Eugenol induced dopamine release from neuron-like PC12 cells after its permeation across the physiological brain barriers simulated in a new organ-on-a-chip platform.

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INTRODUCTION

The **blood-brain barrier (BBB)** and **blood-cerebrospinal fluid barrier (BCSFB)** are critical determinants of central nervous system (CNS) homeostasis, posing challenging barriers to the permeation of circulating solutes, including ions, biomolecules, drugs (1). The study and characterization of multiple transporters endogenously expressed at the BBB and BCSF barriers can be focused on **EFFLUX TRANSPORTERS** that efficiently prevent drugs from attaining therapeutic concentrations in the CNS and/or on **UPTAKE (INFLUX) TRANSPORTERS**, that can facilitate drug delivery (1). **EUGENOL**, cinnamaldehyde and D-limonene, the main components of natural essential oils, are endowed with antioxidant and anti-inflammatory properties on intestinal, cardiac and neuronal levels (Fig. 1). Their pharmacokinetic profiles and aptitude to permeate in the CNS after intravenous and oral administration to rats have been previously characterized (2), evidencing the marked aptitude of Eugenol to permeate in the CSF of rats following both intravenous and oral administrations. Eugenol was therefore recruited for in vitro studies, evidencing its ability to increase cell viability and to induce dopamine release according to a **hormetic behaviour in neuronal differentiated PC12 cells** (2).

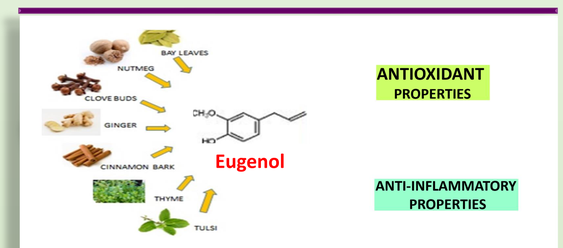


Fig. 1. Eugenol is a phenylpropanoid compound present in cloves and essential oil of spices with anti-fungal, anti-bacterial, anti-inflammatory, anti-mutagenic and anti-oxidant effects.

CELLBLOKS® is a new organ-on-a-chip platform developed by Revivocell®, suitable to test and identify assorted combinations of cells that replicate physiological barriers protecting specific microenvironments. CELLBLOKS® represents an advantageous *in vitro* system in which to easily overview complex physiological phenomena, such as **UPTAKE, DIFFUSION** and **PERMEATION** of a potentially therapeutic compound, through a set of compartments delimited by physiological barriers, such as the **gastrointestinal (GI), BBB** and **BCSF barriers** (Fig. 2).

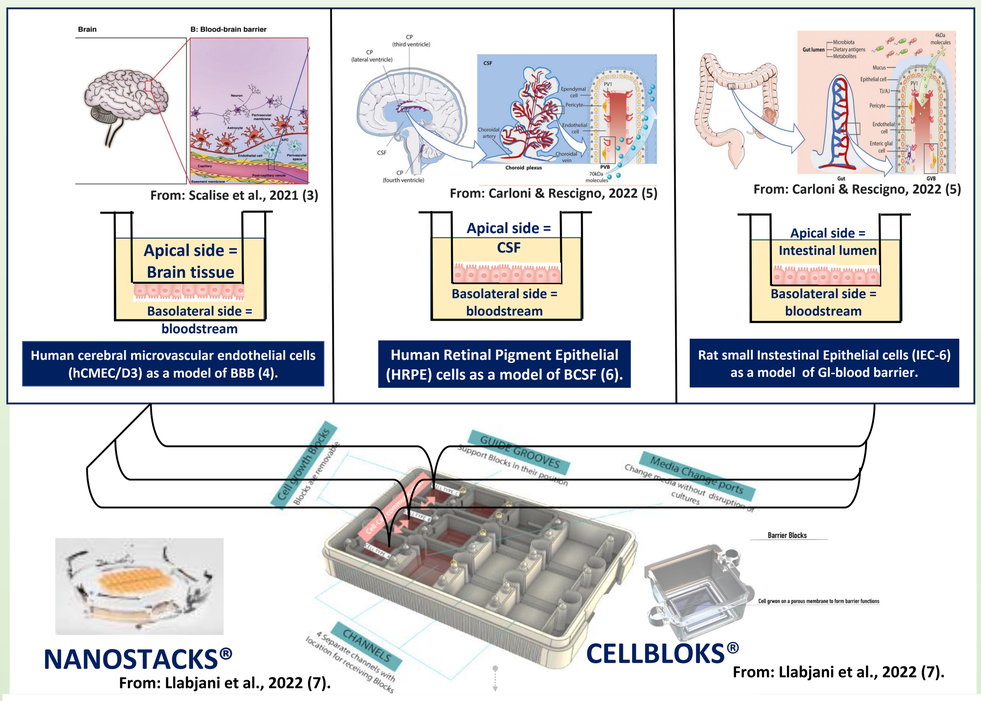


Fig. 2. Schematic comparison between in vivo BBB, BCSFB, and GI and their in vitro simulation in CELLBLOKS system. Neuronal-like PC12 cells were grown in NANOSTACKS inserts

OBJECTIVES

1. Identify the physiological barrier through which eugenol can reach the CNS, i.e., whether it permeates through the BBB or through the BCSFB at the level of the choroid plexuses.
2. Simulation of both its oral administration through the GI (FIG. 3A), and its intravenous administration (FIG. 3B), directly reaching the BBB and BCSFB.
3. Addition on the apical side of BBB and CSFB dopaminergic neuron-like rat PC12 cells seeded on NANOSTACKS™ inserts to replicate the neuronal compartment. Future work will focus on the comparison between BBB and CSFB routes for eugenol-induced dopamine secretion by the PC12 within this model, to maximally improve the therapeutic target in Parkinson's disease (FIG. A1 and B1)

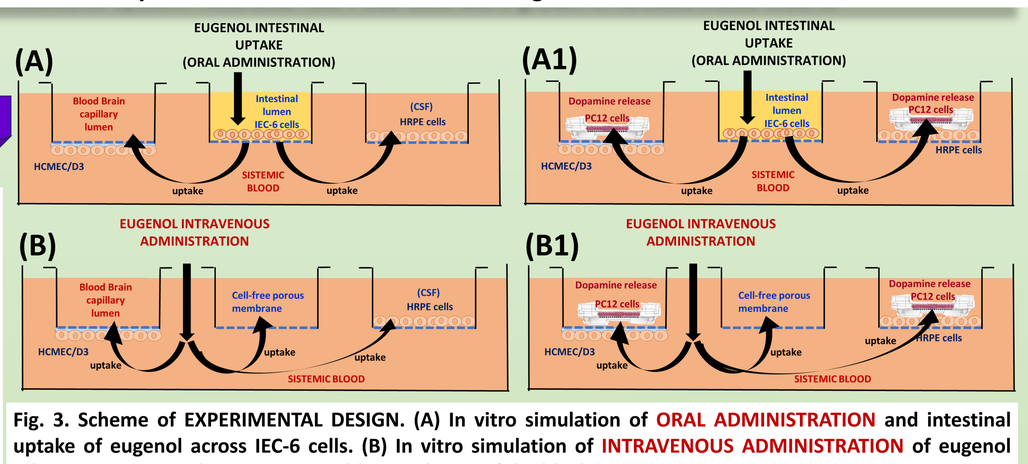


Fig. 3. Scheme of EXPERIMENTAL DESIGN. (A) In vitro simulation of **ORAL ADMINISTRATION** and intestinal uptake of eugenol across IEC-6 cells. (B) In vitro simulation of **INTRAVENOUS ADMINISTRATION** of eugenol taking into account the semi-permeable membrane of the block.

METHODS

All the cell lines were maintained at 37°C, 5% CO_2, in a humidified atmosphere. Endothelial hCMEC/D3 cells were seeded on collagen I coated dishes and grown in MCDB131 medium supplemented with 5% FBS, 1% P/S, 1% glutamine, 1 ng/ml bFGF, 5 µg/ml ascorbic acid, 2 µM hydrocortisone. Epithelial HRPE cells were grown in DMEM/F12 medium supplemented with 10% FBS, 1% P/S, 1% glutamine. Intestinal IEC-6 cells were grown in DMEM containing GLUTAMAX, 10 % FBS, 1% P/S. All reagents were from Thermofisher Life Technologies, Milan, Italy. CELLBLOKS® is a patented (GB2553074B), open-top multi-chambered organs-on-a-chip device designed by Revivocell® (Daresbury, Warrington, UK). Cell density suitable for seeding in Cellbloks was obtained using the cell counter Scepter (Merk-Millipore, Milan, Italy). Trans-epi-endothelial electrical resistance (TEER) values were measured using a Millicell voltmeter (Merk-Millipore, Milan, Italy). TEER values were obtained by applying a transendothelial current to the membrane and then testing the membrane potential generated, and finally translating the value into resistance (current, Ohm) multiplied by the area (cm^2) of the epithelial or endothelial monolayer (Ohm·cm^2). Eugenol concentration in the apical and basolateral compartments was measured by high pressure liquid chromatography (HPLC).

RESULTS AND DISCUSSION

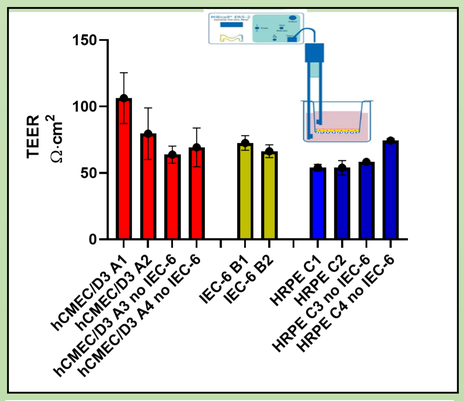


Fig. 4. **Tightness and permeability of IEC-6, HCMEC/D3 and HRPE cell monolayers were evaluated as TEER values**, which after 8 days reached their plateau and were comparable for the three cell types, whether cultured alone or in the same channel of cellblocks.

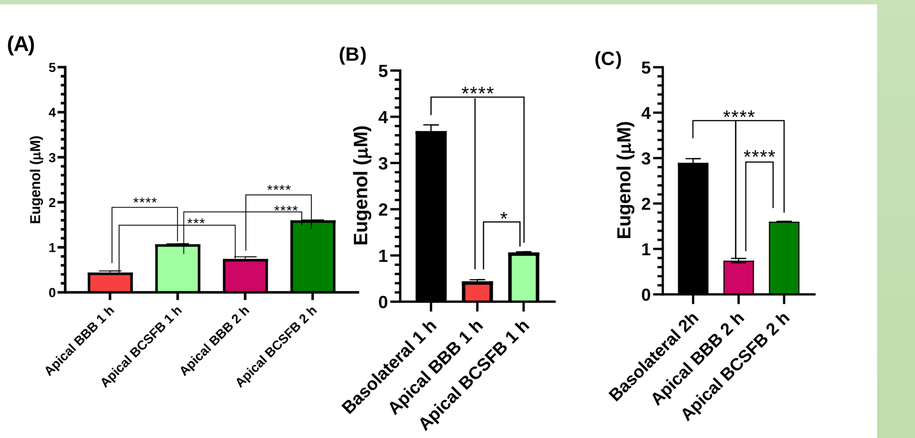


Fig. 5. **ORAL ADMINISTRATION was simulated by addition of 25 µM eugenol to the APICAL compartment of GI-mimetic IEC-6 cells. . (A) From the basolateral compartment (systemic circulation) eugenol can cross both BEE (hCMEC/D3 cells) and BEL (HRPE cells), reaching a quantifiable concentration in both apical compartments, with higher and statistically significant concentration in the apical CSF-mimicking compartment (HRPE cells). 25 µM Eugenol decreased 6-folds (B) and 8-folds (C) passing from the apical to the basolateral compartment of IEC-6 cells, because of the dilution factor and the intestinal barrier features of IEC-6 cells.**

****P<0.0001 ***P<0.0006 *P<0.005 ANOVA followed by Tukey's test for multiple comparisons.

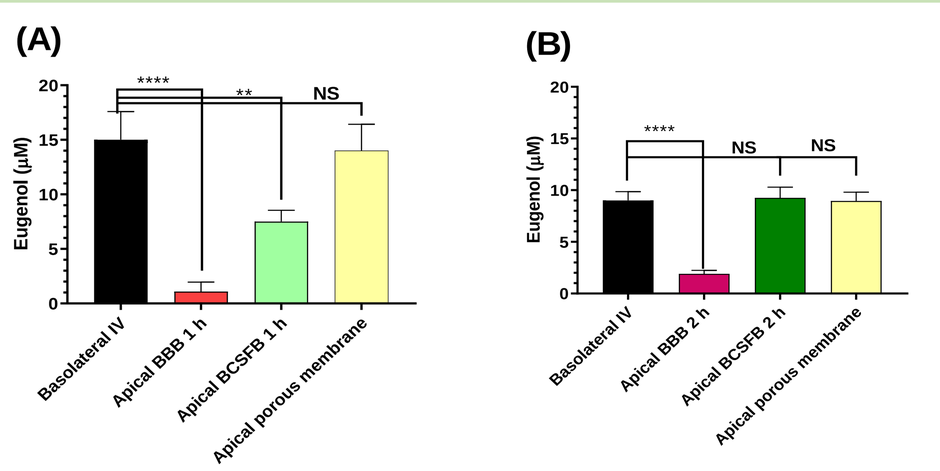
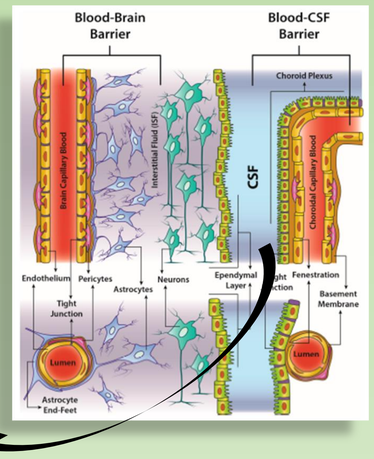


Fig. 6. **INTRAVENOUS ADMINISTRATION (IV) was simulated by direct spiking of 25 µM eugenol to the BASOLATERAL bloodstream-mimetic compartment. 25 µM eugenol crossed both the BBB and the BCSFB, accumulating in the respective apical compartments, therefore basolateral concentration was statistically significantly reduced after 1 h (A) and 2 h (B). As a result, the predominant accumulation of eugenol in the apical compartment of the BCSFB after both 1 h and 2 h was also confirmed in the intravenous-like administration.**

****p<0,0001; **p<0,003followed by Tukey's test for multiple comparisons. NS: not significant

CONCLUSIONS

- In vitro oral administration was simulated spiking eugenol to the apical side of intestinal IEC-6 cell blocks, which reduced eugenol uptake as seen prevoiously in vivo, but did not prevent it from reaching a concentration in the basolateral channel adequate to cross BBB and BCSFB, with a significant prevalence across BCSFB. The systemic administration was simulated spiking eugenol in basolateral channel of the BBB and CSFB, without intestinal IEC-6 cells and the amount of eugenol found in blocks of BBB and CSFB was higher than after oral administration, but still higher in CSFB apical compartment.
- Dopaminergic neuron-like rat PC12 cells, seeded on collagen IV-coated stacks, were inserted in the apical side of BBB and CSFB to replicate brain neuronal compartment, which must be reached by eugenol and stimulated to release dopamine in BBB and CSFB compartments, which remains to be compared.
- The characterization of the INFLUX TRANSPORTERS expressed at the level of the BCSFB could allow to design EUGENOL PRODRUGS aimed to carrier into the brain active compounds but poor permeable, leading them to reach their therapeutic concentration in the CNS.



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