In Vitro Blood-Brain Barrier Models – Latest Advances and Therapeutic Applications in a Chronological Perspective

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Abstract: The first generation of *in vitro* models providing successful isolation of viable brain endothelial cells from different species, which could be maintained in cell culture, have emerged around thirty years ago. However, the time consuming and the difficulty of working with primary culture cells led to the development of simpler models employing cell lines with blood-brain barrier properties. The creation, in late nineties, of a transgenic mouse harboring the temperature sensitive simian virus 40 large T-antigen as a source of conditionally immortalized brain endothelial cell lines circumvented the problems of *in vitro* transfection of tumour inducing gene in primary cells.

These different ways to obtain cultures of brain endothelial cells have profited from the discovery of different cellular factors that allow the growth of differentiated cells on plastic filters. Although cell preparations and culture conditions of brain endothelial cells are based on the same principle, there are two main models for studying the blood-brain barrier: the static and the more recently described dynamic model. Dynamic models were created in order to replicate the physiological *in vivo* environment of the blood-brain barrier. The large pool of *in vitro* models is being enlarged since each laboratory improves its model adding small differences adapted to the research interests. The great impact of blood-brain barrier studies in the development of therapies related to the central nervous system supports the interests of this review about *in vitro* models.

Keywords: Blood-brain barrier, central nervous system, cell culture models, artificial models, drug transport, BBB permeability.

INTRODUCTION

The neuropharmaceutical industry has been searching for an ideal *in vitro* blood-brain barrier (BBB) model for almost 20 years. Such a model would provide a means for a central nervous system (CNS) drug delivery program and would support further investigations on drug discovery [1, 2].

The ultimate goal of such pharmaceutical companies is to develop novel therapeutic drugs to the treatment of diseases. For that purpose, researchers focused on CNS diseases have to know if their drug candidates enter successfully in the brain. The process of discovery and development (D&D) of a drug candidate has several steps in which the BBB screen precedes animal testing and culminates in the submission of the selected drug to the regulator entities and launch phase [1].

Within the competitive drug market, pharmaceutical companies are striving for a faster D&D process to reduce the time required to reach clinical use. For a drug directed to the brain to meet the demands of a faster D&D process, particular attention has to be paid to the *in vitro* cell cultures-based BBB models used as permeability screens for the BBB.

Many potential drug candidates cannot be applied to the treatment of CNS diseases due to their low concentration in the brain even when acceptable doses are administered. In fact, only 5% of the developed therapeutically drugs cross BBB reaching the brain cells [3]. This emphasises on the particularities of the BBB properties.

The physiological concept of the BBB arose in the final of the 19th century when Dr. Paul Ehrlich observed that the intravenous administration of dyes in animals did not stained the brain in contrast to all other studied organs. At present, it is recognized that the BBB separates brain tissue from the bloodstream being functionally the most important global influx barrier [4, 5]. The lumen vessels of this barrier are formed by brain capillary endothelial cells (BCEC). Although capillaries occupy a total surface area of 10-20m^2 in the human brain, the complex tight junctions make the brain practically inaccessible for polar molecules [4]. BCEC differ from the non-neural endothelial cells by the nearly absent intracellular *fenestrae* and fluid phase vesicles-mediated pinocytosis, and the paucity of paracellular leakage [6]. These properties create a high electrical resistance interface (2000–8000 Ω · cm2) that renders BBB permeable only to small molecules which enter the brain by diffusion between cells (paracellular hydrophilic diffusion) or through cells (transcellular lipophilic diffusion). Other molecules can enter the brain crossing two membranes in series, the luminal and abluminal, through a carrier, a receptor or an adsorptive mediated endocytosis [4].

In summary, BBB is a regulatory interface between the CNS and circulatory system with nutritional, homeostatic and communication functions. Furthermore, BBB plays an

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important role preventing the development of pathologies affecting the CNS including neoplasias, multiple sclerosis, viral bacterial and fungal meningitis, dementia caused by human immunodeficiency virus (HIV) and Alzheimer's, and migraines [7]. Thus, the restricted drug crossing makes BBB a clinically important cell barrier to study. This article will describe the different *in vitro* cell cultures and artificial models used as permeability screens for the BBB during the process of drug discovery and development, highlighting the main advantages and disadvantages of each.

IN VITRO CELL CULTURE-BASED BBB MODELS

A valid BBB model is the one that mimics *in vivo* brain endothelial cells (ECs) properties such as its morphology (oval shaped cells), reproducible permeability of reference compounds, expression of complex tight junctions and transporters, and a high transendothelial electrical resistance (TEER) [8]. Generally, sucrose or albumin is used as diffusion marker [9]. The detection of specific transmembrane proteins is used to discriminate brain ECs from other cells types. These proteins (e.g. occludin, claudins and junctional adhesion molecules) are linked to the actin cytoskeleton by a variety of cytoplasmic accessory proteins like zonula occludens (ZO-1, ZO-2 and ZO-3), and cingulin [2, 10]. Specific BBB transporters include the P-glycoprotein (P-gp) encoded by the multidrug resistance gene (MDRI) and glucose transporter (GLUT1). The first is an active efflux transporter while the second is a carrier-mediated transporter [4, 11]. The TEER measures the transcellular opposition to the flow of charge and is one of the most important parameter to tests the model validity giving an estimation of the transmembrane tightness [12]. Often, the final choice of the model is a function of the necessities of the researcher and the laboratory including the time, the cost and how closely a model needs to resemble *in vivo* conditions. Data obtained from *in vitro* models are directly comparable to results from other *in vitro* models but not with data from *in vivo* models since these last are obviously superior in terms of similarity with the human response.

On this chapter we will give a perspective on the differences between dynamic and static BBB models. The diversity of protocols for primary brain cells (a), immortalized brain cells (b) and cell from non-cerebral origin (c) used in static models will be discussed as well as the emergence of pericytes in BBB models.

ISOLATION AND CULTURE OF BRAIN CAPILLARIES

The development of methods for culture cells including the Rose perfusion chamber [13] and the Maximow slide assembly [14] were the main pillars for what now is called BBB research. These investigations started in the fifties and gave birth to numerous publications. In 1973 Yavin and Menkes described a method for the primary culture of dissociated cells from rat cerebral cortex, which allow the culture of a sufficient amount of tissue for metabolic studies [15]. In the same year Joó & Karnushina proposed a procedure for the isolation of capillaries from the rat brain obtaining intact cerebral microvessels dissociated from neurons and glial cells [16].

Primary culture of BCEC is a difficult process involving a first removal of the brain, dissection of the grey matter, followed by the isolation of brain capillaries. Presently, several methods are available for the isolation of capillaries including mechanical homogenization, filtration (separates cells with different diameters e.g. capillaries from venules and arterioles of 5 to 25 μm diameter), density gradient centrifugation (e.g. white matter is not as dense as grey matter), enzymatic dissociation, and column filtration [17].

Primary cultures are more time consuming and laborious, and more susceptible to internal (e.g. pericytes, astrocytes) and external (e.g. bacterial) contaminations than cell lines. However, contaminations can be surpassed by using common cell culture procedures such as the use of growth medium supplemented with antibiotics. Also, the limited growth capability of primary cell cultures can be circumvented by sub-culturing the cells [18] which can be frozen and seeded whenever necessary.

The major advantage in using primary cultures of BCEC is the close resemblance to *in vivo* brain physiological conditions in opposition to immortalized cells and cells from non-cerebral origin [19]. Different sources of BCEC have been used: bovine, porcine, rodent, and human. Bovine brains are used mainly because of the size of the calf brain, resulting in a high yield of brain capillaries. If bovine brain is not available due to bovine spongiform encephalopathy or any other reason, brains from other large animals like porcine brains are an alternative. Rat or mouse brains are broadly used because they are simple to acquire. However, rodent brains produce much lower yield (100 times less yield) and may give rise to a leaky mono-layer [20]. Relating to human brains, their use is very limited due to ethical constraints and to the difficulty in accessing them.

1. Dynamic Models

Dynamic models were created by Janigro and co-workers in 1996 [21] involving a laborious methodology frequently applied to studies that are intended to analyse BBB function [22, 23]. They are concurrent with static models, which offer the best option for BBB-crossing studies.

Dynamic models were produced in order to replicate the physiological environment of BBB *in vivo* using physical stimuli (pulsatile pump) to create a surface tension or shear stress. Shear stress is a tangential force generated by the flow of blood across the apical surfaces affecting the structure and function of brain ECs [24]. In this three-dimensional system peripheral or cerebral ECs are cultured in the lumen of hollow fibres (mimicking capillaries vessels) inside a sealed chamber and are exposed to flow while the astrocytes are seeded in the extraluminal compartment to promote cellular stimuli [21]. The hollow fibre tubes are accessible by ports and the chamber is connected by gas permeable tubing to a growth medium source enabling exchange of oxygen and CO_2. With pulsatile pumping the nutrients are allowed to diffuse through the artificial trans-capillary pores while metabolic products are removed. This type of continuous culture where the change of growth medium gas and metabolic products occurs in an almost fully automated fashion is extremely practical.

On this model the glial/astrocytes cells are seeded at the apparatus and after three to four weeks of co-culture the TEER reaches the maximum value of 736 $\pm$ 38 $\Omega \cdot cm^2$ and is ready for experimentation. Afterwards, the same apparatus may be used for different assays since cultures retain BBB properties (such as low permeability to intraluminal potassium, and BBB-specific ion channels) for several months under sterile and appropriate culture conditions (Table **1**) [21, 25]. However, using one apparatus for sequential experimentation is probably not the most correct procedure. Theoretically pulsatile pumps will remove the waste products including the tested compounds but the radioactive or fluorescent probes may not be completely removed from the system and may camouflage the results.

In addition, with the advance of cell culture protocols, brain ECs are able to retain endothelial specific markers after a prolonged *in vitro* culturing, express several transporters and tight junction proteins, and mimic physiological and structural characteristics of the *in vivo* situation.

Finally, in dynamic models the laminar flow inhibits ECs proliferation through the p53 pathway inducing a cascade of signals in a magnitude and time-dependent manner [26]. P53 is a tumor suppressor protein acting as a transcription factor to mediate a number of cellular functions. In dynamic models the shear stress is induced in long-term at 4 dynes/cm^2 (as expected in brain capillaries) [24] inhibiting ECs proliferation and probably preventing ECs apoptosis [26]. In contrast, static models due to the absence of a laminar flow have an increased cell cycle rate that causes ECs to pile up in a multilayer fashion [27]. However, there are forms to avoid the formation of multilayered ECs by tripsinizing cells followed by diluting them in growth medium.

Consequently, both static and dynamic models are compatible with the growth and differentiation of ECs and high TEER generation making them suitable for pharmacological studies.

2. Static Models

The BBB models are considered static when there is no mimicking of the cerebral blood flow in opposition to dynamic models. This system is a horizontal side-by-side diffusion system for the study of bidirectional transendothelial transport of solutes across the BBB [2]. Static models are by far more cost-effective than dynamic models, more commonly used and simpler to design. The cells (generally BCEC and astrocytes) grow in a limited way, without gaseous exchange tubing and without loading and sampling ports, within a transwell filter [28]. Depending on the source of the cells and the methodology employed, static models may be applied to reproducibly test drug transposition through BBB, test the regulation of BBB permeability, and the disease influence on BBB permeability (Table **1**).

a. Primary Brain Cells

Bovine Brain Endothelial Cells

In 1989 the research group of Cecchelli created a method to isolate BCEC without enzymatic digestion where the microvessels are seeded onto dishes with a selective matrix and within five days the first endothelial cells migrate out of the capillaries [29]. The other novelty of this work was the mass-production method through sub-culturing of BCEC. The cells are cultured and when confluent are sub-cultured and stored in liquid nitrogen at the 3rd passage. Each passage generates at least 75 co-cultures and BCEC can be used from passage 3 to 7 [19, 29]. In order not to loose the phenotypic marker γ–glutamyl transpeptidase (γ-GT) and the characteristic tight junctions of the BBB, the authors described in 1990 a co-culture approach where astrocytes are plated at the bottom of the well without contacting BCEC [18] or plated in direct contact with BCEC [19]. In a month both cell types are stabilized and ready to use for experimentation. In these conditions TEER of the BCEC monolayer reach 661 to 800 $\Omega \cdot cm^2$ and the receptors for low density lipoprotein, transferrin, and P-glycoprotein are expressed [19].

Later the authors developed a mouse syngenic BBB co-culture model to examine inflammatory events [30]. The use of BCEC and astrocytes from the same source (mouse) is required to abrogate the eventual immuno-incompatibility problem [31]. During growth, cells are exposed to an inflammatory mediator, the gram negative bacterial cell wall constituent (lipopolysaccharide). It was shown that this lipopolysaccharide up-regulates the expression of cell adhesion molecules suggested to mediate leukocyte transmigration across the BBB [32]. Therefore, this model reconstitutes the pathological BBB conditions providing a suitable support to study the mechanisms underlying inflammation in brain endothelium.

Recently (2008), the same authors developed a BBB model for high throughput toxicological screening without astrocytes [33]. In this model BCEC are seeded on the upper side of filters, after 3 days the medium is replaced by a specific BBB inducing medium (contains 1% of conditioned medium from the previous co-culture of astrocytes and BCEC), and transport experiments are carried out 24h later. It is claimed as a suitable model adaptable to the needs of pharmaceutical industry with a reducing time for assessing the toxicological profile of chemical substances. However, 4 to 5 days of culture prior to experiments is about the same time described for other BBB models. Moreover, TEER cannot be compared to other models since it was not provided by the authors.

In 2001, the research group of Boer and co-workers established a new method to isolate bovine brain capillaries resulting in high ECs yielding [34]. BCEC and astrocytes are co-cultured for 6 days and before experimentation are transferred to a differentiating medium of 8-(4-Chlorophenylthio) adenosine-3', 5'- cyclic monophosphate sodium salt (8-CPT-cAMP), and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO-20-1724). The role of 8-CPT-cAMP, a membrane permeable cAMP analog, is to activate intercellular membrane channels in cultured cells and this effect is prolonged by RO-20-1724, an inhibitor of cAMP phosphodiesterase, resulting in more junctional coupling mediated by cells factors. The observed high increase in the TEER (approximately 800 $\Omega \cdot cm^2$ [34]) by these substances was firstly described in 1998 by Hurst and co-workers [35]. In addition, the expression of α_v-Integrin, cadherin-5, the phe-

notypic marker γ-GT, and the receptors like the P-selectin receptor and transferring were detected.

Porcine Brain Endothelial Cells

Porcine brains were used by Galla and co-workers in 1999 to create a BBB model based in a mono-culture without serum or astrocytes but in the presence of hydrocortisone [36]. Porcine brain ECs are isolated following the method of Bowman [37], sub-cultivated once, transferred into a filter coated with collagen and ready for *in vitro* transport experiments within 4 days. The results show that when ECs are cultured in a medium without serum but with hydrocortisone reproduce high electrical resistance, reaching 700 $\Omega \cdot cm^2$ (400 $\Omega \cdot cm^2$ in a medium with serum) [36, 38]. Hydrocortisone is added in serum-free medium to induce cell growth and strengthen cell junctions. The effects of this substance on the reinforcement of the BBB properties were firstly described in 1998 by Hoheisel and co-workers [39]. The method described provides an opportunity to work in the absence of serum which is a clear advantage when high performance liquid chromatography sampling is carried affording a direct sample injection and consequently a complete automation of sample analysis. It is also an improvement to detect the transport of labelled proteins without having the interference of the serum proteins.

A syngenic co-culture model of porcine cells from adolescent pigs was described in 2003 [40]. In this model the suspension of porcine cortexes is subjected to successive screens with a progressive tighter pore size and filtered through a separation column with glass beads. The resulting solution will give both BCEC (the beads with the attached capillaries are collected, capillaries are loosen by swirling and finally seeded) and astrocytes (the filtrate is seeded immediately after centrifugation). One week of co-culturing is sufficient to afford the experimental procedure. The use of the same species and age synchronization between the two cell types does not necessarily provide the best model to test drug transposition of the BBB, since transendothelial resistance of the monolayer only reached a maximum of 139 ± 16 $\Omega \cdot cm^2$.

Rat Brain

In 1992, Abbott and co-workers described a protocol that represents a first step in producing an *in vitro* model of the rat BBB [20]. Lately, Perrière and co-workers (2007) described an *in vitro* BBB model based on a co-culture of rat brain ECs and astrocytes [41]. ECs are purified with puromycin to eliminate contaminating cells without affecting their viability or BBB characteristics [42]. The rationale for this methodology is that rat brain ECs expressing high levels of P-gp would resist to puromycin treatment while their counterparts (pericytes and astrocytes) expressing lower or any levels of P-gp would not [42, 43]. After addition of puromycin, ECs are treated with hydrocortisone, seeded on a transwell filter with astrocytes for one week, and transferred to a medium with 8-CPT-cAMP and RO-20-1724 before assays. Optimal culture conditions are obtained in the presence of cAMP/RO and hydrocortisone rendering a TEER of 270 ± 119 $\Omega \cdot cm^2$ while the TEER is near 150 $\Omega \cdot cm^2$ in the presence of only hydrocortisone [41]. This BBB model expresses many different BBB junctional proteins (claudin-5 and ZO-1) and transporters (such as the P-gp and GLUT-1) being suitable for drug transport testing.

Human Brain

Due to the constraints of working with human brains researchers generally opt to address BBB issues with commercially available human brain cell lines [44, 45]. Human brain ECs are achieved from biopsies or autopsies and subsequently are immortalized by transfection with simian virus 40 large T antigen (SV40).

To date human brain cell lines models are leaky and not appropriate for BBB studies. Despite this, some groups have developed strategies to study BBB issues based on these cells. Since 1995, Nagy and co-workers developed a protocol for an *in vitro* co-culture model of human brain ECs and astrocytes to study mainly cerebral vascular diseases like ischemic stroke [46, 47]. In 2002 Megard and collaborators proposed a protocol based on a co-culture of human brain ECs and astrocytes to study drug penetration into the CNS, namely the involvement of P-gp in limiting transport of a HIV-1 protease inhibitor indinavir [48]. Although the TEER obtained was only 260 ± 130 $\Omega \cdot cm^2$ the authors claim that this model is closely related to the *in vivo* situation.

b. Immortalized Cells

Following the pioneer rat brain endothelial cell line [49] several brain cell lines from rat and other species have been described. At present the most commonly used immortalized brain ECs for BBB models are from rat [50].

One interesting work belongs to a Japanese group that in 1999 created a commercial transgenic mouse and rat harboring the temperature sensitive SV40 gene, as a source of conditionally immortalized cell lines (Table **1**) [51]. The gene is stably expressed in all tissues and cell cultures can be easily immortalized by activating the gene at 33ºC. At this temperature the gene is thought to induce cell proliferation by inactivating the growth-suppressive function of the retinoblastoma gene products and p53 [52]. Therefore, it is possible to circumvent the problems of the *in vitro* transfection of tumor inducing gene in primary cells. A year later, Hosoya and co-workers created a conditionally immortalized BCEC line from mice and rat [53]. In addition to immortalized ECs the same group created an astrocyte cell line in 2001 [54] and a pericyte cell line one year later [55] from transgenic rats.

To create transgenic rats or mice, BamH1 DNA fragments of whole SV40 are injected into the pronuclei of fertilized eggs. After, the brain is homogenized, digested and BCEC are seeded until obtaining separate single cells. Following two or three passages, cells are cloned from a single cell, and seeded in transwell filters for 48h being ready for drug transport experiences [53]. These cells have γ-GT, alkaline phosphatase activities, express GLUT1 and P-gp [56]. However, the models have an insufficient tightness because they do not express enough tight junctions to allow transcellular transport studies [57].

In a recent review by Roux & Couraud (2005), it was concluded that none of the immortalized rat brain endothelial

Table 1. Comparison Between the Applications of *In Vitro* BBB Models, Transendothelial Resistance and the Type and Origin of the Cells Used

Model	Application	TEER ($\Omega \cdot cm^2$)	Type and Origin of Cells
Dynamic	BBB function	736±38	Bovine aortic EC line and astrocytes from rat glioma cells [21, 25]
Static	Study of drug penetration	661 to 800	Bovine ECs and rat astrocytes [19]
Static	Study of inflammatory events	777±15	ECs and astrocytes from mouse [30]
Static	High throughput toxicological screening	*	Bovine ECs and rat astrocytes [33]
Static	Study of drug penetration	800	Bovine ECs and rat astrocytes [34]
Static	Study of drug penetration	700	Porcine ECs [36, 38]
Static	Study of drug penetration	139±16	ECs and astrocytes from porcine origin [40]
Static	Study of drug penetration	270±12	ECs and astrocytes from rat [41]
Static	Study of drug penetration	260±13	ECs and astrocytes from human [48]
Static (immortalized cells)	Study of drug transport to the brain and retina	-	Conditionally immortalized capillary endothelial cell lines from mouse brain, rat brain, and rat retina [56]
Static (non-cerebral cells)	Study of drug penetration	600-1000 130-300 100	Caco-2 MDCK ECV304 + Astrocytes [63]
Static (implementation of pericytes)	Study of drug penetration	388±19	ECs, astrocytes and pericytes from rats [65, 66]

*The authors do not mention the TEER although they report to previous results [19] where TEER was 661 to 800 $\Omega \cdot cm^2$.

cells appear to generate the necessary paracellular barrier that would allow transendothelial permeability screening studies [50]. Therefore, cell lines are mainly applied to the study of BBB functions and cell-to-cell interactions. Hopefully in the future these cells with high proliferation capabilities and phenotypic stability would be appropriate for large scale drug screening.

c. Cells from Non-Cerebral Origin

The most commonly used non-cerebral cells lines are from human umbilical vein endothelia (ECV304) [58, 59], from canine kidney (Madin-Darby canine kidney MDCK) [60], and from human epithelial colorectal adenocarcinoma line (caco-2) [61, 62].

In a recent comparative study developed by Garberg *et al.* (2005) these three cell lines were seeded in transwell filter without co-culturing with other cell types except ECV304 (co-cultured with glial cells) to a BBB screening evaluation. Drug transport experiments were taken after 5-6 days post-seeding onto permeable filters (ECV304), 5-20 days (MDCK), and 14-28 days (caco-2). TEER was highest for caco-2 (600-1000 $\Omega \cdot cm^2$) than MDCK (130-300 $\Omega \cdot cm^2$) and ECV304/glia (100 $\Omega \cdot cm^2$), while the lowest permeability to sucrose and other compounds was exhibited by MDCK. The results indicate that MDCK is the most promising cell line for BBB drug screening [63].

Although is possible to obtain non-cerebral cells with a reasonable paracellular resistance, these cells are morphological different from brain ECs, express different transport systems and have different metabolic and growth properties, failing as BBB screens [64]. Therefore, they should be used only when obtaining brain cells is unattainable.

d. The Implementation of Pericytes in Brain Cell Culture

Pericytes embrace the abluminal endothelial surface of arterioles, venules and capillaries being part of a network connection with ECs and astrocytes. Although the identity and lineage of pericytes is still not fully characterized they are intrinsically related to the formation and maintenance of the cerebral microvasculature structure and functions.

Only recently, pericytes were considered critical components to establish BBB models due to the work of Nakagawa *et al.* [65, 66]. The authors evaluated the barrier integrity of different cultures from the same origin (rats with same age and genetic background): 1) mono-culture of ECs, 2) double co-cultures with astrocytes in contact and in non-contact mode with ECs, 3) double co-culture with pericytes in contact and in non-contact mode with ECs, 4) triple co-cultures with ECs, pericytes and astrocytes. They concluded that the TEER is highest ($388 \pm 19\ \Omega \cdot cm^2$) in triple co-cultures, and that double co-cultures present higher electrical resistance than mono-cultures (Table **1**). It was also observed that the tightest barrier is formed when ECs and pericytes are positioned on the two sides of the filter and astrocytes at the bottom of the culture dish mimicking closely the anatomical position of the cells at the BBB *in vivo* [65].

Another study added that pericytes perform better than astrocytes during prolonged oxygen depravation, indicating

that during hypoxia only pericytes maintain barrier function protecting the integrity of cerebral tissue [67].

These up-to-date results clearly states the importance of an *in vitro* BBB model with astrocytes and pericytes which is noticeable considering that both secrete factors that up-regulate expression of tight junction [68] and are intrinsically co-related.

IN VITRO ARTIFICIAL-BASED BBB MODELS

For large series of compounds, e.g., for combinatorial chemistry projects, the measurement of permeation through cell cultures becomes rapidly unpractical and highly expensive with today's technologies. Some simpler yet accurate methods are needed to answer the demands of high throughput screening. On the other hand, the use of the above mentioned methods often hinders a more deeply analysis on quantitative structure-activity relationships for these molecules.

Since that BBB is formed by a continuous layer of the brain endothelium lining brain capillaries, molecular phenomena involving the drugs at the cell membrane-blood interface gain a fundamental role when assessing BBB permeability for those drugs. The distribution of molecules between the two immiscible phases of water and an organic nonpolar solvent was first used to evaluate the lipophilicity of drugs. The most commonly employed systems for predicting the propensity of a solute to insert in a cell membrane is octanol-water [69]. This system originates from the partition-diffusion model for solute transport in which the cell membrane is assumed to be octanol-like in its properties. Octanol, with a polar head and a flexible, nonpolar tail, has hydrogen-bonding capabilities and amphiphilicity characteristics similar to those of the phospholipids found in biological membranes. Although other solvent mixtures have been tested, octanol revealed as the most accurate. Being intensively used in the last decades, this model has proved useful in some aspects [70]. Techniques for the measurement of partition coefficients include the classical shake-flash and stir-flash methods, dual-phase potentiometric titrations, reverse phase planar and liquid chromatographic procedures, cyclic voltammetry, centrifugal partition chromatography, counter-current distribution and rotating diffusion cells, for instance [71]. Several calculation-based predictive methods and their software also appeared to estimate partition coefficients. Being highly accepted and recognized by the scientific community, it is widely used in CNS drug discovery as well as for toxicity evaluation of some systemic treatments towards the brain.

The octanol/water partition coefficient for a neutral substance or an ionisable substance in its neutral form is normally defined as log P:

$$\log P = \log\left(\frac{C_o}{C_w}\right)$$

where C_o is the concentration of compound in the octanol phase and C_w its concentration in the aqueous phase when the system is at equilibrium [72].

Studies with CNS anesthetics highlighted the good correlation between logP and biological activity for several compounds and animal models over an extremely wide range of about 8 log units. [70]). Other authors found that the hypnotic activity of a number of congeneric series of CNS depressants reached a maximum when log octanol-water partition coefficient was near 2. Various researchers confirmed this finding and the "rule of 2" became generally accepted [73]. According to these findings, the optimal log Po/w has been defined as approximately 1.5 to 2.5 [74].

Despite being an extremely useful tool in assessing permeability, logPo/w seems to have, however, a limited performance in predicting brain/blood concentration ratios (logBB). In fact, given the large diversity in biological systems and environmental compartments, it is unlikely that a single solute descriptor could adequately represent the range of expected system properties. Many correlation models are actually restricted to a limited range of solute structures, because the factors responsible for membrane solubility are not the same as those responsible for solvation by wet octanol [72]. Additionally, features such as high binding to plasma proteins, active efflux by P-glycoprotein and involvement of transporters and receptors are not considered in these models.

Thus, the extrapolation of biomembrane/aqueous phase systems to the octanol/water biphasic system in evaluating partition coefficients is an over-simplification. In the 1990s, attempts to a more suitable approximation to the properties of biological membranes lead to the development of models using synthetic phospholipids.

Immobilized artificial membranes (IAMs), for instance, were used as permeability screens [75]. IAMs consist of phosphatidyl choline residues covalently bound to silica propylamine and are used as a chromatographic interface in HPLC [75]. As for logPo/w, IAM relies on the assumption that the rate limiting step for permeation across BBB is partitioning of the drug into the brain membrane. Still, it constitutes a more complete model in mimicking the complexity of factors governing drug permeation, being also applicable when more polar compounds are considered. Nonetheless, the lipid model is on solid support and thus lipid molecular dynamics are not fully displayed.

In recent years, another higher throughput method has gained in popularity; the parallel artificial membrane permeability assay (PAMPA), first introduced in 1998 by Kansy and collaborators. In the PAMPA assay, a "sandwich" is formed from a 96-well microtiter plate and a 96-well microfilter plate, such that each composite well is divided into two chambers: donor at the bottom and acceptor at the top, separated by a 125 μm-thick microfilter disc, coated with a 2% wt/vol of the chosen lipid in an organic solution, under conditions that multilamellar bilayers form inside the filter channels when the system contacts an aqueous buffer solution [76]. PAMPA is suitable for passively permeating compounds, being its greatest potential the screening of large molecule libraries. The lipid composition may be selected to best mimic BBB. From the different lipids studied, porcine polar brain lipid appears to be the more suitable [77]. A recent review analyzed the quality of available data on

PAMPA and made a detailed comparison with other models, concluding that, despite showing good correlation with data from *in vivo* studies, the outcome provided is similar as the one achieved with simpler methods, such as partition coefficient in octanol/buffer system (similar to logP o/w, but instead of water a buffer with pH of 7.4 is used, [78]). Thus, the PAMPA assay may be a very limited asset to a drug discovery effort.

Experimental and computational approaches to estimate solubility and permeability of a drug, i.e. the penetration rates in the brain by passive diffusion, have been developed. The Lipinski's rule of 5 predicts that poor absorption or permeation is more likely when there are more than 5 Hydrogen bond donors, 10 Hydrogen bond acceptors, the calculated log P is greater than 5, and the molecular weight is greater than 500 Da (Lipinski CA *et al*., 1996). Also molecules with rotatable bonds and those that are highly branched have a decreased penetrability potential (Doan KMM *et al*., 2002). These parameters are still useful to test drug penetrability.

The main advantage of these models is the possible rapid evaluation of strategies for achieving drug targeting to the CNS or to appreciate the eventual central toxicity of systemic drug and to elucidate the molecular transport mechanism of substances across the BBB. These factors determine that such assays are generally applied in an early stage of the drug discovery process Fig. (**1**).

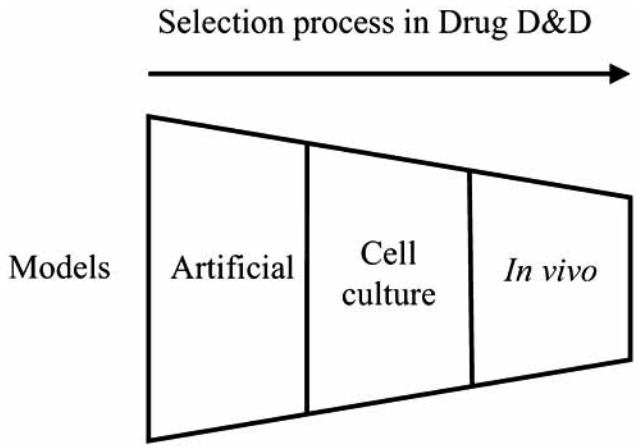

Fig. (1). *In vitro* artificial, cell culture and *in vivo* models used as BBB permeability screens. As the model used turns more realistic, it is accepted that the number of tested positively drugs will progressively decrease. Cell culture and *in vivo* models share similarities with the *in vivo* situation characterized by a highly restrictive BBB.

CONCLUSIONS

In vitro cell cultures and artificial models are used as permeability screens for the BBB in order to assess the drug capability for entering the brain. These models have different advantages for the same purpose. Cell culture models are more closely related to what happens *in vivo* since the cells share many properties with the real BBB, turning that not all the drugs that cross artificial models will cross cell culture models Fig. (**1**). However, artificial models are more time saving and cost-effective than cell culture models which are two important factors in the drug discovery and development process. Thus, as a first approach, artificial models are more suitable for drug high throughput screening. Once that lead compounds are selected, cell culture models should be used to detail the exact mechanisms involved. Therefore, the researcher needs will at least determine the use of either model.

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ABBREVIATIONS

BBB	=	Blood-brain-barrier
CNS	=	central nervous system
D&D	=	discovery and development
BCEC	=	brain capillary endothelial cells
HIV	=	human immunodeficiency virus
ECs	=	endothelial cells
TEER	=	transendothelial electrical resistance
ZO	=	zonula occludens
P-gp	=	P-glycoprotein
MDRI	=	multidrug resistance gene
GLUT1	=	glucose transporter
γ-GT	=	γ–glutamyl transpeptidase
8-CPT-cAMP	=	8-(4-Chlorophenylthio)adenosine-3', 5'-cyclic monophosphate sodium salt
RO-20-1724	=	4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone
SV40	=	simian virus 40 large T antigen
ECV304	=	human umbilical vein endothelia cell line
MDCK	=	Madin-Darby canine kidney cell line
caco-2	=	human epithelial colorectal adenocarcinoma cell line
IAMs	=	immobilized artificial membranes
PAMPA	=	parallel artificial membrane permeability assay

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