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Establishment and Characterization of a Human Model of the Blood-Brain Barrier

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DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Establishment and Characterization of a Human Model of the Blood-Brain Barrier

Dissertação apresentada para a obtenção do Grau de Mestre em Genética Molecular e Biomedicina, pela Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia

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Abbreviations

α-SMA	α-smooth muscle actin
AJ	adherens junction(s)
AB/AM	antibiotic-antimycotic
ABC transporters	ATP-binding cassette transporters
BBB	blood-brain barrier
BMVEC	brain microvascular endothelial cells
CNS	central nervous system
DIV	days in vitro
DMEM-F12	Dulbecco's modified Eagle medium/nutrient mixture F-12 Ham
DMSO	dimethyl sulphoxide
EC	endothelial cells
ECGS	endothelial cell growth supplement
FBS	fetal bovine serum
GLUT-1	glucose transporter-1
HBMVEC	human brain microvascular endothelial cells
HC	hydrocortisone
ISM	isolation supplemented medium
JAM	junctional adhesion molecule(s)
MRP	multidrug resistance-associated protein
PBS	phosphate buffered saline
PTFE	polytetrafluoroethylene
TEER	transendothelial electrical resistance
TJ	tight junction(s)
VE-cadherin	vascular endothelial-cadherin
vWF	von Willebrand Factor
ZO	*zonula occludens*

Abstract

The discovery of the blood-brain barrier has allowed several studies focused on its properties throughout the last decades. The available study methods are both expensive and time-consuming. We describe a new simplified method for isolation of primary cultures of human brain microvascular endothelial cells (HBMVEC). Human samples of temporal lobe, obtained as discarded tissue during operative treatment for epilepsy, were mechanically fragmented and filtered through adequate pore-size polyester meshes. Resulting microvessel fragments were placed on type-I collagen-coated flasks to allow HBMVEC to migrate from vessels and proliferate. Usually, it takes approximately 1 month until confluence. The resulting cultures are highly pure and comprise important barrier properties such as: specific endothelial cell markers (von Willebrand factor and glucose transporter-1), expression of tight and adherens junctions (*zonula occludens*-1 and β-catenin, respectively), vesicular transport machinery (caveolae, through the expression of caveolin-1), ABC transporters (P-glycoprotein), as well as high transendothelial electric resistance, characteristics that are maintained for two passages. Furthermore, we have successfully cryopreserved primary HBMVEC maintaining the expression of the previously mentioned proteins important to barrier properties.

Sumário

A descoberta da barreira hemato-encefálica permitiu, ao longo das últimas décadas, diversos estudos sobre as suas propriedades. Os métodos de estudo disponíveis são dispensiosos e morosos. Descrevemos um método novo e simples de isolamento e cultura de células endoteliais de capilares do cérebro humano (HBMVEC). Amostras humanas obtidas do lobo temporal, retirados do tecido descartado durante tratamentos cirúrgicos para epilepsia, foram mecanicamente fragmentadas e filtradas através de membranas de polyester com poro de tamanho adequado. Os capilares resultantes foram colocados em frascos revestidos com colagénio do tipo I para permitir tanto a migração de HBMVEC a partir dos capilares como a sua proliferação. Por norma, as células ficam confluentes em aproximadamente 1 mês. As culturas resultantes apresentam um grau de pureza elevado e apresentam importantes propriedades de barreira tais como: marcadores específicos para células endoteliais (factor de von Willebrand e transportador da glucose-1), expressão de proteínas de junções justapostas e aderentes (*zonula occludens*-1 e β-catenina, respectivamente), proteínas designadas de caveolinas que são marcadores das cavéolas, invaginações responsáveis por sinalização intracelular e internalização de substâncias (caveolina-1), transportadores ABC (glicoproteina-P), assim como elevada resistência eléctrica endotelial, características que se mantêm durante duas passagens. Adicionalmente conseguimos com sucesso criopreservar HBMVEC de culturas primárias, mantendo a expressão das acima referidas proteínas, importantes para as propriedades de barreira.

Contents

Chapter 1 – Introduction

In 1885, Paul Ehrlich was the first to experimentally demonstrate the existence of the blood-brain barrier (BBB). He observed that vital dyes injected into the circulatory system stain all organs of the mammalian body except the brain and spinal cord (Ehrlich, 1885; Ehrlich, 1906). About 30 years later, an Ehrlich's student,Edwin Goldmann, noticed the opposite phenomenon by injecting trypan blue directly into the cerebrospinal fluid which stained all central nervous system (CNS) and none of the peripheral organs (Goldmann, 1913). Further illustration showed that endothelial tight junctional complexes physically limit solute exchanges between the blood and the brain. This was achieved by injecting horseradish peroxidase intravascularly, showing diffusion between endothelial cells (EC) lining skeletal and cardiac vessels, though it did not pass between EC in the cerebral microvasculature (Reese and Karnovsky, 1967) (Fig. 1.1).

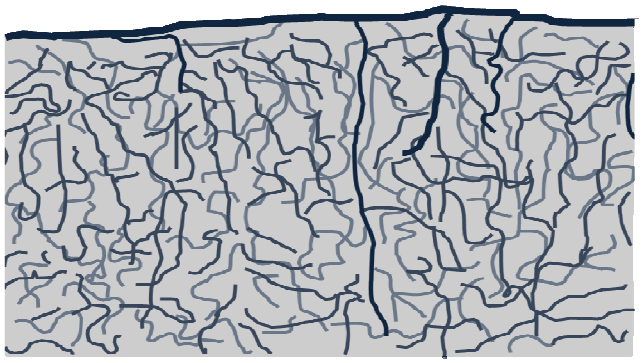


Fig. 1.1 – Injected horseradish peroxidase diffuse through brain vasculature but does not pass through endothelial cells to brain parenchyma. Adapted from Reese and Karnovsky (1967).

The BBB has numerous functions: (1) maintain the CNS homeostasis; (2) protect the brain from extracellular environment; (3) ensure constant supply of nutrients by specific transport systems ; (4) guide the inflammatory cells to respond to changes of local environment (Petty and Lo, 2002; Lee *et al.*, 2006; Persidsky *et al.*, 2006).

Maintenance of homeostasis is achieved by regulating ion balance (Wolburg and Lippoldt, 2002; Hawkins and Davis, 2005; Persidsky *et al.*, 2006) and the influx/efflux of substances (Chaudhuri, 2000; Khan, 2005). This is essential in protection against harmful substances, variations in blood composition and breakdown of concentration gradients (Kniesel and Wolburg, 2000; Petty and Lo, 2002; Wolburg and Lippoldt, 2002).

The BBB is present in all brain regions, except in those regulating autonomic nervous system and endocrine glands of the body, where blood vessels permit diffusion of blood-borne molecules across the vessel wall (Ballabh *et al.*, 2004).

BBB's specific structural and biochemical properties arise from existent layers between the blood and the brain and from interactions between a large variety of cell types (Khan, 2005; Hawkins *et al.*, 2006)

1. Neurovascular unit

In the developing brain, capillaries are differentiated and matured into the BBB (Lee *et al.*, 2006). Only capillary vessels have complete BBB properties. The circumference of the capillary lumen enclosed by a single EC (Hawkins and Davis, 2005) is very important: as the vessel diameter increases, so does the vessel leakiness (Marchi *et al.*, 2004).

Although BBB permeability itself is controlled by the biochemical properties of brain microvascular endothelial cells (BMVEC) (Pardridge, 1999), brain microvascular biology results overall from interactions of these cells with the basement membrane and neighbouring glial cells (Kaur and Ling, 2008), such as microglia and astrocytes, as well as neurons and perivascular pericytes (Wolburg and Lippoldt, 2002; Zlokovic, 2008; Zozulya *et al.*, 2008). Altogether these constitute the neurovascular unit (Persidsky *et al.*, 2006; Choi and Kim, 2008) (Fig. 1.2), essential for both health and function of the CNS (Hawkins and Davis, 2005).

1.1 Basement membrane

The basement membrane, constituted by 3 apposed laminas, is an essential part of the BBB, connecting BMVEC with the surrounding brain resident cells (Persidsky *et al.*, 2006; Weiss *et al.*, 2009). It is composed of different extracellular matrix classes of molecules. These are structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin) and proteoglycans (Adibhatla and Hatcher, 2008; Wolburg *et al.*, 2009). Matrix adhesion receptors are expressed in the vascular cells, neurons and supporting glial cells. These receptors are essential for maintenance of the BBB, as the disruption of extracellular matrix is strongly associated with increased BBB permeability in pathological states. (Hawkins and Davis, 2005; Zlokovic, 2008).

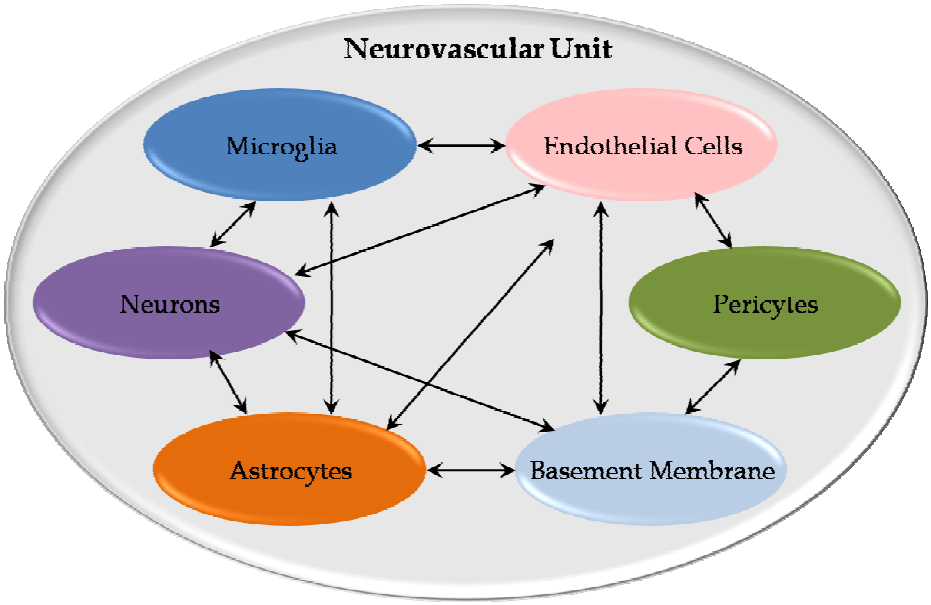


Fig. 1.2 – Schematic overview of the interactions between basement membrane and, neurons, microglia, pericytes, astrocytes and endothelial cells that globally constitute the neurovascular unit. Adapted from Hawkins and Davis (2005).

1.2 Neurons

Little is known about the developmental role that neurons have on the BBB phenotype. However there is some evidence that neurons can regulate the function of blood vessels in response to metabolic requirements by inducing expression of enzymes unique for BMVEC (Persidsky *et al.*, 2006). Also, BMVEC and astrocytic processes are directly innervated by noradrenergic, serotonergic, cholinergic, and GABA-ergic neurons, among others (Hawkins and Davis, 2005). The mature endothelium has a reciprocal function inducing a stable microenvironment that enables proper neuronal activity (Choi and Kim, 2008).

1.3 Microglia

Microglia play a very important role in immune responses of the CNS. These cells present themselves in two forms: resting and activated microglia. When resting, cells have small bodies and long, thin processes. Activated microglia surveys local microenvironment by shifting from long to short processes, assuming a phagocytic morphology (Zlokovic, 2008). Microglia

changes the phenotype in response to homeostatic disturbance of the CNS. According with the macrophage population in other organs, these cells have diverse effector functions (Ransohoff and Perry, 2009). As microglia are found in perivascular space, it is though that their interactions with BMVEC may contribute to the BBB properties (Choi and Kim, 2008). Still, the exact mechanisms on how this proceeds remains unkown.

1.4 Pericytes

Pericytes (also known as vascular smooth muscle cells, mural cells, or myofibroblasts) are important cellular constituents of the capillaries and post capillary venules (Dore-Duffy, 2008), having a close physical association with the endothelium. They share the same basement membrane with the EC (Bagley *et al.*, 2005) and cover 22% to 32% of the capillaries (Kim *et al.*, 2006) (Fig. 1.3). The location of pericytes on the microvessel and the degree of coverage varies considerably between different microvessel types (Allt and Lawrenson, 2001), seeming to correlate with the degree of tightness of the interendothelial junctions (Lai and Kuo, 2005). The vascular pericyte synthesizes most elements of the basement membrane including a number of proteoglycans. Pericyte synthesis and release of laminal proteins is thought to be a critical step in the differentiation of the BBB (Dore-Duffy *et al.*, 2006).

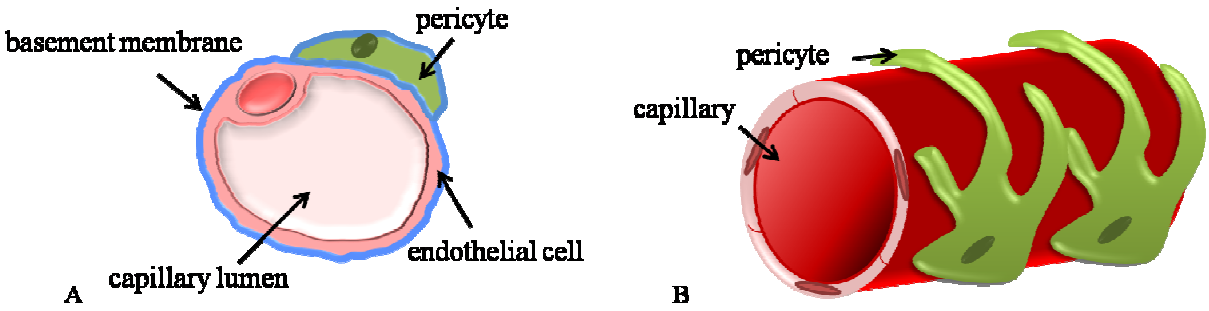


Fig. 1.3 – Pericytes Pericytes have a close physical association with the endothelium, in sharing the basement membrane with the endothelial cells (A) and cover up to 32% of the capillaries (B). Adapted from (Zlokovic, 2008) and Elsevier (2009).

Proper association of pericytes with microvascular endothelia is essential to maintain structural support and junctional integrity (Lai and Kuo, 2005; Braun *et al.*, 2007). Pericytes and endothelial cells share tight junctions (TJ), adhesion plaques (Allt and Lawrenson, 2001) and soluble factors (Bagley *et al.*, 2005).

The association of pericytes to blood vessels has been suggested to regulate EC proliferation, migration and differentiation (Persidsky *et al.*, 2006). There is also evidence that pericytes can induce and up-regulate P-glycoprotein (P-gp) functional activity in these cells (Dohgu *et al.*, 2005). Furthermore, interactions between pericytes and BMVEC are important for the remodelling and maintenance of the vascular system via the secretion of growth factors or modulation of the extracellular matrix (Allt and Lawrenson, 2001). There is evidence that pericytes are involved in the transport across the BBB, as well (Allt and Lawrenson, 2001). Pericytes make regulatory adjustments in response to stress stimuli, as during severe and prolonged O_2 deprivation (Al Ahmad *et al.*, 2009).

Pericytes show rich contents of α-smooth muscle actin (α-SMA) which is a characteristic of the vascular smooth muscle cell, suggesting a contractile ability of pericytes (Lai and Kuo, 2005). The physical contacts of pericytes' cellular processes over interendothelial junctions coupled with the α-SMA contents suggest the functional role of pericytes in controlling blood flow as well as regulating junctional permeability. Pericytes have been shown to migrate away from brain microvessels in rapid response to hypoxia and traumatic brain injury. Both of these conditions are associated with increased BBB permeability (Hawkins and Davis, 2005). The disruption of BBB caused by the detachment of pericytes, reinforces the idea of pericytes' role in junctional permeability (Nishioku *et al.*, 2009).

1.5 Astrocytes

Astrocytes are glial cells whose endfeet form a lacework of fine lamellae closely apposed to the outer surface of the BBB endothelium (Abbott, 2002) (Fig. 1.4). Through contact with their feet, astrocytes are capable of transdifferentiating non-neural EC into the brain type, endowing them with the BBB properties (Hayashi *et al.*, 1997). Therefore, astrocytes and EC interactions influence each other's structure. They induce and modulate the development of the BBB and BMVEC's unique phenotype, enhancing TJ and reducing gap junctional area (Abbott *et al.*, 2006).

Astrocytes could play a major role in promoting proteoglycan synthesis with a resultant increase in BMVEC charge selectivity and playing an important role in the induction of BBB functions (Yamagata *et al.*, 1997; Bernoud *et al.*, 1998). Astrocytes are essential for proper neuronal function and the close proximity of neuronal cell bodies to brain capillaries suggests that interactions between astrocytes and BMVEC are essential for a functional neurovascular unit (Persidsky *et al.*, 2006).

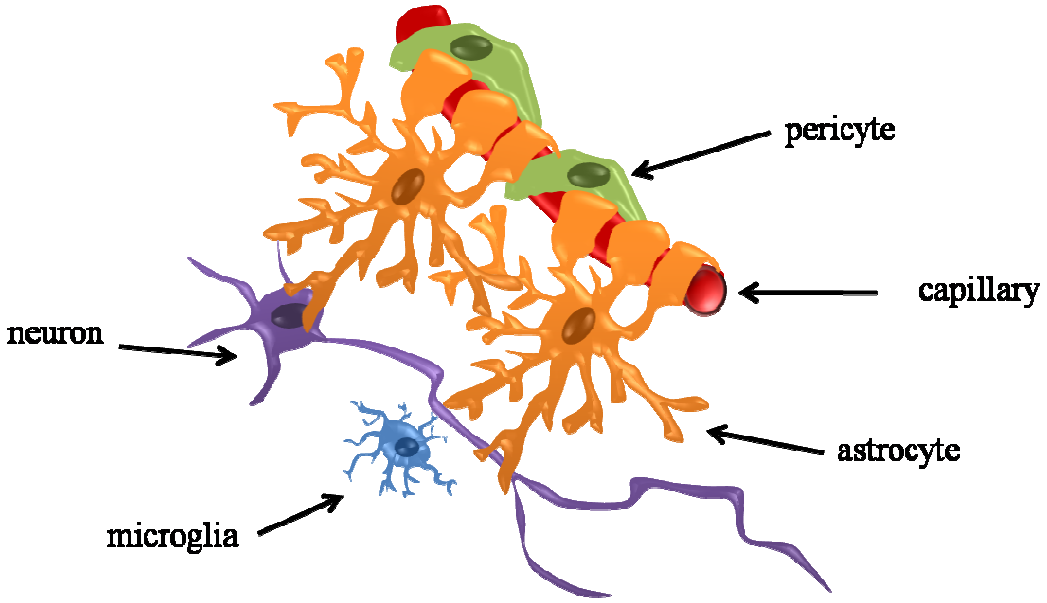


Fig. 1.4 – Astrocytes's endfeet form a fine lamellae closely apposed to the outer surface of the capillary endothelium. Adapted from Best (1990).

In some areas of the CNS there is a blood–tissue barrier with an endothelium that does not contact with astrocytes. Thus, the microvessels lacking astrocytic ensheathment exhibit still some BBB features, likely due to soluble factors acting from the glia limitans or the subarachnoid cerebrospinal fluid (Abbott, 2002). Subsequent studies also showed loss and restoration of barrier integrity *in vivo* following a temporary focal loss of astrocytes (Willis *et al.*, 2004; Persidsky *et al.*, 2006). Attempts to recover BBB properties in BMVEC cultures have included co-culturing BMVEC with astrocytes and/or astrocyte-conditioned medium (Colgan *et al.*, 2008). Astrocytes may therefore modulate the BBB phenotype without being directly involved in the physical BBB properties. The non-contact co-cultivation with astrocytes helps in maintaining the barrier properties of HBMVEC by secreting factors into the medium that upregulate the tight junctional proteins *zonula occludens* (ZO)-1 and occludin, and reduce the transendothelial permeability across the HBMVEC (Siddharthan *et al.*, 2007; Colgan *et al.*, 2008).

1.6 Endothelial cells

EC were demonstrated to be the responsible for BBB impermeability as it was observed that horseradish peroxidase could not pass the endothelial layer from either direction, and that brain capillaries from amphibians have high electric impedance, indicative of restriction to the movement of ions, despite the absence of surrounding astrocytes (Hawkins *et al.*, 2006). It is now accepted that the cerebral endothelium forms the anatomic basis of the BBB in higher animals (Hawkins *et al.*, 2006) and that he capillaries make up the primary part of the BBB (Khan, 2005).

BMVEC interact intimately with other brain cells of the neurovascular unit, and hence can act as mediators between blood and brain (Calabria and Shusta, 2008) (Fig. 1.5). They regulate the selective transport and metabolism of substances from blood to brain as well as in the opposite direction from the parenchyma back to the systemic circulation (Zheng *et al.*, 2003). The BMVEC barrier line is the most critical for preventing toxic substances from entering the brain (Ueno, 2007). Communication between EC and other surrounding cells (Fig. 1.6) enhances the barrier functions consequently resulting in maintenance and elaboration of proper brain homeostasis (Choi and Kim, 2008).

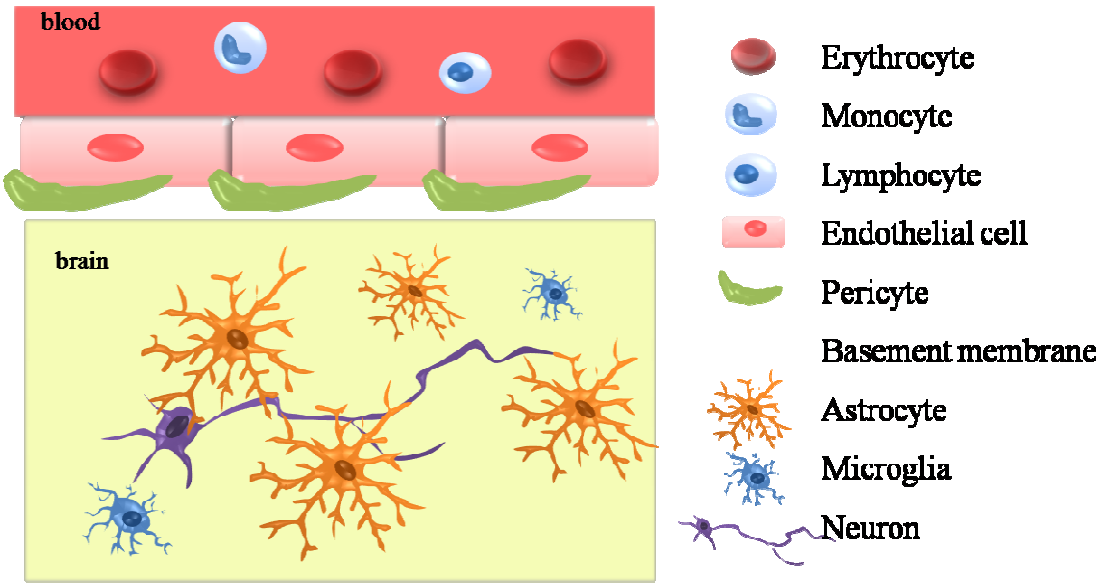


Fig. 1.5 – Brain microvascular endothelial cells establish the contour of blood vessels with erythrocytes and leucocytes, such as monocytes and lymphocytes, and interact intimately with the basement membrane and cells of the neurovascular unit, such as neurons, astrocytes, microglia and peicytes, hence operating as mediators between blood and brain. Adapted from Francis *et al* (2003).

The BMVEC lining the cerebral capillaries differ fundamentally from other vascular endothelia in their capacity to regulate the passage of molecules and cells to and from the neural parenchyma (Ge *et al.*, 2005; Weksler *et al.*, 2005). The capillary endothelium in the brain is 50–100 times tighter than peripheral microvessels as a result of special properties that cause severe restriction of the paracellular pathway for diffusion of hydrophilic solutes (Abbott, 2002).

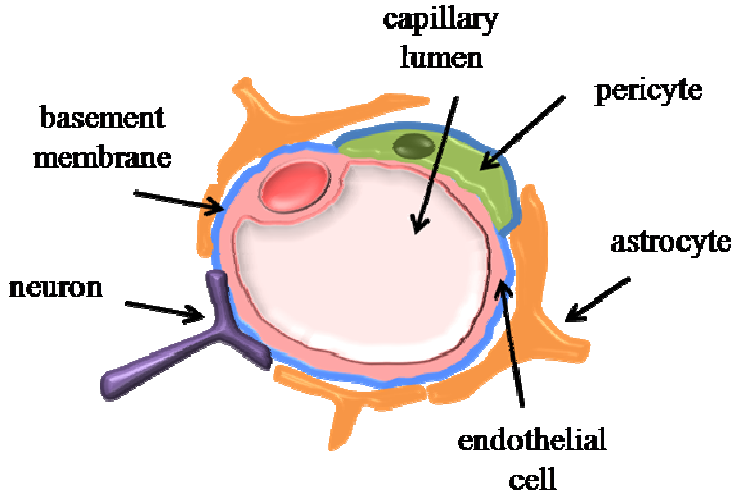


Fig. 1.6 – Interaction between endothelial cells and other neighbouring cells enhance the barrier functions. Adapted from Zlokovic (2008).

BMVEC lining the vascular wall have narrow junctional complexes that eliminate gaps or spaces between cells and prevent any free diffusion of blood-borne substances into the brain parenchymal space (Zlokovic, 2008; Weiss *et al.*, 2009). The EC cytoplasm has uniform thickness with no fenestrate, low pinocytotic activity and a continuous basement membrane (Chaudhuri, 2000; de Boer and Gaillard, 2006). In addition, the BMVEC have a negative surface charge that repulses negatively charged compounds (de Boer and Gaillard, 2006). They have a greater number and volume of mitochondria compared with endothelium in other organs, which enhances the energy potential (Persidsky *et al.*, 2006), and enable enzymes to break down compounds and various selective transport systems allowing active transport of nutrients and other compounds into and out of the brain (de Boer and Gaillard, 2006).

The BBB is more than an impermeable wall. It has a unique pattern of receptors, specific transport systems that facilitate the uptake of important nutrients and hormones, and active pumps that help to regulate the concentrations of ions and metabolites in the brain (Zheng *et al.*, 2003; Weksler *et al.*, 2005; Zlokovic, 2008) that are schematically represented in Figure 1.7. The BMVEC are able to efficiently supply the brain with the metabolites required while contributing

to the maintenance of the brain's ionic homeostasis and protecting the CNS from a large variety of potentially harmful hydrophobic compounds (Betz, 1992; Choi and Kim, 2008).

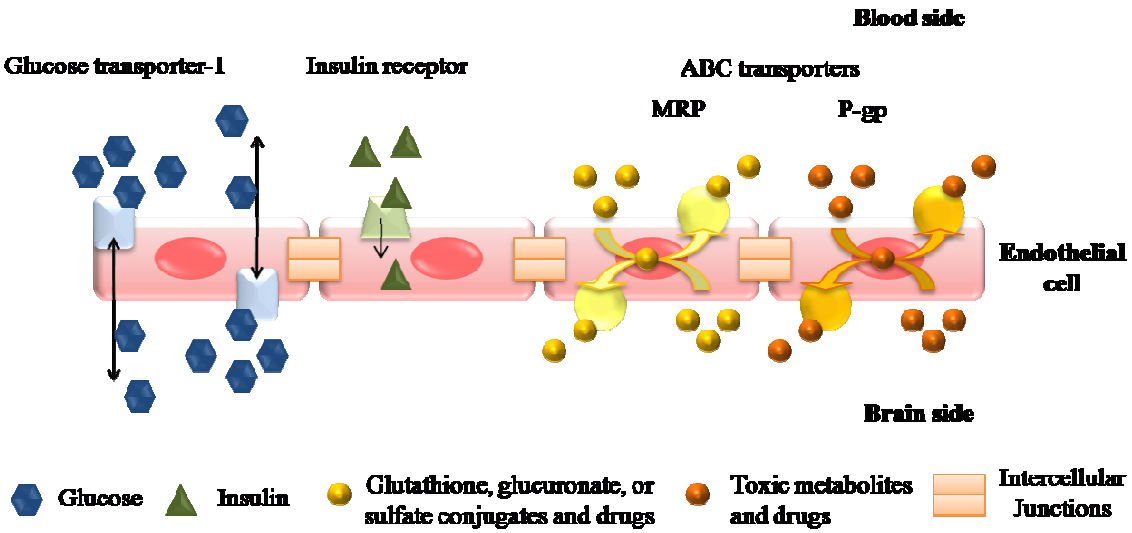


Fig.1.7 – Endothelial cells comprise a large variety of specific transporters and receptors, such as the glucose transporter-1 (GLUT-1), insulin receptor and ABC transporters, as the multidrug resistance-associated protein (MRP) and P-glycoprotein (P-gp) which regulate brain concentrations of nutrients, hormones, ions, metabolites, and xenobiotics. For simplicity, other transporters and receptors are not represented, nor is depicted their differential expression in the luminal or abluminal membrane. Adapted from Zlokovic (2008).

The cerebral microvasculature lining is characterized by the presence of an elaborated junctional complex that includes mainly TJ and adherens junctions (AJ) proteins (Hawkins and Davis, 2005). Gap junctions have also been identified at the BBB, but their role in the barrier function is not clear (Zlokovic, 2008)

1.6.1 Tight junctions

TJ are the main structures responsible for the barrier properties. These are elaborated structures located on the apical region of EC (Fig. 1.8). They function both as a seal that regulates lateral diffusion between the apical and basolateral plasma membrane domains, which enables asymmetric distribution of membrane constituents and as a limit to paracellular permeability (Ge *et al.*, 2005; Hawkins and Davis, 2005; Hawkins and Egleton, 2006; Persidsky *et al.*, 2006). Studies using TJ from different tissues with varying transendothelial and transepithelial electrical resistances show a correlation between increased organization of cytoplasmic fibrils and decreased membrane permeability (Huber *et al.*, 2001).

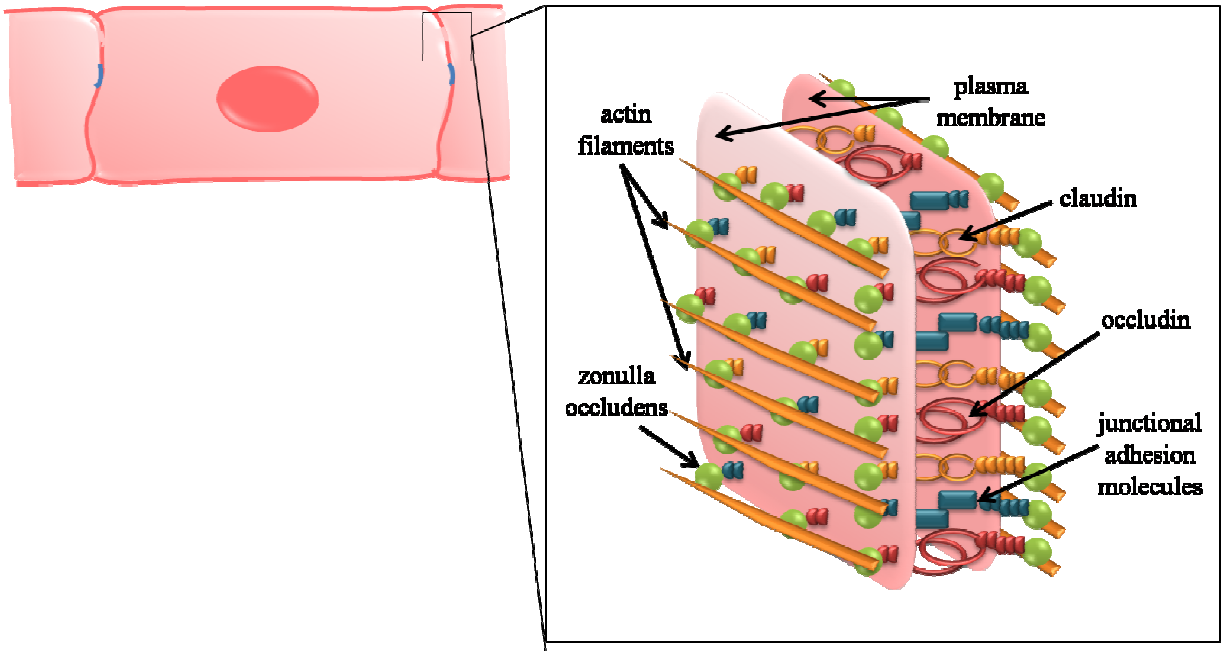


Fig. 1.8 – Tight junctions are located on the apical region of endothelial cells. They form an intricate complex of parallel, interconnected, transmembrane and cytoplasmatic strands of protein arranged as a series of multiple barriers. Based on Schmieder (2007).

The BBB TJ of mammalian species are characterized first of all by the highest complexity found in the vasculature of the body (Kniesel and Wolburg, 2000). Structurally, TJ form an intricate complex of parallel, interconnected, transmembrane and cytoplasmatic strands of proteins arranged as a series of multiple barriers (Wolburg and Lippoldt, 2002). Claudin, occludin, and junctional adhesion molecules (JAM) are integral membrane proteins interacting with those of neighbouring plasma membrane and form TJ barrier (Petty and Lo, 2002). Cytoplasmic TJ accessory proteins such as ZO connect integral TJ to the actin cytoskeleton (Lee *et al.*, 2004).

1.6.1.1 Claudins

The claudins (with approximately 24 kDa) are the TJ molecules that seem to fulfil the task of establishing barrier properties. These proteins are integral membrane proteins that share the four transmembrane domains of occludin, but do not contain any sequence homology to occludin (Wolburg and Lippoldt, 2002). Therefore, claudins and occludins are proteins formed by different aminoacids with similar conformation, without sharing genetic information.

They form dimers and bind homotypically to other claudin molecules in adjacent BMVEC (Huber *et al.*, 2001; Petty and Lo, 2002). These proteins are believed to be responsible

for permeability restriction (Tsukita *et al.*, 2001). Although overexpression of claudins can induce cell aggregation and formation of TJ-like structures (Yamamoto *et al.*, 2008), occludin expression does not result in the TJ formation. Thus, it appears that claudins form the primary seal of the TJ, and occludin acts as an additional support structure (Persidsky *et al.*, 2006).

In mammalian BMVEC only claudin-1, claudin-3 and claudin-5 were detected so far. Interestingly, claudin-5 was shown to be expressed primarily in EC of blood vessels (Petty and Lo, 2002; Mahajan *et al.*, 2008; Neuhaus *et al.*, 2008). Additionally, a novel anti-claudin-3 antibody was recognized in BMVEC TJ (Wolburg and Lippoldt, 2002). Claudin-1 and claudin-5 are associated with maintenance of normal BBB function (Vorbrodt and Dobrogowska, 2003) and are suggested to be important in angiogenesis and in disease processes with increased vessel permeability (Wolburg and Lippoldt, 2002).

1.6.1.2 Occludins

Occludin – a transmembranous TJ protein of approximately 65 kDa – was the first and best-known tight junctional transmembrane molecule discovered (Furuse *et al.*, 1993). It is highly expressed and consistently stains in a distinct, continuous pattern along the cell margins in the cerebral endothelium, whereas it is much more sparsely distributed in non-neural endothelium (Hawkins and Davis, 2005). It has been shown that high levels of occludin ensure decreased paracellular permeability (Huber *et al.*, 2001) and high electrical resistance of the BMVEC monolayers (Persidsky *et al.*, 2006), having therefore an active role in BBB function (Yamamoto *et al.*, 2008). However, it must be seen more in a regulatory context than as a major structural protein in the establishment of the barrier properties (Wolburg and Lippoldt, 2002). Results of several knockout and knockdown experiments indicate that occludin is not essential for the formation of TJ (Hawkins and Davis, 2005) and that normal expression and localization of other junctional proteins well compensate occludin loss (Zlokovic, 2008). Recently, there has been evidence that occludin regulates epithelial cell differentiation (Zlokovic, 2008).

A feature of occludin is a calcium-independent adhesiveness, which is mediated by the first extracellular loop of occludin and depends on the presence of ZO-1 and AJ components (Kniesel and Wolburg, 2000). The cytoplasmic C-terminal domain provides the connection of occludin with the cytoskeleton via accessory proteins ZO-1, ZO-2 and ZO-3 (Shimizu *et al.*, 2008).

1.6.1.3 Junctional adhesion molecules

Adhesion molecules such as the JAM family and the newly discovered EC-selective adhesion molecule are localized at TJ as well. (Wolburg and Lippoldt, 2002). JAM are members of the immunoglobulin superfamily expressed in TJ of EC (Vorbrodt and Dobrogowska, 2003) and mediate homophilic and probably also heterophilic interactions in the tight junctional region (Wolburg and Lippoldt, 2002).

The JAM family consists of JAM-1, JAM-2, and JAM-3. These are 40-kDa proteins, from the IgG superfamily (Persidsky *et al.*, 2006; Bernacki *et al.*, 2008) and are composed by a single membrane-spanning chain with a large extracellular domain (Hawkins and Davis, 2005). JAM-1 is involved in cell-to-cell adhesion organizing the tight junctional structure and in monocytes extravasation through interendothelial junctions *in vivo* and *in vitro* (Stamatovic *et al.*, 2008). It is believed that JAM-1 takes part in the formation of TJ as an integral membrane protein together with occludin and claudins (Vorbrodt and Dobrogowska, 2003). JAM-2 is involved in lymphocyte transmigration and interacts with JAM-3. As for this one, is implicated in cell-cell adhesion and in leukocytes transmigration (Stamatovic *et al.*, 2008). Both JAM-2 and JAM-3 are present in EC of different organs (Wolburg and Lippoldt, 2002; Hawkins and Davis, 2005), whilst JAM-1 is strictly present in the human cerebral cortex (Bernacki *et al.*, 2008).

1.6.1.4 Zonula occludens

Submembranous TJ-associated proteins such as ZO proteins (ZO-1, ZO-2 and ZO-3) belong to the family of membrane-associated guanylate kinase proteins (Persidsky *et al.*, 2006), which serve as recognition proteins for TJ placement and as a support structure for signal transduction proteins (Huber *et al.*, 2001).

Membrane-associated guanylate kinases share three defined core regions: a SRC homology 3 (SH3) domain, a guanylate kinase and a postsynaptic density-95, disc large, and *zonula occludens*-1 domain. The SRC homology 3 domain binds signalling proteins and cytoskeletal elements. Guanylate kinase catalyses the ATP-dependent transformation of GMP to GDP. As for the postsynaptic density-95, disc large, and *zonula occludens*-1 domain, it mediates specific binding to carboxy-terminal cytoplasmic ends of transmembrane proteins (Wolburg and Lippoldt, 2002).

ZO-1, the first TJ-associated protein identified and characterized, is a 220-kDa phosphoprotein mostly expressed in endothelial and epithelial cells that normally form the TJ assembly (Kniesel and Wolburg, 2000; Persidsky *et al.*, 2006). Though it is also expressed in other cell types that do not form TJ, there is no TJ without ZO-1. ZO-1 molecules are located on the cytoplasmic side of the BMVEC plasma membranes delimiting the interendothelial cleft (Vorbrodt and Dobrogowska, 2003) and connecting transmembranous TJ proteins with the actin cytoskeleton. Loss or dissociation of ZO-1 from the junctional complexes is associated with increased barrier permeability (Choi and Kim, 2008). ZO-2, a 160-kDa phosphoprotein, has significant homology to ZO-1. It was thought that ZO-2 was restricted exclusively to the TJ region (Kniesel and Wolburg, 2000); however, it has also been found in non-TJ-containing tissues. Very much like ZO-1, ZO-2 binds to transmembranous proteins of the TJ and transcription factors, and it is localized in the nucleus during stress and proliferation (Persidsky *et al.*, 2006). It is not only an extremely important structural protein, but also a nuclear factor influencing gene expression (Wolburg *et al.*, 2009) and blocking cell cycle progression (Gonzalez-Mariscal *et al.*, 2009).

Initially simply known as a protein that co-precipitated with the ZO-1/ZO-2 complex, ZO-3 has a close homology to ZO-1 and ZO-2. There is evidence that ZO-3 binds occludin and ZO-1 directly, but not ZO-2 (Kniesel and Wolburg, 2000).

The complex formed by ZO-1 protein together with its supplementary components, ZO-2 and ZO-3, is considered to be involved in cadherin-based cell adhesion through their binding to α-catenin and to actin filaments. Binding of ZO proteins to actin suggests that one possible function of these molecules is to form a scaffold to link TJ to the cytoskeleton. Given the evidence of their presence in *zonula adherens*, these proteins may be involved in cell-to-cell signal transduction (Vorbrodt and Dobrogowska, 2003).

1.6.2 Adherens junctions

AJ are composed of transmembrane glycoproteins of the cadherin super family, which are Ca^{2+}-dependent receptors that form homotypic adhesive complexes with neighbouring cells, giving place to a continuous belt – the adhesion belt (Petty and Lo, 2002) (Fig. 1.9).

AJ mediate events such as the adhesion of BMVEC to each other, the contact inhibition during vascular growth and remodelling, the initiation of cell polarity, and the regulation of paracellular permeability (Hawkins and Davis, 2005). Cell-cell adhesion by linking actin

filaments (Cook *et al.*, 2008) involves vascular endothelial (VE)-cadherin and catenins, constituents of AJ (Perrière *et al.*, 2007). The cadherin family and their intracellularly associated catenin proteins form complexes of central importance to the sorting and morphogenic processes of developing animal tissues and in maintaining the integrity and identity of adult tissues (Tao *et al.*, 1996).

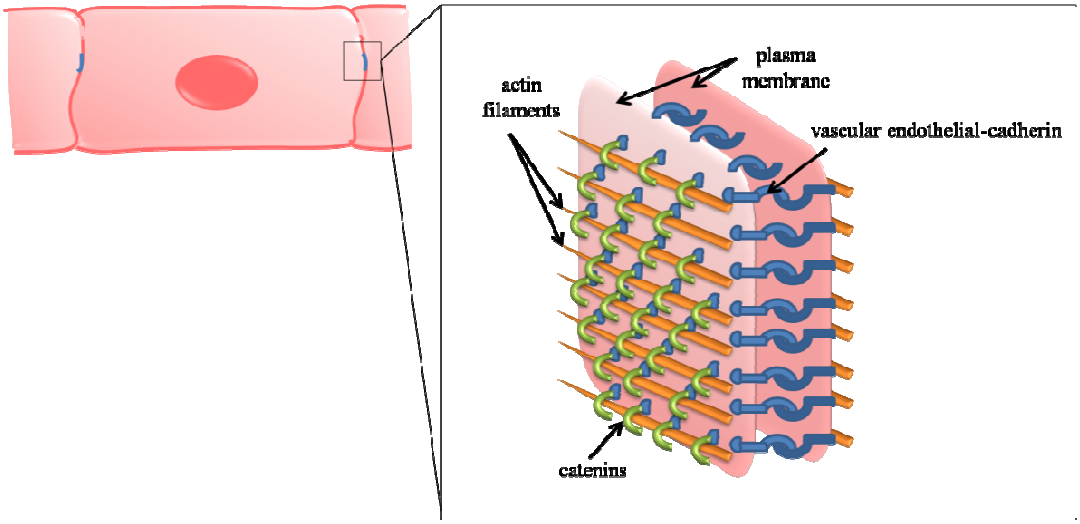


Fig. 1.9 – Adherens junctions are composed of transmembrane glycoproteins of the cadherin super family and by catenins, giving place to the adhesion belt. Adapted from Hill (2008).

1.6.2.1 Cadherins

Calcium-dependent cell-cell adhesion is dependent on a family of transmembrane glycoproteins named cadherins (Navarro *et al.*, 1998), which can also be defined as major transmembrane components of AJ (Vorbrodt and Dobrogowska, 2003). Disruption of the AJ by removal of extracellular Ca^{2+} leads to the opening of the TJ (Hirase *et al.*, 1997).

Cadherins present a certain degree of cell type specificity. Vascular endothelial-cadherin (VE-cadherin), also known as cadherin-5, is an integral membrane glycoprotein expressed exclusively in cells of vascular epithelial origin, whereas Neural-cadherin is expressed in cells of the nervous tissue, vascular smooth muscle cells, and myocytes (Navarro *et al.*, 1998).

VE-cadherin is an important determinant of microvascular integrity both *in vitro* and *in vivo* (Vorbrodt and Dobrogowska, 2003). It clusters at cell junctions and mediates cell adhesion in a calcium-dependent manner, inhibits cell proliferation, and decreases cell permeability and migration when over-expressed in various cell types (Cook *et al.*, 2008).

All cadherins contain a plasma membrane-spanning domain and a cytoplasmic domain associated with other molecular components of the junctional complex, such as catenins (Vorbrodt and Dobrogowska, 2003). VE-cadherin's expression at cell junctions, however, is independent of β-catenin binding, which appears to be required only for junction stabilization (Cook *et al.*, 2008).

1.6.2.2 Catenins

Catenins were first characterized as linking the cytoplasmic domains of cadherin cell-cell adhesion molecules to the cortical actin cytoskeleton. Catenins' major roles is to anchor the cadherin complex to the actin cytoskeleton but they also participate in cell and developmental signalling pathways (Stamatovic *et al.*, 2008).

There are four types of catenin proteins: α-, β-, δ- and γ-catenin. α- and β-catenin are located in interendothelial junctions of BBB-type brain capillaries and their expression is required for cadherins to work as adhesion molecules (Vorbrodt *et al.*, 2009). δ-catenin has been implicated as a regulator of the NF-κB transcription factor (Perez-Moreno *et al.*, 2006), while γ-catenin is closely related to β-catenin and can replace it in the cadherin–catenin complex (Vorbrodt and Dobrogowska, 2003).

β-catenin is essential in endothelial cells for normal vascular patterning. It is a structural protein that participates in cell-cell adhesion and is also involved in the Wingless signalling pathway and in gene expression (Cook et al., 2008; Vorbrodt et al., 2009), serving as a mediator in regulation of P-gp and other multidrug efflux transporters in brain vasculature (Lim et al., 2008). In addition, β-catenin is linked to the cell membrane in a complex with VE-cadherin and platelet-endothelial cell adhesion molecule. This molecule mediates homophilic adhesion (Wolburg et al., 2009). Upon stimulation with growth factors this membrane-associated complex is dissociated, releasing another source of β-catenin for movement to the nucleus and transcription (Petty and Lo, 2002). It is possible that the up-regulation of β-catenin elicits the maintenance of TJ protein assembly and barrier function (Vorbrodt et al., 2009).

2. Pathways across blood-brain barrier

Passage of molecules across the endothelial cells of the BBB can occur between adjacent cells (the paracellular pathway) or through the cells (the transcellular pathway) (Pardridge, 1999). In the endothelium, the relationship of paracellular and transcellular permeability is of crucial importance for the regulation of overall transendothelial permeability (Wolburg *et al.*, 2009). When it comes to the paracellular passage, ions and solutes diffuse between adjacent cells according to their concentration gradient (Petty and Lo, 2002). As for the transcellular pathway (Fig. 1.10), it involves different mechanisms including passive diffusion of lipophilic compounds, receptor-mediated shuttling and transcytosis. Small lipophilic molecules, such as oxygen, CO_2 and ethanol (Abbott, 2002), can pass the BBB freely by diffusion (Zheng *et al.*, 2003), whereas hydrophilic molecules, such as peptides and proteins, may enter the brain through specific transport mechanisms (Norsted *et al.*, 2008). Polar and lipid-insoluble molecules do not cross the BBB (Scherrmann, 2002).

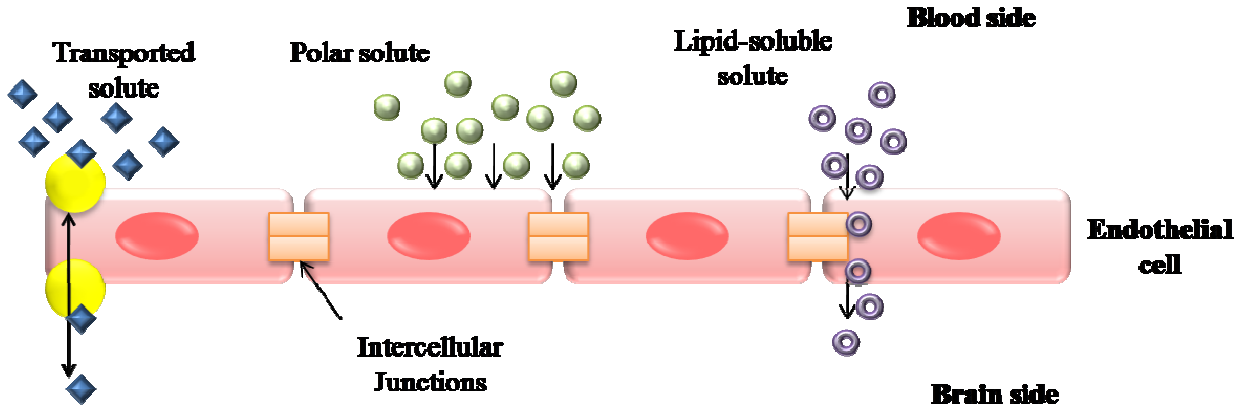


Fig. 1.10 – The transcellular pathway: passage of molecules across the endothelial cells of the blood-brain barrier (BBB) can occur through different mechanisms such as passive diffusion of lipophilic compounds or receptor-mediated shuttling. Polar molecules do not cross the BBB. Adapted from Abbott *et al.* (2006).

An elaborate system of transport proteins, as caveolin-1, glucose transporter-1 (GLUT-1) and ATP-binding cassette (ABC) transporters among others is asymmetrically expressed on the luminal and abluminal EC membranes (Hawkins *et al.*, 2002).

2.1 Caveolae

Caveolae have been suggested as sites of endothelial transcytosis, endocytosis, and signal transduction, and as docking sites for glycolipids and glycosylphosphatidylinositol-linked proteins (Simionescu *et al.*, 2002; Wolburg *et al.*, 2009). Caveolae are invaginations of the plasma membrane rich in the structural and functional coat protein caveolin (Fig. 1.11). Whilst caveolin is the distinct biochemical marker within caveolae domains, caveolae are also rich in cholesterol and sphingolipids (Smith and Gumbleton, 2006). In the BBB, the main caveolin marker proteins are caveolin-1 and -2. The presence of both on brain microvessels was demonstrated for BMVEC of rats, rhesus monkeys, porcine and of normal human samples (Virgintino *et al.*, 2002). Caveolin-1 is the major structural protein of caveolae and is involved in various aspects of vesicular trafficking and signal transduction pathways (Huber *et al.*, 2001). It has also been reported that both occludin and ZO-1 might be organized within TJ by association with caveolin-1 in detergent-insoluble glycolipid rafts (Smith and Gumbleton, 2006). Caveolin-1 has been proposed to not be required for endocytosis but instead to stabilize endocytic raft domains decreasing endocytosis (Nabi and Le 2003).

2.1 GLUT-1

GLUT-1 ensures nutrient delivery, supplying glucose for the brain (Persidsky *et al.*, 2006), the main energy source of the brain. The 55-kDa form of GLUT-1 is highly restricted to the capillary EC in the brain (Wolburg *et al.*, 2009). The density of GLUT-1 at the abluminal membrane is higher than at the luminal. The asymmetrical distribution provides homeostatic control for glucose influx into the brain by preventing glucose accumulation in the brain at levels higher than those in the blood (Zlokovic, 2008). Interestingly, GLUT-1 may also be present at peripheral nerve pericytes transporting D-glucose from the circulating blood into the brain and peripheral nervous parenchyma in cooperation with that on the EC (Shimizu *et al.*, 2008).

2.3 von Willebrand Factor

BMVEC are usually characterized for staining for von Willebrand factor (vWF). Also known as factor VIII-related antigen, it is a carrier protein for coagulation factor VIII stored in large and microvessel endothelium. Although some people consider vWF the most reliable marker for EC, others showed loss of staining after passaging with trypsin (Craig *et al.*, 1998).

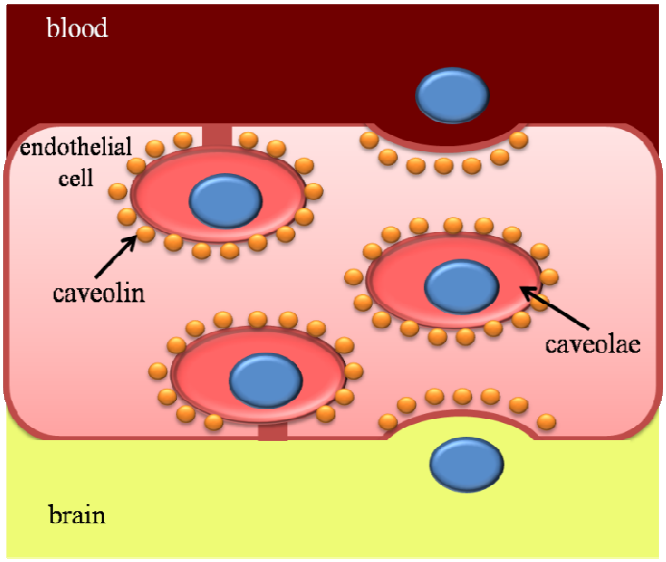


Fig. 1.11 – Caveolin-1 is the major structural protein of caveolae and is involved in various aspects of vesicular trafficking and signal transduction pathways. Adapted from Abbott *et al.* (2006).

2.4 ABC transporters

Efflux of molecules from the brain endothelium can be initiated at the luminal membrane, as in the case of the ABC transporters. P-gp is a transporter for the acquisition of the multidrug resistance phenotype, together with the multidrug resistance-associated protein 1. These proteins are responsible for the active extrusion of nonpolar molecules out of EC and are therefore the focus of research on drug delivery to the brain (Shimizu *et al.*, 2008). There is an association between P-gp and caveolin-1, as the down-regulation of caveolin-1 enhances the transport activity of P-gp (Demeule *et al.*, 2000; Barakat *et al.*, 2007). P-gp in the endothelium prevents entry of blood-borne substances into the brain and facilitates their transport out of the brain parenchyma (Choi and Kim, 2008). The multidrug resistance-associated proteins, including the breast cancer resistance protein, mediate the efflux of anionic compounds (Zlokovic, 2008). These transporters have the potential to reduce penetration of many drugs into the brain and increase their efflux from the brain.

3. Methods to evaluate BBB characteristics

BBB characteristics are often assessed by monitoring certain properties. There are various methods to study the properties of the BBB *in vivo* (Mano *et al.*, 2002), *ex-vivo* (Ebert and Svendsen, 2005), *in silico* (Garg and Verma, 2006) and *in vitro* (Hawkins *et al.*, 2006), giving valuable information on a large variety of matters.

3.1 Study systems

3.1.1 Studies *in vivo*

In vivo studies provide the most reliable reference information for testing and validating other models. They take into account not only a section but the whole brain microenvironment. The most recent studies on BBB disorders include, for example, information on Parkinson's disease with primates (Astradsson *et al.*, 2009), and multiple sclerosis (Coisne *et al.*, 2009) or cerebral ischemia (Abulrob *et al.*, 2008) with mice. Nonetheless, they can be applied strictly to animals and require expensive equipment (Malina *et al.*, 2009).

3.1.2 Studies *ex vivo*

Ex-vivo studies are performed on living tissue in an artificial environment outside the organism. These studies may also be performed on post-mortem tissues. This allows experimentation under highly controlled conditions impossible in the intact organism, for example in Alzheimer's disease studies (Ebert and Svendsen, 2005).

3.1.3 Studies *in silico*

In silico prediction methods are cheaper and less limited than obtaining experimental data through other methods, and are very useful in permeability studies. However, these studies are performed strictly via computer simulation (Malina *et al.*, 2009; Mensch *et al.*, 2009).

3.1.4 Studies *in vitro*

Even though *in vitro* models of the BBB continue to be labour-intensive and difficult to reproduce, they permit to mimic the BBB microenvironment while studying not only a larger range of properties and functions (Aschner *et al.*, 2006), and to perform studies in different animal species. Most reports fall upon brain tissue from rat (Perrière *et al.*, 2007) and mouse (Coisne *et al.*, 2005), as well as from feline (Fletcher *et al.*, 2009), porcine (Zhang *et al.*, 2006) and bovine (Zhang *et al.*, 2009) models. However, the results obtained must take into account the differences between species. Furthermore, experiments with human tissue are extremely difficult and require methods both time-consuming and expensive (Hewett, 2009). Thus, cell lines became the most obvious alternative.

3.1.4.1 Cell lines

Establishment of a human model of the BBB has been proven difficult to achieve. To accomplish this, normal HBMVEC were transduced by lentiviral vectors incorporating human telomerase or SV40 T antigen, giving rise to cell lines. Human cell lines allow a faster and more frequent access to HBMVEC, as well as larger amount of cells per experience. These cells achieve confluence in a few days and maintain their phenotype and karyotype for several passages (Weksler *et al.*, 2005). Whilst a range of immortalized BBB cell lines are commercially available, they commonly present leaky intercellular junctions and a poorly differentiated phenotype compared to their *in vivo* siblings, characteristics which limit their effective use as robust and predictive *in vitro* BBB models (Smith *et al.*, 2007).

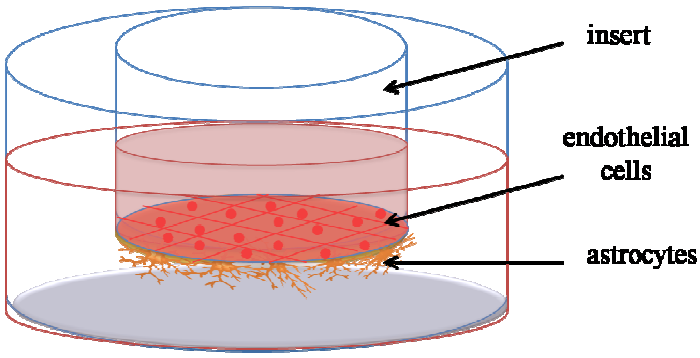
3.1.4.2 Primary cultures

Primary cell cultures of BMVEC, though technically challenging to isolate and maintain, represent the closest possible phenotype to the *in vivo* BBB cell providing a convenient model and the optimal choice for BBB research (Smith *et al.*, 2007). Several attempts to develop *in vitro* models of the BBB have been previously reported, using BMVEC from different species (Zenker *et al.*, 2003; Siddharthan *et al.*, 2007). Although primary cultures of HBMVEC have been shown to retain some phenotypic characteristics of brain endothelium, many problems have been encountered. Some relate to the difficulty to obtain pure endothelial cultures, as the basal

membrane surrounding the microvascular endothelium also encloses pericytes that are difficult to remove from the endothelial fraction (Calabria *et al.*, 2006). Therefore, these cells often contaminate BMVEC cultures. Another problem relates to loss of many of the functions observed *in vivo*, assigned to the removal of BMVEC from local brain microenvironment (Calabria *et al.*, 2006). They rapidly lose their specific characteristics in culture (Nakagawa *et al.*, 2009) undergoing dedifferentiation and senescence even upon limited passaging, thus hampering usefulness as *in vitro* models of the human BBB (Weksler *et al.*, 2005). Therefore, data generated from *in vitro* systems have to be carefully interpreted since it is unclear to what extent the de-differentiation is occurring (Calabria and Shusta, 2008).

3.1.4.3 Co-cultures

Research on BBB functionality/recovery has been very much enhanced by the availability of *in vitro* BBB co-culture systems (Ramsauer *et al.*, 2002; de Boer and Gaillard, 2006) (Fig.1.12). The discovery of cell-culture inserts with porous filter membranes allowed the use of BMVEC for permeability studies *in vitro* (Nakagawa *et al.*, 2007). In addition, the use of such systems allows the study in detail of BBB related phenomena at the sub-cellular level in the absence of feedback systems from the rest of the body (Megard *et al.*, 2002; de Boer and Gaillard, 2006). With co-cultures it has also been possible to evaluate effects on BMVEC under flow or shear stress, in the presence of other types of cells (Kim *et al.*, 2006). There have been studies performed with co-cultures of BMVEC and astrocytes both from either mouse (Ghazanfari and Stewart, 2001) or rat (Perriere *et al.*, 2007), as well as from bovine (Gaillard *et al.*, 2001) and porcine (Jeliazkova-Mecheva and Bobilya, 2003) models. Up until now, studies with HBMVEC are performed by co-culturing these cells with non-human astrocytes (Santaguida *et al.*, 2006). Most recently, there have also been studies with triple cultures of BMVEC, astrocytes and pericytes, though all extracted from rat brain (Nakagawa *et al.*, 2009).



3.2 Function assessment

3.2.1 TEER

Transendothelial electrical resistance (TEER) is a useful indicator of paracellular ion flux and BMVEC barrier function (Calabria *et al.*, 2006). There are several methods used in TEER measuring, including the epithelial voltage-ohm meter (EVOMX) and the STX2 electrode (Fig. 1.13**)**, in an EndOhm chamber, or even connected to a computer to monitor TEER continuously (Davidson *et al.*, 2009; Malina *et al.*, 2009).

It has been difficult to reproduce in cultured cells the high electrical resistance normally found across BBB EC *in situ* (Santaguida *et al.*, 2006). In BMVEC monocultures TEER is often 100 $\Omega.cm^2$ or lower (Calabria and Shusta, 2008).

3.2.2 Permeability

Transcellular permeability to small molecule tracers can also yield valuable information regarding barrier integrity (Calabria and Shusta, 2008). Most tracers are labelled by a fluorescent dye or isotope that helps the quantification of the molecule. Albumin (a 67kDa protein), for example, is a marker of transendothelial permeability and it has been observed in endothelial vesicles (Abbott, 2000; Deli *et al.*, 2005). Elevated permeability of the normally highly restrictive BBB accompanies a variety of CNS afflictions, including inflammation, infection, ischemia, seizures, and trauma (de Boer and Gaillard, 2006; Song *et al.*, 2007).

Similarly to TEER, the tightness of the barrier *in vivo* and permeability to polar molecules is less stringent than the BBB *in vivo*, allowing compounds that would normally poorly penetrate across BBB *in vivo* to readily diffuse across the endothelial monolayer in the static model (Santaguida *et al.*, 2006).

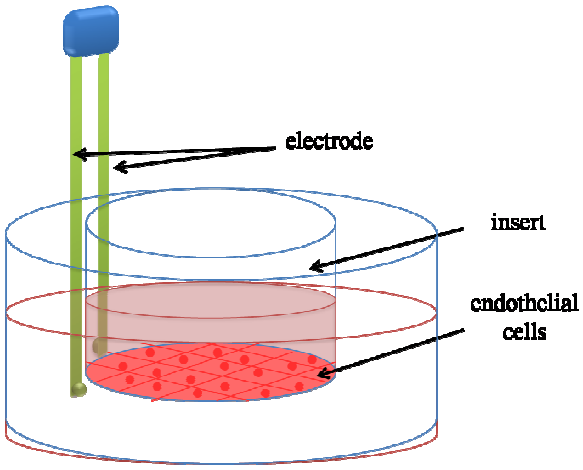


Fig. 1.13 – Schematic representation of the transendothelial electrical resistance measurement with STX2 electrodes.

3.2.3 Intercellular junctions

The main features of the BBB are the presence of tight intercellular junctions which strictly limit the diffusion of blood-borne solutes and cells into the brain. Several studies have been performed regarding these junctions. The most recent of these focus on the relation between β-catenin expression and Down's syndrome in a mouse model (Vorbrodt *et al.*, 2009), the disorganization of ZO-1 during hypoxia (Lu *et al.*, 2009), the ischemic preconditioning effects on TJ and cell adhesion of BMVEC (An and Xue, 2009), the caveolae-mediated internalization of occludin and claudin-5 during TJ remodelling in BMVEC (Stamatovic *et al.*, 2009) and the depletion of intercellular junctions at the site of bacteria-host cell interaction (Coureuil *et al.*, 2009).

3.2.4 Transport across the BBB

Membrane transporters and vesicular mechanisms protect the healthy brain, shielding it from toxic substances and allowing the entrance of others which are necessary. Some of the most

recent studies regarding this barrier include the regulation of major efflux transporters under inflammatory conditions (von Wedel-Parlow *et al.*, 2009), differential expression and function of ABC transporters during development and ageing (Bojanic *et al.*, 2009) and their regulation by cytokines (Poller *et al.*, 2009). Also, related to ABC transporters, comparative gene expression profiles have been done in BMVEC and brain in five species including the human one (Warren *et al.*, 2009).

4. Aim of the thesis

The main goal of this project was to implement a model of the BBB, relying on the use of confluent monolayers of HBMVEC. The procedure should maintain the *in vivo* barrier characteristics and serve as a robust *in vitro* cell culture tool for research programs addressing the BBB in a human model. Given the limited availability of human tissue samples, it will also be evaluated whether cells can be stored as frozen stocks without phenotypic drift, which shall constitute a remarkable benefit to research studies in the area. In order to further improve the exploitation of a single cell culture, it will be further ascertained the number of passages in culture with no loss of BMVEC phenotype.

Chapter 2 – Materials and Methods

1. Chemicals

Collagen type I and endothelial cell growth supplement (ECGS) were acquired from BD Biosciences (Bedford, MA, USA); DPX mountant for microscopy was obtained from BDH Prolabo (Poole, UK); Hank's Balanced Salt Solution (HBSS) and glutamax were purchased from Invitrogen (Carlsbad, CA, USA); Dulbecco's modified Eagle medium/nutrient mixture F-12 Ham (DMEM-F12), fetal bovine serum (FBS) and heparin were obtained from Biochrom (Berlin, Germany); Triton X-100 was obtained from Roche Diagnostics (Germany); antibiotic--antimycotic solution (AB/AM), bovine serum albumin (BSA), Hoechst dye 33258, hydrocortisone (HC) and Trypsin-EDTA solution (0.05% Trypsin, 0.02% EDTA in Hank's Balanced Salt Solution) were acquired from SIGMA (St Louis, MO, USA). Other chemicals were of analytical grade and were purchased from Sigma or Merck (Darmstadt, Germany).

Mouse anti-α-SMA was purchased from ABD Serotec (Oxford, UK). Rabbit anti-caveolin-1 was acquired from Cell Signaling (Danvers, MA, USA). Mouse anti-VE-cadherin and rabbit anti-vWF were purchased from Chemicon (Billerica, MA, USA). Rabbit anti-β-catenin, mouse anti-claudin-5, rabbit anti-occludin and rabbit anti-ZO-1 were obtained from Invitrogen (Carlsbad, CA, USA). Rabbit anti-GLUT-1 was purchased from Santa Cruz Biotechnology (CA, USA). Secondary antibodies goat anti-rabbit cyanine dye (CY)2 and goat anti-mouse CY3 were acquired from GE Healthcare (Chalfont St. Giles, UK).

2. HBMVEC isolation and culture procedure

Cells were isolated and cultured according to the technique developed by Bernas *et al*, (2009) (Fig. 2.1). Human samples (5-10 mm^3) of temporal lobe were obtained as discarded tissue during operative treatment for epilepsy, after informed consent and approval by the Ethics Comission of the Hospital Santa Maria, Lisbon. This technique was also applied to Wistar rats (cortex from both hemispheres). The brain sample was transported in a dry tube at room temperature to the laboratory, in up to 15 minutes. The sample was placed in a Petri dish containing ~5 ml of isolation medium (10 ml FBS, 1 ml Glutamax, 1 ml AB/AM, 88 ml DMEM-

F12) and the meninges and visible larger vessels were carefully removed under a stereomicroscope (Zeiss, model Stemi DV4; Jena, Germany). The tissue was fragmented using repeatedly sterile pipettes of 25, 10 and 5 ml, until the sample could be passed easily back and forth through the 5 ml pipette. The resulting tissue fragments were filtered thought a series of polyester meshes with pore-sizes of 500 µm and 30 µm. The collected fragments were transferred in medium to a centrifuge tube and spin at 400 rpm (~30 g) for 10 min. The resulting pellet was resuspended in 1 ml isolation supplemented medium (ISM) [10 ml FBS, 1 ml Glutamax, 1 ml AB/AM, 2 ml ECGS (50µg/ml), 3.4 ml heparin (1 mg/ml) and 82.6 ml DMEM-F12]. The suspension was transferred to a T-25 flask previously coated with 2.5 ml collagen type I solution (50 µg/ml of collagen in 0.02 N acetic acid), shaking to ensure coverage of the flask, and placed to adsorb for 1 hour in incubator. Afterwards, 3 ml of ISM were carefully added to the bottom of the T-25 flask without shaking. The flask was incubated at 37 ºC with 5% O_2 (Heraeus, model Function line). Three to four days later the medium was changed for the first time and afterwards once a week. Cells were monitored under contrast phase microscopy (Olympus optical microscope with phase-contrast equipment, model CK2-TR and Nikon digital camera, model L1; Japan).

3. HBMVEC passage

At confluence (approximately 1x 10^6 cells) , ISM was removed from the T-25 flask and a wash was performed with 2 ml versene solution **(**8.00 g NaCl, 0.40 g KCl, 0.20 g EDTA, 0.02 g phenol red in 1 l H_20 and adjusted pH to 7.4). 2 ml trypsin-EDTA solution were added and the flask placed for 10 minutes in incubator. DMEM/Ham's F12 + 10 % FBS (37ºC) was added to the flask (3 x trypsin volume). All fluid containing cells was harvested to a 50 ml tube and centrifuged at 800 rpm (~130 g) for 10 min. After decanting-off the fluid, the pellet was resuspended in ISM. The suspension was plated 4 ml per T-25 flask and 500 µl per 12 mm glass cover slip previously coated with 2.5 ml or 0.2 ml collagen type I solution, respectively.

4. HBMVEC cryopreservation

Following trypsinization, cells were resuspended in 1 ml ISM. After cell count, equal volumes of cell suspension (maximum of 1x 10^6 cells) and freezing medium **[**200 µl FBS (20%),

10 µl Glutamax, 10 µl AB/AM, 20 µl ECGS (50µg/ml), 34 µl heparin (1 mg/ml), 526 µl DMEM-F12 and 200 µl dimethylsulphoxide] were placed in a cold cryotube. Cryotubes where then placed in cryo freezing container from NALGENE (Rochester, NY, USA). After 4 h to overnight at -80 ºC, cryotubes were transferred to liquid nitrogen for 30 days. Following this time period, cells were thawed at 37 ºC and harvested to a Falcon tube. Up to a maximum of 4 ml of thawing medium [2 ml FBS (20%), 100 µl Glutamax, 100 µl AB/AM, 200 µl ECGS (50µg/ml), 340 µl heparin (1 mg/ml), 7.26 ml DMEM-F12] was added to the suspension and all fluid was transferred to a T-25 flask previously coated with 2.5 ml collagen solution and placed in incubator at 37 ºC with 5% CO2.

Fig. 2.1 – Representative steps of the isolation and culture of human brain microvascular endothelial cells. (A) Brain sample placed in a 100 mm dish containing isolation medium; (B) removal of meninges and large vessels using sterilized surgical forceps and a stereomicroscope to facilitate the visualization; (C) fragmentation of the tissue by repeatedly sterile pipettes of 25 and 10 ml until (D) the sample can be passed effortlessly back and forth through a 5 ml pipette; (E) passage of the sample through a 500 µm polyester screen for removal of large fragments; (F) filtration of the collected fluid through a 30 µm polyester screen placed over a wire frame for support; (G) collection of the fragments retained on the screen by washing into a new dish; (H) pellet of fragments following centrifugation; (I) resuspended microvessels and introduction in a collagen coated T-25 flask.

5. TEER assessment

To assess TEER, rat BMVEC were grown on Millicell polycarbonate 0.4 μm culture plate insert 12 mm diameter for attachment-dependent cell culture acquired from Millipore (Billerica, MA, USA). HBMVEC (either fresh or cryopreserved) were grown either on Millicell hydrophilic polytetrafluoroethylene (PTFE) or on Millicell polycarbonate 0.4 μm culture plate insert 12 mm diameter for attachment-dependent cell culture acquired from Millipore (Billerica, MA, USA). TEER was measured firstly after 2 days in vitro (DIV) and daily from then on, using the epithelial voltage-ohm meter (EVOMX) and STX2 Electrode (Fig. 1.12), both purchased from World Precision Instruments, INC (Hertfordshire, UK).

6. Immunofluorescence

Immunofluorescence was performed both on fresh and cryopreserved HBMVEC, when confluence was reached. Supernatant was removed and cells were fixated with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature. Percentage of triton X-100 in permeabilization solution and incubation time varied according to primary antibody, as well as whether cells were from cell line or primary cultures (Table 2.1). For all antibodies, 3% BSA in PBS was used as blocking for 1 h at room temperature. Cells were incubated overnight at 4ºC with the relevant primary antibody (for antibodies dilution, see Table 2.1). Secondary antibodies were incubated at room temperature for 1 h (Table 2.1). Both primary and secondary antibodies were diluted in blocking solution. Nuclei were stained with Hoescht dye 33258 in PBS (1:1000) for 2 minutes. DPX was used as mountant medium. Coverslips were observed under upright microscope fitted with widefield epi-fluorescence (Zeiss, model Scope.A1) with integrated digital camera (Leica, model DFC490). In order to have negative controls, some coverslips were incubated only with secondary antibody. This assured us that the signal obtained for coverslips incubated with both primary and secondary antibodies was a result of the expression of the target proteins.

Table 2.1 – Immunofluorescence permeabilization conditions and antibodies dilution

Target Protein	Primary Antibody						Secondary antibody	
	HBMVEC line			HBMVEC primary culture				
	Permeabilization		dilution	Permeabilization		dilution	antibody	dilution
	Triton (%)	Time (min)		Triton (%)	Time (min)			
ACTA2	0.2	3	1:1000	0.2	3	1:1000	CY3	1:1000
β-catenin	0.2	3	1:100	0.2	3	1:100	CY2	1:1000
Caveolin 1	0.2	3	1:100	0.2	3	1:100	CY2	1:1000
Claudin-5	0.2	15	1:100	0.2	3	1:100	CY3	1:1000
vWF	0.2	3	1:100	0.2	3	1:100	CY2	1:1000
GLUT-1	0.2	3	1:100	0.2	3	1:100	CY2	1:1000
Occludin	0.2	15	1:100	0.2	3	1:100	CY2	1:1000
P-gp	0.2	3	1:100	0.2	3	1:20	CY3	1:1000
VE-cadherin	0.5	15	1:10	-	-	-	CY3	1:1000
ZO-1	0.5	15	1:100	0.2	3	1:100	CY2	1:1000

Abbreviations: α-SMA – α-smooth muscle actin; GLUT-1 – glucose transporter-1; HBMVEC - human brain microvascular endothelial cells; P-gp – P-glycoprotein; VE-cadherin – vascular endothelial-cadherin; vWF – von Willebrand factor; ZO-1 – *zonula occludens*-1.

Chapter 3 – Results

1. Cell culture implementation

It was a very hard working and time-consuming process to implement a primary culture of HBMVEC. We have originally adopted the isolation protocol reported by (Liu *et al.*, 2002). This uses complicated methods such as Percoll gradients and 3D gels, which require not only expensive means and available time, but also expertise. After several tries and failures, as well as attempts to contact the authors of the previously referred paper, we established contact with Dr. Michael Bernas (University of Tucson, Arizona), who was very helpful in implementing a new, simplified and less time-consuming protocol. As human brain tissue is very difficult to obtain we have initially used Wistar rat whole brain to implement the protocol. The results here presented both for rat and human cells were all originated using the protocol developed under the collaboration with Dr. Bernas.

2. Cell culture characterization

2.1. Morphological analysis of rat and human BMVEC by contrast phase microscopy

A week after the day of the isolation procedure, the first resulting cells were visible under contrast phase microscopy. Early BMVEC were spindle shaped and with random growth direction (Fig. 3.1 A). At the end of 30 DIV, however, these cells were apposed to each other and formed monolayers (Fig. 3.1 B). HBMVEC, on their hand, took at least a fortnight to be visible under contrast phase microscopy. In resemblance to rat BMVEC, these cells presented random growth direction. It appeared as though they were multiplying from within agglomerates formed at cell seeding during culture procedure (Fig. 3.1 C). Within 30 DIV, confluence was achieved and cells formed a monolayer, though with different morphology. Under contrast phase microscopy, confluence HBMVEC appeared hexagonal-like (Fig. 3.1 D).

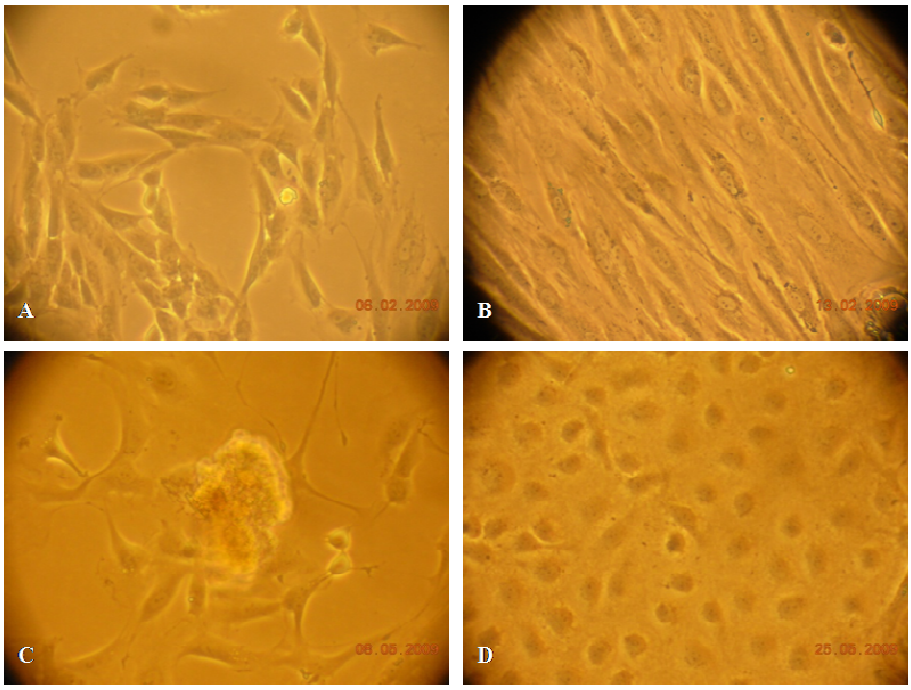


Fig. 3.1 – BMVEC primary cultures from Wistar rat at (A) 8 days and (B) 30 days, and from human brain tissue at (C) 14 days and (D) 30 days, under contrast phase microscopy. Original magnification: 100 x.

2.2. TEER and ZO-1 expression in rat and human BMVEC cultures

To better mimic barrier properties, the number of DIV after trypsinization in which studies should be performed was determined. Rat BMVEC at cell passage 1 (P1) were seeded onto polycarbonate inserts previously coated with collagen type I solution. TEER was measured daily from 2 to 11 DIV and compared to control (insert with both collagen coating and ISM, and no cells) (Fig. 3.2). TEER increased continuously through time, assuming mostly values around 100 $\Omega.cm^2$ and over, with no thresholds. Therefore, further studies were needed.

Rat BMVEC at P1 were grown on collagen coated glass coverslips with ISM, parallel to TEER measurements. Immunostaining for ZO-1 was performed on these cells after 2 DIV, 7 DIV, 9 DIV, 11 DIV, 16 DIV and 20 DIV (Fig. 3.3). A larger number of cells were visible after 7 DIV, achieving its maximum at 11 DIV. After 16 DIV cells showed spaces between them, evolving to a lesser number of cells per field at 20 DIV. These cells, however, did not express ZO-1 properly in the plasma membrane contour.

The result of 104 $\Omega.cm^2$ at 4 DIV is out of frame with those obtained for previous and following days. This might be due to a misreading in control, once in Figure 3.2 A this result is under average, whilst BMVEC TEER is in frame with the remaining results.

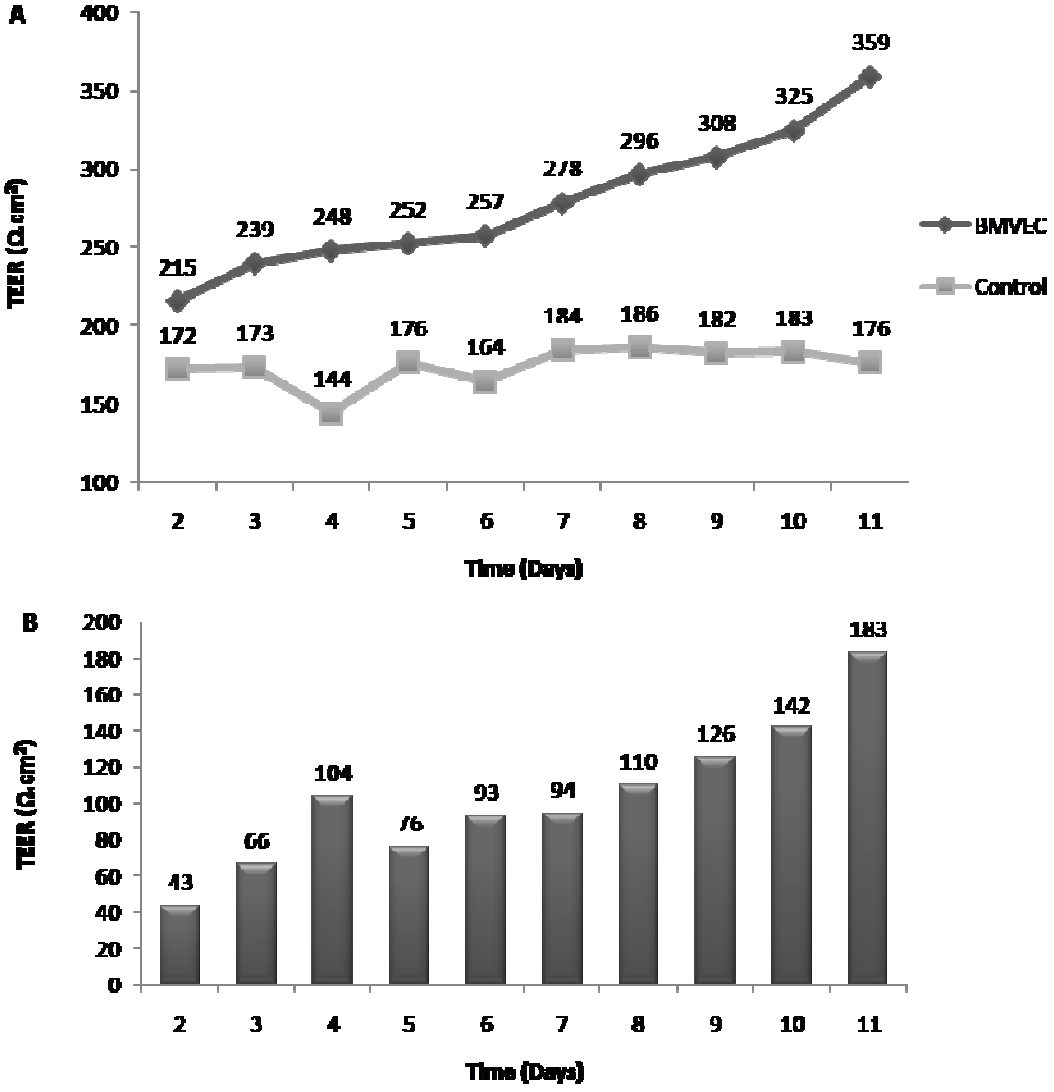


Fig. 3.2 – Daily transendothelial electrical resistance (TEER) measuring for rat brain microvascular endothelial cells (BMVEC) in primary culture at cell passage 1, with isolation supplemented medium (ISM) after 2 days *in vitro*. (A) Absolute values for BMVEC and control (insert with ISM and no BMVEC). (B) Difference between TEER values for BMVEC and control.

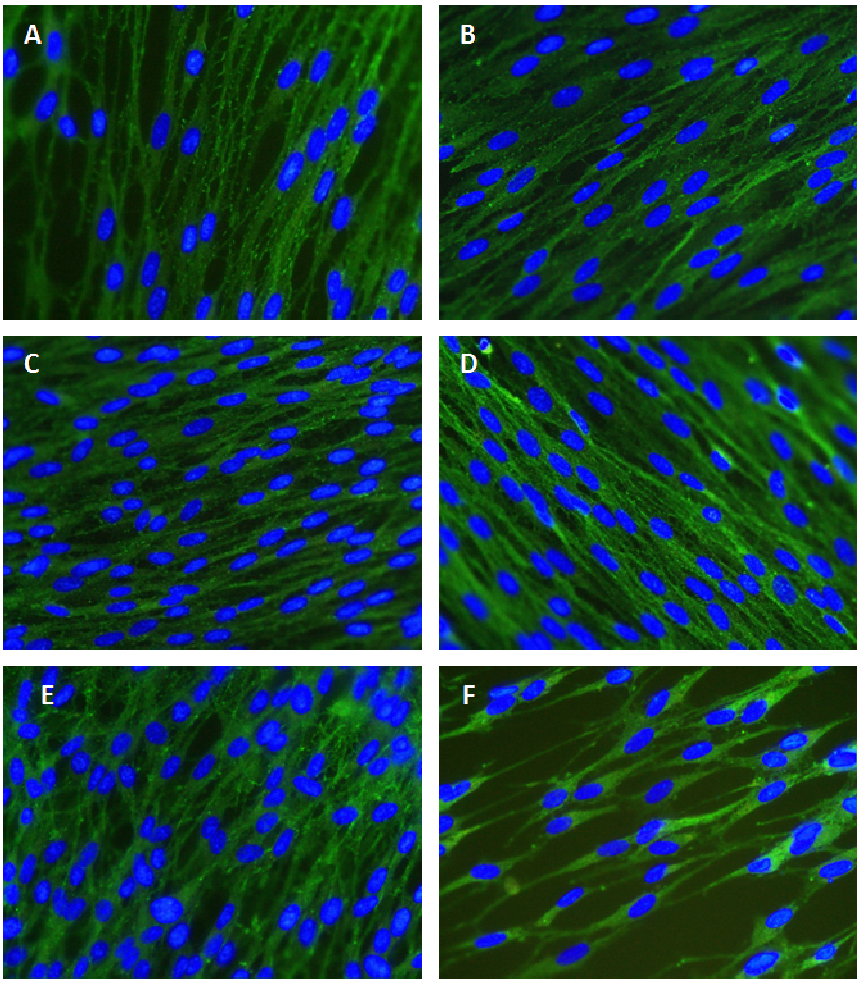


Fig. 3.3 – Immunofluorescence microscopy of rat brain microvascular endothelial cells (BMVEC) in primary culture at cell passage 1, for *zonula occudens*-1 after (A) 2 days *in vitro* (DIV), (B) 7 DIV, (C) 9 DIV, (D) 11 DIV, (E) 16 DIV and (F) 20 DIV with isolation supplemented medium. Original magnification: 630 x.

There are studies which claim that, after cells achieve confluence, the addition of HC to growth medium increases expression of TJ proteins and, therefore, enhance barrier properties (Förster *et al.*, 2008). So, after 11 DIV, rat BMVEC on both inserts and glass coverslips were incubated another 5 DIV with i) ISM, ii) ISM without ECGS, iii) ISM without ECGS and with HC, and the resulting TEER measurements were compared to control (Fig. 3.4). After 1 DIV cells had similar TEER values in spite of the different mediums, even though those without ECGS in medium composition (either with or without HC) showed a slight increase. This became more evident after 2 and 3 DIV, with ISM with HC reporting the highest values of 291 $\Omega.cm^2$ (50 $\Omega.cm^2$ over ISM without ECGS and 132 $\Omega.cm^2$ over ISM). After 4 DIV TEER values decreased, dropping to less than 100 $\Omega.cm^2$ at the end of 5 DIV.

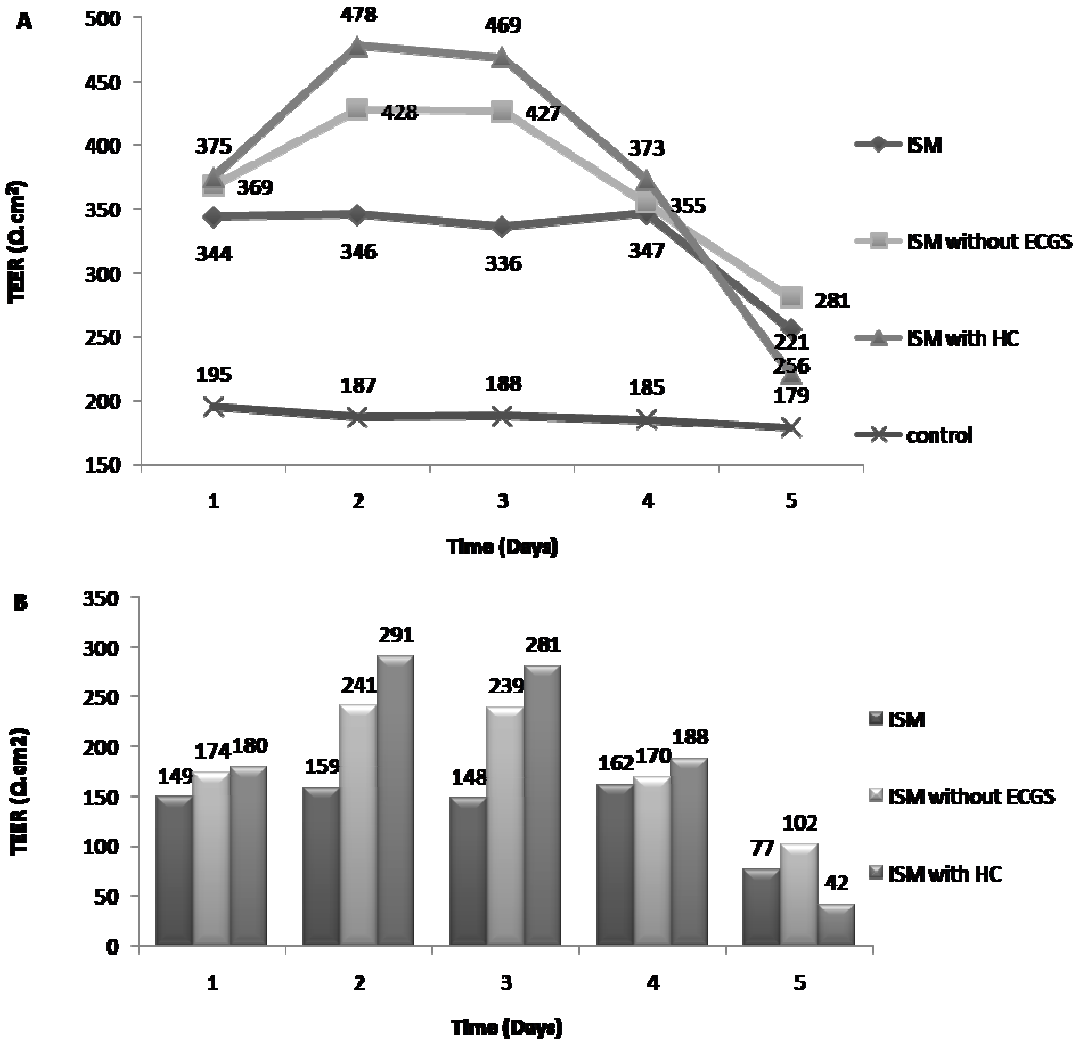


Fig. 3.4 – Daily transendothelial electrical resistance (TEER) measuring for rat brain microvascular endothelial cells (BMVEC) in primary culture at cell passage 1, with different media (after 11 days *in vitro*). (A) Absolute values for BMVEC with isolation supplemented medium (ISM), BMVEC with ISM without endothelial cell growth supplement (ECGS), BMVEC with ISM without ECGS and with hydrocortisone (HC), and control (insert with ISM and no BMVEC). (B) Difference between TEER values for BMVEC and control for each medium.

To confirm the enhancement of barrier properties, rat BMVEC grown in parallel on glass coverslips were stained for ZO-1 (Fig. 3.5). With ISM, as in previous results (Fig. 3.3), cells did not express ZO-1 in the plasma membrane and showed spaces between them. When ECGS was removed from medium after 11 DIV, not only was ZO-1 expressed at TJ site, as well as BMVEC were closer apposed to each other, enhancing barrier properties. As for the addition of HC to medium, there was no expression for ZO-1 and the majority of cells had died.

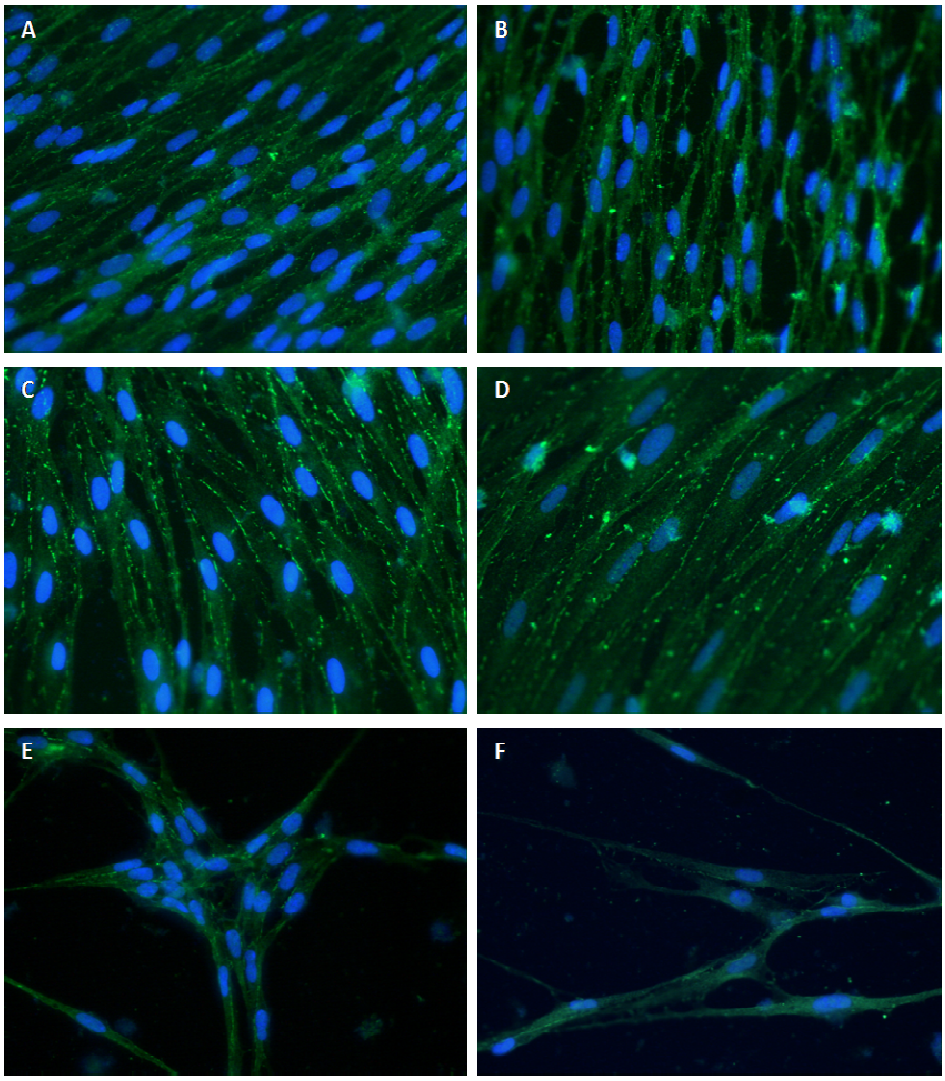


Fig. 3.5 – Immunofluorescence microscopy of rat brain microvascular endothelial cells (BMVEC) in primary culture for ZO-1, after 11 days *in vitro (*DIV) with isolation supplemented medium (ISM) followed by (A) 3 DIV and (B) 5DIV with ISM; (C) 3 DIV and (D) 5 DIV with ISM without endothelial cell growth supplement; (E) 3 DIV and (F) 5 DIV with ISM with hydrocortisone. Original magnification: 630 x.

Given the above mentioned results, we then proceeded to HBMVEC (at P1) studies. First TEER measurements were performed on PTFE inserts with collagen coating and with ISM. We have chosen to perform TEER measurements for HBMVEC in these inserts as once these are clear, it would allow to also monitor cell expansion under contrast phase microscopy. Studies showed maximum values of around 15 $\Omega.cm^2$ (difference between TEER values for HBMVEC and control) at 3 DIV (Fig. 3.6 A,B), quickly dropping afterwards to results similar to those measured for control (coated inserts with no cells). Under contrast phase microscopy, at the time of seeding, cells were apposed to each other in resemblance to monolayer. However, just after 4 DIV, a larger number of cells had died and detached from the insert surface (Fig. 3.6 C,D).

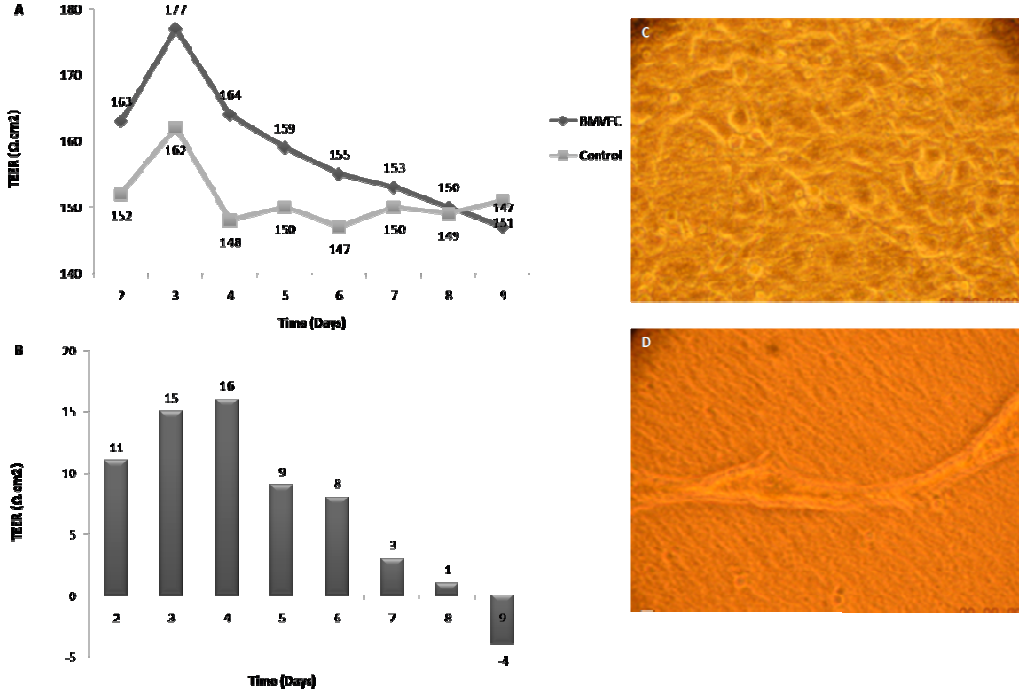


Fig. 3.6 – Daily transendothelial electrical resistance (TEER) measuring for human brain microvascular endothelial cells (HBMVEC) in primary culture after 2 days *in vitro*. (A) Absolute values for HBMVEC and control (insert with isolation supplemented medium and no HBMVEC). (B) Difference between TEER values for HBMVEC and control. HBMVEC primary culture under contrast phase microscopy on the day of seeding on the inserts is represented in C and 4 days later in D. Original magnification: 100 x.

New TEER measurements were then taken regarding another type of inserts, with different membrane composition (polycarbonate) (Fig. 3.7), used previously in rat BMVEC TEER measurements. TEER increased gradually ranging from a minimum of 46 $\Omega.cm^2$ at the time of seeding to 94 $\Omega.cm^2$ after 6 DIV, reaching a threshold from herein on. To evaluate whether HBMVEC barrier properties resembled those in rat BMVEC, ECGS was removed from medium for 2 DIV for both inserts and coverslips. TEER was measured daily and HBMVEC on coverslips were stained for ZO-1. Unlike rat BMVEC, TEER for HBMVEC did not increase for both mediums, maintaining the same values as on the previous days. Immunofluorescence results showed that with regular ISM and at the time of threshold, HBMVEC were apposed to each other, presented a hexagonal-like shape and expressed ZO-1 at all TJ sites, delimiting cell boundaries (Fig. 3.8 A). On the other hand, those with the same medium and more DIV, though expressing ZO-1 at TJ sites, showed larger spaces between cells (Fig. 3.8 B). Such spaces tend to enlarge when removing ECGS from medium (Fig. 3.8 C). Therefore and according with these results, to better mimic barrier properties, we have worked with 8 DIV cells after seeding with ISM.

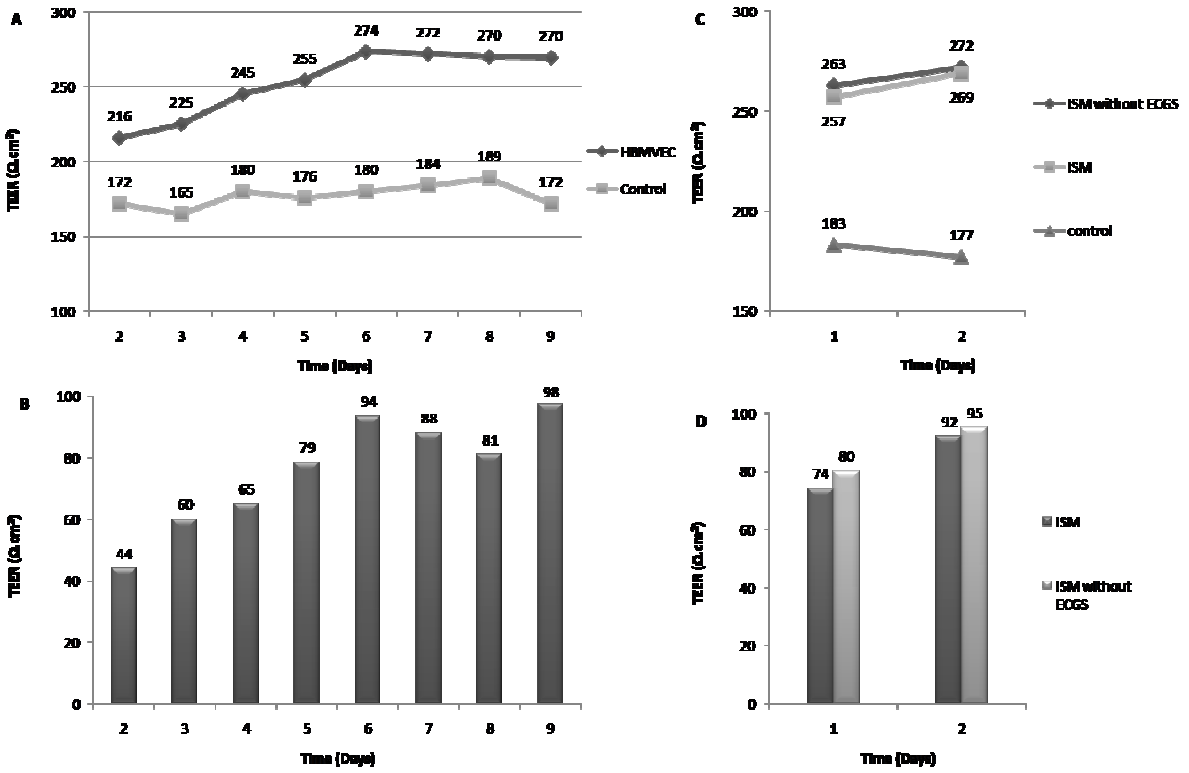


Fig. 3.7 – Daily transendothelial electrical resistance (TEER) measuring for human brain microvascular endothelial cells (HBMVEC) in primary culture cell passage 1, after 2 days *in vitro* (DIV). (A) Absolute values for HBMVEC and control [insert with isolation supplemented medium (ISM) and no HBMVEC]. (B) Difference between TEER values for HBMVEC and control. (C) Absolute values for HBMVEC and control (insert with ISM and no HBMVEC) incubated for 2 DIV with either ISM or ISM without ECGS, following 9 DIV with ISM in A. (D) Difference between TEER values for HBMVEC and control when incubated with either ISM or ISM without ECGS, following 9 DIV with ISM in B.

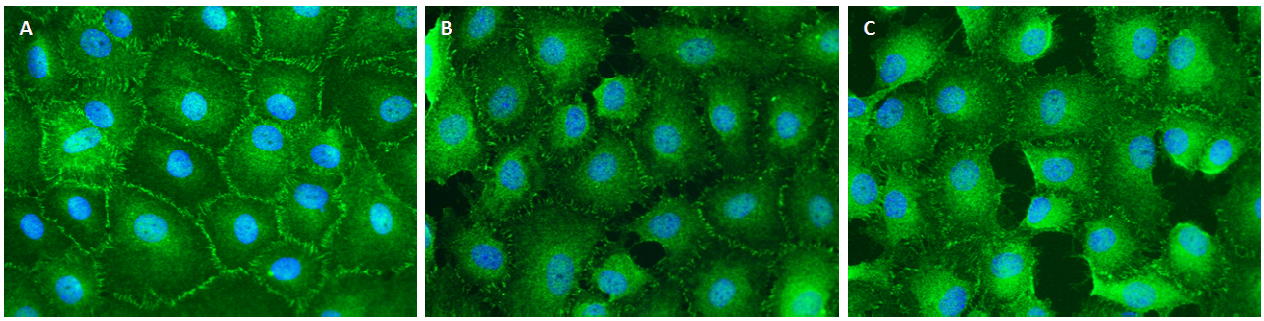


Fig. 3.8 – Immunofluorescence microscopy of human brain microvascular endothelial cells in primary culture. (A) Staining for *zonula occludens*-1 (ZO-1) after 9 days *in vitro* (DIV). (B) Staining for ZO-1 after 9 DIV followed by 2 DIV with isolation supplemented medium (ISM). (C) Staining for ZO-1 after 9 DIV followed by 2 DIV with ISM without ECGS. Original magnification: 630 x.

2.3. Immunofluorescence analysis of purity and phenotype of HBMVEC cultures

To characterize HBMVEC primary culture, cells were stained for specific EC markers GLUT-1 and vWF, and for pericytes marker α-SMA. At P1, HBMVEC highly expressed GLUT-1 and vWF, with little contamination from pericytes (Fig. 3.9). In fact, analysis of 50 randomly selected fields revealed that only 3% of the cells stained for α-SMA.

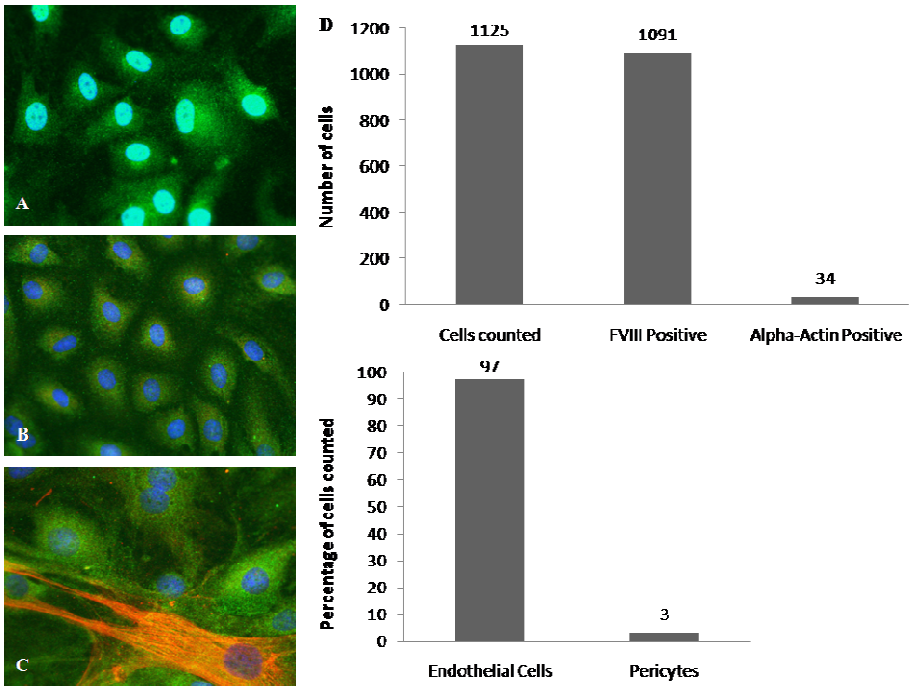
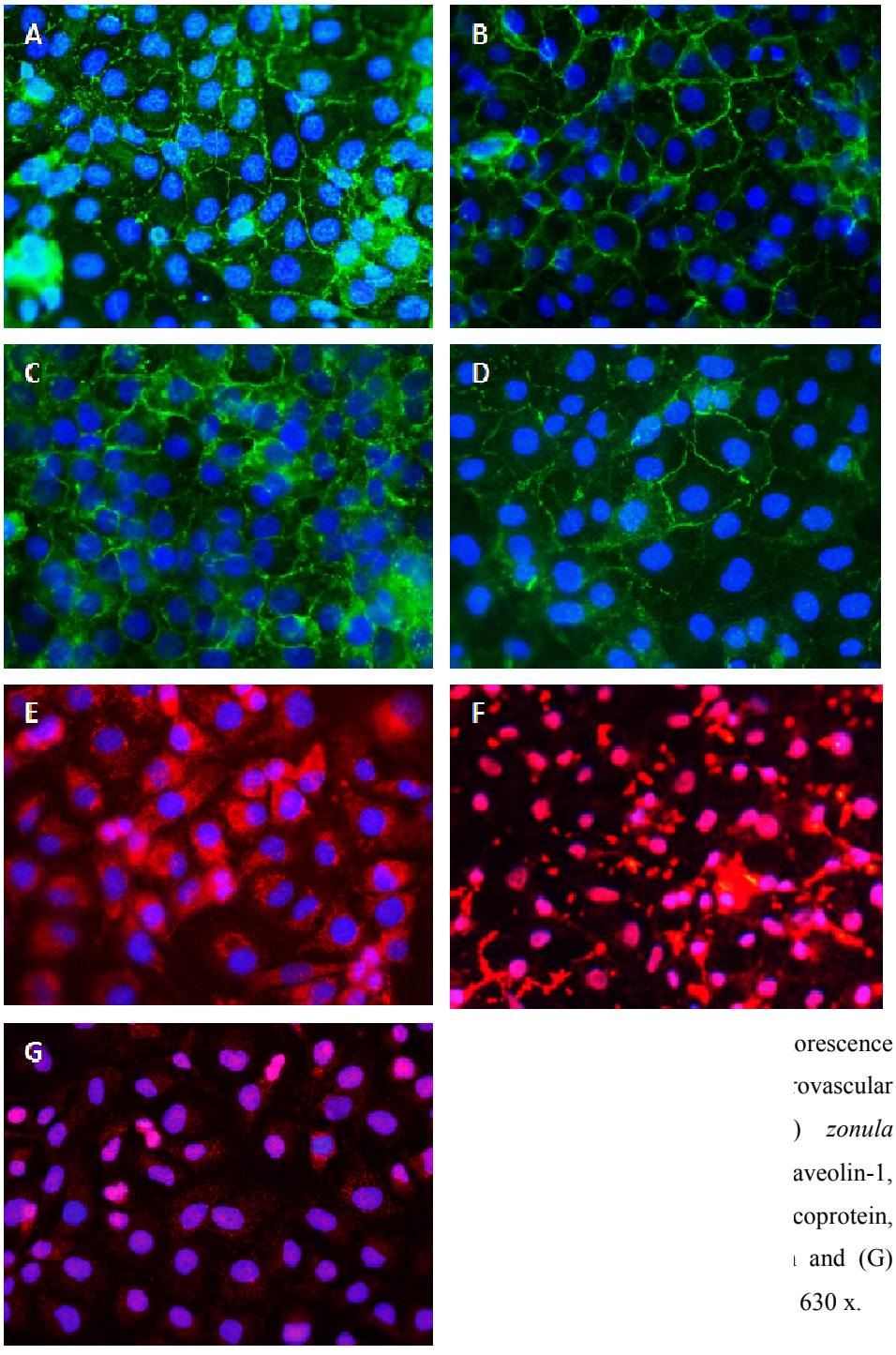


Fig. 3.9 – Characterization of human brain microvascular endothelial cells primary culture. Immunofluorescence microscopy for (A) glucose transporter-1 and (B, C) double staining for von Willebrand factor and α-smooth muscle actin (α-SMA). In (C) it is shown in red a pericyte with positive staining for α-SMA. For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for vWF and for α-SMA, was counted in 50 randomly selected fields (D). Original magnification: 630 x.

As stated earlier, human samples are difficult to obtain. Bearing this into account, the optimization process for immunofluorescence analysis was on a HBMVEC line available in the laboratory. To determine barrier properties, cells were stained for TJ proteins ZO-1, occludin and claudin-5. AJ components β-catenin and VE-cadherin were also determined, as well as transport molecules caveolin-1 and P-gp (Fig. 3.10). Immunofluorescence conditions were optimized for ZO-1, β-catenin, caveolin-1, occludin and P-gp. Results for VE-cadherin are represented in Figure 3.10 F. Only after the addition of calcium to the antibody solution fine lines were visible

delimiting the cells. Several variations were made during optimization for claudin-5: percentage of Triton X-100 and time of exposure to the permeabilizing solution, composition of blocking solution, and incubation time with both primary and secondary antibodies. However, in spite of all the variations applied we were unable to obtain proper staining for this protein (Fig. 3.10 G). Therefore, we have discarded these two proteins from HBMVEC characterization.



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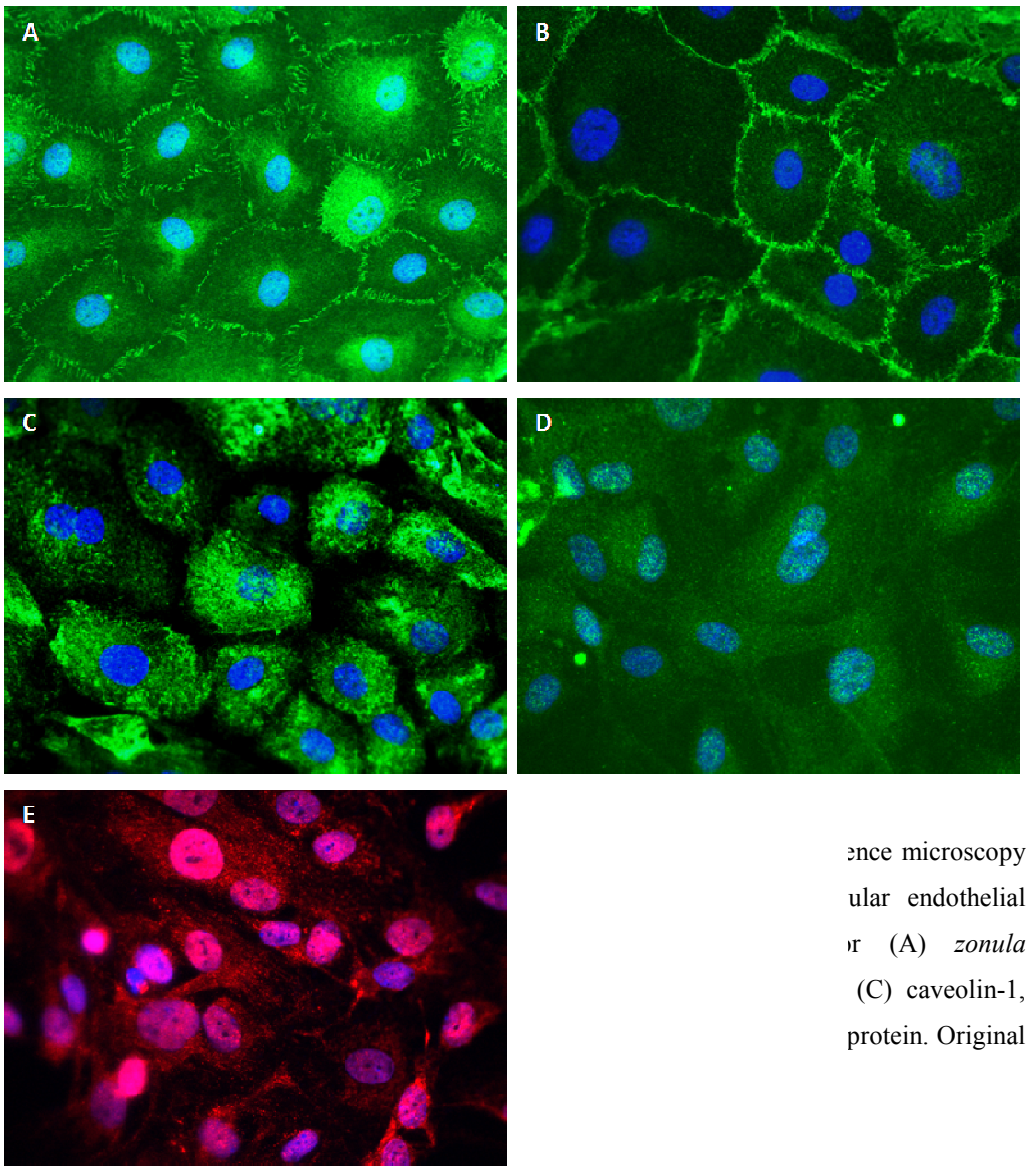
When applying immunofluorescence results to primary HBMVEC, our results showed that in P1 (Fig. 3.11) these cells expressed both ZO-1 (as already shown in Figure 2.2.) and β-catenin at TJ and AJ sites, respectively, and perfectly delimiting cell boundaries and connecting neighbouring cells. Caveolin-1 was also highly expressed. P-gp was also expressed by these cells. Occludin, in spite of several attempts of optimization, was not found in primary HBMVEC contour but disperse in the cytoplasm.



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ular endothelial
or (A) *zonula*
(C) caveolin-1,
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3. Evaluation of TEER and phenotype in HBMVEC along passages

To characterize the cell passages following P1, HBMVEC derived from primary cultures were observed under contrast phase microscopy to determine cell morphology. These were also stained for specific EC markers GLUT-1 and vWF, and for the pericytes marker α-SMA to determine cell contamination. To evaluate barrier properties, TEER was assessed and expression of ZO-1, β-catenin, caveolin-1 and P-gp determined by immunofluorescence.

3.1. Cell passage 2 (P2)

At P2, HBMVEC under contrast phase microscopy showed no apparent difference in morphology from cells at P1, with hexagonal-like shape and forming monolayers. Immunofluorescence results showed expression for both GLUT-1 and vWF, though with a strong decrease in total cell number and with a few spaces in between cells. Still, there was little contamination from pericytes as only 5% of the cells stained for α-SMA, in 50 randomly selected fields (Fig. 3.12).

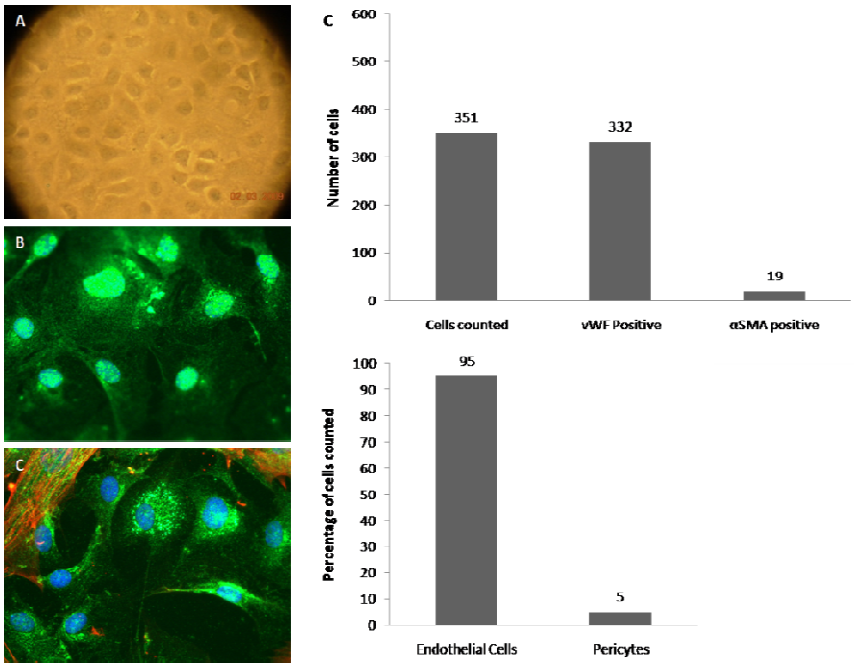

Fig. 3.12 – Characterization of human brain microvascular endothelial cells primary culture at P2 by (A) contrast phase microscopy and immunofluorescence microscopy for (B) glucose transporter-1 and (C) double staining for von Willebrand factor and α-smooth muscle actin (α-SMA). In (C) it is shown in red a pericyte with positive staining for α-SMA. For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for vWF and for α-SMA, was counted in 50 randomly selected fields (D). Original magnification: 630 x.

TEER assessment results for P2 (Fig. 3.13) were very similar to those in P1. TEER increased gradually ranging from a minimum of 53 $\Omega.cm^2$ at the time of seeding to a maximum of 98 $\Omega.cm^2$ after 7 DIV. As for immunofluorescence assay (Fig. 3.14), our results showed that HBMVEC also express both ZO-1 and β-catenin at TJ and AJ sites, respectively, delimiting cell boundaries and connecting neighbouring cells. Even though not as highly as in P1, both caveolin-1and P-gp were expressed in these cells.

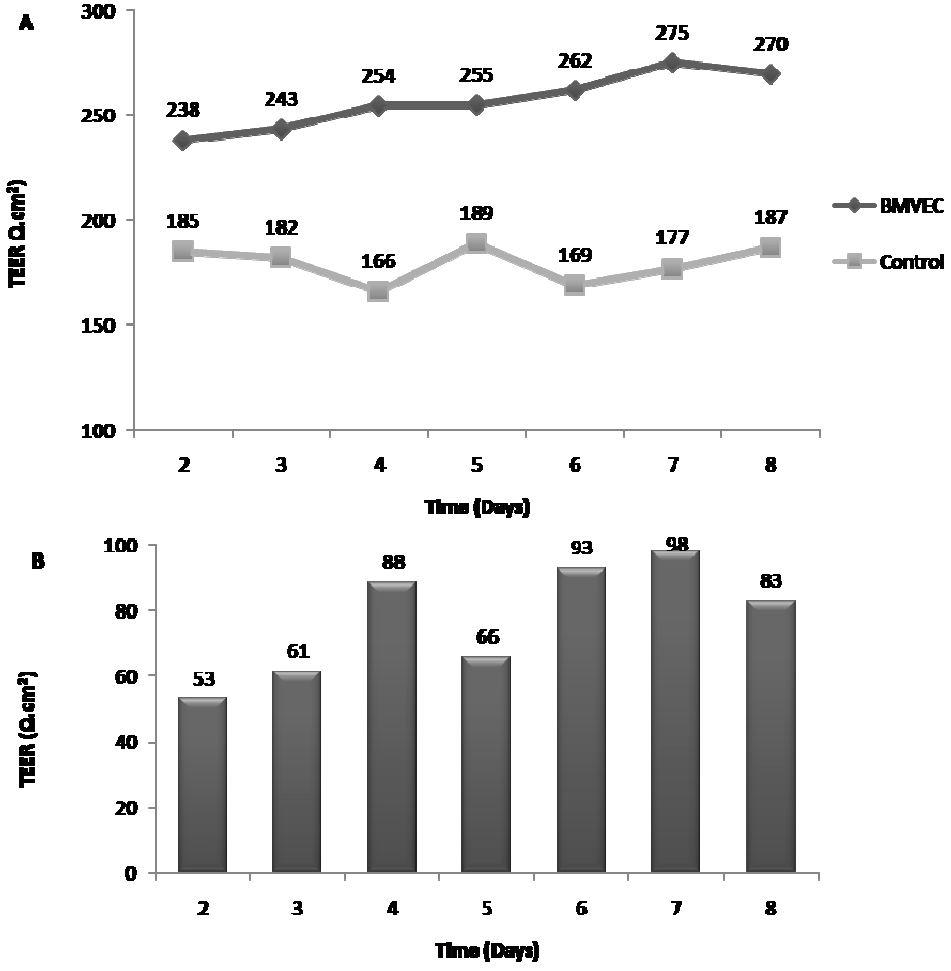

Fig. 3.13 – Daily transendothelial electric resistance (TEER) measuring for human brain microvascular endothelial cells (HBMVEC) primary culture at P2, after 2 days *in vitro*. (A) Absolute values for HBMVEC and control (insert with isolation supplemented medium and no HBMVEC). (B) Difference between TEER values for HBMVEC and control.

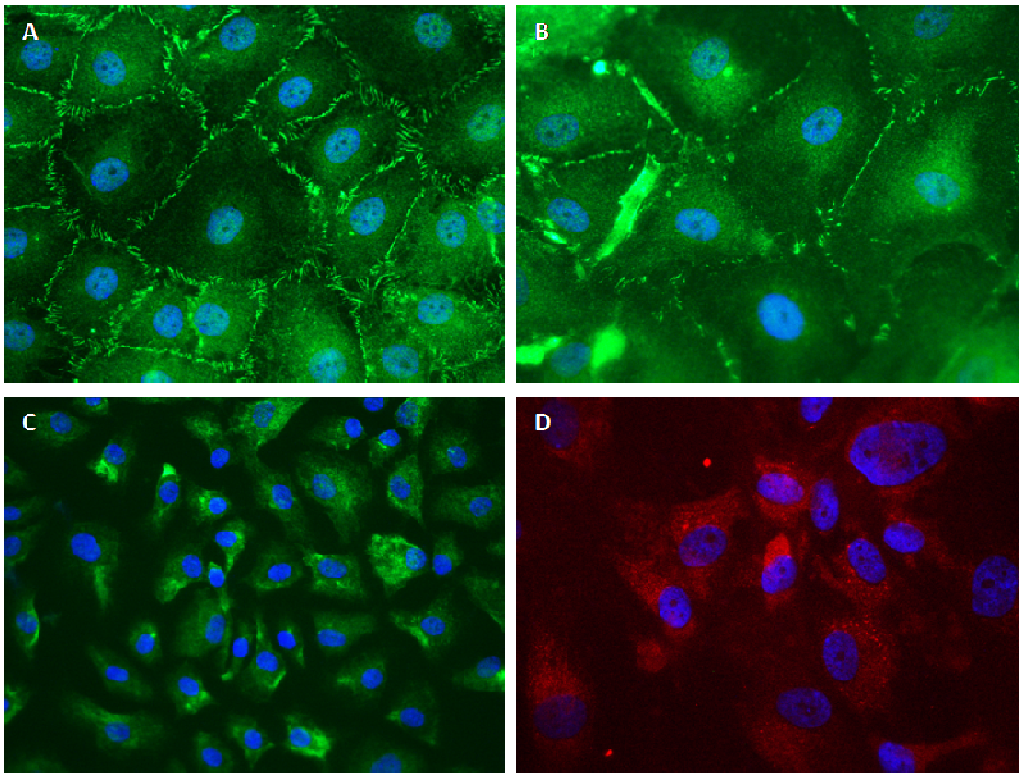

Fig. 3.14 – Immunofluorescence microscopy of human brain microvascular endothelial cells primary culture at P2 for (A) *zonula occludens*-1, (B) β-catenin, (C) caveolin-1 and (D) P-glicoprotein. Original magnification: 630 x.

3.2. Cell passage 3 (P3)

Following the third trypsinization, HBMVEC began to show differences in morphology from P1 and P2 under contrast phase microscopy. Most cells were still hexagonal-like shaped and did form monolayers. Nevertheless, a large number of endothelial cells with sickled-like morphology and not apposed to each other were visible. Immunofluorescence results showed expression for both GLUT-1 and vWF. There were in total 377 cells in 50 randomly selected fields which is similar to the previous passage (351 cells). Contamination by pericytes was of 7% (Fig. 3.15).

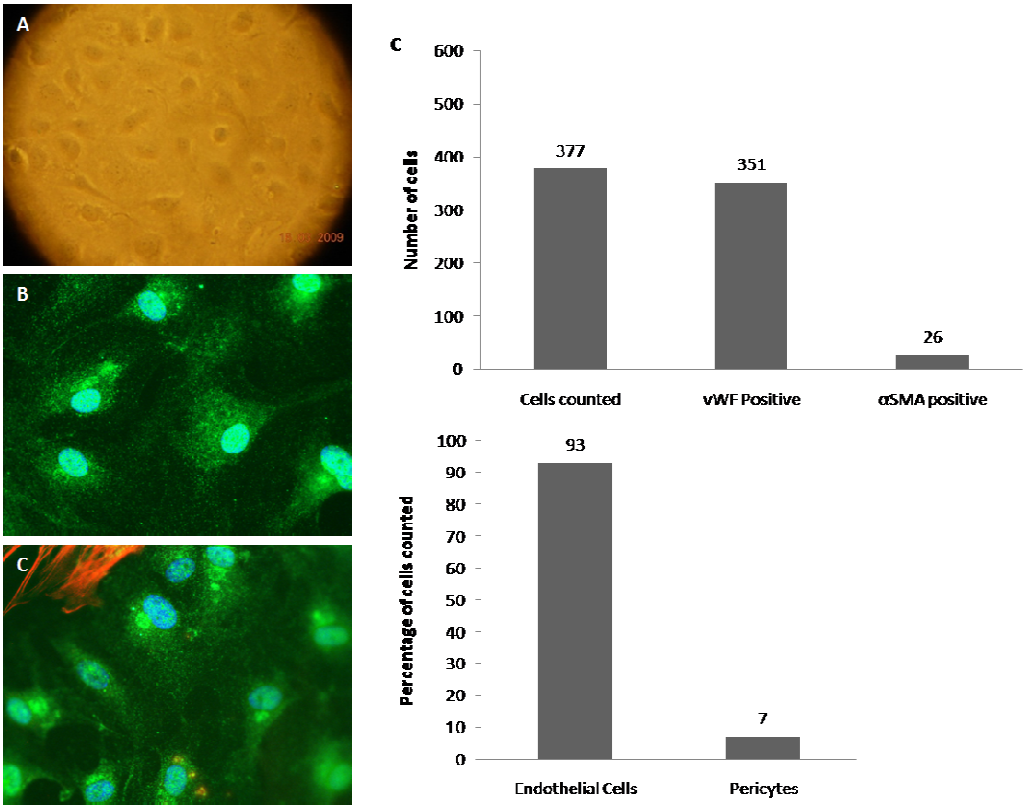

Fig. 3.15 – Characterization of human brain microvascular endothelial cells primary culture at P2 by (A) contrast phase microscopy and immunofluorescence microscopy for (B) glucose transporter-1 and (C) double staining for von Willebrand factor and α-smooth muscle actin (α-SMA). In (C) it is shown in red a pericyte with positive staining for α-SMA. For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for vWF and for α-SMA, was counted in 50 randomly selected fields (D). Original magnification: 630 x.

TEER assessment results for P3 (Fig. 3.16) revealed lower values than in previous passages. TEER increased gradually from a minimum of 28 $\Omega.cm^2$ at the time of seeding to a maximum of 78 $\Omega.cm^2$ after 7 DIV. This result is 20 $\Omega.cm^2$ less than P1 and P2, but the first TEER measurement also decreased approximately 20 $\Omega.cm^2$. Therefore, this result is still similar to those obtained for previous passages. Our immunofluorescence results (Fig. 3.17) showed that ZO-1 and β-catenin still remained in TJ and AJ sites delimiting cell boundaries of neighbouring cells. However, it was visible the presence of few spaces between some of the cells. Caveolin-1 and P-gp seemed to have not suffered notorious differences in expression.

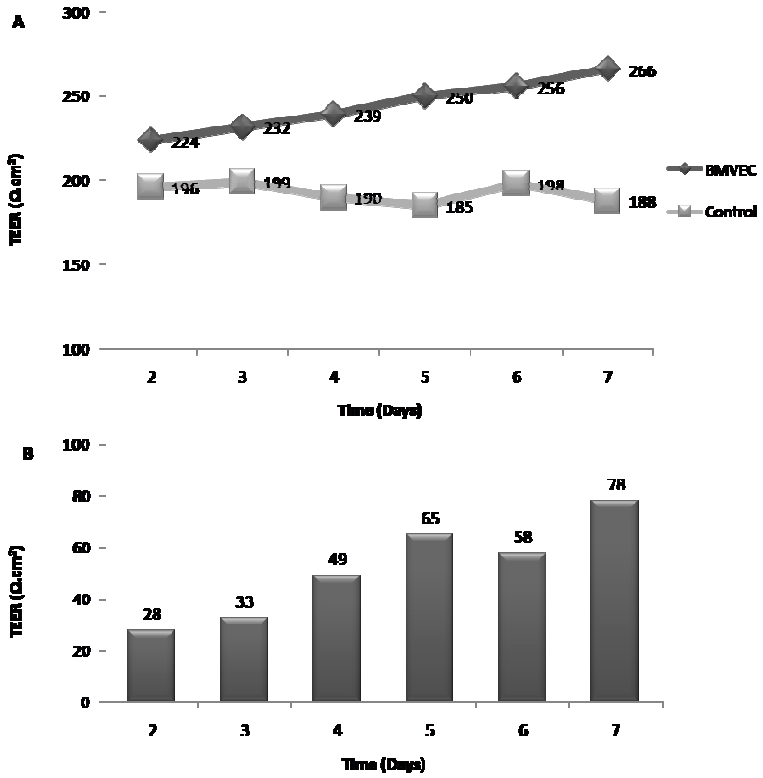

Fig. 3.16 – Daily transendothelial electric resistance (TEER) measuring for human brain microvascular endothelial cells (HBMVEC) primary culture at P3. (A) Absolute values for HBMVEC and control (insert with isolation supplemented medium and no HBMVEC). (B) Difference between TEER values for HBMVEC and control.

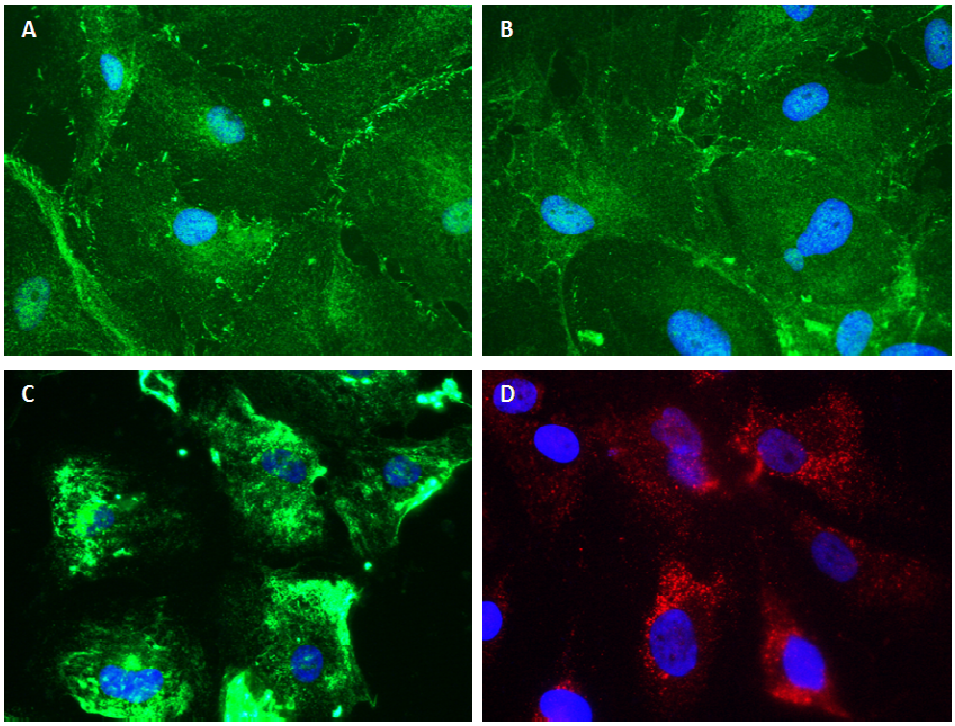

Fig. 3.17 – Immunofluorescence microscopy of human brain microvascular endothelial cells primary culture at P3 for (A) *zonula occludens*-1, (B) β-catenin, (C) caveolin-1 and (D) P-glicoprotein. Original magnification: 630 x.

3.3. Cell passage 4 (P4)

Under contrast phase microscopy, HBMVEC were in its majority sickled-like shaped and further separated from each other. Immunofluorescence results showed expression of GLUT-1 and a decrease in expression of vWF. Total number of cells was of 301, of which 38 were positive for α-SMA, resulting in a contamination by pericytes of 13% (Fig. 3.18).

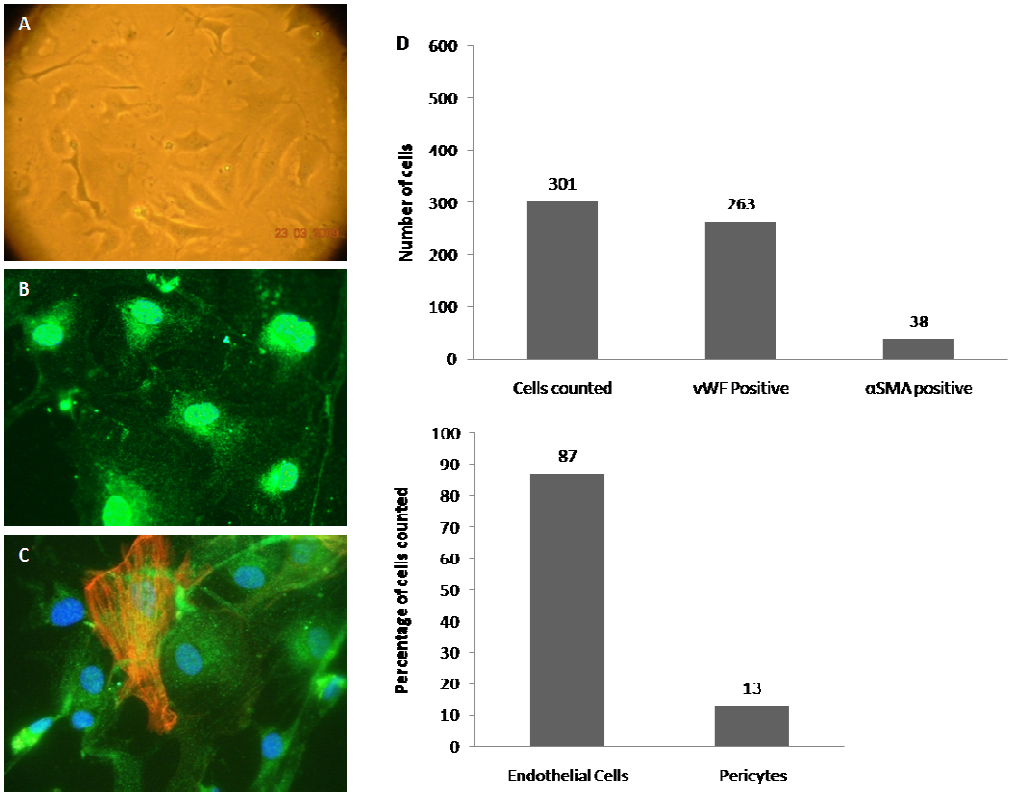

Fig. 3.18 – Characterization of human brain microvascular endothelial cells primary culture at P2 by (A) contrast phase microscopy and immunofluorescence microscopy for (B) glucose transporter-1 and (C) double staining for von Willebrand factor and α-smooth muscle actin (α-SMA). In (C) it is shown in red a pericyte with positive staining for α-SMA. For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for vWF and for α-SMA, was counted in 50 randomly selected fields (D). Original magnification: 630 x.

For P4, TEER slowly increased from a minimum of 23 $\Omega.cm^2$ at the time of seeding to a maximum of 52 $\Omega.cm^2$ after 9 DIV (Fig. 3.19). In P3 this value was reached at the end of 4 DIV. Immunofluorescence revealed that ZO-1 expression underwent a larger decrease than β-catenin (Fig. 3.20). Caveolin-1 and P-gp had also decreased their expression.

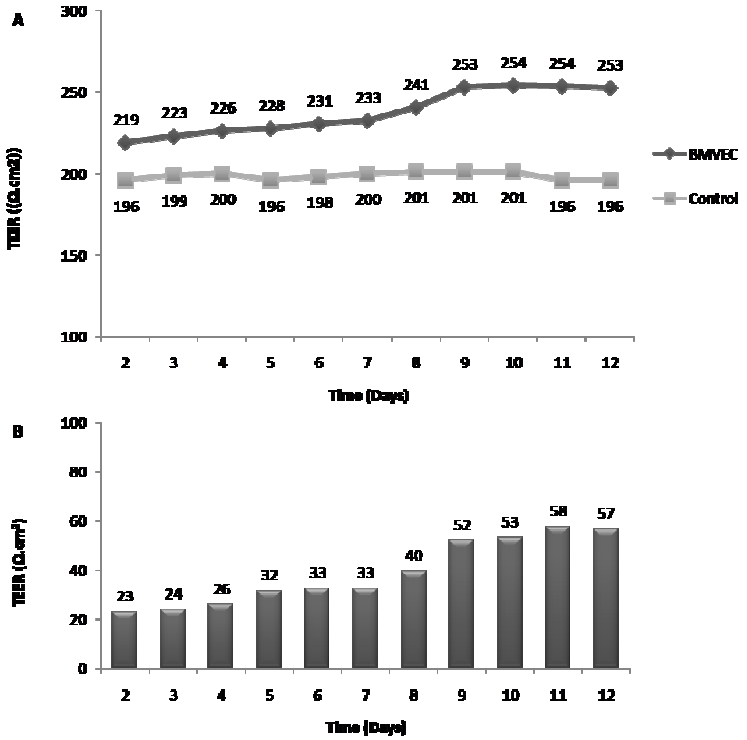

Fig. 3.19 – Daily transendothelial electric resistance (TEER) measuring for human brain microvascular endothelial cells (HBMVEC) primary culture P4, after 2 days *in vitro*. (A) Absolute values for HBMVEC and control (insert with isolation supplemented medium and no HBMVEC). (B) Difference between TEER values for HBMVEC and control.

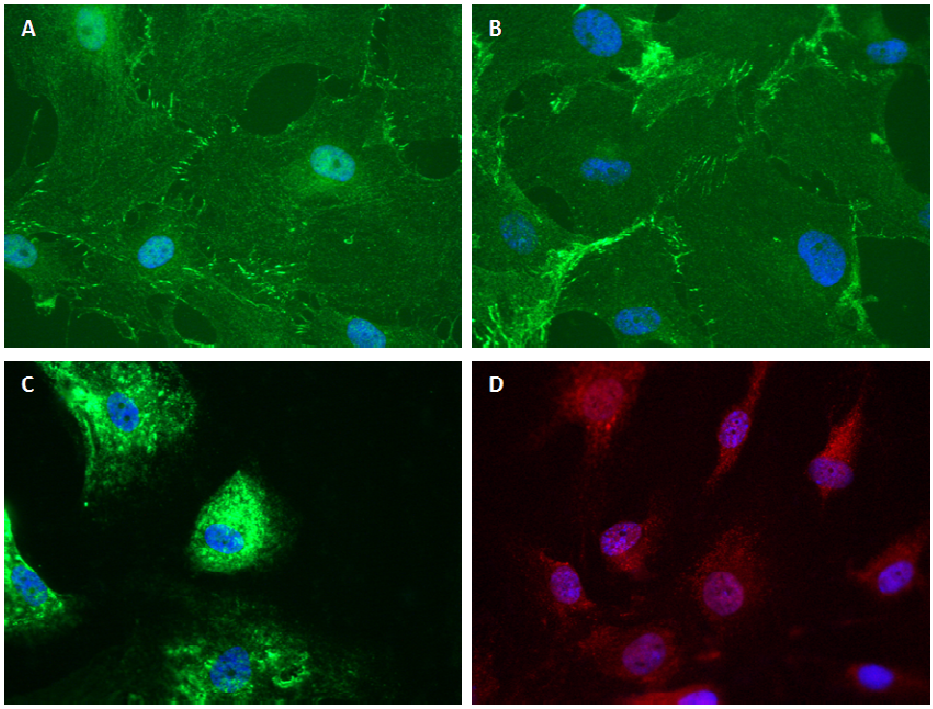

Fig. 3.20 – Immunofluorescence microscopy of human brain microvascular endothelial primary culture at P4 for (A) *zonula occludens*-1, (B) β-catenin, (C) caveolin-1 and (D) P-glicopreotein. Original magnification: 630 x.

3.4. Cell passage 5 (P5)

HBMVEC in P5 appeared to grow in random directions and further separate from one another, under contrast phase microscopy. GLUT-1 expression was very dim and total number of cells decreased to 227 of which 39 were positive for α-SMA. This resulted in a contamination by pericytes of 17% (Fig. 3.21).

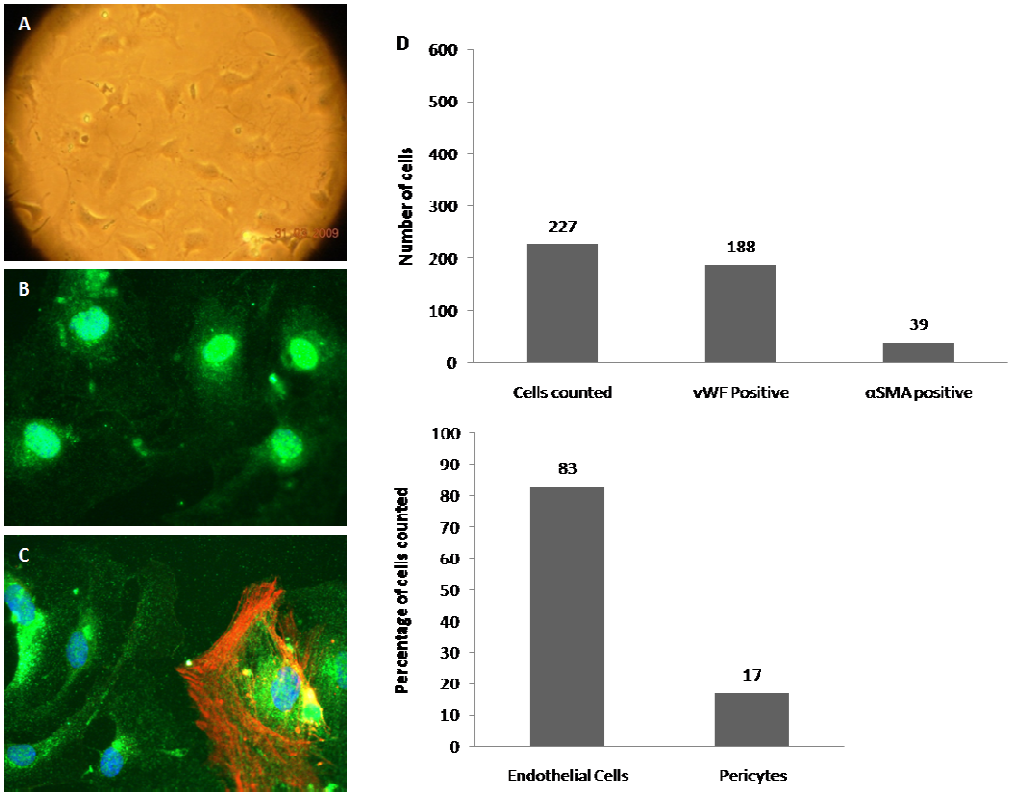

Fig. 3.21 – Characterization of human brain microvascular endothelial cells primary culture at P2 by (A) contrast phase microscopy and immunofluorescence microscopy for (B) glucose transporter-1 and (C) double staining for von Willebrand factor and α-smooth muscle actin (α-SMA). In (C) it is shown in red a pericyte with positive staining for α-SMA. For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for vWF and for α-SMA, was counted in 50 randomly selected fields (D). Original magnification: 630 x.

Results for P5 TEER assessment (Fig. 3.22) revealed similar maximum values to P4 (78 $\Omega.cm^2$ at 6 DIV), though with a minimum of 13 $\Omega.cm^2$. Immunofluorescence (Fig. 3.23) revealed barely any expression for ZO-1. Nevertheless, β-catenin was still expressed at AJ sites, connecting neighbouring cells. Caveolin-1 and P-gp seemed to have not diminished expression from one passage to another.

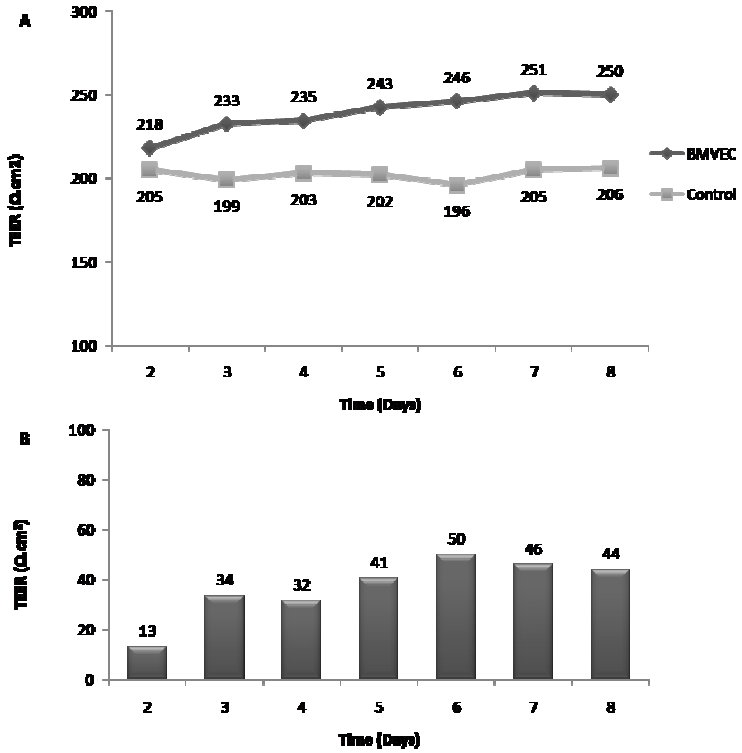

Fig. 3.22 – Daily transendothelial electric resistance (TEER) measuring for human brain microvascular endothelial cells (HBMVEC) primary culture P5, after 2 days *in vitro*. (A) Absolute values for HBMVEC and control (insert with isolation supplemented medium and no HBMVEC). (B) Difference between TEER values for HBMVEC and control.

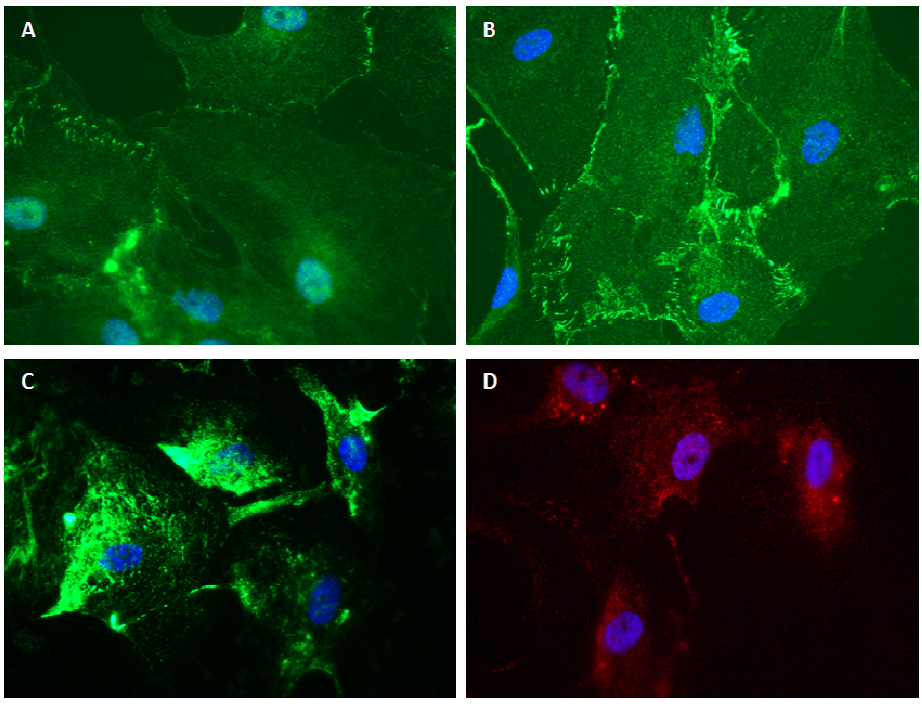

Fig. 3.23 – Immunofluorescence microscopy of human brain microvascular endothelial cells primary culture P5 for (A) *zonula occludens*-1, (B) β-catenin, (C) caveolin-1 and (D) P-glicoprotein. Original magnification: 630 x.

4. HBMVEC cryopreservation

Following trypsinization, HBMVEC were cryopreserved at -80 ºC. After 30 days, cells were thawed and seeded onto a collagen coated T-25 flask. Given that human brain samples are difficult to obtain, as stated before in this thesis, all cryopreserved cells were used in immunofluorescence studies once the small amount of cells obtained did not allow us to perform both immunofluorescence and TEER measurements. As cells were thawed at P1, this passage could only be characterized by its morphology under contrast phase microscopy. Confluence was achieved 15 DIV after seeding (Fig. 3.24). As previous to cryopreservation, HBMVEC were closely apposed to each other forming a monolayer. Nonetheless, most cells were not hexagonal-like shaped as in comparison to P1 before cryopreservation, but more elongated.

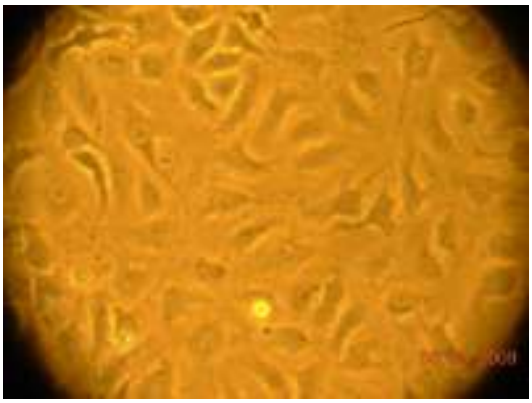

Fig.3.24 – Contrast phase microscopy from human brain microvascular endothelial cells primary culture cryopreserved for 30 days in liquid nitrogen at -80 ºC. Confluence was achieved 15 days after thawing. Original magnification: 100 x.

4.1. Morphological analysis of cryopreserved HBMVEC by contrast phase microscopy

Following trypsinization, HBMVEC at P2 under contrast phase microscopy showed differences in morphology from P1 cells. EC did form monolayers, but their shape had evolved more sickled-like. P3 cryopreserved HBMVEC were very similar to P2. P4 and P5 cryopreserved HBMVEC appeared to have large breaches between them (Fig. 3.25).

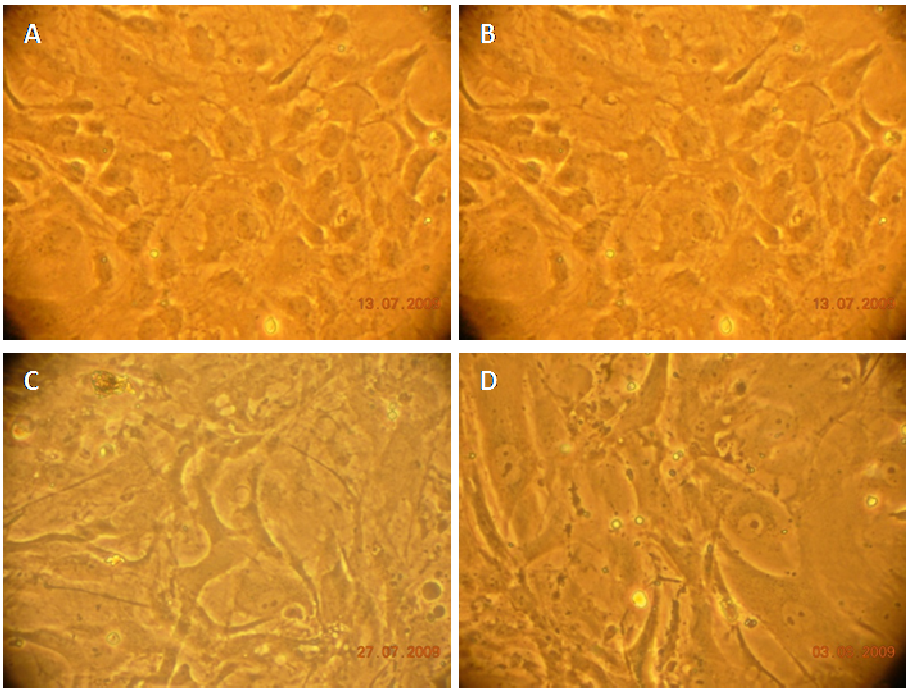

Fig.3.25 – Contrast phase microscopy from human brain microvascular endothelial cells primary culture cryopreserved for 30 days in liquid nitrogen at -80 ºC, at cell passages P2 (A), P3 (B), P4 (C) and P5 (D). Original magnification: 100 x.

4.2. Immunofluorescence analysis of purity of HBMVEC cultures

Cryopreserved cells at P2 had little contamination from pericytes as only 2% stained for α-SMA. There was still little contamination from pericytes at P3 given that only 28 cells were positive for α-SMA, representing 4% of contamination. At P4, the level of contamination was the same as in non-cryopreserved cells at P3, with 7% of pericytes. The main difference appeared at P5 as 37 out of 354 cells stained for pericytes marker. This meant a 10% contamination with cells other than HBMVEC.

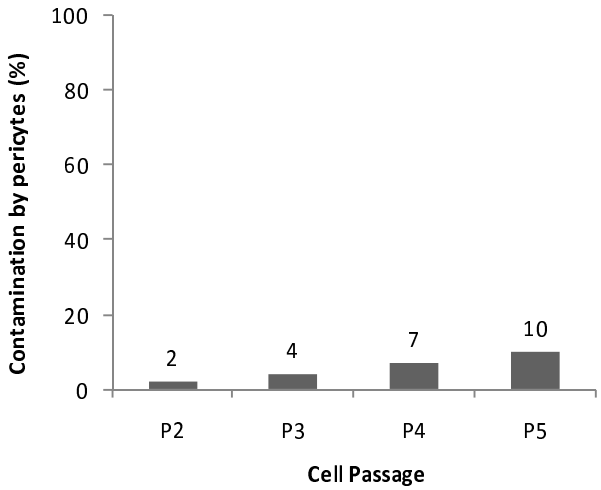

Fig. 3.26 – For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for von Willebrand factor and for α-smooth muscle actin, was counted in 50 randomly selected fields (D). Original magnification: 630 x.

4.3. Immunofluorescence analysis of phenotype of HBMVEC cultures after cryopreservation

To determine the phenotype of cryopreserved HBMVEC, cells were stained for endothelial cell marker vWF, TJ protein ZO-1, AJ component β-catenin, vesicular transport protein caveolin-1 and ABC transporter P-gp. Immunofluorescence results showed strong expression for vWF at P2, slightly decreasing for HBMVEC at P3. At the stage of P4, vWF signal was weaker, rapidly decreasing to P5 where it was extremely dim. P2 cryopreserved HBMVEC expressed ZO-1 around all cell limits at TJ site with a very strong signal. Along the passages, there seemed to be a decrease in ZO-1 expression mainly after P4, though cell contours were still visible. As for β-catenin, it was expressed at P2 though only at few AJ sites, whilst P3 appeared to have slightly increased its expression. At P4 and P5 β-catenin presented several breaches in signal. As for caveolin-1, it presented very high signal both at P2 and P3. It seemed to weaken its expression after P4, though it might be related to the spaces in between cells. When it comes to P-gp, opposing the previous proteins, it seemed to maintain its expression along cell passages (Fig. 3.27).

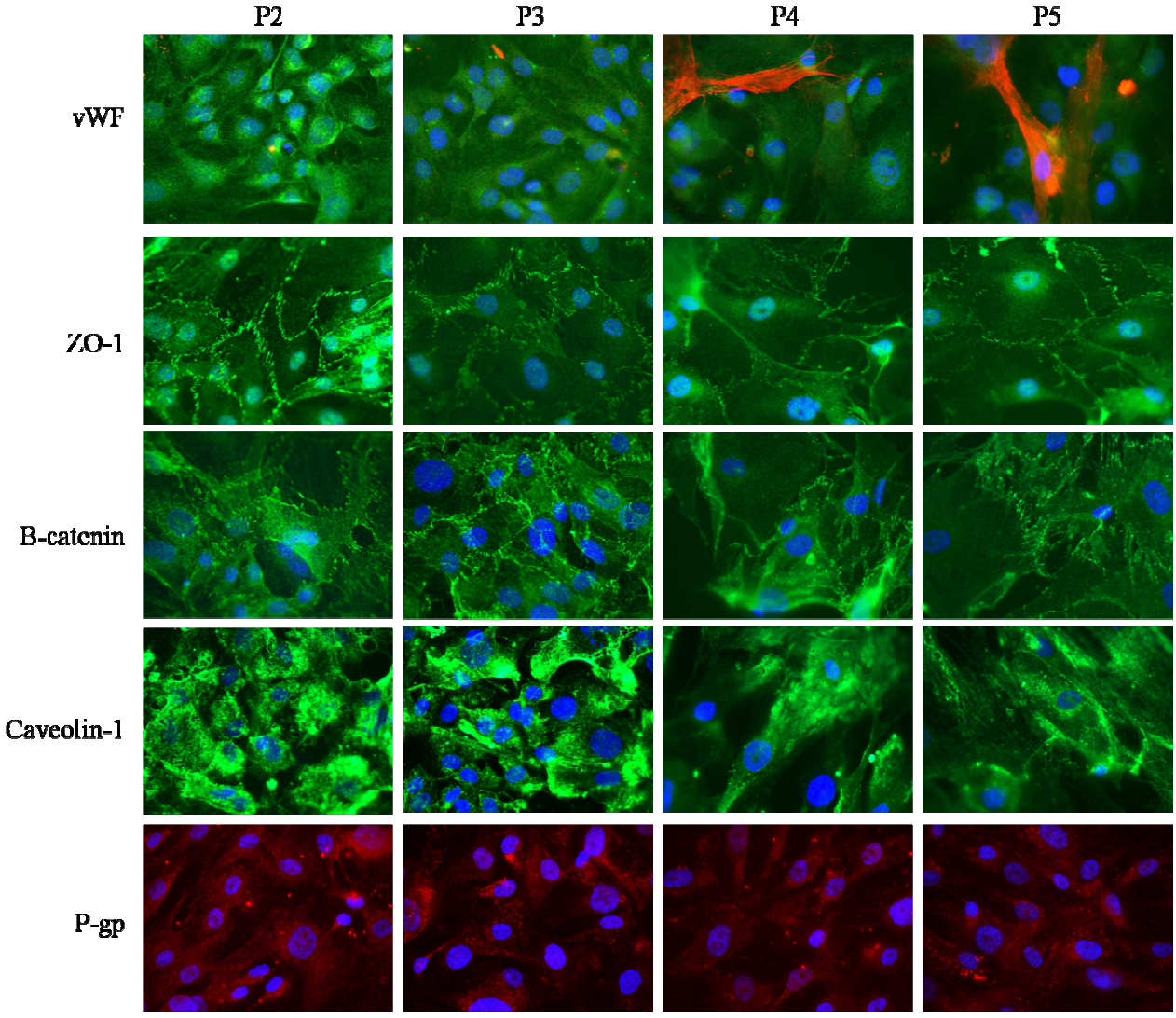

Fig. 3.27 – Immunofluorescence microscopy of human brain microvascular endothelial cells primary culture at P2, P3, P4 and P5. Immunostaining for: endothelial cell marker von Willebrand Factor (vWF), tight junction protein *zonula occludens* (ZO)-1, adherens junctions component β-catenin, vesicular transport protein caveolin-1 and ATP-binding cassette transporter P-glicoprotein (P-gp). Original magnification: 630 x.

Chapter 4 – Discussion

The discovery of the BBB has allowed several studies focused on its properties throughout the last decades. These may be performed under a large variety of study models, either *in vivo*, *ex vivo*, *in silico* or *in vitro*. However, these lack important characteristics. They require more means than those at hand, like *in vivo* studies (Malina *et al.*, 2009), and apply to few circumstances, such as i*n silico* prediction methods which are mainly used for permeability studies (Mensch *et al.*, 2009). *In vitro* studies permit to mimic the BBB microenvironment studying a larger range of properties and functions (Aschner *et al.*, 2006). The majority of these studies fall upon brain tissue from rat (Perrière *et al.*, 2007), mouse (Coisne *et al.*, 2005), feline (Fletcher *et al.*, 2009), porcine (Zhang *et al.*, 2006) and bovine (Zhang *et al.*, 2009) models. Therefore, the results obtained must take into account the differences between species. The better alternative would be to work directly with human tissue. However, the existing methods are both time-consuming and expensive (Hewett, 2009).

Given these facts, we have developed a method for HBMVEC isolation which requires lesser equipment and can be done in little over 2 hours. We have studied the barrier properties through cell passages. Moreover, to circumvent the difficulty to obtain human samples, we have carried out cryopreservation studies.

It is known that EC properties are determined by their location in the organism (Hayashi *et al.*, 1997; Craig *et al.*, 1998). Primary cultures of rat BMVEC isolated by methods reported in literature are spindle shaped, apposed and form monolayers (Dux *et al.*, 1991; Hiu *et al.*, 2008; Vajda *et al.*, 2008). Primary HBMEVC, in resemblance to the previous cells, also present themselves in monolayers and juxtaposed (Jain *et al.*, 2006; Wong *et al.*, 2007), but with different morphology. With our method, we were able to obtain juxtaposed monolayered cell cultures for both species. Furthermore, we were able to evidence that the morphology of rat and human EC was specific and relative to its species.

TEER is usually over 100 $\Omega.cm^2$ (Calabria and Shusta, 2008). We have been able to assess rat TEER measurements of over 150 $\Omega.cm^2$ with our cells. This result is slightly above the expected, and there is no threshold. This might be due to the fact that measurements were taken up at 11 DIV because corresponding immunofluorescence showed that cells did not reach confluence before that period of time.

It has been stated that TEER measurements could be further improved by adding HC and removing ECGS to the medium used in the inserts (Calabria *et al.*, 2006). In line with this, we verified that 2 days after such procedure, TEER assumed a difference of 100 $\Omega.cm^2$ over the original results. Nonetheless, shortly after, cells lost their barrier properties and eventually died. HC affected differently cells isolated with our method in comparison to others, probably needing the use of a more diluted solution. This experiment has allowed us to understand that rat BMVEC TJ expression could be enhanced, and therefore barrier properties, by removing ECGS from the growth medium ISM. ECGS contains a large number of growth factors, and these seem to have to be removed from medium for cells to express TJ proteins properly.

When assessing TEER in HBMVEC, after an initial value of little over 15 $\Omega.cm^2$ at 2 DIV, TEER would suddenly decrease to results very similar to those in control. BMVEC are usually seeded with a concentration of nearly 1 x 10^5 cells per cm^2 (Inglis *et al.*, 2004; Nakagawa *et al.*, 2009). To rule out the hypothesis of low initial cell concentration, cells were seeded at a concentration of 8.5 x 10^4 cells per cm^2 onto the insert. As seen is Figure 3.6, after 4 DIV most cells had died and detached from the insert surface. This may be a result of the inserts themselves. The inserts used in this experiment were clear PTFE inserts and therefore with different membrane composition from those used in rat BMVEC TEER assessment (polycarbonate). In our experience, HBMVEC had a greater difficulty both to attach to the surface of the insert and to form a proper monolayer.

New TEER assessment was performed with equal inserts to those used with rat BMVEC, this time with results around 100 $\Omega.cm^2$ as expected (Calabria and Shusta, 2008). Unlike what was determined for rat BMVEC, when removing ECGS from medium HBMVEC grew further apart from each other. Besides growth factors in its composition, ECGS is also effective in cell attachment and proliferation, what might explain the need of its presence in the medium for HBMVEC to better express TJ. Therefore, immunofluorescence results indicate that with our method we have been able to isolate EC expressing TJ protein ZO-1 with no need to remove ECGS and consequently with enhanced barrier properties.

To confirm the endothelial phenotype, specific EC markers were assessed, such as vWF and GLUT-1 (Craig *et al.*, 1998; Shimizu *et al.*, 2008), and to determine the degree of contamination, also the pericyte marker α-SMA (Lai and Kuo, 2005). Our results indicate that with our method 97% of the cells in culture are EC. This reflects a highly pure culture.

To validate the HBMVEC monolayer as a reliable BBB model, proteins specific to TJ (ZO-1, occludin and claudin-5) and AJ (β-catenin) were determined, alongside with cellular

vesicular transport machinery marker caveolin-1 and the ABC transporter P-gp. Given that cell lines are an easier method to access HBMVEC (Tai *et al.*, 2009), we have used optimized immunofluorescence for these proteins on a human cell line available in our lab. It was expected that the cell line would express caveolin-1 and P-gp on the inner surface (Smith and Gumbleton, 2006), and ZO-1, occludin, claudin-5 and β-catenin in the plasma membrane along the peripheral cell limits (Vorbrodt and Dobrogowska, 2003; Vorbrodt *et al.*, 2009). With our immunofluorescence protocol we have seen some breaches in signal for all proteins. For both claudin-5 and VE-cadherin, there was little signal. However Western blot assay confirmed that all proteins are expressed in these cells (data not shown). Therefore, it is possible that the optimal conditions for immunofluorescence detection of claudin-5 and VE-cadherin were still not achieved. The available HBMVEC line does not exhibit growth inhibition by cell-cell contact, and consequently neither does it form monolayer. Thus, the poor expression of these proteins may also be related to these characteristics.

Primary cultures' characteristics are different in each isolation but still present more primary endothelial traits than cell lines (Bouis *et al.*, 2001). With our isolation method primary HBMVEC expressed ZO-1 and β-catenin in the plasma membrane across the peripheral cell limits. Whereas in cell line the signal for these proteins had breaches, in the primary culture the signal delimited the entire cell contour and were noticeable cell-cell interactions. Furthermore, HBMVEC also expressed P-gp on the cell's inner surface, as well as caveolin-1. This protein is also expressed as microdomains in the cytoplasm, working as cellular vesicular transport machinery (Huber *et al.*, 2001). As for the lack of signal for occludin in the cell contour, this might be due to internalization via a caveolae-dependent pathway. A recent study showed that during TJ remodelling of the BMVEC, occludin and claudin-5 might be internalized becoming sorted into recycling endosomes until a new signal causes their return to the cell membrane to re-establish junctional interactions (Stamatovic *et al.*, 2009). Further tests, as Western blot assay, need to be performed in order to determine whether our primary HBMVEC express this protein or if further immunofluorescence optimization is required.

To better evaluate the relevance and innovation of the implementation of our method, we have studied HBMVEC through cell passages. Several tests presented a contamination by pericytes of nearly 8% (Wagner *et al.*, 2003). There are studies described which involve the use of puromycin to decrease such contamination (Calabria *et al.*, 2006). However, extreme caution with puromycin's concentration is required as this may be toxic to cell (Perrière *et al.*, 2005). With our method, culture cell purity varies from 97% to 83%. P1, P2 and P3 are considered to be

pure cultures with only 2%, 5% and 7%, respectively, of contamination by pericytes. P4 and P5 have contaminations over 10%, showing that pericytes' growth is favoured after each trypsinization. Given these facts, we have been able to implement an isolation method that results in highly pure cultures without the need of using puromycin.

For primary cultures, TEER measurements were expected to be of approximately 100 $\Omega.cm^2$ (Calabria and Shusta, 2008). For our primary cultures, the first three passages had high TEER with results very similar to that in literature, though for P3 TEER values were lower. This behaviour was similar in both contrast phase microscopy and immunofluorescence results for all proteins. The difference between the number of total cells in P1, P2 and P3 might be due to a loss of cells during trypsinization. Therefore with our isolation method we have been able to implement a model which could be used not only in P1 but also in P2, maintaining cells phenotype. Moreover, if we take into account both that trypsin digests the proteins that facilitate adhesion to the container and the results obtained for P3, by using a gentler substitute (TrypLE™) this cell passage might also be used. However, further studies are needed.

There are studies that claim that the expression of caveolin-1 increases with BBB breakdown, and at the same time decreases the expression of both occludin and claudin-5 (Nag *et al.*, 2007). This could explain the fact that even though barrier properties such as TEER and TJ breakdown along cell passages, we have not noticed a decrease in caveolin-1 expression.

As it was previously referred in this work, obtaining a reliable supply of human brain tissue is an extremely difficult task. With the article on primary porcine BMVEC cryopreserved for up to 12 months (Smith *et al.*, 2007), came the idea that the same might be achieved for primary HBMVEC. With the original protocol, cells were stored at -80 ºC and, when thawing, centrifuged to remove DMSO. This however reflected in no cellular growth after plating. The problem in focus may have been caused by the freezing temperature for the time required, as well as for a bigger sensibility to centrifuge whilst thawing. To attempt to solve it, cells were stored at -80 ºC in isopranol overnight and transferred to liquid nitrogen for 30 days. When thawing cells were immediately seeded onto a collagen coated T-25 flask. Medium was replaced the following day to remove remaining DMSO. After a fortnight, cells had reached confluence and under contrast phase microscopy resembled non-cryopreserved primary HBMVEC. After trypsinization, cryopreserved P2 had only 2% contamination by pericytes in a total of 854 cells, which reflect a pure culture. The immunofluorescence obtained for ZO-1, β-catenin, caveolin-1 and P-gp, was very similar to that obtained in P2 non-cryopreserved HBMVEC. Passages P3 and P4 were pure with 4% and 7% of contamination by pericytes, though P4 showed breaches in

signal for the studied proteins. The morphological differences that cryopreserved HBMVEC had from cells that had not undergone cryopreservation, might be due to long exposure to extremely low temperature. A bigger susceptibility to trypsin could also be an effective cause, which might be solved in further studies with a trypsin substitute. As for the decrease in expression of vWF, there are studies that corroborate our results. According to Craig *et al.* (1998), vWF staining has many variables: the higher the passage numbers the more cells stained faintly, and cells which had been recently trypsinized were negative for this factor.

Even still, we have been able to successfully cryopreserve primary HBMVEC and recover those 30 days later with high culture purity and expressing TJ and AJ proteins, as well as vesicular transport machinery and ABC transporters.

With this work we have been able to implement a new method to isolate and culture primary HBMVEC, which comprises important barrier properties such as high TEER, presence of TJ and AJ, as well as vesicular transport machinery and at least one ABC transporter. We have then established a new simplified human model of the BBB, which maintains its phenotype and barrier properties up to P2. Furthermore, we have successfully cryopreserved primary HBMVEC maintaining the expression of proteins important to barrier properties. The availability of cryopreserved primary HBMVEC that can be recovered when required will therefore be of significant benefit to researchers.

Chapter 5 – Future perspectives

The success of the implementation of our method has inspired us to continue studying the BBB properties even further. In future studies we aim to elucidate the role of microglia in the neurovascular unit, namely the conditions determining protective or injurious effects of microglia, upon stimulation, on the BBB. An in vitro model of human BBB, composed by double (microglia + endothelial cells) and triple co-cultures (plus astrocytes or pericytes), will be implemented to assess if microglia influence structure and properties of the BBB and whether microglia reactivity is modulated by the presence of other neurovascular unit components. In rats acutely and chronically injected with lipopolysaccharide we will assess the patterns of microglial activation, as well as of BBB disruption. Additionally, we will use several compounds known to modulate microglial reactivity, in both in vitro and in vivo models, to evaluate their influence on the tightness of BBB integrity. Understanding the microglia-mediated signalling processes that regulate BBB properties shall provide new targets for potential CNS medicines to acute or chronic neuroinflammation. This work shall constitute the PhD thesis entitled "Has microglia a role in blood-brain barrier function and dysfunction?" which shall hopefully start in January 2010, provided that FCT supports the submitted PhD grant proposal SFRH/BD/62959/2009. To a better accomplishment of the proposed project we count with the valuable collaboration of Maria Deli, who implemented a triple co-culture system (Nakagawa *et al.*, 2009), through a training in her lab.

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