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Effect of Iron Overload on Tight Junctions and Adhesion Molecules in ECV304 Cells

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Abstract

Blood vessels are essential for the body's tissues and organs to receive oxygen and nutrition. The Blood-Brain Barrier (BBB) is a special feature of the blood arteries that vascularise the Central Nervous System (CNS) which enables these vessels to tightly control the flow of ions and molecules between the blood and the brain. The accurate regulation of CNS homeostasis facilitates appropriate neuronal performance and safeguards neural tissue from toxins and pathogens. Modifications to these barrier characteristics play a significant role in the development of many neurological disorders. BBB contains tight junction transmembrane proteins, integral membrane proteins, occludin, claudins, IgG-type proteins, junction adhesion molecules and scaffold proteins. Numerous biological functions require iron in the CNS, including neurotransmitter synthesis, myelin formation and mitochondrial function. However, excess iron can lead to oxidative stress and damage, which are implicated in neurodegenerative diseases. In this research, the impacts of iron accumulation on Cadherin 5 (CDH5), Claudin 5 (CLDN5), Intercellular Adhesion Molecule 1 (ICAM-1), Occludin (OCL), p-selectin (P-SEL), Vascular Cell Adhesion Molecule 1 (VCAM-1) and Zonula occludens-1 (ZO-1) genes expressions in Human Umbilical Vein Endothelial Cells (ECV 304) cells were investigated. It was found that in human umbilical vein endothelial cell line cells, iron overload enhanced the expression of CDH5 and P-SEL genes while reducing the expression of VCAM1, Cldn5, ICAM-1, OCL, and Zo-1 genes.

Keywords: Blood- Brain Barrier, ECV-304, Gene Expression Analysis, Iron Overload

1. Introduction

The Blood–Brain Barrier (BBB) refers to the distinct characteristics of the Central Nervous System (CNS) microvasculature. The brain capillary endothelium's TJ-controlled paracellular impermeability suggests that hydrophilic molecules must pass through the endothelium transcellularly to reach their neuronal destinations or exit the brain. The five primary types of transcellular bidirectional transport across the BBB are receptor-mediated transport, ion transport, carrier-mediated transport and caveolae-mediated transport[1,2].

Due to their strong ability to restrict blood flow, BBB Endothelial Cells (ECs) are able to maintain the central nervous system's homeostasis, which is essential for normal neuronal function. They can also shield the central nervous system from infections, toxins, inflammation, damage and disease. Since the BBB's restrictions make it difficult to transfer drugs to the CNS, significant efforts have been made to develop

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strategies for modulating or avoiding the BBB for the flow of medicines[3].

Ion dysregulation, altered signalling homeostasis, immune cell and molecule entrance into the CNS, and other processes that result in neuronal dysfunction and degeneration can all be caused by BBB dysfunction[4]. BBB contains Tight Junction (TJ) transmembrane proteins, integral membrane proteins, occludin and claudins (claudin-5, -3, -12, -1) an IgG type protein, Junction Adhesion Molecule (JAM) and scaffold proteins (ZO-1, ZO-2)[5]. Occludin is a functional component of intercellular tight junctions. Scaffold and actin proteins interact with the cytoskeleton[5,6].

Claudins are among the TJ proteins. There are now 27 members of the Claudin family known to exist. At least eight related integral membrane proteins with four transmembrane domains each make to the claudin family. All of the family members are parts of the TJ strands seen in a variety of tissues. Claudin-5 is the dominant TJ component in the BBB[5-7].

The Membrane-Associated Guanylate-Kinase (MAGUK) protein family includes the Zonula Ocludens (ZO-1, ZO-2 and ZO-3) proteins. The actin cytoskeleton, adherens and TJ proteins are all bound by binding domains found in the MAGUK family. Interaction with the scaffolding proteins ZO-1, ZO-2 and F-actin is critical for the development of the endothelial barrier[5].

The primary endothelial adhesion molecule that regulates cellular interactions and blood vessel development is called Vascular Endothelium (VE)-cadherin. A highly endothelial-specific adhesion protein, VE-Cadherin is present at the junctions between the EC[8].

Nontransferrin-Bound Iron (NTBI) has been found in iron overload disorders, including heart failure, cirrhosis of the liver, cardiac arrhythmia and arthritis, among others. This particular form of iron could impact cellular activity, the inflammatory response and pro-oxidant effects[9]. The selectin family mediates leucocyte rolling during inflammation and leucocyte transendothelial migration through vascular endothelium and monocyte/macrophage adherence are significantly influenced by the expression of Cell Adhesion Molecules (CAMs) such as ICAM-1 and vascular adhesion molecule-1 VCAM-1[9].

In this study, the effects of iron accumulation on CDH5, CLDN5, ICAM-1, OCL, P-SEL, VCAM-1 and ZO-1 gene expressions in ECV304 cells were investigated.

2. Materials and Methods

2.1 Human Umbilical Vein Endothelial Cells (ECV304)

The ECV304 (ATCC® CRL¬1998™) has been used in the scope of this research. Dulbecco's Modified Eagled Medium (DMEM) including 10% fetal bovine serum and 1% penicillin-streptomycin was used as the ECV304 medium.

2.2 Iron Overload Treatment

Two experimental groups were established for this study: 1) Control Group: In this group, ECV304 cells were subjected to incubation solely with the medium. 2) Treatment Group ($FeSO_4$-only incubation group): A total of 250 µl from a 1mM $FeSO_4$ solution and 750 µl of DMEM were combined and subsequently incubated with ECV304 cells overnight. Following a 24-hour duration, Ribonucleic Acid (RNA) isolations were performed on all groups to enable subsequent qPCR experiments[10].

2.3 Trypan Blue Cell Viability

The cells of both the experimental groups were rinsed with a serum-free medium. Then, for five to fifteen minutes, they were incubated in a trypan blue solution. Following this incubation period, the cells were subjected to three washes using DMEM containing 0.1% Bovine Serum Albumin (BSA). Finally, the number of cells exhibiting blue staining, indicative of cell death, was quantified using a phase-contrast microscope[11].

2.4 Ribonucleic Acid (RNA) Isolation

The RNeasy-Mini kit Qiagen Part 1 (74104) was used to isolate RNA in accordance with the directions provided by the manufacturer.

2.5 Real-Time PCR

Analysis of the expression of CDH5, Cldn5, ICAM-1, OCL, P-SEL, VCAM-1, ZO-1 and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) genes was performed with the GoTaq 1-Step RT-qPCR System Technical Manual (catno: A6020) kit. The expression of the GAPDH gene in cells was measured for normalisation. The primer sequences are shown in Table 1. To calculate alterations of gene expression levels $2^{-\Delta\Delta CT}$method was used[12].

Table1. Primer list of target genes

Gene Name	Oligonucleotide Sequences (5′–3′)
OCLN	f: TCAGGTGAATTGGTCACCGAG
	r: GATAAACCAATCTGCTGCGTC
Cldn5	f: TCAGGTGAATTGGTCACCGAG
	r: GATAAACCAATCTGCTGCGTC
ZO-1	f: TGCCATTACACGGTCCTCTG
	r: TCTGCTTTCTGTTGAGAGGCT
CDH5	f: CCGGCGCCAAAAGAGAGA
	r: CTGGTTTTCCTTCAGCTGGAAGTGGT
VCAM-1	f: TTACACATTGATGAAATGGATTCTGT
	r:AAGGTGAGAGTTGCATTCTCAGAAAG
ICAM-1	f: GCAGCCGCAGTCATAA
	r: CCCAATAGGCAGCAAGT
P-SEL	f: GGTCAACTACCGTGCCAACCT
	r: GACTTACCACCGCTCCA
GAPDH	f: CGACTTCAACAGCGACACTCAC
	r: CCCTGTTGCTGTAGCCAAATTC

2.6 Statistical Analysis

The statistical analysis of the data was done using the Statistical Product and Service Solutions (SPSS) Statistic 23 application. Using the Least Significant Difference test (LSD test) as a post-hoc test and one-way analysis of variance (ANOVA) in the SPSS software, statistical differences between the groups were examined.

3. Results

The consequences of iron sulfate-induced damage on the BBB connections were examined in this study. The viability findings of Trypan blue cells are displayed in Figure 1.

Gene expression analysis results of CDH5, Cldn5, ICAM-1, OCL, P-SEL, VCAM-1 and ZO-1 genes of the Control and Treatment groups are shown in Figure 2. Statistically significant changes were observed in mRNA levels in VCAM, ZO-1, P-SEL and OCL genes. It has been observed that the expression levels of CDH5 and P-SEL are upregulated in 1,2 and 3,02 fold than control groups in Figure 2, respectively. Nevertheless, for Cldn5, ICAM-1, OCL, VCAM-1 and ZO-1, the expression levels are down-regulated in 0.95, 0.52, 0.99, 0.98 and 0.43 fold than control groups in Figure 2, respectively.

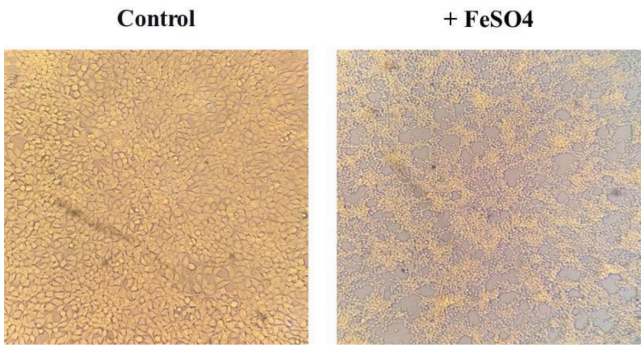


Figure1. Trypan blue cell viability results.

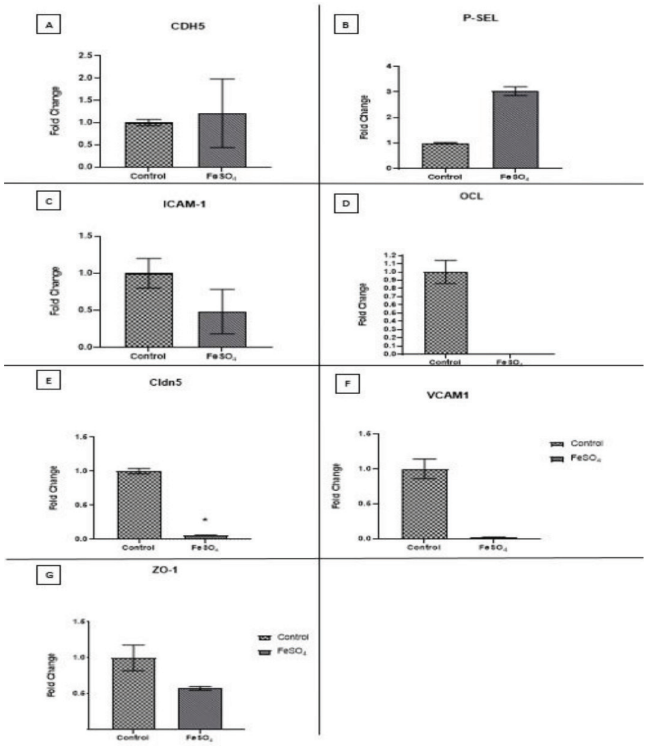


Figure 2. Target gene expression levels for the two experimental groups; (**A**) CDH5, (**B**) P-SEL, (**C**) ICAM-1, (**D**) OCL, (**E**) Cldn5, (**F**) VCAM1, (**G**) ZO-1.

4. Discussions

Iron is necessary for the brain to carry out various biochemical processes, including cell division, myelination, nitric oxide metabolism, neurotransmitter synthesis and oxygen transport. Despite all this, the brain is extremely susceptible to oxidative damage caused by iron. Excessive iron accumulation and loading create pathological problems for the normally ageing brain. In addition, excessive iron accumulation causes

neurodegeneration by affecting brain mitochondrial functions. Memory loss, learning impairments and other brain dysfunctions may result from the buildup and loading of iron in the brain[13].

Iron promotes oxidative damage and degradation in the in vitro BBB model, as demonstrated by Lockman *et al*[14]. Iron sulfate increases barrier permeability[10]. Reduced levels of Matrix Metalloproteinase (MMP-1, -2 and -9) and Protein Tyrosine Kinase (PTK) inhibitors are another way that oxidative stress affects the BBB. It is well known that the disruption of TJ proteins is accompanied by a rise in MMPs and PTK activity[15]. Decreasing intercellular TJ increases the permeability of BBB.

Age-related increases in brain iron buildup are more common in patients with neurodegenerative illnesses like Parkinson's Disease (PD)[16]. It has been observed that PD patients have higher substantia nigra iron levels than controls[17].

Our prior investigation demonstrated that an excess of iron leads to an augmentation of permeability in the ECV304 cell line[10]. To facilitate a further investigation into this matter, the mRNA expression levels of proteins responsible for intercellular metabolite transfer such as CDH5, CLDN5, ICAM-1, OCL, P-SEL, VCAM-1 and ZO-1 genes were compared in ECV304 cell line in the presented study.

CDH5 is a protein that is found on the surface of endothelial cells. It is known for its role in promoting the formation of new blood vessels and maintaining the integrity of BBB[18,19]. In recent years, research has shown that CDH 5 also plays a role in regulating the permeability of the BBB. This means that it helps to control the movement of molecules between the blood and the brain[20-23]. Ischemic stroke is a condition that occurs when the blood supply to the brain is blocked. This can damage the BBB and lead to the leakage of molecules into the brain[24]. A recent study found that patients with acute ischemic stroke had higher levels of CDH5 Circulating Endothelial Cells (CECs) than healthy controls. This suggests that CDH5 may be released from damaged EC in the brain during an ischemic stroke. The increased levels of CECs may contribute to the disruption of the BBB and the development of symptoms of stroke[25].

The main task of CDH5 is to control the permeability to ions. Suppression of the CDH5 gene at the BBB may cause Alzheimer's, multiple sclerosis, neuro-inflammatory disorders, depression and schizophrenia[26]. On the other hand, expression levels of the CDH5 gene in treated groups are upregulated in our study.

An accumulation of iron-dependent lipid peroxides results in ferroptosis, a type of cell death associated with several illnesses, including cancer and ischemic organ damage[27,28]. Wu *et al*., demonstrated that CDH5 is an important protein that regulates ferroptosis in epithelial cells[29]. Madsen *et al*., showed that Fe+3-exposed EC were elongated and stress-related proteins were upregulated. Also, they reported that VE-Cadherin proteins were increased in endothelium in response to iron-induced stress[30]. Our findings demonstrate a pivotal role for the CDH5 protein within EC under conditions of iron accumulation.

Members of these lectin family, VCAM-1 and ICAM-1, are adhesion molecules involved in monocyte recruitment to the endothelium. During inflammation, the expression of these cell adhesion molecules plays an important role[9]. VCAM-1 and its ligand α4β1 integrin are important molecules in mononuclear leukocyte recruitment and lesion induction[31]. It provides control of immune cell trafficking across the BBB of the human brain microvascular endothelium[32]. In addition, VCAM-1 is a molecule identified in Multiple Sclerosis (MS) lesions due to its role in CNS inflammation[33]. Therefore, modulation of the expression of VCAM-1 and ICAM-1 is very important for the treatment of MS. In this study, it was determined that iron application decreased ICAM-1 expression, while VCAM-1 expression was almost completely suppressed as shown in Figure 2.

ZO-1 proteins are important proteins for the maintenance of TJ in the mature BBB. Therefore, studies on the analysis of changes in occludin expression will provide an important insight into the TJ properties in the BBB[34]. As demonstrated in this study, the addition of iron was found to be effective in regulating the ZO-1 gene. The expression level of the ZO-1 gene decreased by 0.48 fold.

A debilitating condition known as Germinal Matrix Haemorrhage (GMH), which affects iron metabolism, frequently causes periventricular leukomalacia, post-haemorrhagic hydrocephalus and subsequent neurocognitive abnormalities like ischemic stroke in preterm infants. Since P-SEL is a vascular adhesion molecule involved in the recruitment of leukocytes, it might be expected to contribute to these secondary inflammatory responses following GMH, as demonstrated by Hatchell *et al.*'s observation that P-SEL expression was increased in periventricular, hippocampal and white matter regions following GMH[35]. However, during a stroke, the expression of P-SEL and ICAM-1, two proteins that promote the

adhesion of leukocytes to endothelial cells, is increased in brain capillary EC. Leukocytes can bind to the BBB more firmly because of the upregulated expression of P-selectin and ICAM-1. This permits leukocytes to pass through the damaged BBB and enter the ischemic brain parenchyma. To prevent brain damage from ischemia/reperfusion, Dong *et al*. created neutrophil membrane-coated nanoparticles that can administer anti-inflammatory agents to the brain[36].

The ferrous sulfate group showed a significant decrease in the expression of the ZO-1 and ICAM-1 gene by approximately 0.5 compared to the control (P<0,05). In addition, iron accumulation appears to almost completely suppress VCAM-1, Cldn5 and OCL gene expressions. P-SEL is encoded by the SELP gene in humans. It functions as an adhesion molecule in activated platelet and endothelial cells lining the inner surface of blood vessels. Expression of P-SEL, an adhesion molecule, is much lower in CNS EC than in peripheral endothelial cells. However, it has been proven to be elevated in neuroinflammatory diseases such as stroke and MS[33]. According to our findings, the ferrous sulfate group's P-SEL gene expression increased three times (p<0.05) in comparison to the control group.

5. Conclusions

The accumulation and sequestration of iron within the cerebral environment has been associated with cognitive decline, affecting memory, learning and various cognitive functions. Consequently, it is necessary to investigate the underlying molecular pathways and gene expression patterns involved in these molecular processes. In conclusion, it is important to note that this study serves as a preliminary investigation and further in-depth research is needed to elucidate the molecular mechanisms underlying these variations in gene expression.

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