Inês Tavares Pinto de Sá Pereira

Licenciatura em Biologia

Basement membrane alterarions in kernicteric brain microvasculature and pericyte response to bilirubin

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Maria Alexandra de Oliveira Silva Braga Pedreira de Brito, Professora Auxiliar, Faculdade de Farmácia, Universidade de Lisboa

Co-orientador: Dora Maria Tuna de Oliveira Brites, Investigadora Coordenadora e Professora Catedrática Convidada, Faculdade Farmácia, Universidade de Lisboa

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Two publications are in preparation, ensuing from the work developed in this thesis

Sá Pereira I, Brites D, Brito MA.
Neurovascular unit: a focus on pericytes (Review)

Sá Pereira I, Fernandes A, Palmela I, Brites D, Brito MA.
Unconjugated bilirubin induces apoptotic cell death, cytokine secretion and nitrosative stress in human brain vascular pericytes

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ABSTRACT

Kernicterus is a neuropathological condition characterized by deposition of unconjugated bilirubin (UCB) in specific brain regions that can lead to permanent sequelae and death, particularly in premature infants. UCB-induced toxicity has been studied in nerve and glial cells and, more recently, in brain microvascular endothelial cells. However, the effects of UCB on pericytes or on the basement membrane were never reported.

We performed *in vitro* studies to assess apoptotic death, nitrosative stress and inflammatory reaction elicited by human brain vascular pericytes exposed to UCB. We also assessed the basement membrane component, collagen type IV, in brain sections of cortex, basal nuclei, hippocampus and cerebellum, collected at autopsy of a kernicteric preterm newborn.

Using the pericyte marker, α-smooth muscle actin, we characterized the cells and confirmed the normal outgrowth towards a typical morphology with long processes. UCB induced an early secretion of interleukin-6, followed by that of vascular endothelial growth factor. mRNA upregulation preceded the secretion and confirmed the precocious profile of IL-6. UCB also caused the release of nitrites, which was maximum at 72 h incubation. The earlier upregulation of endothelial nitric oxide synthase expression confirmed the induction of nitric oxide production by UCB, although not excluding that other isoforms of the enzyme are also involved. Probably as a corolary of all these events, apoptotic cell death occurs in a time- and concentration-dependent manner. Through immunohistochemistry we examined the area occupied and the immunoreactivity of collagen type IV, which were reduced in the kernicterus case as compared with a non-icteric control.

These findings are the first to demonstrate the compromise of pericytes and the impairment of collagen IV by hyperbilirubinemia and raise some basis for creation of possible target-directed therapy against pericyte and basement membrane damages as a result of UCB exposure.

Keywords: Basement membrane; blood-brain barrier; cytokines; nitrosative stress; pericytes; unconjugated bilirubin.

RESUMO

Kernicterus é uma condição neuropatológica caracterizada pela deposição de bilirrubina não conjugada (BNC) em regiões específicas do encéfalo que pode resultar em danos permamentes e na morte, especialmente em recém-nascidos prematuros. A toxicidade da BNC já foi estudada em células nervosas e da glia e, mais recentemente, em células endoteliais da microvasculatura do cérebro. No entanto, os efeitos da BNC em pericitos e na membrana basal nunca foram descritos.

Neste trabalho, avaliámos a morte por apoptose, stress oxidativo e reacção inflamatória de pericitos humanos expostos à BNC. Avaliámos também um dos componentes da membrana basal, o colagénio tipo IV, em cortes humanos de cortex, núcleos da base, hipocampo e cerebelo provinientes de uma autópsia de um recém-nascido permaturo com *kernicterus.*

Usando α-actina do músculo liso como marcador de pericitos, procedemos à sua caracterização e confirmámos um crescimento normal no sentido de uma morfologia típica com longos prolongamentos. A BNC induziu a secreção precoce de interleucina-6 e mais tardia do factor de crescimento endotelial vascular. A sobrexpressão do mRNA antes da libertação confirmou ambos os perfis de secreção. A BNC levou também à libertação de nitritos, atingindo valores máximos às 72 h de incubação. A sobrexpressão precoce da enzima endotelial de síntese do óxido nítrico comprovou a indução da produção do mesmo. Porém, não podemos excluir o facto de que outras isoformas da enzima poderão estar envolvidas. Provavelmente como uma sequência destes eventos, a morte por apoptose ocorreu de uma maneira dependente do tempo e da concentração. Através de uma análise imunohistoquímica do caso de *kernicterus*, observámos uma redução da área ocupada e da intensidade do colagénio tipo IV, comparativamente a um caso controlo não ictérico.

Estes resultados são os primeiros a demonstrar o comprometimento dos pericitos e do colagénio tipo IV causado por hiperbilirrubinémia e a levantar algumas bases para a criação de uma possível terapia direccionada a alvos contra danos nos pericitos e na membrana basal resultantes da exposição à BNC.

Termos-chave: Barreira hemato-encefálica; bilirrubina não conjugada; citocinas; membrana basal; pericitos; stress nitrosativo.

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ABBREVIATIONS

α-SMA	α-smooth muscle actin
ABC	ATP-binding cassette
Ang	angiopoietin
AJ	adherens junction
BBB	blood-brain barrier
bFGF	basic fibroblast growth factor
BM	basement membrane
BMVEC	brain microvessel endothelial cell
BSA	bovine serum albumin
CAM	cell adhesion molecule
CNS	central nervous system
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
ECs	endothelial cells
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
Glut-1	glucose transporter-1
HBVP	human brain vascular pericytes
HSA	human serum albumin
IL	interleukin
JAM	junctional adhesion molecule
LPS	lipopolysaccharide
MHC	major histocompatiblity complex
MMP	matrix metalloproteinase
MRP	multidrug resistance-associated proteins
Na-F	sodium fluorescein
NG-2	nerve Glial-2
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PDGF-β	platelet-derived growth factor-β
PDGFR-β	platelet-derived growth factor-β receptor
P_e	transendothelial permeability coefficient
P-gp	P-glycoprotein
PN-1	protease nexin-1
RT-PCR	real-time Polymerase chain reaction
S1P	sphingosine-1-phosphate
TEER	transendothelial electrical resistance

TGF-β	transforming growth factor-β
TJ	tight junction
tPA	tissue plasminogen activator
UCB	unconjugated bilirubin
VEGF	vascular endothelial growth factor
ZO	zonula occludens

I. INTRODUCTION

I. INTRODUCTION

1. Neurovascular unit

It was in 1885, that Paul Ehrlich injected vital dyes into the circulatory system and observed that all organs except the brain and spinal cord were stained (Ehrlich, 1885). Latter on, Edwin Goldmann, an Ehrlich's student, injected trypan blue into cerebro-spinal fluid and noticed that it only stained the central nervous system (CNS) (Goldmann, 1913) (Fig. I.1). These evidences pointed to the existence of a barrier separating the CNS and the circulation that was named *bluthirnschranke* (blood-brain barrier, BBB) by Lewandowsky (1900) who further noticed the absence of pharmacological actions of bile acids and ferrocyanide in the CNS. However, this barrier was related with endothelial cells (ECs) and their tight junctions (TJ) only with the advance of electron microscopy (Reese and Karnovsky, 1967).

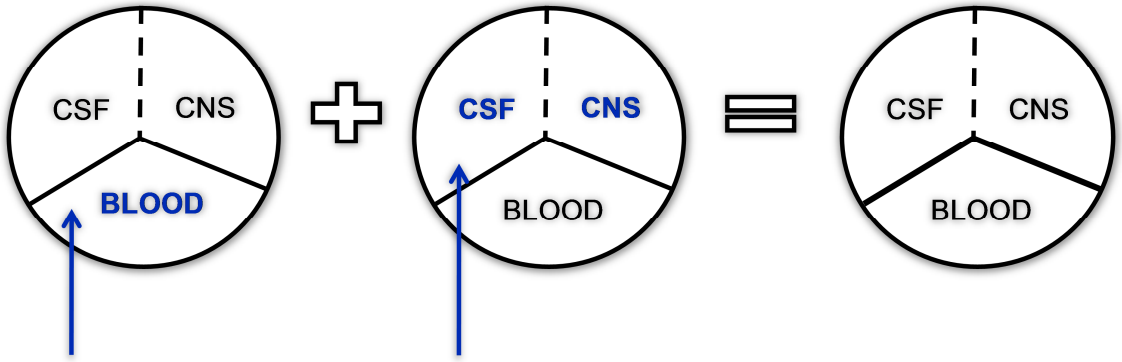

Figure I. 1: The discovery of the blood-brain barrier. Paul Ehrlich injected vital dyes into the circulatory system and all organs except the brain and spinal cord were stained. Edwin Goldman injected trypan blue into cerebro-spinal fluid and all central nervous system (CNS) was stained. These evidences led to the discovery of a barrier between CNS and blood.

All organisms with a developed CNS have a BBB that shields the CNS from toxic and harmful substances in the blood and from free paracellular diffusion of water-soluble molecules, especially through endothelial TJs. This barrier allows the uptake of water-soluble nutrients, metabolites and required molecules into the CNS microenvironment and filters harmful compounds from the brain to the bloodstream through expression of specific transport systems, specifically by the ECs (Persidsky et al., 2006; Kim et al., 2008; Nishioku et al., 2009). BBB also limits the entry into the brain of red blood cells and leukocytes (Zlokovic, 2008) (Fig. II.2).

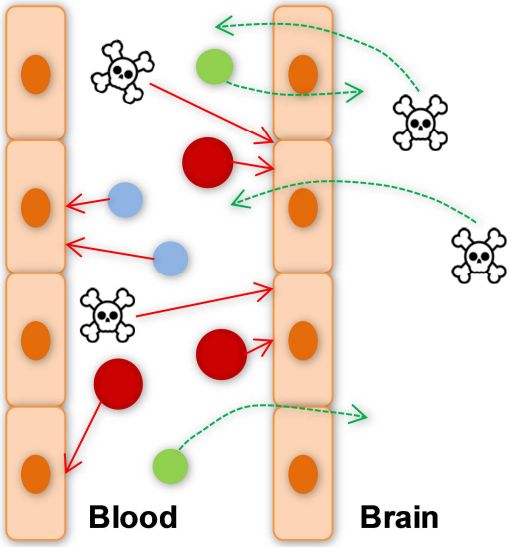

Figure I. 2: Scheme of the main roles of the blood-brain barrier (BBB). The BBB protects the central nervous system (CNS) from toxic and harmful substances (toxic symbol) and from paracellular diffusion of water-soluble molecules (blue). BBB also limits the entry of cells from the blood (red), allows the uptake of molecules needed for CNS (green) and eliminates harmful compounds from the brain to blood (toxic symbol).

The only regions where there is no BBB are those that regulate autonomic nervous system and endocrine glands of the body since blood vessels allow diffusion of blood-borne molecules across the vessel wall (Ballabh et al., 2004). This barrier is mainly formed by ECs, astrocytes end-feet, basement membrane (BM) and pericytes. All these elements, together with microglia and neurons are part of the functional neurovascular unit (Cardoso et al, 2010). More specifically, a differentiated BBB is composed by the highly specialized ECs, surrounded by a BM in which a large number of pericytes are embedded. These last cells are also covered by the BM, which is ensheathed by astrocytic endfeet (Kim et al., 2008; Engelhardt and Sorokin, 2009) (Fig. I.3).

The interactions of ECs with the other components of the BBB provide a stable environment for neural function. On the other hand, the TJs between ECs, together with the enzymes and diverse transport systems, make the transport across the BBB strictly limited that translate a restricted permeability (Persidsky et al., 2006). Thus, the BBB is also seen as a limiting factor and obstacle for the delivery of therapeutic agents and drugs into the CNS (Kim et al., 2006b; Zlokovic 2008; Abbott et al., 2010).

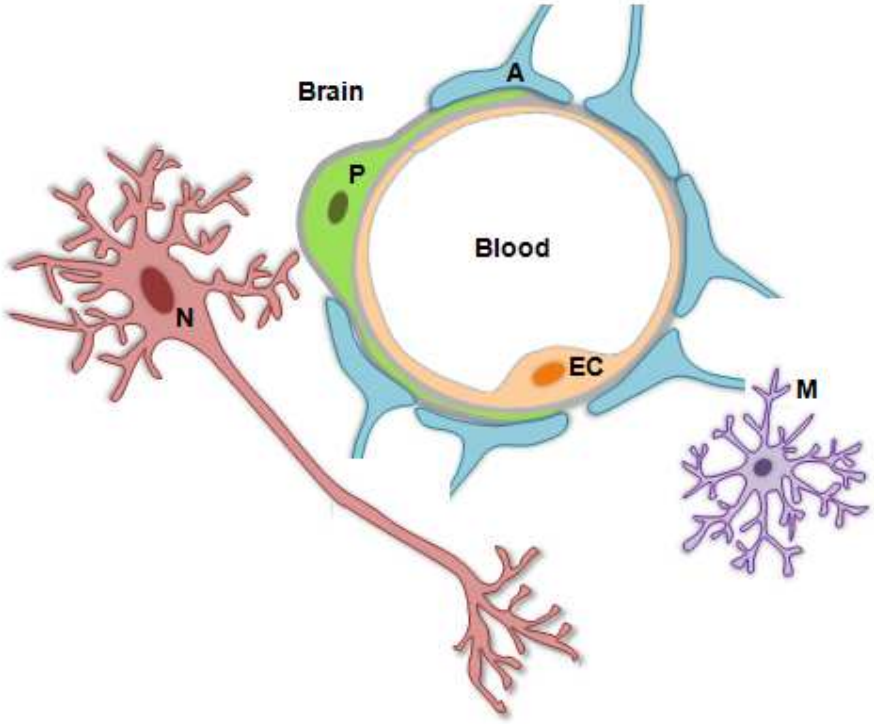

Figure I. 3: Schematic representation of the neurovascular unit. EC, endothelial cell; P, pericyte; A, astrocyte endfeet; N, neuron; M, microglia and in grey the basement membrane.

1.1. Endothelial cells

In developed animals like mammals the cerebral endothelium is considered the anatomic basis of the BBB (Hawkins et al., 2006) and its interactions with others brain cells determine and turn possible the correct function of the BBB (Ballabh et al., 2004; Cardoso et al., 2010).

The brain ECs are located at the interface between the blood and the brain (Fig. I.3) and have a morphology, biochemistry and functional properties that make these cells unique and distinguishable from all other ECs in the body (Engelhardt and Sorokin, 2009). Brain capillary endothelium is 50-100 times tighter than peripheral microvessels (Abbott, 2002) and has 0.2 to 0.3 µm of thickness (Zlokovic, 2008). Compared to ECs in other tissues, BBB endothelium has long TJs (Kniesel and Wolburg, 2000), sparse pinocytic vesicular transport systems (Sedlakova et al., 1999) and no fenestrations in their cytoplasm (Fenstermacher et al., 1988). Moreover, these cells have a negative surface charge that repulses negatively charged compounds (de Boer and Gaillard, 2006). Brain microvascular ECs (BMVECs) also have a greater number and volume of mitochondria that enhance their energy potential for enzymes and transport systems activity (Oldendorf et al., 1977). Endothelial specific transport systems and receptors have a unique pattern that facilitates the uptake of nutrients and hormones required for brain function (Zlokovic, 2008; Cardoso et al., 2010). Solute carrier family of transport proteins assures the uptake of water-soluble molecules like glucose (Boado and Pardridge, 1993) or aminoacids (Lyck et al., 2009). Members of ATP-binding cassettes (ABC) transporter family,

such as P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRP), move out of the brain harmful hydrophilic and hydrophobic molecules (Begley, 2004). The expression of P-gp is one of the specialized characteristics of BMVECs (Tatsuta et al., 1992; Tatsuta et al., 1994). Ecs also express enzymes that can modify and change a range of molecules, which otherwise could pass through the BBB and affect neuronal function (Persidsky et al., 2006; Zlokovic, 2008). Enzymes concentration is high in cerebral microvessels and includes γ-glutamyl transpeptidase, alkaline phosphatase, and aromatic acid decarboxylase (Persidsky et al., 2006). Besides all these, BMVECs also have other active pumps that help in regulation of ions, metabolites and xenobiotics concentrations in the brain (Cardoso et al., 2010).

To eliminate spaces between ECs and prevent free paracellular diffusion of blood-borne substances into the brain parenchymal space, BMVECs of capillaries and postcapillary venules have junctional complexes that include mainly TJs and adherens junctions (AJs). The first are located on the apical region of ECs and AJs are below TJs. TJs proteins include the transmembranar proteins claudins, occludin, junctional adhesion molecules (JAMs) and the cytoplasmic proteins like zonula occludens (ZO). The claudins family include by now 27 members (Mineta et al., 2011), which have 20-27 kDa and four domains. Occludin was the first TJ protein discovered. It has 65 kDa and four domains like claudins but with a different amino acid sequence (Furuse et al., 1993). JAMs are proteins with approximately 40 kDa that have been identified in 1998. They belong to the immunoglobulin superfamily and have a single transmembrane domain (Martin-Padura et al., 1998). There are three JAMs, including JAM-1 predominantly expressed in the brain (Aurrand-Lions et al., 2001) that is involved in cell-to-cell adhesion and takes part in the formation of TJs as an integral membrane protein together with occludin and claudins. Recent data obtained in our lab revealed the presence of another transmembarne TJ protein in BMVECs, which is particularly concentrated at tricellular TJs, though also distributed along bicellular TJs (Mariano et al., unpublished). Finally, cytoplasmic proteins include ZO-1 that ensures the linkage of TJs to the cytoskeleton. AJs include cadherins that have a plasma membrane-spanning domain and a cytoplasmic domain associated with catenins, the other AJs proteins (Vorbrodt and Dobrogowska, 2003). These last make the link between cadherins and actine cytoskeleton (Cardoso et al., 2010). AJs also include nectin-afadin complex where afadin, also known as AF-6, anchors nectins to the cytoskeleton (Niessen, 2007).

Thus, TJs and AJs limit the paracellular flux of hydrophilic molecules but not of small lipophilic substances such as O_2 and CO_2, which can diffuse freely across plasma membranes along their concentration gradient (Ballabh et al., 2004) (Fig. I.4).

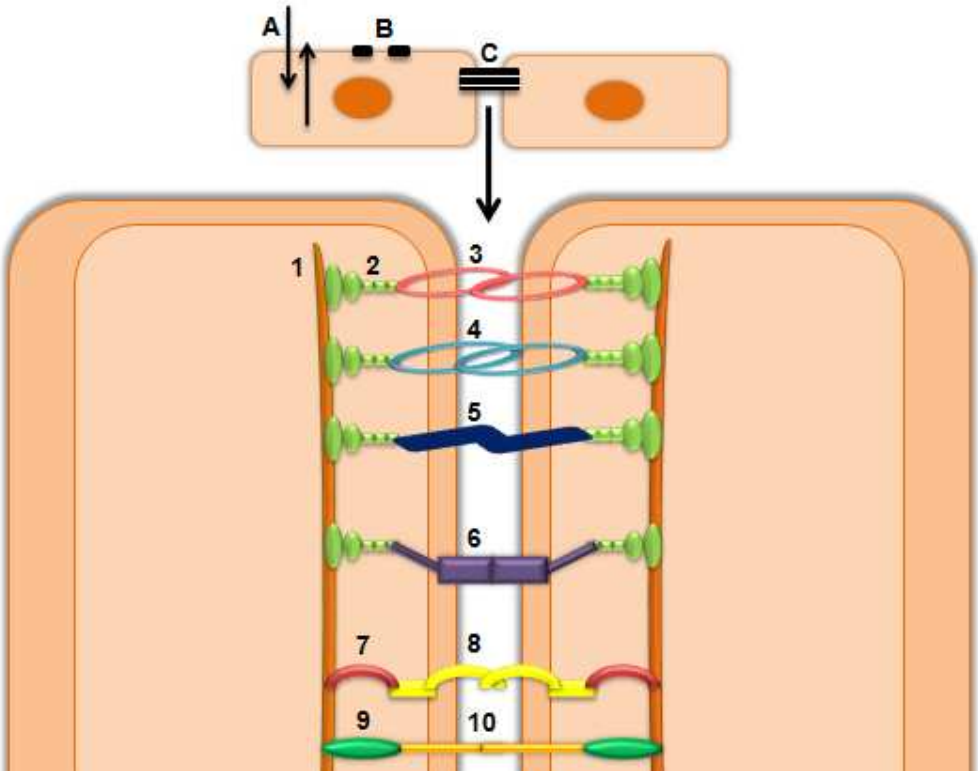

Figure I. 4: The main characteristics of endotheial cells and their junctional complexes. A, transport systems; B , specifics receptors; C, tight and adherens junctions: 1, actin filament; 2, zonula occludens; 3, claudin; 4, occluding; 5, junctional adhesion molecule; 6, tricelullin; 7, catenins; 8, vascular endothelial cadherin; 9, afadin; 10, nectin.

ECs also adhere to the BM through transmembrane proteins that are classified into three families of cell adhesion molecules (CAMs): selectins, immunoglobulin superfamily and integrins (Cardoso et al., 2010). Thus, these molecules may contribute to BBB integrity (Engelhardt and Sorokin, 2009).

1.2. Basement membrane

The BM, surrounding ECs and pericytes, is an essential component of the BBB (Fig. I.5). Its formation and maintenance is assured by ECs, pericytes and astrocytes (Zlokovic, 2008). Like all BMs, it is formed by tightly interwoven protein sheets of 20-200 nm tickness (Timpl, 1989), constituted by structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin) and proteoglycans, organized in three apposed laminas (Cardoso et al., 2010). This membrane has an important role, contributing to tissue/cells organization, stability and differentiation (Yurchenco and Patton, 2009).

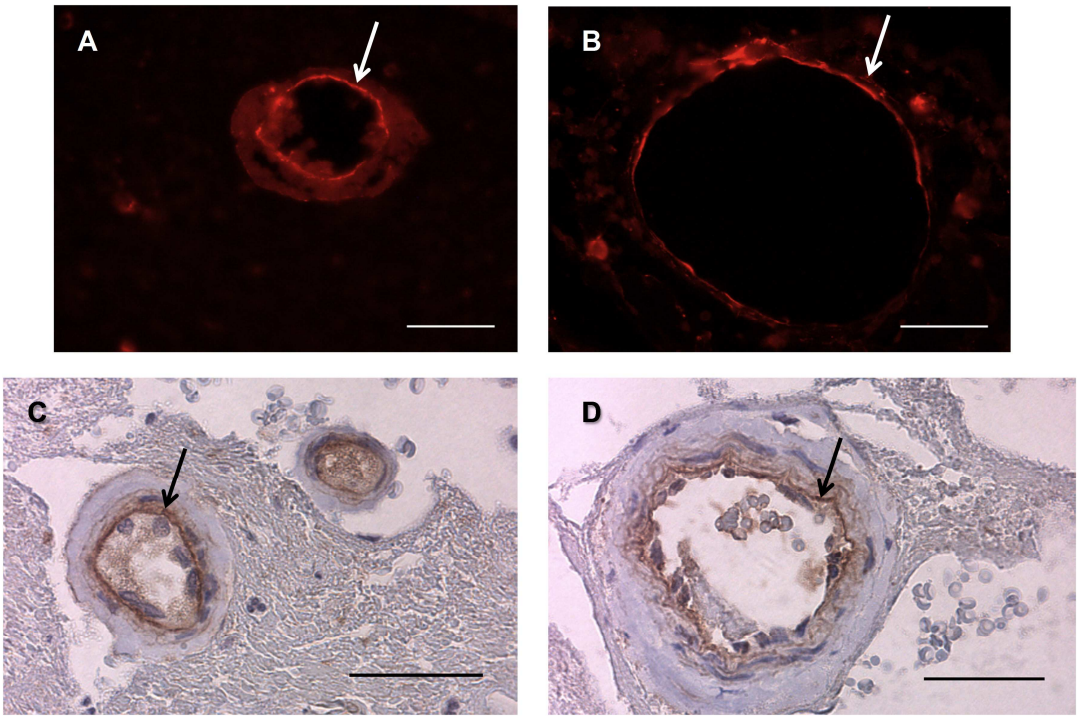

Figure I. 5: Basement membrane (BM) of human hippocampus sections. Arrows point to collagen IV, a main component of the BM, visible by immunofluorescence (red) (A,B) and imunohistochemistry (brown) (C,D). Scale bars: 40 µm.

The collagen type IV, is a covalently-stabilized network polymer and is one of the proteins most important for structural integrity of small vessels. However, collagen type IV is dispensable for initiation of its assembly during early development (Pöschl et al., 2003). The laminins, with a branched structure (Yurchenco and Patton 2009), present in CNS BM are the laminin α4 and α5 produced by ECs (Sixt et al., 2001). Together, the collagen type IV and the laminin form two overlapping polymeric networks (Coelho et al., 2011). Moreover, perlecan is the predominant heparan sulfate proteoglycan in the BM (Engelhardt and Sorokin, 2009). The attachment of BM to the cells is done primarily by laminins to cell surface sulfated glycolipids and transmembrane receptors (Yurchenco and Patton, 2009). Laminins are also critical for the organization and scaffolding of BM (McKee et al., 2007; Yurchenco and Patton, 2009), while collagen type IV enhances the stability of the membrane (Poschl et al. 2004; Yurchenco and Patton, 2009) (Fig. I.6).

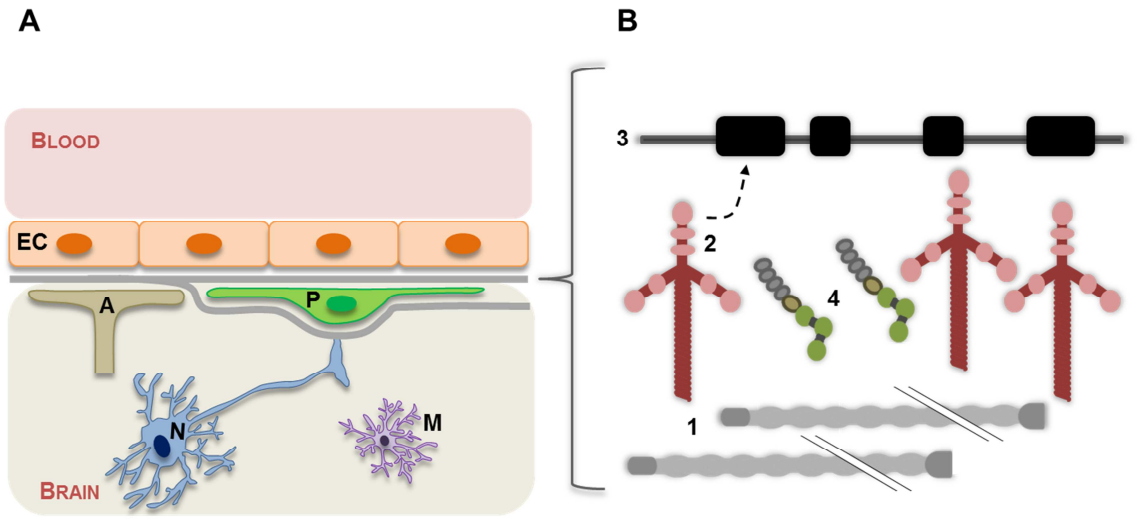

Figure I. 6: Simplified representation of the blood-brain barrier (BBB) focusing on the basement membrane. A, components of the BBB: endothelial cell (EC), basement membrane (gray), astrocyte (A), pericyte (P), neuron (N) and microglia (M). B, the main elements of basement membrane: the collagen type IV (1), laminins (2) that are the principal responsible for the attachment of basement membrane to the cells receptors (3), and the perlecan (4) that is the predominant heparan sulfate proteoglycan in the endothelial cell basement membrane (adapted from Yurchenco and Patton, 2009).

Beyond all these matrix molecules the BM is also composed by matrix adhesion receptors, known as CAMs, as well as signaling proteins, which form an extensive and complex extracellular matrix (ECM) (Carvey et al., 2009). These molecules are expressed in the vascular cells, neurons and supporting glial cells and are important for BBB's maintenance (del Zoppo et al., 2006). Integrin and dystroglycan families of CAMs are expressed by cells within cerebral microvessels. Integrins participate in mediating endothelial signaling, cell migration and brain capillary tube formation during angiogenesis. These and all CAMs have an essencial role in the maintenance of BBB integrity (Zlokovic, 2008). The receptors on the cell surface, in turn, allow the link between the ECM and the underlying cytoskeleton (Yurchenco and Patton, 2009).

A perfect BM besides provide anchoring and structural integrity to capillaries may also be involved in pericytes function and differentiation. Pericytes encased in the BM or exposed to laminal proteins do not usually differentiate (Dore-Duffy, 2011).

Matrix metalloproteinases (MMPs) have the capacity to digest the BM, consequently reducing anchoring of brain ECs, and to affect TJs integrity that lead to alteration in BBB integrity (Carvey et al., 2009). MMP-2 and MMP-9 are two gelatinases capable of proteolyze some membrane compounds including collagen type IV, glicoptotein like fibronectin and vitronectin, the proteoglycan aggrecan and elastin. MMP-2 can also cleave laminin. Serine proteases, cysteine proteases, and heparanase are other families of proteases that can affect the BM (del Zoppo, 2010).

The disruption of BM can promote alterations in the cytoskeleton of ECs that affect TJs and barrier's integrity (Cardoso et al., 2010). Changes like these can be due to proteases expression by pericytes (Du et al., 2008). BM can also thicken, in response to stress stimuli (Dore-Duffy, 2011).

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1.3. Neurons

There are many neurons that directly innervate ECs and astrocytic processes, like noradrenergic (Cohen et al., 1997), serotonergic (Cohen et al., 1996), cholinergic (Tong and Hamel, 1999) and GABA-ergic neurons (Vaucher et al., 2000). Furthermore, there are evidences that neurons can regulate the blood vessel's function through inducing expression of enzymes unique for ECs in response to metabolic requirements (Tontsch and Bauer, 1991). Thus, neurons may have an important role on the BBB phenotype but little is known about this. On the other hand, ECs and a BBB well developed are important to create a stable environment to neural function (Abbott et al., 2006). However, evidences about the role of neurons in BBB properties begin to arise. Minami (2011) through an ischemic model proved that presence of neurons increase the transendothelial electrical resistance (TEER) and decrease the permeability of ECs in an *in vitro* model of the BBB.

1.4. Astrocytes

Astrocytes are glial cells whose endfeet ensheath the BM on the outer surface of the BBB endothelium. These cells cover more than 99% of the endothelium (Persidsky et al., 2006), contributing to the BBB properties and development, and to the unique endothelial phenotype. These roles are made through interactions with ECs, expression and release of soluble factors (Persidsky et al., 2006; Cardoso et al., 2010) and throught their anatomic proximity to ECs (Lai and Kuo, 2005). For example, the astrocytes interacting with ECs enhance their TJs and reduce gap junctional area (Tao-Cheng and Brightman, 1988), so these cells have an important role in the permeability and integrity of the BBB. Other studies by Siddharthan et al. (2007) and Malina et al. (2009) proved that the presence of astrocytes elevate the TEER and decrease the permeability of BBB endothelium. The interactions between astrocytes and ECs are also essential in regulating brain water and electrolyte metabolism under physiologic and pathologic situations (Zlokovic, 2008).

Besides their role in the BBB, astrocytes are essential for proper neuronal function and the interactions astrocytes-BMVECs are very important for a functional neurovascular unit since neuronal cell bodies are very close to brain capillaries (Persidsky et al., 2006).

1.5. Microglia

Microglia are a distinct class of glial cells that constitute the brain's immunocompetent cells. Over time, their nature has been extensively discussed but now is accepted that microglia are ontogenetically related to cells of the mononuclear phagocyte lineage contrary to the other cells of CNS (Kreutzberg, 1996). Monocytes enter into the brain during embryonic development and differentiate into brain resident microglia displaying surface antigens of macrophages (Kim and Vellis, 2005).

Microglia are characterized by two principal forms according to the brain conditions. In physiologic situation, the resting microglia have small bodies and long, thin processes, known as ramified morphology. In case of pathology activated cells have a phagocytic morphology with short processes named ameboid microglia. The evolution from one form to another is associated with changes in

surface antigen and cytokine release (Kim and Vellis, 2005; Zlokovic, 2008). This ability to quikly change from one to other form makes their resting form the vigilant cells to the homeostatic disturbance in the CNS (Kreutzberg, 1996).

Since microglia are located in perivascular space, it is suggested that their interactions with ECs may influence BBB's properties (Cardoso et al., 2010). However, the mechanisms behind this remain unknown.

2. Pericytes

It was in 1873 that Rouget described for the first time a population of cells on capillary walls and distinguished them from migratory leucocytes (Rouget, 1873) (Fig. I.7). Fifty years later these cells were named pericytes by Zimmermann (Krueger and Bechmann, 2010). The term pericyte arises from "peri-" around and "cyto-" cell and reflects their location at the abluminal side of the microvessels (Balabanov and Dore-Duffy, 1998). These cells are presently known as perivascular cells with multifunctional activities (Braun et al., 2007; Krueger and Bechmann, 2010).

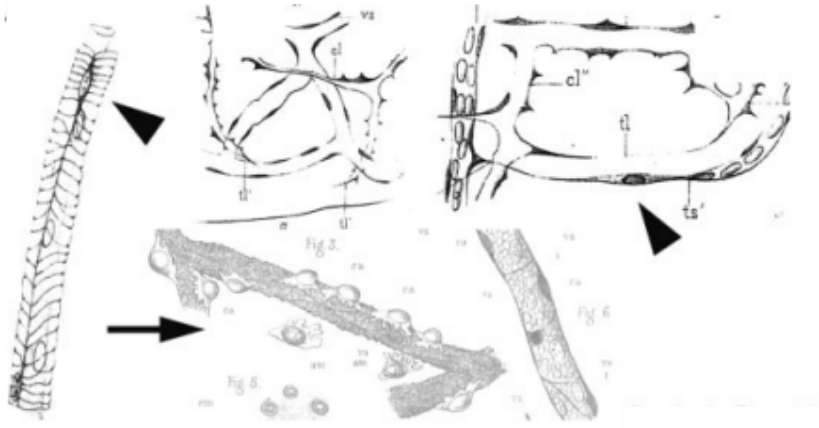

Figure I. 7: Original draw of pericytes by Rouget in 1873. Rouget (1873) described the cells of the capillary walls and divided into amoeboid migratory cells (arrow) and fusiform contractile cells (arrow heads). (Krueger and Bechmann, 2010)

2.1. Characteristics of pericytes

In most BBB research the pericytes are often devalued and the astrocyte-endothelial interactions more investigated. This is mainly due to the difficulty in extracting pericytes from their location, as well as to the lack of pericyte-specific markers, which vary with the type of tissue (Engelhardt and Sorokin, 2009). Therefore, it is necessary to look for new markers that allow not only to find them, but also to deepen our knowledge of their functions and role in the neurovascular unit. Currently, location and identification of pericytes require a series of stains with a combination of positive and negative immunoreactivity. Pericytes express surface antigens allowing their identification, including the pAPN/CD13, platelet-derived growth factor-β receptor (PDGFR-β), α-smooth muscle actin (α-SMA), desmin, nerve glial-2 (NG-2), the promoter trap transgene XlacZ4 and the regulator of G-protein signling-5 (Krueger and Bechmann, 2010). Besides these, pericytes are immunoreactive for γ-glutamyl

transpeptidase, alkaline phosphatase, nestin and vimentin (Fisher, 2009; Engelhardt and Sorokin, 2009; Krueger and Bechmann, 2010). However, most of these molecules are expressed in neighboring cells as well (Fisher, 2009; Krueger and Bechmann, 2010). On the other hand, in the brain, only pericytes close to arterioles are immunoreactive for α-SMA (Dore-Duffy, 2008). Another difficulty associated with pericytes is the fact that they are tricky to identify because they are often confused with adjacent cells, such as vascular muscle cells, perivascular cells and juxtavascular microglia (Krueger and Bechmann, 2010). .

Brain pericytes are multifunctional and polymorphic cells (Guillemin and Brew, 2004; Lai and Kuo, 2005) but normally are star-shaped and have long cytoplasmic processes that are oriented along the axis of the blood vessel, while smaller circumferential arms engirdle the vascular wall (Fisher, 2009; Krueger and Bechmann, 2010) (Fig. I.8). There may be up to 90 processes with a width of 300 to 800 nm per 100 µm of capillary length (Zlokovic, 2008). However, processes morphology tends to be heterogenous and is likely to represent functional differentiation of pericytes (Dore-Duffy and Cleary, 2011). Moreover, they have cytoplasmic lysosomes that give to pericytes granularity (Fisher, 2009). These cells can be seen on the abluminal surface of ECs of capillaries, venules and arterioles (Fig. I.9). They cover 22-32% of cerebral capillary surface and tend to arise over endothelial TJ regions with one layer of BM between them as stated above. Another layer of BM lies between pericytes and astrocyte endfeet (Fisher, 2009; Krueger and Bechmann, 2010). The membrane surrounds all pericytes even their projections (Dore-Duffy and Cleary, 2011). During development and angiogenesis pericytes deposit components of this membrane (Dore-Duffy, 2008) and contribute to its maintenance (Zlokovic, 2008).

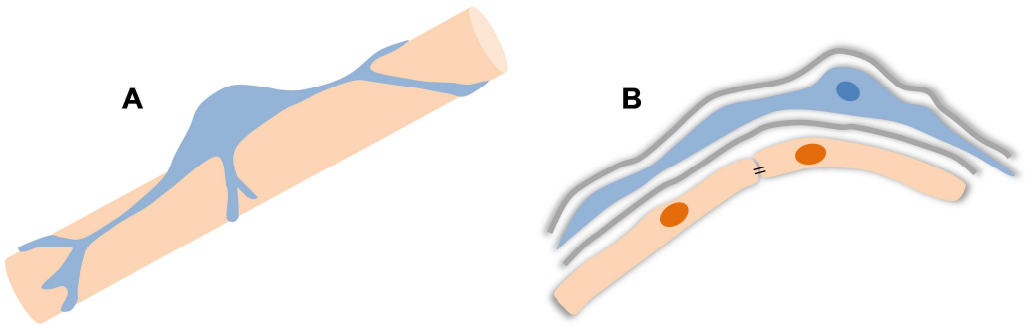

Figure I. 8: Schematic representation of a pericyte (blue) ensheathing a blood vessel (orange). A, Longitudinal view showing the long processes of a pericyte along a blood vessel; B, Transversal view with a pericyte over endothelial cells and the basement membrane (gray) between them.

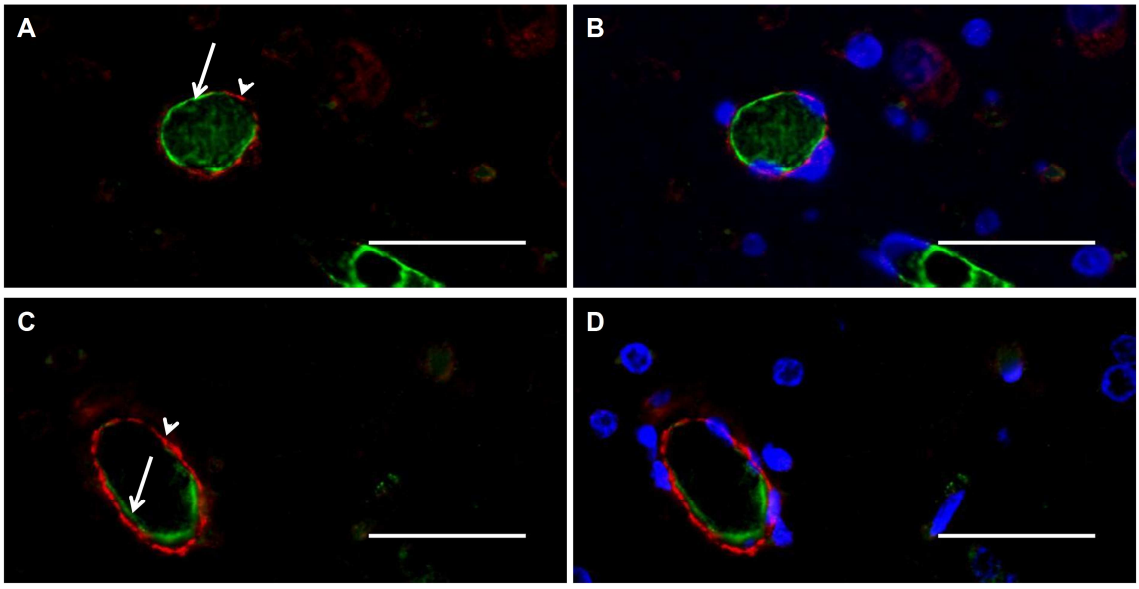

Figure I. 9: Double labeling immunofluorescence analysis of endothelial cells and pericytes in human hippocampus. Endothelial cells were labeled for von Willebrand factor (green, arrow) and pericytes for α-smooth muscle actin (red, arrowhead). Nuclei were identified by DAPI (blue) (B,D). Scale bars: 40 µm.

Pericytes-to-endothelia ratio in the brain is higher than in other organs (1:3 compared with 1:100 in striated muscles), and the location of pericyte on microvessels and their abundance varies according microvessels types (Dore-Duffy, 2003). The degree of coverage appears to correlate with the degree of tightness of the interendothelial junctions (Shepro and Morel, 1993).

Pericytes are cells from the vascular smooth muscle lineage (Nishioku et al., 2009) and it is generally considered that they have a mesodermal origin (Guillemin and Brew, 2004). It is possible that they migrate into the tissue during the latter stage of vascularization then assuming their characteristics. Their precursor cells settle on newly formed capillary sprouts and differentiate into pericytes as they become enclosed within basal lamina (Rhodin and Fujita, 1989).

2.2. Functions

In recent years a variety of studies, mainly in cell cultures, set various functions of pericytes. These include contractile, immune and phagocytic, migratory and angiogenic functions. In addition, pericytes contribute to the BBB and perform a regulatory role in brain hemostasis (Fisher, 2009).

2.2.1.Contribution to BBB properties

The short distance, about 20 nm, between ECs and pericytes (Zlokovic, 2008), the high density of pericytes in the CNS and the intimate association through gap junctions (Cuevas et al. 1984), peg-and-socket (Rucker et al., 2000) and adhesion plaques junctions between these cells and ECs make pericytes as key players in maintenance and stabilization of the BBB and in the development of BBB TJs, therefore contributing to the low paracellular permeability (Dohgu et al., 2005; Nakagawa et al., 2007). Gap junctions allow communication through exchange of ions and small molecules and are

constituted by N-cadherin, a variety of adhesion molecules, β-catenin, ECM molecules such as fibronectin, and a number of integrins. Adhesion plaques support transmission of contractile forces from pericytes to other cells (Cuevas et al., 1984; Nakamura et al., 2008). Peg-and-socket contacts prevent pericytes penetrating through basal membrane and contacting with other cells and vessels (Rucker et al., 2000). It was thought that these functions related to the BBB stabilization were in charge of astrocytes, but today we find data describing the development of endothelial TJs independently of astrocytes (Krueger and Bechmann, 2010). Al Ahmad et al. (2011) studied the role of astrocytes and pericytes in TJs and AJs formation and observed the essencial role of each cell in the establishment of BBB-specific TJ complexes in ECs. Thus, we must consider a dialogue between several populations of the neurovascular unit and the possible existence of compensatory mechanisms that may take over each other's role in case of impaired function (Fisher, 2009). The ECs are not the only ones that express TJ molecules. Pericytes express several TJ molecules including claudin-12, JAM, ZO-1, ZO-2 and occludin. These cells also express several barrier-related transporters like ABCG2, P-gp, MRP-1, and glucose transporter-1 (Glut-1) (Shimizu et al., 2008). Once again, pericytes are essential for BBB properties, namely, permeability and hemostasis of the brain.

Doghu et al. (2005) evaluated BBB function based on the transendothelial permeability coefficient (P_e) to sodium fluorescein (Na-F) and on the cellular accumulation of rhodamine 123 in mouse brain capillary ECs, as markers of paracellular permeability and functional activity of P-gp, respectively, and proved that pericytes participate in tightening the intercelular junctions and facilitating P-pg function in brain ECs through production of soluble factors including TGF-β and by cell-to-cell contact. Besides this factor, others are also derived from pericytes, the VEGF that increases the permeability of brain ECs, the bFGF that tights the intercellular junctions and induces the expression of MRP and the angiopoietin (ang) -1, an antipermeabitily factor. These factors are also produced by astrocytes, which suggest that the four factors are involved in the interaction between ECs, pericytes and astrocytes under physiological and pathophysiological situations (Dohgu et al., 2005).The integrity of the BBB can also be evaluated through measurement of TEER. Nakagawa et al. (2007) constructed, for the first time, a rat primary culture based syngeneic triple co-culture BBB model, using brain pericytes besides brain ECs and astrocytes and showed that the contact between the three different cells increased the TEER and consequently tightened TJs. Recently, Armulik et al. (2010) demonstrated the pericytes role at the BBB *in vivo*, correlating reduced pericyte densities with increased vessel diameter and reduced vessel density and established a correlation between pericytes density and BBB permeability for a range of tracers of different molecular masses (Fig. I.10).

All these evidences turn possible that pericytes regulate brain's endothelial barrier by collaborating with astrocytes (Dohgu et al., 2005). Recently**,** Daneman et al. (2010) proposed a model for BBB formation: ECs are induced to express BBB-specific genes by interactions with neural progenitors, and then the functional integrity of the BBB is regulated by pericytes during development and by astrocytes in adulthood.

Pericytes also contribute to BM formation by synthesizing collagen type IV, glycosaminoglycans, and laminin (Engelhardt and Sorokin, 2009; Fisher, 2009), and by inducing ECs to secrete BM components (Brachvogel et al., 2007).

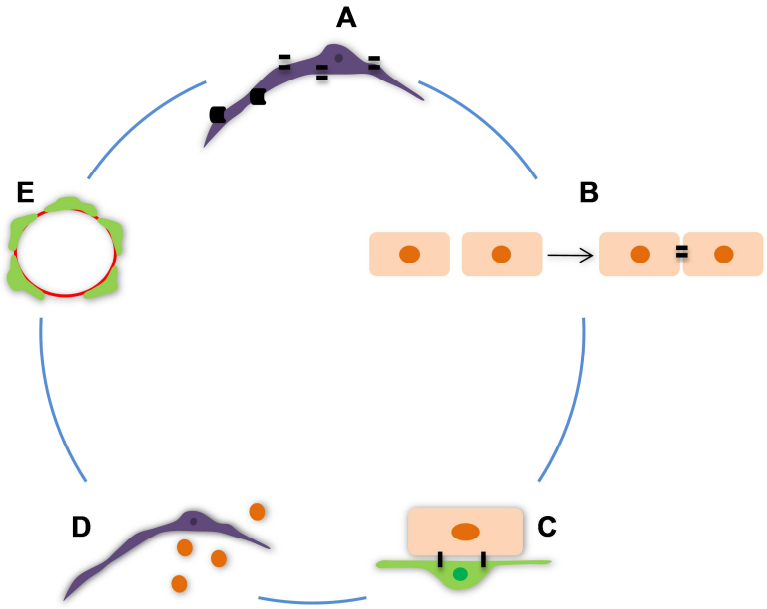

Figure I. 10: The main features of pericytes that determine the blood-brain barrier properties. A, Expression of tight junctions and barrier-related transporters; B, Role in the formation of tight junctions between endothelial cells (ECs); C, Short distance to endothelial cells and gap, peg-and-socket and adhesion plaques junctions with ECs; D, Production of soluble factors; E, High density of pericytes around brain microvessels.

2.2.2.Participation in vascular development

During vertebrate embryo development, the first functional organ is the vascular system, whose growth must be continuous. The vasculature forms by vasculogenesis (new vessel formation from angioblasts or stem cells) and angiogenesis (sprouting, bridging and intussusceptive growth from existing vessels). Mesenchymal cells differentiate into endothelial tubes that form a primitive blood vessel network from which new blood vessels develop. After formation of the first endothelial tubes they become associated with mural cells that include pericytes and vascular smooth muscle cells (Hellström et al., 2001).

Pericytes have an important role on angiogenesis. This role has been demonstrated in models of brain injury and brain hypoxia that are strong stimuli for angiogenesis (Dore-Duffy et al., 1999). In ultrastructural studies, it was shown that pericytes are the first cells that respond to brain hypoxia and suffer morphology alterations in cats. These alterations were interpreted as initial steps of migration as the abluminal surface of the cells formed characteristic “peaks” pointing and extending towards the parenchyma. At the same time, the luminal BM between ECs and pericytes begins to thicken and the abluminal one thinned out (Gonul et al., 2002). Then occurs the elongation of pericytes and the disappearance of the basal lamina at the leading edge of migrating cells. Migratory pericytes express and show in the leading tips urokinase plasminogen activator and its receptor that are charactetistic and mediate their activation and migration (Dore-Duffy et al., 2000).

VEGF is very important in this process as it initiates vessel formation and activates a chain of molecular and cellular events that lead to mature vasculature (Jain, 2003). VEGF, which is produced by pericytes under hypoxic (Yamagishi et al., 1999) and hypoglycemia (Hellström et al., 2001) conditions, is crucial to communication with ECs. VEGF induces angiogenic sprouts, positive for the CD31 marker of mature and embryonic ECs, to display α-SMA and desmin, characteristic of pericytic phenotype. This observation indicates that ECs transform into pericytes or smooth muscle cells and that VEGF plays an important role in this transformation. Furthermore, the number of pericytes covering new capillaries can be increased by VEGF (Hagedorn et al., 2004). Prove of the role of VEGF in angiogenesis is the blockage of its receptor VEGFR2 can temporally normalize a tumor vessel structure (Winkler et al., 2004). Interleukin (IL) -6 is also an important cytokine to pericytes differentiation and may be responsible for recruiting ECs and promoting angiogenesis (Kale et al., 2004).

After formation, the nascent vessels are stabilized by recruiting mural cells, including pericytes, and by generating ECM. This stabilization process is regulated by at least four molecular pathways. The recruitment/differentiation of mural cells, namely pericytes, to sites of angiogenesis or neurovascularization is mediated by the platelet-derived growth factor-β (PDGF-β) produced by ECs (Hellström et al., 2001; Lai and Kuo, 2005; Abramsson et al., 2007), presumably in response to VEGF (Jain, 2003) and by the corresponding receptor PDGFR-β expressed by pericytes (Hellström et al., 2001; Lai and Kuo 2005; Abramsson et al., 2007). The essential role of this factor was demonstrated by Hellström et al. (2001) through PDGFR-β knockout mice that lacked pericytes along the vessels and by Abramsson et al. (2007) who showed that the absence of an amino acid motif of PDGF-β produces defective investment of pericytes in microvascular system. Angs are also important for vascular development and stabilization. Ang-1 produced by pericytes and perivascular cells binds to receptor Tie-2 fostered vascular stabilization and ang-2, expressed by ECs, binds to the same receptor and acts like a destabilizing factor (Jain, 2003; Ballabh et al., 2007; Krueger and Bechmann, 2010) in the absence of VEGF, and restricted to ECs in areas of vascular remodeling (Jain, 2003). Thus, the development of vasculature remains unstable and immature until pericytes or its precursors are recruited (Ballabh et al., 2007; Krueger and Bechmann, 2010). Like mentioned above, in the presence of VEGF, ang-2 facilitates vascular sprouting (Jain, 2003). Therefore, the presence of pericytes regulates negatively ECs proliferation, determining their number, morphology and microvessel architecture (Hellström et al., 2001). Vascular stability is sustained also before pericytes arrive to their position, by binding of sphingosine-1-phosphate (S1P) to its endothelial receptor $S1P_1$ (Krueger and Bechmann, 2010) (Fig. I.11). After binding, small GTPase Rac is activated in ECs (Paik et al., 2004) and N-cadherin, found in peg-socket contacts between ECs and pericytes, and vascular endothelial-cadherins that are normally found in junction complexes between neighboring ECs are expressed (Krueger and Bechmann, 2010). Finally, TGF-β is a multifunctional cytokine that also promotes vessel maturation through stimulation of ECM production and differentiation of mesenchymal cells to mural cells (Jain, 2003). TGF- β is also important, like ang-1 and its receptor, in the formation of pericytes and vascular smooth muscle cells from the mesenchyme surrounding the endothelium (Hellström et al., 2001)

Al Ahmad et al. (2011) through a novel 3D *in vitro* model that closely mimics barrier formation and morphology showed the proangiogenic role of pericytes. Pericytes can participate in angiogenesis through NG-2, which is expressed by immature pericytes. Both blocking by antibodies as well as knocking out the gene of NG-2 cancel vascular growth in various models of induced angiogenisis, but it is necessary to have in mind that NG-2 is expressed in more cells of the CNS. Due to their important role in angiogenesis, pericytes may be suggested as a target to pharmacological therapy for tumors (Krueger and Bechmann, 2010)

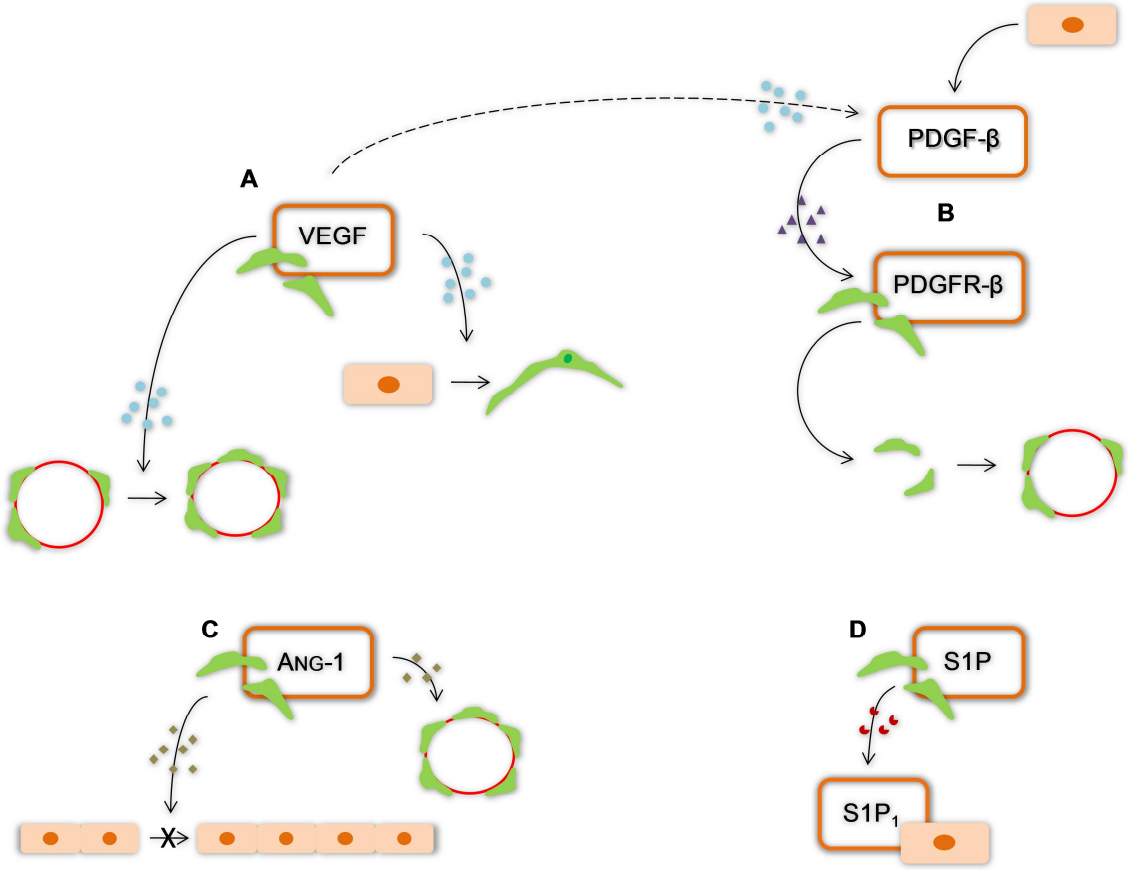

Figure I. 11: Factors and receptors produced by pericytes that contribute to angiogenesis. A, Vascular endothelial growth factor (VEGF) allows the transformation of endothelial cells into pericytes or smooth muscle cells and increase the number of pericytes covering new capillaries; B, platelet-derived growth factor receptor-β (PDGFR-β) recognizes the platelet-derived growth factor-β (PDGF-β) produced by endothelial cells presumably in response to VEGF. This interaction is responsible for the pericyte recruitment; C, angiopoetin (ang)-1 inhibits endothelial proliferation and is responsible for vascular stabilization; D, sphingosine-1-phosphate (S1P) connects to its endothelial receptor and promotes the continuous stability.

2.2.3.Contractile function

As already mentioned above, Rouget (1873) was the first to describe a pericyte and distinguished them from migratory leucocytes. This distinction was based in the elements found in pericytes. However, the expression of these elements varies according to the local and the species. Brain

pericytes express α-SMA (Herman and D'Amore, 1985), with more robust expression in pre-capillaries compared to mid- and post-capillaries (Nehls and Drenckhahn, 1991; Boado and Pardrige, 1994; Fisher, 2009; Kruger and Bechmann, 2010) (Fig. I.12). Pericytes also possess tropomyposin and myosin that contribute to their contractile capacity (Joyce et al., 1985a, b). However not all pericytes express α-SMA. Bandopadhyay et al. (2001) proved that in CNS pericytes α-SMA is the predominant contractile protein but was not present in all brain pericytes, which supports the idea that the pericytes constriction ability is not universal.

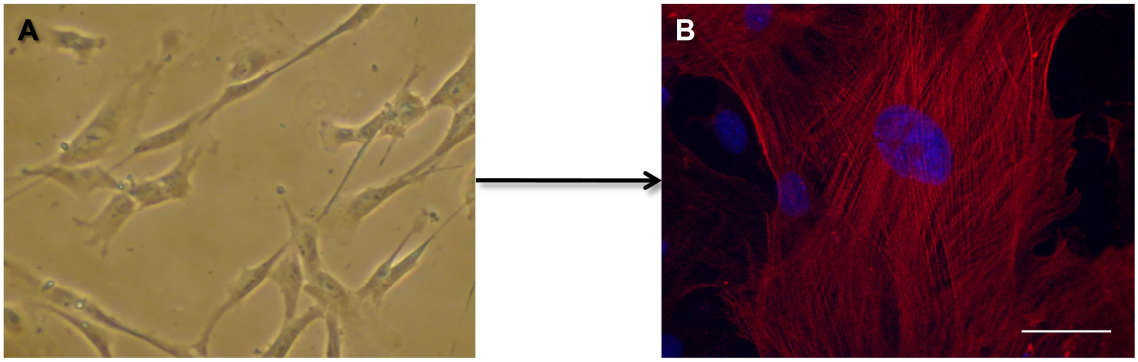

Figure I. 12: Morphological features of human brain vascular pericytes (HBVP). A, HBVP in primary culture observed by phase contrast microscopy shows the typical morphology. Original magnification: 100x. B, Immunofluorescence analysis of the pericyte marker α-smooth muscle actin (α-SMA). Nuclei were stained with Hoechst 33258 dye. Scale bar: 40μm.

Pericytes also can express receptors for vasoactive substances such as prostacyclin (Fisher, 2009; Kruger and Bechmann, 2010), angiotensin II, endothelin-1, catecholamines, and vasopressin. Ang-2 is a vasoctive peptide that most of the times cause vasoconstriction. Ferrari-Dileo et al. (1996) demonstrated that pericytes have specific ang-2 binding sites. They also found binding sites for vasoactive intestinal polypeptide. Zwieten et al. (1988) discovered the presence of binding sites for vasopressin. The receptors for endothelin-1, other vasoactive peptide, were also located (Yamagishi et al., 1993). Nitric oxide (NO) also affects pericytes, causing their relaxation trough cyclic guanosine monophosphate (Haefliger et el., 1994). With all these evidences, it is considered that pericytes have a contractile function and blood flow regulatory capabilities, especially in pre-capillary arterioles (Balabanov and Dore-Duffy, 1998; Kruger and Bechmann, 2010).

Like already said pericytes are associated to microvessels and have some features of smooth muscle cells, so the absence of these last cells in microvessels turn pericytes their possible contractile substitutes (Bandopadhyay et al., 2001).

Yemisci et al. (2009) showed that pericytes contract during ischemia and even after reopening of the occluded middle cerebral artery. Pericytes also cause segmental narrowing of capillaries, turn erythrocytes trapped in the capillary constrictions and obstruct microvirculation. These authors also proved that peroxynitrite leads to pericytes constriction, wheareas the suppression of oxygen and nitrogen radicals formation can reverse this situation (Fig. I.13).

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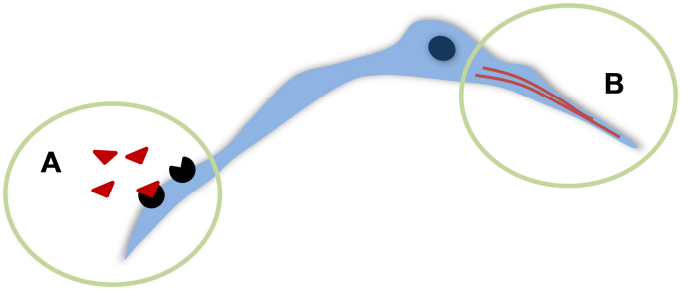

Figure I. 13: Pericytes properties that turn them into cells with contractile functions. A, Receptors for vasoactive substances; B, Expression of α-smooth muscle actin.

2.2.4.Immune and phagocytic function

Brain pericytes constitutively express low levels of adhesion molecules (intercellular adhesion molecule-1 and vascular CAM-1), which have potential stimulatory activity in major histocompatiblity complex (MHC) -class II dependent antigen presentation (Veerbek et al., 1995). Thus, pericytes may have the capacity to present antigens to T-lymphocytes. Dore-Duffy and Balabanov (1998) showed the response of primary rat CNS pericytes to interferon y with upregulation of the MHC class II molecule and present antigen to primed lymphocytes (Balabanov and Dore-Duffy, 1998). Brain pericytes also produce immunoregulatory cytokines like IL-1β and IL-6 (Fabry et al., 1993). TGF-β produced by pericytes may also function as immunoregulator at the BBB (Dore-Duffy et al., 1996; Balabanov and Dore-Duffy, 1998)

Pericyte lysosomes express acid phosphatase that implies a phagocytic function of pericytes (Fisher, 2009). Pericytes also have components that are recognized by macrophage-selective monoclonal antibodies EBMS/11 and ED2 (Esiri et al., 1986; Balabanov et al., 1996) (Fig. I.14).

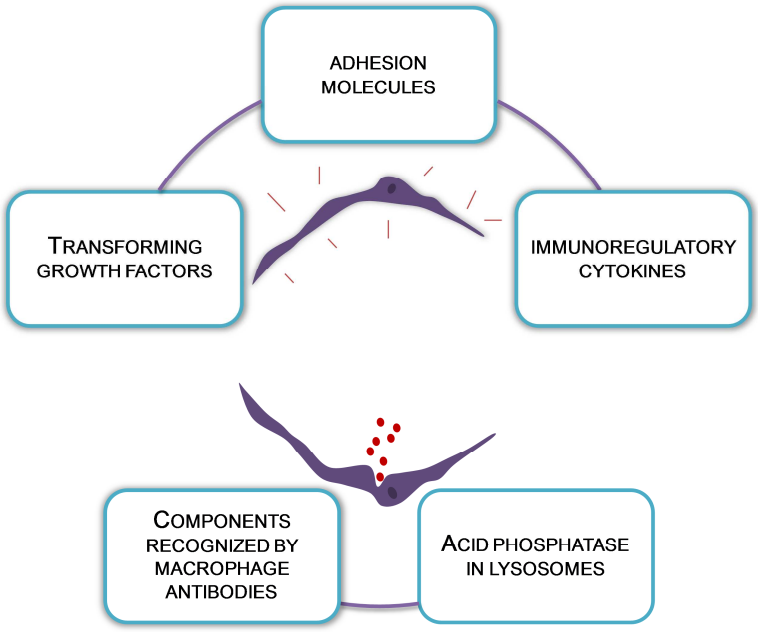

Figure I. 14: Characteristics of pericytes that are responsible for their immune and phagocytic role. The production of transforming growth factors, adhesion molecules and immunoregulatory cytokines characterize the

pericytes like immune cells. The expression of acid phosphatase in lysosomes and the possession of components recognized by antibodies for macrophages turn pericytes into phagocytic cells.

2.2.5.Roles on hemostasis

Pericytes appear to be cells with pro- and anti- coagulant activity [60] (Fig. I.15). In fact, Kim et al. [104] showed that pericytes decrease endothelial tissue plasminogen activator (tPA), a serine protease that processes plasminogen into proteolytically active plasmin thus allowing fibrinolysis to occur, and suggested that this effect is mediated by a soluble-derived factor since it was observed in a non-contact ECs-pericytes co-culture model. They also showed that pericytes amplify the LPS-induced enhanced release of plasminogen activator inhibitor-1 (PAI-1), and further showed that pericytes express robust amounts of the antithrombin and antifibronolytic molecule serpin protease nexin-1 (PN-1). Thus, these evidences indicate that pericytes have a complex and important role in brain microvascular hemostasis, with effects that are largely antifibrinolytic. Besides inducing an anti-coagulant response through PN-1, pericytes can differentially regulate expression of pro-coagulant enzyme complexes involved in the extrinsic pathway of blood coagulation. Bouchard et al. [105] have defined functionally active tissue factors on the surface of human brain pericytes that are the primary generator of the coagulation cascade.

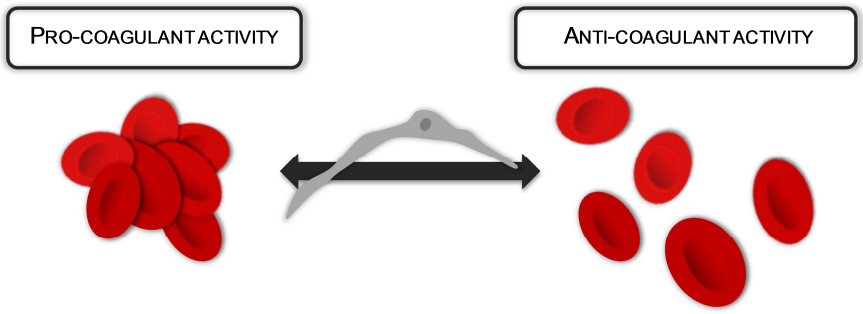

Figure I. 15: Pericytes have the capacity to regulate blood clotting through a pro-and anti-coagulant activity.

2.2.6.Multipotent cells

Besides all these functions, pericytes can be seen like multipotent cells. Pericytes may be a source of multipotent stem cells that differentiate along multiple lineage and may provide trophic support and maintenance in the adult brain (Dore-Duffy et al., 2006). They can differentiate into osteoblasts, chondroblasts, fibroblasts, adipocytes, and smooth muscle cells (Lai and Kuo 2005, Dore-Duffy et al., 2006, Dore-Duffy, P. 2008) (Fig. I.16).

Human brain pericytes express CD146, a molecule present at some endothelial surface (Li et al., 2003) and NG-2, a proteoglycan and a neural progenitor cell marker (Ozerdem et al, 2002). Crisan et al. (2008) also confirmed the presence of these antigens in brain pericytes, as well as in pericytes from skeletal muscle, myocardium, pancreas, bone marrow, abdominal fat and placenta. The same

authors showed that cultured pericytes express mesenchymal stem cells markers, including CD44, CD73, CD90 and CD105.

Recently Dore-Duffy characterizate of CNS capillary pericytes from the transgenic mice harboring a temperature-sensitive mutant of the SV40 virus target T-gene. These Immortopericyte (IMP) are stable and do not differentiate for long periods of time and, at 33℃ in the presence of interferon γ. These authors showed that these cells are α-SMA negative and pluripotent. However they can be induced to differentiate along mesenchymal and neuronal lineage at 37ºC.

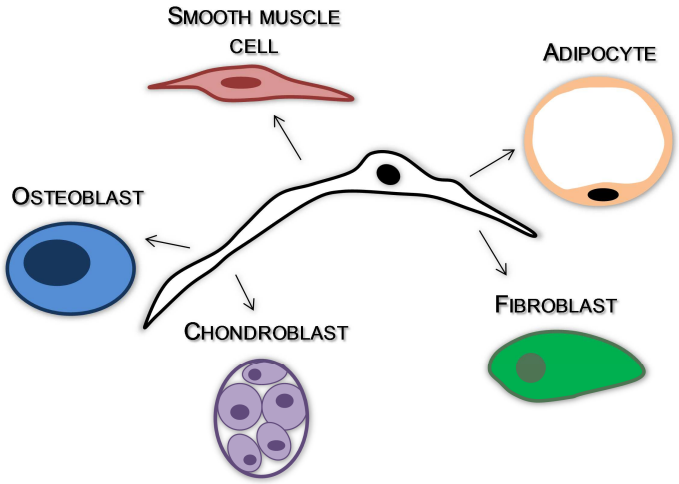

Figure I. 16: Pericytes can differentiate into osteoblast, chondroblast, fibroblast, adipocytes and smooth muscle cells.

3. Neurovascular unit pathology

3.1. Involvement of pericytes in BBB dysfunction

Actually, there are already numerous diseases associated with BBB dysfunction, including hypoxia and ischemia (Kaur and Ling, 2008), multiple sclerosis (Correale and Villa, 2007) edema (Rosenberg and Yang, 2007), Parkinson's and Alzheimer's diseases (Desai et al., 2007), epilepsy (Remy and Beck, 2006), tumors (Bronger et al., 2005) and glaucoma (Grieshaber and Flammer, 2007). BBB dysfunction can range from a simple transient opening of TJs (Fig. I.17B) to chronic barrier breakdown. Changes in transport systems and enzymes can also occur (Abbott et al., 2010) (Fig. I.17C). One of the consequences of the BBB breakdown is the increase of permeability that leads to the penetration of plasma into extracellular space of the brain causing vasogenic brain edema (Kaal and Vecht, 2004). Blood components, as red blood cells and leukocytes can also enter into the brain due to ischemic injury, intracerebral hemorrhage, trauma, neurodegenerative diseases, inflammation or vascular disorder and promote production of neurotoxic products that can influence and compromise synaptic and neuronal functions (Zlokovic, 2008). The increase of the BBB permeability can be due to chemical mediators that are released in pathologic conditions and include glutamate, aspartate, taurine, ATP, NO, IL-1β, histamine, thrombin, platelet-activating factor, free radicals, among

others. Some of these last are released by ECs and endothelium itself responds to the released agents (Ballabh et al., 2004). On the other hand, the length of brain capillaries is reduced in neurodegenerative diseases, like Alzheimer´s disease, which diminishes the transport of energy substrates and nutrients across the BBB and the elimination of neurotoxins from the brain (Zlokovic 2008).

Pericytes dysfunction or their loss also play an important role in the pathogenesis of some diseases. Reduction of pericytes has already been observed after stroke (Duz et al., 2007), multiple sclerosis (Zlokovic, 2008), brain tumor (Ho, 1985), diabetic retinopathy (Hammes et al., 2002), aging (Frank et al., 1990), and in a variety of angiopathies (Szpak et al., 2007) (Fig. I.14D).

Eberhard et al. (2000) observed different degrees of pericyte recruitment in six different types of malignant human tumors (glioblastomas, renal cell carcinomas, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas) indicating differences in the functional state of the tumor vasculature that can reflect variations in maturation degrees of the tumor vascular bed, since pericytes confer stability to the vasculature when present in a large number. These evidences support the possibility of a pericytes targeted therapy against the progression of tumors, especially when they are still immature.

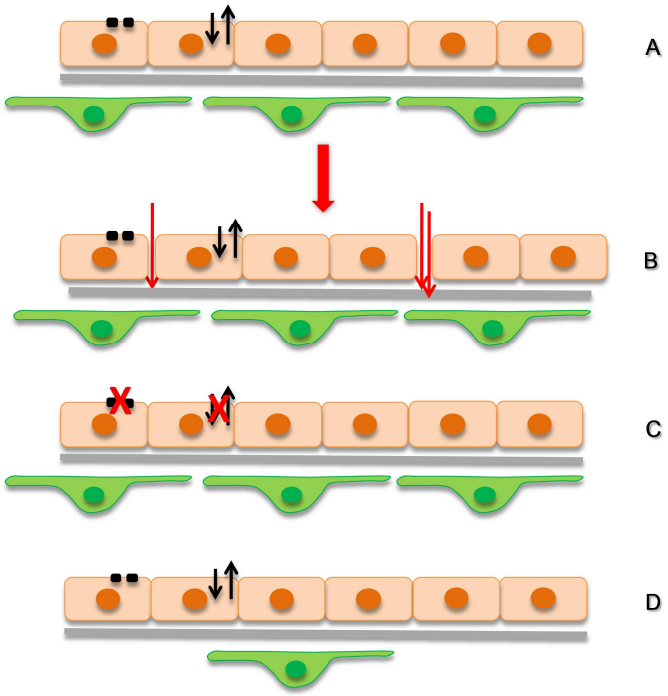

Figure I. 17: Dysfunction of the blood-brain barrier (BBB). A, Normal BBB. The BBB dysfunction includes tight junctions opening (B), changes in transport systems (C) and reduction of pericytes number (D).

Hypoxia is a common disease where pericytes are able to protect BBB (Hayashi et al., 2004; Hawkins and Davis, 2005). Hayashi et al. (2004) showed that pericytes contact co-culture with ECs counteracted the increased permeability of the endothelial monolayer following short duration of hypoxia. Thus, it was proposed that pericytes may have a protective role against BBB disruption

following ischemia through direct contact with ECs. Al Ahmad et al. (2011) through a 3D BBB model in hypoxia condition showed that EC monolayer exhibited significantly disrupted TJ protein expression, namely claudin-5 and ZO-1, and that the presence of astrocytes and pericytes is essential for optimal localization of these two proteins in cell borders.

3.2. Bilirubin neurotoxicity

3.2.1. Hyperbilirubinemia

3.2.2. Acute bilirubin encephalopathy vs. kernicterus or chronic bilirubin encephalopathy

Unconjugated bilirubin (UCB) is the end product of heme catabolism (Dennery et al., 2001) and is a tetrapyrrole with propionic side groups. These groups form internal hydrogen bonds with the distal lactam groups that render this molecule nearly insoluble in aqueous medium at physiologic pH. Therefore, UCB circulates in serum bound to albumin. Serum albumin has a binding site for UCB with high affinity and two or more binding sites with lower affinity (Berk and Noyer, 1994; Ostrow et al., 1994, Wennberg, 2000).

During embryonic life bilirubin is produced starting from 12 weeks gestation and is eliminated through maternal circulation. After birth this placental protection is lost, which contributes to UCB accumulation (Brito et al., 2006). Moreover, in neonatal life the short red blood cell lifespan leads to an UCB overproduction, the immaturity of hepatic cells decreases UCB conjugation and the lack of bacterial intestinal flora impairs bilirubin excretion (Porter and Dennis, 2002). These facts, together with the characteristic neonatal hipoalbuminemia (Kaplan and Hammerman, 2005) that causes disturbances of the albumin-bilirubin equilibrium (Ahlfors and Parker, 2010), lead to neonatal hyperbilirubinemia, which occurs in up to 60% of full term newborns and 80% of pertems (Denneryet al., 2001). In addition, other factors may arise and contribute for even higher levels of UCB, including metabolic acidosis, infection, hyperoxia, as well as prematurity and drugs or preservatives (Kaplan and Hammerman, 2005). UCB has the capacity to enter into the brain and in high concentrations can affect and turn yellow specific locations (Ostrow et al., 2004), as the globus pallidus and subthalamic nucleus, as well as the auditory and oculomotor brainstem nuclei (Shapiro, 2010). The cerebellum (Brito et al., 2011) and hippocampus may also be affected (Shapiro, 2010). If untreated, hyperbilirubinemia can develop into UCB encephalopathy or kernicterus depending on neurological damage progress to chronic and permanent or even death (American Academy of Pediatrics, 2004).

3.2.3. Effects of unconjugated bilirubin in the neurovascular unit

There are already several studies indicating diverse effects of UCB in neural cells, including death, nitrites release and increased endothelial nitric oxide synthase (eNOS) expression and cytokines production. In fact, UCB causes death in astrocytes (Silva et al., 2001b, Silva et al., 2002), neurons (Silva et al., 2002), oligodendrocytes (Genc et al., 2003), microglia (Silva et al., 2010) and ECs (Akin et al., 2002). UCB can also affect the production of cytokines by glial cells and neurons (Fernandes et al., 2004; Falcão et al., 2005; Falcão et al., 2006; Silva et al., 2010) and by the BBB BMVECs

(Palmela et al. 2011). Finally, increases in the neuronal form of NO synthase and nitrites production were shown to occur in the presence of elevated levels of UCB (Brito et al., 2008; Vaz et al., 2010; Brito et al., 2010). Palmela et al. (2011) also showed the upregulation of eNOS expression, increase of nitrites production, decrease of the TEER and increase the permeability in human BMVECs and that UCB crosses the ECs monolayer in a time- and concentration dependent manner (Palmela et al., unpublished).

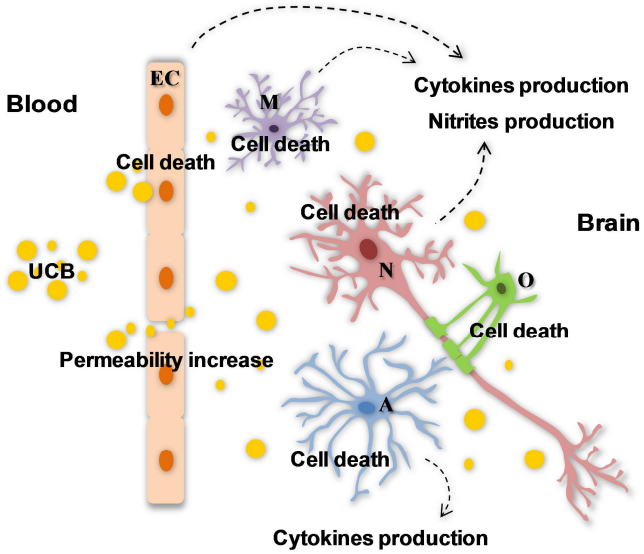

Figure I. 18: Some effects of unconjugated bilirubin (UCB) in central nervous system cells. EC, Endothelial cell; M, microglia; N, neurons; A, astrocyte; O, oligodendrocytes. The presence of UCB increases the permeability of endothelial cells, leads to death of all cells represented, secretion of cytokines by endothelial cells, microglia, astrocytes and even by neurons, as well as to production of nitrites by endothelial cells, microglia and neurons.

4. Aims

Pericytes and a proper BM play key roles in the maintenance of the BBB properties, and their impairment have been reported in several pathologies. On the other hand, hyperbilirubinemia is known for its disturbing effects in neural and BMVECs but its reflex on pericytes and BM has never been reported. Therefore, the aims of this thesis are: (1) to examine whether UCB induces cell death, disrupts the redox status and elicits an inflammatory response in primary cultures of human brain perivascular pericytes; and (2) examine collagen IV immunoreactivity and thickness in brain parenchyma of a kernicterus case. Collectively, the data obtained in this thesis will add new insights into the cellular and molecular events occurring during moderate to severe neonatal jaundice and reveal targets to prevent UCB encephalopathy.

II. MATERIALS AND METHODS

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1. Materials

Human brain vascular pericytes (HBVP) and Trypsin/EDTA solution were acquired from ScienCell Research Laboratories™ (Carlsbad, California, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS), were purchased from Biochrom AG (Berlin, Germany). Antibiotic antimycotic solution (20X), Trypsin/EDTA 1X, human serum albumin (HSA; fraction V, fatty acid free), bovine serum albumin (BSA), Hoechst 33258 dye, poly-L-lysine, poly-D-lysine and organosilanecoated slides were from Sigma Chemical Co. (St. Louis, MO, USA). Triton X-100 was acquired from Roche Applied Science (Mannheim, Germany). DPX mountant for microscopy was obtained from BDH Prolabo (Poole, UK). Mouse anti-α-SMA was purchased from ABD Serotec (Oxford, UK). Mouse anti-eNOS Type III antibody was acquired from BD Biosciences (Erembodegem, Belgium). Fluorescein isothiocyanate (FITC)-labeled horse anti-mouse antibody was acquired from Vector laboratories (Burlingame, CA, USA). Antibody Alexa Fluor 488 goat anti-mouse was from Invitrogen Corporation™ (Carlsbad, CA, USA). Primary antibody mouse anti-collagen IV came from Sigma Chemical Co. (St. Louis, MO, USA). DuoSet ELISA kits were from R&D systems® (Minneapolis, MN, USA). TRIzol Plus RNA Purification Kit was from Invitrogen Corporation™ (Carlsbad, CA, USA). Primers for real-time Polymerase chain reaction (RT-PCR) analysis were purchased from Thermo Scientific (Soeborg, Denmark). RevertAid H Minus First Strand cDNA synthesis and Maxima SYBR Green qPCR Master Mix (2x) were obtained from Fermentas (Burlington, Ontario, Canada).

Vector® antigen unmasking solution was from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.) and Dako REAL™ EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse was acquired from Dako,Glostrup, Denmark). Mayer's hematoxilin, H_2O_2 solution and all other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2. *In vitro* studies – pericytes

2.1. Primary culture

The HBVP used for this study were isolated from human brain tissue, cryopreserved at secondary culture after purification and delivered frozen. Each vial contains > 5 x 10^5 cells in 1 ml volume. These cells are guaranteed to further expand for 15 population doublings in the condition provided by ScienCell Research Laboratories. HBVP were carefully resuspended and cultured in DMEM medium supplemented with 20% FBS and 1% antibiotic-antimycotic solution. For this T-75 flask coated with poly-L-lysine or poly-D-lysine were used. After distribute the cells evenly the flask was placed in the incubator at 37℃ in a humid atmosphere enriched with 5% CO_2. The medium was changed the next day to remove the unattached cells and every other day thereafter until the culture achieved approximately 80% confluence. Then, cells were incubated 3 min with 5 ml of trypsin/EDTA solution or trypsin/EDTA 1x to detach them. Next, 5 ml of growth medium was added to neutralize trypsin, the flask was rinsed and cells were harvested. The suspension was centrifuged at 75 g for 5 min and ressuspended in 1 ml of growth medium. Finally, the cells were counted to be seeded at a density of

5000/cm^2 in new coated vessels. For immunofluorescence analysis, cells were plated on poly-D-lysine-coated coverslips, previously placed in 24-well culture plates.

2.2. Treatment

UCB was purified according to the method of McDonagh and Assisi (1972). For UCB treatment, a 10 mM stock solution was prepared in 0.1 N NaOH and used immediately after preparation. The pH value was restored to 7.4 by addition of equal amounts of 0.1 N HCl, and all the procedures were performed under light protection to avoid photodegradation. Confluent HBVP were incubated with 50 or 100 µM UCB, or no addition (control), in the presence of 100 µM HSA (UCB/HSA molar ratios of 0.5 and 1.0, respectively), for 1, 4, 24 or 78 h at 37ºC to the cell death, cytokines and nitrites release analysis (Fig. II.1).

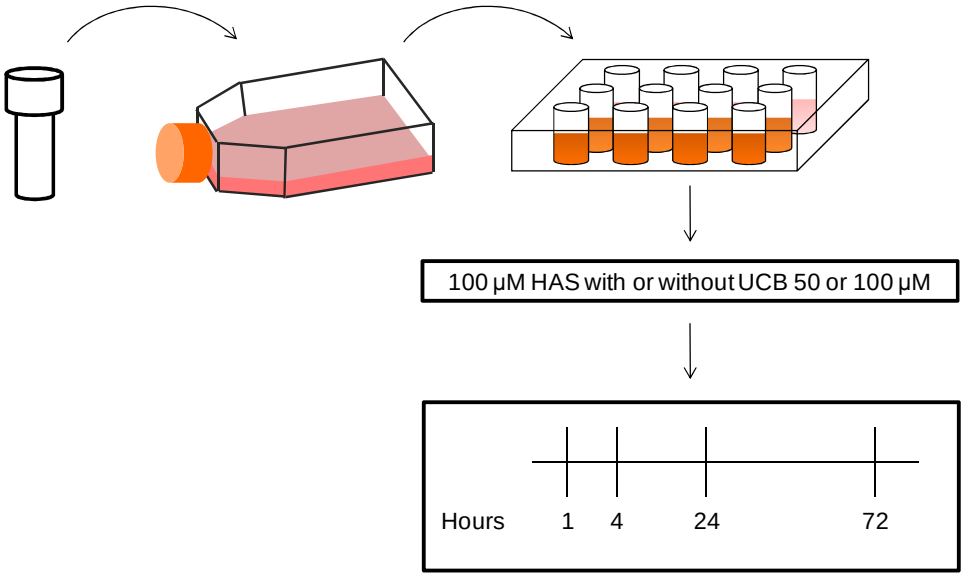

Figure II. 1: Schematic representation of the pericytes primary culture and UCB treatment. After being thaw the cells were cultured in DMEM medium supplemented with 20% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution until the culture achieved approximately 80% confluence. Then they were detached and incubated with 50 or 100 µM UCB, or no addition (control), in the presence of 100 µM HSA (UCB/HSA molar ratios of 0.5 and 1.0, respectively), for 1, 4, 24 or 78 h at 37ºC to the cell death, cytokines and nitrites release analysis.

2.3. Characterization

Characterization of untreated HBVP was performed by a standard indirect immunocytochemical technique. After 1, 4, 24 and 72 h cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature. Next, permeabilization was performed using 0.2% triton X-100 during 3-5 min and 3% BSA in PBS was used as blocking solution for 1 h at room temperature. Cells were incubated overnight at 4ºC with the mouse anti-α-SMA primary antibody (1:100) and with the secondary antibody Alexa Fluor 488 goat anti-mouse (1:500) for 1 h at room

temperature. Both primary and secondary antibodies were diluted in blocking solution. Nuclei were stained with Hoechst 33258 dye in PBS (1:1000) for 2 minutes. DPX was used as mountant medium. In order to have negative controls, some coverslips were incubated only with secondary antibody. This assured that the signal obtained for coverslips incubated with both primary and secondary antibodies was a result of the expression of the target proteins. Fluorescence was visualized using a Leica DFC 490 camera adapted to an AxioScope.A1 microscope (Zeiss). Fifteen red-fluorescence images of random microscopic fields were acquired per sample under 100x magnification. At the end of each time point images were also acquired using a phase contrast microscopy (Olympus, model CK2-TR).

2.4. Assessment of apoptosis

To determine if pericyte death occurs by apoptosis, we evaluated the nuclear morphology after Hoechst staining as previously described (Silva et al., 2001). Fluorescence was visualized using a Leica DFC 490 camera (Leica, Wetzlar, Germany) adapted to an AxioScope.A1 microscope (Zeiss, Göttingen, Germany). Apoptotic nuclei were identified by condensed chromatin, as well as nuclear fragmentation, and were counted in twenty random microscopic fields per sample under 400x magnification.

2.5. Quantification of Cytokine Release

Cytokine release by HBVP was quantified following UCB treatment for 1, 4, 24 or 72 h in 12-well plates. The cytokines IL-6 and VEGF were assessed in duplicate, using the supernatants free from cellular debris by a specific DuoSet ELISA development kits according to the manufacturer's instructions. Measurements were obtained at a wavelength of 450 nm, with a reference filter of 620 nm, using a microplate reader. The results were expressed as relative secretion vs. control samples.

2.6. Measurement of Cytokine mRNA Expression

IL-6 and VEGF mRNA expression was measured by quantitive RT-PCR using mRNA cells from control (HSA) and UCB treatment. The time points were chosen according to the results of the release of these cytokines. For IL-6 were used the time periods 30, 45 and 60 min, and for VEGF 1, 4 and 8 h. Total RNA was extracted using the TRizol reagent according to the manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer. One microgram of total RNA was reverse transcribed into cDNA using a RevertAid H Minus First Strand cDNA synthesis kit under the recommended conditions. It was performed quantitive RT-PCR using β-actin as an endogenous control to normalize the expression levels of cytokines mRNAs. The following sequences were used as primers: IL-6 sense, 5'-GACAGCCACTCACCTCTTCA-3' and anti-sense, 5'-TTCACCAGGCAAGTCTCCTC-3' (Wang et al., 2006); VEGF sense, 5'CCCTGATGAGATCGAGTACA-3' and anti-sense, 5'-CCTCGCCTTGCAACGCGAGT-3' (Park et al., 2009); β-actin sense, 5'-ACAGAGCCTCGCCTTTGCCG-3' and anti-sense, 5'-TGGGCCTCGTCGCCCACATA-3' (NM_001101.3).

RT-PCR detection (Applied Biosystems 7300 Fast Real-time PCR System, Applied Biosystem, Madrid, Espain) was performed using a SYBR Green quantitive PCR Master Mix (2x). The PCR was

performed in 8-strip individual tubes with each sample performed in triplicate, and a no-template control was included for each amplificate. The melting temperature of the PCR program was adjusted to 62ºC. Standard curves using a "calibrator" cDNA (chosen among the cDNA samples) were prepared for each target and reference gene. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. The relative quantification was made using the Pfaffl modification of the $\Delta\Delta C_T$ equation (C_T, cycle number at which the fluorescence passes the threshold level of detection), taking into account the efficiencies of individual genes. The results were normalized to β-actin and the initial amount of the template of each sample was determined as relative expression *vs.* control samples (reference). The relative expression of each sample was calculated by the formula $2^{-\Delta\Delta CT}$. ΔC_T is a value obtained, for each sample, by the difference between the mean C_T value of each cytokine and the mean C_T value of β-actin. $\Delta\Delta C_T$ of one sample is the difference between its ΔC_T value and ΔC_T value of the sample chosen as reference.

2.7. Quantification of Nitrite Levels

In order to evaluate if UCB effects on HBVP include the activation of signalling pathways leading to NO production and ultimately to nitrosative stress, the levels of nitrite, a stable end product of NO, were measured in the incubation medium after cell treatment. Supernatants free from cellular debris were mixed with Griess reagent [1% (w/v) sulfanilamide in 5% H_3PO_4 and 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride (v/v) in a 1:1 ratio] in 96-well tissue culture plates for 10 min at room temperature in the dark. The absorbance at 540 nm, with a reference filter of 620 nm, was determined using a microplate reader. Solutions of sodium nitrite (0-200 µM) were used as standards.

2.8. Evaluation of eNOS Expression

The production of nitrites was further confirmed by evaluation of the expression of one of the enzymes responsible for its formation in pericytes, the eNOS (Loesch et al., 2010). Detection of this enzyme was performed by a standard indirect immunocytochemical technique using a mouse anti-eNOS Type III antibody (1:100) as the primary antibody and a horse FITC-labelled anti-mouse antibody (1:227) as the secondary antibody. Fluorescence was visualized using a Leica DFC 490 camera adapted to an AxioScope.A1 microscope. Five to ten green-fluorescence images of random microscopic fields were acquired per sample using a 63X objective.

3. *Ex vivo* studies – Basement membrane

3.1. Subjects

The patient, a girl, was born preterm at 32(5/7) weeks with a weight of 1600 g, and Apgar score of 6 at 1 min and 7 at 5 min, requiring artificial ventilation. A cranial ultrasound scan did not reveal cerebral pathology. Evidence of focal pulmonary infiltration was noted on the X-ray. Total bilirubin levels on the first, second and fourth postnatal days were 13.1 mg/dL, 28.8 mg/dL and 21.4 mg/dL respectively, and the serum albumin was 3.3 g/dL on day 2 and 3.2 g/dL on day 4. Intensive phototherapy (blue light) was initiated on the second day and antibiotic therapy (penicillin, gentamicin

and flucloxacillin) on the first day of life because of sepsis suspection. The clinical course was dominated by a rapid deterioration of the clinical condition with high pitch cry, poor suckling, hypotonia, hypotension, bradycardia, metabolic acidosis, development of generalized seizures, which did not respond to the pharmacological treatment initiated on the third postnatal day (phenobarbital, midazolam, clonazepam and lidocaine), and progressive cardiorespiratory problems. The child died on the fourth day of life.

Autopsy examination revealed signs of meconium aspiration. No other evidence of infection or hemolysis was found in any of the other organ systems. No developmental abnormalities were detected. Brain tissue was formalin-fixed and, following macroscopic assessment, was paraffin embedded, sectioned at 6 μm and mounted on organosilanecoated for microscopic examination. Autopsy material from a male preterm born at 30 weeks was used as a matched control specimen. With a weight of 1450 g, this child died from cardiorespiratory failure on the 14 th day of life, with sepsis and without history of hyperbilirubinemia. Sections from cortex, basal nuclei, hippocampus, and cerebellum of both the icterus and the unicteric control case were processed for conventional stains, for immunocytochemical reactions. The brain was of normal size for the patient's age, weighing after fixation 260 g.

Brain material was obtained from the files of the department of neuropathology of the Academic Medical Center (University of Amsterdam). Tissue was obtained and used in a manner compliant with the Declaration of Helsinki, after appropriate maternal consent for brain autopsy.

3.2. Immuhistochemistry

Immunostaining was performed in buffered formaldehyde-fixed embedded tissue sections. After the deparaffinization with xilol and rehydration procedures, the sections were rinsed in distilled water for 5 min and then a 3% H_2O_2 solution was used for the inhibition of endogenous peroxidase during 15-20 min. Antigen recovery was achieved by treatment with citrate buffer for 10 min at 650 W plus 10 min at 850 W in microwaves. After cooling and washing with PBS, sections were directly incubated with primary antibody mouse anti-collagen IV (1:100) for 4^0C overnight, washed again in PBS and incubated with EnVision detection system for 1.30/2 hours at room temperature. Finally, the sections were developed in 3, 3'-diaminobenzidine tetrahydrochloride and H_2O_2 solution and were counterstained with Mayer's hematoxylin. Images were acquired using a Leica DFC 490 camera adapted to an AxioScope.A1 microscope (Zeiss). Fifty random pictures of each brain area of two experiments were taken under 630x magnification. Next, using ImageJ software (National Institutes of Health, USA) we measured the area, the immunoreactivity and the perimeter of the exterior and interior (lumen) limits of the collagen staining of fifty vessels (Fig. II.2). Then, we calculated the area occupied by collagen IV using exterior area minus the lumen area and normalized to the lumen perimeter since we looked for vessels with diverse sizes. For immunoreactivity of collagen type IV we used the exterior minus lumen intensity. Next we analysed the intensity of the imunostaining using the absolute values and a three-point rating scale considering 1 for mild immunoreactivity, 2 for moderate immunoreactivity and 3 for strong immunoreactivity (Büttner et al., 2005) corresponding to 0 to 50, 50 to 100 and highest than 100 of the intensity obtained in ImageJ, respectively.

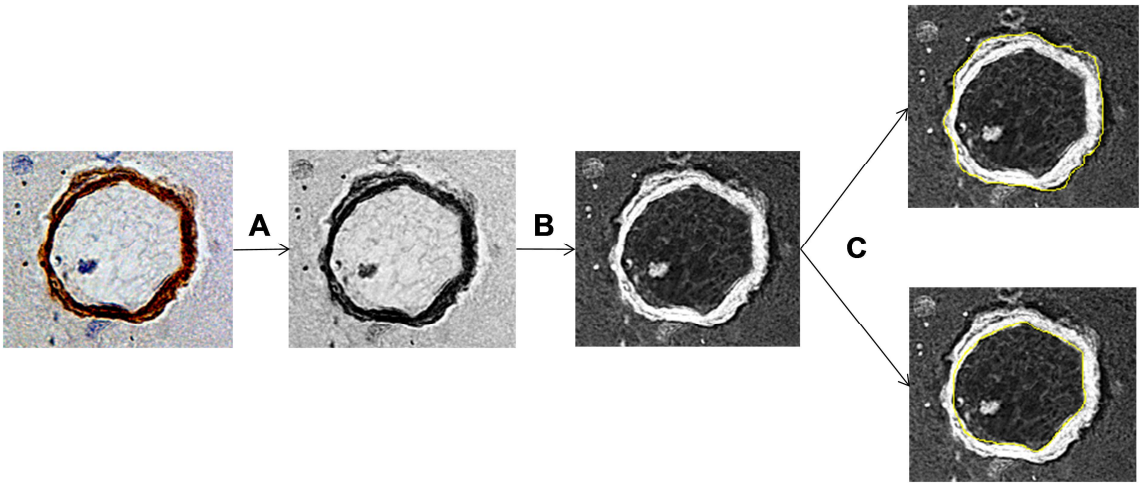

Figure II. 2: ImageJ analysis of vessels stained for collagen type IV. After turn the pictures into 8-bits (A) and inverted (B) the exterior and interior collagen tyoe IV limits were delimited (C) for perimeter, area and staining intensity measure.

4. Statistical analysis

Results from *in vitro* and *ex vivo* studies are expressed as means ± SEM and means ± SD values from, at least, two separate experiments performed in duplicate, respectively. Statistical analysis was performed by the Student's *t*-test and a P value lower than 0.05 was considered statistically significant.

III. RESULTS

III. RESULTS

1. *In vitro* studies – Pericytes

1.1. Characterization

Since it was the first time we worked with pericytes, we initially characterized these cells in culture. Using a primary antibody for α-SMA, one of the pericytes markers, we examined their morphology (Fig. III.1A). As shown in figure III.1B, pericytes were extending their processes over the time in culture, as revealed by immunocytochemistry and by phase contrast morphology. We next counted the number of extended cells and calculated the percentage of these cells in the total cell count (Fig. III.1C), which confirmed that pericytes tended to turn more extended with time.

1.2. Assessment of apoptosis

Previous studies showed that UCB induce cell demise in neural cells (Fernandes et al., 2004; Falcão et al., 2006). So, we next assessed the extent of cell death by exposure of pericytes to a moderate or severe hyperbilirubinemia, corresponding to UCB/HSA 0.5 or 1.0, respectively. We analysed morphologic features of apoptosis as indicator of cell death, as seen in figure III.2A that represents apoptosis induced by 72 h incubation with UCB. Looking at figure III.2B we also see that the number of apoptotic cells increased over time, reaching maximum levels at 72 h incubation (1.2-fold and 1.3-fold, $P<0.05$ for UCB/HSA 0.5 and 1.0, respectively).

1.3. Quantification of cytokine release

The effects of UCB in the production of IL-6, IL-8, monocyte chemoattractant protein-1 and VEGF by human BMVECs (Palmela et al. 2011), as well as in the secretion of pro-inflammatory cytokines by glial cells and neurons were already established (Fernandes et al., 2004; Falcão et al., 2005; Falcão et al., 2006; Silva et al., 2010). So we decided to look at the release of IL-6 and VEGF by HBVP. This last is one of the cytokines produced by pericytes (Yamagishi et al., 1999) that has an important role in BBB integrity, including increase of permeability (Fischer et al., 1999, Doghu et al., 2005).

We can see in figure III. 3A exposure of pericytes to UCB led to an early secretion of IL-6, already visible at 1 h incubation for both UCB concentrations (1.5-fold and 1.7-fold, $P<0.05$ for UCB/HSA 0.5 and 1.0, respectively; UCB/HSA 1.0 ~15 pg/ml vs. control ~12 pg/ml) followed by a less marked elevation thereafter and approaching control values at 72 h **despite the levels released into medium increased in a time-dependent manner (~39 pg/ml at 4 h up to ~506 pg/ml at 72 h for UCB/HSA 1.0).**

The VEGF secretion only began after 4 h incubation and reached its peak at 24 h for both UCB concentrations (1.2-fold, $P<0.01$ and 1.3-fold, $P<0.01$ for UCB/HSA 0.5 and 1.0, respectively; UCB/HSA 1.0 ~21 pg/ml vs. control ~16 pg/ml). At 72 h incubation the production arrived to levels below control **(UCB/HSA 0.5 ~94 pg/ml vs. control ~106 pg/ml)** corresponding to production inhibition (Fig. III.3).

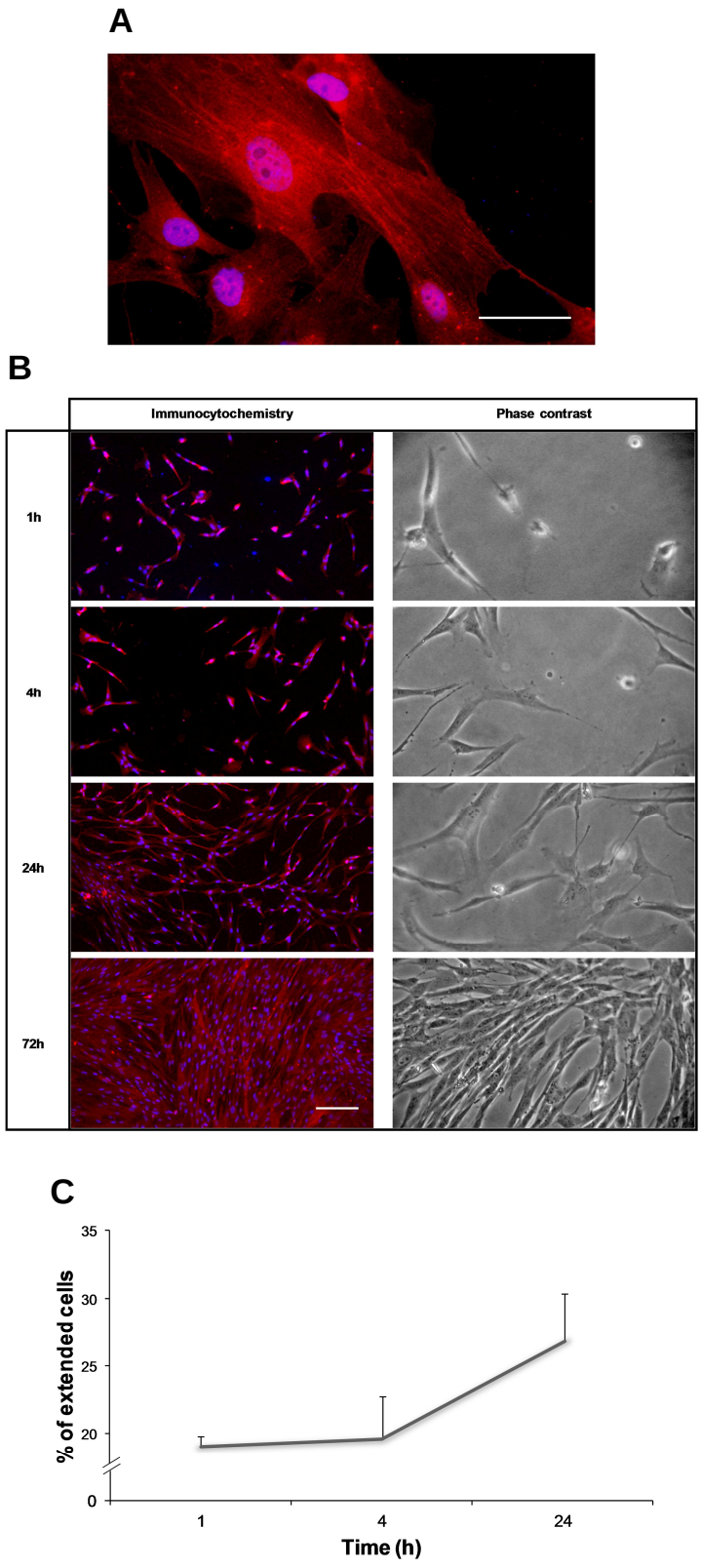

Figure III. 1: Characterization of human brain vascular pericytes in primary culture. After culturing pericytes in their growth medium for 1, 4, 24 and 72 h immunocytochemistry was performed using α-smooth

muscle actin antibody (A), cell morphology was analyzed by immunocytochemistry and by contrast phase microscopy (B), and the number of extended pericytes in the immunocytochemistry images was counted and their percentage relatively to the total cell count was calculated (C). Results are expressed as means ± SEM from two independent experiments performed in duplicate. Scale bar: 40 µm (A) and 200 µm for immunocytochemistry images and 100x magnifications for contrast phase images.

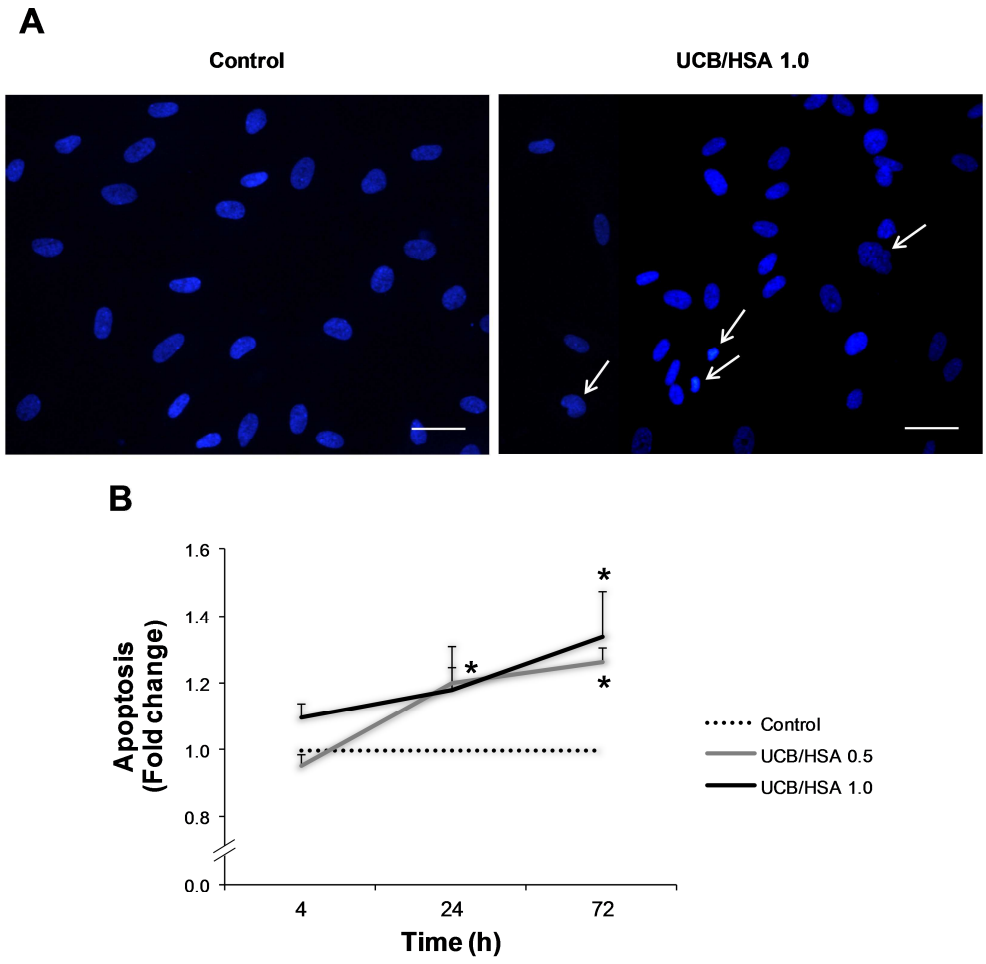

Figure III. 2: Apoptosis of human brain vascular pericytes. Pericytes in primary culture were incubated without (control) or with UCB at 50 or 100 µM, in the presence of 100 µM HSA (UCB/HSA 0.5 and 1.0, respectively) for the indicated time periods and apoptosis was evaluated based on the nuclear morphology after Hoechst 33258 staining. Representative photos of one experiment are shown (A), corresponding to 72 h incubation (arrows point to apoptotic features of apoptosis). The number of apoptotic nuclei was counted and results are expressed as folds ± SEM from three independent experiments performed in duplicate (B). *P<0.05 vs. respective control. Scale bar: 40 µm.

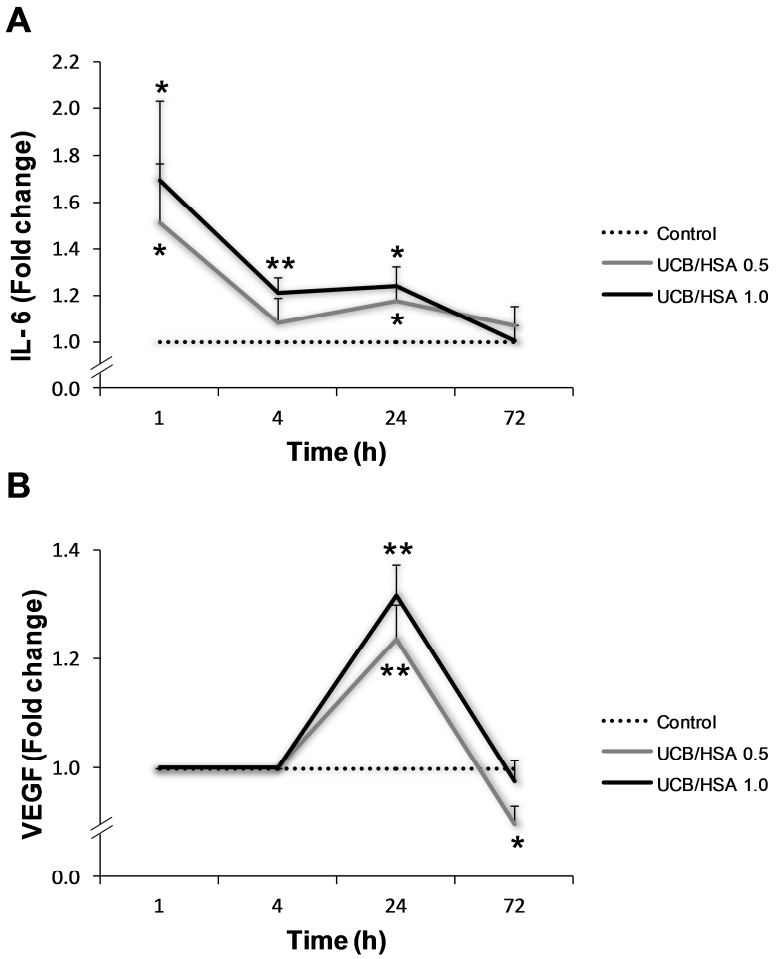

Figure III. 3: Secretion of VEGF and IL-6 by human brain vascular pericytes exposed to UCB. Pericytes in primary culture were incubated without (control) or with UCB at 50 or 100 µM, in the presence of 100 µM HSA (UCB/HSA 0.5 and 1.0, respectively) for the indicated time periods and secretion of cytokines was determined using specific DuoSet ELISA development kits. VEGF (A) and IL-6 (B). Results are expressed as folds ± SEM from at least three independent experiments performed in duplicate. *P<0.05 and **P<0.01 vs. respective control.

1.4. Measurement of Cytokine mRNA Expression

We next examined if UCB-induced secretion of cytokines was preceded by increased cytokine mRNA expression. These experiments were performed at earlier time points according to the secretion profile. For Il-6 we choose 30, 45 min and 1 h and for VEGF 1, 4 and 8 h.

We can see in figure III.4 that UCB/HSA 1.0 led to a IL-6 mRNA expression peak at 45 min incubation (1,2-fold, P<0,05), whereas UCB/HSA 0.5 caused a slower and progressive expression until 1 h incubation. In contrast, both UCB concentrations caused the same VEGF mRNA expression profile, with a peak at 4 h incubation, with a higher fold change for UCB/HSA 1.0 (1.7-fold and 2.0-fold, P<0.05 for UCB/HSA 0.5 and 1.0, respectively).

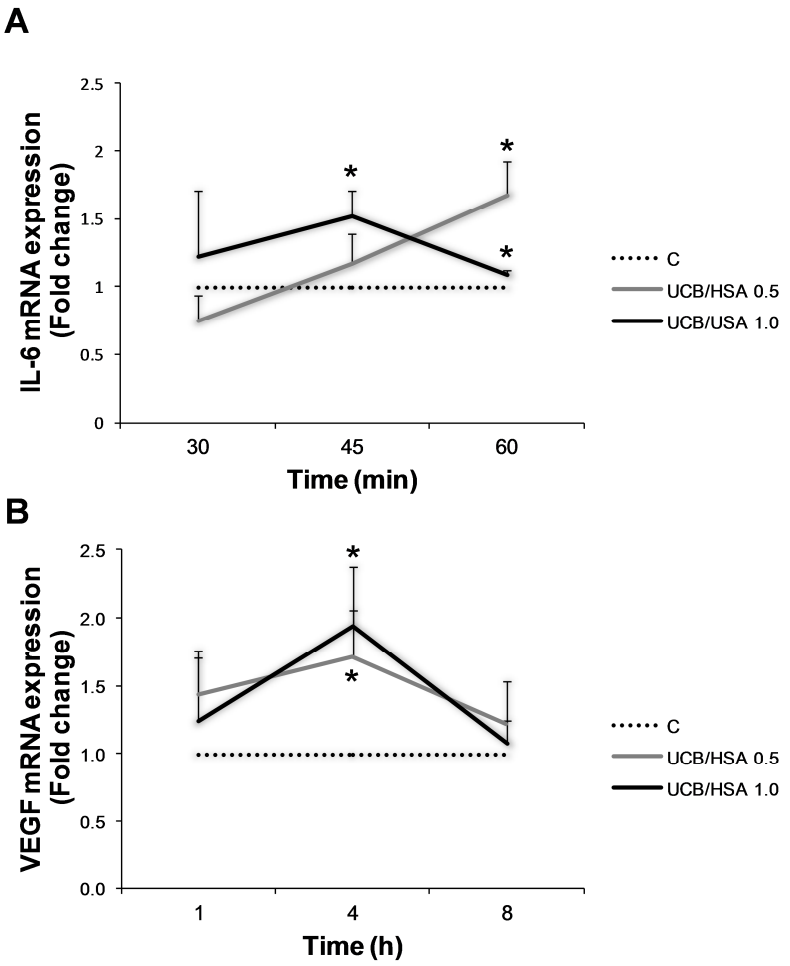

Figure III. 4: mRNA expression of VEGF and IL-6 by human brain vascular pericytes exposed to UCB. Pericytes from primary culture were incubated without (control) or with UCB at 50 or 100 μM, in the presence of 100 μM HSA (UCB/HSA 0.5 and 1.0, respectively) for the indicated time periods. Cytokine mRNA expression of IL-6 (A) and VEGF (B) was analysed by qRT.PCR with the $\Delta\Delta C_T$ method. Results are expressed as folds ± SEM from at least three independent experiments performed in duplicate. *P<0.05 vs. respective control.

1.5. Quantification of Nitrite Levels and Evaluation of eNOS Expression

Previous studies showed that UCB induces the production of NO by neurons and human BMVECs, as a result of the overexpression of the neuronal and endothelial NO synthase isoforms, respectively (Brito et al., 2008; Vaz et al., 2010; Brito et al., 2010; Palmela et al., 2011). Since pericytes express the endothelial NO synthase, we examined the expression of this isoform following UCB-treatment and determined nitrite levels in the medium to assess whether UCB induces nitrosative stress in cultured HBV.

Looking at figure III.5 we can see that nitrites were secreted into the medium in a time and concentration-dependent manner, reaching maximum levels at 72 h incubation (1.2 μM, P<0.05 and

2.0 μM, P<0.01 for UCB/HSA 0.5 and 1.0, respectively), which corresponds to a 2.1-fold and 3.4-fold for UCB/HSA 0.5 and 1.0, respectively.

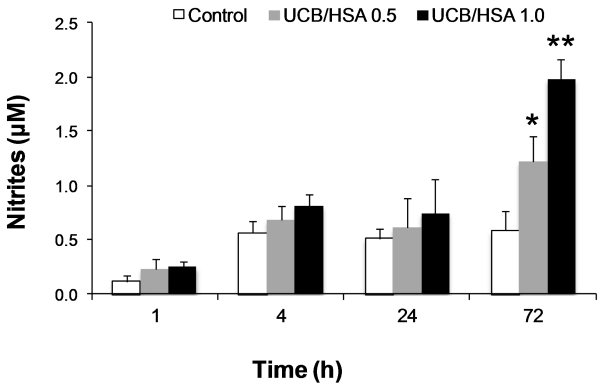

Figure III. 5: Nitrite production by human brain vascular pericytes exposed to UCB. Pericytes in primary culture were incubated without (control) or with UCB at 50 or 100 μM, in the presence of 100 μM HSA (UCB/HSA 0.5 and 1.0, respectively) for the indicated time periods, and nitrite levels were determined. Results are expressed as folds ± SEM from at least three independent experiments performed in duplicate. *P<0.05 and **P<0.01 vs. respective control.

Examination of eNOS expression (Fig. III.6) revealed an initial increase of the fluorescence intensity, especially at 4 h incubation, related with an upregulation of eNOS expression. At 24 h we also observed the increase of eNOS expression with a diference between the control and UCB less notable. At 72 h incubation there was no effect of UCB in eNOS expression. Thus, the expression peak may be between 4 and 24 h or even at 4 h incubation. The overlapping of pericites, particularly at 24 and 72 h treatment, together with the impossibility of delimitation of individual cells per microscopic field, did not allow the quantification of the fluorescence intensity.

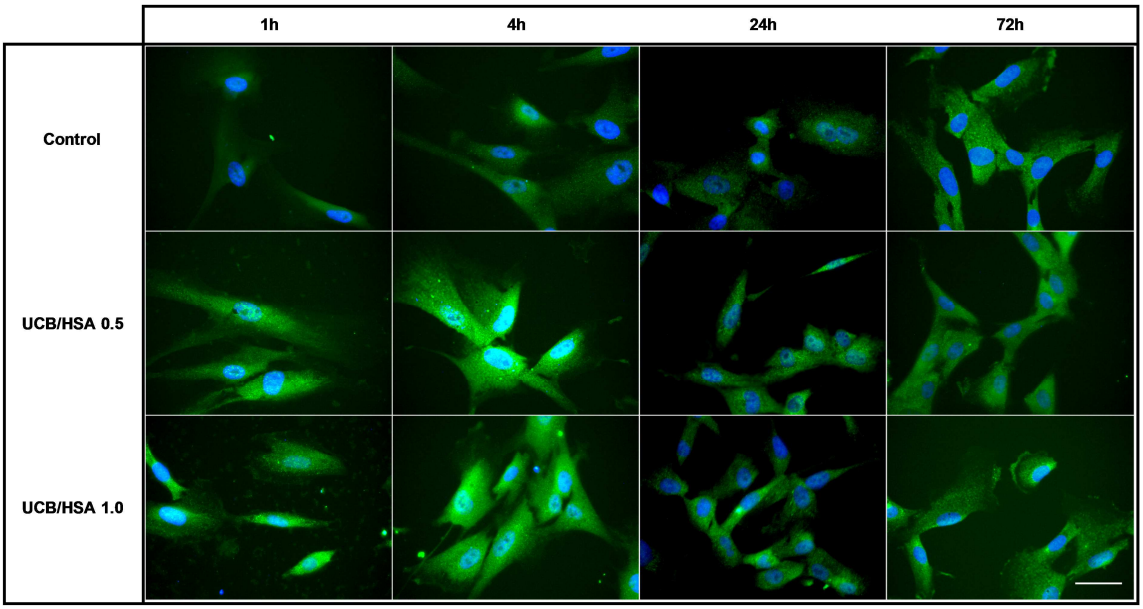

Figure III. 6: Expression of eNOS by human brain vascular pericytes exposed to UCB. Pericytes in primary culture were incubated without (control) or with UCB at 50 or 100 µM, in the presence of 100 µM HSA (UCB/HSA 0.5 and 1.0, respectively) for the indicated periods, and eNOS expression was analysed by immunocytochemistry. Representative results of one experiment are shown. Scale bar: 40µm.

2. *Ex vivo* studies – Basement membrane

At a first look to randomly selected fields, we saw a decrease of immunoreactivity and thickness of the collagen IV of the kernicterus case as compared to the non-icteric control (Fig. III.7). To confirm this hypothesis we analysed the vessels by determining the collagen type IV area and immunoreactivity levels.

The results of figure III.8 proved that the bilirrubin in brain causes the decrease of the area per vessel occupied by the BM represented by its most abundant constituent, the collagen IV. All the four brain regions analysed suffered a reduction in the area immunostained for collagen type IV, being the cortex the most affected area (0.7-fold, $P<0.05$). The hippoccampus was the area with the lowest decrease in the area though still significantly different from the control (0.9-fold, $P<0.05$).

For the collagen type IV immunoreactivity we also have seen the reduction of the intensity in all brain regions in the same damage order. Looking at figure III.9 we see the mean intensity per vessel decreasing in all regions with the greatest difference once again in cortex (0.7-fold, $P<0.05$). Other analysis (Fig. III.10), through percentage of the 1 (0 to 50 mean intensity), 2 (50 to 100 mean intensity) and 3 (higher than 100 of mean intensity), demonstrated the same evidence that in all regions there are a reduction of raking points 2 and 3, corresponding to the highest reactivity, and an increase of the 1 raking point, the lower immunoreactivity.

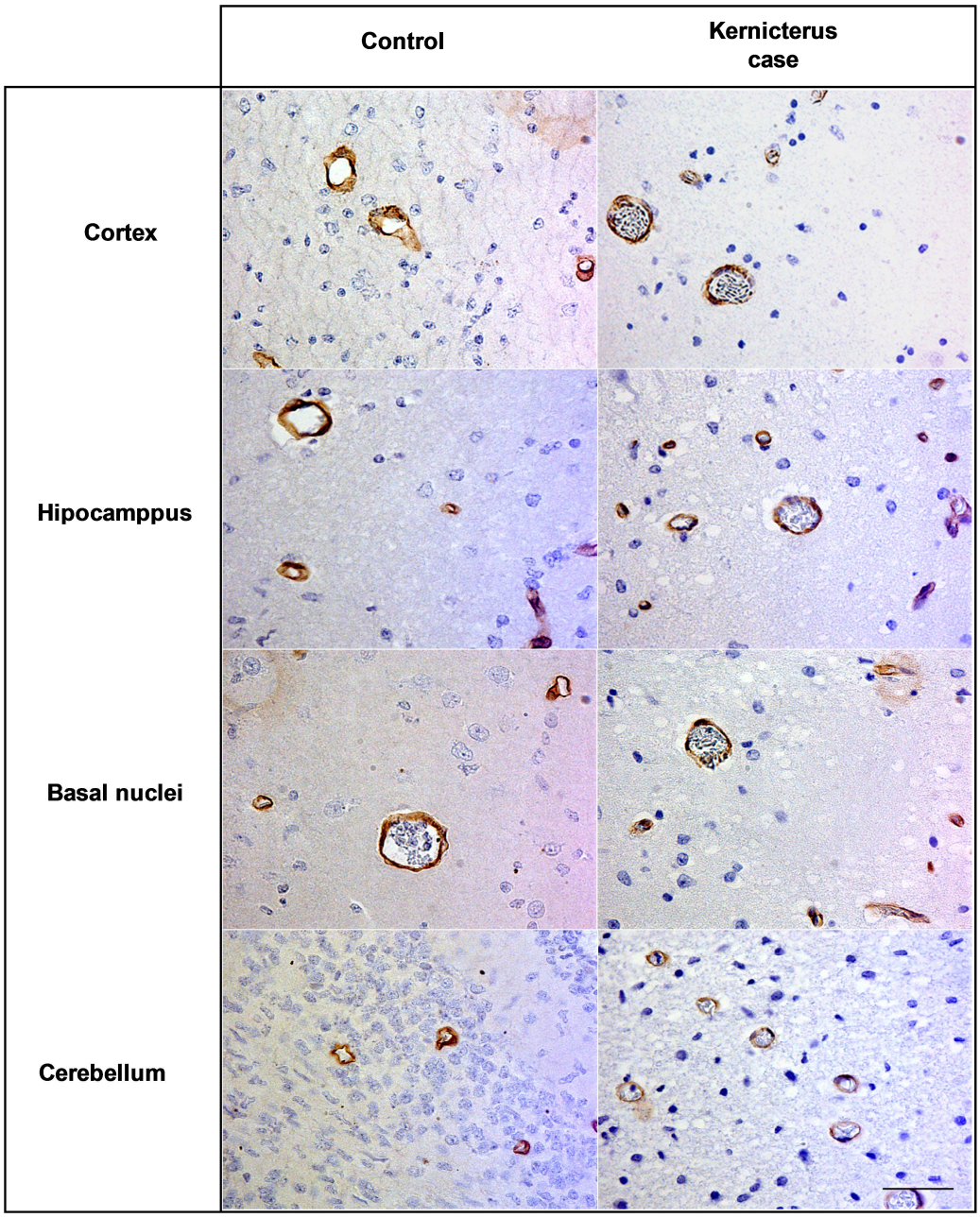

Figure III. 7: Immunohistochemistry for collagen type IV. Immunohistochemistry for collagen type IV was performed in four brain areas (cortex, hippocampus, basal nuclei and cerebellum) of a non-icteric control and the kernicterus case, and reduction in immmunoreactivity and thickness were observed in two different paraffin sections. Representative results of one experiment are shown. Scale bar: 40µm.

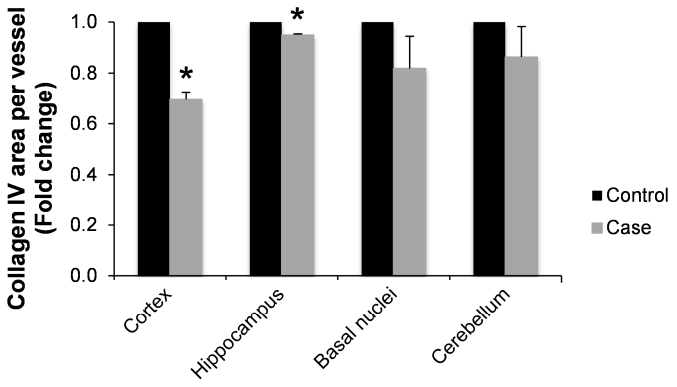

Figure III. 8: Area per vessel occupied by collagen type IV. Immunohistochemistry for collagen type IV was perfomed in four brain areas (cortex, hippocampus, basal nuclei and cerebellum) of a non-icteric control and the kernicterus case. The area of collagen type IV in each brain region was obtained by analysis of fifty vessels using ImageJ software. Results are expressed as folds ± SD from two independent experiments. *P<0.05 vs. respective control.

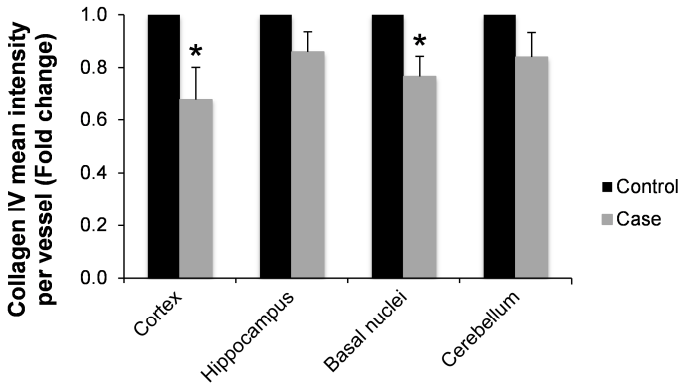

Figure III. 9: Collagen type IV immunostaning intensity. Immunohistochemistry for collagen type IV was performed in four brain reagions (cortex, hippocampus, basal nuclei and cerebellum) of a non-icteric control and the kernicterus case. The mean intensity was obtained from measurement of fifty vessels in each brain region unsing ImageJ software Results are expressed as folds ± SD from two independent experiments using the absolutes values. *P<0.05 vs. respective control.

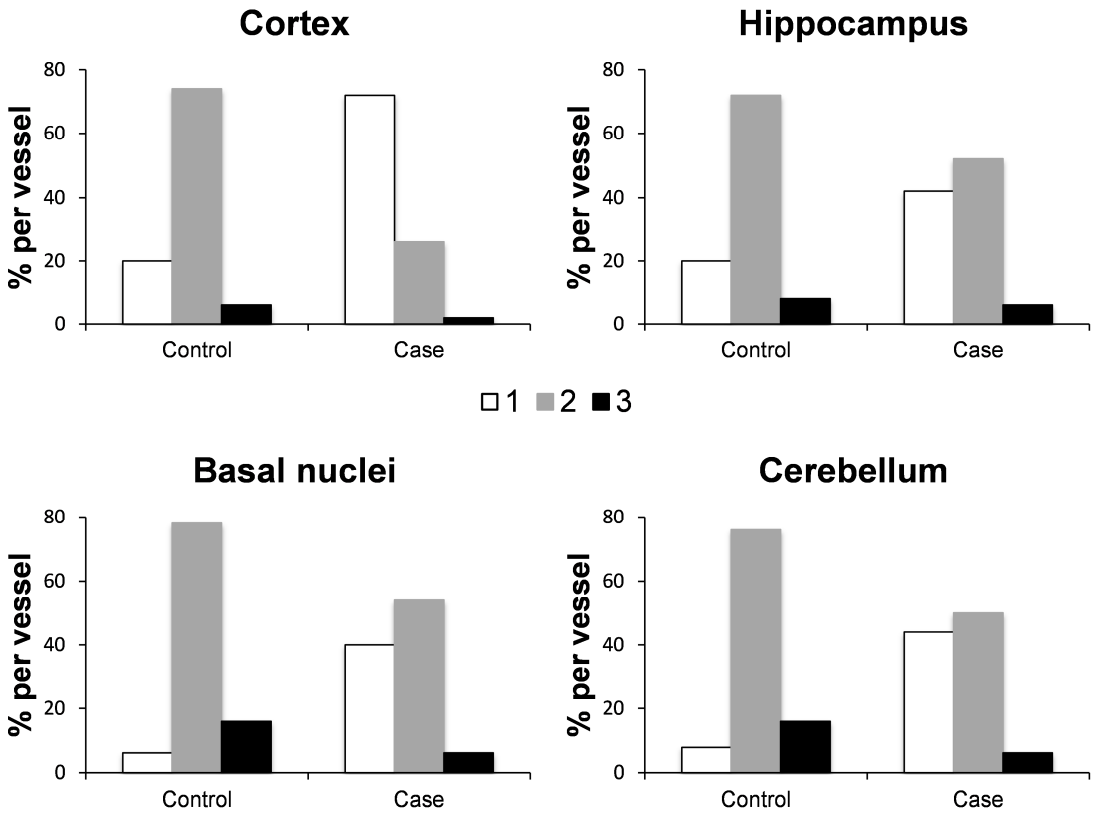

Figure III. 10: Collagen type IV immunostaning intensity raking. Immunohistochemistry for collagen type IV was performed in four brain regions (cortex, hippocampus, basal nuclei and cerebellum) of a non-icteric control and the kernicterus case. The mean intensity was obtained from measurement of fifty vessels in each brain region unsing ImageJ software and the percentual distribution of vessels immunoreactivity was examined in the same vessels based on a three ranking point scale, where 1, 2 and 3 correspond to mild, moderate and strong immunoreactivity (0-50, 50-100 and higher than 100 mean intensity, respectively). Results of one tissue section are represented.

IV. DISCUSSION

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Over the years several studies have shown diverse effects of UCB in brain cells, including neurons (Grojean et al., 2000; Falcão et al., 2007; Fernandes et al., 2009; Brito et al., 2010; Vaz et al., 2010; Vaz et al., 2011) astrocytes (Fernandes et al., 2004; Falcão et al., 2005; Fernandes et al., 2006; Fernandes et al., 2007a; Fernandes et al., 2007b), microglia (Gordo et al., 2006; Silva et al., 2010), oligodendrocytes (Genc et al., 2003) and BBB ECs (Akin et al., 2002; Palmela et al., 2011; Cardoso et al., 2011 submitted). In this study we explore, for the first time, the UCB effects in HBVP and BM. For that we exposed pericytes in primary culture to two pathophysiologically relevant conditions of jaundice, corresponding to moderate (UCB/HSA 0.5) and severe hyperbilirubinemia usually associated with kernicterus (UCB/HSA 1.0). We also analysed sections from cortex, basal nuclei, hippocampus, and cerebellum of a non-icteric control and a kernicterus case, to investigate the influence of UCB in the BM.

Since the pericytes are new in our group we began with their characterization based on α-SMA that can be present in pericytes as a contractile protein (Herman and D'Amore, 1985; Bandopadhyay et al., 2001) and is normally used for this purpose (Nishioku et al. 2009; Al Ahmad et al., 2011). We know that not all brain pericytes *in vivo* express α-SMA (Bandopadhyay et al., 2001) but *in vitro*, 100% of pericytes eventually become αSMA positive (Dore-Duffy, 2003). Like in other cultures (Dore-Duffy, 2003; Berrone et al., 2009; Phil et al., 2010) our pericytes grew along the time to reach their typical morphology with long processes (Dore-Duffy, 2011). We also saw an evident outgrowth of the pericytes in 7 days according with a previously studies (Phil et al., 2010; Dore-Duffy, 2003). Therefore, pericytes in our lab were growing well and ready to be used for the studies with UCB.

We first looked at cell death that may occur when cells come into contact with toxic substances. We observed a time-dependent increase in the number of apoptotic bodies and nuclear fragmentation upon exposure to UCB. These results are in agreement with others obtained by our group where UCB induced cell death by apoptosis in neurons (Fernandes et al., 2004), astrocytes (Falcão et al., 2006), microglia (Silva et al., 2010) and BMVECs (Palmela et al., 2011). Furthermore there are also other studies reporting pericytes death by apoptosis induced by diverse stimulus (Katychev et al., 2003; Geraldes et al., 2009; Walshe et al., 2011).

One of the events that we have previously demonstrated to occur following UCB exposure is the production of cytokines (Fernandes et al., 2004; Gordo et al., 2006; Fernandes et al., 2006; Silva et al., 2010; Palmela et al., 2011). Thus, we examined whether UCB elicits an inflammatory reaction by pericytes, through evaluation of the mRNA expression and secretion of cytokines, such as IL-6 and VEGF. For IL-6, UCB induced a very rapid response as the greatest increase in IL-6 secretion was observed at 1 h, the shorter time-point examined, in contrast with our previously studies showing an early inhibition of IL-6 secretion in astrocytes (Fernandes et al., 2004; Fernandes et al., 2006), microglia (Silva et al., 2010) and BMVECs (Palmela et al., 2011). There is also a study using brain pericytes that release IL-6 after 3 days in presence of stimulus like LPS and IL-1β (Fabry et al., 1993).

With our results we can hypothesize that the secretion of IL-6 may be on of the first responses of perycites to UCB compensating the late secretion of the other cells. As many compounds, IL-6 can be either neuroprotective/anti-inflamatory or neurotoxic/pro-inflammatory, depending of its concentration (Gadient and Otten, 1997). In this case more studies will provide evidences of the real effect of IL-6 that can include a role in maintenance of BBB function (Krizanac-Bengez et al., 2003) or in the disruption of the BBB (de Vries et al., 1996) increasing the transport of immune cells across the BBB, which in turn are capable of inflammatory mediators secretion (Sumi et al., 2010). IL-6, together with other cytokines, may also enhance the UCB cytotoxicity regarding ECs viability (Yeung et al., 2001). Concerning IL-6 mRNA expression following exposure to the highest UCB concentration, it peaks before the cytokine release as would be anticipated. Upon interaction of pericytes with the lowest UCB concentration the upregulation of IL-6 mRNA was slower, indicating a delayed response.

Different of IL-6 is the VEGF secretion profile that begins after 4 h incubation and peaks at 24 h, attaining the greatest variation by exposure to UCB/HSA 1.0. Our group also showed a similar profile of VEGF released by BMVECs (Palmela et al., 2011), which attained 55 pg/ml following incubation with UCB/HAS 1.0 for 24 h. Taken together with the 21 pg/ml released by pericytes incucbated in the same experimental conditions, the release of VEGF surpasses 40 pg/ml, a concentration of the cytokine referred to be active in vitro (van der Flier et al., 2001). Like UCB, hypoxia and hipoglycemia (Hellström et al., 2001) can also promote VEGF release by pericytes. Consequently, VEGF will increase BBB permeability (Fischer et al., 1999; Doghu et al., 2005) that can be through BBB disruption (Shimizu et al., 2011), decrease of TEER (Thanabalasundaram et al., 2010) and decrease of occludin levels (Wang et al., 2001; Murakami et al., 2009; Thanabalasundaram et al., 2010). Due to its elevated concentration, VEGF can contribute to brain edema (van der Flier et al., 2001), as observed in kernicterus (Wennberg, 2000) and consistent with the edematous changes observed in the presently evaluated kernicterus case. The VEGF secretion peak occurs later than IL-6 according to the induction of VEGF mRNA expression by IL-6 (Cohen et al., 1996) and in line with our previous observations in human BMVECs (Palmela et al., 2011). The VEGF mRNA expression also occurs after the IL-6 mRNA expression.

Like neurons (Brito et al, 2010; Vaz et al., 2011) and BMVECs (Palmela et al., 2011), pericytes also release the free radical NO. NO is known to increase the BBB permeability (Shukla et al., 1996; Fischer et al., 1999; Mayhan et al., 2000; Yamauchi et al., 2007), which can occur through changes and redistribution in the expression of TJs and AJs proteins (Yamagata et al., 2004) or phosphorylation of occludin, claudin-5 and ZO-1, therefore leading to TJs and cytoskeletal rearrangements (Haorah et al., 2005). It was also demonstrated that NO can mediate MMPs activation (Haorah et al., 2007), which in turn digest molecules of the BM (Carvey et al., 2009). After BBB opening, NO-induced permeability can lead to vasogenic edema and secondary brain damage (Thiel and Audus, 2001; Krizanac-Bengez et al., 2003, Krizbai et al., 2003; Mohammadi et al., 2011). However, there are other studies that showed NO with a protective role, decreasing the BBB permeability (Utepbergenov et al., 1998; Wong et al., 2004). Thus, to further confirm the real role of NO produced by pericytes in response to UCB more studies have to be realized, namely by using ECs together with pericytes. We can also consider that NO effects may be protective or toxic according to

its concentration, since in our study NO production by pericytes occurs in a time- and concentration-dependent manner. By immunocytochemistry we can see an early upregulation of eNOS followed by the production of nitrites that progressively increases along the 72 h incubation period. Thus, eNOS may be responsible for NO production. However, pericytes can also express iNOS that can further contribute to NO production (Chakravarthy et al., 1995).

Together, NO, IL-6 and VEGF will concur to enhance hyperbilirrubinemia severity since they promote BBB disruption and, consequently, UCB entrance.

To study the influence of UCB in BM we used sections of a non-icteric control and a kernicterus case that were already used in a previous study (Brito et al., 2011). The brain regions analysed are those characterized by the yellow discoloration characteristic of kernicterus: hippocampus, basal nuclei and cerebellum. We also used cortex, as the region with no visible yellow staining, exhibiting normal features of cortical organization. After immunohistochemical analysis of collagen type IV, visible changes in the staining and the loss of immunoreactivity were the most notable differences. We demonstrated a reduction in the area and immunoreactivity of collagen type IV in all regions, being the cortex the most affected, in contrary to our expectations. The least affected was the hippocampus. With these results we may hypothesize that cortex can be affected in a different and still unclear way. The large cortex area may be a reason for the non visible changes like the yellow staining. On the other hand, the edematous changes observed in the histologic evaluation of the presently evaluated kernicterus case may support these basement membrane alterations, since the entrance of blood plasma is only possible when the BBB is disrupted.

The global loss of the BM around brain vessels is in agreement with other brain diseases, like, ischemia (Baumann et al., 2009; Kwon et al., 2009), Alzheimer's disease (Donahue et al., 1999), subarachnoid hemorrhage (Schöller et al., 2007) as well as after injurious conditions as drug abuse (Büttner et al., 2005). Like discussed above, pericytes and other cells of neurovascular unit produce IL-6, VEGF and NO that may contribute to BM disruption. Moreover, BM can also be digested by MMPs that may come from ECs (Cardoso et al., 2011 submitted) and microglia (Silva et al., 2010) after UCB induction. The pericytes themselves can increase the MMP-9 produced by ECs by interacting with them and MMPs can also be activated by VEGF (Winkler et al., 2004) and NO (Haorah et al., 2007). However, MMPs also participate in the VEGF activation process since there is a decrease in VEGF levels after inhibition of MMP (Thanabalasundaram et al., 2010). Thus, it is conceivable that all these events create a cycle that will culminate in BM disruption and consequently in the lost of BBB integrity.

This study leads us to conclude that UCB affects pericytes through nitrosative stress and cytokine release, as well as BM through loss of collagen type IV. All the findings turn pericytes and BM essential members for the BBB integrity and function and provide a basis for a target-directed therapy against UCB-induced injury to pericytes and BM. Some targets may be the enzymes responsible for NO production, the receptors of the cytokine studied and the cells capable to produce collagen type IV.

In the future, more studies should be made to better understand the presently explored and other UCB effects. Co-cultures with pericytes and ECs may lead us to discover possible protective effects of pericytes to BBB integrity. Related to the *ex vivo* study, double staining for pericytes and BM or ECs will provide indications about the eventual alterations on pericyte vascular coverage during hyperbilirrubinemia. Immunohistochemical analysis using antibodies for others BM elements and for adhesion molecules can be done to investigate more possible modifications induced by UCB.

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