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Effects of iron and inflammation on astrocytes functions

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การสะสมของธาตุเหล็กภายในเซลล์ และการอักเสบเป็นพยาธิสภาพที่มักพบในโรคเสื่อมของระบบประสาท ตัวอย่างเช่น โรคอัลไซเมอร์ และโรคพาร์กินสัน ซึ่งในปัจจุบันสาเหตุที่ทำให้เกิดโรคเสื่อมของระบบประสาทเหล่านี้ยังไม่ทราบแน่ชัด โดยเป็นไปได้ว่ามีปัจจัยหลายชนิดที่ก่อให้เกิดโรคเหล่านี้ การศึกษาในครั้งนี้ มีวัตถุประสงค์เพื่อทดสอบผลของสภาวะที่มีธาตุเหล็ก หรือ pro-inflammatory cytokines หรือ ภาวะที่มีทั้งเหล็กและ pro-inflammatory cytokines ร่วมกัน ต่อการเปลี่ยนแปลงการทำงานของแอสโทรไซต์ ผลจากการศึกษาพบว่า ในสภาวะแวดล้อมที่มีเหล็กสามารถก่อให้เกิดการสะสมของเหล็กและมีการสะสมของเหล็กเพิ่มมากขึ้นอย่างเห็นได้ชัดเมื่อมีเหล็กร่วมกับ cytokines อีกทั้งยังมีผลทำให้เกิดการการเพิ่มขึ้นของสร้างสารอนุมูลอิสระภายในเซลล์ ในขณะเดียวกันพบว่าสภาวะที่มีเหล็กร่วมกับ cytokines มีความสามารถในการยับยั้งการแบ่งตัวของแอสโทรไซต์ นอกจากนี้ผลการทดลองยังแสดงให้เห็นว่าในสภาวะที่มีเหล็กมากทำให้แอสโทรไซต์มีการเปลี่ยนแปลงการแสดงออกของยีนต่างๆ ที่เกี่ยวข้องกับการควบคุมสมดุลธาตุเหล็ก โดยพบว่าแอสไซต์มีการแสดงออกของยีนในการนำเหล็กเข้าเซลล์และเก็บสะสมเหล็กเพิ่มมากขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบกับกลุ่มควบคุม ในขณะที่การแสดงของยีนในการนำเหล็กออกนอกเซลล์มีการลดลงอย่างมีนัยสำคัญทางสถิติ และนอกจากนี้ยังพบว่าแอสโทรไซต์มีการเปลี่ยนแปลงการแสดงของยีน GFAP และ vimentin ซึ่งเป็นยีนที่เกี่ยวข้องกับโครงสร้างของเซลล์โดยความเปลี่ยนดังกล่าวนี้แสดงให้เห็นภาวะเริ่มต้นของแอสโทรไซต์ที่กำลังจะเข้าสู่ภาวะถูกกระตุ้น และยิ่งไปกว่านั้นสภาวะที่มีเหล็กร่วมกับ cytokines ยังสามารถกระตุ้นให้แอสโทรไซต์แสดงออกยีนที่เกี่ยวข้องกับการตอบสนองต่อการอักเสบเพิ่มมากขึ้น ในขณะเดียวกันสภาวะดังกล่าวนี้ยังมีผลทำให้การหลั่งของ cytokines ประกอบด้วย IL-1β, IL-6 และ TNF-a รวมไปถึงการหลั่งของกลูตาเมตจากแอสโทรไซต์เพิ่มมากขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุมอีกด้วย จากผลการศึกษาทั้งหมดนี้แสดงให้เห็นว่า แอสโทรไซต์ในโรคเสื่อมของระบบประสาท อยู่ภายใต้ภาวะเครียดออกซิเดชั่น ซึ่งเป็นไปได้ว่าอาจจะเป็นผลมาจากการที่มีเหล็กสะสมภายในเซลล์ ทำให้แอสโทรไซต์มีการหลั่ง cytokine และกลูตาเมตเพิ่มขึ้น ซึ่งสามารถเป็นอันตรายต่อเซลล์ประสาทได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

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KEYWORDS: ASTROCYTES / NEURODEGENERATIVE DISORDER / BRAIN IRON ACCUMULATION / NEUROINFLAMMATION

WORANITTHA CHAIYANA: Effects of iron and inflammation on astrocytes functions. ADVISOR: ASSOC. PROF. POONLARP CHEEPSUNTHORN, Ph.D., CO-ADVISOR: ASST. PROF. CHALISA LOUICHAROEN CHEEPSUNTHORN, Ph.D., 69 pp.

Iron accumulation and inflammation are present in a variety of neurodegenerative diseases include Alzheimer's disease and Parkinson's disease. Nowadays it has not yet known what the specifically causes neurodegeneration. There is probably not one single, but several factor are important to describe the etiology of these diseases. In this study, ferric ammonium citrate, pro-inflammatory cytokines including interleukin 1β and tumor necrosis factor alpha and combined were investigated the altered astrocytes functions. The results showed that FAC and combination of cytokines were markedly induced intracellular iron accumulation, free iron and reactive oxygen species, while the proliferation was inhibited in the presence of combination. In addition, the results demonstrated that iron enriched astrocytes alters the genes expression profiles involved in iron homeostasis by increased the expression of iron importer and storage genes couple with decreased in iron exporter gene. According to this condition, the rearrangement of astrocytes cytoskeletons were observed in an alteration of intermediate filaments, GFAP and vimentin, which refer to an initially activated astrocytes. Moreover, the combination of FAC and cytokines was able to induce the expression of the inflammation response genes including IL-1β, IL-6 and TNF-a. At the same time, the presence of FAC and combination of cytokines were significantly increased cytokines levels including IL-1β, IL-6 and TNF-a and glutamate secretion compared to unexposed control cells. Taken together, these findings suggest that astrocytes in neurodegenerative disorders are oxidatively stressed due to their tendency to accumulate iron, express and release more of cytokines and glutamate that can be harmful to neuron.

Field of Study: Medical Science Student's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Iron is essential for brain development and function [1]. Iron is a required cofactor for many cellular enzymes involved in energy metabolism and the syntheses of DNA, lipid and several neurotransmitters [2]. However, high levels of iron can be toxic to all brain cells. This is due to the ability of iron to catalyze the Fenton reaction generating the hydroxyl radicals that cause cell damage via lipid peroxidation, protein aggregation and nucleic acid modification [3, 4].

Astrocytes are the most abundant type of glial cells in the brain that support neuronal function. Astrocytes can modulate synaptic activity by releasing molecules such as Adenosine triphosphate (ATP), gamma-Aminobutyric acid (GABA) and glutamate [5]. Astrocytes help removing excessive glutamate from the synapse, therefore protecting neurons from glutamate excitotoxicity, a pathological process responsible for neuronal cell death in stroke and neurodegenerative disorders[6]. Astrocytes also play a key role in brain iron homeostasis after iron is transported across the blood-brain barrier into the brain[7]. Because of their significant roles in neuronal survival and function, astrocyte dysfunction can lead to neuronal cell death.

Neurodegeneration is primarily characterized by progressive loss of neurons and is strongly linked to brain iron accumulation and inflammation [8, 9]. For example, elevated levels of brain iron have been consistently reported in the brains of patients with Alzheimer's diseases (AD) and Parkinson's diseases (PD) [10, 11]. Increased levels of several brain inflammatory cytokines including Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) has been reported in AD and PD [9, 12, 13]. It is well established that astrocytes under pathological conditions, so-called reactive astrocytes, are cellular source of these cytokines.

Astrocytes has been reported that involved in brain iron homeostasis and inflammation. They have expanded endfeet that ensheath the abluminal surfaces of

capillary endothelial cells that is the reason why they can transport iron from blood into brain. Astrocytes act as an immune cell in brain. They also responses to inflammation by releasing pro-inflammatory cytokines. Increasing evidences suggest that functions of astrocytes might be altered during neurodegenerative diseases. However, the mechanism underlying this alteration remains elusive. Therefore, all experiments conducted in this study aimed to investigate modulatory role of iron accumulation and inflammation in astrocyte functions.

1.2 Research Question

Does iron and inflammation alter astrocyte functions?

1.3 Objectives of This Research

1.3.1 To determine cytotoxic range of iron and pro-inflammatory cytokines in cultured astrocytes

1.3.2 To determine effects of exposure to iron and pro-inflammatory cytokines on intracellular accumulation of iron in cultured astrocytes

1.3.3 To determine effects of exposure to iron and pro-inflammatory cytokines on cell proliferation, expression of astrocyte cytoskeleton (GFAP, VIM) and iron homeostasis (TfR, DMT1, Ft and FPN), intracellular ROS levels and production of glutamate and inflammatory cytokines (IL-1β, IL-6 and TNF-α) in cultured astrocytes

1.4 Hypothesis

Iron accumulation in astrocytes could be enhanced by elevation of extracellular iron levels and the presence of inflammatory cytokines. It is speculated that iron accumulation and inflammation could alter cellular iron metabolism and shift function of normal astrocytes toward cytotoxic astrocytes.

1.5 Keywords

Astrocytes

Neurodegenerative disorder

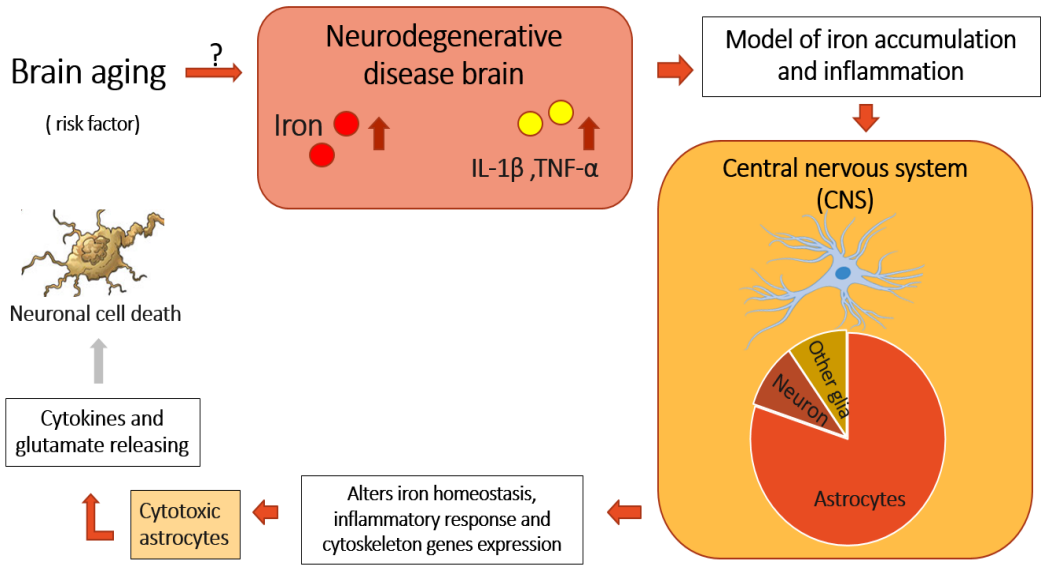
Brain iron accumulation

Neuroinflammation

1.6 Benefits and Applications

The new knowledge from this study provide valuable insight into the role of astrocytes in neurodegeneration with brain iron accumulation and inflammation.

1.7 Conceptual framework



CHAPTER II

Literatures Review

2.1 Overview iron and iron metabolism

Iron, an essential element, acts as cofactor in many pathways including oxygen transportation of haemoglobin (Hb), carbohydrate metabolism, immune system and brain cognitive function [1, 14, 15]. Iron also supports brain function via many mechanisms such as oxygen transportation, DNA synthesis, mitochondrial respiration, myelin synthesis, and neurotransmitter synthesis and metabolism [2]. However, deregulation of iron homoeostasis can cause neurotoxicity [16]. An excess iron in the labile iron pool causes of cellular oxidative damage and cell death [17]. Accumulation of iron in brain regions mostly found in elders, especially in neurodegenerative disorder patients [18]. It has still been argued whether iron accumulation is a primary cause or secondary effect of neurodegenerative disorders. However, aging is the major risk factor for neurodegeneration. Therefore, age-related accumulation of iron might be an important factor to promote neurodegenerative processes [19].

There are two derivatives of iron in human body; ferrous (Fe^{2+}) and ferric (Fe^{3+}). Fe^{2+} can be found in Hb and myoglobin but Fe^{3+} found in foods. Fe^{2+} can be easily absorbed in intestine in contrast with Fe^{3+} is unable to absorb. However, Fe^{3+} can be converted to Fe^{2+} by ferric reductase for absorption and reverted back to Fe^{3+} for transferring to other organs via blood circulation [20].

2.2 Labile iron pool

The cellular labile iron pool (LIP) is defined as a pool of redox-active iron complexes. It was first suggested by Jacobs [21] that LIP acts as a transitory or chelatable pool of intracellular irons that comprises both Fe^{2+} and Fe^{3+} bound to low-molecular-mass intracellular chelates such as citrate, various peptides, ATP, AMP or pyrophosphate [22]. LIP, scavenged by chelators, participates in cellular redox-cycling. Normally, the amount of LIP in quiescent condition presents around 5% of total

cellular iron or 50–100 μM. However, it can handle biochemical stimulations under homeostatic changes. Nevertheless, extreme fluctuations of iron including overload or deficiency might affect LIP capacity in maintenance of cellular homeostasis [23].

2.3 Brain iron and oxidative stress

The first protection against iron overload of brain is blood-brain barrier (BBB), regulating from membrane transport system [24]. *In vitro* studies, an excess of iron in brain could generate oxidative stress, increasing of the level of reactive oxygen and nitrogen species (ROS, RNS) [20]. Normally, to the ability of iron is a donation of electrons to oxygen. Therefore, increased iron levels can lead to formation of hydroxyl radicals (OH·) and hydroxyl anions (OH^-) via the Fenton Reaction ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^-$) [25]. These ROS can damage cellular macromolecules including proteins, lipids, and DNAs [26]. Under normal conditions, several intracellular detoxification systems and antioxidant defense mechanisms exist to prevent these damages for example; catalase and glutathione peroxidase quickly convert hydrogen peroxide (H_2O_2) to water, reducing harmful of ROS [27]. Iron-induced oxidative stress is particularly dangerous because it causes iron releasing from further iron-containing proteins such as ferritin (Ft), heme proteins and iron-sulfur (Fe-S) clusters, exacerbating the toxicity of iron overload in brain [28].

2.4 Intracellular iron import, storage and export

Iron import

Transferrin-bound iron (TBI)

Transferrin is iron-binding blood plasma glycoproteins that control the level of iron in biological fluids. Most cell types intake transferrin mediated iron (Fe-Tf) in form of Fe^{3+} using transferrin receptor 1 (TfR1), which ubiquitously expressed. TfR1 is a major route in uptake iron (Figure 1). Fe-Tf binds to TfR1 on the cell surface and Fe-Tf/TfR1 complex localizes to clathrin-coated pits to form specialized endosomes [29]. Decreasing of endosomal pH causes conformational changes resulting in the release of iron from Tf. TfR1 expression is regulated by the level of intracellular iron and a

presence of a specific RNA hairpin structure called iron-responsive elements (IREs). High level of intracellular iron inhibits iron respond proteins (IRPs) in binding with IREs, localizing in the 3' untranslated region (UTR) of TfR1 mRNA [30]. TfR1 mRNAs without IRPs are easily degraded leading to decrease TfR1 expression (Figure 2).

Non- Transferrin-bound iron (NTBI)

Iron uptake in the NTBI pool requires carrier molecules. Divalent Metal Transporter 1 (DMT1) or Divalent Cation Transporter 1 (DCT1) or Nramp2 is responsible for intestinal uptake of NTBI or non-heme iron [20]. DMT1 is a highly hydrophobic integral membrane glycoprotein transporting Fe^{2+} and other divalent cations [31] (Figure 1). Regulation of DMT1 expression depends on the presence IRE within the 3' UTR of DMT1 mRNA (Figure 2). However, there is another isoform of DMT1 mRNA, lacking IRE occurs from alternative splicing [32].

Iron storages

Ferritin

Ferritin serves as a non-toxic form of iron (Fe^{3+}) storage, which iron could be transported to the requiring areas [33] (Figure 1). There are several functions and structures of ferritin for different type of cells. Ferritin consists of two subunits namely based on their molecular weight: L “light”, H “heavy” subunits. In the middle of H-type or iron binding site has a ferroxidase activity or property in converting of Fe^{2+} to Fe^{3+}, which able to reduce the production hydroxyl radical. In contrast, L-type has no ferroxidase activity but may be responsible for the electron transfer across the protein cage. The presence of iron could trigger an expression of ferritin by controlling IRE within 5′UTR of ferritin mRNA (Figure 2) [34]. When cells need iron, it will be released from ferritin via degradation of ferritin, mainly performed in lysosomes [35].

Iron export

Ferropotin 1 (FPN1)

FPN1 is a transmembrane protein, exporting intracellular iron to outside. After iron is absorbed into the intestinal cells, FPN1 will export iron from those cells to the bloodstream [36] (Figure 1). Hepcidin can inhibit FPN1 by binding with FPN1 and internalizing into the cell. Hepcidin is released from the liver in response to inflammatory cytokines, consequent decreasing of plasma iron levels [37]. Mechanism in regulation of FPN1 expression involves IRPs, since FPN1 mRNA harbors IRE at 5′UTR [30]. Low level of iron concentration is insufficient to inhibit IRP, subsequently blocks translation of FPN1 from IRP-IRE interaction at 5′UTR (Figure 2).

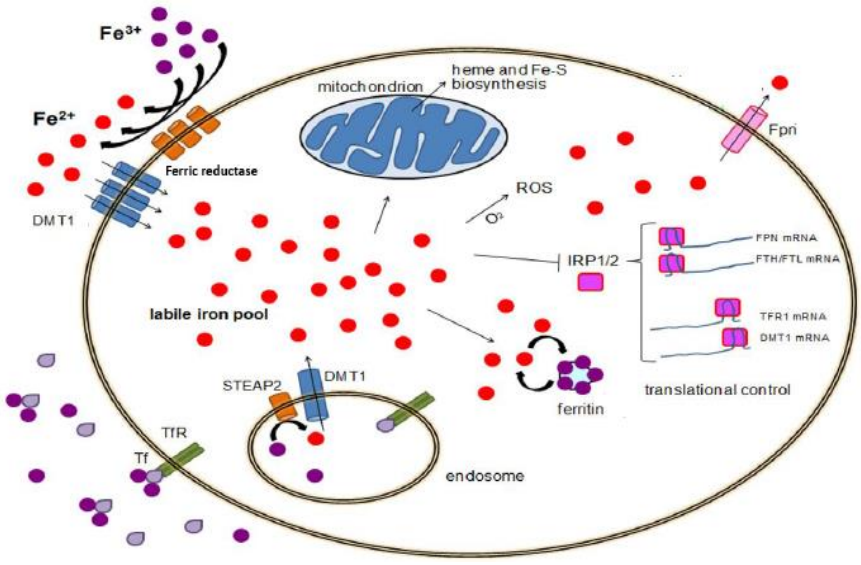


Figure 1 Diagram showing a generalized view of cellular iron homeostasis in humans. Iron import can occur via endocytosis of transferrin receptor 1 or ferrous iron importers (DMT1). Intracellular iron, stored in ferritin, generates ROS and regulates transcription of iron related proteins via iron-responsive element-binding proteins (IRP1/2). Iron export occurs through ferroportin.
(Adapted:https://commons.wikimedia.org/wiki/File:Cellular_iron_homeostasis.png#/media/File:Cellular_iron_homeostasis.png)

2.5 Regulation of cellular iron content

Coordination of genes in iron transport, export and storage regulate cellular iron homeostasis. There are two cytoplasmic iron regulatory proteins; IRP1 and IRP2 and iron responsive elements (IREs) on mRNAs of related proteins involve in controlling cellular iron content, which occurs during post transcription. IRE is a phylogenetically conserved hairpin structure, constituting of IRP1 or IRP2 binding sites [38]. Under iron overload, IRPs stimulate the expression of ferritin and FPN1 by stabilizing mRNAs via IRPs- IREs interaction at 3'UTR (Figure 2). In the other hand, IRPs inhibit translation of TfR1 and DMT1 by blocking ribosome binding site at IRE 5'UTR. IRE/IRP system also controls the expression of additional IRE-containing mRNAs, encoding proteins for iron and energy metabolism. The activities of IRP1 and IRP2 are regulated by cellular iron levels. In iron-replete cells, IRP1 assembles an iron-sulfur cluster, preventing its IRE binding capacity, while IRP2 undergoes proteasomal degradation. IRP1 and IRP2 also respond to iron-independent signals such as hydrogen peroxide, hypoxia or nitric oxide.

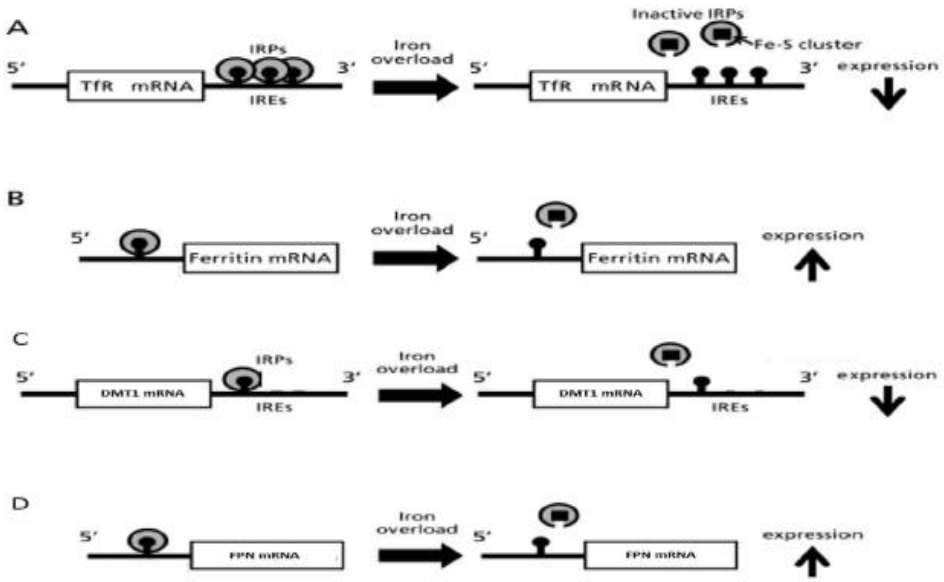


Figure 2 The IRP-IRE regulatory system

At high level of iron, IRP containing Fe-S cluster, is unable to interact with IREs of mRNA. A and C represent the regulation of TfR1 and DMT1 expression, respectively. Binding of IRPs at IREs in the 3' UTR of TfR1 and DMT1 mRNA protects against mRNA degradation, subsequently promotes translation of TfR1 and DMT1. B and D represent

the regulation of ferritin and FPN expression, respectively. Binding of IRP within IRE at 5' UTRs of ferritin and FPN mRNA blocks the translation of ferritin and FPN [39].

2.6 Iron and neurodegenerative disorders

Changing of regional or cellular iron concentration and iron metabolism relating proteins that found in neurodegenerative disorders (Figure 3) enhances hydroxyl radical formation, lipid peroxidation, protein aggregation, glutathione consumption, nucleic acid modification, iron homeostasis disruption, mitochondrial dysfunction and cell death [10, 18, 40]. Therefore, iron has directly associated with aberrant peptide aggregation [41]. An important pathological hallmark of neurodegenerative disorders is aberrant aggregation of beta amyloid (Aβ)-rich plaques in AD, and alpha (α-) synuclein-rich Lewy bodies in PD.

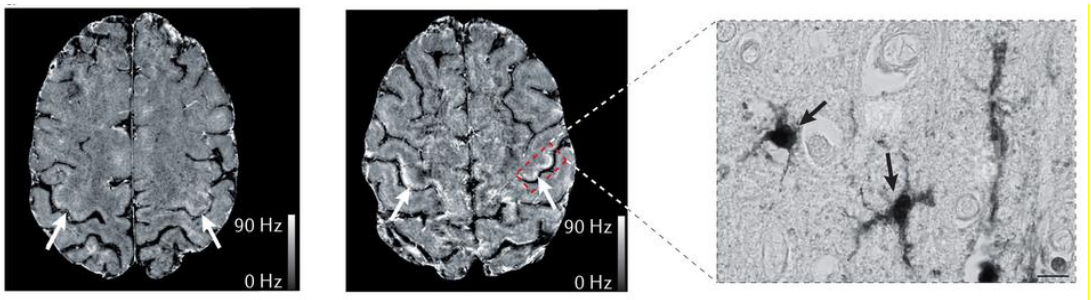


Figure 3 MRI has revealed iron overload in the motor cortices of patients with ALS on $R2^*$ maps of 7T MRI scans, raising the possibility that iron overload is important in pathogenesis [42].

2.7 Neuro-inflammation

Central nervous system (CNS) is an immunologically privileged site because peripheral immune cells are unable to access through BBB, a specialized structure composed of astrocytes and endothelial cells. In CNS, microglia is an innate immune cell, activated in response to infection, traumatic brain injury, toxic metabolites, aging and autoimmunity [43]. Although the response aims to protect CNS from the infectious

agents, sustainably activation of glial cells mediate neuro-inflammation or chronic inflammation in CNS, resulting recruit other immune cells into the brain [44]. Crossing of circulating peripheral immune cells through compromised BBB encounters neurons and glial cells to express major histocompatibility complex (MHC) molecules, perpetuating the immune response.

2.8 Cytokines

Cytokines are a class of proteins that regulates inflammation, cell signaling, and various cell processes such as growth and survival [45]. Chemokines are a subset of cytokines that regulate cell migration of immune cells to an infection or injury sites. Various cell types in the brain can produce cytokines and chemokines such as microglia, astrocytes, endothelial cells, and other glial cells [46]. Common cytokines respond to brain injury including interleukin-6 (IL-6), produced during astrogliosis, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), inducing neuronal cytotoxicity [47]. Although the pro-inflammatory cytokines may induce cell death, they are necessary to repair damaged tissue. For example, TNF-α causes of neurotoxicity at early stages of neuro-inflammation, but contributes tissue growth at later stages of inflammation [48].

2.9 Interleukin 1 beta (IL-1β)

Interleukin-1α and β are prototypic pro-inflammatory cytokines that exert pleiotrophic effects on a variety of cells and play key roles in acute or chronic inflammatory and autoimmune disorders. IL-1β, mediating neuro-inflammation, is released from Schwann cells, microglias and astrocytes [46]. Although IL-1β has an important function in homeostasis of organism, overproduction of IL-1β in brain affects pathophysiological changes during progression of diseases including multiple sclerosis (MS), PD and AD [9, 47]. There are two IL-1 receptors; IL-1 type 1 receptor (IL-1RI) and

IL-1 type 2 receptor (IL-1 RII). IL-1β signals through IL-1RI and others receptors; IL1 receptor accessory protein (IL-1RAcP) and IL-1 receptor antagonist (IL-1ra), which competes with IL-1 receptors [49].

2.10 Tumor necrosis factor alpha (TNF-α)

TNF-α has pro-inflammatory properties, which play crucial roles in the innate/ adaptive immunity, cell proliferation and apoptotic processes This cytokine is produced by microglia, astrocytes and other glial cells [46]. TNF-α signals through two structurally distinct receptors; tumor necrosis factor receptor type I (TNF-RI) and type II (TNF-RII), presenting on cellular membrane of all cell types except erythrocytes [50]. These two receptors have different binding affinity and intracellular signaling pathway. The intracellular domain of TNF-RI binds to TNF receptor-associated death domain (TRADD) protein further activates either the apoptotic pathway or the pro-inflammatory pathway. TNFR1 mediates apoptotic pathway by Fas-associated death domain (FADD) protein. In contrast, TNFR1 mediated pro-inflammatory pathway signals through TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein, resulting in the activation of nuclear factor-κB (Figure 4). NF-κB is an important regulator of inflammatory responses including the producing of TNF-α itself [45].

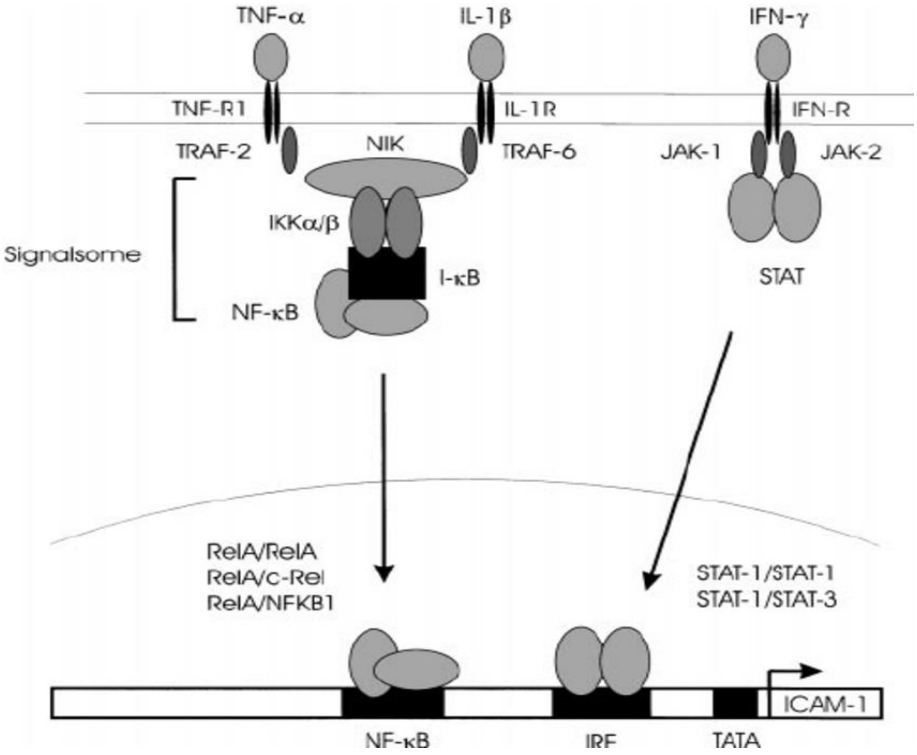


Figure 4 A simplified diagram illustrating Toll-like receptor (TLR), IL-1R and TNFR1 signaling pathways [51].

2.11 Neuro-inflammation and neurodegenerative diseases

AD has been characterized by two major hallmarks; neurofibrillary tangles, insoluble aggregates of tau proteins and amyloid-beta plaques, extracellular deposits of the amyloid-beta protein [52]. It has been currently proposed that neuro-inflammation plays a role in AD due to activated microglia has been numerously found in post-mortem AD brains. Moreover, inflammatory cytokine activated microglia is unable to clear amyloid-beta, contributing to plaque accumulation [53]. In addition, IL-1β, upregulated in AD, is associated with synaptic loss which consequent from decreasing of synaptophysin [54].

PD, a common neurodegenerative disease, is characterized by the degeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta. Prominent features of PD consist of T cell infiltration, increasing of inflammatory cytokines and

toxic mediators derived from activated glial cells [55]. Promoting neuro-inflammation by increasing of cytokines, which further propel degeneration of nigral dopaminergic neurons is a key pathogenesis of PD [56]. Recently, *in vitro* studies have suggested that chronic release of pro-inflammatory cytokines by activated astrocytes and microglia leads to the exacerbation of dopaminergic neurons degeneration [57].

2.12 Association between iron and inflammation

Iron acts as prosthetic group of many enzymes in synthesis of neurotransmitter. For example, monoamine oxidases A and B in dopamine catabolism, tryptophan hydroxylase in serotonin synthesis, tyrosine hydroxylase in dopamine and norepinephrine synthesis, glutamate decarboxylase in GABA synthesis and glutamate transaminase in L-glutamate synthesis [58]. Iron accumulation and inflammation in brain are hallmark of several neurodegenerative disorders [9, 10], although it is not clear whether iron accumulation or inflammation is a primary cause or a consequence of the disorder. Inflammation affects iron homeostasis through control of iron transporters expression. In previous report found transcription factor NF-κB from TNF-α and IL-1 signaling pathways induces DMT1 expression, which supports the relationship between inflammation and iron homeostasis [59]. Moreover, it has been reported that TNF-α and IL-6 directly increase DMT1 mRNA and protein levels and decrease FPN1 protein, thus generating an increment of iron content in neurons and microglia [60]. In recent study using primary cultures of ventral mesencephalic neurons demonstrated that TNF-α or IL-1β induce an increment of DMT1 and TfR1 protein levels, together with a reduction of FPN1 levels, resulting in an increasing of ferrous iron influx and decreasing of iron efflux in neurons [61]. Moreover, stimulation of macrophage cell lines with IFN-γ and TNF-α increase IRE-IRP1/IRP2 complex and DMT1 mRNA expression [62]. Indeed, TNF-α was detected in glial cells in the SN of PD patients

but not in control subjects, together with immunoreactivity for TNF-α receptors in dopaminergic neurons of both control and PD patients [63]. These findings support that activation of nigral microglia, resulting in TNF-α secretion, increase iron uptake by dopaminergic neuron via NF-κB-induced DMT1 expression. Furthermore, an increase in the nuclear immunoreactivity of NF-κB has been observed in PD brains [64]. Therefore, activation of NF-κB via inflammatory stimuli contributes to iron accumulation in PD.

2.13 Astrocytes

Astrocyte (astrocytic glial cell or astroglia) is a star-shaped cell surrounding neurons in the brain and spinal cord. Astrocytes is the numerous cells in brain with ratio between astrocytes and neurons around 50:1. It is a subtype of glial cells or non-neuronal cells in the nervous system. There are many functions of glial cells, including building the micro-architecture of brain parenchyma, creating the brain environment, maintenance homeostasis in the brain, storage energy, controlling the development of synaptogenesis, synaptic maintenance, neural cells and brain defense [7, 65]. Main functions of astrocytes are buffering CNS potassium, removing and recycling toxic of glutamate, balance water homeostasis and modulating synaptic activity. Astrocytes also produce neurotrophins and anti-inflammatory cytokines [6]. Astrocytes have been divided into two main subtypes based on cellular morphologies and anatomical locations; protoplasmic and fibrous. Protoplasmic astrocyte is found throughout all gray matter and exhibit morphology of several stem branches that give rise to many finely branching processes in a uniform globoid distribution. Fibrous astrocyte is found throughout all white matter and exhibit morphology of many long fiber-like processes. Classical and modern neuroanatomical studies also indicate that both astrocyte subtypes make extensive contacts with blood vessels [7]. Electron microscopic analyses revealed that the processes of protoplasmic astrocytes envelop synapses and

the processes of fibrous astrocytes contact nodes of Ranvier. Both types of astrocytes form gap junctions between distal processes of neighboring astrocytes [66].

Expression of glial fibrillary acid protein (GFAP) has become a prototypical marker for immunohistochemical identification of astrocytes (Figure 5). GFAP is a family of intermediate filament proteins that serve largely cyto-architectural functions [67]. Studies in transgenic mice indicated that GFAP is not necessary for normal function of astrocytes in healthy CNS of transgenic mice, but is essential for the process of reactive astrogliosis and glial scar formation [68, 69]. GFAP is absolutely absent in the finely branching astrocyte processes and is often undetectable in the cell body [68].

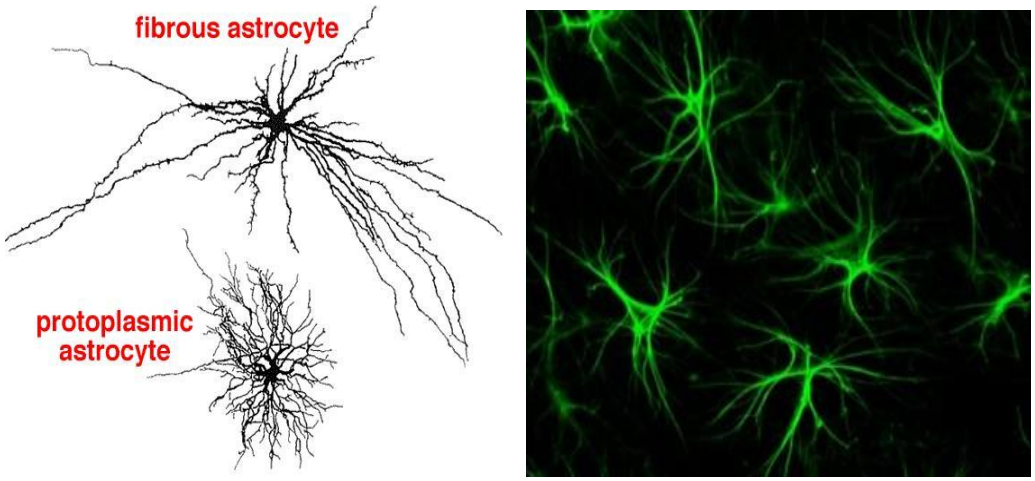


Figure 5 Astrocytes morphology and GFAP expression as a marker of astrocytes (From: https:wiki.brown.edu/confluence/display/BN0193S04 and www. astrocytes/info)

Astrocytes support the plasticity of the nervous system [70]. They play direct roles in synaptic transmission by releasing synaptically active molecules including glutamate, purines (ATP and adenosine), GABA, D-serine and excitatory neurotransmitter [5]. Releasing of gliotransmitters from astrocytes during communication with neurons affects neuronal synaptic activity, neuronal and astrocyte excitability via a calcium ion dependent mechanism [71]. Astrocytes also respond to

CNS injury or disease via reactive astrogliosis [72, 73]. They have involved in the pathology of Huntington's disease, AD, concussions, stroke and CNS infections.

Astrocytes have expanded endfeet that ensheath the abluminal surfaces of capillary endothelial cells. These endfeet allow astrocytes to act as gatekeepers, controlling the transport of metabolites between blood and neuropil. Endfeet of astrocytes also protect brain cells from metal toxin (e.g. lead and mercury) and provide brain cells the glycolysis metabolites, neurotransmission and antioxidant defense [74]. Astrocytes also transfer iron from the periphery into the neuropil. *In vitro* study, it has been found that astrocytes excessively uptake after exposed to large concentration of iron, which overwhelming challenge to oxidative stress [17]. It has been found an increasing of ferritin and iron accumulation in astrocytes AD brain [75].

Blood brain barrier (BBB)

BBB is an essential regulatory component of the neural interface with the brain vasculature. It regulates interstitial fluid (ISF) and prevents the formation of additional injuries during cerebrovascular accidents [76]. It maintains ion, hormone and transmitter homeostasis in the brain by regulating the movement of ions, nutrients and cells between neural cells and blood using specific ion transporters [76, 77]. BBB is composed of brain capillary endothelial cells (ECs) associated with astrocytic endfeet processes and mesenchymal-like cells pericytes. Astrocytes endfeet links between the endothelial blood flux and neurons (Figure 6) [78]. BBB dysfunction has been associated with cerebral ischemia, brain trauma, glioblastoma, stroke, MS, epilepsy, AD and PD [79, 80].

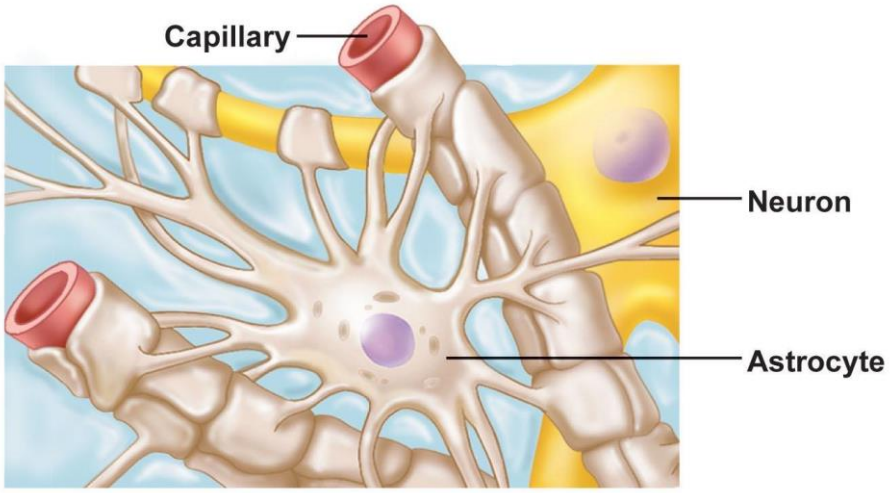


Figure 6 Astrocytes is a component of BBB by sending astrocytes end-feet to wrap on capillary. (From:https://www.emaze.com/@AOTWRTFW/Central-Nervous-System-copy0)

2.14 Astrocytic glutamate transport and modulation of neurotransmission

One of the major functions of astrocytes is glutamate uptake, which influences excitatory neurotransmission and prevents excitotoxicity. Glutamate uptake is converted to glutamine by glutamine synthase in glutamate-glutamine cycle. Then, it is reversed to be glutamate in neuron [7]. Glutamate transporter proteins; GLAST and GLT-1 (EAAT2) are predominant transporters for glutamate-glutamine cycle [81]. GLAST is expressed primarily in radial glia as well as cortical astrocytes during development and persist in the adult brain especially in Bergmann glial cells of the cerebellum, fibrous astrocytes of the ventral white matter tracts of the spinal cord and the progenitor cells of the subgranular layer of the dentate gyrus [82]. GLT-1 is the predominant glutamate transporter expressed in the adult brain and is highly active in both protoplasmic and fibrous astrocytes [83]. It has been reported that dysfunction of glutamate transporter leads to excessive of glutamate, found in many neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [84].

2.15 Role of astrocytes in brain iron homeostasis

Astrocytes have directly accessed to the capillary endothelial cells via their endfeet, making them ideally positioned to take up iron from the vasculature and transport it into the neuropil [80]. Astrocytes have a high capacity for iron accumulation and are able to store the iron in ferritin, but they do not normally contain large amounts of iron or ferritin. This situation strongly suggests that astrocytes are involved in trafficking iron to other brain cells, similar to the way that they are involved in transporting other essential nutrients into the brain. When astrocytes are exposed to large concentrations of iron in vitro, their rate of uptake of iron temporarily exceeds the storage capacity of their ferritin and the cells experience transient oxidative stress [17, 31]. In addition, it has been reported the antioxidant defenses of astrocytes are compromised, astrocytes are damaged by exogenous and endogenous peroxides in an iron-dependent process.

2.16 Role of astrocytes in brain inflammation

Astrocytes are the best-characterized innate immune neuroglia. After activation of Toll-like receptor (TLR) signals, astrocytes generate innate inflammatory mediators, including complements (IL-1β, IL-6) and chemokines (CCL2, CXCL1, CXCL10, CXCL12). [85]. High concentration of this cytokine impairs the capacity of astrocytes to detoxify glutamate, resulting in neuronal loss termed “excitotoxicity” [86]. Microglial-astrocyte interactions are also critical in CNS innate immunity. In mouse model, the inflammatory transcriptional regulator NF-κB was silenced in astrocytes by transgenic over expression of NF-κB inhibitor [86]. The blocking of NF-κB signaling in astrocytes showed reduced retinal ganglion cell death after ischemic injury, improved recovery of spinal cord trauma, along with increased axonal sparing and regeneration and lessened inflammation in a rodent model of the human inflammatory demyelinating disease (MS) [87].

2.17 Astrocytes pathology

Reactive astrogliosis is widely used as a pathological hallmark of diseased in CNS tissue. GFAP is performed as a marker for astrocytes to test overestimate of astrocyte proliferation to reactive astrogliosis [68]. During mild or moderate astrogliosis, overexpression of GFAP and cellular hypertrophy without cell proliferation are responsiveness of astrocytes [88]. Many different types of intercellular signaling molecules are able to trigger reactive astrogliosis including (1) large polypeptide growth factors and cytokines such as IL1, IL6 and TNF-α (2) mediators of innate immunity such as lipopolysaccharide (LPS) and other Toll-like receptor (TLR) ligands (3) neurotransmitters such as glutamate and noradrenalin (4) purines such as ATP (5) ROS including nitric oxide (NO) (6) hypoxia and glucose deprivation (7) products associated with neurodegeneration such as β-amyloid [7]. Such molecular mediators of reactive astrogliosis can be released by all cell types in CNS tissue including neurons, microglia, oligodendrocyte lineage cells, pericytes, endothelia and other astrocytes. Reactive astrocytes can release a wide variety of extracellular molecules including inflammatory modulators, chemokines and cytokines and various neurotrophic factors. These factors can be either neuroprotective (e.g., cytokines such as IL-6 and transforming growth factor-β [TGF-β]) or neurotoxic such as IL-1β and TNF-α [48]. The interplay between neuroprotective and neurotoxic effects of reactive gliosis is exemplified by the process of glial scar formation. The expression of vimentin (VIM), an intermediate filament protein, was found during glial scar formation [89]. The glial scar serves to isolate the damaged area and prevents the spread of damage by restricting the infiltration of inflammatory cells.

2.18 Astrocytes and neurodegenerative diseases

Reactive astrocytes are found at Aβ deposit sites in postmortem of human AD brain and also in animal models of AD [90]. In AD mouse model, astrocytes plated on Aβ-bearing brain sections and decrease Aβ levels [91]. In addition, fluorescence labeled astrocytes, transplanted into AD mouse model, and wild-type (WT) astrocytes could migrate to deposited Aβ and clear Aβ plaques [92, 93]. Moreover, Aβ-associated tissue damage correlates with highly amount of Aβ in astrocytes and reactive gliosis [72]. Reactive astrogliosis is generally mild to moderate in autopsy specimens of substantia nigra from PD patients [94]. Astrocytes have been implicated as potential manifestation for both neurotoxic and neuroprotective activities in PD.

Sporadic ALS is characterized by loss or dysfunction of glutamate transporters in astrocytes, which isolated from spinal cord and cerebral cortical areas that loss of lower and upper motor neurons [95, 96]. Suggesting that increased glutamate excitotoxicity may contribute to motor neuron death. Stimulation of glutamate transporters expression in astrocyte and enhance glutamate sufficient uptake could reduce excitotoxicity and to provide neuroprotection in animal models of stroke and ALS [97].

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Penicillin, streptomycin, L-glutamine, pyruvate and sodium bicarbonate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Culture plates and flasks were from Nunc (Naperville, IL, USA). TRIzol reagent and SYBR Green were purchased from Thermo Scientific (Waltham, MA, USA). Human recombinant tumor necrosis factor (rh-TNF-α) and Human recombinant interleukin1 beta (rh-IL-1β) were purchased from PeproTech (Rocky Hill, NJ, USA).

3.2 Cell cultures

Human astrocyte 1321N1 cell line was a kind gift from Professor James R Connor, The Pennsylvania state University, USA. The cell line was maintained in DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and supplemented with 10% FBS at 37 ℃ in 95% relative humidity and 5% CO_2 environment. Cell culture medium was replaced every three days with subculturing.

3.3 Cell treatments

Cells were replated at density 1 x 10^6 in T25 flasks for 24 h. Then, cells were treated with fresh medium serum free containing ferric ammonium citrate (FAC), IL-1β or TNF-α cytokines, either alone or combined, for 24 h. Untreated cells served as control group. After treatment, cell pellets and cell-free supernatant were collected and processed for qPCR, ELISA and glutamate assay, respectively.

3.4 Cell viability assay

The cell viability of astrocytes was determined using MTT colorimetric assay, which involves the conversion of the water soluble 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (St. Louis, MO, USA) to an insoluble formazan by cellular oxidoreductase enzymes. In brief, the cells were plated as previously described at density $4x10^4$ cells/well for 24 h in 96-well plates. Then, the medium in each well was removed and freshly prepared serum free-medium containing either iron (FAC; 50-1000 μg/ml) or cytokines (IL-1β and TNF-α; 1, 10, and 100 ng/ml) were added. After incubating cells for 24 h, MTT with a final concentration of 2 mg/ml was added and incubated for 3 h at 37 °C. Then, the medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve purple crystals of formazan at 37 °C for 10 min. Finally, the absorbance was read at 570 nm using a microplate reader (BioTek, USA).

3.5 Visualization of intracellular iron deposit

Intracellular iron deposit was visualized using a modified Perls' stain [98]. Briefly, cells were plated at density $4x10^4$ cells/well for 24 h in 24-well plates. Then, the medium in each well was removed and cells were incubated with each freshly condition medium with serum free (see treatment conditions above). After incubation, cells were fixed with 10% Neutral Buffered Formalin (see appendix), then washed with PBS, followed by 5% (v/v) hydrochloric acid (HCl) and 5% (w/v) potassium ferrocyanide ($K_4Fe(CN)_6$). Then, cells were washed with PBS and incubated with 3, 3'-diaminobenzidine (DAB) substrate Chromogen System (Dako, USA) for 5 min. The reaction was stopped by washing with distilled water. Cells were examined using light microscopy (Nikon, Japan). Photomicrographs of cells were taken.

3.6 Measurement of labile iron pool

To measure intracellular free iron, calcein (CA) assay was performed. CA binds to soluble (free) forms of intracellular iron and represents as iron-bound (quenched) form. In contrast, excessed CA or unbound form having no iron interacting with

provides the basal fluorescence. Therefore, fluorescence intensity depends on intracellular concentration of labile iron. Briefly, cells were seeded at density $4x10^4$ cells/well in black-walled clear bottom 96 well-plates for 24 h and treated in various conditions as described previously. After incubation in treatment conditions, medium were discarded. Then, fresh media containing 100 µl of 1 µM CA was added and incubated for 40 min at 37° C. Then, the solution was completely removed and washed with PBS. Fluorescence intensity at 490 nm excitation and at 520 nm emission was measured.

3.7 Measurement of intracellular levels of ROS

To evaluate the accumulation of intracellular ROS during iron (FAC), IL-1β and TNF-α treatments, 5, 6 choloromethyl -2',7'- dichlorodihydro fluorescein diacetate, acetylester probe (CM-H_2DCFDA) (Thermo scientific, USA) was used. CM-H_2DCFDA enters the cells and is hydrolyzed to be non-fluorescent dichlorofluorescein (DCF). DCF then reacts with ROS to form the highly fluorescent dichlorofluorescein. Thus, fluorescence intensity reflects the accumulation of intracellular ROS. Cells were seeded at density $4x10^4$ cells/well for 24 h. Then, 10 mM CM-H_2DCFDA was added to each well. After incubation for 30 min, the solution was completely removed and then cells were exposed to the conditions medium as described for 24 h. Finally, the measured fluorescence intensity at 495 nm excitation and 525 nm emission was recorded.

3.8 Measurement of gene expression

3.8.1 RNA extraction and cDNA synthesis

After treatment for 24 h, the medium was removed followed by adding 1 ml of Trizol reagent to extract the RNA, according to the manufacturer's protocol. The RNA pellets were air-dried at room temperature for half an hour and dissolved in RNase free water. The RNA purity and concentration were measured using a NanoDrop™ spectrophotometer (Thermo scientific, USA). The RNA was kept at -80 °C, until use. Extracted RNAs was converted to cDNA using RevertAid First Strand cDNA Synthesis Kit

(Thermo scientific, USA), according to the manufacturer's protocol. Then, the cDNA products were used for quantitative PCR (qPCR).

3.8.2 Polymerase Chain Reaction (PCR) and Quantitative Real-time PCR (qPCR)

PCR mixture reaction consisted of 2 ul of cDNA template, 11 µl of sterile water, 2 µl of 1X PCR buffer, 0.4 µl of 0.2 mM dNTP, 0.4 µl of 1 mM MgCl, 0.1 µl of 0.5 U Taq polymerase and 2 µl of 200 nM forward and revers primers. The PCR reaction was firstly pre-denature at 95°C for 2 min, then was repeated for 35 cycles with the following steps; denature at 95°C for 30 sec, anneal at an appropriated temperature for each pair of primer for 30 sec extension at 72°C for 30 sec; and finally the reaction was extended at 72°C for 3 min and holded at 4°C. The PCR products were then detected by 1% agarose gel electrophoresis. Then, the results were photographed using Gel Doc™ XR + imaging system (Biorad, USA)

To determine the expression of target genes, qPCR was performed. Briefly, qPCR mixture reaction was composed of 5 µl of 1 µl of cDNA template, 2X SYBR Green, 2 µl of distilled water and 1 µl of primers; as follows: TfR1, F: 5′- CAGCCCAGAAGCATT -3′, R: 5′-CCAAGAGCTTTATCC -3' (143 bp); DMT1, F: 5′- GAACAATAGCAGGAA GTT-3′, R: 5′-GGATGGCAATAGAGCGAGT -3′ (419 bp); Ft-H, F: 5′- GGTCCAGA ACTACC -3′, R: 5′-CATCATGGTCAAAGTAG -3′ (116 bp); Ft-L, F: 5′-CCAGCAC CGTTGTGGTT -3′, R: 5′-TAGGAGGCCTGGTACAA -3′ (128 bp); FPN1, F: 5′- GGG TCTCCTACAACCAG -3′, R: 5′-GGCAACACGGAGATCAC -3′ (286 bp); GFAP, F: 5′- GGCCCCACTGCAGGATACC -3′, R: 5′-CTTCTGCTGGCCCTCATGAG -3′ (328 bp); VIM, F: 5′- CGTCTTGACCTTGAACG -3′, R: 5′-GTGAGGTCAGGCGAAAC -3′ (149 bp); IL-1β, F: 5′- CCTGTGGCCTTGGGCCTCA -3′, R: 5′ GGTGCTGATTACCA GTTGGG -3′ (204 bp); IL-6, F: 5′-TTCGGTCCAGTCTT-3′, R: 5′-TCTTTGCTTTCAC-3′;(233 bp) TNFα, F: 5′- GGTCCCTTCAAGGCCAAG -3′, R: 5′-CTCACAGGGAATG ATCCCA -3′(293 bp) and GAPDH, F: 5′-CACT CCTCCACCTTTGACG-3′ R: 5′-CTT GTGCTCTTGCTGGGGC-3′(179 bp). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. qPCR reaction was firstly pre-denature at 95°C for 2min, then repeated the reaction for 35 cycles under the following steps;

denature at 95°C for 30 sec, anneal at an appropriated temperature for each pair of primer for 30 sec**,** extension at 72°C for 30 sec and finally extend the reaction at 72°C for 3 min and holding at 4°C using real time thermocycler (Thermo Scientific, USA) The relative amount of target gene expression was calculated as $2^{\Delta Ct}$ where **Δ**Ct is the difference between Ct of GAPDH and of target gene. Fold changes were determined by dividing $2^{\Delta Ct}$ of treatment with that of control.

3.9 Enzyme-linked immunosorbent assay (ELISA)

Quantification protein level of IL-1**β**, IL-6 and TNF-**α** was performed using ImmunoTool ELISA kit (ImmunoTools, Germany). The detection range of these assays is 0-1000 pg/ml. According to the manufacturer's instructions, 100 µl of standard dilutions or samples was added into precoated 96-well plates and incubated for 2 h at room temperature followed by incubations with biotinylated detector (2 h at room temperature), HRP conjugated-streptavidin (30 min at room temperature) and TMB substrate solution (30 min at room temperature). The reaction was stopped by adding 2M HCl into each well. Plates were read at 450 nm excitation and 630 nm emission using microplate reader.

3.10 Glutamate assay

The amount of glutamate released from astrocytes was determined using an Amplex Red Glutamic acid assay kit (Thermo Scientific, USA). According to the manufacturer's instructions, 50 µl of standard dilutions and cell-free media were mixed with 50 µl of a working solution containing 100 µM Amplex Red, 0.25 U/ml horseradish peroxidase, 0.08 U/ml L-glutamate oxidase, 0.5 U/ml L-glutamate-pyruvate transaminase and 200 µM L-alanine and incubated in the dark at room temperature for 30 min. Then, the fluorescence intensity was measured at excitation 540 nm and emission 590 nm using microplate reader. Values were corrected for background fluorescence by subtracting values derived from control samples without glutamate. Results were expressed in µM of glutamate from a standard curve generated from known molar concentrations of glutamate.

3.11 Statistical analysis

The data are presented as mean ± SD of 3-5 independent experiments. Statistical analysis was analyzed by One-way ANOVA with post-hoc Bonferroni test using IBM SPSS statistic version 22. The *P*-values of <0.05 was considered significant by comparing with untreated group.

CHAPTER IV

RESULTS

4.1 Cytotoxic effect of iron in cultured human astrocytes

To determine the cytotoxic range of iron in human astrocytes, cells were exposure to FAC in various concentrations ranging from 50-1000 µg/ml for 24 h and subjected to MTT assay. Results showed a significantly decreased in astrocyte cell viability at 200 µg/ml of FAC (94%; $p<0.05$), compared to that of unexposed control cells. Cytotoxic effect of FAC in cultured astrocytes increased in a concentration-dependent manner (Figure 7). The minimal non-toxic concentration of FAC at 50 µg/ml was selected for subsequent experiments aiming to determine its effects on intracellular accumulation of iron and astrocyte functions.

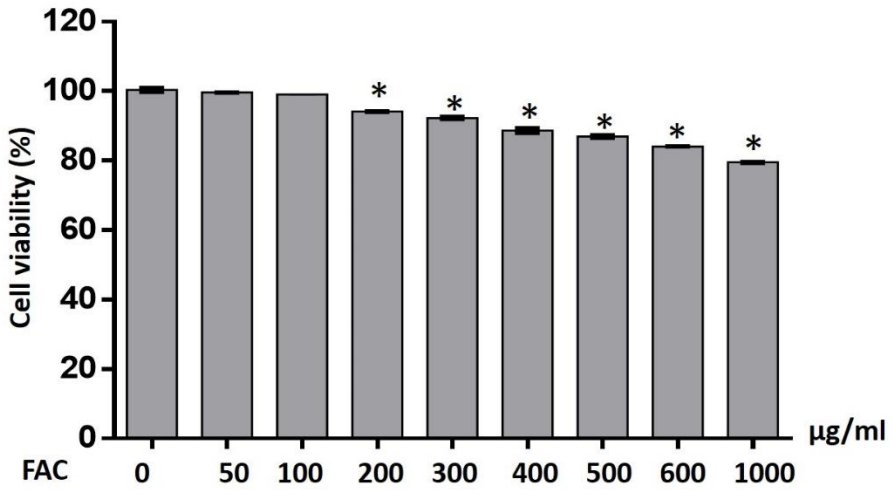


Figure 7. The cytotoxic range of iron in cultured human astrocytes. After exposure to various concentrations of FAC for 24 h, cell viability was determined by the MTT assay. Results were presented as percent of unexposed control cells. The experiments were performed in triplicate for each condition and repeated three times. Bars display mean ± SD. * compared to control values ($p<0.05$).

4.2 Cytotoxic effects of IL-1β and TNF-α in cultured human astrocytes

To determine the cytotoxic range of IL-1β and TNF-α in human astrocytes, cells were exposed to various concentrations of IL-1β and TNF-α (1, 10 and 100 μg/ml) for 24 h. The cytotoxicity was examined by the MTT assay. Results showed that all concentrations of IL-1β tested were not toxic to cultured astrocytes. At a concentration of 1 ng/ml, IL-1β exhibited proliferative effect on astrocytes, compared to unexposed control cells (Figure 8a). In contrast to IL-1β, cytotoxicity of TNFα was observed at 100 ng/ml, compared to unexposed control cells (Figure 8b). Because of its non-cytotoxic and non-proliferative effects, a concentration of 10 ng/ml was selected for both IL-1β and TNF-α for the subsequent experiments aiming to determine their effects on intracellular accumulation of iron and astrocyte functions.

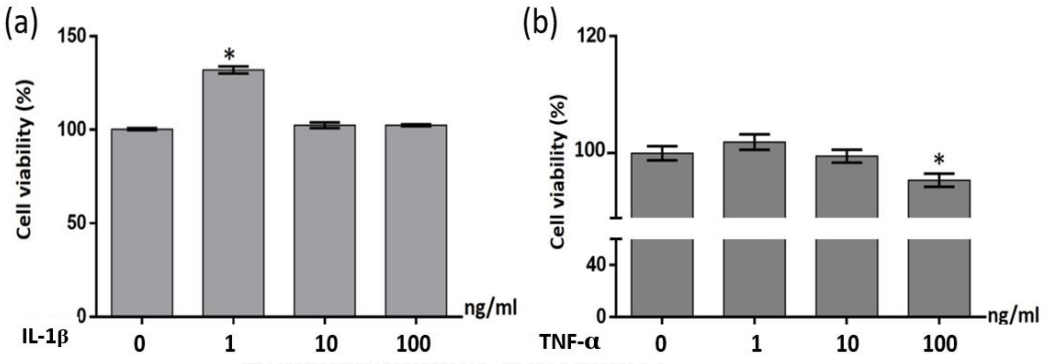


Figure 8. Effects of IL-1β and TNF-α cell viability of cultured human astrocytes. Cell viability was determined by the MTT assay after 24 h exposure to various concentrations of IL-1β (a) and TNF-α (b). The experiments were performed in triplicate for each condition and repeated three times. Bars display mean ± SD. * compared to control values ($p<0.05$).

4.3 Effects of exposure to iron and pro-inflammatory cytokines on intracellular accumulation of iron in cultured human astrocytes

To investigate whether exposure to iron and pro-inflammatory cytokines can cause astrocytes to deposit iron, cells were incubated with iron (50 μg/ml FAC), IL-1β (10 ng/ml) and TNF-α (10 ng/ml) alone or combined for 24 h. Using the modified Perls' staining method, results showed that exposure of astrocytes to 50 μg/ml FAC for 24 h caused iron deposition, compared to unexposed control cells. (Figure 9a, b). Exposure of astrocytes to 10 ng/ml of cytokines either alone or combined for 24 h slightly increased intracellular iron levels in astrocytes compared to unexposed control cells (Figure 9c, e, g). When presented simultaneously, FAC and cytokines alone or combined led to excessive iron deposition in astrocytes (Figure 9d, f, h) compared to their appropriated control cells (Figure 9a, b, c, e, g). These results demonstrate that IL-1β and TNF-α can enhance iron accumulation in human astrocytes.

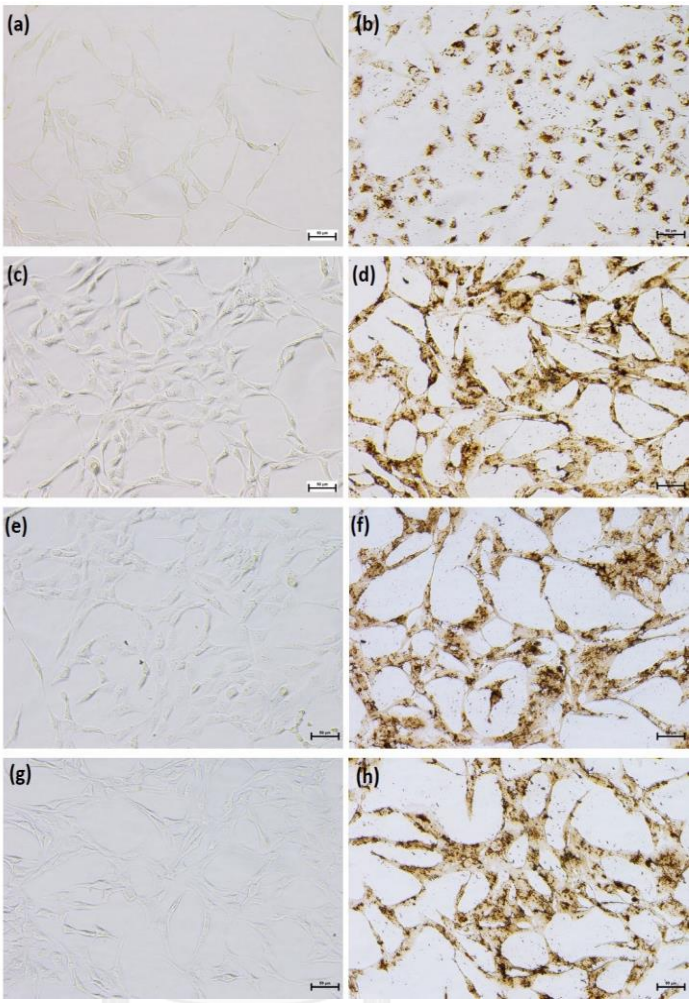


Figure 9. Representative photographs showing histochemical staining of intracellular iron deposits (Perls' stain) in cultured human astrocytes at 24 h. (a) fresh medium alone, (b) 50 μg/ml FAC, (c) 10 ng/ml IL-1β, (d) a combination of FAC and IL-1β, (e) 10 ng/ml TNF-α, (f) a combination of FAC and TNF-α, (g) a combination of IL-1β and TNF-α and (h) a combination of FAC, IL-1β and TNF-α. The experiments were performed in triplicate for each condition and repeated three times. Scale bars = 50 um

4.4 Effects of iron and pro-inflammatory cytokines on expression of genes involved in iron homeostasis in human astrocytes

To investigate whether exposure to iron and pro-inflammatory influences mRNA expression levels of genes involved in iron homeostasis in astrocytes including iron transporters (TfR1 and DMT1), iron storage (ferritin) and iron exporter (FPN1), cells were exposed to various treatment conditions as described above. As shown in Figure 10a, qPCR revealed that exposure to IL-1β and TNFα alone or combined induced a significant increase in TfR1 mRNA expression levels in astrocytes, compared to untreated control cells. When FAC was presented either alone or combined with either IL-1β or TNF-α, a significant decrease in TfR1 mRNA expression levels was observed, compared to untreated control cells. Results in Figure 10b revealed that exposure to IL-1β and TNF-α alone or combined induced a significant increase in DMT1 mRNA expression levels in astrocytes, compared to untreated control cells. When FAC was presented either alone or combined with either IL-1β or TNF-α, a significant increase in DMT1 mRNA expression levels was observed, compared to untreated control cells. Results in Figure 10**c** showed that all treatment conditions examined significantly increased mRNA expression levels of ferritin H and L subunits with a dramatic increase in expression of ferritin H subunit. As shown in Figure 10d, exposure to FAC alone induced a significant increase in FPN1 mRNA expression levels in astrocytes. This result was in contrast to what obtained when cells were exposed to IL-1β and TNF-α alone or combined with FAC. Taken together, these results suggest a mechanism in which exposure to iron and pro-inflammatory cytokines such as IL-1β and TNF-α promote iron accumulation in human astrocytes.

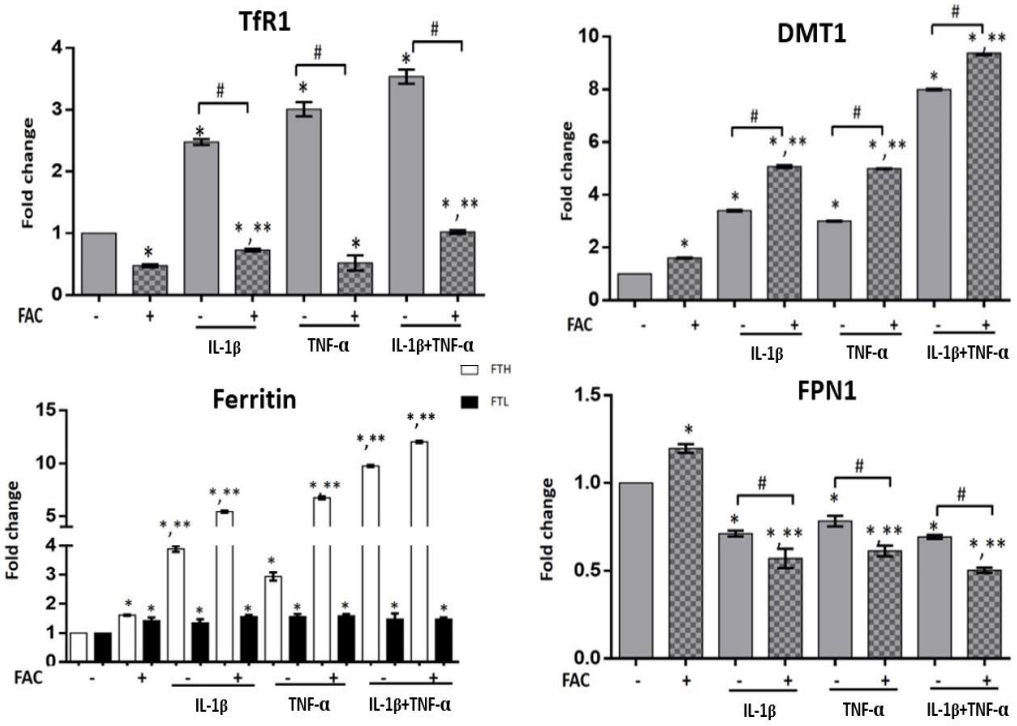


Figure 10. Expression of genes involved in iron homeostasis of astrocytes at 24 h after exposure to fresh medium alone, 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α. The expression of (a) TfR1, (b) DMT1, (c) FT-H and L and (d) FPN1 levels were determined by qPCR. The experiments were performed in triplicate for each condition and repeated three times. Bars display mean ± SD. * compared to control values ($p<0.05$), (**) compared to FAC alone and (#) compared to individual cytokines or combined cytokines.

4.5 Effects of iron and pro-inflammatory cytokines on intracellular levels of free iron in human astrocytes

To examine whether exposure to iron and pro-inflammatory cytokines influences intracellular levels of free iron, cells were exposed to various treatment conditions as previously described. Intracellular levels of free iron in astrocytes were measured using calcein fluorescence probe. It should be noted that the fluorescence intensity of calcein is quenched by free iron in cells. As shown in Figure 11, exposure

to either FAC or cytokines led a significant increase in intracellular levels of free iron in astrocytes, compared to untreated control cells (p<0.05). More dramatic effects were observed when cells were exposed to FAC and cytokines simultaneously. These results indicate that exposure to iron and pro-inflammatory cytokines can lead to elevation of free iron levels in human astrocytes.

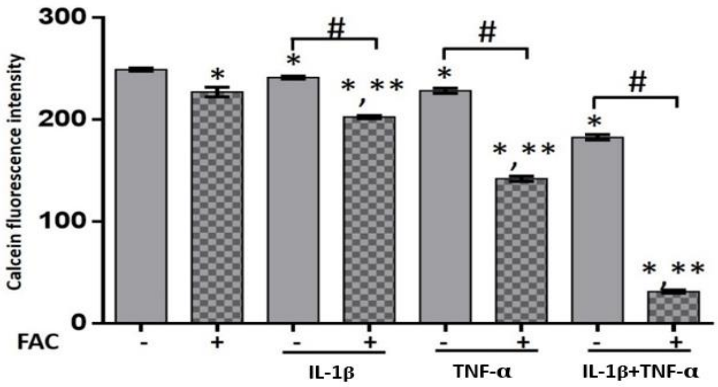


Figure 11. Effects of exposure to iron and pro-inflammatory cytokines on intracellular levels of free iron in astrocytes. Cells were exposed to 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α for 24 h. The experiments were performed in triplicate for each condition and repeated three times. Bars display mean ± SD. * compared to control values (p<0.05), (**) compared to FAC alone and (#) compared to individual cytokines or combined cytokines.

4.6 Effects of iron and pro-inflammatory cytokines on intracellular levels of ROS in human astrocytes

To examine whether exposure to iron and pro-inflammatory cytokines influences intracellular levels of ROS in astrocytes, cells were exposed to FAC, IL-1β and TNF-α either alone or combined as indicated in figure legend. ROS levels were measured using CM-H2DCFDA fluorescence probe. As shown in Figure 12, a significant elevation of intracellular ROS in astrocytes compared with unexposed control cells (p<0.05) was observed only when FAC was presented. These results suggest that

exposure to iron rather than pro-inflammatory cytokines is responsible to elevation of intracellular ROS levels in human astrocytes

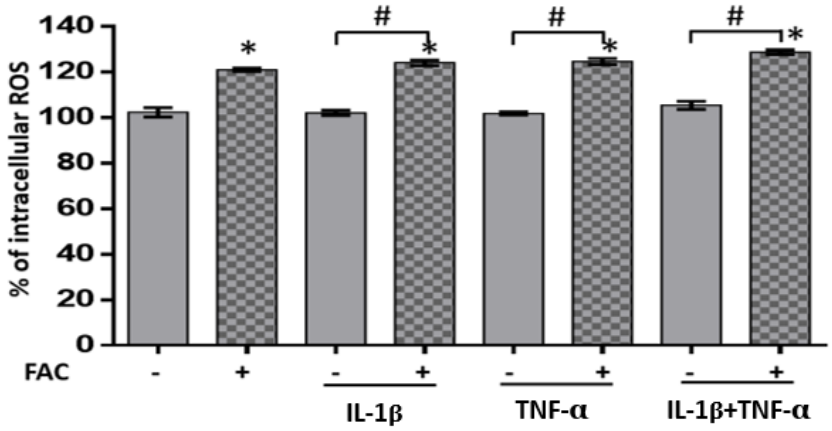


Figure 12. Effects of iron and pro-inflammatory cytokines on intracellular levels of ROS in human astrocytes. Cells were exposed to 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α for 24 h before assaying ROS levels using CM-H2DCFDA fluorescence probe. The experiments were performed in triplicate for each condition and repeated three times. Bars display mean ± SD. * compared to control values ($p<0.05$), (**) compared to FAC alone and (#) compared to individual cytokines or combined cytokines.

4.7 Effects of iron and pro-inflammatory cytokines on astrocytes cell proliferation

To investigate effects of exposure to iron and pro-inflammatory cytokines on astrocytes cell proliferation, cells were exposed to FAC, IL-1β and TNF-α as indicated in figure legend for 24 h. Cell proliferation was examined by MTT assay. As shown in Figure 13, results demonstrated that exposure to FAC alone, individual cytokines or individual cytokines with FAC did not affect astrocyte proliferation, compared to unexposed control cells. Exposure to combined cytokines without or with FAC significantly decreased astrocyte proliferation (95.7% and 93% respectively; $p<0.05$),

compared to unexposed control cells. These results suggest that exposure to a combination of iron and pro-inflammatory cytokines might interfere with the proliferative ability of human astrocytes.

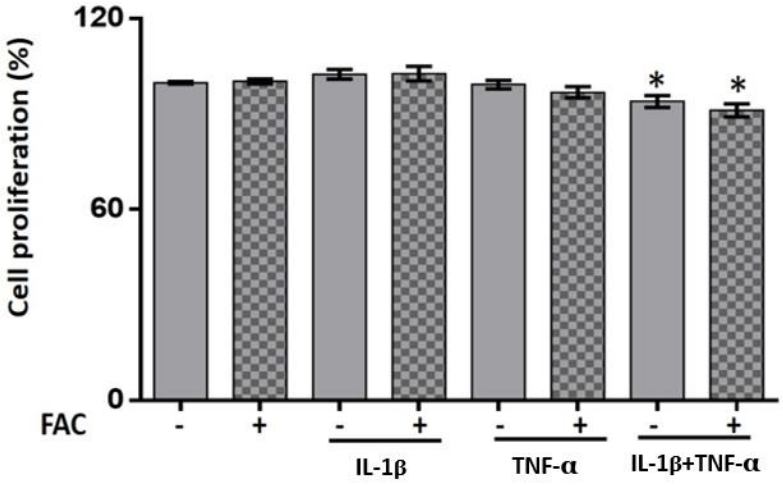


Figure 13. Effect of iron and pro-inflammatory cytokines on astrocytes cell proliferation. Cell proliferation was determined by the MTT assay at 24 h after exposure to 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α. Results are presented as percent of unexposed control (n=3, Mean ± SD). (*) $p<0.05$ compared to unexposed control values.

4.8 Effects of iron and pro-inflammatory cytokines on astrocyte cytoskeleton gene expression

To examine effects of iron and pro-inflammatory cytokines on astrocytes cytoskeleton gene expression, which include GFAP and VIM. Quantitative PCR analysis was performed. Cells were exposed to FAC, IL-1β and TNF-α for 24 h as indicated in figure legend. Results shown in Figure 14 demonstrated that exposure to FAC did not change mRNA expression levels of GFAP and VIM, compared to unexposed control cells. This was opposite to what observed for the effects of exposure to individual cytokines or combined cytokines with or without FAC. Exposure to individual or

combined cytokines with or without iron significantly decreased mRNA expression levels of GFAP, while increased mRNA expression levels of VIM. Moreover, FAC appeared to enhance the ability of individual cytokines to increase VIM mRNA expression, compared to individual cytokine control (p<0.05). These results suggest that exposure to pro-inflammatory cytokines in combination with iron could lead to cytoskeletal rearrangement in human astrocytes.

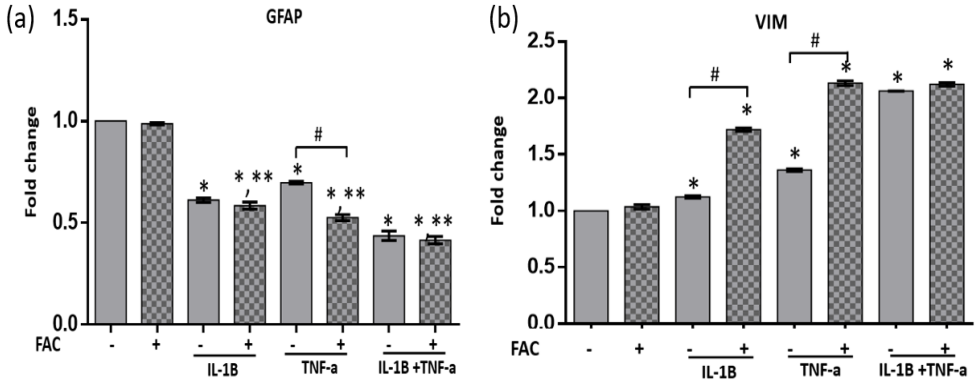


Figure 14. Effects of iron and pro-inflammatory cytokines on astrocyte cytoskeleton gene expression. The mRNA expression levels of (a) GFAP and (b)VIM mRNA were analyzed by qPCR. Cells were exposed to fresh medium alone, 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α for 24 h. The experiments were performed in triplicate for each condition and repeated three times. Data are expressed as Mean ± SD. (*) p<0.05 compared to unexposed control, (**) compared to FAC alone and (#) compared to individual cytokines or combined cytokines.

4.9 Effects of iron and pro-inflammatory cytokines on genes and proteins involved in astrocyte inflammation responses

To examine effects of iron and pro-inflammatory cytokines on astrocytes inflammatory responses, mRNA expression levels and release of IL-1β, IL6 and TNF-α from human astrocytes were determined by qPCR and ELISA, respectively. As shown

in Figure 15, results all together demonstrated that exposure to a combination of pro-inflammatory cytokines was more effective than exposure to individual cytokines in up-regulation and release of IL-1β, IL6 and TNF-α from human astrocytes. Moreover, the presence of iron during the exposure to individual or combined cytokines further enhanced up-regulation and release of IL-1β, IL6 and TNF-α in human astrocytes.

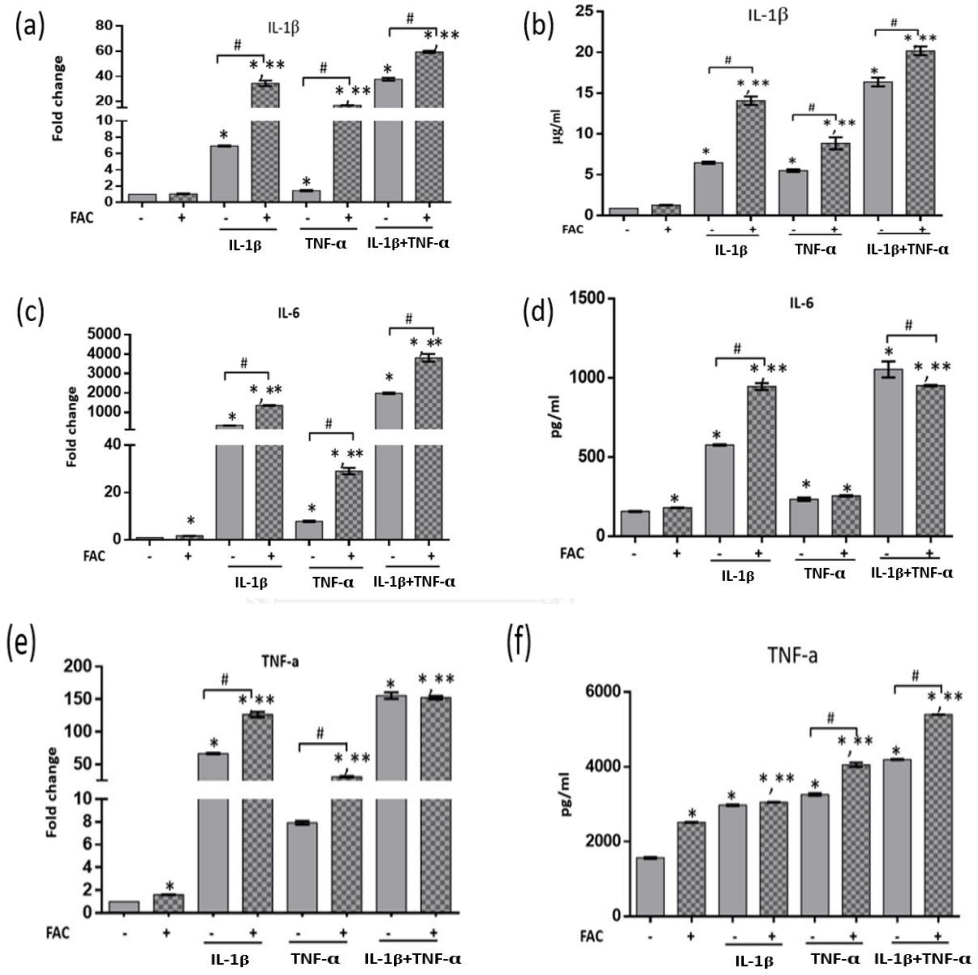


Figure 15. Astrocyte inflammatory responses after 24 h of exposure to fresh medium alone, 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α . IL-1β mRNA expression and protein released (a, b), IL-6 mRNA expression and protein released (c, d) and TNF-α mRNA expression and protein released (e, f). The mRNA expression levels were determined by qPCR and proteins released were measured by ELISA. The experiments were performed in

triplicate for each condition and repeated three times. Data are expressed as Mean ± SD. (*) $p<0.05$ compared to control, (**) compared to FAC alone and (#) compared to cytokines alone or combined cytokines.

4.10 Effects of iron and pro-inflammatory cytokines on glutamate released from human astrocytes

To examine the effect of iron and pro-inflammatory cytokines on glutamate release from astrocytes, cells were exposed to FAC, IL-1β and TNF-α for 24 h. Then, the amount of glutamate in the medium from each condition was quantified. As shown in Figure 16, results revealed that exposure to FAC alone did not affect glutamate release from astrocyte, compared to unexposed control cells. Exposure to individual or combined cytokines significantly increased glutamate release from astrocytes, compared to unexposed control cells. FAC when presented simultaneously with individual or combined cytokines further enhanced glutamate release from astrocytes, compared to individual or combined cytokine controls. These results indicate that exposure to pro-inflammatory cytokines in combination with iron causes human astrocytes to release more glutamate.

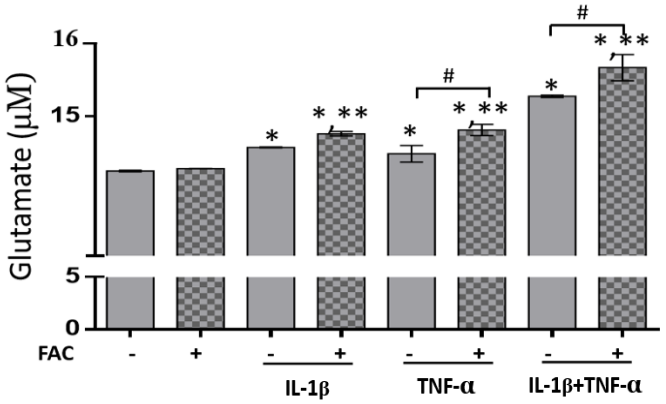


Figure 16. Effects of iron and pro-inflammatory cytokines on astrocyte glutamate release. After 24 h of exposure to 50 μg/ml FAC, 10 ng/ml IL-1β, a combination of FAC and IL-1β, 10 ng/ml TNF-α, a combination of FAC and TNF-α, a combination of IL-1β and TNF-α and a combination of FAC, IL-1β and TNF-α for 24 h, the amount of glutamate in the media was determined by Amplex Red glutamic acid assay kit. The experiments were performed in triplicate for each condition and repeated three

times. Data are expressed as Mean ± SD. (*) $p<0.05$ compared to control (**), compared to FAC alone (#) and compared to individual cytokines or combined cytokines.

CHAPTER V

DISCUSSION AND CONCLUSION

Iron accumulation, inflammation and glial activation are a common features of several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis [99-102]. Iron plays an important role in the development of the central nervous system (CNS) and in neuronal functions, and acts as an intrinsic producer of reactive oxygen species which can cause of oxidative stress. [103, 104]. Cellular iron homeostasis is regulated by the iron transporters TfR1, DMT1, FPN1 and by the iron storage protein ferritin. The main iron uptake transporter is DMT1, while FPN1 functions essentially as an iron efflux transporter [105]. Inflammation is mediated by reactive microglia and astrocytes that become neurotoxic through the secretion of inflammatory cytokines such as IL-1, IL-6, and TNF-a [106-108]. Indeed, the brains of PD patients contain elevated levels of cytokines, including IL-1β, IL2, IL4, IL-6, and TNF-α [9, 55], while in AD microglia surrounding Ab plaques show increased secretion of IL-1, IL-6, and TNF-a [12, 13]. Recently, knowledge on how iron accumulation and inflammation cross-talk in glial cells particularly astrocytes is still in its incipient stage. In this study, we investigated a potential role of iron and pro-inflammatory cytokines in mediating functions of astrocytes, which constitute the most abundant type of glial cells in the brain.

To generate the model of neurodegenerative diseases by iron accumulation and inflammation, a non-toxic concentration of FAC (50 µg/ml; approximately 200 µM) was selected for the following experiments. This concentration of iron is in range of the concentration of non-haem iron reported in human cerebral cortex [109] and in neuropil iron content in the elderly [110] which might be encountered in normal physiology. The results from the present study also showed that iron-enriched

environment was toxic to human astrocytes. It has been accepted that exposure to high concentration of iron compromised the integrity of astrocyte cell membrane [17, 111] due to the ability of iron to catalyze the generation of ROS through the Fenton reaction [26].

Previous studies have been shown the increasing levels of IL-1β and TNF-α which commonly found in the brain of neurodegenerative diseases patients such as AD and PD [9, 13, 55]. These two common cytokines were used to perform in the inflammation model. A low concentration of both cytokines was found to be increase a cell viability. This possibility due to acting as growth factors [112, 113] whereas, high concentration of cytokines especially TNF-α showed to decrease cell viability [113]. A non-toxic concentration of IL-1β and TNF-α were demonstrated as 10 ng/ml which are commonly used to activate astrocytes [85].

The finding of this study showed that the concentration of iron or cytokines described above was capable to induce iron accumulation in astrocytes especially in combination of iron and cytokines. As demonstrated by the utilization of modified Perl's staining method, a common stain used to detect the presence of iron deposit (Fe^{3+}) in ferritin which composes of H and L subunits. We also investigated the free iron in its reduced form (Fe^{2+}) inside the cell constitutes the "labile iron pool" (LIP) by using calcein assay. The increasing of iron deposit by Perl's staining are consistent with an increasing of labile iron pool in calcein assay. It has been accepted that the accumulation of iron in microglia was commonly found in neurodegenerative diseases [114]. The result indicated that astrocytes in iron enrich environment also has ability to accumulation of iron which might play an important role in pathological process of neurodegenerations.

According to the iron accumulation, it was found the alteration of genes involved in iron homeostasis. The results showed that exposure to iron decreased the expression of iron importer TfR1 and also increased iron storage FT-H and L and iron

exporter FPN1 to maintain iron homeostasis in astrocytes. The mechanism involved in genes iron homeostasis synthesis was regulated by the IRE/IRP regulatory system [111, 115, 116]. In addition, another iron importer DMT1 was investigated which expressed two alternative transcripts of the 3′ UTR, which were identified as one with an iron response element (+IRE) and one without an IRE (–IRE) [117]. It's has been report that –IRE DMT was not regulated by IRP/IRE system [118]. So it's possible that the increasing of DMT1 expression may be due to –IRE DMT1 expression effect. These results suggesting that DMT1 considered as a main rout of iron influx which led to iron accumulation.

Moreover, when combination of iron and cytokines with strongly increase in mRNA expression levels of DMT1 and Ft-H but slightly increase in FT-L couple with strongly decrease in FPN1and TfR1. These results are in general agreement with a recent study, which reported that pro-inflammatory cytokines can influence astrocytes iron homeostasis [60]. The cause for observed alteration of DMT1 and FPN1 expression in astrocytes is unknown. Probably, the increase in DMT1 was probably a direct consequence of the activation of DMT1 transcription by IL-1β and TNF-α signaling pathways through transcription factor NF-κB [119]. Increasing of H ferritin mRNA expression levels may be the mechanism of cells responsible for against oxidative damage from cytosolic LIP since this protein expressed ferroxidase enzyme that convert the toxic form of iron (Fe^{2+}) in LIP to non-toxic form (Fe^{3+}) and storage [120]. However, it has been reported that overexpressing of ferritin proteins are associated with several neurodegenerative diseases [121]. In contrast, a decrease in FPN1 could be because of a transcriptional and post-transcriptional control by inflammatory stimuli through transcription factor NF-κB as described in previous study [36] and cause of the effect of hepcidin regulates FPN1 by directly binds to cells expressing FPN1 and results in the internalization and lysosomal degradation of FPN1, thus providing a link between cytokines and regulation of FPN1 expression [122]. The conservation of the

iron export capacity is probably the main reason why iron accumulation was observed as a result of inflammatory stimuli. These results suggested that presence of pro-inflammatory cytokines such as IL-1 β and TNF-α can be enhanced intracellular iron accumulate in astrocytes by alters the express of DMT1 and FPN1 genes. In agreement with many previous studies that an increase in the iron content has potentially toxic due to iron ability to catalyze the generation of reactive oxygen species through the Fenton reaction [26, 123] cause of oxidative stress. As expected, the accumulation of iron can induce ROS production in astrocytes. It is speculated that astrocytes in neurodegeneration are under oxidative stress which may toxic and become activated, Increasing evidence suggest that astrocyte functions may be altered during activation stage especially their capacity to support neurons. This speculation may be relevant to neurodegenerative diseases, where there is a reduction in neuronal cell number.

Reactive astrocytes is a responsible of astrocytes to CNS injury. It has been used as a marker of pathology in a variety of CNS disease including multiple sclerosis and Alzheimer's disease [67, 124]. Reactive astrocytes are characterized by cell hypertrophy, increased numbers of cell processes, increased expression of the intermediate filament proteins including glial fibrillary acidic protein (GFAP), vimentin, and nestin, and have altered expression of many other genes [88]. Which discordance with our study showed decreasing expression of GFAP and increasing expression of vimentin in response to IL-1β and TNF-α, and the strongly changes were observed when simultaneously present of FAC with each cytokine and combination. However, these results were consistent with a previous study which reported that the human fetal astrocytes in response to recombinant IL-1β showed a marked transient reduction in GFAP and increase in vimentin mRNA content by 24 h as reflect initially activated state of astrocytes by rearrange their cytoskeleton and recover to increase after 24 h post-stimulation [125]. We extended times of astrocytes treated with cytokine up to 48 and 72 h showed an increase of GFAP expression compared to control (data not

shown). A decrease of GFAP expression result was consistent with a decrease of astrocytes cell proliferation. It's has been reported that GFAP associated with cell mitosis [126]. In fact, the demand for intermediate filaments is highly increased in Schwann cell after damage. Both GFAP and vimentin are upregulated, perhaps to provide an efficient cytoskeleton rearrangement necessary for proliferation and differentiation [127-129]. The result suggesting that astrocytes treated with cytokines for 24 h induced change of astrocytes cytoskeleton observed by the expression of GFAP and VIM resulted in cell proliferation decreased. It's likely that their cytoskeleton change reflects their functional status.

Astrocytes belong to neuroglia in the central nervous system (CNS) which play active roles in many neuronal functions by producing various cytokines, chemokines, growth factors, and metabolites, all of which act as a molecular coordinator of neuron-glia communication. In the brain, activated astrocytes are the predominant source of cytokines such as IL-1β, IL-6 and TNF-α [130]. However, several studies have reported that an increase in these cytokines levels are associated with many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease [131, 132]. It has been shown that chronic exposure to IL-6 during neuronal development results in cellular damage and cell death in a subpopulation of developing granule neurons [133]. Moreover, the blocking of IL-1β receptor markedly attenuates tau pathology, and partly reduces certain fibrillary and oligomeric forms of amyloid-b in Alzheimer's disease Model [134].

In the present study, the result demonstrated that astrocytes exposure to FAC for 24 h slightly increased mRNA expression and production of three major cytokines, IL-1β, IL-6 and TNF-α but statistical significant only in IL6 and TNF-α compared to unexposed cells. Moreover, exposure to IL-1β or TNF-α alone significantly increased mRNA expression and production of three cytokines in astrocytes, compared to control cells. This finding is consistent with the report showing that IL-1β or TNF-α up-regulates

protein expression of themselves and IL6 via NF-κB in astrocytes [85]. Accordingly, binding of IL-1β and TNF-α to its receptors on astrocytes will result in NF-κB activation followed by the production of more IL-1β, TNF-α and IL6 mRNA and protein [135]. It has been reported that non-stimulated human astrocytes do not constitutively produce IL-1β or TNF-α, while activated human astrocytes could produce both [85]. We observed that the highest levels of three cytokines mRNA and production were obtained when simultaneously exposed FAC with IL-1β, TNF-α or combined cytokines.

In this study, It's speculated that iron could act directly on NF-κB to synergize IL-1β, TNF-α and IL6 production initiated by IL-1β and TNF-α. This is due to the fact that iron is required as a cofactor for NF-κB activation. Previously, it has been shown that iron chelator effectively blocks NF-κB activation. [136]. Thus, increasing intracellular iron levels could lead to transcription factor NF-κB activation and production of cytokines [119, 137]. Therefore, our results suggest a synergistic effect of iron accumulate and cytokines on expression and release of IL-1β, IL-6 and TNF-α in astrocytes that can occur through the activation of NF-κB signaling.

Next, glutamate production in astrocytes was examined. In fact, astrocytes help removing excess glutamate from the synapse to maintain extracellular levels of glutamate and to protect neuron from glutamate induced excitotoxicity [81]. On the other hand, astrocytes can release chemical transmitters, including glutamate, ATP, and D-serine, which in turn control synaptic activity [138]. Glutamate is the main excitatory neurotransmitter in the brain and is involved in many aspects of normal brain function. It's has been reported that elevated of glutamate level in cerebrospinal fluid in Amyotrophic lateral sclerosis (ALS) patients (Rothstein et al., 1990) and excessive of glutamate cause of degeneration of dopamine nigral neuron in PD [139]. Here, the result observed that glutamate production in astrocytes displayed similar pattern to those of cytokines following exposure to FAC, IL-1β, TNF-α alone and combined. The result suggested that iron and cytokine identified as an important

player in mediating glutamate over-production which consequently induce neurotoxicity and apoptosis leading to neurodegeneration.

The results suggest that astrocytes partially lose their function and neuroprotective ability during brain iron accumulation, inflammation and may contribute to exacerbating neuronal injury related neurodegenerative processes. In summary, astrocytes in normal condition has diverse functions in brain especially to support neuronal function and survival. Our studies demonstrated that iron-enriched environment can be either toxic or non-toxic to astrocytes. The result demonstrate that astrocytes exposed to sub-lethal concentration of iron have the capacity to accumulate iron, which can be enhanced by the presence of pro-inflammatory cytokines such as IL-1β and TNF-α. These iron-laden change their iron homeostasis genes expression and astrocytes become activated by rearrange their cytoskeleton and alters pro-inflammatory cytokines genes expression. Theses alterations reflect their functions changes. This activated astrocytes that can be harmful to neurons by releasing more cytokines; IL-1β, TNFα, IL6 and glutamate. Results obtained from this study provide insights into the role of astrocytes in neuro-degeneration with brain iron accumulation.

CONCLUSION

1. Iron-enriched environment can be either toxic or non-toxic to astrocytes depend on concentrations.

2. The exposure to non-toxic concentration of iron was sufficient to induce intracellular accumulate iron in astrocytes.

3. Intracellular accumulate iron in astrocytes can be enhanced by the presence of pro-inflammatory cytokines such as TNF-α.

4. When presented simultaneously, FAC and cytokines can be mediated astrocytes activates.

5. Normal functions of astrocytes has been changed when they become activated by FAC and cytokines treatment with alter the expression of their genes.

6. Activated astrocytes can be harmful to neurons by releasing more cytokines and glutamate.

APPENDIX

APPENDIX A

Abbreviation

1. DMSO = dimethyl sulfoxide
2. FAC = ferric ammonium citrate
3. M = molar
4. mM = millimolar
5. µM = micromolar
6. ml = milliliter
7. µl = microliter
8. mg = milligram
9. µg = microgram
10. DMEM = Dulbecco's Modified Eagle Medium
11. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5—diphenyltetrazolium bromide
12. OH^- = hydroxyl radical
13. qPCR = quantitative polymerase chain reaction
14. ROS = reactive oxygen species
15. RT-PCR = reverse transcription polymerase chain reaction
16. SD = standard deviation
17. GFAP= glial fibrillary acidic protein
18. VIM = vimentin
19. TfR1 = transferrin receptor 1
20. DMT1 = divalent metal transporter 1
21. FTH = ferritin heavy chain
22. FTL = ferritin light chain
23. FPN1 = ferroportin 1
24. IL-1β = interleukin 1 beta
25. IL-6 = interleukin 6
26. TNF-α = tumor necrosis factor alpha

APPENDIX B

1% Agarose gel (w/v) 100 ml

Agarose	1 g
1x TBE	100 ml

Dissolve by heating until no granules of agarose are visible.

10x TBE buffer (pH 8.0) 1 liter

Tris base	108 g
EDTA $2H_2O$ (pH 8.0)	40 ml
dH_2O	800 ml
Slowly add the boric acid, anhydrous	55 g

Adjust the pH to 8.0 with conc.HC1

Adjust the volume to 1 liter with dH_2O

1x TBE buffer 1 liter

10x TBE buffer	100 ml
dH_2O	900 ml

Adjust the pH to 7.4 with conc.HC1

Adjust the volume to 1 liter with dH_2O

Make culture media for human astrocytes cell lines (Powder media, pH7.2)

Reagent	**Volume**
DMEM	13.4 g
Sodium bicarbonate	3.7g/L
Sodium pyruvate	110 mg
Adjust pH 7.2	
HEPES	10 ml
Antibiotics(penicillin/streptomycin)	10 ml
Total Volume	1L

Add FBS to get 5% FBS DMEM before use

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