a possibilidade de um efeito direto do PNV sobre as vias de transporte transcelular. No entanto, a quebra da BHE no hipocampo de ratos pelo PNV (i.v.) pode ter como fator co-adjuvante um efeito hemodinâmico, como a hipertensão, causada pelo veneno circulante.

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