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*In vitro*blood-brain barriermodel to study the permeation of nanoformulated fluorescent molecules: the case ofFITC-loaded ferritin nanoparticles

AUTHORS:
Fiandra, L
Dipartimento di Scienze Biomediche e Cliniche “Luigi Sacco”
Università di Milano
Milano, Italy
luisa.fiandra@guest.unimi.it

Mazzucchelli, S
Dipartimento di Scienze Biomediche e Cliniche “Luigi Sacco”
Università di Milano
Milano, Italy
serena.mazzucchelli@gmail.com

Truffi, M
Dipartimento di Scienze Biomediche e Cliniche “Luigi Sacco”
Università di Milano
Milano, Italy
marta.truffi@unimi.it

Bellini, M
Dipartimento di Biotecnologie e Bioscienze
Università di Milano-Bicocca,
piazza della Scienza 2,
Milano, Italy
michi.bellini@gmail.com

Corsi, F
Dipartimento di Scienze Biomediche e Cliniche “Luigi Sacco”
Università di Milano
Milano, Italy
fabio.corsi@unimi.it

CORRESPONDING AUTHOR:*(required; this is the official corresponding author for publication, but may differ from the author used for submission in the Editorial Manager system)*

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SHORT ABSTRACT:
Here is described the setting and validation of an *in vitro* model of blood brain barrier based on a co-culture of rat brain microvascular endothelial cells and astrocytes. This system has proved to be a valid tool to study the effect of nanoformulationon the trans-barrierpermeation of fluorescent molecules.

LONG ABSTRACT:
In the blood brain barrier (BBB), the brain microvascular endothelial cells, reinforced by an endfeet of pericytes and astrocytes, are responsible for the low permeation of large hydrosoluble drugs from blood circulation into the brain.In the last years, different strategies have arisen with the aim to promote brain targeting and improve drug activity in this site.Among them, many nanotechnologicalstudies have been directed to identify innovative nanostructured devicesfor the delivery of drugs across the BBB. In this context, an in vitro approach based on a simplified cell model of BBB can represent a usefultool to investigate the effect of nanoformulation on the trans-BBB permeation of drugs. Here is described the protocol for the development of a double-layer BBB, starting from a co-culture of immortalized primary rat brain microvascular endothelial cellsand astrocytes, commercially available frozenat passage one. A multiparametricapproach for the validation of the model,based on the measurement of the transendothelialelectrical resistance and the apparent permeability of a high molecular weight dextran,is also described. As proof of concept for the employment of this BBB model to study of the effect of nanoformulation on the translocation of fluorescent molecules across the barrier, we describe the case of the fluorescein isothiocyanate (FITC)loaded into ferritin nanoparticles. The ability of ferritins to improve the trans-BBB permeation of FITC, likely by a transferrin receptor1-mediated endocytosis of the nanosystem into the endothelial cells, is demonstrated by flux measurements and confocal microscopy assays.

INTRODUCTION:
The resistance ofcentral nervous system (CNS) diseases (i.e. cancer, epilepsy, depression,schizophrenia and HIV-associated neurological disorder) to the pharmacological therapies is due to different mechanisms, also including arduous drug permeation across the blood brain barrier (BBB). The BBB is the boundary that isolates brain tissues from the substances circulating in the blood, while allowing water and small lipophilic molecules to access this compartmentaccording to their concentration gradients. Within this barrier, the layer of Brain Microvascular Endothelial Cells (BMECs), strengthened by an end-feet of pericytes and astrocytes, is responsible for the highly selectivity of the BBB to thosehydrosoluble drugs with a molecular weight higher than 400 Da. Another drug-related resistance mechanism is linked to the presence on BMECs of drug efflux transporters (P-glycoprotein and multidrug resistance proteins),which co-operate to reduce drug penetration into the CNS and facilitate their extrusion from brain[1].
In the last decade, a huge number of nanotechnological approaches have been developed to face the clinical and biological challenge of drugs delivery across the BBB[2-4]. In this context, ferritinnanospheres (FnN) represent a completely innovative andpromising solution. FnNare 12 nm cave spheres of 24 self-assembling ferritin (Fn) monomerswhich are arranged in a hollow

spherical structure of 8 nm in inner diameter. Ferritin subunits can be disassembled at acidic pH and reassembled bringing the pH to neutrality in a shape-memory fashion, and so encapsulate various organic molecules. Therefore, FnN representan interesting model for the development of a multifunctional drug delivery system[5,6]. Moreover, FnN may interact with BMEC thanks to the specific recognition of TfR1, which is overexpressed on the luminal membrane of these cells[7].
So far, different *in vitro* models of BBB have been developed to test the interaction between the drugs and the barrier in order to elucidate toxicity, permeability or interaction of the molecule with efflux transporters. Indeed, these models have been recognized as valid *in vitro* approaches for a rapid screening of active molecules before proceeding with *in vivo* studies. These models were based on a single endothelial layer of BMECs or a co-colture of BMECs and astrocytes (more rarely pericytes), obtained from animal (rat, mouse, pig and bovine) or human cell lines[8,9]. The TransEndothelial Electrical Resistance (TEER) and the apparent permeability (P_{app}) of tracers with a defined molecular weight represent two critical parameters to determine the quality of the *in vitro* model: high TEER and low P_{app} values are the requisites of a good BBB model. Here we describe an example of BBB *in vitro* model, based on a co-culture of rat BMECs (RBMECs) and Cortical Astrocytes (RCAs). This model, whose TEER and P_{app} of the high dimension tracer FITC-dextran 40 (40 kDa) are in agreement with the production of a suitable BBB, can be employed to investigate endothelial cells uptake of nanoformulated fluorescent molecules and their trans-BBB translocation. The case of ferritin nanocages encapsulating the fluorescein isothiocyanate (FITC) is reported as proof of concept.

PROTOCOL:

1. BBB setting

For BBB set up, use purchase immortalized primary.All steps must be performed with sterile reagents and disposables, handled in laminar flow hood.

1.1) Cell cultures

1.1.1) Thaw 1 x 10^6RCAs and 5 x 10^5 RBMECs in Endothelial Cell Medium (ECM) and seed them in 1 T175 flask and 1 T75 flask, respectively; before seeding, flasks need to be coated with poly-L-lysine 100 µg/ml (1 hr at RT) or fibronectin 50 µg/ml (1 hr at 37 °C) to promote the attachment of RCAs or RBMECs, respectively.

Thawing and seeding passages of RCAs and RBMECs have to be adjusted, in terms of cell density and time in culture, according to the number of experimental conditions to be tested on the BBB model. Starting with 1 vial of 1 x 10^6RCAs and 1 vial of 5 x 10^5 RBMECs, it is possible to obtain up to 20 BBBs.

1.1.2) Keep the cells at 37 °C and 5% CO_2 in a humidified atmosphere for approximately 6 days, up to reach about 80% confluence for RCAs and over 90% confluence for RBMECs; then, detach the cells with trypsin for 5 min, stop the trypsin activity with FBS-containing medium , centrifuge cell suspensions at 750 × g for 5 min and resuspend the pellets in ECM.

1.1.3) Split RBMECs in 3 T175 flasks and culture them in ECM for other 3 days before seeding on inserts. Count the total number of living RCAs, by observing a cell suspension diluted 1:1 with trypan blue under an optical microscope in a Burker chamber.

1.2) Cell seeding on inserts

1.2.1) Treat differentially the two sides ofthe PolyEthyleneTerephtalate (PET) membrane of 6 multi-well transparent inserts with poly-L-lysine 100 µg/ml and fibronectin 50 µg/ml, to allow RCAs and BMECs attachment, according to the following steps: 1) allocate the inserts into the plate and add the fibronectin solution (minimum 500 µl) into the upper chamber; 2) after 1 hr of incubation at 37 °C, remove the fibronectin solution, take the inserts off the multi-well plate and put them upside down on the bottom of a 150 cm^2 Petri dish, used here as a sterile support for already coated inserts; 3) displace gently 800 µl of poly-L-lysine on the bottom side of the insert (as shown in Figure 1A referred to RCAs seeding), and keep the solution on the inserts for 1 hr at RT; 4) remove the solution and let dry the inserts at RT for 15-30 min. The inserts are now ready for the cell seeding, but can also be stored into the multiwell for several days at 4 °C, before proceeding with BBB production.

Remember to keep at least 3 coated inserts free from cells, to be used as controls for the procedures of BBB validation.

1.2.2) Seed 1,5 x 10^5 RCAs on the bottom side of poly-L-lysine-coated insert, by dropping 800 µl of cells suspension on the upside down system (Figure 1A), and leave the RCAs solution on the inserts for 4 hr at RT, in order to provide an efficient attachment of the cells to the membrane. Aspire residual solution, put the inserts into the wells containing 2 ml of ECM and keep the multiwellinstandard culture conditions, changing the ECM every 2 days.

1.2.3) After 3 days, when the RCAs have coated the lower face of the insert, seed RBMECs on the upper side of the insert, following these steps: 1) detach RBMECs from the T175 flasks and count the total living cells, as reported in paragraph 1.1.2 and 1.1.3; 2) add 2,5 x 10^5RBMECs onto the upper surface of the insert membrane in ECM (1000 µl) (Figure 1B) and put the multiwell instandard culture conditions. Leave the system in culture for at least 3 days, by changing the ECM in the inner and lower chamber every 2 days.

Handle the inserts with tweezers in order to avoid as much as possible any contact with the PET membrane.

2 BBB validation

2.1) TEER measurements

Starting from the 3rd day of co-colture, check the TEER by inserting the BBB-bearing inserts in a Endohm-24SNAP cup containing 4 ml of ECM, connected to an EVOM2 Epithelial Tissue Volt/Ohmmeter. Together with the TEER measurements of BBB-systems, record the TEER values of 3 empty inserts, that will be subtracted from the values obtained with the BBBs. Multiply the resulting TEER values for the surface of the insert (4.2 cm^2) in order to express the results as $\Omega \times cm^2$. Record TEER every day, until the values are stable for at least two consecutive days, before performing flux experiments.

This procedure can be performed on the same BBB-systems devoted to the following permeability experiments (paragraph 3). Operate in sterility.

2.2) Trans-BBB flux of FITC-dextran 40 (FD40)

Measure the FD40 flux from the upper to the lower chamber of the BBB models compared to that across the 3 empty inserts employed for TEER background (2.1.2), according to the

following steps:
2.2.1) Add 1 mg/ml FD40 in the upper compartment of BBBs, and after 1, 2 and 3 hrwithdraw 200 µl samples from the 2 ml of ECM in lower chamber and measure the fluorescence intensity by spectrofluorimeter(λ_{ex} 488 nm and λ_{em} 515 nm).
2.2.2) After subtraction of background ECM fluorescence, determine the amount of permeated FD40 by using a calibration curve produced with known amounts of the compounds dissolved in ECM and calculatethe apparent permeability coefficient (P_{app}) from the mean flux values according to:
$P_{app} = J/AC$
where J is the flux of the molecule (moles/hr), A is the permeation area (cm^2) and C is the concentration of the molecule in the upper compartment ($moles/cm^3$).
A number of 3 or more BBB-systems that have reached suitable TEER values (usually between the 5th and the 7th day of co-colture), must be exclusively devoted to this validation procedure, and will not be employed for the following permeability experiments (paragraph 3); sterility is not mandatory.

3Trans-BBB permeation of FITC-loadedferritins (FnN)

A recombinant variant of human ferritin (Fn), produced in *Escherichia coli* and assembled in nanocages (FnN) for the encapsulation of different fluorescent molecules, is available in the NanoBioLab of Prof. Prosperi (University of Milan-Bicocca, Italy)[1]. FnN are loaded with fluorescein isothiocyanate (FITC), according to a previously described protocol[10] and the concentration of both ferritin and loaded molecule is accurately determined.

3.1) Trans-BBB flux of FITC and FITC-FnN
Measure the FITC-FnN flux from the upper to the lower chamber of validated BBB models (usually at the 5th-7th day of co-colture), compared to that of the free dye at 7 and 24 hr of incubation, according to the following steps:
3.1.1) Add FITC-FnN (50 µg/ml FnN, 1.1 µM FITC) or equal amount of free FITC into the upper chamber of the validated BBB systems, using at least 6 inserts for each formulation, in order to withdraw the 2 ml of ECM solution from the lower chamber of at least 3 inserts after 7 hr and, after 24 hr, the lower solution of the other 3 inserts.
3.1.2) Measure the fluorescence intensity of collected samples by spectrofluorimeter (λ_{ex} 488 nm and λ_{em} 515 nm) and, after subtraction of background ECM fluorescence, determine the concentration of permeated FITC or FITC-FnN by using two different calibration curves produced with known amounts of the compounds dissolved in ECM.

3.2) FITC-FnN localization in RBMECs
3.2.1) Once completely removed the ECM from the upper and lower chamber of the BBB systems exposed to FITC or FITC-FnN for 7 or 24 hr, wash with PBS and fix the RBMECs on at least a couple of inserts for each experimental group by adding paraformaldehyde (4% in phosphate buffer saline-PBS)in the upper compartment for 10 min at RT; wash three times to remove residual paraformaldehyde. From this point on, sterility is not necessary.

3.2.2) Cut fragments of the PET membrane, and proceed with the immunodecoration of the cells according the following steps: 1) permeabilize RBMECs with 0.1% Triton X-100 in PBS for 10 minutes; 2) perform the blocking step for 1 hr at RT with a solution containing 2% bovine serum albumin (BSA), 2% goat serum in PBS; 3) incubate samples with an anti-CD31 at a 1:20 dilution, for 2 hr at RT; 4)after three times washing with PBS, reveal anti-CD31 by exposing the cells for 2 hr at RT to a 2% BSA, 2% goat serum solution containing an adequate secondary antibody conjugated to AlexaFluor 546 at a 1:300 dilution.
3.2.3) Mount the fragments of inserts with the immunodecorated cells in Prolong Gold antifade reagent on microscope slides and, analyze the samples at the confocal microscope.

REPRESENTATIVE RESULTS:
During BBB setting, all steps of cell attachment and growth on the inserts can be monitored at the light microscope thanks to the transparence of the insert PET membrane. RCAs, seeded at a density of 35000 cells/cm^2, attach efficiently to the bottom side of the insert after 4 hr of incubation at RT (Figure 2A) and grow up to cover the membrane surface in 3 days, taking a spindle-shaped morphology (Figure 2B). RBMECs, seeded at a density of 60000 cells/cm^2, are visibly attached to the upper face of the PET membrane after about 3 hr of incubation at 37 °C (Figure 2C). The developing RBMECs layer is not really distinguishable over the following days at the optical microscope, because of the overlapping with underlying RCAs layer (Figure 2D).

A correct validation of the BBB model always requires TEER measurements (1), and can be confirmed by evaluating the trans-BBB P_{app} of a low permeability tracer, such as the FD40 (2).
1) The TEER values, recorded over the co-culture period, represent the first clear indication of a correct formation of the endothelial barrier. On the 3^{rd}day from the seeding of the RBMECs, the recorded TEER, subtracted of the TEER of empty inserts, is mainly due to the contribution of the non-electrogenic RCAs layer and of the developing RBMECs layer. At this time point, our BBBs gives values ranging between 40 and 60 Ω x cm^2.On the following days, usually between the $4^{th}$and the 5th day of co-culture, the TEER values arise thanks to the formation of tight junction between the endothelial cells[11], reaching values usually between 75 and 130 Ω x cm^2, and exceptionally over[11]. The values measured at the 4th/5th day of co-culture remain stable at least until the 7th-8th day before starting to fall, to indicate that a very narrow time window is available for the trans-BBB flux experiments.
2) Between the 5th and the 7th day of co-culture, if BBBs reach acceptable TEER values, the integrity of the experimental models can be confirmed by evaluating the FD40 trans-BBB permeability. Figure 3 shows an example of trans-BBB flux ofFD40 (1 mg/ml),compared to the flux across empty inserts, over 3 h of incubation; BBBs are at the 6th day of co-culture and the recorded TEER is 75.6 ± 15.8 Ω cm^2 (mean ± SE, n = 3). The flux is linear between 1 and 3 hr of incubation and the mean P_{app} calculated between 1 and 2 hr, and 2 and 3 hr of incubation is $0.12 \pm 0.01 \times 10^{-6}$ cm sec^{-1} (± SE, n = 6).
Once obtained at least three consecutive successful BBBs in independent experiments, in term of TEER and FD40 P_{app}, the measurement of tracer permeability can be avoided, while the TEER needs to be always recorded before each experiment.

The trans-BBB permeability of fluorescent molecules and the effect of nanocomplexation on their delivery can be investigated using the rat BBB models described above. Figure 4 describes the permeation of the model dye FITC upon encapsulation in FnN across BBBs with TEER of 120.4 ± 3.5 Ω cm^2 (n= 16) at the 7th day of co-culture. The histograms, representing FITC concentration in lower chamber after 7 and 24 hr from the addition of free or nanoformulated dye in the upper compartment, indicate that FnN is able to significantly increase the delivery of the cargo across the BBB.

Confocal microscope images of the upper side of the insert after 7 h of incubation with FITC (Figure 5 A,B) or FITC-FnN(Figure 5 C,D) show that nanoconjugation of FITC with FnN increases its internalization in RBMECs.
At the end of the incubations, a further check of the TEER is necessary before processing the inserts for the confocal microscopy, in order to assess the absence of any FnN-mediated effect on BBB integrity on the BBB endothelial cells.

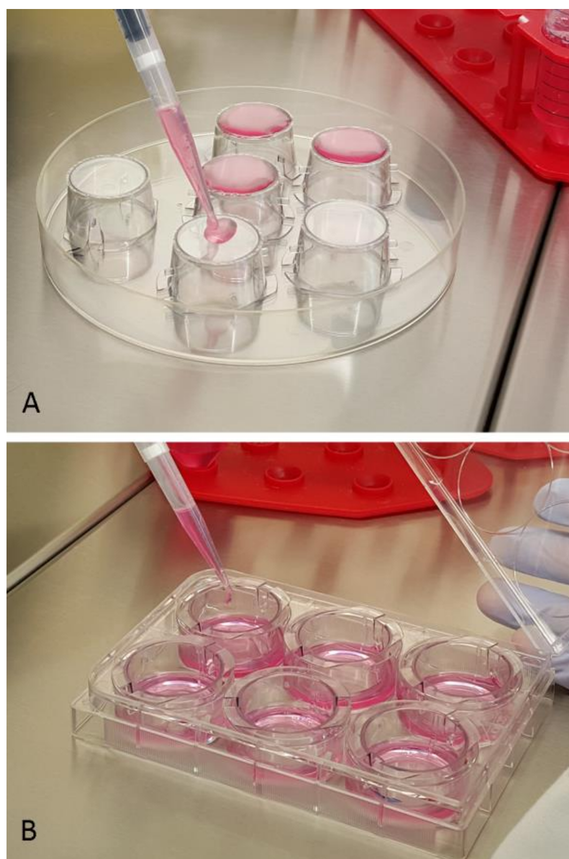

Figure 1: Cell seeding on inserts. Procedure of seeding of RCAs (A) and RBMECs (B) on the bottom and upper face of the inserts, respectively.

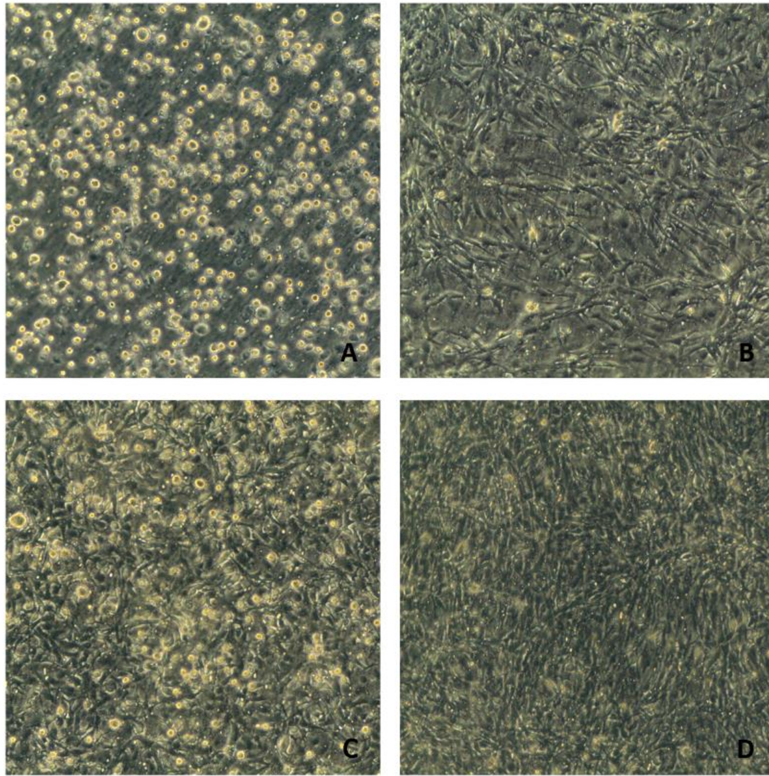

Figure 2: Optical microscope images of RCAs and RBMVECs on inserts. Insert seeded with RCAs and RBMECs are observed at the light microscope(20 × optical zoom). A) After 4 hr seeding, round and translucent RCAs are visibly attached on the bottom face of the insert; B) Spindle-shaped RCAs are visible at the 3rd day of culture on the insert; C) Round and translucent RBMECs are attached on the upper side of the insert after 3 hr of incubation; D) At the 4th day of co-culture both the faces of the insert are completely covered by cells, and the two layers are not perfectly distinguishable.

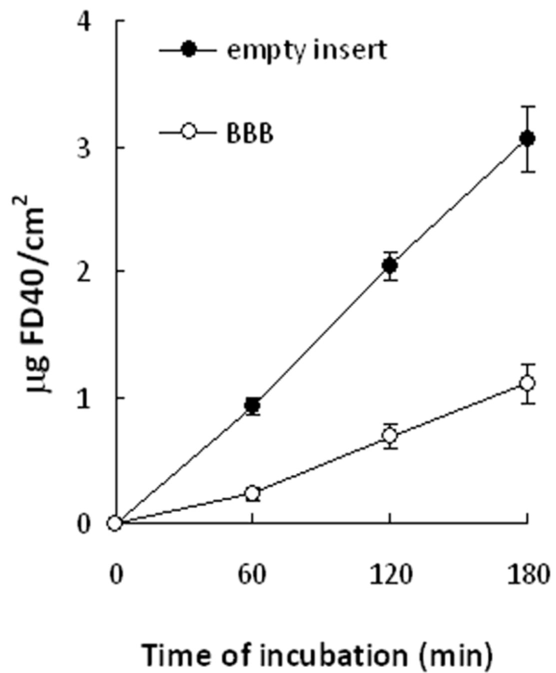

Figure 3: FD40 flux across the BBB. Time course of the FD40 (1 mg/ml) flux from the upper to the lower side of the BBB in vitro system, compared to that across the empty insert. Means ± SE; n° inserts = 3.

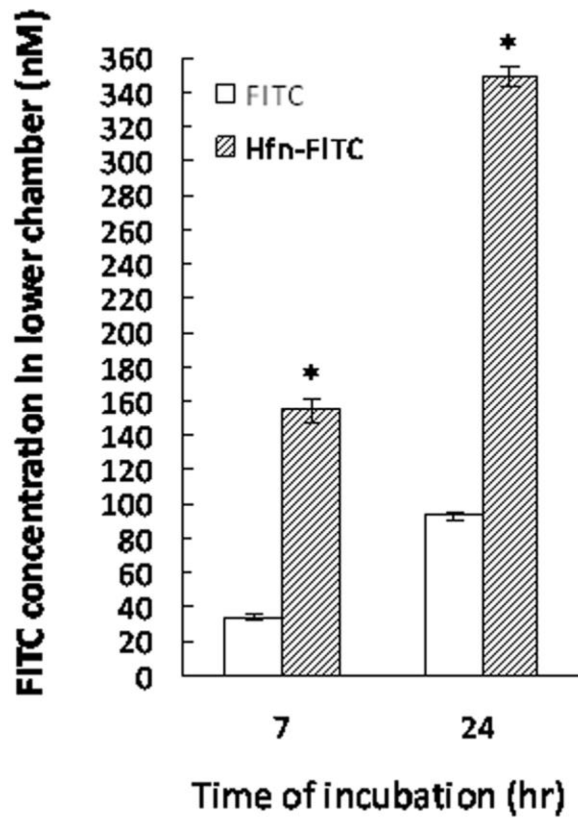

Figure 4: Effect of FnN encapsulation on FITC permeation across the BBB. Concentration of FITC in the lower chamber of the BBB *in vitro* system calculated at 7 and 24 hr from the addition of FITC or FITC-FnN into the upper chamber. Mean ± SE of 4-5 replicates; *P<0.0005, FITC-FnN vs FITC (Student's t-test).

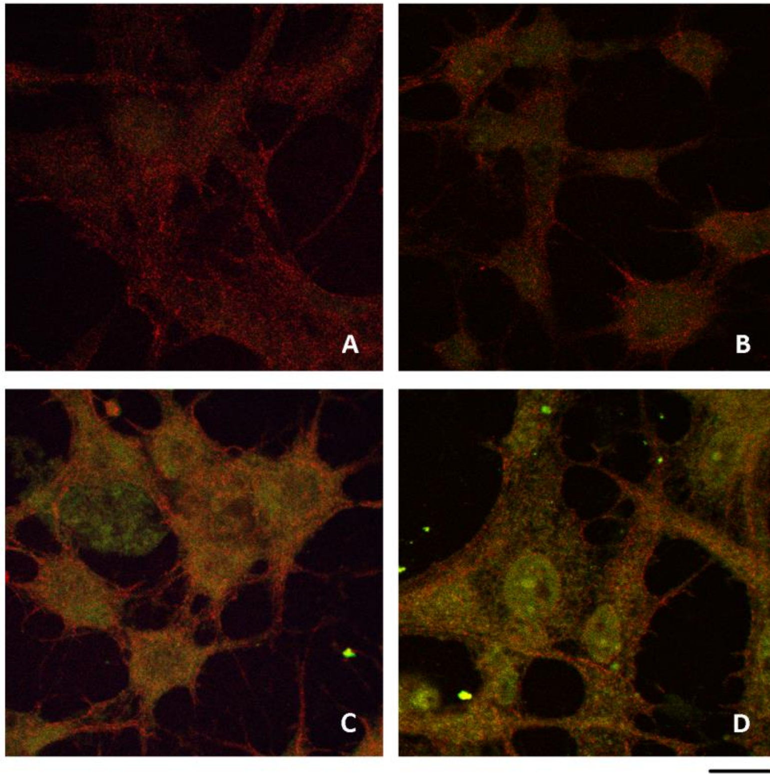

Figure 5: Confocal microscopy of RBMECs on inserts. Confocal laser-scanning micrographs (single optical sections) of RBMECs after 7 hr (A, C) or 24 hr(B, D) of incubation with free FITC(A, B) or FITC-FnN (C, D). FITC is green; endothelial cells are immunodecorated with anti-CD31 antibody (red). Images represent merge of the green and red channels. Bar: 10 µm.

DISCUSSION:

1. BBB model quality

The BBB *in vitro* model here presented, based on a co-culture of rat BMECs and astrocytes, represents a good quality model. Indeed, even when the recorded TEER is under the best standards suggested in literature (150-200 Ω cm^2)[8], the trans-BBB permeability of the high dimension tracer Dextran 40 is totally in agreement with the formation of a tight barrier. In this sense, it is interesting the comparison with other BBB models obtained with human BMECs with TEER >270 Ω cm^2, whose FD40 P_{app} values[12,13]are considerably higher than those obtained with our 75 Ω cm^2 model. Therefore, even if a certain correlation between TEER and P_{app} of tracers has been suggested in a wide comparative study on different types of BBBs[8], a significant selectivity to high dimension molecules could be itself an index of good quality for the BBB model.

2. Limitation and advantages of the model

The most important limitation of the present model is correlated to the high costs of the non-immortalized primary RBMECs, which must be newly purchased (frozen at passage one) for each

experimental setting. Indeed, our preliminary proofs of BBB production demonstrate that the ability of endothelial cells to produce a tight BBB is strictly associated to the number of passages in culture after the first post-purchase thawing: when the RBMECs are subjected to greater number of passages and/or to additional freezing and thawing steps compared to those described in Protocol section, they are no more able to develop an optimal BBB. In that case, TEER never exceeds the 40 Ω cm^2 during the whole period of co-culture and the FD40 P_{app} is over $10x10^{-6}$ cm sec^{-1}.

The advantages in developing BBBs from these rat BMECs and astrocytes, are (1) an optimal growth efficiency of the endothelial cells, (2) a suitable time for the production of the final model (no over 13 days) and (3) the compliance with ethical issues, avoiding to use cells gained from human brain tissue (autopsy material, surgical specimen, and fetal tissue).

3. Advantages of a multidisciplinary approach

The present technique allows exerting different assays on a single BBB model, with the aim to obtain several data from each experimental setting. In detail, on a single BBB system exposed to free or nanocomplexed fluorescent molecules, it is possible: 1) to measure the trans-barrier flux of the molecule by analyzing the fluorescence intensity of ECM aliquots collected from the lower chamber at different time points of incubation; 2) to investigate the nano-mediated internalization of the molecules in RBMECs and their intracellular trafficking by confocal microscopy analysis of the cells on inserts; 3) to determine the status of BBB cells upon exposure to the nanoformulations, by measuring TEER at the end of the experiment or by analyzing endothelium integrity by electron microscopy.

4. Future perspectives for the application of this model in nanodrug delivery studies

The *in vitro* method here described represents a useful standardized and validated approach to study the trans-BBB delivery of fluorescent molecules (i.e model dyes, labeled drugs etc..) upon nanoformulation with different types of organic or inorganic nanoparticles. FnN represents a good candidate for the translocation of molecules across the BBB because of the high biocompatibility, the high affinity of ferritin for the TfR1 receptor overexpressed on BMECs luminal membrane and the efficient mechanism of encapsulation of low dimension hydrosoluble molecules. However, many other types of nanoparticles could be employed with our model. Another example is represented by a polymer-coated iron oxide nanoparticle, which has been recently investigated as trans-BBB delivery system for fluorescence-labeled antiretroviral drugs[11]. In this case, as it could be for other inorganic nanoparticles, electron microscopy localization of nanoformulations in RBMECs can be associated to the fluorescence detection, and other highly sensitive diagnostic methods can be employed to evaluate the nanosystem delivery across the BBB (i.e. Inductively Coupled Plasma (ICP)-MS). On the other hand, in that case, a more accurate evaluation of NPs impact on BBB cells should be performed to ensure the absence of any toxic effect of the inorganic component.

In conclusion, the methodology here described achieves the production of a high-quality BBB *in vitro* model, which allows to study the permeation of nanocomplexed-fluorescent dyes such as FITC. The fluorescent cargo can be delivered loaded into the nanodevice, as described in the present article, or conjugated on the NPs surface[11]. As perspectives, this model could be a useful

approach also to study the effect of nanocomplexation on drug delivery across the BBB.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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