Cleveland Clinic Science Internship Program

In vitro assessment of cigarette smoke toxicity on blood-brain barrier associated cells

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Abstract

Active and passive cigarette smoking are associated with dysfunction of normal endothelial physiology [1]. Cigarette smoke is known to contain high concentrations of free radicals and oxidants. However, virtually nothing is known about the oxidative damage associated with cigarette smoke on *human* brain microvasculature and more specifically on the cellular components of the blood-brain barrier (BBB). Such a study can provide us with an understanding of whether smoking is associated with BBB damage, which is known to result in brain disorders such as epilepsy, Alzheimer's, and multiple sclerosis.
In this study, we decided to: **(1)** investigate the effect of soluble cigarette side smoke extract (CSSE) on BBB integrity using a dynamic *in vitro* model, **(2)** test the effects of CSSE on human brain microvascular endothelial cells (HCMEC/D3), astrocytes, and monocytes, and **(3)** see if the presence of antioxidants (i.e. Vitamin C, Vitamin E) can counteract oxidants in the CSSE and minimize oxidative damage to the cells. Specifically, we looked at cell survival following the exposure to superoxide and other reactive oxygen species in the smoke extract. Furthermore, we tested the protective effect and efficacy of both vitamin C and vitamin E.

Hypothesis

Exposure to free radicals and superoxide contained in cigarette smoke can disrupt endothelial tight junctions, decreasing the integrity of the BBB. Also, oxidative damage from CSSE can decrease cell viability and cause endothelial cells (EC) to release pro-inflammatory cytokines, triggering an inflammatory response. The presence of antioxidants such as Vitamin C and Vitamin E can prevent cigarette smoke induced oxidative damage of proteins and increased proteolysis.

Rationale

Because cigarette smoke contains superoxide and other reactive oxygen species (free radicals), some of the adverse effects of smoking may result from oxidative damage to ECs [2], such as DNA strand breakage [3;4], which results in shortages of nitric oxide (NO) and antioxidants. The hypothesis is strongly supported by the fact that antioxidant supplementation prevents the oxidation and inflammation induced by cigarette smoke in animals and cells [5;6]. A controlled trial demonstrated the ability of 3 grams of vitamin C, taken by non-smokers two hours prior to being exposed to cigarette smoke, to reduce the free radical damage and LDL cholesterol oxidation associated cigarette smoke exposure [7].

References

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4. DeMarini, et al., Mutat. Res. 567: 447-474; 2004.
5. Koul,A. et al., Inhal. Toxicol. 15: 513-522; 2003.
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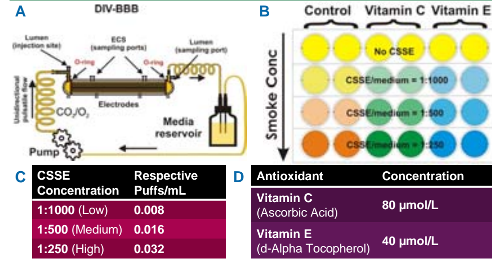
Experimental setup



CSSE Concentration	Respective Puffs/mL
1:1000 (Low)	0.008
1:500 (Medium)	0.016
1:250 (High)	0.032

Antioxidant	Concentration
Vitamin C (Ascorbic Acid)	80 μmol/L
Vitamin E (d-Alpha Tocopherol)	40 μmol/L

Figure 1: **(A)** Schematic representation of the DIV-BBB apparatus, **(B)** Setup of 24 well plate used to test effects of CSSE and/or antioxidants, **(C)** Concentrations of CSSE in cell medium and equivalence in puffs/mL **(D)** Concentrations of antioxidants used to treat cells

Dynamic *in vitro* blood-brain barrier (DIV-BBB) model setup

Human brain microvascular endothelial cells (HBMEC) were intraluminally co-cultured under dynamic conditions with abluminal human astrocytes in pre-coated artificial capillaries embedded in a polycarbonate hollow chamber. The luminal compartment was connected to a circuit containing a medium reservoir and a pulsatile pump and BBB integrity was assessed by measuring trans-endothelial electrical resistance (TEER) **(Fig. 1A)**.

24 Well Plate setup

Human endothelial cells (HCMEC/D3), astrocytes, and monocytes were seeded into 24 well plates and exposed to three different concentrations of CSSE. Cells were either left untreated or treated with Vitamin C or Vitamin E, at the concentrations shown **(Fig. 1B,1C,&1D).**

Sampling Protocol

200μL of supernatant from EC and astrocyte cell cultures was collected 1h, 3h, 6h, 24h, 48h, 72h following treatment with CSSE and/or antioxidants. 200μL of monocyte cultures were collected 6h and 24h following treatment. All samples were stored at -20°C. The sampled volume was replaced with the appropriate media. Cell morphology was assessed by direct inspection with an inverted light microscope.

Data

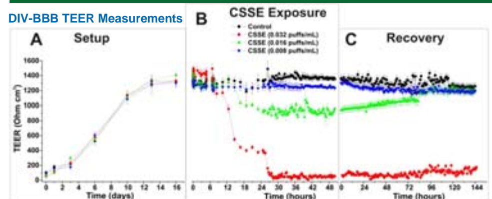


Figure 2: Effect of CSSE exposure on BBB integrity. Panel A: TEER measurements reveal the establishment of viable BBBs *in vitro* in the DIV-BBB system. Panel B: Measurements of TEER shows a deterioration of the BBB integrity following the exposure to CSSE. The effect is dose dependent and becomes evident between 8 and 24 hours following the initial exposure. Panel C: BBB integrity can be restored if cells are still viable.

Data (cont'd)

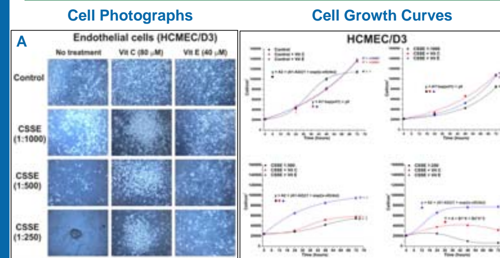


Figure 3: Effect of CSSE exposure on BBB endothelial cells. Brain microvascular endothelial cell viability decreases as CSSE concentration increases **(A,B)**. These preliminary data strongly suggest that treatment with vitamins C and E has a protective effect on cell viability and stimulates cell growth under control conditions **(B)**.

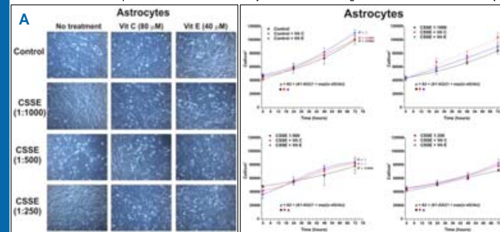


Figure 4: Effect of CSSE exposure on BBB astrocytes. Astrocytes are only minimally affected by CSSE exposure, even at concentrations lethal to ECs **(A, B)**. Pre-treatment with vitamins C and E positively affects the cellular growth as previously seen in endothelial cells **(A)**.

Zymography Gels

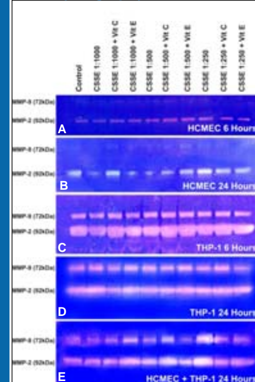


Figure 5: Assessment of MMP-2 and MMP-9 Activity Using Zymography. Zymography was used to study the activity of Matrix metalloproteinases 2 and 9, (MMP-2 and MMP-9) in cell culture supernatant samples of ECs and monocytes collected 6h 24h following treatment **(Panels A&B)** and also in monocyte culture samples taken at the same time intervals **(Panels C&D)**. Further, supernatant from ECs and monocytes co-cultured was collected 24h following exposure **(Panel E)**. The data suggest that endothelial cells show little noticeable MMP-9 activity, and that MMP-2 activity is significantly less in ECs than in monocytes. Monocytes show uniform MMP-9 activity with slight variation in MMP-2 activity. Monocytes and endothelial cells co-cultured appear to release both MMP-2 and MMP-9, but data collected from the other gels suggest that monocytes are solely responsible for MMP-9 release.

Results

The exposure to increasing concentrations of CSSE resulted in a proportional loss of BBB integrity (**Fig 2**). Antioxidants exhibited a protective effect on the ECs. Although vitamin E appeared to be more effective in protecting the cells in the long term and at higher concentrations of CSSE, vitamin C appeared to be more effective at lower concentrations of CSSE and at earlier time intervals. This can be attributed to the chemical composition of the antioxidants. Vitamin C is hydrophilic, so it can neutralize the CSSE in the medium, providing a short term protective effect. Contrastingly, Vitamin E's lipophilic nature allows it to pass through the membrane and enter the cells, providing better protection to the ECs **(Fig. 3)**. Astrocytes were not adversely affected by cigarette smoke. The presence of antioxidants showed an increase in astrocyte viability **(Fig. 4)**. The monocytes did not show a significant response to smoke (figure not shown). This suggests that monocytes are not activated by CSSE exposure alone; rather, they are activated by the endothelium. This is supported by zymography data **(Fig. 5)**.

Conclusions & Suggestions

Our preliminary data confirm that oxidative damage due to cigarette smoking results in a loss of BBB integrity due to endothelial cell death rather than astrocyte death **(Fig. 2)**. The presence of antioxidants protects the cells from oxidative stress caused by superoxide and free radicals present in the cigarette smoke. Additional experiments with a more complex *in vitro* setup will be needed to confirm our preliminary data. The DIV-BBB system in combination with primary human brain endothelial cells, abluminal astrocytes and circulating white blood cells, can be successfully used to mimic the BBB physiology *in vivo,* thus providing a valuable tool to evaluate the effects of exposure to potentially damaging xenobiotic substances (such as cigarette smoke) on the BBB integrity. The same system can be also used to evaluate the efficacy of current and novel drug treatments aimed at protecting the BBB viability.

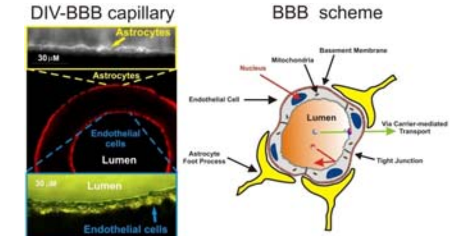


Figure 6: The BBB: *In vitro* vs. *in vivo*. Cross section view of a DIV-BBB artificial capillary *(left)*. Schematic of the human BBB *(right)*. In the DIV-BBB capillary, endothelial cells grow in a typical mono-layer fashion with astrocytes co-cultured on the abluminal side of the hollow fiber, thus mimicking the physiology of the human BBB.