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Protective effects of Folic acid and Vitamin C against iron overload at the *in vitro* Blood-Brain Barrier

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ABSTRACT

Brain iron accumulation increases with age and this is more common in patients with neurodegenerative diseases such as Parkinson's disease. Also iron overload in addition to zinc accompanies with plaques containing β-amyloid (AP) of Alzheimer's Disease. The blood-brain barrier (BBB) is a dynamic interface between the blood and the brain that plays an important role in maintaining central nervous system (CNS) homeostasis.

In our study, protective effects of vitamin C and folic acid against iron overload in the *in vitro* blood-brain barrier model were investigated. Four different groups were created for the experimental procedure: 1) Control, 2) $FeSO_4$, 3) $FeSO_4$ + Vit C, 4) $FeSO_4$ + Folic acid for toxicity experiments. After iron overload, permeability differences of Vitamin C and folic acid *in vitro* BBB model were assayed using Bovine Serum Albumin (BSA) Bradford protein assay. Both substances were found to have a protective effect against iron sulphate-induced damage. Also, vitamin C and folic acid significantly decrease the permeability after increasing caused by iron sulfate in BBB model. Considering the toxic effects of high concentrations of vitamin C, systemic effects of folic acid should also be investigated by in vivo studies to compare with vitamin C in ageing.

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Introduction

The blood-brain barrier (BBB) is a dynamic interface between the blood and the brain that plays an important role in maintaining central nervous system (CNS) homeostasis. Endothelial cells are interconnected by tight junctions that are critical both for the control of BBB vascular permeability and for the protection of the brain from various circulating toxins and other harmful molecules [1, 2]. Astrocytes and pericytes also contribute to the formation and maintenance of BBB [3].

The major functions of BBB are: (1) maintaining different ionic composition required for neuronal function, (2) special neurotransmitter pool, (3) low protein concentration, (4)

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preventing toxin exposure leading to neuronal damage, (5) reducing the traffic of molecules and inflammatory cells to prevent local inflammation [4, 5].

Iron ions serving as transition metal molecules that catalyze hydroxyl radical production through the Fenton reaction and Haber-Weiss cycle, accumulate in neurons, astrocytes and microglia, causing neuron death [4]. It is known that damage due to reactive oxygen species (ROS) and nitric oxide (NO) causes diseases such as cancer and Alzheimer's disease (AD) [3, 6].

Vitamin C, which cannot be physiologically synthesized by humans and primates, can be synthesized endogenously by some mammals (e.g., mouse and rat) [7]. Vitamin C, a water-soluble vitamin, should be taken in a daily diet. Vitamin C is involved in critical pathways for the body, such as protecting the integrity of vascular and connective tissue, inhibition of lipid peroxidation and acts as a scavenger of free radicals in the brain [7, 8].

Folate plays an important role as a cofactor or coenzyme in the maintenance and repair of the genome, regulation of gene expression, amino acid metabolism, myelin formation and neurotransmitter synthesis [8]. Folate is hydrophilic vitamin of the B complex, deficiency of which results in some disease such as hyperhomocysteinaemia, cerebral folate deficiency syndrome [8].

In our study, protective effects of vitamin C and folic acid against iron overload in the *in vitro* blood-brain barrier model were investigated.

Materials and Methods

in vitro Blood-Brain Barrier

ECV304 (ATCC® CRL¬1998 ™) human umbilical vein endothelial cell line was used for the *in vitro* blood-barrier model. DMEM (Dulbecco's Modified Eagled Medium/High Glucose, DMEM/High, Gibco 41966) including 10% fetal bovine serum (Gibco 10270106) and 1% penicillin-streptomycin (Gibco 15070063) was used as ECV304 medium. To produce monolayer-barrier models, $5x10^4$ ECV304 cells were seeded in 1 ml of DMEM into the top well of the transwell plate (Costar 3450, 6-well polyester filter). 3 ml of DMEM was added to the bottom well of the plate [9].

Toxicity Studies

Four different groups were created for the experimental procedure: 1) Control, 2) $FeSO_4$, 3) $FeSO_4$ + Vit C, 4) $FeSO_4$ + Folic acid. For first group, ECV304 cells incubated with

only medium. On the other groups, 250 µl from 1mM $FeSO_4$ solution and 750 µl DMEM were added and incubated with ECV304 cells during o/n. While in the third group firstly 100 µg/ml Vitamin C (Selenovita-C) for 24 hours then $FeSO_4$ were added, in the last group after 100µg/ml folic acid (Folbiol (5 mg) resolved in 1 ml DMSO and diluted with DMEM medium) 24 hours incubation, $FeSO_4$ was added. All group were incubated 24 hours.

Trypan Blue Cell Viability

All goups were washed with serum-free medium. Then incubated with trypan blue solution for 5–15 min. Cells were then washed three times with DMEM with 0.1% BSA, and the number of dead cells staining blue was counted under a phase-contrast microscope [10].

Permeability Measurement

After iron overload, permeability differences of Vitamin C and folic acid *in vitro* BBB model were assayed using Bovine Serum Albumin (BSA) Bradford Protein Assay Kit protocol according to manufacturer instructions (Bio Basic Inc, SK3031). After creating the BSA standard curve, 110 µq/ml BSA, serum free medium solution was added to the upper well of the transwell. In the 30^{th} and 60^{th} minutes, 20 µl sample was collected from both the upper and lower wells to the eppendorfs. 200 µl of Bradford Reagent (BioBasic) was added to each sample. It was also turned in a shaker for 30 seconds and incubated for 10 minutes at room temperature. After incubation, their absorbances were measured in 595 nm.

Permeability was analyzed according to the formula below [11].

Papp (cm/s) = (Concentration of basal compartment ($[C]_B$) x Volume of basal compartment (V_B)) / the surface area available for permeability (A) x Concentration of apical compartment ($[C]_A$) x t)

Results and Discussion

In this study, protective efffects of vitamin C and folic acid against iron sulfate-induced damage in the blood-brain barrier model were investigated. Trypan blue cell viability results of 4 groups were shown in Figure 1. As shown in Figure 1B by the arrows on the barrier model after $FeSO_4$ exposure, decreased cell adhesion and dead cell density stained with trypan blue indicate the damage caused by $FeSO_4$.

Lockman et al. showed that iron causes oxidative damage and destruction in the *in vitro* blood-brain barrier model [12]. Another effect of oxidative stress on BBB decreases matrix metalloproteinases (MMP-1, -2 and -9) inhibitors and protein tyrosine kinase (PTK) inhibitors. It is known that the increase in MMPs and PTK activities is parallel to the disruption in tight junction (TJ) proteins [13]. Decreasing of intercellular tight junctions increases the permeability in blood-brain barrier.

Brain iron accumulation increases with age and this is more common in patients with neurodegenerative diseases such as Parkinson's disease [14]. Parkinson's patients have been shown that their iron levels in the substantia nigra increase in the patients compared to controls [3]. The elevation of chronic ferritin has been shown to result in age-related progressive neurodegeneration of neurons containing midbrain dopamine. Iron overload in addition to zinc accompanies with plaques containing β-amyloid (AP) of AD [3].

In addition to Parkinson's Disease, it has been observed that oxidative stress leads and aggravates the course of the disease in diseases such as Alzheimer's, ALS, and MS. It has been reported that brain damage is triggered by increased damage and permeability in the BBB [15].

Regulation of iron homeostasis includes many steps that control iron transport, storage and regulation. Although iron ion plays an important role in many biochemical processes such as neurotransmitter synthesis, myelination, oxygen transport and cell division, it is known to cause toxic effects at high doses [15]. Increased amount of iron ions in cells contributes to the production of reactive oxygen species (ROS) through Fenton reaction [4].

Vitamin C has been shown to have a protective effect on the blood-brain barrier [16]. Vitamin C, in the form of deaccorbic acid, is known to increase the antioxidant capacity in the central nervous system by crossing the blood-brain barrier via glucose transporter protein 1 (GLUT1) [17]. Folic acid, a water-soluble vitamin, is essential for DNA synthesis, repair and methylation in the cell. It is known that folic acid has anticancer, cardiovascular and neuroprotective effects. Folic acid deficiency can affect various processes in the body, including loss of extracellular matrix component collagen and disruption of the blood-brain barrier [18].

In Figure 1C and 1D, barrier models that are exposed to metal after 24 hours incubation with vitamin C and folic acid, respectively, are shown. Compared to the damage seen in Figure 1B, both substances have protective effects against iron sulphate-induced damage.

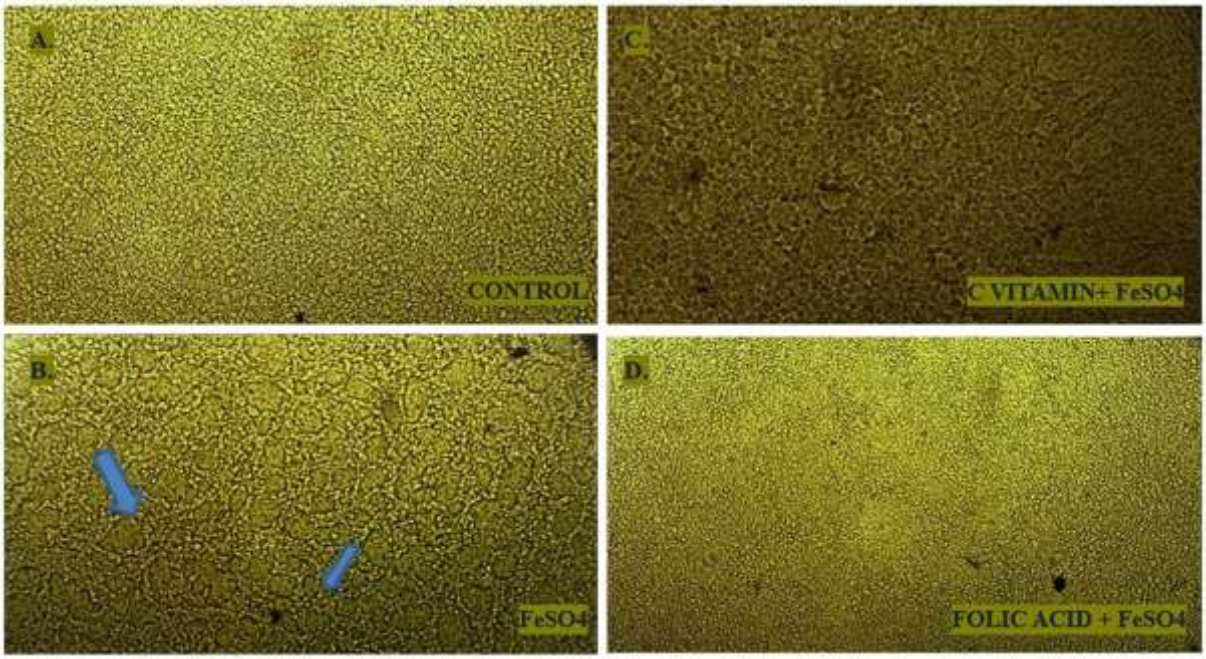

Fig 1 A. Microscope image of a monolayer blood-brain barrier model after trypan blue staining, B. ECV304 cells incubated with $FeSO_4$, C. After vitamin incubation for 24 hours, $FeSO_4$ was added. D. Folic acid incubation for 24 hours then $FeSO_4$ was added

As seen in the graph given in Figure 2, iron sulfate increases barrier permeability. Increased BSA transition indicates reducing cell-cell interactions and barrier damage. Also it is seen that vitamin C and folic acid significantly decrease the permeability after increasing caused by iron sulfate. Both folic acid and Vitamin C were found to have similar protective effects compared to each other.

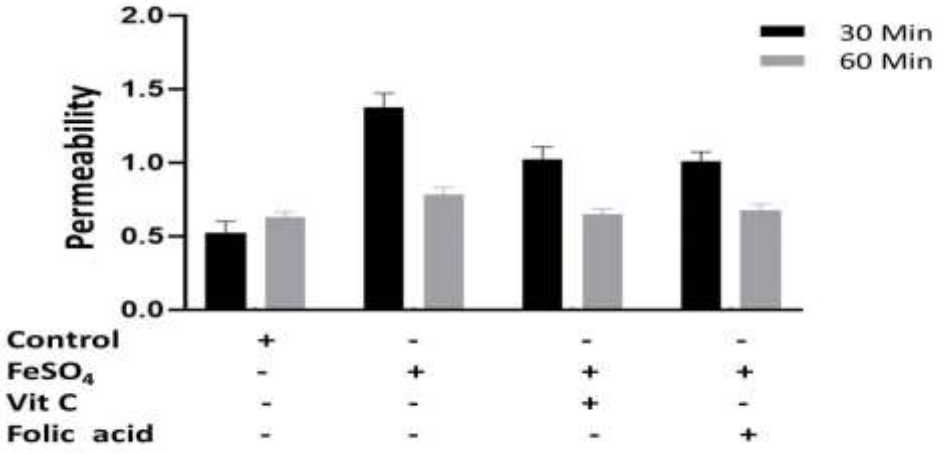

Fig 2 Effects of iron overload, vitamin C and folic acid on BSA permeability in a monolayer blood-brain barrier model

Conclusion

Vitamin C accumulates in the central nervous system and its level in the brain is much higher than in plasma or other organs. Plasma vitamin C levels are lower in AD patients than healthy individuals. Vitamin C has been shown to have a protective effect on the blood-brain barrier. However, mechanisms of folic acid against iron overload in blood-brain barrier are unknown. It has been shown *in vitro* that folic acid supplementation may have a protective effect against increasing Fe concentration in the brain with aging. Considering the toxic effects of high concentrations of vitamin C, systemic effects of folic acid should also be investigated by in vivo studies to compare with vitamin C in ageing.

Abbreviations
AD: Alzheimer's disease; BBB: The blood-brain barrier; BSA: Bovine Serum Albumin

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