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Neuroprotective effects of gamma-glutamylcysteine ethyl ester on an in vivo moderate traumatic brain injury-mediated model and in vitro in cortical astrocytes and neurons

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NEUROROPROTECTIVE EFFECTS OF GAMMA-GLUTAMYLCYSTEINE ETHYL ESTER ON AN *IN VIVO* MODERATE TRAUMATIC BRAIN INJURY-MEDIATED MODEL AND *IN VITRO* IN CORTICAL ASTROCYTES AND NEURONS

By

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for the degree of
MASTER OF SCIENCE
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DEDICATION

This thesis is dedicated to my parents and brother

Mr. Sanghyun Cho

조 상 현

And

Mrs. Pyungsoon Kim

김 평 순

And

Mr. Jaekwan Cho

조 재 관

who always there for me at every step and eternally love me.

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ABSTRACT

Neurodegeneration is the loss of neuronal structures or functions, while neuroprotection is the delay or prevention of neurodegeneration. In traumatic brain injury (TBI), neurodegeneration can occur as the result of oxidative stress, the imbalance of oxidants and antioxidants levels; therefore, antioxidant approaches can be effective therapeutic methods for neuroprotection by attenuating oxidative stress. Glutathione (GSH), a naturally occurring antioxidant, plays an important role in the maintenance of intracellular redox homeostasis by scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS). In this thesis, we attempted to evaluate the abilities of a GSH precursor, gamma-glutamylcysteine ethyl ester (GCEE), to prevent neurodegeneration by combating intracellular oxidative stress in neurons and astrocytes. For *in vivo* experiments, controlled cortical impact (CCI) was performed on Wistar rats to simulate moderate TBI, and GCEE (150 mg/kg) or saline was administrated 30 min or 60 min after the brain injury. Fluoro Jade-B (FJB), an ionic fluorescein derivate, was adapted to selectively stain the degenerating neurons on the brain tissues, and FJB-positive neurons were quantified. Administration of GCEE (150 mg/kg) post- injury decreased the number of FJB positive neurons that were significantly increased in saline treated groups. Next, the protective roles of GCEE *in vitro* in rat primary cortical astrocytes were investigated using various concentrations of tert-butyl hydroperoxide (tBHP) in order to induce oxidative stress and further toxicity. Cell viability was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay.

The status of oxidative stress was determined by measuring intracellular ROS levels through dichlorofluorescein (DCF) assay. GCEE restored cell viability that was significantly decreased in untreated cells by decreasing oxidative stress. Lastly, the antioxidant properties of GCEE in neurons and astrocytes were investigated in a time-dependent manner. GCEE was able to immediately attenuate intracellular oxidative stress both in neurons and astrocytes. Such decreased oxidative stress was progressively increased in neurons, whereas decreased oxidative stress remained in astrocytes. Overall, our current findings suggest the protective roles of GCEE in both neurons and astrocytes may contribute to the potential therapeutic effects on oxidative stress-associated neurodegeneration following traumatic brain injury.

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LIST OF ABBREVIATIONS

3-NT	3-nitrotyrosine
4-HNE	4-hydroxynonenal
ApN	Aminopeptidase N
BBB	Blood brain barrier
Cys	Cysteine
CysGly	Cysteinylglycine
ETC	Electron transport chain
GCEE	Gamma-glutamylcysteine ethyl ester
GCS	Gamma-glutamylcysteine synthetase
Glu	Glutamate
Gly	Glycine
GPx	Glutathione Peroxidase
GR	Glutathone reductase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Oxidized glutathione
LP	Lipid peroxidation
PN	Peroxynitrite
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
tBHP	tert-butyl hydroperoxide
TBI	Traumatic brain injury
γ-GlyCys	Gamma-glutamylcysteine
γ-GT	Gamma-glutamyltranspeptidase

CHAPTER 1

INTRODUCTION

The brain is composed of two main types of cells: neurons and glia. Glia is further divided into astrocytes, oligodendrocytes, and microglia. The concentrations and distributions of cells are different depending on the regions of the brain and the types of the cells. Moreover, the functions of each cell are varied. Neurons have the ability to sense changes in environment, communicate with surrounding neurons, and respond to these changes due to their unique structure. Neurons contain axons and synapses which help transport and release chemical neurotransmitters (Fig.1.1). Neurons are the main cells contributing to the special functions of the brain, such as memory, learning, emotions, and perception. Neuronal damage can alter these functions and ultimately lead to overall brain dysfunction in these aforementioned areas. Glial cells fill the space between neurons regulate extracellular space and protect neurons. In particular, astrocytes play an important role in regulating glutamate concentrations to prevent glutamate neurotoxicity because glutamate is toxic at high concentrations and initiates glutamate-neurotoxicity although it is an essential excitatory neurotransmitter at normal concentrations. Another crucial role of astrocytes is supplying glutathione precursors in the extracellular space so that neurons can utilize them for glutathione synthesis, which ultimately protects neurons against oxidative stress (Dringen 2000). Additionally, astrocytes are involved in synaptogenesis and synaptic plasticity by releasing growth factors and neuromodulators (Ullian et al. 2004, Allen and Barres 2005,

Hu et al. 2007). Astrocytes also rapidly remove debris in the extracellular space. Thus, it is important to protect not only neurons, but also astrocytes because they interact with each other.

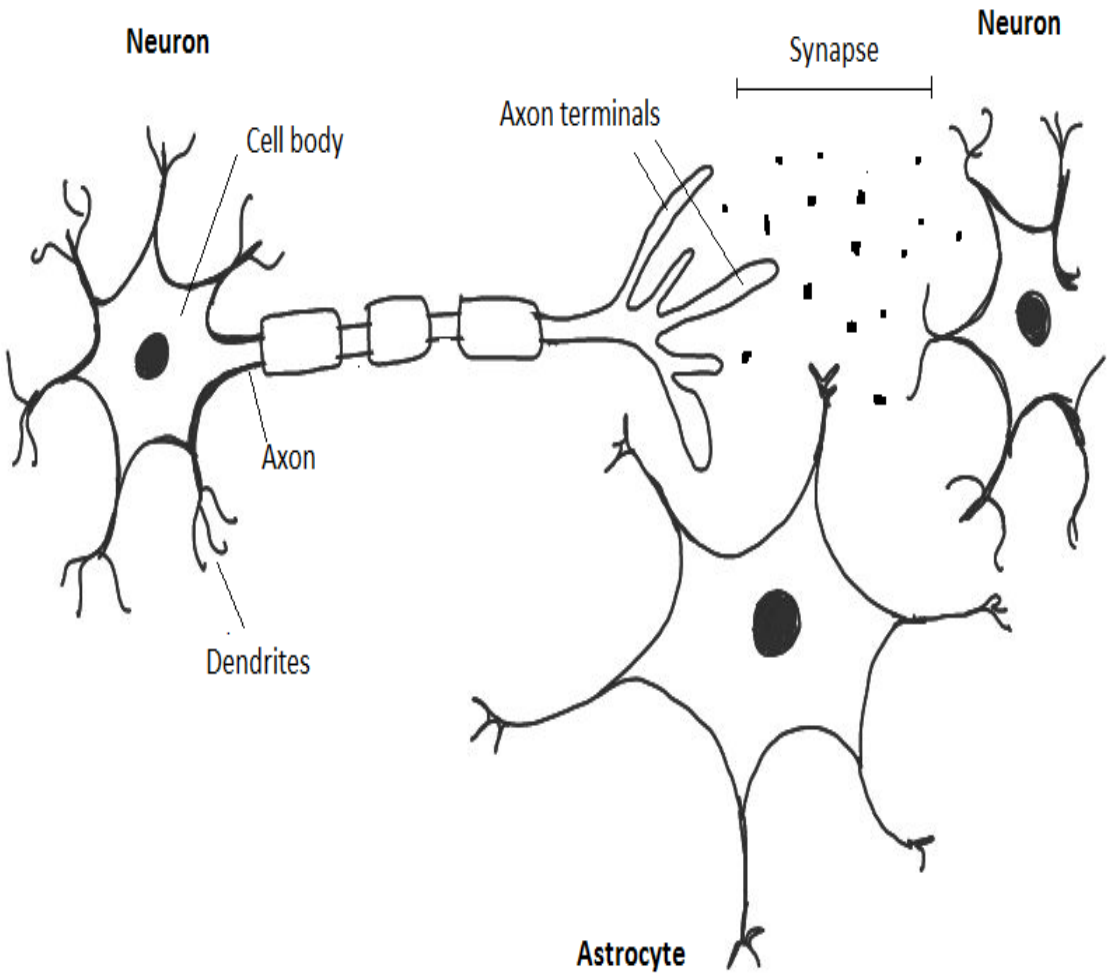


Fig.1.1 Illustration of the interaction between neurons and astrocytes

Traumatic brain injury (TBI) is a serious health problem in all age groups. It is caused by a spontaneous, sudden accident. Once an accident occurs, the brain sustains primary physical damage, which can alter biochemical and cellular pathways. Primary injury then initiates acute or delayed secondary injury in which neuronal structures and functions are progressively altered. Accumulation of degenerating neurons is a pathway of neurological disorders. This entire process can be short term, occurring in hours to days, or long term, occurring in months to years. Therefore, development of therapeutic strategies to stop delayed secondary injury is necessary to prevent neurodegeneration and further neurodegenerative disorders.

A large number of studies have investigated pathological consequences of traumatic brain injury at cellular and molecular levels to develop methods for neuroprotection. More specifically, the energy generated by the mechanical impact during the brain injury is immediately delivered to the brain tissue and the depolarization of neurons is subsequently induced, which causes an uncontrolled release of glutamate in the synaptic gap (Andriessen, Jacobs, and Vos 2010). The excessive amounts of glutamate bind to glutamate receptors on the membrane and consequently activate Ca^{2+} ion channels, thereby a massive influx of Ca^{2+} into neuronal cells occurs (Choi et al. 2012, Choi 1985, Choi, Koh, and Peters 1988). As a result, several pathophysiological processes are initiated within cell, and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are subsequently overproduced in response to rapid elevation of extracellular glutamate and subsequently intracellular Ca^{2+} (Bullock et al.

1998, Choi 1985). In early experimental TBI models, significantly increased superoxide ($\bullet O_2$) and hydroxyl radicals (•OH) have been found as early as 5 minutes after TBI and lasted for few hours (Kontos and Povlishock 1986, Kontos and Wei 1986, Hall, Andrus, and Yonkers 1993). Nitric oxide synthase, an enzyme generating nitric oxide (•NO), has been shown to be activated in response to glutamate neurotoxicity following TBI, increasing levels of •ON; however, delayed overexpression of nitric oxide synthase has been observed (Griffith and Stuehr 1995, Orihara et al. 2001). Moreover, •ON reacts with $\bullet O_2$ and generates the toxic species peroxynitrite (ONOO-). Peroxynitrite then undergoes further reactions, producing highly cytotoxic free radicals, including nitrite ($\bullet NO_2$), carbonate ($\bullet CO_3$), and •OH. Such dramatically increased levels of reactive oxidants exceed the capacity of the endogenous antioxidant defense system, and thereby ROS/RNS cannot be immediately removed, but instead are left inside the cell. Oxidative stress is implicated in the imbalance between ROS/RNS levels and antioxidants levels due to either overproduction of ROS/RNS or the depletion of antioxidants or both, resulting in the interruption of redox homeostasis, which can have long lasting effects on overall brain function and energy metabolism because highly reactive radicals rapidly react with cellular components, such as lipids, proteins, and DNA (Awasthi et al. 1997, Freire 2012). Subsequently, cellular components change their structure and function, ultimately leading to cellular death. Especially, peroxynitrite which interacts with proteins and lipids and increases levels of protein carbonyls, 3-nitrotyrosine (3-NT), and 4-hydroxynonenal (4-HNE), as the markers of protein oxidation, protein nitration and lipid peroxidation, respectively (Hall et al. 2004, Reed et

al. 2009). Also, peroxynitrite reacts with mitochondria and oxidatively damage the membrane (Radi 1998). All this deleterious oxidative damage on the cellular components contributes to cellular death.

Although pathological consequences of traumatic brain injury have been elucidated by a great amount of studies, a promising therapeutic agent is not available yet due to its complexity and heterogeneity. However, an increasing number of studies have shown that antioxidant approaches effectively prevent neurodegeneration by protecting neurons against oxidative stress as oxidative stress has been suggested as a major underlying cause of almost all neurodegenerative diseases. In particular, glutathione is the most abundant thiol (-SH) containing antioxidant in the brain. The essential role of glutathione is to maintain redox status within the cells by scavenging toxic reactive oxidants. Since a dramatic elevation of reactive oxygen and nitrogen species levels has been well characterized as a consequence of traumatic brain injury, promoting glutathione levels has been suggested as an effective neuroprotective strategy by effectively attenuating oxidative stress due to the ability of glutathione to eliminate ROS/RNS; however, the concentration of glutathione is limited due to both the limited availability of substrates and the regulation of biosynthesis by feedback inhibition (Richman and Meister 1975). Thus, administration of chemical substances that can increase intracellular glutathione has been suggested since glutathione itself is not effective because it is degraded into amino acids before it reaches the brain and it cannot easily cross the blood brain barrier (BBB) (Meister and Anderson 1983).

Gamma-glutamylcysteine ethyl ester (GCEE), an analogue of gamma-glutamylcysteine (γ-GluCys) is a substrate for glutathione synthesis, has been shown to up-regulate total brain glutathione levels by overcoming feedback inhibition as well as mitochondrial glutathione (Drake et al. 2002, Drake et al. 2003). Up-regulated glutathione by GCEE has shown antioxidant capabilities to combat oxidative stress and decrease the makers of oxidative damage (Boyd-Kimball, Sultana, Poon, et al. 2005, Joshi et al. 2007) Since oxidative stress is one of the major causes of neurodegeneration in traumatic brain injury, we hypothesized that treatment with GCEE could combat increased reactive oxygen and nitrogen levels by boosting cellular glutathione levels in the brain, which may attenuate oxidative stress and damage, and ultimately preventing neurodegeneration (Fig.1.2).

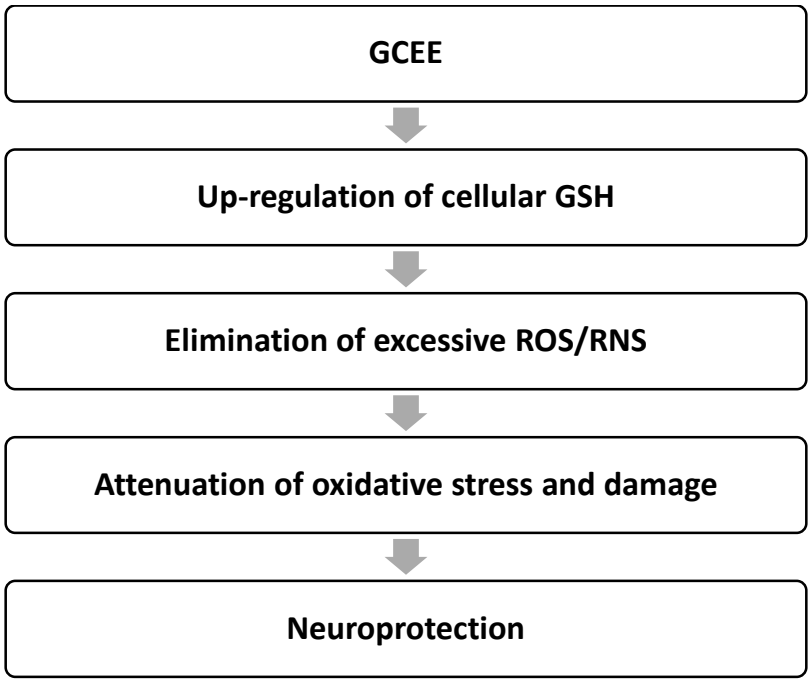


Fig.1.2 Schematic representation of hypothesis in this thesis

The overall aim of my work was to investigate the neuroprotective effects of GCEE as a potential therapeutic agent in moderate traumatic brain injury. The first study (Chapter 3) was conducted to determine if administration of GCEE post-injury decreased the number of degenerating neurons in experimental TBI rat brain due to its ability to increase glutathione and attenuate oxidative stress in neurons. The second study (Chapter 4) was performed in rat primary cortical astrocytes in order to see whether GCEE could enter astrocytes and protect these structures against oxidative stress and oxidative stress-induced cytotoxicity. My final experiment (Chapter 5) was performed in rat primary cortical astrocytes and neuronal culture separately in order to assess any time course effects of GCEE in different cells types.

CHAPTER 2

BACKGROUND

2.1 Traumatic Brain Injury

2.1.1 Overview

According to the National Center for Injury Prevention and Control at the Centers for Disease Control and Prevention (CDC), approximately 2.5 million people in the United States reported emergency department visits, hospitalizations, or deaths due to traumatic brain injury in 2010, involving 2.2 million emergency department visits, 280,000 hospitalizations, and 50,000 deaths. Unpredictable suddent accidents, such as falls (40.5%), hits by/against (15.5 %), motor vehicle crashes (14.3%), and assaults (10.7%), or unknown causes (19.0%) mainly contribute to traumatic brain injury according to the CDC. Traumatic brain injury is also a serious health concern for military soldiers and athletes. In the military population, blast explosions are one of the main risk factors that cause TBI, especially for those who are deployed in combat regions (Rosenfeld et al. 2013). Athletes who are involved in sports, such as contact-collisions sports (i.e. boxing, football, ice hockey, rugby) and high-velocity sports (i.e skiing and motor racing), are more frequently and repeatedly exposed to TBI (Jordan 2013).

2.1.2 Classification

The Glasgow Coma Scale (GCS) classifies traumatic brain injury severity into three classifications: mild, moderate, and severe by measuring the level of consciousness. Scores range from 1 to 15: mild (GCS 13-15), moderate (GCS 9-12), and

severe (GCS ≤8) (Teasdale and Jennett 1974). The majority of TBI incidents are characterized as mild TBI (80%), and the rest are either considered moderate TBI (10%) or severe TBI (10%). There is a strong correlation between TBI severity and the risk of death. Approximately 1 % of mild TBI, 15 % of moderate TBI, and 40 % of severe TBI die as a result of their brain injury (Andriessen, Jacobs, and Vos 2010, af Geijerstam and Britton 2003, Boto et al. 2009).

Depending on the distribution of the damage produced by collision forces on the brain, TBI can be classified as focal or diffuse. Focal injury is limited to the site of impact or the opposite site of the impact, whereas diffuse injury involves extensive and widespread damage including axonal injury, or both focal and diffuse injuries may occur together (Andriessen, Jacobs, and Vos 2010). The location and severity of impact to the brain is related to neurological pathologies following TBI.

Depending on the point of onset of the injury, TBI is characterized into two types: primary injury and secondary injury. The damage that occurs immediately by initial external physical impact is defined as a primary injury, while those initiated by primary injury are defined as secondary damage. Once TBI occurs, there is no way to reverse the primary injury, which indicates there is an increased risk for secondary injuries to occur following TBI, which could take as little as a few hours to days or months to years (Zaloshnja et al. 2008). In other words, although people can survive from a traumatic brain injury and not show any immediate symptoms, long-term disabilities can arise due to delayed secondary injuries and reduce their quality of life.

2.1.3 Pre and post injury treatment

Pre-injury treatment is related to preventative measures, using safety devices, such as helmets, seatbelts, and airbags. In contrast, post-injury treatment is related to providing therapeutic treatments after injury occurs to prevent delayed secondary injuries. Pre-injury treatment could reduce TBI severity as well as primary injury on the brain; however, pre-injury treatment itself cannot prevent the accidents from occurring. Once an accident happens, there is no way to reverse primary damage regardless of the severity of TBI. Thus, post-treatment strategy is a more effective approach and essential to preventing secondary injury.

Application of hypothermia has been widely used in TBI patients as a post therapeutic option for neuroprotection; however, the effectiveness of this strategy is controversial to whether outcomes are improved or not (Sydenham, Roberts, and Alderson 2009, Urbano and Oddo 2012, Crossley et al. 2014). Although the necessity and importance of early post-TBI treatment are obvious, there are no known promising therapeutic strategies due to the complex and heterogeneous consequences. Thus, a plethora of studies have tried to elucidate the pathological processes following traumatic brain injury and develop strategies to prevent secondary injuries, including oxidative stress which accounts for neurodegeneration following TBI.

2.2 Reactive Oxygen Species and Reactive Nitrogen Species

2.2.1 Overview

Reactive oxygen species and reactive nitrogen species (ROS/RNS) are produced from normal biochemical processes in very small amounts. These toxic species are classified into radicals and non-radicals based on the presence of un-paired electron(s) on the outermost shell. Free radicals, such as superoxide ($\bullet O_2$), hydroxyl radical ($\bullet OH$), nitric oxide ($\bullet NO$), carbonate radical ($\bullet CO_3$), and nitrite radical ($\bullet NO_2$), have un-paired electron(s), while non-radicals, such as hydrogen peroxide (H_2O_2) and peroxynitrite (PN: $ONOO^-$), do not have un-paired electron(s) (Table 2.1). Free radicals are highly reactive due to un-paired electrons and readily react with adjacent molecules to form stable electron pairs. Although non-radicals do not have un-paired electrons, they are still reactive because toxic free radicals are derived from non-radicals through several reaction processes, such as the Fenton reaction and the decomposition of peroxynitrite.

The cells have an endogenous defense system against ROS/RNS, which either prevents ROS/RNS formation or eliminates existing ROS/RNS, thereby the concentrations of ROS/RNS are maintained below cytotoxic levels during normal physiological conditions. The endogenous defense system includes enzymes and low-molecular weight antioxidants. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are the main protective enzymes, while glutathione is categorized as an antioxidant molecule.

Table 2.1 Reactive oxygen species and reactive nitrogen species

	ROS/RNS	Symbols	Source
Free radicals	Superoxide	$\bullet O_2$	Mitochondrial leakage
	hydroxyl radical	•OH	Fenton reaction
	nitric oxide	•NO	Nitric oxide synthase (NOS)
	carbonate radical	$\bullet CO_3$	Decomposition of peroxynitrite
	Nitrite	$\bullet NO_2$	Decomposition of peroxynitrite
Non-radicals	hydrogen peroxide	H_2O_2	Superoxide dismutase
	peroxynitrite	ONOO-	The reaction of $\bullet O_2$ and •NO

Source: Hall, E. D., R. A. Vaishnav, and A. G. Mustafa. 2010. "Antioxidant therapies for traumatic brain injury." Neurotherapeutics 7 (1):51-61

2.2.2 Superoxide

Superoxide ($\bullet O_2$) is produced by reducing one molecule of oxygen (O_2) with a single electron (Fig.2.1). During normal biochemical processes, 1-2% of total oxygen consumed by mitochondria is reduced to $\bullet O_2$ in the electron transport chain (ETC) on the mitochondrial membrane (Boveris 1977, Drose and Brandt 2012, Kowaltowski and Vercesi 1999).

$$O_2 + e^- \xrightarrow{\text{NAD(P)H oxidase}} \bullet O_2$$

Fig.2.1 Formation of superoxide radical

2.2.3 Hydrogen peroxide

Although $\bullet O_2$ is highly reactive, the chances of oxidative damage by $\bullet O_2$ itself are low because its activity with cellular components is relatively weak in aqueous conditions (Hall, Vaishnav, and Mustafa 2010). Moreover, once $\bullet O_2$ is formed, superoxide dismutase (SOD), immediately converts the free radical to hydrogen peroxide (H_2O_2) which is less reactive (Fig.2.2, step 1).

Moreover, H_2O_2 is eliminated by either catalase or glutathione. Catalase reacts with two molecules of H_2O_2, producing two molecules of water (H_2O) and O_2 (Fig.2.2, step 2). Two molecules of glutathione scavenge one molecule of H_2O_2, generating two molecules of water and oxidized form of glutathione (GSSG), which is catalyzed by GPx (Fig.2.2, step 3).

$$2(\bullet O_2) + 2H^+ \xrightarrow{\text{Superoxide dismutase (SOD)}} H_2O_2 + O_2 \quad \text{(step 1)}$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad \text{(step 2)}$$

$$H_2O_2 + 2GSH \xrightarrow{\text{Glutathione Peroxidase (GPx)}} 2H_2O + GSSG \quad \text{(step 3)}$$

Fig.2.2 Formation and elimination of hydrogen peroxide

2.2.4 Hydroxyl radical

Although H_2O_2 is less reactive and cytotoxic, the hydroxyl radical (•OH), the most toxic oxidizing agent due to its ability to react with almost every biological molecule found in the cells, is generated from H_2O_2 through the Fenton reaction in which ferrous iron (Fe^{2+}) is oxidized into ferric iron (Fe^{3+}) (Fig.2.3, step 1) (Rodopulo 1951, Haber and Weiss 1934). Ferric iron can be reduced back to ferrous iron by superoxide and ferrous iron then can be reused for another Fenton reaction (Fig.2.3, step 2). During normal physiological conditions, iron containing proteins, such as transferritin, ferritin, and hemoglobin, store iron with a high affinity at neutral pH so that iron concentrations can be maintained with non-toxic levels. However, when the pH is lowered by external impact on the cell during injury, the affinity of iron containing proteins towards iron is reduced, thereby excessive iron are released, leading to the overproduction of •OH (Hall, Vaishnav, and Mustafa 2010, Bains and Hall 2012).

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+} \quad \text{(step 1)}$$

$$\bullet O_2 + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad \text{(step 2)}$$

Fig.2.3 Fenton reaction showing the hydroxyl radical formation and recycling of iron

2.2.5 Peroxynitrite

Peroxynitrite (PN: $ONOO^-$) is formed through the reaction between nitric oxide (•ON) and $•O_2$ (Fig.2.4, step 1) (Beckman 1991, Wada et al. 1998). Furthermore, when PN is protonated it can produce peroxynitrous acid (ONOOH), which is then decomposed into nitrite radical ($•NO_2$) and •OH (Fig.2.4, step 2) (Beckman 1991). Another reaction of PN with carbon dioxide (CO_2) produces nitrosoperoxocarbonate ($ONOOCO_2$), which is decomposed to $•NO_2$ and the carbonate radical ($•CO_3$) (Fig.2.4, step 3) (Bains and Hall 2012). Although PN itself is not highly reactive due to the absence of an un-paired electron, its derivatives, including •OH, $•NO_2$, and $•CO_3$ are highly reactive and toxic.

$$•O_2 + •NO \rightarrow ONOO^- \text{ (step 1)}$$

$$ONOO^- + H^+ \rightarrow ONOOH \rightarrow •NO_2 + •OH \text{ (step 2)}$$

$$ONOO^- + CO_2 \rightarrow ONOOCO_2 \rightarrow •NO_2 + •CO_3^- \text{ (step 3)}$$

Fig.2.4 Formation and decomposition of peroxynitrite

2.3 Cellular and Molecular Processes Following TBI

2.3.1 Overview

Following traumatic brain injury, complex cellular and molecular processes immediately occur and ultimately lead to neurological disorders. Glutamate, an important neurotransmitter at normal levels, but a key factor for initiating pathophysiological processes at high concentrations, is significantly increased at extracellular space following TBI. In response to glutamate neurotoxicity, $\bullet O_2$ and •ON are overproduced inside cells and peroxynitrite is subsequently generated from the reaction of $\bullet O_2$ and •ON. Peroxynitrite then diffuses within cells and is decomposed to produce reactive •OH, $\bullet NO_2$, and $\bullet CO_3$. Once it is decomposed, •OH, $\bullet NO_2$, and $\bullet CO_3$ readily react with biological molecules such as proteins and lipids, leading to deleterious oxidative damage on cellular components through lipid peroxidation (LP), protein nitration, and protein carbonylation (Hall et al. 2004). Consequently, cellular and mitochondrial membranes are disrupted and proteins are aggregated. All this oxidative damage is irreversible and can promote neurodegeneration.

2.3.2 Overproduction of ROS/RNS

The mitochondria begin to overproduce $\bullet O_2$ in response to the accumulation of intracellular Ca^{2+} following TBI (Kontos and Povlishock 1986, Kontos and Wei 1986). Mitochondria take intracellular Ca^{2+} into the matrix to modulate ionic homeostasis on cytoplasm, which alters the structure of the electron transport chain on the mitochondrial membrane, thereby the damaged ETC boosts the production of $\bullet O_2$

(Kowaltowski, Castilho, and Vercesi 1995). Thus, the electron transport chain becomes the main location where the majority of $\bullet O_2$ is generated after TBI. Besides the ETC on the mitochondrial membrane, a variety of other sources are also involved with the overproduction of $\bullet O_2$ during brain injuries, such as the arachidonic acid cascade via prostaglandin synthase and 5-lipoxygenase activity, enzymatic or autoxidation of biogenic amine neurotransmitters (i.e. dopamine, norepineprine, 5-hydroxytryptamine), mitochondrial leakage , xanthione oxidase activity, oxidation of extravasted hemoglobin, and delayed activation of microglia, infiltration of neutrophils, and macrophages (Hall, Vaishnav, and Mustafa 2010, Moochhala et al. 2005, Rubinek and Levy 1993).

2.3.3 Lipid peroxidation

The brain has a high concentration of lipids, especially polyunsaturated fatty acids. Unstable ROS/RNS can readily react with lipids and induce lipid peroxidation (LP) through three steps: initiation, propagation, and termination. In particular, •OH mainly initiates lipid peroxidation. Initiation starts when •OH withdraws a hydrogen atom from lipids, thereby •OH itself becomes H_2O and lipids themselves form lipid radicals (•L). Since •L is also unstable, •L reacts with O_2, which yields a lipid peroxyl radical (•LOO) (Fig 2.5, step 1). Following initiation, •LOO propagates a series of chain radical formation reactions. Previously produced •LOO deprotonates adjacent lipids, generating another •LOO with formation of lipid hydroperoxide (LOOH). Propagation is continuously carried out until no more lipids are available or until •LOO is transformed into a stable molecule by reacting with another radical or an antioxidant.

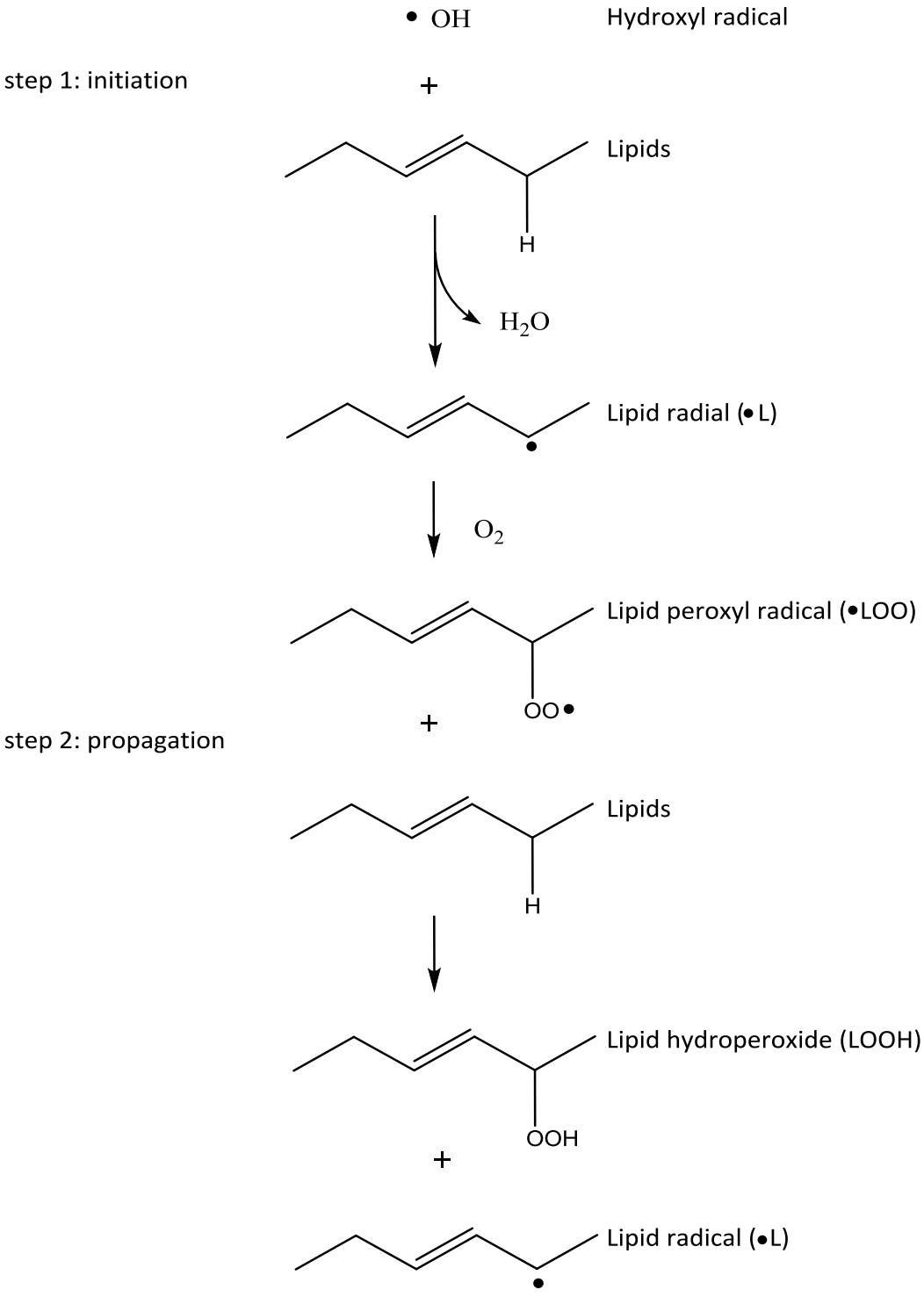


Fig.2.5 Schematic representation of lipid peroxidation process

Elevated levels of •OH and $•O_2$ have been observed in early TBI models (Hall, Andrus, and Yonkers 1993, Hall et al. 1994, Kontos and Wei 1986). Since the cellular membrane, mitochondrial membrane, and blood-brain barrier (BBB) are made of lipids or polyunsaturated fatty acids, the elevation of •OH or $•O_2$ in the brain after TBI leads to disruption of those membranes and BBB in the rats (Smith et al. 1994). As a result of lipid peroxidation, reactive aldehydes, such as acrolein, malondialdehyde, and 4-hydroxynonenal (4-HNE) are produced. These are frequently used as an indicator of lipid peroxidation in many experimental TBI models to measure of levels of oxidative stress or damage (Fig.2.6). 4-HNE can further react with proteins, resulting in conformation and functional changes of proteins.

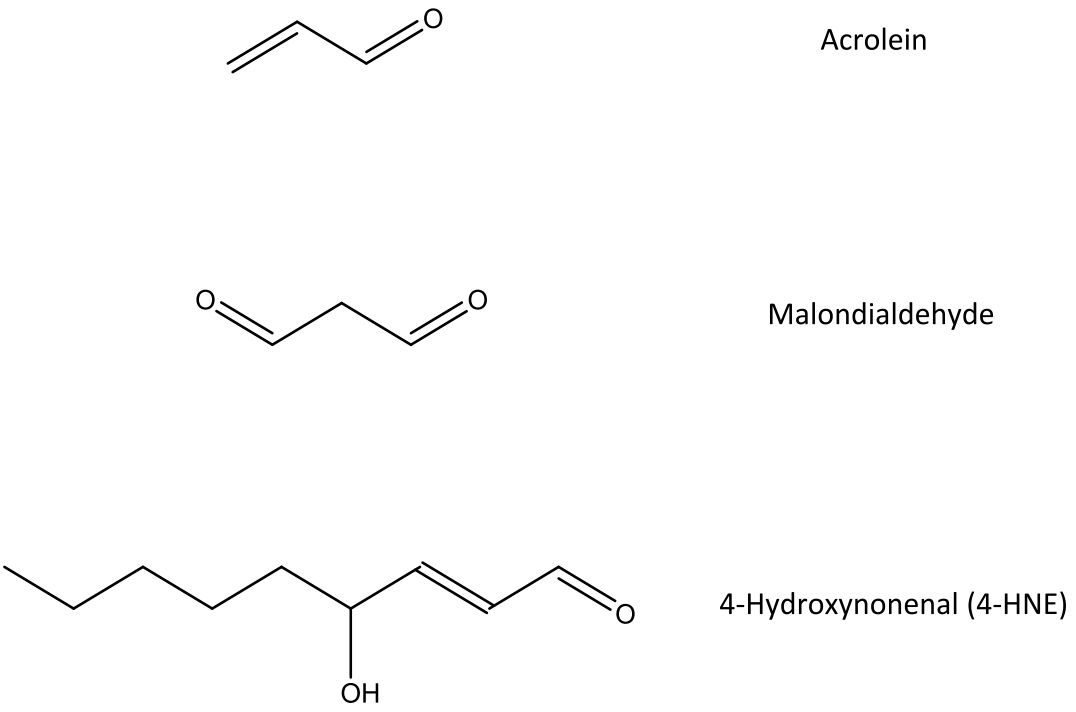
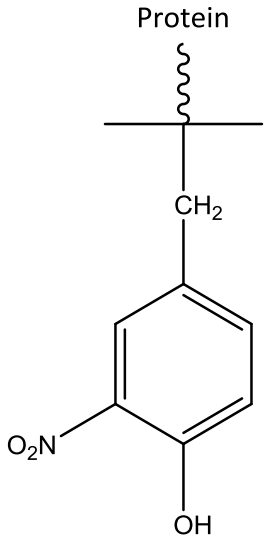


Fig.2.6 Structures for several common products of lipid peroxidation

2.3.4 Protein nitration

The derivatives of peroxynitrite, such as $\bullet NO_2$ and $\bullet CO_3$, react with proteins and produce oxidation products. Some proteins are more susceptible to oxidative damage than other proteins due to specific functional groups in their amino acids. For example, hydroxyl-containing amino acids (tyrosine) and amine-containing amino acids (histidine, arginine, proline, and lysine) are targeted by ROS/RNS and undergo nitration and carbonylation, respectively. In particular, 3-nitrotyrosine (3-NT), the end product of the nitration of a tyrosine residue, has been widely used as an index of oxidative stress and protein oxidation (Fig.2.7). This irreversible oxidative modification of proteins lead to conformational changes, thereby proteins may be aggregated or unfolded, and ultimately loss of function.



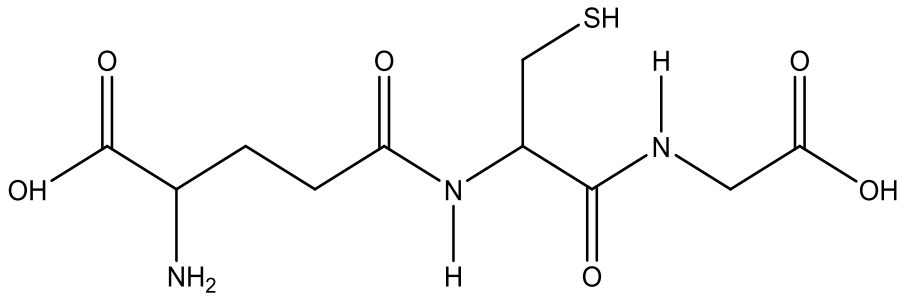
3-nitrotyrosine (3-NT)

Fig.2.7 Structure of 3-nitrotyrosine, a product of protein nitration

2.4 Glutathione

2.4.1 Overview

Glutathione (γ-glutamylcysteinylglycine), a tripeptide, is composed of glutamate (Glu), cysteine (Cys), and glycine (Gly) (Fig.2.8). The γ-peptide linkage between Glu and Cys is not easily hydrolyzed comparing to α-peptide bonds frequently found between two amino acids, thereby the stability of glutathione is enhanced.



Glutathione (GSH)

Fig.2.8 Structure of glutathione

Moreover, the thiol group (-SH) of Cys can be deprotonated and reacts with other chemical molecules, contributing to several important functions of glutathione in the brain. One of the most important functions of glutathione is to scavenge ROS/RNS as an antioxidant, especially H_2O_2. One molecule of H_2O_2 is reduced to one molecule of water while two molecules of GSH are oxidized to form one molecule of oxidized glutathione (GSSG) which is catalyzed by the enzyme, glutathione peroxidase (GPx). Besides H_2O_2, glutathione can react with hydroperoxides and ONOO- to remove them from the cell (Sies et al. 1972, Srivastava, Awasthi, and Beutler 1974, Radi et al. 1991).

The advantage of glutathione is that it can be recycled because GSSG can be easily reduced to two molecules of GSH through the glutathione reductase (GR) enzyme in the presence of NADPH and H^+ (Fig.2.9).

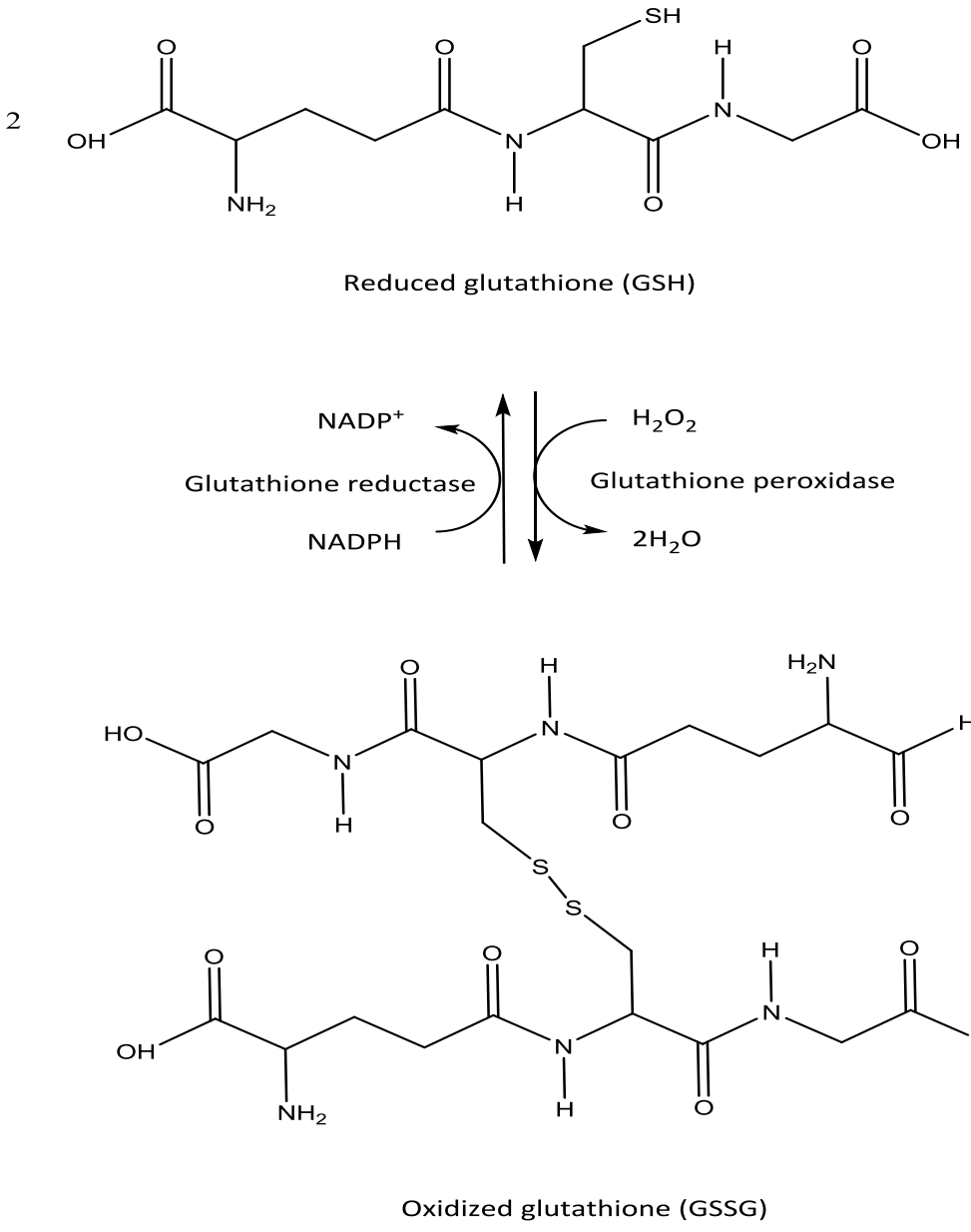


Fig.2.9 Schematic representation of intracellular glutathione recycling process

2.4.2 Synthesis

Glutathione is naturally synthesized in the cytoplasm of most cells, including neurons and astrocytes, through a two-step process. The biosynthesis of glutathione involves two ATP-dependent enzymes, gamma-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) for each step (Fig.2.10). The first step is the formation of gamma-glutamylcysteine (γ-GluCys) through a γ-peptide linkage between Glu and Cys, which is catalyzed by GCS. Upon the formation of γ-GluCys, GS catalyzes the next step to generate the final product, glutathione by adding Gly to γ-GluCys which was produced from the previous step (Snoke, Yanari, and Bloch 1953).

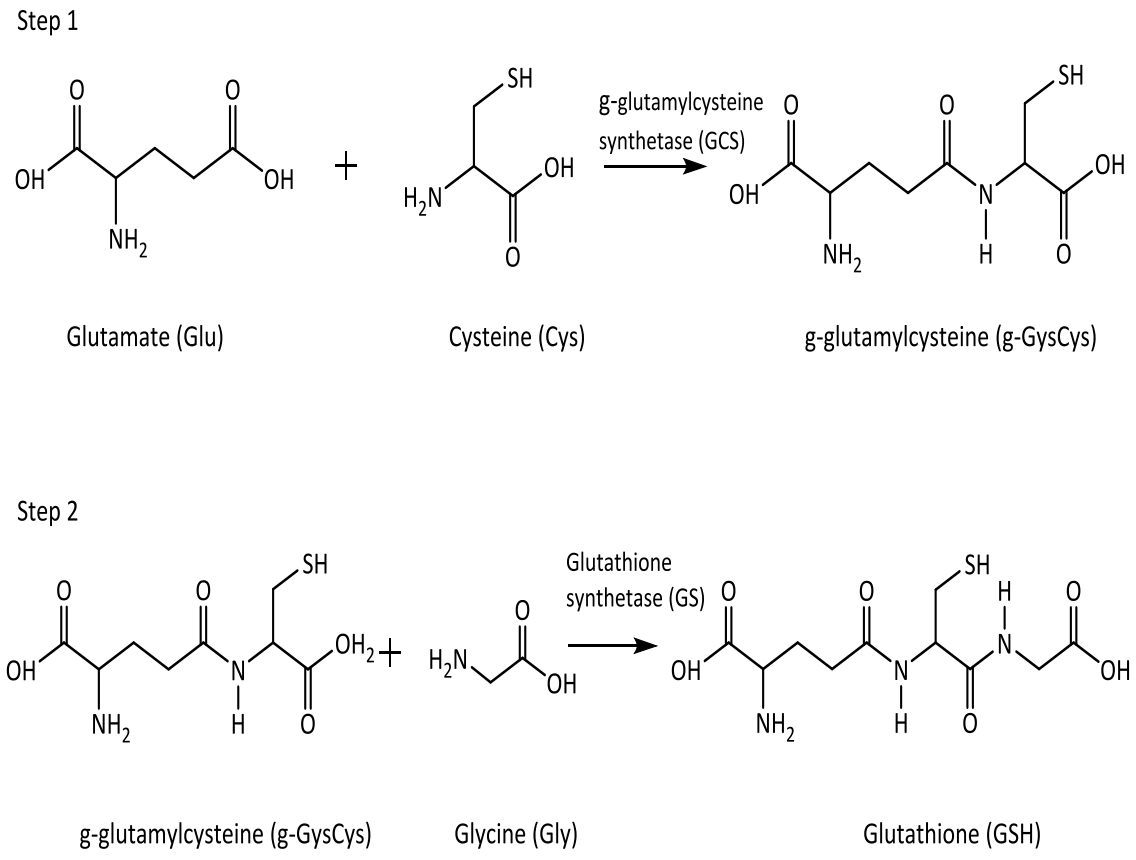


Fig.2.10 Schematic representation of intracellular glutathione synthesis

Two main factors regulate biosynthesis of glutathione. First, non-allosteric feedback inhibition prevents continuous synthesis of glutathione in which glutathione itself inhibits the first reaction that is catalyzed by GCS by competing for the Glu or/and Cys sites on GCS, thereby the formation of γ-GluCys is reduced and the levels of glutathione is subsequently decreased (Richman and Meister 1975). The availability of Cys is another factor effecting the biosynthesis of glutathione because the concentration of Cys is relatively less than those of Glu and Gly (Raps et al. 1989). Thus, Cys is the rate determining substrate, which makes Cys-involving first step be the rate determining step in glutathione synthesis.

2.4.3 Metabolism

Although glutathione is synthesized within the cells, it is also found in the extracellular space. Astrocytes, one type of glial cell, release intracellular glutathione into extracellular space, which ultimately supplies glutathione precursors to other cells in the brain, including neurons (Fig.2.11) (Sagara, Makino, and Bannai 1996). In particular, extracellular glutathione is cleaved into glutamate and cysteinylglycine by γ-glutamyltranspeptidase (γ-GT), an enzyme found on the cellular membrane of the astrocytes (Hird and Springell 1954, Meister, Tate, and Griffith 1981). Cysteinylglycine is then taken up by neurons or further hydrolyzed into cysteine and glycine by aminopeptidase N (ApN) enzyme found on the cellular membrane on the neurons (Meister 1988). Neurons take up extracellular cysteine through the excitatory amino acid transporters and utilize to synthesize intracellular glutathione (Dringen, Pfeiffer,

and Hamprecht 1999, Wang and Cynader 2000). Astrocytes also reuse extracellular cysteine by accepting as cystine through the cystine/glutamate transporter and convert it to cysteine inside the cell (Meister 1995, Anderson 1998, Bender, Reichelt, and Norenberg 2000). Therefore, the stores of extracellular glutathione secreted by astrocytes are good sources for neurons to synthesis intracellular glutathione (Sagara, Miura, and Bannai 1993).

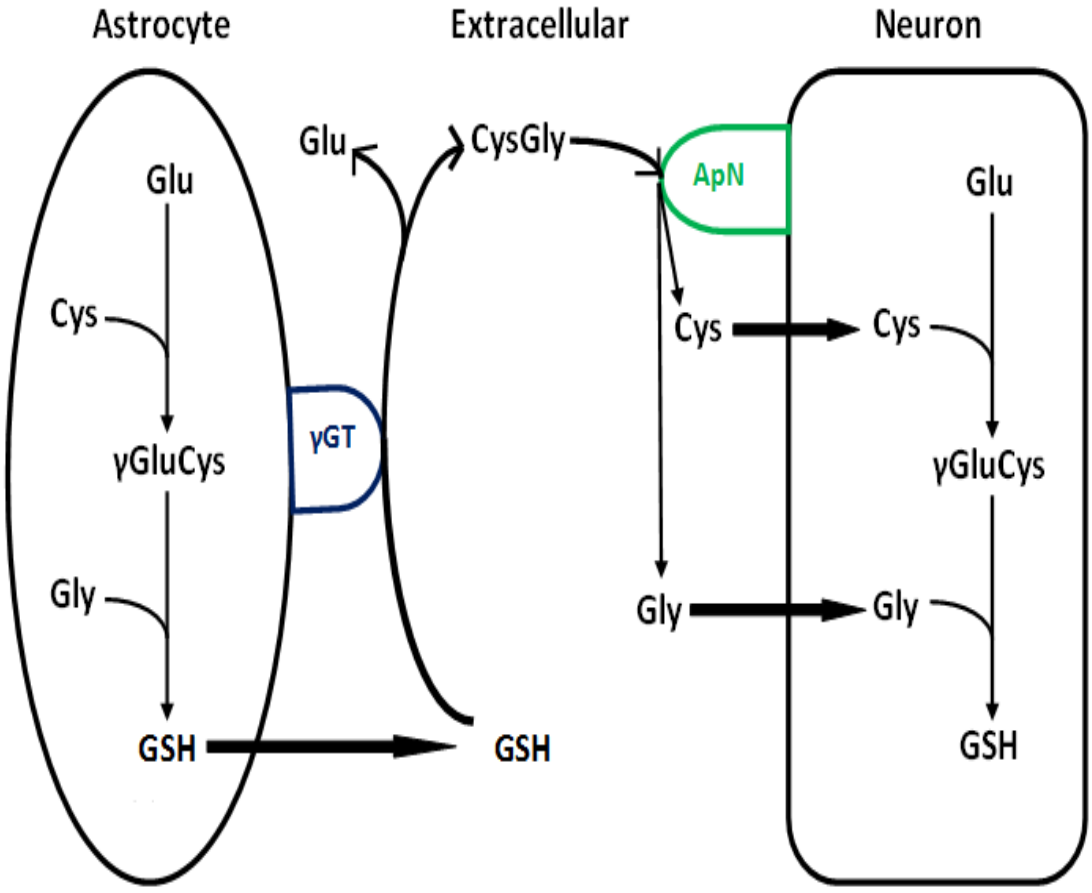


Fig.2.11 Schematic representation of glutathione recycling process. Glutamate (Glu), Cysteine (Cys), Glycine (Gly), γ-glutamylcysteine (γGluCys), glutathione (GSH), Cysteinylglycine (CysGly), γ-glutamyltranspeptidase (γGT), aminopeptidase N (ApN)

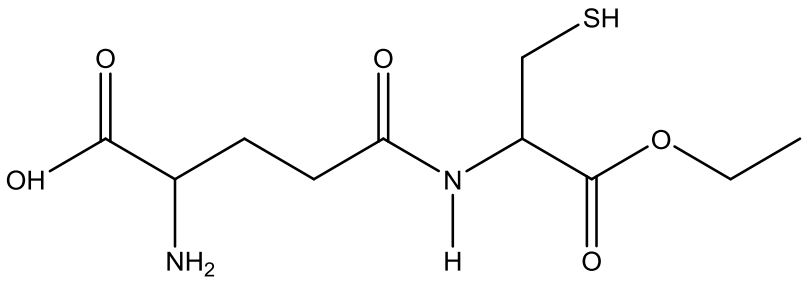
2.4.4 Up-regulation of glutathione

As an antioxidant, glutathione plays a critical role in protecting the brain against oxidative stress by maintaining redox homeostasis; however, the depletion of glutathione causes oxidative stress due to relatively high levels of ROS/RNS. Oxidative stress is a pathway of almost all neurological pathways in the brain. Hence, elevation of glutathione levels may attenuate oxidative stress and protect the cells against oxidative damage, thereby preventing cellular death. However, provision of glutathione itself is not up-regulating cellular glutathione levels, because glutathione is rapidly degraded in the gut and circulated before it reaches the target location; it also is not transported into cells. Therefore, several glutathione precursors have been investigated as an alternative way to boost cellular glutathione levels, including glutathione ethyl ester and N-Acetylcysteine which provide glutathione and cysteine, respectively. Although both can increase cellular glutathione levels, the amount is limited due to feedback inhibition. Thus, provision of γ-glutamylcysteine has been suggested as an alternative way to boost glutathione by avoiding feedback inhibition.

2.4.4.1 Gamma-glutamylcysteine ethyl ester

Gamma-glutamylcysteine ethyl ester (GCEE) is a modified form of γ-glutamylcysteine with an ester moiety, which is de-esterified by intracellular cellular esterase (Fig.2.12). The addition of ethyl ester moiety to γ-glutamylcysteine facilitates transport of GCEE across BBB as well as the plasma membrane (Anderson and Meister 1989, Anderson et al. 1985, Levy, Anderson, and Meister 1993). It has been shown that

administration of GCEE increases total brain GSH levels by circumventing feedback inhibition (Drake et al. 2002).



γ-glutamylcysteine ethyl ester (GCEE)

Fig.2.12 Structure of γ-glutamylcysteine ethyl ester

A large number of studies have demonstrated the antioxidant abilities of GSH up-regulated by GCEE to protect the brain against a variety of oxidative stress. More specifically, GCEE has been shown to protect hippocampal neurons against ONOO- induced damage, in part preventing the loss of mitochondrial functions by increasing mitochondrial GSH levels *in vivo* and *in vitro* (Drake et al. 2003). Neuronal GSH levels have been increased *in vitro* by GCEE, which in turn protects neurons against Aβ(1-42)-induced cytotoxicity, such as protein oxidation, mitochondrial dysfunction, and fragmentation of DNA, by decreasing oxidative stress (Boyd-Kimball, Sultana, Abdul, et al. 2005). Oxidative stress induced by Aβ(1-42) has been documented as the key factor causing Alzheimer's disease like symptoms were are decreased by administration of GCEE (Boyd-Kimball, Sultana, Poon, et al. 2005). The degeneration of dopaminergic neurons has been also prevented by GCEE in a model of Parkinson's disease which is

characterized by the reduction of GSH levels in selected brain regions (Chinta et al. 2006).

GCEE also has demonstrated its protective capability against oxidative stress related side effects of adriamycin, a chemotherapeutic drug used for treating solid tumors (Joshi et al. 2007, Aluise et al. 2009). Kainic acid, an excitatory neurotoxic molecule, also has been known to generate oxidative stress in the brain by disrupting ion homeostasis and stimulating production of oxidants; however, GCEE reduces oxidative stress-associated neuronal death by increasing the levels of glutathione (Turunc, Kanit, and Yalcin 2010, Yalcin et al. 2010)

Furthermore, the capability of GCEE as an antioxidant to attenuate oxidative stress in TBI has been investigated. Post-treatment with GCEE following TBI reduced levels of protein carbonyls, 3-nitrotyrosine, and nitrated proteins, all biomarkers of oxidative damage in TBI (Reed et al. 2009). Also, GCEE showed protective effects on cerebral endothelial cells in TBI models (Lok et al. 2011).

2.5 Experimental Methods

2.5.1 Controlled Cortical Impact

Human TBI is a heterogeneous disease, involving physical, physiological, behavioral, emotional, and histopathological changes. Although all these changes observed in human TBI cannot be perfectly reproduced in animal models, studying in animal models is an essential step to elucidate pathophysiological processes occurring after TBI, in order to develop new therapeutic strategies and assess the efficacy of

potential pharmaceutical therapies. Several types of TBI animal models have been investigated, such as controlled cortical impact, fluid percussion, weight drop, inertial acceleration, blast, and so forth (O'Connor, Smyth, and Gilchrist 2011). Depending on the types of techniques, advantages and disadvantages are involved. In this thesis, controlled cortical impact (CCI) was used and will be discussed.

Controlled cortical impact (CCI) has been widely used in a variety of brain injury models. CCI was initially developed to investigate experimental TBI on the midline brain in ferrets by Lighthall and later adapted in rats by Dixon (Lighthall 1988, Dixon et al. 1991). This approach uses a pneumatic impactor consisting of a small-ball to directly contact and impact the exposed intact brain. CCI has several advantages over other techniques. 1) Cortical contusions that are most commonly observed in human TBI can be induced by lateral controlled cortical impact; 2) Neurological changes that contribute to secondary injuries and chronic diseases following TBI can be observed; and 3) The velocity of an impactor can be calibrated to produce deformations with various depths and volumes, which is related to the severity of injury, such as mild, moderate, and severe. The faster the velocity of the impactor, the deeper the deformation in the brain (Dixon et al. 1991).

2.5.2 Fluoro-Jade B Staining

Fluoro-Jade B (FJB) is a common technique used for histochemical studies in brain tissue due to its ability to selectively stain degenerating neurons, including cell bodies, dendrites, axons, and axonal terminals (Schmued, Albertson, and Slikker 1997).

FJB positive neurons are observed under a fluorescence microscope and appear bright green against a dark background. FBJ has been widely used in experimental TBI models to study neurodegeneration (Anderson et al. 2005).

Fluoro-Jade (FJ) was originally developed prior to FJB by Schmued, showing advantages over other staining methods in terms of sensitivity and simplicity (Schmued, Albertson, and Slikker 1997). The results were reliable and easy to interpret. Later, FJB modified from FJ later shows greater affinity and contrast than FJ (Schmued and Hopkins 2000). FJB can be combined with other staining methods, such as immunofluorescence to detect activated glial cells, thereby the effects of activated glial on the degenerating neurons or vice versa can be investigated (Schmued, Albertson, and Slikker 1997).

The exact mechanism of how FJB can selectively stain degenerating neurons and not healthy neurons is unclear. However, it is assumed that degenerating neurons may release strongly basic molecules, thereby highly acidic FJB could possibly bind to those basic molecules (Schmued, Albertson, and Slikker 1997). Three possible fluorescent compounds that are responsible for the ability of FJB to detect degenerating neurons were discovered (Xu et al. 2005). They are 1) 5-(6'-hydroxy-3'-oxo-3H-xanthen-9'-yl)benzene-1,2H-tricarboxylic acid; 2) 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(2,4-dihydroxybenzoyl)terephthalic acid; and 3) 4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-6-(2,4-dihydroxybenzoyl)isophthalic acid.

2.5.3 Tert-butyl hydroperoxide

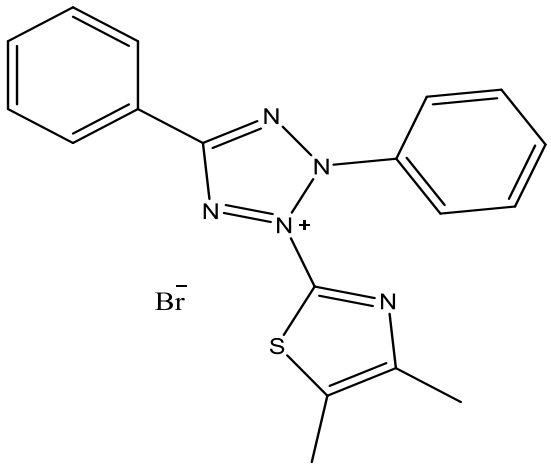
Tert-butyl hydroperoxide (tBHP) is an oxidizing agent, which is converted into hydroperoxides, such as t-butylperoxy radicals (t-BuOO•) and t-butox radicals (t-BtO•) by endogenous iron once it is transported into the cell (Cadenas and Sies 1982, Abe and Saito 1998). It has been widely used over H_2O_2 to induce oxidative stress and assess the roles of endogenous GSH or exogenous GSH in a variety of *in vitro* studies because addition of H_2O_2 makes it difficult to exclude the role of catalase in removing H_2O_2. Instead, tBHP destroys catalase, which allows it assess the roles of GSH because GSH can react with not only H_2O_2, but also organic hydroperoxides (Pichorner, Jessner, and Ebermann 1993, Sies et al. 1972, Awasthi et al. 1997). It has also been found that toxicity of tBHP can be attenuated by GPx with glutathione, but not by superoxide dismutase nor catalase (Abe and Saito 1998).

2.5.4 MTT assay

The MTT assay is a colorimetric assay for determining cell viability based on their ability to reduce MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) into formazan crystals (Fig2.13). Since the MTT assay was originally developed by Mossman to quantify survival cells, it has been successfully applied to the determination of cytotoxicity in compounds as well (Mosmann 1983). The body consists of a variety of cell types, and each cell type has a different resistance to the same compound. One specific cell type can show various viabilities to different types of compounds. Thus, the MTT assay is an essential step in *in vitro* studies for establishing the proper

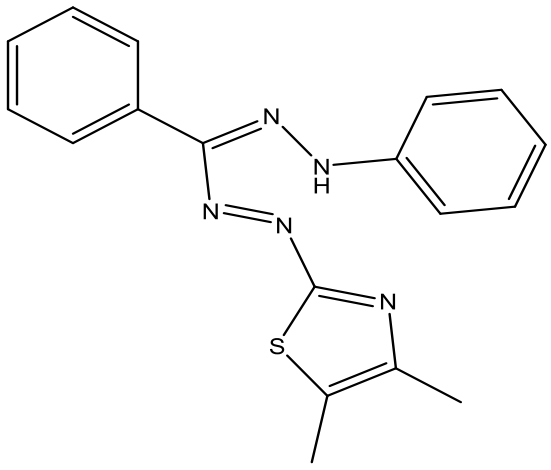
concentrations of the compound of interest and incubation times before evaluating the efficacy of the potential therapies so that the results can be independent on the numbers of living cells.

MTT is initially colorless and water soluble. Once it is reduced by mitochondrial esterase inside the cells, formazan crystal products, become intensely purple colored (Fig2.13). The concentrations of formazan are measured by a microplate absorbance reader. Since the number of active mitochondria is related to the number of living cells, the concentration of formazan is proportional to the number of viable cells. The disadvantage of MTT assay is that formazan is insoluble. Thus, dissolving formazan in dimethyl sulfoxide or some other solution is a crucial step, which has been suggested as the major technical problem because of the efficacy of solution. Despite this disadvantage, the results of the MTT assay are more sensitive and reliable than other similar staining techniques.



3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT)

Mitochondrial reductase



Formazan

Fig.2.13 Reaction of MTT assay. Mitochondrial reductase in living cells reduces MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) and forms formazan.

2.5.5 DCF assay

The DCF assay has been widely used as a fluorometric assay for measuring intracellular ROS/RNS formations (Wardman 2008). Since the DCF assay is based on the conversion of non-fluorescent to highly fluorescent substances, an increase in fluorescence is measured instead of a decrease in fluorescence, which can diminish estimations. Also, the DCF assay itself is very sensitive to changes in the redox state, easy to use, requires a small amount of sample, and is inexpensive.

DCFDA (2',7'-Dichlorofluorescein diacetate) was originally developed to quantify H_2O_2 and hydroperoxides in the cell (Keston and Brandt 1965, Brandt and Keston 1965). DCFDA, the non-fluorescent compound, is permeable to the cell membrane. Upon the influx of DCFDA into the cells, the two ester bonds of DCFDA are hydrolyzed by cellular esterase, producing non-fluorescent DCFH (Fig.2.14). Since DCFH is relatively polar and has poor permeability to cross the cell membrane, DCFH is not effused extracellularly, but instead is accumulated within the cells for a few hours. DCFH is then oxidized into fluorescent DCF by various ROS/RNS and oxidizing enzymes (Fig.2.14). For example, $\bullet OH$, $\bullet NO$, $\bullet NO_2$, and $\bullet CO_3$ non-enzymatically oxidize DCFH, while xanthine oxidase and hypoxanthine oxidase enzymatically oxidize DCFH (Zhu et al. 1994, Gunasekar et al. 1995). Thus, the level of fluorescence measured by a fluorescence microplate reader at the end of a kinetic run of the assay is a useful indicator of not just ROS levels, but the overall oxidative stress state in the cells (Wang and Joseph 1999, LeBel, Ischiropoulos,

and Bondy 1992). In other words, the DCF assay can be applied to evaluate the efficacy of antioxidants against oxidative stress in the cells (Wang and Joseph 1999).

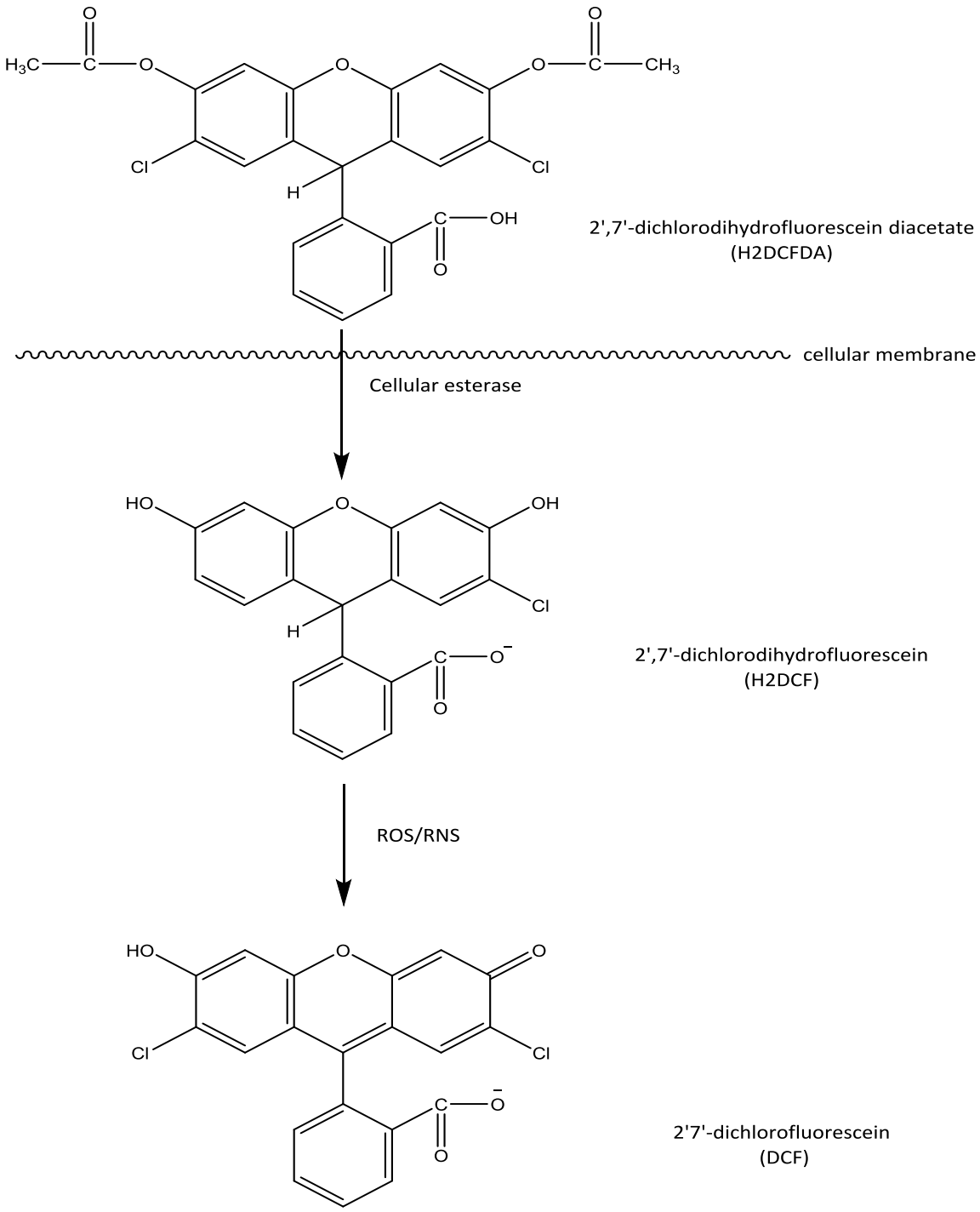


Fig.2.14 Reaction of DCF assay. Non fluorescent H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) crosses the cellular membrane. H2DCFDA is then hydrolyzed into H2DCF (2',7'-dichlorodihydrofluorescein) by cellular esterase. H2DCF is then converted into fluorescent DCF (2',7'-dichlorofluorescein) by ROS/RNS.

CHAPTER 3

NEUROPROTECTIVE EFFECTS OF GAMMA-GLUTAMYLCYSTEINE ETHYL ESTER ON CONTROLLED CORTICAL IMPACT-INDUCED MODERATE TRAUMATIC BRAIN INJURY

3.1 Overview

Traumatic brain injury (TBI) is a risk factor for neurodegeneration. Oxidative stress is one of the underlying causes leading to neurodegeneration in TBI. Glutathione (GSH) is the most abundant endogenous antioxidant in the brain and plays an important role in preventing oxidative stress by scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS). Biosynthesis of glutathione is regulated by non-allosteric feedback inhibition, thereby elevated ROS/RNS levels by TBI exceeds the capability of brain glutathione levels. Gamma-glutamylcysteine ethyl ester (GCEE) is a glutathione precursor. Administration of GCEE *in vivo* and *in vitro* has been shown to up-regulate cellular glutathione levels in the brain by circumventing feedback inhibition, and up-regulated GSH decreases oxidative stress and oxidative damage. In this study, we investigated the protective role of GCEE against neurodegeneration in traumatic brain injury. Controlled cortical impact (CCI) was performed on male Wistar rats to simulate a moderate traumatic brain injury. Fluoro-Jade B (FJB) staining was adapted to detect degenerating neurons on brain tissue. The number of FJB-positive neurons was measured for quantitative analysis. Administration of GCEE post TBI by intraperitoneal (i.p) injection decreased the total number of FJB-positive neurons in the brain compared to untreated controls. These results suggest that GCEE protects neurons against oxidative stress-induced neurodegeneration in traumatic brain injury. The

neuroprotective efficacy of GCEE as a potential therapeutic agent may contribute to the prevention of delayed neurological pathologies in traumatic brain injury.

3.2 Introduction

Neurons are distributed throughout the brain and are responsible for unique functions of the brain. Neurodegeneration is the progressive loss of neuronal structures and functions, which is a pathway of almost all neurological disorders. In the brain, oxidative stress is one of the leading causes of neurodegeneration, attributing to many neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Lee et al. 2010, Jomova et al. 2010). Traumatic brain injury is an unpredictable sudden accident, accompanying with irreversible damage to the brain. This phenomenon involves primary and secondary injuries. At the moment of an incident, the external physical impact on the brain initially generates primary injury, such as swelling and shearing of neurons as well as the changes in cellular and molecular pathways. Subsequently, primary injury initiates immediate or delayed secondary injury, including the breakdown of the blood brain barrier (BBB), the initiation of excitotoxicity, the accumulation of extracellular glutamate, the disruption of intracellular ion homeostasis, and the overproduction of toxic radicals, resulting in oxidative stress (Giza and Hovda 2001, Barkhoudarian, Hovda, and Giza 2011, Freire 2012).

The brain has the ability to combat excess levels of reactive oxygen and nitrogen species, using glutathione, which is a tripeptide containing a thiol (-SH) group and highly abundant in brain. However, the depletion of glutathione has been well documented in

a variety of TBI models. Therefore, it is highly required to boost cellular glutathione levels post brain injury to protect neurons against oxidative stress and it has been shown that administration of GCEE up-regulates glutathione levels in the brain, including neuronal cells and neuronal mitochondria by avoiding feedback inhibition (Drake et al. 2002, Drake et al. 2003). Such up-regulated glutathione by GCEE has shown antioxidant ability to combat oxidative damage induced by a variety of oxidizing agents, including Aβ(1-42), adriamycin, MPTP, and ONOO- (Boyd-Kimball, Sultana, Abdul, et al. 2005, Boyd-Kimball, Sultana, Poon, et al. 2005, Joshi et al. 2007, Chinta et al. 2006, Drake et al. 2002). Furthermore, the antioxidant properties of GCEE in TBI models have been shown by others (Reed et al. 2009, Lok et al. 2011).

In this study, we investigated whether administration of GCEE post-traumatic brain injury in experimental rats could prevent neurodegeneration due to its antioxidant ability to decrease oxidative damage as a precursor of glutathione in the brain. For quantitative analysis, Fluoro-Jade B (FJB) was selected to stain degenerating neurons and changes in the number of degenerating neurons were measured (Schmued and Hopkins 2000).

3.3 Materials and Procedures

3.3.1 Chemicals and Materials

All chemicals and gel-coated slides used for FJB staining were provided by Dr. Bardgett at Northern Kentucky University. GCEE was purchased from Bachem (Torrance, CA).

3.3.2 Animal Surgical Procedures and Treatments

For this study, sixteen Wistar adult male rats (Harlan Laboratories, Indianapolis, IN, USA) were used whose average weight was approximately 215g. Animals were divided into three groups (Sham, Saline, GCEE). A naïve rat, in which no craniotomy, injury or treatment, was used as well. The sham group was treated as the control group, so neither injury was induced nor treatment was administrated; however, a craniotomy was performed. Both Saline and GCEE groups were subjected to a moderate TBI (1.5 mm depth injury). Saline groups were treated with saline (150 mg/kg) instead of GCEE, while GCEE groups were treated given 150 mg/kg GCEE for treatment. Saline and GCEE groups were further divided into multiple groups (Saline 30, Saline 60, GCEE 30, and GCEE 60) depending on the time delay of treatment. Saline or GCEE was administrated either 30 min or 60 min after TBI was performed. Each group had three rats (n= 3) except the naïve group which had one rat.

All rats (except for the naïve rat) were completely anesthetized with 3.0 % isoflurane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Surgery and injury were completed as previously described (Sullivan et al. 2002). First, a craniotomy was performed in which the hair on the scalp was shaved, the skin retracted, and the skull cap was gently removed. Controlled cortical impact (CCI) was then induced onto the exposed brain to produce cortical contusions by an impactor containing a metal ball which was calibrated to make a 1.5 mm deep deformation and simulate moderate TBI as previously described, except for sham groups (Sullivan et al.

2002). After craniotomy and surgery, a 4 mm disk made from dental cement was placed over the craniotomy site and adhered to the skull with cyanoacrylate. The skull cap was placed back and the skin was sutured. The rats were placed onto a heating pad until they regained consciousness and started to move normally. Saline or 150 mg/kg GCEE was administrated through i.p. injection 30 min or 60 min after CCI for non-treatment or treatment groups, respectively. The rats kept alive for 24 hour. For histochemical analysis, the rats were sacrificed after 24 hour by overdose of pentobartital and perfusion was performed. The whole brains were rapidly removed after decapitation and stored in 10% formalin until use. Depending on whether CCI was performed or not, the difference was observed on the surface of the brains. On the surface of all CCI-induced brains cortical contusions appeared with red color regardless of the administration of GCEE or saline, whereas no cortical contusions were shown on the brain in naïve and sham groups (Fig.3.1).

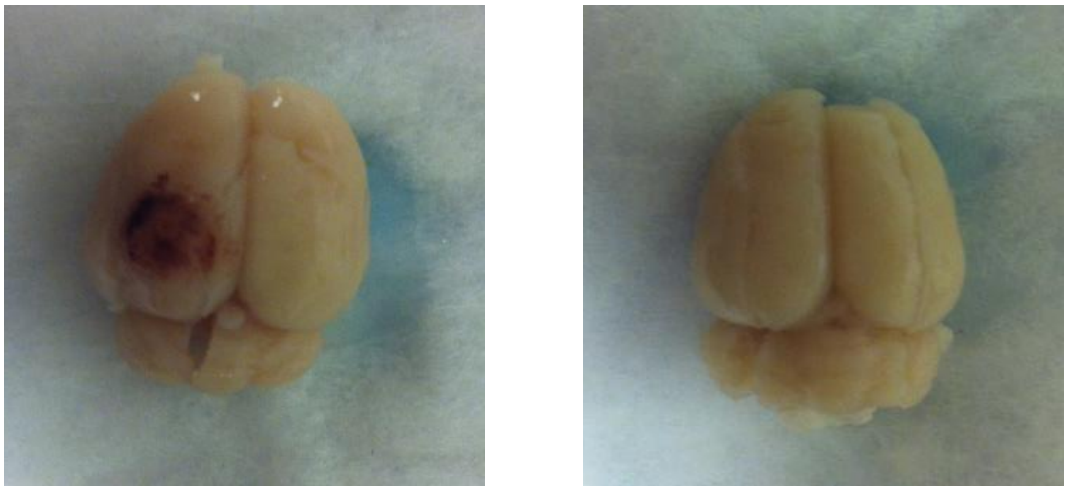


Fig.3.1 Images of rat brains with CCI (left) and without CCI (right). Cortical contusion was not produced when CCI was not performed, while a 1.5mm deep cortical contusion was produced when CCI was performed. The red color shows the injury site for CCI treated animals.

3.3.3 Tissue preparation

To prepare brain tissues, each brain sample was completely frozen and sectioned into 40 microns thickness at -25°C, using a cryostat (Leica CM 1850). Every other brain section was collected into a 24-well plate containing 0.1M potassium phosphate buffer solution (KPBS) + 0.05% sodium azide. The sections were mounted on gelatin coated slides and dried overnight at room temperature for FJB staining.

3.3.4 Fluoro-Jade B staining

The slides were first immersed into a solution containing 100% ethanol for 3 min, next transferred into a solution containing 75% ethanol for 1 min, and then rinsed with distilled water for 1 min. The slides were then placed into a solution containing 0.06% potassium permanganate ($KMnO_4$) for 15 min on a shaker and rinsed with distilled water for 1 min. The sections were stained with a solution containing 0.001% FJB (Histochem Inc., Jefferson, AR) for 30 min by gently shaking on a shaker in a dark room. The slides were washed with distilled water for 1 min three times and dried overnight at room temperature in a completely dark room. The slides were then immersed into 100 % xylene for 2 min three times and coverslipped with DPX mounting media.

3.3.5 Statistical Analysis

Experiments were performed with three animals per group (n = 3) except naïve (n=1). All data were analyzed using ANOVA and expressed as mean ± SD. Statistical significance was set at $p < 0.05$.

3.4 Results

3.4.1 Craniotomy injury causes neurodegeneration

Craniotomy is the mandatory step before performing CCI, which may possibly affect neurons somehow because the brain is exposed to molecular oxygen while a small piece of skull is removed. Hence, craniotomy was also performed on control groups, including sham, but not naïve, although the skull was immediately placed back and the wound was sutured. The difference in the number of FJB-positive neurons was actually observed between sham and naïve groups. While FJB-positive neurons did not appear on the brain tissues of the naïve group, FJB-positive neurons were observed on the cortex of brain tissue in sham groups although CCI was not performed on sham groups (Fig.3.2). This result suggests that the craniotomy process itself can induce neurodegeneration without CCI. Thus, sham group was selected as control group over naïve group for following analysis.

3.4.2 GCEE decreases neurodegeneration in controlled cortical impact-induced rats

After craniotomy, CCI was performed to induce moderate TBI on GCEE and Saline treated groups. GCEE (150 mg/kg) was subsequently administrated 30 min or 60 min after CCI, while for non-treatment groups, saline was administrated 30min or 60 min after CCI. Prepared brain tissues were stained with FJB. The brain tissues obtained from non-treatment groups were more deformed compared to those of GCEE-treated groups. In addition, FJB-positive neurons were more widely distributed on the brain tissues in non-treatment groups compared to GCEE-treated groups (Fig.3.3). The effects of GCEE

on degenerating neurons were measured in different treatment groups by quantifying the number of fluorescent neurons stained by FJB. Injured groups had increased numbers of FBJ-positive neurons in the cortex compared to sham groups, whereas GCEE treated rats after brain injury decreased in the number of FJB-positive neurons than that of non-treated rats (Table 3.1).

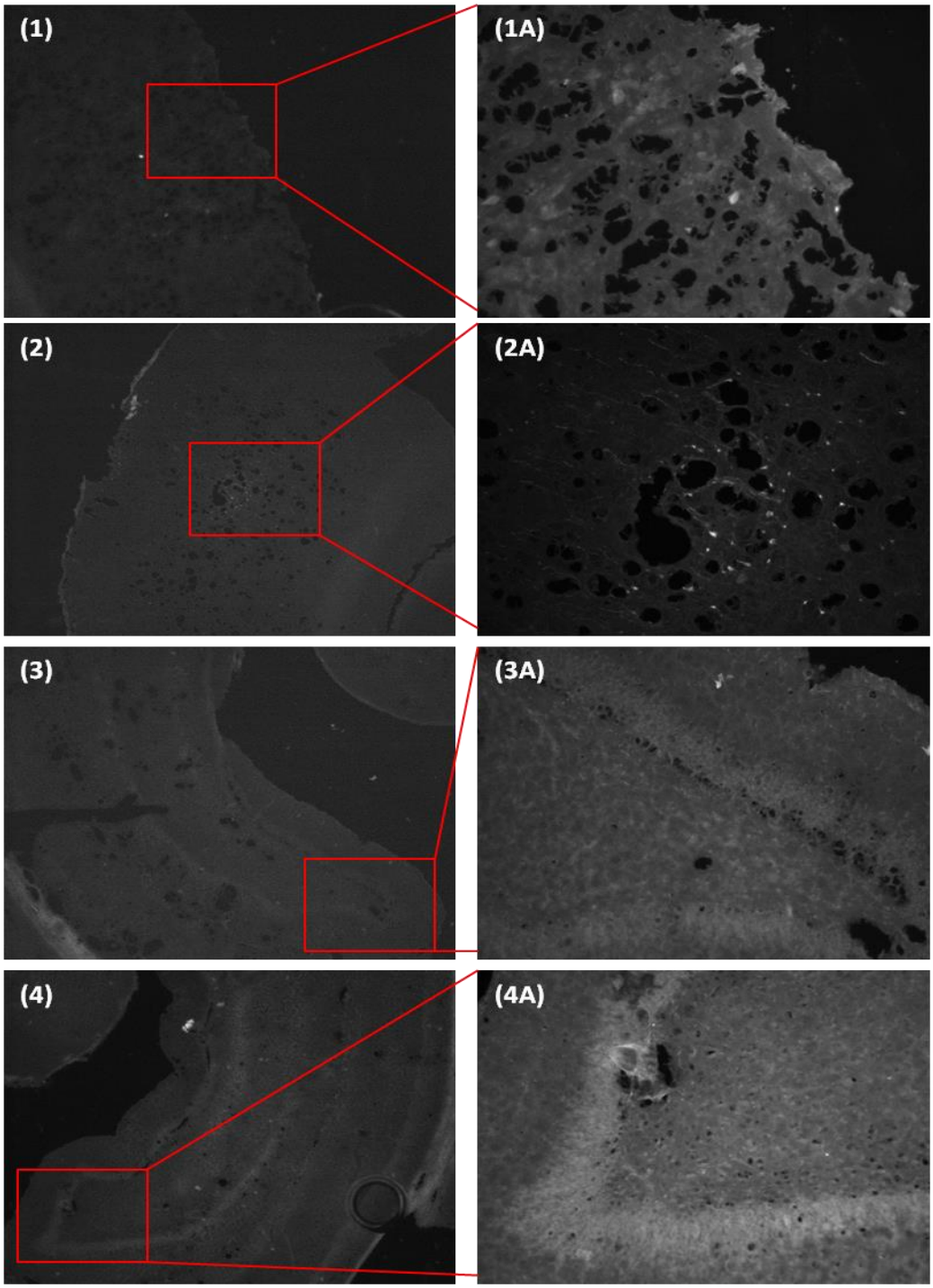


Fig.3.2 Images of FJB stained cortex and hippocampus from naïve and sham. Cortex of naïve ((1), (1A)), cortex of sham ((2), (2A)), hippocampus of naïve ((3), (3A)), and hippocampus of sham ((4), (4A)). Images ((1), (2), (3), (4)) are captured at 2.5X magnification and images ((1A), (2A), (3A), (4A)) are captured at 10X magnification. Craniotomy-induced rats showed FBJ-positive neurons in the cortex.

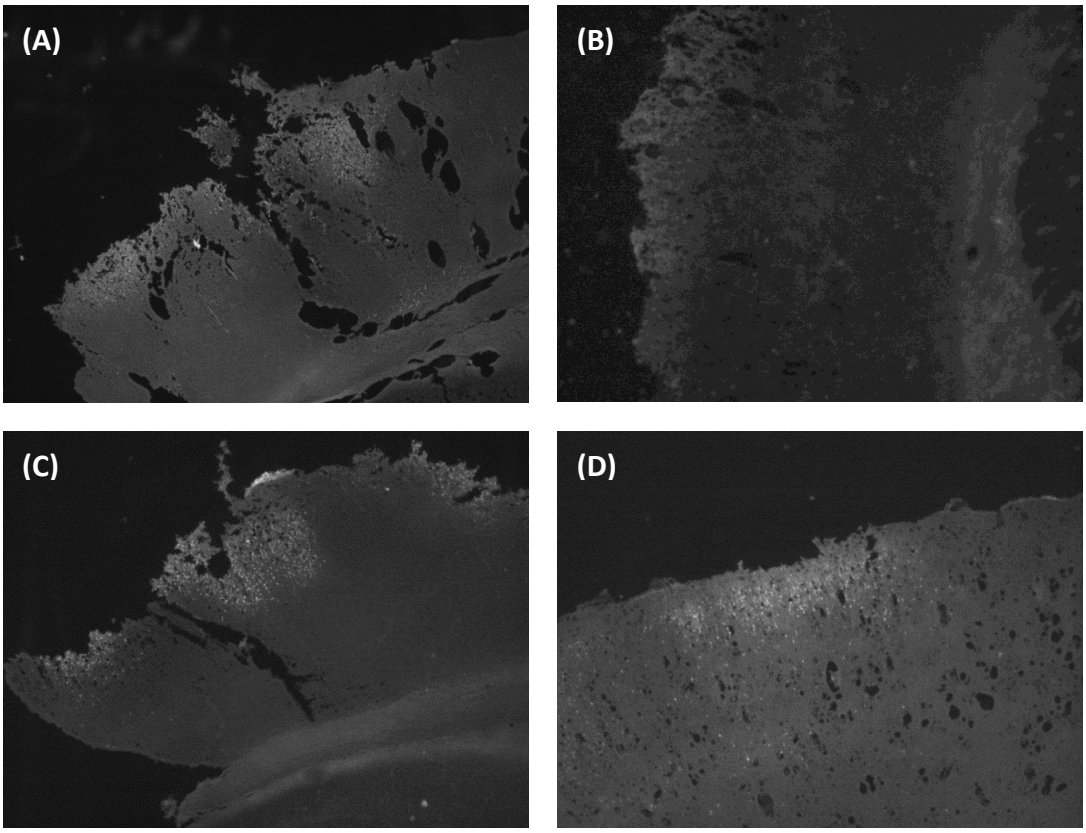


Fig.3.3 Images of the brain tissues stained by FJB after CCI and treatments. Saline 30 (A), GCEE 30 (B), Saline 60 (C), and GCEE 60 (D). Images are captured at 10X magnification.

Table 3.1 Average number of degenerating neurons stained by FJB on rat brain tissues. GCEE (150 mg/kg) or saline was administrated to rats 30 min or 60 min after controlled cortical impact. The quantity of FJB positive neurons appeared on the images of the brain tissues captured at 2.5X magnification was measured within a rectangle 0.1 mm x 0.2 mm (n=3). Values are expressed as mean ± SD.

Groups	Average number of FJB-positive neurons (in 0.02 mm^2)
Sham	17 ± 21
Saline 30	22 ± 7
GCEE 30	18 ± 2
Saline 60	27 ± 5
GCEE 60	20 ± 5

3.5 Discussion

Traumatic brain injury is a risk factor for neurodegeneration (Sato et al. 2001, Anderson et al. 2005, Hall et al. 2008, Hall et al. 2005). Neurodegeneration contributes to neurodegenerative disorders, such as Alzheimer's diseases following TBI (Van Den Heuvel, Thornton, and Vink 2007, Sivanandam and Thakur 2012). Oxidative stress has been suggested as the underlying cause of neurodegeneration in TBI by overproducing reactive oxygen and nitrogen species (Awasthi et al. 1997, Tyurin et al. 2000, Kontos and Povlishock 1986). Administration of GCEE post-TBI increases brain glutathione levels and decreases the markers of oxidative damage (Lai et al. 2008, Reed et al. 2009).

In the present study, the total number of degenerating neurons was higher in TBI rats compared to non-injury rats (Table 3.1), proving that TBI causes neurodegeneration. It also has been proven in a variety of experimental TBI models by histochemical studies although the concentrations, contributions, and time of appearance are different depending on the types of injury-inducing methods and severity of injury (Anderson et al. 2005). In the fluid percussion method, degenerating neurons are initially observed in cortex as early as 3 h post-TBI and the maximum numbers are measured 24 h after an injury in TRBI rats (Sato et al. 2001). In CCI induced-TBI mice and rats, degenerating neurons are immediately seen in the ipsilateral cortex around the site of impact, and the number of degenerating neurons has a time dependent increase until 48 h post-injury (Hall et al. 2005, Hall et al. 2008). However, in our study, administration of GCEE after CCI decreased the number of degenerating

neurons compared to non-treatment rats, showing that treatment with GCEE post-TBI prevented neurodegeneration and early treatment was more effective. This could be due to the antioxidant role of gamma-glutamylcysteine ethyl ester in TBI as previously investigated in experimental TBI models by other groups. Administration of GCEE in CCI-induced mice preserved total brain glutathione levels which were significantly decreased in non-treatment mice as well as markers of oxidative stress-induced autophagy were significantly decreased (Lai et al. 2008). Similarly, administration of GCEE in TBI rats significantly decreased the levels of 3-NT and 4-HNE which were increased in non-treatment groups as the markers of oxidative damage by peroxynitrite (Reed et al. 2009). Similarly, GCEE administration in CCI-induced mice decreased acute blood-brain barrier (BBB) permeability by protecting endothelial cells against oxidative stress (Lok et al. 2011). Taken together, the results from this study suggest that early treatment with GCEE post-TBI can protect neurons against oxidative stress-related degeneration in traumatic brain injury. Neurons contribute to the critical functions of the brain, including learning, memory, emotions, and perception. Thus, protecting neurons is important to prevent the subsequent neurological changes and pathologies.

In conclusion, this study has shown that the GCEE's antioxidant capabilities protect neurons against oxidative stress-related degeneration in TBI. This neuroprotective efficacy of GCEE may contribute to the prevention of delayed neurological disabilities in traumatic brain injury as a potential therapeutic agent.

CHAPTER 4

GAMMA-GLUTAMYLCYSTEINE ETHYL ESTER PROTECTS ASTROCYTES AGAINST TERT-BUTYL HYDROPEROXIDE MEDIATED-OXIDATIVE STRESS AND CYTOXICITY

4.1 Overview

Glutathione is a naturally occurring antioxidant in the brain. The capability of glutathione to scavenge intracellular reactive oxygen and nitrogen species protects the cells against oxidative damage during normal conditions. However, cells are susceptible to oxidative stress during brain injury because oxidants are immediately overproduced and the availability of glutathione is reduced. Hence, increasing the level of glutathione is essential to decrease oxidative stress and protect cells. Gamma-glutamylcysteine ethyl ester has been known to increase neuronal and mitochondrial glutathione in the brain as a precursor of glutathione. In this study, we investigated whether GCEE could up-regulate glutathione in astrocytes specifically, and if such upregulation could attenuate oxidative stress in astrocytes and further protect astrocytes against oxidative stress-induced cytotoxicity. Oxidative stress was induced to rat primary cortical astrocytes by adding an oxidizing agent, tert-butyl hydroperoxide. The status of oxidative stress was measured by adapting the dichlorofluorescein assay. Treatment of astrocytes with GCEE significantly reduced tBHP-induced oxidative stress. GCEE treatment also prevented a decrease in cell viability in a dose dependent manner. These results suggest that GCEE can protect astrocytes against oxidative stress-induced cytotoxicity, which may be due to its ability to increase glutathione. These beneficial effects of GCEE on astrocytes may contribute to its role as a neuroprotective agent in traumatic brain injury.

4.2 Introduction

Overproduction of cellular reactive oxygen and nitrogen species depletes antioxidant levels. The imbalance between the levels of oxidants and antioxidants results in oxidative stress. Oxidative stress is accompanied with oxidative damage because unstable free radicals rapidly react with cellular components, such as cellular and mitochondrial membranes, proteins, and nucleic acids, which alter their conformations and functions. As a result, the permeability of the cellular membrane and BBB is increased, proteins are aggregated or unfolded, and DNA is mutated, which consequently contributes to cellular death.

Astrocytes are the most abundant glial cells in the brain, outnumbering the neurons in the brain. The distribution of brain glutathione is uneven, but localized in non-neuronal cells (Slivka, Mytilineou, and Cohen 1987). In particular, much higher concentrations of intracellular glutathione has been found in astrocytes (3.8 mM) than in neurons (2.5mM) (Raps et al. 1989, Rice and Russo-Menna 1998). Thus, the majority of glutathione in the brain is related to astrocytes. In addition, the substrates required for biosynthesis of neuronal glutathione, such as cysteine, are supplied by astrocytes through the γ-glutamyl cycle in which astrocytes release intracellular glutathione into extracellular space (Walz 1989, Sagara, Makino, and Bannai 1996). Such released glutathione is cleaved into glutamate and the dipeptide, cysteinylglycine, by γ-glutamyl transpeptidase, an enzyme found on the membrane of astrocytes. Cysteinylglycine is subsequently cleaved into cysteine and glycine by aminopeptidase N, which is found on

plasma membrane of neurons (Meister, Tate, and Griffith 1981, Meister 1995). However, astrocytes are targeted to attacks of reactive oxygen and nitrogen species during traumatic brain injury. Subsequently, astrocytic glutathione levels are diminished and oxidative stress prompts cellular death through irreversible oxidative damage.

In this study, we investigated whether GCEE could increase glutathione in astrocytes and protect astrocytes against oxidative stress as GSH precursor. Oxidative stress was induced in rat primary cortical astrocytes by adding tert-butyl hydroperoxide, an oxidizing agent. tBHP has been widely used as an oxidizing agent in various *in vitro* models because tBHP is stable and has a greater membrane permeability in astrocytes (Abe and Saito 1998). The levels of intracellular oxidative stress were measured by dichlorofluorescein assay which is based on the conversion of non-fluorescent compounds into fluorescent compounds through reaction of intracellular reactive oxygen and nitrogen species (Keston and Brandt 1965, Wang and Joseph 1999). The toxicity of substances and cell viabilities were measured by MTT assay based on the formation of formazan from MTT through the activation of mitochondrial cellular enzymes in living cells (Mosmann 1983).

4.3 Materials and Methods

4.3.1 Materials

All reagents required for culturing astrocytes, such as Dulbecco's Modified Eagle Medium (DMEM; high glucose), Fetal Bovine Serum (FBS), and Accutase were purchased from Invitrogen (Carlsbad, CA, USA). All reagents required for experiments, such as

Dulbecco's Phosphate Buffered Saline (D-PBS; without Ca^{2+}, Mg^{2+}, and phenol red), Hank's Balanced Salt Solution (HBSS), and MTT assay kit were also purchased from Invitrogen (Carlsbad, CA, USA). Both H2DCFDA (2,7-dichlorodihydrofluorescein diacetate) and tBHP (tert-butyl hydroperoxide) were purchased from Acros Organics. GCEE was purchased from Bachem (Torrance, CA, USA).

4.3.2 Cell Culture

Rat primary cortical astrocytes purchased from Invitrogen (Carlsbad, CA, USA), were isolated from the cortex of Sprague-Dawley at day E19 of gestation. Complete medium containing the basic nutrients as well as the supplement nutrients required for cellular growth was prepared by adding 15 % FBS to DMEM. Complete medium was always pre-warmed to 37^{o}C before being used. Astrocyte cultures were prepared according to the protocol obtained from Invitrogen (http://www.lifetechnologies.com/order/catalog/product/N7745100?ICID). Briefly, cells were initially plated on T-25 cm^2 flask at a density of 1 x 10^4 cells/cm^2 with complete medium and incubated at 37^{o}C, 5% CO_2, and 90% humidity. When the culture was 100% confluent, cells were expanded and transferred onto an uncoated tissue-culture treated 24-well plate at a seeding density of 2x10^4 cells/cm^2 with complete medium and incubated at 37^{o}C, 5% CO_2, and 90% humidity until the culture was 100% confluent.

4.3.3 Measurement of cell viability

The MTT assay was performed in triplicate on 24-well plates to determine the cytotoxicity levels and incubation times of GCEE and tBHP as well as the cell viability

under tBHP-mediated cytotoxic conditions in the presence of GCEE by measuring the number of living cells. The reagents required for MTT assay were prepared according to the protocol obtained from Invitrogen (https://www.lifetechnologies.com/order/catalog/product/V13154?ICID). Briefly, a 12 mM MTT stock solution was prepared in PBS and a SDS solution was prepared in 0.01 M HCl. To determine the cytotoxicity of GCEE, astrocytes were treated with vehicle, 750 µM GCEE, or 900 µM GCEE in serum-free DMEM and incubated for 30 min or 60 min at 37^{o}C, 5% CO_2, and 90% humidity. To determine cytotoxicity of tBHP, astrocytes were treated with vehicle, 100 µM tBHP, or 200 µM tBHP in serum-free DMEM and incubated for 30 min or 60 min at 37^{o}C, 5% CO_2, and 90% humidity. To determine the effects of GCEE on decreased cell viability due to tBHP-mediated cytotoxicity, astrocytes were treated with 0 µM, 750 µM or 900 µM GCEE for 60 min prior to exposure of 200 µM tBHP for 60 min at 37^{o}C, 5% CO_2, and 90% humidity. Upon the completion of each experiment, the medium containing different conditions of treatment was removed. The cells were then loaded with 250 µl of HBSS and 25 µl of 12 mM MTT, which was then incubated at 37^{o}C, 5% CO_2, and 90% humidity for 4 hours. After the 4 hour incubation, 250 µl of the SDS-HCl solution was added to each well and incubated for an additional 4 hours at 37^{o}C, 5% CO_2, and 90% humidity. The formazan levels were measured by reading the absorbance at 570 nm, using a microplate reader (Epoch, BioTeck, Winooski, VT, USA). The levels of formazan were directly proportional to the number of living cells.

4.3.4 Measurement of intracellular ROS formation

The DCF assay was used to determine the ability GCEE to attenuate tBHP-mediated intracellular oxidative stress in primary cortical astrocytes by measuring intracellular ROS levels on a 24-well plate. The DCF assay was performed in triplicate for each group. Before experiments, HBSS and DPBS were pre-warmed to 37^{o}C. Once cells were 100 % confluent, the experiment was performed. Spent complete medium was removed. The cells were washed with 500 µl 1X DPBS per well and used 1X DPBS was rapidly removed from the wells. The cells were then incubated with 500 µl HBSS containing 10 µM H2DCFDA per well for 30 min at 37^{o}C, 5% CO_2, and 90% humidity. After 30 min, H2DCFDA-containing HBSS was removed and the cells were washed with 500 µl 1X DPBS per well. After removing 1X DPBS, the cells were loaded with 500 µl HBSS alone or pre-treated with 500 µl HBSS containing 750 µM GCEE per well for 1 h at 37^{o}C, 5% CO_2, and 90% humidity. After 1 hour, HBSS and GCEE-containing HBSS was removed from all wells and the cells were washed with 500 µl 1X DPBS per well. After removing 1X DPBS, 500 µl HBSS alone or 500 µl HBSS containing 200 µM tBHP was added to the cells in each well and incubated for 30 min or 60 min at 37^{o}C, 5% CO_2, and 90% humidity. After addition of 200 µM tBHP (at 30 or 60 minutes), the levels of fluorescence were measured by using a fluorescence plate reader (GENios, TECAN, Männedorf, Switzerland) with at excitation at 488 nm and emission at 525 nm. The levels of fluorescence were directly related to the status of intracellular oxidative stress.

4.3.5 Statistical Analysis

All data were analyzed using ANOVA and expressed as mean ± SD. Statistical significance was set at $p < 0.05$.

4.4 Results

4.4.1 Cytotoxicity of GCEE on cortical astrocytes

The cytotoxicity of GCEE on cortical astrocytes was determined by MTT assay. Rat primary cortical astrocytes were incubated with different concentrations of GCEE (0, 750, and 900 µM) for 30 min or 60 min, and the number of living cells in the different groups was measured. Treatment with 750 µM GCEE for 30 min increased the viability of astrocytes, whereas treatment with 750 µM GCEE for 60 min decreased the viability of astrocytes (Fig.4.1). Treatment of cortical neurons with 900 µM GCEE for 30 min as well as for 60 min increased the viability of astrocytes (Fig.4.1). However, the differences were not statistically significant compared to controls. Thus, concentrations (750 µM and 900 µM) and incubation times (30 min and 60 min) of GCEE were found to be non-toxic to astrocytes.

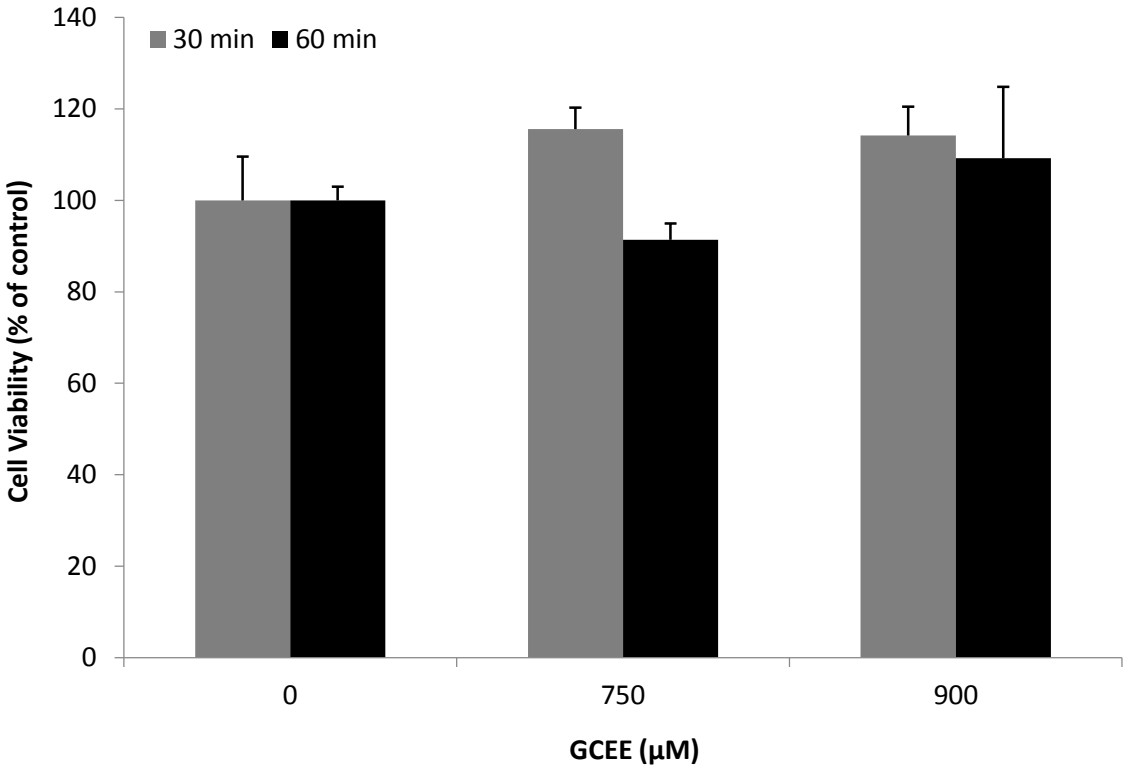


Fig.4.1 Cytotoxicity of GCEE on cortical astrocytes. Astrocytes were treated with vehicle or varying concentrations of GCEE (0 µM, 750 µM, 900 µM) during different incubation times (0 min or 60 min). Cell viability was measured by MTT assay.

4.4.2 Cytotoxicity of tBHP on cortical astrocytes

The cytotoxicity of TBHP on cortical astrocytes was also tested by MTT assay. Rat primary cortical astrocytes were exposed to different concentrations of tBHP (0, 100, and 200 μM) for 30 min or 60 min, and the number of living cells in the different groups was measured by MTT assay. The results showed that exposure of cortical astrocytes to tBHP (100, and 200 μM) for 30 min increased viabilities of astrocytes compared to control although differences were not statistically significant (Fig.4.2). In contrast, when astrocytes were exposed to tBHP (100, and 200 μM) for 60 min, a dose dependent decrease in the cell viabilities was observed (Fig.4.2). Specifically, incubation with 200 μM tBHP for 60 min significantly decreased the number of viable cells by 33% compared to that of control (Fig.4.2). Thus, 200 μM tBHP at 60 min was selected as the cytotoxic level, while the rest of conditions were considered non-toxic levels to cortical astrocytes for following experiments in this study.

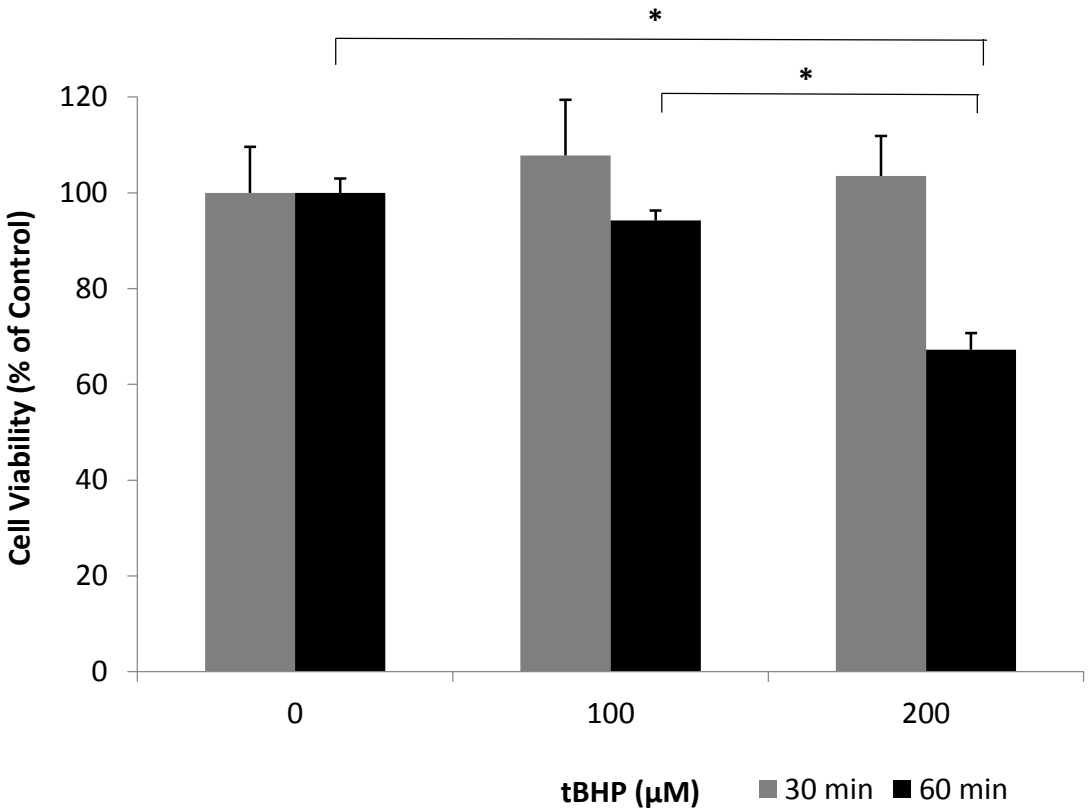


Fig.4.2 Cytotoxicity of tBHP on cortical astrocytes. Astrocytes were exposed to vehicle or various concentrations of tBHP (0 μM, 100 μM, and 200 μM) during different incubation times (30 min or 60 min), and cell viability was measured by MTT assay. (*$p<0.05$ compared to the control)

4.4.3 GCEE attenuates tBHP-induced oxidative stress in cortical astrocytes

DCF assay was used to investigate the ability of GCEE to attenuate oxidative stress in cortical astrocytes. The levels of fluorescence were directly proportional to intracellular oxidative stress status. Rat primary cortical astrocytes were pre-treated with 750 µM GCEE for 1 hour and exposed to oxidative stress by adding 200 µM tBHP for 30 min. Exposure of cortical astrocytes to 200 µM tBHP alone for 30 min increased fluorescence levels greater than control groups, which were not statistically significant (Fig.4.3). In contrast, treatment with 750 µM GCEE 1 hour prior to addition of 200 µM tBHP for 30 min significantly decreased fluorescence levels by 35% than untreated astrocytes (Fig.4.3). The concentrations and incubation times of tBHP and GCEE selected for this experiment were found to be non-toxic to cortical astrocytes in this study (Fig.4.2). Hence, the reason why oxidative stress was not significantly increased in astrocytes after addition of tBHP in this study may be because astrocytes' intrinsic property of rapidly clearing tBHP through the glutathione system according to the study conducted by Dringen (Dringen, Kussmaul, and Hamprecht 1998). Taken together, this data suggested that GCEE was able to enter into astrocytes and attenuate oxidative stress by enhancing the endogenous glutathione system as GSH precursor.

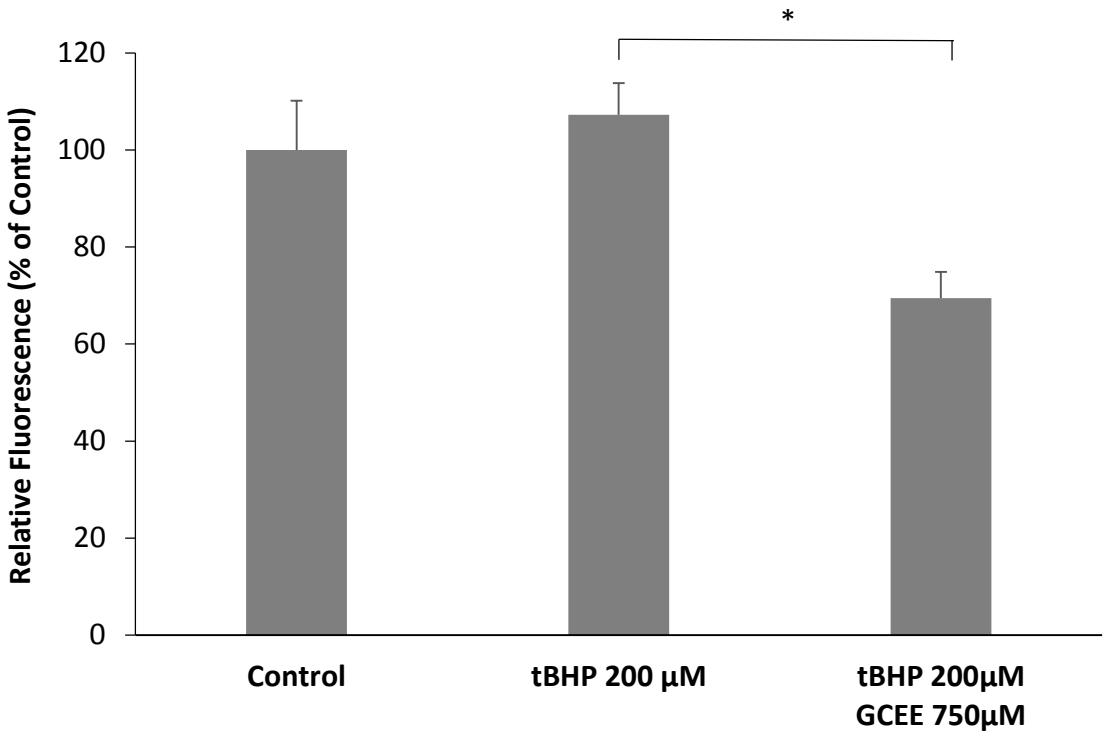


Fig.4.3 Levels of intracellular oxidative stress in cortical astrocytes exposed to non-cytotoxic tBHP and treated with GCEE. Astrocytes were treated with vehicle or 750 µM GCEE prior to exposure to vehicle or 200 µM tBHP for 30 min, and intracellular fluorescence levels were measured by DCF assay. (*$p<0.05$ compared to the control).

4.4.4 GCEE protects cortical astrocytes against tBHP-mediated cytotoxicity

The ability of GCEE to protect cells from oxidative stress-related cytotoxicity was investigated by measuring the number of living cells via MTT assay. Rat primary cortical astrocytes were pre-treated with different concentrations of GCEE (0, 750 and 900 µM) for 60 min, followed by incubation with 200 µM tBHP for 60 min. The concentration and incubation time of tBHP was selected because addition of 200 µM tBHP for 60 min significantly decreased cell viability (Fig.4.4). Exposure of cortical astrocytes to 200 µM tBHP for 60 min significantly reduced the number of viable cells by 21% compared to control groups, consistent with the result of MTT assay with tBHP alone in this study (Fig.4.4). However, cortical astrocytes that were treated with GCEE (750 and 900 µM) for 60 min prior to addition of 200 µM tBHP showed a dose dependent increase in cell viability (Fig.4.4).

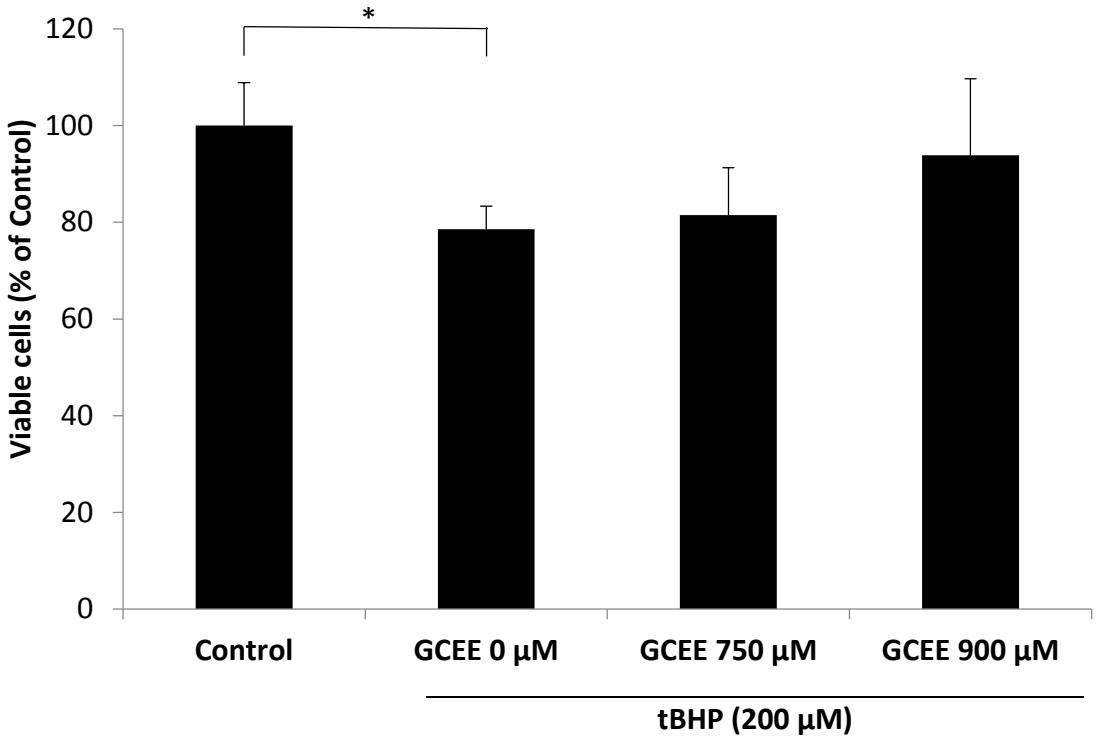


Fig.4.4 Viability of cortical astrocytes treated with GCEE against tBHP-mediated death. Astrocytes were treated with vehicle or various concentrations of GCEE (750 µM and 900 µM) for 1 h prior to addition of 200 µM tBHP for 60 min. Cell viability was measured by MTT assay. (*$p<0.05$ compared to the control).

4.4.5 GCEE attenuates oxidative stress in cortical astrocytes during tBHP-mediated cytotoxicity

Similarly, the antioxidant capability of GCEE to reduce intracellular oxidative stress was investigated, but cortical astrocytes were under tBHP-induced cytotoxicity, whose concentration and incubation times were determined by MTT assay in this study (Fig.4.2). Rat primary cortical astrocytes were pre-treated with 750 µM GCEE for 1 hour and exposed to oxidative stress by adding 200 µM tBHP for 60 min. Exposure of cortical astrocytes to 200 µM tBHP alone for 60 min significantly decreased fluorescent levels by 35% than control groups (Fig.4.5), which may be due to the decreased number of viable astrocytes expressing fluorescent levels as addition of 200 µM tBHP for 60 min significantly reduced cell viability in the previous experiment (Fig.4.2 and Fig.4.4). In contrast, treatment with 750 µM GCEE for 1 hour significantly decreased fluorescent levels by 24% compared to untreated cortical astrocytes (Fig.4.5) although treatment with GCEE increased the number of viable astrocytes in a dose-dependent manner which was significantly reduced after addition of 200 µM tBHP for 60 min (Fig.4.4). Since the fluorescence level obtained from DCF assay is related to the status of oxidative stress in intracellular space, these results showed treatment with GCEE significantly decreased oxidative stress in *in vitro* in astrocytes even during cytotoxic conditions.

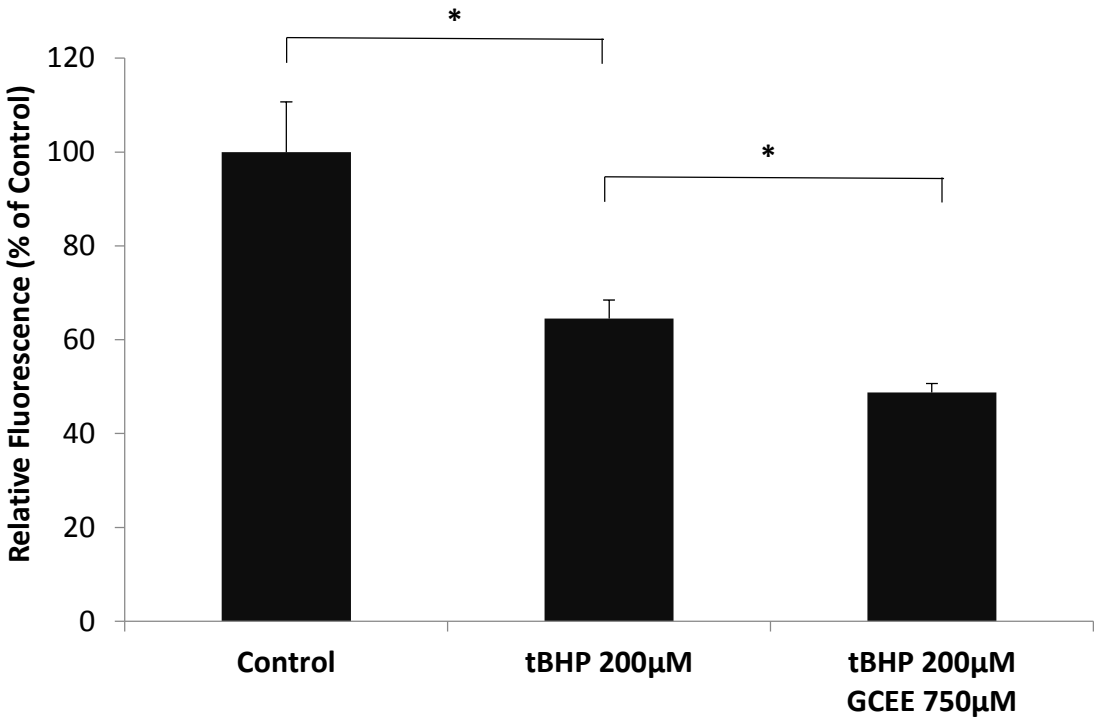


Fig.4.5 Levels of intracellular oxidative stress in cortical astrocytes exposed to cytotoxic tBHP and treated with GCEE. Astrocytes were treated with vehicle or 750 µM GCEE prior to exposure to vehicle or 200 µM tBHP for 60 min, and intracellular fluorescence levels were measured by DCF assay. (*p<0.05 compared to the control)

4.5 Discussion

The important roles of astrocytes in traumatic brain injury for neuroprotection have been investigated in TBI-models through histochemical studies. Both degenerating astrocytes and neurons are detected following TBI; however, the loss of astrocytes appears first, and prolonged neurodegeneration occurs later, which suggests that the loss of astrocytes contribute to neurodegeneration (Zhao et al. 2003). It has been confirmed by another study that the presence of astrocytes prevents the loss of brain tissue and protects neurons from damage, whereas the absence of astrocytes significantly increases the loss of brain tissue with increased number of degenerating neurons and inflammation (Myer et al. 2006).

Although GCEE has shown beneficial effects on neurons, this may be the first study to investigate the roles of GCEE in astrocytes. In this study, we tested whether GCEE could increase glutathione levels in astrocytes and could protect astrocytes against oxidative stress and cytotoxicity in which tBHP was used to induce oxidative stress. Addition of tBHP increased cytotoxicity in cortical astrocytes in time- and dose-dependent manner. In contrast, treatment of cortical astrocytes with GCEE restored cell viability and decreased tBHP-mediated intracellular oxidative stress levels in astrocytes. Taken together, these results suggest that GCEE can enter cortical astrocytes and increase glutathione levels, which in turn, attenuate intracellular oxidative stress and protects cortical astrocytes from oxidative stress-related cytotoxicity.

Our finding, the ability of GCEE to increase intracellular glutathione levels in astrocytes, is particularly important for neuroprotection because although the presence of astrocytes enhances the ability of neurons to defend themselves against oxidative stress and promotes neuronal survival during oxidative stress, GSH depleted astrocytes lose their neuroprotective roles, which causes neuronal toxicity and consequently decreases cell viability (Drukarch et al. 1997, Drukarch et al. 1998, Chen et al. 2001, Pizzurro, Dao, and Costa 2014). In addition, the depletion of glutathione in astrocytes is also harmful to the astrocytes themselves (Im, Paik, and Han 2006). Astrocytes contribute to other functions of the brain besides supporting neurons. They remove debris in extracellular space and form the blood-brain barrier which prevents large molecules or foreign toxic substances from entering the brain (He and Sun 2007). In other words, the loss of astrocytes results in not only neurodegeneration, but also overall brain dysfunction (Seifert, Schilling, and Steinhauser 2006). Thus, maintenance of astrocytes glutathione is crucial in protecting neurons against oxidative stress because astrocytic GSH plays an important role in maintenance of neuronal glutathione levels as well as to prevent brain dysfunction by protecting astrocytes themselves.

In conclusion, this study has shown that GCEE can decrease oxidative stress in astrocytes and protect astrocytes from oxidative stress-mediated cytotoxicity due to its ability to increase cellular glutathione levels. Astrocytes have shown neuroprotective roles during oxidative stress by supplying GSH precursors to neurons. Thus, the beneficial effects of GCEE on astrocytes may enhance the endogenous ability of

astrocytes to protect neurons against oxidative stress by increasing the availability of GSH precursors, which would contribute to the neuroprotective effects of GCEE in TBI.

CHAPTER 5

TIME COURSE ANALYSIS OF GAMMA-GLUTAMYLCYSTEINE ETHYL ESTER ON ATTENUATION OF TERT-BUTYL HYDROPEROXIDE MEDIATED-OXIDATIVE STRESS *IN VITRO* IN CORTICAL NEURONS AND ASTROCYTES

5.1 Overview

Traumatic brain injury has been well documented to accompany oxidative stress in the brain as an early event by immediately elevating cellular reactive oxygen species and reactive nitrogen species levels. Oxidative stress causes deleterious cellular damage due to a rapid reaction between free radicals and cellular molecules, which consequently attributes to cellular death. Glutathione plays an important role in the maintenance of intracellular redox homeostasis by scavenging oxidants. Gamma-glutamylcysteine ethyl ester has been found to increase intracellular glutathione levels in the brain. In this study, we assessed the effects of GCEE on combating oxidative stress both in neurons and astrocytes in a time dependent manner. We prepared rat primary cortical neurons and rat primary cortical astrocytes separately. Cells were exposed to oxidative stress for 30 minutes by adding tert-butyl hydroperoxide and a change in the levels of oxidative stress was measured by dichlorofluorescein assay. At the early point of oxidative stress, treatment with GCEE significantly decreased intracellular oxidative stress both in neurons and astrocytes. As oxidative stress proceeded, while GCEE-treated neurons gradually increased in oxidative stress as indexed by tBHP levels, GCEE-treated astrocytes maintained lowered intracellular oxidative stress. These results suggest GCEE can up-regulate glutathione both in neurons and astrocytes and attenuate

oxidative stress; however up-regulated neuronal glutathione by GCEE is not maintained, whereas up-regulated astrocytic glutathione by GCEE is maintained. Since astrocyte glutathione has been known to contribute to neuronal glutathione *in vivo*, the beneficial effects of GCEE in astrocytes may keep neurons from losing up-regulated glutathione by GCEE *in vivo*, which could prevent neurodegeneration in traumatic brain injury.

5.2 Introduction

The brain is composed of different types of cells, such as neurons and glial cells. Each type of cell has unique functions and further interacts with each other. Neurons, the most important cells in the brain, are responsible for playing a major role in learning, memory, emotions, and perception. The main role of glial cells is to support surrounding neurons. In particular, astrocytes, the most abundant glial cells, play an important role in protecting neurons against oxidative stress through several ways: 1) preventing glutamate-mediated neurotoxicity by regulating extracellular glutamate concentrations and 2) supplying glutathione precursors to neurons by the γ-glutamyl cycle, and 3) releasing neuroprotective factors in extracellular space during oxidative stress (Dringen, Kranich, and Hamprecht 1997, Dringen, Pfeiffer, and Hamprecht 1999, Dringen, Gutterer, and Hirrlinger 2000, Chen et al. 2001, Volterra et al. 1994). As oxidative stress is a major underlying cause of neurodegeneration, neuroprotective properties of astrocytes are critical to keep neurons from losing their functions, and ultimately preventing delayed neurodegeneration.

Traumatic brain injury is a sudden incident, producing physical damage to the exterior of the brain. The levels of reactive oxygen and nitrogen species are subsequently elevated following traumatic brain injury, which exceed the capability of the endogenous glutathione, and thereby both neurons and astrocytes are susceptible to oxidative stress during the brain injury. However, as the functions differ between neurons and astrocytes, their resistance to oxidative stress differ as astrocytes are more resistant to oxidative stress than neurons (Makar et al. 1994). It may be because neurons and astrocytes have different concentrations of glutathione, 2.5mM and 3.8mM respectively (Raps et al. 1989). Therefore, it is crucial to boost neuronal glutathione levels post brain injury in order to enhance the capability of neurons to defend against oxidants and prevent oxidative stress-related neurodegeneration in which astrocyte glutathione plays an essential role through γ-glutamyl cycle. In other words, up-regulation of glutathione in astrocytes is also important as much as increasing neuronal glutathione for neuroprotection.

Although the ability of GCEE to enrich cellular glutathione in neurons and astrocytes has been proven by others and by our work in Chapter 4 (Fig.4.3), respectively, this may be the first study investigate the antioxidant capability of GCEE to attenuate oxidative stress by preventing intracellular reactive oxygen and nitrogen species formation in a time dependent manner. In this study, we prepared rat primary cortical neuronal cultures and astrocytic cultures separately prepared and tert-butyl hydroperoxide was added into neuronal and astrocytic cultures to induce oxidative

stress. It has been known that oxidative stress induced by tBHP can be attenuated by glutathione system only in both neurons and astrocytes (Abe and Saito 1998). In other words, addition of tBHP leads to a rapid depletion of cellular glutathione (Rush and Alberts 1986, O'Connor et al. 1995). Dichlorofluorescein assay was adapted to measure intracellular oxidative stress level over time. The levels of fluorescence were related to the levels of intracellular oxidative stress.

5.3 Materials and Procedures

5.3.1 Materials

All reagents required for culturing neurons and astrocytes, such as Neurobasal Medium, 200 mM GLUTAMAX, 50X B27 supplement, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Accutase were purchased from Invitrogen (Carlsbad, CA, USA). All reagents required for experiments, such as Dulbecco's Phosphate Buffered Saline (D-PBS; without Ca^{2+}, Mg^{2+}, and phenol red) and Hank's Balanced Salt Solution (HBSS) were also purchased from Invitrogen (Carlsbad, CA, USA). Both H2DCFDA (2,7-dichlorodihydrofluorescein diacetate) and tBHP (tert-butyl hydroperoxide) were purchased from Acros Organics. GCEE was purchased from Bachem (Torrance, CA, USA).

5.3.2 Neuronal Cell Culture

Rat primary cortical neurons purchased from Invitrogen (Carlsbad, CA, USA), were known to be isolated from day 18 Fisher-344 rat embryos. Complete medium used for culturing neurons was prepared by adding 2% of B27 supplement and 0.5 mM of

GLUTAMAX to Neurobasal Medium. Before culturing, complete medium was pre-warmed to 37^{o}C. Neuronal cultures were prepared according to the protocol obtained from Invitrogen (https://www.lifetechnologies.com/order/catalog/product/A1084002?ICID). Briefly, cells were plated on poly-D-lysine coated 48 well-plate at a density of 1 x 10^5 cells/well with complete Neurobasal medium supplemented with B27 and GLUTAMAX, and incubated at 37^{o}C, 5% CO_2, and 90% humidity. After a 24 h incubation, half of complete medium was aspirated from each well and the same amount of fresh complete medium was added. Afterwards, spent medium was replaced with fresh complete medium every third day until cells were ready for use.

5.3.3 Glial Cell Culture

Rat primary cortical astrocytes purchased from Invitrogen (Carlsbad, CA, USA), were known to be isolated from the cortex of Sprague-Dawley at day E19 of gestation. Complete medium for astrocyte cultures was prepared by adding 15% FBS to DMEM (high glucose). Before culturing, complete medium was pre-warmed to 37^{o}C. Astrocyte cultures were prepared according to the protocol obtained from Invitrogen (https://www.lifetechnologies.com/order/catalog/product/N7745100?ICID). Briefly, cells were initially plated on T-25 cm^2 flask at a density of 1 x 10^4 cells/cm^2 with DMEM (high glucose) complete medium and expanded at 37^{o}C, 5% CO_2, and 90% humidity. When the culture was 100% confluent, cells were transferred onto an uncoated tissue-culture treated 24-well plate at a seeding density of 2x10^4 cells/cm^2 with DMEM

complete medium and incubated at 37^{o}C, 5% CO_2, and 90% humidity until the culture was 100% confluent.

5.3.4 Measurement of intracellular ROS/RNS formation

The ability of GCEE to prevent formation of intracellular ROS/RNS and eliminate increased ROS in cortical neurons was determined through DCF assay by measuring overall oxidative stress status. The DCF assay was conducted on primary cortical neurons and astrocytes separately, but applied concentrations and incubation times were the same. The experiment was performed at least in triplicate for each treatment. Before experiments began, HBSS and DPBS were pre-warmed to 37^{o}C. Spent complete medium was removed. The cells were washed with 500 µl 1X DPBS per well and used 1X DPBS was rapidly removed from wells. The cells were then loaded with 500 µl HBSS alone or pre-treated with 500 µl HBSS containing 900 µM GCEE per well for 1 h at 37^{o}C, 5% CO_2, and 90% humidity. After 1 hour, HBSS and GCEE-containing HBSS solutions were removed from all the wells and the cells were washed with 500 µl 1X DPBS per well. After removing 1X DPBS, the cells were incubated with 500 µl HBSS containing 10 µM H2DCFDA per well for 30 min at 37^{o}C, 5% CO_2, and 90% humidity. After 30 min, H2DCFDA-containing HBSS was removed and the cells were washed with 500 µl 1X DPBS per well. After removing 1X DPBS, 500 µl HBSS alone or 500 µl HBSS containing 100 µM tBHP was added to the cells in each well. Five minutes after addition of tBHP, the levels of fluorescence were measured by using a fluorescence microplate reader with excitation and emission wavelengths set to 488 nm and to 525 nm, respectively.

Intracellular ROS formations were recorded at 2.5 minutes intervals for 30 min (neurons) and at 1.5 min intervals for 30 min (astrocytes).

5.3.5 Statistical Analysis

All data were analyzed using ANOVA and expressed as mean ± SD. Statistical significance was set at $p < 0.05$.

5.4 Results

5.4.1 Antioxidant effects of GCEE on tBHP-induced oxidative stress in cortical neurons

Rat primary cortical neurons were pre-treated with vehicle or 900 µM GCEE 1 hour prior to addition of 100 µM tBHP, and a change in the levels of intracellular oxidative stress was observed. Addition of tBHP alone to cortical neurons initially decreased intracellular oxidative stress level compared to control neurons; however, the level of decreased intracellular oxidative stress was progressively increased and approximately 20 minutes after addition of tBHP, intracellular oxidative stress level began to exceed that of control neurons (Fig.5.1). Treatment with GCEE for 1 hour prior to addition of tBHP also initially decreased intracellular oxidative stress level, which was lower compared to untreated neurons and lasted for approximately 17 to 22 minutes after addition of tBHP; however, the level of intracellular oxidative stress began to progressively increase and exceed that of untreated neurons (Fig.5.1). Since tBHP-induced oxidative stress can be reduced by the glutathione system, initially decreased oxidative stress levels indicated that naturally occurring neuronal glutathione effectively eliminated tBHP-induced radicals at the early point of oxidative stress and GCEE was

able to augment its endogenous glutathione ability as a glutathione precursor. However, progressively increased intracellular oxidative stress levels suggest that the effects of glutathione were not maintained in neurons.

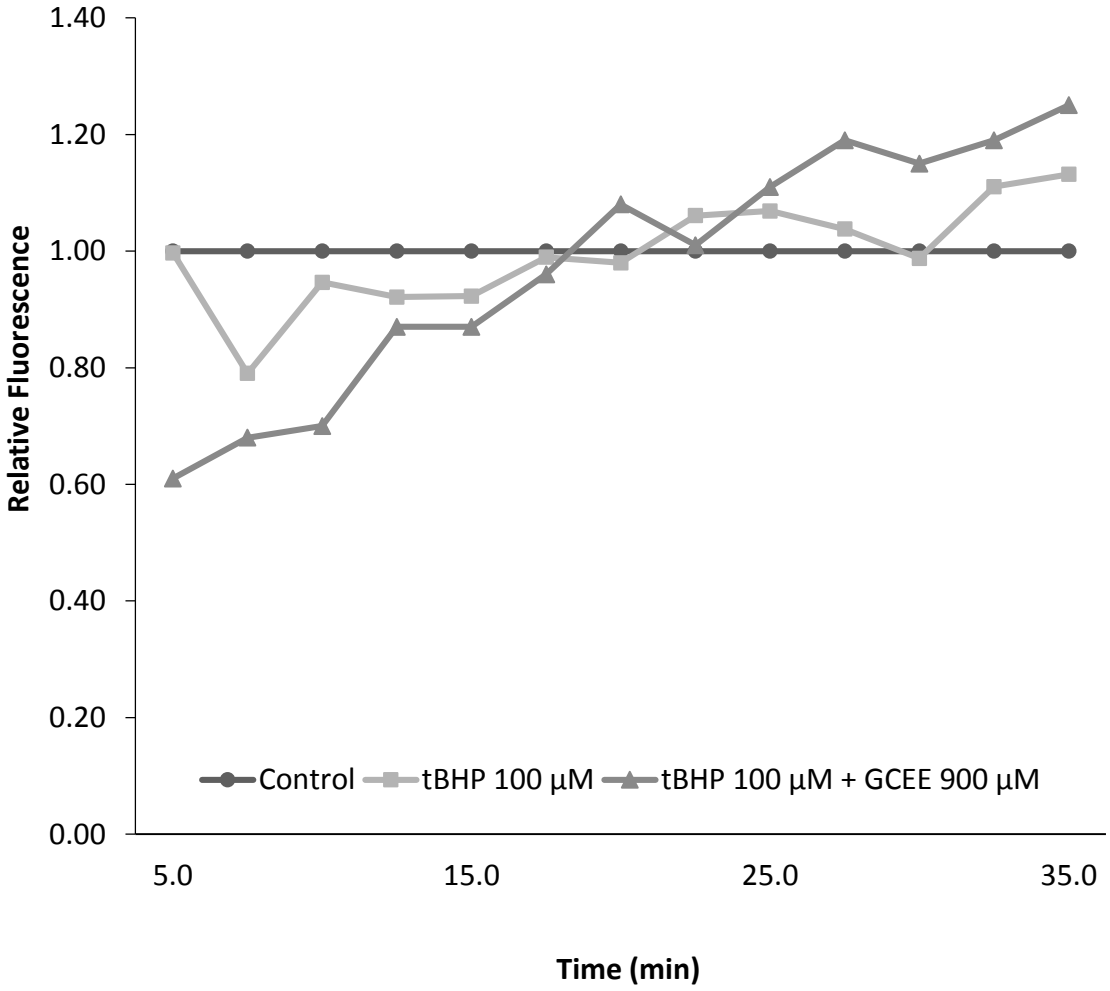


Fig.5.1 Levels of temporal intracellular oxidative stress in GCEE-treated cortical neurons. Cortical neurons were pre-treated with either vehicle or 900 µM GCEE for 1 h and exposed to vehicle or 100 µM tBHP. The intracellular fluorescence levels were measured 5 min after addition of tBHP by DCF assay at 2.5 min intervals for 30 min.

5.4.2 Antioxidant effects of GCEE on tBHP-induced oxidative stress in cortical astrocytes

Rat primary cortical astrocytes were also pre-treated with vehicle or 900 μM GCEE for 1 hour before adding 100 μM tBHP, and a change in intracellular oxidative stress levels was observed. Exposure of cortical astrocytes to 100 μM tBHP alone, which was found not to decrease viability of astrocytes from Chapter 4 (Fig.4.2), immediately increased intracellular oxidative stress level compared to control astrocytes, which lasted throughout 30 minutes (Fig.5.2). Although it has been well known that astrocytes have a higher concentration of glutathione than neurons, the reason why astrocytes were not able to effectively decrease oxidative stress compared to neurons (Fig.5.1) may be because astrocytes are more dependent on the activity of catalase than neurons, but tBHP inhibits the activity of catalase (Abe and Saito 1998). However, as oxidative stress was not significantly increased, this data suggested that naturally occurring astrocytic glutathione was still able to defend against tBHP-mediate radicals. Treatment of cortical astrocytes with 900 μM GCEE for 1 hour, which was found to slightly increase viability of astrocytes from Chapter 4 (Fig.4.1), significantly reduced intracellular oxidative stress level compared to untreated astrocytes as early as 5 minutes after addition of tBHP (Fig.5.2). Similarly, in normal conditions, treatment with GCEE still significantly decreased intracellular oxidative stress level compared to control astrocytes (Fig.5.2). Although decreased intracellular oxidative stress level began to progressively increase, it reached a plateau at half of the baseline level at approximately 35 minutes. Levels were lower than that of untreated astrocytes (Fig.5.2). This data

supports the hypothesis that the ability of GCEE to attenuate oxidative stress in cortical astrocytes, which had been already shown in Chapter 4 (Fig.4.3), could be maintained over time.

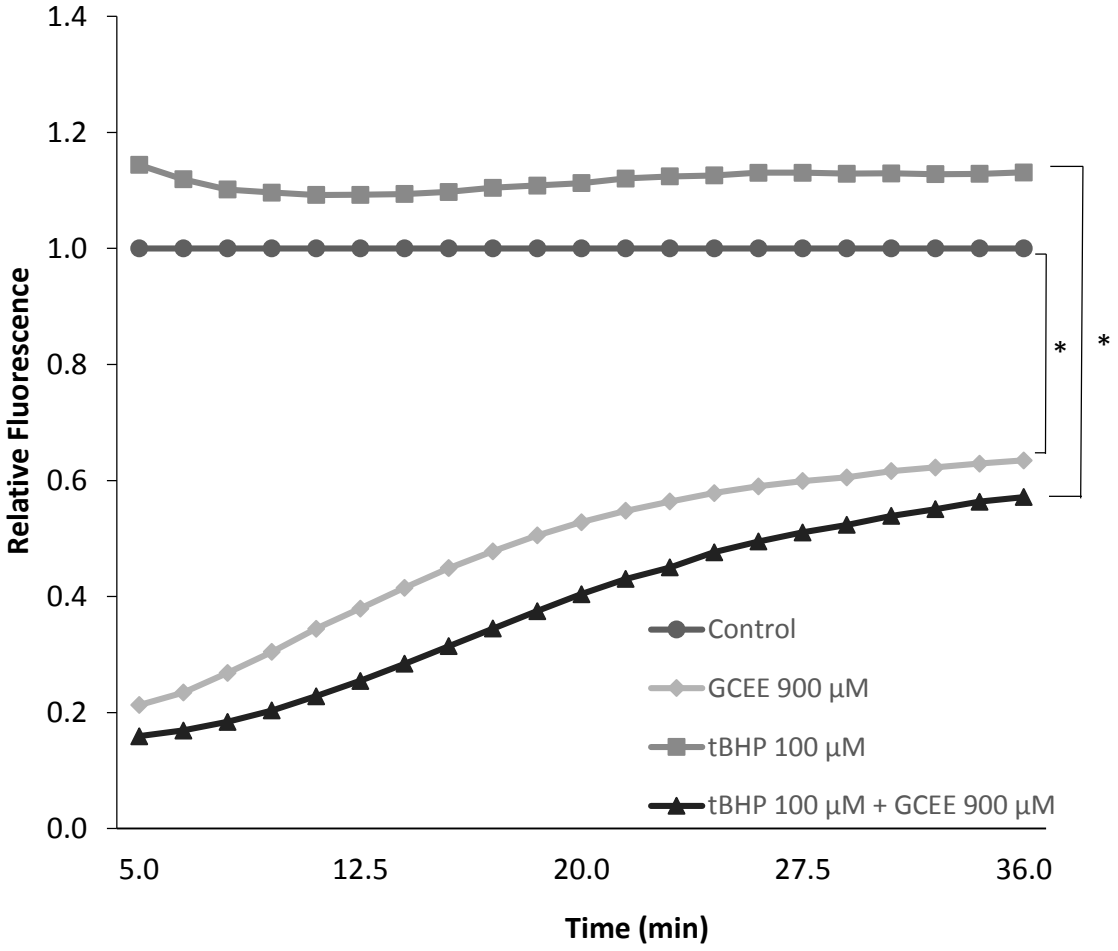


Fig.5.2 Levels of temporal intracellular oxidative stress in GCEE-treated cortical astrocytes. Cortical astrocytes were pre-treated with either vehicle or 900 µM GCEE for 1 h and exposed to vehicle or 100 µM tBHP. The intracellular fluorescence levels were measured 5 min after addition of tBHP by DCF assay at 1.5 min interval for 30 min(*$p<0.05$ compared to the control)

5.5 Discussion

5.5.1 The effects of GCEE on neurons

As soon as neurons were exposed to tBHP, neurons initially decreased overall intracellular oxidative stress levels below the baseline (Fig.5.1), indicating that endogenous glutathione rapidly removes tBHP-mediated radicals independent of the absence of catalase. Treatment with GCEE further decreased intracellular oxidative stress levels compared to neurons treated with tBHP alone (Fig.5.1), showing that GCEE can up-regulate neuronal glutathione levels and remove tBHP-induced radicals. However, as oxidative stress continued, oxidative stress gradually increased in both GCEE treated and untreated neurons, indicating that both endogenous GSH and exogenous GSH levels decreased. Altogether, this result suggests that GCEE can increase neuronal glutathione and attenuate oxidative stress, but up-regulated-GSH by GCEE is not maintained, but decreased within neurons.

Neuronal GSH is affected by astrocyte GSH through gamma-glutamyl cycle which is initiated when astrocytes release glutathione into extracellular and provide GSH precursors (Walz 1989, Sagara, Makino, and Bannai 1996, Dringen, Gutterer, and Hirrlinger 2000, Sagara, Miura, and Bannai 1993). Numerous studies have shown that astrocyte-mediated neurons increase glutathione levels and decrease ROS/RNS levels, thereby increasing cell viability, whereas neurons alone decrease glutathione and increase ROS, leading to an increase in cell death (Rathinam et al. 2012, Pizzurro, Dao, and Costa 2014, Chen et al. 2001). Thus, increased oxidative stress in neurons in this

study may be due to the absence of astrocytes, especially astrocyte glutathione, which has been demonstrated by others (Drukarch et al. 1998, Drukarch et al. 1997, Gegg, Clark, and Heales 2005).

5.5.2 The effects of GCEE on astrocytes

As soon as astrocytes were exposed to tBHP, oxidative stress was initially increased. Over time, the initially increased oxidative stress remained constant; however, initially increased oxidative stress was not significantly different from base levels (Fig.5.2), indicating that astrocytes were resistant to tBHP-induced oxidative stress due to glutathione, which is consistent to Dringen's work in which astrocytes rapidly remove tBHP through endogenous GSH system (Dringen, Kussmaul, and Hamprecht 1998). Moreover, it has been suggested that transcriptional up-regulation of γ-GC, an enzyme required for glutathione synthesis in astrocytes, can boost intracellular GSH levels and increase the resistance of astrocytes against oxidative stress (Iwata-Ichikawa et al. 1999).

Treatment with GCEE significantly decreased oxidative stress compared to non-treated astrocytes (Fig.5.2), indicating that GCEE can effectively up-regulate GSH in astrocytes and such up-regulated GSH can actively reduce oxidative stress, which is consistent with the results observed in Chapter 4 (Fig.4.3). Furthermore, the data from this study showed that such decreased oxidative stress was remained at constant levels over the time (Fig.5.2), indicating that up-regulation of GSH by GCEE was not reduced as oxidative stress proceeded. These results suggest that the GCEE can constantly enhance

the resistance of astrocytes against oxidative stress through glutathione. These beneficial effects of GCEE in increasing astrocyte GSH may be beneficial for neurons because GSH released from astrocytes is essential to protecting neurons against oxidative stress (Shih et al. 2003, Chen et al. 2001).

In conclusion, increasing neuronal glutathione is important to prevent oxidative stress following TBI because neurons are more vulnerable to oxidative stress (Raps et al. 1989, Makar et al. 1994). However, increasing astrocyte glutathione is also important for the maintenance of neuronal glutathione because astrocytes release glutathione into extracellular space which supply glutathione precursors to neurons and protect neurons against oxidative stress (Desagher, Glowinski, and Premont 1996, Mena et al. 1996). This study has shown that GCEE can increase cellular glutathione both in neurons and astrocytes, which, in turn, decreases oxidative stress. Astrocytes, not neurons, can maintain up-regulated GSH by GCEE. Altogether, the results from this study suggest that the beneficial effects of GCEE in astrocytes may enhance the beneficial effects of GCEE in neurons, which could ultimately prevent neurodegeneration and support GCEE as a potential therapeutic agent against traumatic brain injury.

CHAPTER 6

CONCLUSIONS AND FUTURE STUDIES

6.1 Conclusions

Oxidative stress is an early event in traumatic brain injury. It can cause acute or delayed neurodegeneration following TBI. Therefore, it is important to immediately combat oxidative stress by using neuroprotective measures. Glutathione, a naturally occurring antioxidant, plays an important role in scavenging reactive oxygen species and reactive nitrogen species in the brain. Gamma-glutamylcysteine has been shown to increase cellular glutathione levels in the brain. In this thesis, we investigated the antioxidant capabilities of GCEE to reduce oxidative stress and protect cells against oxidative stress-induced degeneration as a GSH precursor.

The goal of the first study was to test our hypothesis that GCEE could decrease the quantity of degenerating neurons in the brain due to its ability to increase glutathione and decrease the index of oxidative stress as described in other studies (Reed et al. 2009, Lok et al. 2011). In this study, controlled cortical impact (CCI) was utilized to simulate moderate TBI on rat brains and Fluoro-Jade B (FJB) was adapted to stain degenerating neurons on the brain tissue. We have found that there is a protective role of GCEE in TBI-induced neurodegeneration. Administration of GCEE post-TBI decreased the distributions and concentrations of degenerating neurons on the brains compared to untreated rat brain. This protective effect of GCEE on neurons may prevent

the loss of neurons and reduce the acceleration of neurodegenerative disorders during TBI.

The goal of second experiment was to test whether GCEE could enter astrocytes and protect these structures from oxidative stress and oxidative stress-induced cytotoxicity. Primary cortical astrocytes were cultured and tert-butyl hydroperoxide (tBHP) was selected to induce oxidative stress to cell culture. DCF and MTT assays were conducted to measure intracellular redox status and cell viability, respectively. Treatment with GCEE was able to decrease tBHP-induced oxidative stress and by increasing GSH and such antioxidant capability of GCEE was able to protect astrocytes from tBHP-induced death. This protective effect of GCEE on cortical astrocytes may enhance neuroprotective properties of astrocytes and ultimately protect neurons during oxidative stress.

The goal of the last study was to assess time course effects of GCEE on different types of cells: neurons and astrocytes. Primary cortical neurons and astrocytes were separately cultured from embryonic rats. As performed in another study, tBHP was added to cell cultures in order to induce oxidative stress after pre-treatment with GCEE. The changes in the intracellular oxidative stress levels were then measured by DCF assay. We have found the effects of GCEE on astrocytes last longer, but the effects of GCEE on neurons alone decrease over time. GCEE was able to significantly reduce oxidative stress up to 30 min in astrocytes, whereas GCEE was able to reduce oxidative stress for only 20 min. This may be because astrocytes contain higher concentrations of

glutathione than neurons (Raps et al. 1989). Neuronal decrease in intracellular GSH makes them more vulnerable to oxidative stress in the absence of astrocytes (Rathinam et al. 2012, Gegg, Clark, and Heales 2005, Drukarch et al. 1997). Therefore, co-culture of neurons with astrocytes could protect neurons and also enhance the protective effects of GCEE on neurons with support of protective effects of GCEE on astrocytes.

Collectively, this work has shown that GCEE is capable of protecting both neurons and astrocytes against oxidative stress induced degeneration. These findings support the potential therapeutic roles of GCEE as neuroprotective agent in TBI.

6.2 Future Studies

1. (Re-analysis) In our *in vivo* experiment, the total number of degenerating neurons was quantified. In the future, spatial analysis can be tested to compare the effects of GCEE on ipsilateral (same side as impact) versus contralateral because degenerating neurons have been observed not only in the ipsilateral hemisphere, but also in the contralateral hemisphere although the contralateral hemisphere did not receive direct mechanical force during the brain injury (Hall et al. 2005). Additionally, the effects of GCEE on specific brain regions, particularly the hippocampus which is directly responsible for memory, can be investigated because degenerating neurons have been detected other brain regions in TBI-induced rats by others (Anderson et al. 2005, Zhou et al. 2012, Gao et al. 2008).
2. The early loss of astrocytes after TBI has been observed on the brain tissue through histochemical study (Zhao et al. 2003). In our *in vitro* study, GCEE protected cortical

astrocytes from oxidative stress and increased cell viability. Therefore, an *in viv*o experiment should be conducted to investigate whether administration of GCEE after TBI could protect astrocytes and decrease the quantity of degenerating astrocytes through histochemical methods, as we previously identified degenerating neurons in our animal experiment using FJB staining techniques. For identification of astrocytes, glial fibrillary acidic protein which is mainly expressed on astrocytes can be used as a marker (Eng 1985). Protecting astrocytes would be important because the loss of astrocytes contribute to increasing neurodegeneration, whereas the presence of activated astrocytes protects neurons from degeneration following traumatic brain injury (Zhao et al. 2003, Myer et al. 2006).

3. In our *in vitro* study, the ability of GCEE to decrease tBHP-induced oxidative stress in cortical astrocytes cultures lasted the time course. In contrast, the ability of GCEE to combat tBHP-induced intracellular oxidative stress was gradually decreased in cortical neuronal cultures as the exposure time increased. It has been demonstrated in numerous studies that neurons are vulnerable to oxidative stress in the absence of astrocytes due to decreased glutathione levels, whereas astrocyte-mediated neurons are resistant to oxidative stress due to the neuroprotective role of astrocytes through the glutathione system (Rathinam et al. 2012, Pizzurro, Dao, and Costa 2014). Therefore, this experiment can be repeated with a co-culture system of neurons and astrocytes to investigate whether astrocytes prevent GCEE-treated neurons from increasing oxidative stress, but instead enhance neuroprotective effects of GCEE on neurons. Levels of intracellular GSH and extracellular GSH can be

also measured both in neurons and astrocytes during co-culture system to investigate whether increased glutathione levels in astrocytes by GCEE administration could increase intracellular GSH in neurons based on the fact that astrocytes supply GSH precursors to neurons (Dringen, Gutterer, and Hirrlinger 2000, Wang and Cynader 2000).

4. Although GCEE was able to decrease intracellular oxidative stress in astrocytes, intracellular glutathione levels were not measured. Therefore, measurement of intracellular glutathione levels is necessary. Also, extracellular glutathione level is another important key factor because extracellular GSH is used as neuronal GSH precursors. If GCEE can either increase extracellular glutathione levels by up-regulating intracellular GSH so that more GSH is released or GCEE itself is cleaved and utilized by neurons, both ways can have the potential for showing neuroprotective effects on neurons.

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APPENDIX

DATA

Table 3.1b Average number of degenerating neurons stained by FJB on rat brain tissues (Supporting data)

Groups	Numbers of FJB positive neurons (in 0.02 mm^2)		
(n=3)	1	2	3
GCEE 30	0	0	20
			16
			18
GCEE 60	27	18	17
	14	23	26
		18	21
		16	
Saline 30	0	16	14
		23	
		14	
		30	
		30	
Saline 60	0	21	25
		26	22
		34	
		32	
		26	
Sham	0	41	2
			7

Table 4.1b Cytotoxicity of GCEE on cortical astrocytes (Supporting data)

30 min				60 min			
	Control	GCEE 750 µM	GCEE 900 µM		Control	GCEE 750 µM	GCEE 900 µM
A	0.073	0.086	0.084	A	0.091	0.085	0.094
B	0.086	0.092	0.094	B	0.097	0.092	0.117
C	0.087	0.093	0.091	C	0.094	0.09	0.087
Blank	0.035	0.036	0.036	Blank	0.036	0.036	0.036
N	3	3	3	N	3	3	3
Mean	0.082	0.090	0.090	Mean	0.094	0.089	0.099
Std. Deviation	0.0078	0.0038	0.0051	Std. Deviation	0.0030	0.0036	0.0157
Std. Error of Mean	0.0045	0.0022	0.0030	Std. Error of Mean	0.0017	0.0021	0.0091
% Control	100	116	114	% Control	100	91	109
T test		P value		T test		P value	
Control vs GCEE 750 µM		0.17		Control vs GCEE 750 µM		0.14	
Control vs GCEE 900 µM		0.23		Control vs GCEE 900 µM		0.59	
GCEE 750 µM vs GCEE 900 µM		0.87		GCEE 750 µM vs GCEE 900 µM		0.33	

Table 4.2b Cytotoxicity of tBHP on cortical astrocytes (Supporting data)

30 min				60 min			
	Control	tBHP 100 µM	tBHP 200 µM		Control	tBHP 100 µM	tBHP 200 µM
A	0.073	0.08	0.079	A	0.091	0.091	0.073
B	0.086	0.098	0.089	B	0.097	0.094	0.079
C	0.087	0.094	0.092	C	0.094	0.09	0.079
Blank	0.035	0.04	0.038	Blank	0.036	0.037	0.038
N	3	3	3	N	3	3	3
Mean	0.082	0.091	0.087	Mean	0.094	0.092	0.077
Std. Deviation	0.0078	0.0095	0.0068	Std. Deviation	0.0030	0.0021	0.0035
Std. Error of Mean	0.0045	0.0055	0.0039	Std. Error of Mean	0.0017	0.0012	0.0020
% Control	100	108	104	% Control	100	94	67
T test			P value	T test			P value
Control vs tBHP 100 µM			0.29	Control vs tBHP 100 µM			0.33
Control vs tBHP 200 µM			0.48	Control vs tBHP 200 µM			3.02E-03
tBHP 100 µM vs tBHP 200 µM			0.58	tBHP 100 µM vs tBHP 200 µM			3.27E-03

Table 4.3b Levels of intracellular oxidative stress in cortical astrocytes exposed to non-cytotoxic tBHP and treated with GCEE (Supporting Data)

	Control		tBHP 200 µM		tBHP 200µM GCEE 750µM	
C1	31851	33573	33795	41510	26823	25767
C1	29789	39003	38627	33091	34266	30761
C1	39725	46667	44133	39081	34384	32056
B1	10504	8951	9204	9502	12193	11551

Treatment	Mean	N	Std. Deviation	Std. Error of Mean	% of Control
Control	27041	6	6726.1	2745.9	100
tBHP 200µM	29020	6	4322.8	1764.8	107
tBHP 200µM GCEE 750µM	18804	6	3569.0	1457.0	70

T test	P values
Control vs tBHP 200 µM	5.58E-01
tBHP 200 µM vs tBHP 200 µM + GCEE 750 µM	1.20E-03

Table 4.4b Viability of cortical astrocytes treated with GCEE against tBHP-mediated death (Supporting data)

	C1	C2	C3	B4	B5	B6
Control	0.131	0.129	0.143	0.043	0.043	0.042
tBHP 200 µM + GCEE 0 µM	0.118	0.124	0.115	0.048	0.047	0.046
tBHP 200 µM + GCEE 750 µM	0.117	0.128	0.136	0.051	0.054	0.052
tBHP 200 µM + GCEE 900 µM	0.126	0.156	0.135	0.054	0.055	0.050

	Mean	N	Std. Deviation	Std. Error of Mean
Control	0.134	3	0.00757	0.00437
tBHP 200 µM + GCEE 0 µM	0.119	3	0.00458	0.00265
tBHP 200 µM + GCEE 750 µM	0.127	3	0.00954	0.00551
tBHP 200 µM + GCEE 900 µM	0.139	3	0.01539	0.00889

T test	P value
Control vs tBHP 200 µM + GCEE 0 µM	0.0399
Control vs tBHP 200 µM + GCEE 750 µM	0.356
Control vs tBHP 200 µM + GCEE 900 µM	0.662
tBHP 200 µM + GCEE 0 µM vs tBHP 200 µM + GCEE 750 µM	0.356
tBHP 200 µM + GCEE 0 µM vs tBHP 200 µM + GCEE 900 µM	0.0972
tBHP 200 µM + GCEE 750 µM vs tBHP 200 µM + GCEE 900 µM	0.315

Table 4.5b Levels of intracellular oxidative stress in cortical astrocytes exposed to cytotoxic tBHP and treated with GCEE (Supporting data)

	Control	Control	tBHP 200 µM	tBHP 200 µM	GCEE 750 µM +tBHP 200 µM	GCEE 750 µM +tBHP 200 µM
A	26704	43724	28671	27344	26368	24653
B	35778	47782	34150	31574	22526	26272
C	47292	47447	28113	24991	24020	23426
D	8740	8629	8236	7750	8086	9059

	Control	tBHP 200 µM	GCEE 750 µM +tBHP 200 µM
N	6	6	6
Mean	32770	21148	15972
Std. Deviation	8581.7	3152.1	1545.5
Std. Error of Mean	3503.5	1286.8	631.00
% of Control	100	65	49

T test	P value
Control vs tBHP 200 µM	1.10E-02
tBHP 200 vs tBHP 200 + GCEE 750	4.80E-03

Table 5.1b Levels of temporal intracellular oxidative stress in GCEE-treated cortical neurons (Supporting data)

	Control	tBHP 100 µM	tBHP 100 µM + GCEE 900 µM	tBHP 100 µM + GCEE 900 µM
C1	1067	1140	1154	798
C1	884	688	801	702
C1	781	679	820	886
B1	594	520	659	672
C2	1174	1182	1121	803
C2	962	738	871	760
C2	880	706	859	908
B2	662	604	672	632
C3	1196	1265	1185	870
C3	1018	779	926	760
C3	958	760	886	989
B3	691	588	666	694
C4	1337	1321	1332	952
C4	1106	870	1008	829
C4	1030	830	1040	1100
B4	781	660	719	716
C5	1454	1466	1470	1071
C5	1231	939	1049	883
C5	1158	908	1098	1190
B5	858	714	781	735
C6	1704	1553	1617	1122
C6	1370	1017	1172	1083
C6	1297	1015	1224	1349
B6	984	727	851	767
C7	1839	1774	1756	1333
C7	1604	1171	1339	1190
C7	1475	1149	1400	1480
B7	1111	847	911	784
C8	2144	1925	1937	1438
C8	1827	1257	1360	1360
C8	1674	1345	1534	1585
B8	1284	875	998	867
C9	2305	2099	2105	1696
C9	2004	1481	1519	1465
C9	1926	1478	1702	1753
B9	1471	1037	1129	936
C10	2687	2312	2376	1885
C10	2242	1624	1726	1720
C10	2190	1615	1820	1948
B10	1703	1155	1232	999
C11	3099	2525	2577	2083
C11	2597	1841	1868	1868
C11	2447	1873	2134	2113
B11	1932	1307	1350	1069

Table 5.1b (continued)

	Control	tBHP 100 µM	tBHP 100 µM + GCEE 900 µM	tBHP 100 µM + GCEE 900 µM
C12	3414	2826	2953	2285
C12	2857	2003	2021	2147
C12	2765	2105	2388	2319
B12	2175	1382	1484	1235
C13	3910	3097	3163	2635
C13	3114	2346	2224	2275
C13	3143	2277	2595	2561
B13	2497	1564	1667	1256

	Mean			Std. Deviation			Std. Error of Mean		
	Control	tBHP	T+G	Control	tBHP	T+G	Control	tBHP	T+G
1	317	316	195	145	264	159	83.6	152	64.9
2	343	271	235	152	266	115	87.6	154	47.1
3	366	347	256	124	286	151	71.5	165	61.8
4	377	347	326	160	273	168	92.3	157	68.5
5	423	390	369	154	314	186	89.0	181	75.9
6	473	468	452	217	310	183	125	179	74.6
7	528	518	569	185	355	171	107	205	69.7
8	598	634	603	240	363	201	138	210	82.0
9	607	649	674	200	358	215	116	207	87.7
10	670	695	797	273	400	242	158	231	98.8
11	782	773	898	341	386	249	197	223	102
12	837	929	993	351	449	303	203	259	124
13	892	1009	1114	451	455	348	261	263	142

	Relative mean			P values	
	Control	tBHP	tBHP + GCEE	Control vs tBHP	tBHP vs tBHP + GCEE
1	1	1.00	0.61	0.996	0.409
2	1	0.79	0.68	0.705	0.774
3	1	0.95	0.70	0.918	0.541
4	1	0.92	0.87	0.879	0.888
5	1	0.92	0.87	0.879	0.898
6	1	0.99	0.96	0.983	0.924
7	1	0.98	1.08	0.965	0.770
8	1	1.06	1.01	0.892	0.870
9	1	1.07	1.11	0.869	0.896
10	1	1.04	1.19	0.932	0.642
11	1	0.99	1.15	0.976	0.568
12	1	1.11	1.19	0.793	0.806
13	1	1.13	1.25	0.767	0.710

Table 5.2b Levels of temporal intracellular oxidative stress in GCEE-treated cortical astrocytes (Supporting data)

	Control	GCEE 900 µM	tBHP 100 µM		tBHP 100 µM + GCEE 900 µM	
C1	6391	701	7654	7695	637	583
C1	747	738	611	750	603	690
C1	614	677	612	657	584	562
B1	132	183	169	214	240	199
C2	6535	759	7702	7746	691	628
C2	841	838	679	843	653	743
C2	701	767	683	733	639	613
B2	134	188	177	225	251	206
C3	6645	880	7758	7791	761	689
C3	965	970	768	962	726	816
C3	803	886	778	831	707	677
B3	139	197	187	236	262	216
C4	6687	1015	7859	7880	848	769
C4	1110	1115	875	1100	810	897
C4	925	1011	886	944	795	765
B4	141	205	201	249	273	228
C5	6739	1179	7997	7975	963	867
C5	1282	1291	1004	1261	919	1011
C5	1073	1163	1019	1082	907	872
B5	149	218	218	266	288	243
C6	6777	1350	8144	8145	1090	991
C6	1473	1472	1152	1450	1047	1148
C6	1239	1324	1173	1241	1038	995
B6	154	241	238	289	309	262
C7	6822	1536	8336	8309	1246	1134
C7	1676	1677	1322	1661	1196	1300
C7	1433	1501	1347	1421	1198	1146
B7	162	264	263	315	334	284
C8	6855	1722	8524	8451	1417	1292
C8	1894	1889	1505	1898	1356	1479
C8	1625	1683	1552	1625	1372	1309
B8	171	289	293	345	363	311
C9	6885	1913	8749	8692	1601	1469
C9	2125	2094	1710	2149	1542	1666
C9	1846	1871	1760	1840	1559	1495
B9	183	317	327	382	398	344
C10	6947	2107	8986	8895	1795	1664
C10	2367	2329	1935	2422	1742	1877
C10	2079	2068	2008	2082	1765	1702
B10	197	349	369	424	437	379
C11	7005	2314	9226	9098	2012	1858
C11	2611	2548	2168	2696	1948	2095
C11	2330	2263	2264	2340	1998	1911
B11	214	385	409	470	477	419
C12	7067	2498	9467	9402	2234	2066

Table 5.2b (continued)

	Control	GCEE 900 µM	tBHP 100 µM		tBHP 100 µM + GCEE 900 µM	
C12	2862	2793	2426	2994	2171	2316
C12	2588	2452	2535	2603	2218	2126
B12	231	422	456	519	526	460
C13	7190	2694	9739	9642	2457	2278
C13	3120	3029	2701	3297	2405	2556
C13	2848	2657	2830	2888	2476	2346
B13	252	462	503	569	578	500
C14	7326	2894	10034	9906	2694	2504
C14	3369	3279	2983	3606	2639	2792
C14	3124	2878	3129	3193	2740	2595
B14	269	508	555	630	635	555
C15	7490	3094	10340	10268	2930	2739
C15	3634	3525	3272	3923	2893	3042
C15	3416	3087	3452	3496	2988	2818
B15	298	551	610	690	692	611
C16	7693	3298	10691	10516	3184	2963
C16	3899	3798	3588	4254	3141	3292
C16	3703	3292	3774	3800	3244	3044
B16	324	602	666	747	754	661
C17	7916	3521	11067	10896	3441	3231
C17	4204	4070	3904	4601	3401	3538
C17	4033	3511	4102	4123	3543	3267
B17	354	656	725	815	819	721
C18	8141	3736	11398	11207	3699	3474
C18	4471	4368	4232	4936	3643	3804
C18	4322	3746	4443	4448	3830	3558
B18	381	706	786	880	884	778
C19	8391	3951	11755	11544	3979	3729
C19	4755	4656	4589	5298	3932	4086
C19	4661	3988	4796	4763	4100	3790
B19	415	761	851	943	957	836
C20	8622	4191	12144	11918	4268	4001
C20	5062	4965	4924	5649	4203	4349
C20	5005	4217	5179	5126	4413	4074
B20	448	820	911	1018	1025	896
C21	8894	4405	12553	12296	4532	4246
C21	5353	5256	5282	6014	4476	4623
C21	5314	4479	5548	5482	4677	4322
B21	481	880	976	1086	1094	963

	Mean				Std. Deviation			
	Control	GCEE	tBHP	T+G	Control	GCEE	tBHP	T+G
1	2452	522	2805	390	3298	31	3624	53
2	2558	600	2863	433	3329	43	3610	54
3	2665	715	2937	490	3327	50	3584	57

Table 5.2b (continued)

	Mean				Std. Deviation			
	Control	GCEE	tBHP	T+G	Control	GCEE	tBHP	T+G
4	2766	842	3032	564	3275	59	3573	55
5	2882	993	3148	658	3213	70	3561	58
6	3009	1141	3287	766	3132	79	3559	62
7	3148	1307	3444	894	3044	93	3557	63
8	3287	1476	3607	1034	2945	109	3536	70
9	3436	1642	3796	1184	2832	119	3543	72
10	3601	1819	3992	1350	2731	141	3530	77
11	3768	1990	4193	1522	2622	152	3513	83
12	3941	2159	4417	1696	2511	185	3513	86
13	4134	2331	4647	1860	2432	205	3497	104
14	4337	2509	4883	2066	2358	227	3487	99
15	4549	2684	5142	2250	2292	251	3501	104
16	4774	2861	5397	2437	2249	290	3492	113
17	5030	3045	5679	2634	2194	320	3518	116
18	5264	3244	5944	2837	2163	362	3512	124
19	5521	3437	6227	3040	2127	397	3513	133
20	5782	3638	6526	3258	2072	440	3525	135
21	6039	3833	6832	3451	2056	471	3542	143

	Relative mean				P values		
	Control	GCEE	tBHP	T+G	Control vs GCEE	Control vs tBHP	tBHP vs T+G
1	1	0.2130	1.1440	0.1592	0.368	0.892	0.134
2	1	0.2345	1.1192	0.1691	0.366	0.906	0.130
3	1	0.2683	1.1017	0.1840	0.367	0.916	0.126
4	1	0.3044	1.0962	0.2037	0.366	0.917	0.121
5	1	0.3445	1.0921	0.2282	0.366	0.917	0.118
6	1	0.3792	1.0925	0.2546	0.360	0.912	0.113
7	1	0.4152	1.0938	0.2841	0.354	0.906	0.110
8	1	0.4489	1.0973	0.3145	0.347	0.897	0.105
9	1	0.4780	1.1047	0.3447	0.335	0.884	0.101
10	1	0.5052	1.1085	0.3748	0.322	0.873	0.097
11	1	0.5281	1.1127	0.4040	0.306	0.860	0.092
12	1	0.5478	1.1207	0.4302	0.287	0.842	0.087
13	1	0.5639	1.1241	0.4500	0.270	0.829	0.080
14	1	0.5785	1.1257	0.4763	0.252	0.817	0.076
15	1	0.5901	1.1304	0.4947	0.234	0.801	0.071
16	1	0.5992	1.1305	0.5105	0.218	0.790	0.065
17	1	0.6053	1.1289	0.5235	0.196	0.782	0.060
18	1	0.6163	1.1293	0.5390	0.186	0.771	0.056
19	1	0.6226	1.1280	0.5506	0.171	0.762	0.051
20	1	0.6292	1.1287	0.5634	0.154	0.750	0.047
21	1	0.6347	1.1312	0.5714	0.144	0.736	0.042

VITA

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