Neuroprotective Effects of Sulforaphane on Oxygen/Glucose Deprived Neurons and Astrocytes

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Abstract

Cerebral Palsy (CP) constitutes the primary outcome of premature nerve injury. Impairment of oxygen and glucose supply during pregnancy to the fetus can induce neuron damage and death. Mental retardation, seizures, learning disabilities, and other mental diseases can occur as a result of this insufficient nutrient delivery to the fetus. Therapeutic interventions that are efficacious for the injured newborn are limited mainly because the majority of insults (90%) resulting in CP occur during pregnancy and current therapies only address those injuries that occur during labor and delivery or after birth, therefore addressing only 10% of the injured newborns. This investigation was aimed to determine neuroprotective effects of sulforaphane. Sulforaphane is an isothiocyanate found in vegetables such as broccoli sprouts, Brussels sprouts, and cabbage that have anti-inflammatory, anti-oxidant, anti-apoptotic effects.

Neurons and astrocyte cell death was decreased with sulforaphane treatment during oxygen-glucose deprivation. Cell death was analyzed through trypan blue viability assay, lactate dehydrogenase assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, DNA quantification, and FAM-DEVD-FMK caspase 3 and 7 inhibitor immunofluorescence quantification.

This study demonstrates neuroprotective effects of sulforaphane for neurons and astrocytes and its potential as a preventative treatment for pregnant mothers.

Dedicated to family and friends

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List of Abbreviations

ADHD	Attention deficit hyperactivity disorder
AEDs	Antiepileptic drugs
AMPK	AMP-activated protein kinase
ARE	Antioxidant-response element
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BrSps	Broccoli sprouts
CAM	Complementary and alternative medicine
CMV	Cytomegalovirus
CP	Cerebral palsy
EMG	Electromyography
EPIIs	Phase II detoxifying enzymes
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
Hb	Haemoglobin
HI	Hypoxia ischemia
HIE	Hypoxia ischemic encephalopathy
ITCs	Isothiocyanates
Hp	Haptoglobin

HO-1	Heme oxygenase -1
H_2O_2	Hydrogen peroxide
Keap 1	Kelch-like ECH –associated protein
LBW	Low birth weight
LDH	Lactate dehydrogenase
LP	Lipid peroxidation
MACS	Manual ability classification system
MCAO	Middle cerebral artery occlusion
MEK	Mitogen protein kinase
$MgSO_4$	Magnesium sulphate
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHE	Na^+/H^+ exchanger
NMDA	N-methyl-D-aspartate
NHPs	Natural Health Products
Nrf2	Nuclear factor (erythroid-derived 2)-like
NSE	Neuronal Specific Enolase
OGD	Oxygen glucose deprivation
PARP	Poly ADP ribose polymerase
PDL	Poly-D-lysine
PIS	Prenatal Ischemic Stroke

ROS	Reactive oxygen species
SFN	Sulforaphane
TRLs	Toll-like receptors
TNF – α	Tumour necrosis factor – α
TSC1/2	Tuberous sclerosis complex 1/2
VEGF	Vascular endothelial growth factor
4-HNE	4-hydroxy-2-nonenal

Chapter 1
Introduction

1.0 Introduction

1.1 Cerebral Palsy (CP)

1.1.1 Definition

Cerebral palsy (CP) is a life-long neurodevelopmental disorder caused by non-progressive disturbances that occur in the developing fetal or infant brain. The end results are disorders in the development of movement and posture, which includes disturbances of sensation, cognition, communication, perception, behaviour, and seizures [1]. Lifetime costs associated with a person with CP is approximately $1,300,000 for males and $1,200,000 for females, and the economic cost associated with CP is $11.5 billion [2, 3].

Children born with very low birth weight (LBW) after neonatal complications are more likely to develop neurosensory disorders including CP [4, 5]. LBW, in accordance with the World Health Organization, is defined as an infant born weighing <2500g [6]. Children born with normal birth weights (2500 – 5000 g) can also develop CP [7]. However, normal birth weight children with CP are more likely to have severe motor impairments [8].

Frequency of CP in newborns is relatively stable despite significant advances in obstetric and neonatal care [9]. Overall prevalence of CP is reported to be about 2 per 1000 live births for full term infants while it ranges from 55 to 150 per 1000 live births for preterm infants [10], where preterm infants are defined as those born at <37 weeks from the first day of the last menstrual period [6].

1.1.2 Diagnosis

There are two early detection tools of CP: General Movements Assessment (GMS) and Test of Infant Motor Performance (TIMP). Using the classification systems can provide a more in-depth and holistic understanding and how CP is affecting the patient.

1.1.2.1 General Movements Assessment (GMS)

The GSM is a qualitative analysis of videotapes of spontaneous or self-initiated movements of preterm and term infants used for CP diagnosis. Healthy infants at 6 to 9 weeks after birth are able to move their neck, trunk, and limbs in small circular ways to execute goal-oriented tasks. In contrast, children who have suffered brain injuries have rigid limb and trunk movements [11].

1.1.2.2 Test of Infant Motor Performance (TIMP)

TIMP has high interrater reliability and validity and can identify motor delays early as 7 days after birth. It is successful in diagnosing 80% of children with CP by 2 months. Diagnosis is made through evaluation of children's functional motor control and organization of posture and movements [12]. After diagnosis, the severity of CP can be classified through movement and clinical classifications.

1.1.3 Classifications

1.1.3.1 Movement Classification

Positive and negative signs clinically characterize movement disorders. Positive signs are abnormalities due to absence of inhibition from cortical circuits which includes spasticity, dyskinesia, hyper-reflexia, retained developmental reactions, and secondary musculoskeletal malformation. Negative signs reflect poor coordination of movement, balance, absent or loss of proper sensorimotor control mechanisms [13]. The positive and negative signs manifest in children with CP as disturbed motor control during walking.

1.1.3.2 Walking Classification

In those with CP, the cortical neurons that project from the motor cortex to limbs were altered or did not develop properly, which resulted in impairments of voluntary movements [14]. Difficulties in walking/walking dysfunction arise from insufficient activation of agonist muscles

with hyper activation of antagonist muscles. Quantification of electromyography (EMG) activity can be used to evaluate relative contributions of paresis, spasticity, excessive co-activation, or hypoextensibility of walking. These analyses are used to classify walking patterns as to hemiplegic or diplegic.

Although walking classifications are useful in individualizing therapeutic approaches, they do not provide insight into the underlying dysfunction, which limits its use as a clinical classification instrument. However, gait analysis can be used to evaluate physiotherapy or surgical intervention effectiveness [13].

1.1.3.3 Clinical Classifications

Gross Motor Function Classification Systems (GMFCS) and Manual Ability Classification System (MACS) are used to classify and categorize motor functions in children with CP. These classification systems help to ensure that the care provided is appropriate.

1.1.3.3.1 Gross Motor Function Classification System (GMFCS)

The Gross Motor Function Classification System (GMFCS) is a standardized, clinically applicable system that is based on sitting, walking, and the need for assistive devices. GMFCS is used internationally to best represent the child's abilities and limitations using a five-level system. Children in Level I can walk without restrictions with limited advanced motor skills, while children in Level V have very limited movement even with the use of assistive technology [13].

Relationship between Brain Magnetic Resonance Imaging (MRI) and GMFCS

MRI is the imaging system of choice for defining relationships between brain structure and motor outcomes in children with CP. MRI analysis revealed that brain malformations occur during the first and second trimesters resulted in spastic hemiplegia, followed by spastic

quadriplegia, spastic diplegia, then non-spastic CP subtypes. Brain malformations make up 9% of all lesions and subjects were classified as GMFCS III-V.

The most common brain lesions are those in the white matter during early third trimester or in preterm infants, which make up 46% of all lesions. White matter lesions resulted in spastic diplegia, spastic hemiplegia, spastic quadriplegia, and then non-spastic subtypes. Two thirds of these children had mild functional disabilities in the level I and II of the GMFCS.

Grey matter injuries accounting for 25% of all lesions and the majority of patients with grey matter lesions were classified as GMFCS III-V. Prenatal strokes and other postnatally acquired lesions make up 1% of all classified lesions. 12% of patients with CP who had normal MRI findings were most likely to have mild motor impairment with over two-thirds in the GMFCS I-II group [15].

In addition, MRI has also helped in revealing the association between location and extent of brain injury and functional motor deficits. Lesions in both grey and white matter has been shown to lead to more severe motor disabilities compared to those with lesions in either grey or white matter. Also, more severe injuries with greater reduction in white matter lead to significantly lower limb function [15].

1.1.3.3.2 Manual Ability Classification System (MACS)

MACS is a five level system that classifies the ability of children with CP to handle every-day objects. Those in Levels I and II are more independent while Levels III require assistance. Children in Levels IV and V require additional support and assistance. MACS helps in developing therapies for children to maximize rehabilitation outcomes. Although MACS, GMFCS, and typical diagnostic classifications are independent of one another, MACS and GMFCS levels are highly correlated with one another [13, 16]. Thus, using both classification

systems can provide a more holistic picture of children with CP for clinical, therapeutic, and research purposes.

1.1.4 Clinical Outcome Measures

After diagnosis and initial classification of the severity of CP, Gross Motor Function Measure (GMFM) can be used for long-term clinical evaluation. GMFM is a test with high intra- and interrater reliability that quantifies the changes in gross motor abilities over time and thus can be used to evaluate effectiveness of therapy or surgery. It is composed of 88 items that is measured by observation [17].

1.1.5 Aetiology

CP results from synergy of predisposing factors, prenatal and postnatal events to alter the developing and maturing brain. Prenatal risks factors are associated with 70-80% of CP, while intra-partum complications make up 10-20% [18]. Fetal brain injury from prenatal and postnatal complication account for roughly 40% of all cases of intellectual disability or global developmental delay [19].

1.1.5.1 Prenatal Hypoxic-Ischemic Insult

Prenatal brain damage from hypoxia-ischemia (HI) and/or inflammation is one of the leading causes of lifelong disabilities including CP, seizure disorders, sensory impairment, and cognitive limitations [20]. A late prenatal or prenatal hypoxic-hemodynamic insult is one of the major contributors of CP [21]. Children who suffered prenatal brain injury show increased incidence of developmental delays, motor disabilities, anxiety disorders, and autism spectrum disorders [22]. The ischemia results from decreased cardiac output as a result of decreased myocardial contractility from severe hypoxemia [18]. Hypoxemia can be caused by prenatal arterial

ischemic stroke, which is defined as to occur between 28 weeks gestation and 28 days of postnatal life [23]. Prenatal ischemic stroke (PIS) arises as a result of fetal or maternal arterial or venous thrombosis or embolization in the fetus. PIS is 17 times more likely to occur in the prenatal period due to a combination of maternal, neonatal, and placental conditions [24]. Some maternal risk factors include smoking, preeclampsia, and thrombophilia, while fetal risk factors include congenital heart disease, hypoglycaemia, and infection [25]. Placental risk factors, which include placental lesions, abruption, and chorioamnionitis that reduce perfusion, have been associated with prenatal stroke, hypoxia ischemia encephalopathy (HIE) and CP [26]. A combination of these risks factors that reduce blood flow and result in ischemic insult, can result in motor, cognitive, or behavioural difficulties can arise including CP [24].

The etiology of more than half of all children with CP is related to some form of cerebrovascular focal or global insult. In preterm infants, hypoxic-ischemic injury adversely affects white-matter tract development and predisposes the brain to periventricular white-matter injury and CP. Subplate neurons, transient during human fetal brain development, are vulnerable to HI in preterm babies with long-term consequences. In full term newborns, the striatum, thalamus, and cortex are vulnerable and often affected [26]. More mature fetuses are less vulnerable to white matter injury or are differentially vulnerable depending on the type of insult [18].

1.1.5.2 Birth Asphyxia

Birth asphyxia is the interruption of placental blood flow during labour [27]. Up to 60% reduction of blood flow can occur leading to intra-partum hypoxia [28]. For “near total asphyxia”, a brief but intense insult injures cerebral cortex, basal ganglia, and the brainstem leading to rigidity of movement [18]. Lesions of the extrapyramidal tracks in the basal ganglia and the subsequent disruption of the neurological circuits involved in motor activation are

responsible for the rigidity of movements [29]. On the other hand for less severe but sustained asphyxia, referred to as "partial or prolonged asphyxia", injury is more multifocal and diffuse within the cerebral cortex. Partial asphyxias cause less prominent neuronal damage, and are more restricted to subcortical white matter resulting in spastic movements and upper motor neuron abnormalities involving the four extremities [18]. Cortical upper neurons affected in partial asphyxia disinhibit thalamocortical projections leading to hyperkinetic spastic movements [29].

1.1.5.3 Infection &Inflammation during Pregnancy and Birth

Both bacterial and viral infections can lead to CP. Bacterial chorioamnionitis associated with *E. Coli* or group B *streptococci* infection is most frequently associated with CP. Almost 30% of CP is attributed to chorioamnionitis in preterm infants [30]. Cytomegalovirus (CMV) is the most frequent virus implicated in brain damage during pregnancy. The viral exposure may be associated with hypertensive disorders, preterm delivery, and CP [31]. The infections from bacterial chorioamnionitis and CMV cause inflammation, and inflammation of the foetal membranes has been associated with CP [9].

Inflammation is caused by oxidative stress, brought on by PIS and asphyxia, when there is an imbalance between oxidant production and scavenging [32]. During inflammation, there is an up-regulation of inflammatory cytokines. To this end, IL-1β and TNF-α have been shown to be upregulated in the brains of human newborns afflicted with CP. The molecular cascade associated with IL-β and TNF-α has been predicted to lead to prenatal brain damages [30]. Mechanistically, cytokines released from endotoxin-activated macrophages or by glial cells activate monocytes infiltrating the brain. The cytokine-activated cells release reactive oxygen species (ROS) and toxic granules enhancing cellular and tissue damage. Cytokines can also activate cytotoxic T cells, natural killer cells, and lymphokine-activated cells [20]. The cytokines released within the brain leads to apoptosis of oligodendrocytes, microglial activation,

haemorrhage, and myelin damage [30]. The neurotoxic effects of cytokines are due to potentiation of noxious effects of excitotoxic cascades or oxidative stress by converting a sub threshold insult to a seriously damaging event [31].

Preterm infants are more highly susceptible to ischemic related inflammatory pathway activation with higher prevalence rate of developmental disabilities such as CP [33].

1.1.5.4 Genetic factors of CP

There is an increased risk of CP in some families. Although uncommon, familial CP accounts for almost 2% of all CP cases [34]. Thrombophilia has underlying genetic basis that involves polymorphic variations of Factor V Leiden, Prothrombin G20210A, and MethylenetetrahydrofolateReductase C677T genes [35]. Those with predisposing genetic factors to thrombophilia are more likely to suffer from thrombosis, and in turn, stroke. About half the infants who suffered a stroke were found to be thrombophilic. Underlying genetics factors involved in thrombophilia in combination with viral or bacterial infection causes vascular thrombosis leading to prenatal strokes and secondary CP. In addition, preterm birth, placental abruption, preeclampsia have also been shown to have genetic association [36]. Further, polymorphisms of genes encoding inflammatory proteins and coagulation factors are also associated with CP in some children [31]. During pregnancy, coagulation factors such as von Willebrand factor, and plasma fibrinogen concentrations are found at a higher concentration in the mother's blood to protect against haemorrhage during delivery [37]. Genetic polymorphisms of prothrombotic factors such as methylene tetrahydrofolatereductase (MTHFR), Factor V Leiden, and prothrombin increase risk of neonatal stroke. Further, mutations in COL4A1, a gene that encodes the α-1 subunit of collagen type IV has been shown to be increase incidences of intracerebral haemorrhages [26].

1.2 Pathology of Hypoxic-Ischemic Brain Damage

1.2.1 Cytoplasmic Acidification

Insufficient oxygen supply serves as a switch from aerobic to anaerobic metabolism which increases lactate formation. This results in acidification of cellular cytoplasm, which hyper activates the Na^+/H^+ exchangers (NHE). Hyper activation of NHE leads to alkaline shift in pH which results in a Na^+ overload with increased Ca^{2+} entry and subsequent cell death. Increased intracellular Ca^{2+} leads to generation of oxygen and nitrogen free radicals, and other toxic free radicals, which promotes apoptosis, and necrosis [38].

1.2.2 Necrosis and Apoptosis

Necrosis is considered as the predominant early neuronal death mechanism immediately following ischemic insult, while apoptosis and autophagy has been related to delayed cell death [26].

The first phase of brain cell death, necrosis, involves energy depletion, excitotoxicity, and free radical damage. Insufficient oxygen and energy depletes high-energy phosphate compounds such as phosphocreatinine and adenosine triphosphate (ATP). Without ATP, ion gradients cannot be maintained resulting in influx of Na^+ and Ca^{2+}. Water follows the influx of these ions resulting in cytotoxic oedema and the subsequent cell rupture lysis [39]. Also, the hypoxic-ischemic brain releases large amounts of the excitatory amino acid, glutamate. Activation of glutamate receptors found to peak in the early neonatal period in both neuronal and glial cells, leads to excessive depolarization, NaCl and water influx, swelling, and cell death. N-methyl-D-aspartate (NMDA) receptors, a type of glutamate receptor, are predominant mediators for calcium-mediated exocitotoxicity in neonatal hypoxic-ischemic brain injury. Influx of Ca^{2+}through NMDA receptors activates phospholipase A_2 and free radicals from arachidonic acid damaging the cellular membrane [21].

The second phase of cell death, which occurs hours to days post injury is apoptosis. Ischemia triggers apoptosis by stimulating caspase or BAX activation. BAX is a member of the Bcl-2 gene family with proapoptotic functions that regulates and release of cytochrome C from the mitochondria to contribute to apoptosis [39]. Up regulation of the FAS death receptor and over activation poly ADP ribose polymerase (PARP) also play a role in triggering apoptosis. Free radicals that are formed during the first phase of cell death causes mitochondrial dysfunction activating the intrinsic apoptotic pathway by activating proapoptotic genes [31].Neuronal apoptosis is more commonly observed in the immature brain as apoptotic pathways are more readily activated in the immature brain [26].

During reperfusion, reactions from primary ischemic insult are exacerbated including excess oxygen free radicals, intracellular Ca^{2+} accumulation, microvascular endothelial dysfunction, nitric oxide formation, and activation of apoptotic pathways. Reperfusion and oxygenation can also promote further mitochondrial injury and neuronal apoptotic death [27]. Infarct region of 8 hours of reperfusion compared to 2 hours after 60 minutes of middle cerebral artery occlusion (MCAO) was found to be significantly greater in mice [40].

1.2.3 Microglial Activation

Microglia make up 10% of overall number of cells in the brain during development. Microglia can be activated by various factors including cytokines, by-products of bacteria, and disruption of the tissue after trauma, and release of intracellular contents after ischemia [41]. In turn, the activated microglia produces inflammatory mediators, free radicals, and other toxic molecules to promote the immune response, which is injurious in the ischemic brain [26]. For example, matrix metalloproteinases (MMPs) and plasminogen activators that digest damaged tissue, cytokines and chemokines that increase proliferation of microglia, and toll-like receptors (TLRs) that

increase cytokine and chemokine productions are activated and up-regulated after upon microglial activation [41].

Inhibition of microglia activation was found to have beneficial effects. The anti-inflammatory drug, minocycline, which inhibits microglial activation, was shown to reduce levels of inflammatory mediators (IL-1β, TNF-α), reduced neuro-inflammation and brain tissue loss after HI in neonatal rats [42].

However, a second role of microglia in phagocytosis is beneficial in repairing the extracellular matrix after stroke [43]. Microglial phagocytosis of dead cells after HI/stroke event helps to limit and prevent further inflammation and hence limits extension to unaffected regions [41]. Thus, microglia may have a dual role after ischemic injuries.

1.2.4 Reactive Oxygen Species (ROS)

Cerebral ischemia leads to accumulation of ROS. ROS include compounds such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH), nitrosoperoxocarbonate ($ONOOCO_2^-$), oxygen free radical, hydroxyl radical, nitrogen dioxide radical, and carbonate radical. H_2O_2 is formed through NADPH oxidase that catalyzes the reaction of oxygen and NADPH to superoxide (O_2^-), hydrogen ion, and $NADPH^+$. As shown in Figure 1, once oxygen is reduced to superoxide, it can react with itself to form H_2O_2 [44]. ROS production also causes peroxidation of lipids, protein oxidation, DNA cleavage, mitochondrial dysfunction and altered signal transduction. Collectively, these events are referred to as ROS-mediated oxidative stress, which can culminate into cell death of all brain cells [45].

ROS is a crucial facet of microglial activation as well. Upon microglial activation, chemotactic molecules, metalloproteases, and pro-inflammatory cytokines are produced. Furthermore, activated microglia can itself also release ROS through activation of NADPH oxidase, which

results in further expression of pro-inflammatory cytokines. Persistent ROS and cytokine release activates apoptotic programs leading to neuronal death [46].

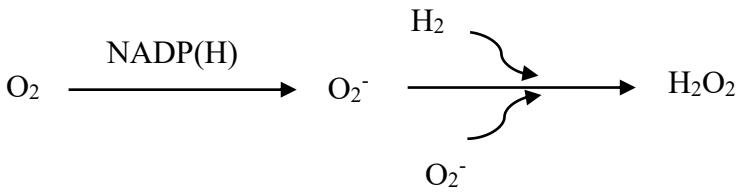

Figure 1 – ***Schematic representation of hydrogen peroxide formation***

ROS mediated lipid peroxidation (LP)

LP involves oxidation of polyunsaturated fatty acids that subsequently undergoes phospholipase-mediated hydrolysis. LP is normally initiated by a radical species interacting with polyunsaturated fatty acids that removes an allylic carbon to form an alkyl radical. The alkyl radical then reacts with oxygen to form a lipid peroxyl radical, which then reacts with another allylic carbon to form a second generation of alkyl radical and a lipid hydroperoxide. Once lipid hydroperoxides are formed, either ferrous iron (Fe^{++}) or ferric iron (Fe^{+++}) reacts to form alkoxyls or a peroxyl radicals, respectively [47]. The result of LP is severe disruption/damage of cellular membranes due to the loss of membrane phospholipid architecture, loss of proper function of phospholipid-dependent enzymes, ion channels, and structural proteins.

The presence of a peroxidized fatty acid breakdown product, 4-hydroxy-2-nonenal (4-HNE) is indicative of oxidative stress induced LP [48]. 4-HNE is highly reactive with amino acids and mitochondria to impair cellular proteins and mitochondrial respiration. 4-HNE is able to react

with basic and sulfhydryl groups in amino acids and directly inhibit mitochondrial respiration by binding to mitochondrial proteins. Specifically in the mitochondria, 4-HNE targets pyruvate dehydrogenase and complex-I mitochondrial redox carrier associated proteins [49]. Free radical-mediated oxidative damages in CNS injuries result in protein oxidation and mitochondria dysfunctions [50].

1.3 Therapeutic options for CP and their Limitations

1.3.1 Prenatal Therapies

Some clinical trials have suggested that antenatal maternal administration of magnesium sulphate ($MgSO_4$) protected babies born preterm against CP. $MgSO_4$, taken by pregnant women at high risk of early preterm delivery was shown to reduce CP rates by 32% compared to the placebo-trial group [51]. However, long-term motor, cognitive, and behavioural outcomes in children were insignificant. MgSO4's protective mechanisms of reducing neural dysfunction are still unclear [52]. A possible mechanism of neuroprotection is initiated by brain-derived neurotrophic factor (BDNF). BDNF inhibits of apoptosis and inflammation and increases neurogenesis. Cord blood BDNF level drawn from preterm infants was higher with $MgSO_4$ administration before birth compared to preterm infants born without antenatal $MgSO_4$. BDNF acts as the first line of defense against CP in premature infants by preventing the release of cytokines like IL-1β, IL-6, and TNF-α by binding to NDMA receptors to prevent glutamate binding [53].

In contrast, using other types of NMDA receptor antagonists have shown to have detrimental effects. For example, when NMDA receptor antagonists, MK-801, phencyclidine, ketamine, or carboxypiperzain-4-yl-propyl-phosphoric acid (CPP) were injected to neonatal rat pups, neurodegeneration was potentiated negatively affecting neurodevelopment [54]. Ketamine

administration on postnatal day 10 mice induced cortical cell degeneration lead to deficits in learning, retention of memory, and motor capabilities [55].

Similarly, antiepileptic drugs (AEDs) that inhibit glutamatergic neurotransmission by blocking NMDA receptors have been used for CP [56]. Most commonly used AEDs have been shown to have undesirable effects. AEDs cause neurons to undergo apoptotic death in the developing rat brain affecting intellectual skills [57]. Depending on the stage of brain development, AEDs can have consequences on the developmental mechanisms of neuronal circuitry and, eventually the expression of neurobehavioral disorders, such as attention deficit hyperactivity disorder (ADHD) [55].

In addition to inconclusive and negative findings of prenatal therapies, the public resistance to any risk from prescribed pharmaceuticals during pregnancy and the rigorous licensing procedure add to the difficulty of finding effective prenatal therapeutics for CP[58].

1.3.2 Postnatal Therapies

Therapies available for CP in the posnatal period are very limited in their efficacy. The main reason for this is that currently used therapeutics at this stage address putative recovery after brain injury has already occurred.

The introduction of assisted ventilation of preterm infants born with CP was shown to increase their survival in the early 1980s. Postnatal steroids and lung surfactant therapies have also been introduced but their long-term outcomes on development have not been studied [9]. Although postnatal administration of corticosteroids was shown to be partially protective against early death, treatment may be associated with long-term risks with abnormal neurological exam and CP [9]. Hydrocortisone is a corticosteroid that is administered to preterm infants for its anti-

inflammatory effects. Infants who were administered with hydrocortisone had significantly lower mean full-scale intelligence quotient (IQ) compared to the control group not treated with hydrocortisone at 5 years of age[59]. Corticosteroids are believed to reduce myelin basic protein expression, which results in neurological deficits through the processes of hypomyelination [60]. Hypothermia is currently the only neuroprotective treatment for injury resulting from HIE. Hypothermia has been demonstrated to reduce mortality without increasing major disability in survivors, and its effectiveness has been shown from mice to sheep. Cooling of the brain is thought to potentially induce anti-inflammatory effects [26]. However, there seems to be no consensus yet concerning the extent and degree of cooling required for neuroprotection. Hypothermia has been shown to significantly improve short-term outcomes including reducing rate of severe neuromotor disability but long-term outcomes has not been reported [9]. Despite reduction in death and disability with therapeutic hypothermia, only 40% of infants survive with normal neurodevelopmental functions [38].

1.4 Natural Health Products

Natural health products (NHPs) are defined as vitamins and mineral supplements, herbal therapies, traditional medicines, probiotics, dietary supplements, or drugs [61]. They are considered to be natural because they contain ingredients that occur naturally from plants, mammals, and microorganisms. Use of NHPs for the prevention of injury for the fetal and newborn brain offers an innovative way to prevent prenatal brain injury with great promise for success. NHPs are considered to be safe for consumption during pregnancy by nurses, obstetricians, and midwives [62]. NHPs can be given prophylactically to mothers during pregnancy to prevent injury. Complementary and alternative medicine (CAM) is defined as the use of products and practices outside the conventional allopathic drugs and practices, which

includes NHPs [63]. CAM is already used by 57% of pregnant women for various reasons [64]. CAM is considered to be safe, pregnant women want to be in control of their own health, and they may be worried about side effects of pharmaceuticals [65]. Uses of CAMs are also considered safe and advocated by the majority of obstetricians [62]. In addition, because CAM are not subjected to the same rigorous and strict regulations of conventional pharmaceuticals, they are more accessible and available. In Canada, there are over 70,000 NHPs that have been licensed by Health Canada [61].

1.4.1 Cruciferous Vegetables

Cruciferous vegetables such as cauliflower, broccoli, cabbage, and Brussels sprouts have been shown to contain high amounts of antioxidants, vitamins, and fibre. Broccoli contains high amounts of selenium and glucosinolates such as glucoraphanin and isothiocyanate sulforaphane (SFN). Glucosinolates are hydrolyzed into isothiocyanates (ITCs) by myrosinase [66]. SFN is the isothiocyanate metabolite of the glucosinolate [67]. SFN can induce the antioxidant Nrf2/ARE pathway [32], and it is an inducer of enzymes important in protecting cells against oxidative stress such as heme oxygenase-1 (HO-1), glutathione (GSH) reductase, and GSH peroxidase [68]. Thus, SFN has potential use in the setting of a compromised fetal environment.

Benefits of ingesting broccoli have been demonstrated in rats. When rats were fed with broccoli for 30 days, the broccoli fed group appeared to rescue cardiomyocytes in the ischemic reperfused heart. An involvement of the PIK3-Akt survival pathway was implicated in this protective mechanism. Cardio protection was evident by improvement of ventricular function, reduction of myocardial infarction and apoptosis compared to the control. Broccoli fed animal heart tissue showed increased phosphorylation of Akt, which in turn likely blocked cell death [32]. Broccoli ingestion was also shown to decrease inflammation in kidney and cardiovascular tissues of hypertensive stroke-prone rats. After 14 weeks of broccoli ingestion, the rats had greater

glutathione levels, reduced number of infiltrating macrophages, and lower blood pressure which indicates improved renal function and hemodynamics [69].

1.5 Sulforaphane (SFN)

NHPs such as broccoli sprouts (BrSps) have been widely studied in the field cancer, cardiovascular disease, and hypertension. These diseases are also associated with enhanced inflammation and oxidative stress. BrSps have been identified as the richest source of SFN, an isothiocyanate produced by the action of myrosinase on the glucosinolate, glucoraphanin, compared to other crucifers. Sulforaphane is known to act as a cytoprotective agent in multiple organ systems in models of oxidative stress and inflammation, including cancer, cardiovascular disease and stroke.

1.5.1 Mechanism of Action

SFN is a powerful anti-inflammatory and anti-oxidant. Mechanistically, SFN enhances endogenous anti-oxidant capabilities by activating nuclear factor (erythroid-derived 2)-like (Nrf2). SFN's interaction with the Nrf2 pathway in turn induces phase II detoxifying enzymes (EPII) and anti-oxidant enzymes that are important in protecting cells against oxidative stress.

Nrf2 pathway

The brain has an endogenous defense mechanism against effects of ROS, and Nrf2 plays an important role in this defense [40]. Under resting conditions, Nrf2 is sequestered in the cytoplasm, repressed by Kelch-like ECH-associated protein (Keap1). Keap1 sends Nrf2 for proteasomal degradation via ubiquitination.

However, oxidative stress and Nrf2 inducers can cause alterations in Keap1 structure, which leads to dissociation of Nrf2-Keap1 complex and altered E3 ubiquitin ligase function to reduce Nrf2 degradation. Some examples of Nrf2 inducers include curcumin, plumbagin, and *tert*-

butylhydroquinone. Alternatively, phosphorylation of serine/threonine residues in Nrf2 also allows it to dissociate from Keap1 [40]. Nrf2 is released from Keap1 by a hinge-latch mechanism that allows for nuclear translocation into the nucleus where it heterodimerizes with small musculoaponeurotic fibrosarcoma (Maf) proteins and binds to induce transcription and consequent production of defense proteins [50].

In the nucleus, Nrf2 induces phase II antioxidant defense enzymes like heme oxygenase-1 and glutathione (Figure 2). Protective genes regulated by Nrf2, protects astrocytes and neurons by regulating expression of inflammatory markers and antioxidant enzymes [40]. These genes contain a common cis-acting promoter element called antioxidant-response element (ARE) that assists the binding to Nrf2[50, 70].

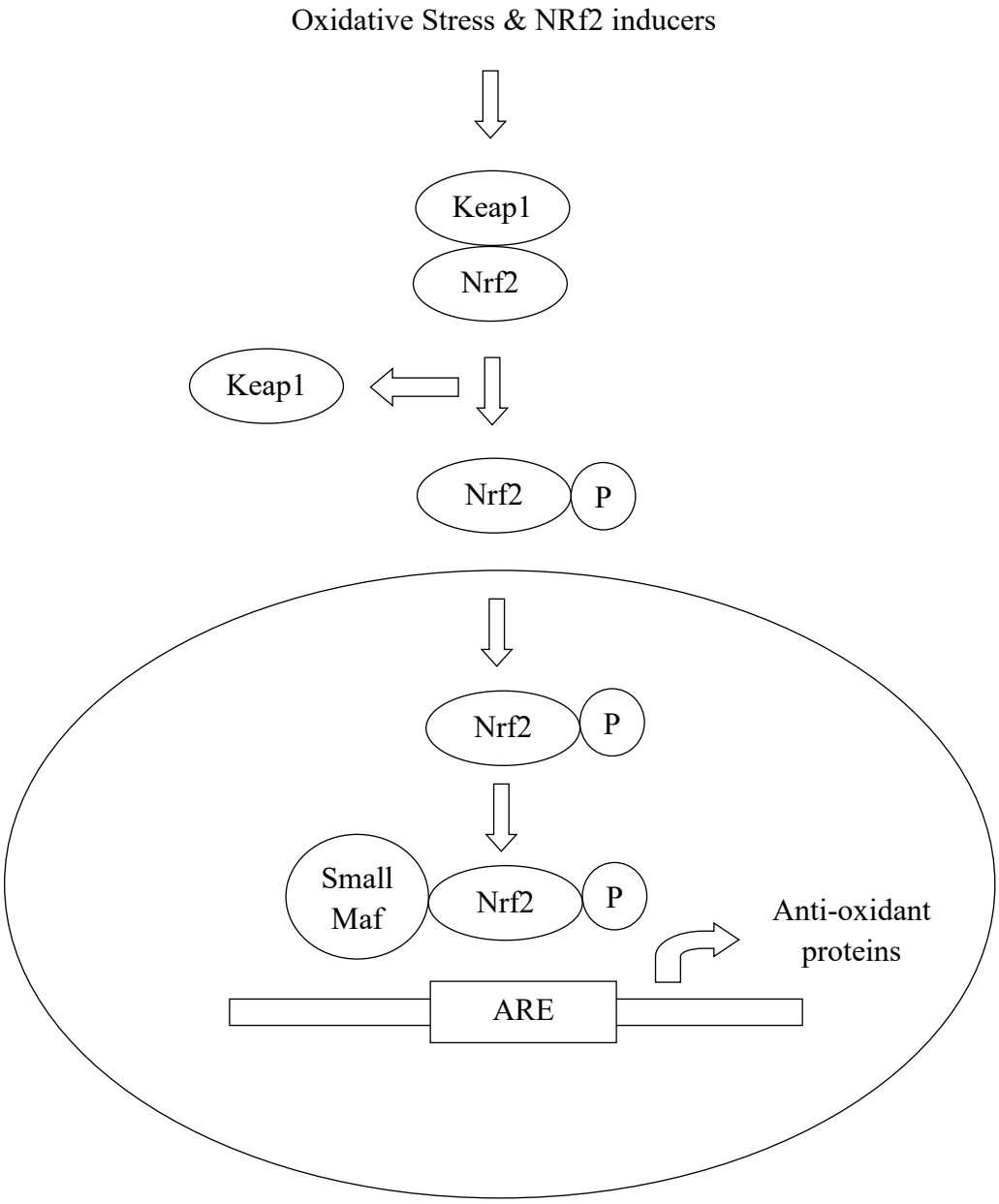

Figure 2 – ***Schematic representation of activation of the Nrf2 pathway***

SFN & Nrf2 pathway

SFN crosses the blood brain barrier (BBB) and is a well-known activator of Nrf2 [40]. SFN interacts with thiol groups of Keap1 in a concentration-dependent manner to change conformation, resulting in dissociation of Nrf2-Keap1 complex, and Nrf2 transition into the nucleus [48]. The conformational changes results from the reaction of the highly electrophilic central carbon atom of SFN with the sulfhydryl groups of cysteine residues called the diothiocarbamate group in Keap1. Formation of dithiocarbamate results in the disruption of Keap-1 Nrf2 interactions and induction of ARE genes [71]. Nrf2 is essential for EPII expression by SFN [48].

SFN's mechanism of action through the Nrf2 pathway

Nrf2 is up regulated with oxidative stress resulting in increased production of target antioxidants and enzymes that protect against apoptosis in both neurons and astrocytes[72].

Activation of Nrf2-ARE decreases lipid peroxidation (LP) and this is significant as neuronal mitochondria are highly susceptible to LP-mediated oxidative damage. SFN was shown to increase expression of HO-1 mRNA through the Nrf2-ARE pathway. As a result, it decreased the level of 4-hydroxynonenal (4-HNE), a neurotoxic byproduct of LP [50]. SFN also suppressed LPS-mediated expression of cytokines such as IL-1β, IL-6, and TNF-α in addition to decreasing the release of inducible nitric oxide synthase (iNOS), COX-2, and NO from LPS-stimulated microglia [73].

Protein levels of Nrf2-regulated genes encoding antioxidant enzymes, HO-1 and NAD(P)H dehydrogenase quinone 1 (NQO1), were up regulated with SFN treatment in rats. As a result of this increase in detoxifying and antioxidant enzymes through SFN, rats had reduced neuronal death, contusion volume, and neurological dysfunction after cortical injury[45]. On the other

hand, Nrf2 knock out mice did not benefit from SFN treatment but rather had more severe injuries from ischemia.

SFN induction of Nrf2 pathway is also beneficial to microglia and the BBB. In one study, LPS induced inflammation in mouse hippocampus. SFN administration for 4 days resulted in decreased microglial activation compared to the Nrf2-knock out mice. The decreased number of activated microglia attenuated microglial inflammation as evident by reduced levels of IL-6 and TNF-α[46]. SFN induction of Nrf2 also helps to reduce the loss of endothelial cell makers and tight-junctions proteins to preserve BBB function [74].

1.5.1.1 Mitochondria

Mitochondria under oxidative stress and exposure to elevated levels of Ca^{2+} leads to opening of mitochondrial permeability transition pore (PTP). PTP opening inhibits ATP formation as a result of membrane depolarization, uncoupling of oxidative phosphorylation, and mitochondrial osmotic swelling resulting in metabolic failure [75]. In addition, the release of mitochondrial pro-apoptotic proteins such as BAX and BID results in cell death [76]. SFN increases resistance of brain mitochondria to inner membrane permeability transition pore (PTP), which are responsible for causing bioenergetics failure and subsequent cell death [75].

1.5.1.2 Phase II Enzymes (EPIIs)

EPIIs, which include NADPH quinoneoxidoreductase 1 (NQO-1), epoxyde hydrolase (EPHX), glutathione reductase, glutathione-S-transferase, glutathione peroxidase, thioredoxinreductase, and Heme oxygenase-1 (HO-1), play a central role against oxidant and inflammatory injuries [48, 77].

HO-1, one of the EPIIs is an endogenous antioxidant that serves as the first rate-limiting enzyme in catabolism of heme [78]. HO-1 cleaves heme to biliverdin, consequently forming carbon monoxide (CO) and releasing chelated Fe^{2+}. Biliverdin is reduced to bilirubin, which serves as a

free radical scavenger to protect against oxidative stress [79]. HO-1 increases the rate of free heme catabolism to inhibit apoptosis and has been shown to be cytoprotective for neurons and other types of cells [78, 80]. Overexpression of HO-1 in cerebral neurons was shown to increase resistance to glutamate toxicity as a result of oxidative stress [81].

Increase in HO-1 expression with SFN pre-treatment was shown in rats exposed to hepatic ischemic reperfusion injury (HIRI) [82]. Similar findings were observed in retinal ischemia-reperfusion rat models where administration of SFN 24 hours before ischemia elevated level of HO-1 expression [83]. SFN's ability to increase levels of HO-1 can be observed in other types of oxidative stress caused by doxorubicin, a chemotherapeutic agent [84].

1.5.2 SFN's effect on activity and integrity of neurological system

1.5.2.1 SFN on Neurons

SFN has been shown to have protective properties against cytotoxic and ischemic insult of neuronal cell cultures. 24 hour pre-treatment with SFN protected primary neuronal cultures from rat striatum against treatment with H_2O_2. SFN increased cell viability likely through inducing an increase in nuclear translocation of Nrf2. Increased nuclear levels of Nrf2 increased the expression of γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme for glutathione synthesis. SFN also increased HO-1 expression in neurons [79]. In another study, mouse hippocampal neurons were exposed to oxygen glucose deprivation (OGD). OGD was achieved by transferring neurons to an incubator with 0% O_2, 10% H_2, 5% CO_2, 85% N_2 and using a glucose free media. When SFN was added for 24 hours during recovery, there was an increase in the mRNA levels of Nrf2/ARE genes, NQO1 and HO-1 compared to the control [85]. Similarly in primary cortical neurons, SFN treatment during OGD and recovery increased cell viability,

decreased percentage of dead/dying cells, decreased cleaved caspase-3 and increased anti-apoptotic protein Bcl-2 [86].

SFN treatment in animal models also shows neuronal protection. 7-day rat pups were subjected to left common carotid artery ligation and hypoxia. The effect of intraperitoneal SFN administration, prior to the insult, was examined in the cortex and hippocampus. SFN increased expression of Nrf2 and HO-1 while reducing caspase-3 activity and LP. The area of damage was significantly reduced with SFN treatment when infarct regions were visualized using 2, 3, 5-triphenyltetrazolium chloride (TCC) staining [87].

1.5.2.2 SFN on Astrocytes

Astrocytes have important roles in BBB function, metabolism, uptake of glutamate, and formation of glial scars during injury [88]. They also play an important role in axonal development, secretion of growth factors, regulating ions and neurotransmitters, and providing energy substrates for neurons [76].

Astrocytes are more efficient in synthesis of glutathione and antioxidant response element (ARE)-linked gene expressions, thus more protected than neurons against basal levels of oxidative stress. They provide protection to neurons by releasing glutathione (GSH) and glutamine, which serves as a precursor for GSH synthesis in neurons [40]. In addition, activation of ARE is seen predominantly in astrocytes as ARE-genes are preferentially activated[89, 90]. SFN was shown to activate ARE in astrocytes [89]. 48 hour SFN preconditioning followed by treatment after 4 hours of OGD protected rat cortical astrocytes against death. SFN increased nuclear localization of Nrf2 which through the Nrf2-ARE pathway increased mRNA and protein levels of NQO1 and HO-1. Increased levels of antioxidant genes were then able to protect astrocytes from cell death [91]. Further, even brief SFN treatment lead to accumulation of HO-1,

which then remained elevated for over 24 hours to protect astrocytes against peroxide-induced damage [92].

1.5.2.3 SFN on Microglia

SFN administration has also been shown to suppress activation of microglia. When 7-day old rat pups were administered with SFN 30 minutes before HI insult the numbers of activated microglial cells were significantly reduced. Suppression of microglial activation has beneficial role, as microglial activation is responsible for generation of ROS and inflammatory cytokines [87].

In addition, 30 minute SFN pre-treatment was shown to reduce microglial activation in response to LPS. Primary rat microglial cells had suppressed expression of IL-1β, IL-6, and TNF-αwith SFN treatment. The cytokines activate NF-κB, which in turn further potentiates expression of cytokines by a positive feedback loop. SFN is able to reduce NF-κB activation and levels of inflammatory cytokines by inhibiting ERK1/2 and JNK phosphorylation [73].

Upon injury, microglia undergo morphological change from a ramified shape to an amoeboid shape. 1 hour of pre-treatment with SFN before LPS in primary microglial cultures, reduced the number of microglia in the more rounded amoeboid shape caused by LPS administration, indicating decreased microglial activation [93].

1.5.2.4 SFN on Oligodendrocytes

Secondary injuries such as stroke that culminate after primary brain injuries play an important role in causing oedema and worsening of neurological damages. Key contributors of secondary brain damage are components released upon lysis of red blood cells (RBCs). RBCs lyse to release haemoglobin (Hb), which degrades into heme and iron, and cytotoxins. Hb acts as a neurotoxin damaging cellular components including DNA, lipids, and proteins. Hb is also able to

induce apoptosis, BBB disruption, and neuronal death. When Hb binds to haptoglobin (Hp) to form the Hb-Hp complex, it prevents oxidative and cytotoxic effects of Hb.
SFN has been shown to induce production of Hp in oligodendrocytes by activating the Nrf2 pathway, which in turn upregulates the Hp gene in oligodendrocytes. Upregulation of the Hp gene is not observed in neurons, astrocytes, or microglia [94].

Because of the benefits of SFN on neurons, astrocytes, microglia, and oligodendrocytes in hypoxic ischemic environment *in vitro*, investigation of SFN's potential use to minimize the in utero hypoxic-ischemic insult to the fetal brain is warranted. However, because SFN also has been used to cause apoptosis of cancer cells, query into cytotoxicity of SFN in the OGD environment also needs to be investigated.

1.6 Hypothesis

i. SFN protects neurons and astrocytes against oxygen-glucose deprivation (OGD).

ii. SFN may be cytotoxic to neurons and astrocytes at a certain dosages.

1.7 Objectives

i. To determine the optimal OGD times for neurons and astrocytes.

ii. To determine effect of SFN on neurons and astrocytes under normal conditions.

iii. To determine effect of SFN dosages on neurons and astrocytes exposed to OGD.

Chapter 2

Materials and Methods

2.0 Materials and Methods

2.1 Isolation of Neurons

Rat cortical neurons were prepared from postnatal day 2 Long-Evan rats of either sex (Health Science Lab Animal Services, U of Alberta). Brains were dissected and cortices were isolated. Cortical tissues were enzymatically digested by 1mg/ml papain for 10 minutes at 37°C. DNase was added to the digestion mix in the last 5 minutes of incubation. After mechanical dissociation using a flame polished Pasteur pipette, the cell suspension was filtered through a cell strainer (40 μm Nylon; Falcon), neurons were plated on poly-D-lysine-coated wells at a density of 1.5×10^5 cells/well in 24 well plates. The cell culture medium used consisted of NeurobasalA medium (Gibco, Cat# 10888-022) supplemented with B27 (Gibco, #17504-044), penicillin Streptomycin (Gibco, #3505-061). Experiments started at day 7 in culture.

2.2 Isolation of Astrocytes

2.2.1 Method 1

Postnatal day 2 Long-Evans cortices are isolated and transferred to a Petri dish containing calcium and magnesium free (CMF) Hank's Balanced Salt Solution (HBSS). Cortices are digested using DNase and trypsin and incubated at 37°C for 5 minutes. Cortices are triturated using a glass Pasteur pipette and incubated at 37°C for 5 minutes again. Growth media containing 10% horse serum, 30% w/v D-glucose in Minimum Essential Media (MEM) added to the cortices to filter through a 70 μm cell strainer. Cell suspension is centrifuged and re-suspended in 1 mL of growth media. Astrocytes are plated at a cell density of 2×10^4 cells/well. Media is changed 24 hours after plating, and every 3 days after for 2 weeks.

2.2.2 Method 2

Rat cortical astrocytes were prepared postnatal day 2 Long-Evan rats of either sex (Health Science Lab Animal Services, U of Alberta). Rat cortices were washed with PBS and dissociated with 0.1% (w/v) trypsin at 37°C for 5 minutes. BME-glucose media with 10% FBS was added to stop the action of trypsin. The cortices were then mechanically dissociated using a Pasteur pipette. Cells were filtrated through a cell strainer (70μm Nylon; Falcon) and plated 8 x 10^4 cells/well in 24 well plates. After 24 hours, BME-glucose media was replaced with BME-glucose-free media supplemented with sorbitol and 10% FBS. Media was changed every 3 days and experiments started at day 12 in culture.

2.3 Hypoxic-Ischemic Insult

The oxygen sensor (Pro Ox: 110, BioSpherix) for the hypoxia chamber is set at 0% oxygen level. Oxygen in the incubator is replaced by a mixture of gas that is 95% nitrogen and 5% carbon dioxide at 37°C. Regular growth media is replaced by DMEM with no glucose during the hypoxic-ischemic insult. DMEM is degassed for an hour using a vacuum to remove the oxygen within the media. Once the oxygen has been removed from the both incubator and the media, the plates of cells are then placed in the hypoxia chamber for the appropriate amount of time. Oxygen levels were monitored throughout with an oxygen sensor. At all times, oxygen level was below 1%.

2.4 Recovery

DMEM is replaced with regular growth media then the cells are placed in an incubator at 37°C with 5% carbon dioxide and 95% air.

2.5 Drug Treatment

2.5.1 SFN

R, S-Sulforaphane, not dissolved in any solvent, is obtained from LKT Laboratories, Inc. 25mM stock, which was diluted using ddH_2O stored at -20°C. From the 25 mM stock, 5mM aliquots are diluted with ddH_2O. 5mM stocks are diluted again into appropriate concentrations for each experiment.

2.5.2 Treatment of cells with SFN

Cells were treated with SFN at various dosages of 0.5, 1, 2, 2.5, 5, 10, 25, and 50μM. SFN is added to degassed DMEM for OGD and added to regular growth media for recovery.

2.5.3 Staurosporine induced cell death

100 nM of staurosporine was added to induce massive cell death. Staurosporine was diluted from 1 mM stock and incubated with cells for 25 hours at 37 °C.100 nM of staurosporine was reported to induce 70 to 80% cell death in neurons and glial cells [95, 96].

2.6 Preparation of Cell Lysates

Cells were scrapped with a mini-cell scraper (Biotium) then washed twice with phosphate buffered saline (PBS). PBS containing protease inhibitor was added to prevent protein degradation. Cells and PBS were collected then homogenized with a 26 gauge syringe, centrifuged at 4°C for 5 minutes at 13.2 rpm. The pellet was discarded and the supernatant was collected for protein concentration determination.

2.7 Immunoblotting

Equivalent amount of proteins were resolved by 8% Tricine polyacrylamide gel electrophoresis. Proteins were then transferred onto polyvinylidenedifluoride (PVDF) membranes (Milipore).

After the transfer, membranes were blocked in 3% Bovine Serum Albumin (BSA) and incubated with various antibodies at 4°C overnight. Membranes are then washed with TBST then incubated with secondary antibodies (GE Healthcare UK Limited) at 37°C for an hour. Western blots were visualized using Western Lightning® Plus-ECL (PerkinElmer, LAS Inc.). Antibodies to the following proteins were used for this study: Anti-Neuronal Specific Enolase (Abcam®), Anti-Glial Fibrillary Acidic Protein (Abcam®), and Actin (Santa Cruz Biotechnology Inc.).

2.8 Trypan Blue Viability Assay

Trypan blue viability assay is used to determine number of viable and non-viable cells. Viable cells with intact cell membranes exclude the dye, while cells with compromised cell membranes are unable exclude the dye and appear blue. Briefly, cells are collected gently by using a mini-cell scraper. The media containing the scrapped cells placed in a 1ml eppendorf tube then centrifuged for 2 minutes at 200g. Supernatant is removed then 1:1 trypan blue and PBS is added. Cells are gently triturated using a pipette. Dead and live cells are counted using a haemocytometer.

2.9 LDH Cytotoxicity Assay

Upon injury, cell membranes are damaged, rupture and become permeable and LDH leaks from the injured cells. This permeability also allows fluids to enter the cells resulting in cell swelling, rupture and necrosis. The rupturing of intracellular organelles and the deterioration of the plasma membrane allow LDH to be released to the cytosol [97]. The LDH that is released can be measured and quantified. LDH converts lactate into pyruvate. During this conversion, NAD^+ is reduced into NADH. In the calorimetric assay for LDH quantification, WST-1 tetrazolium dye is reduced as NADH is oxidized back to NAD^+. Because WST-1 is cell impermeable, reduction

occurs outside the cell membrane. WST-1 tetrazolium dye reduction generates an orange/yellow color, and the intensity (read by a spectrometer at 450 nm) of which is directly proportional to amount of LDH released (Figure 3).

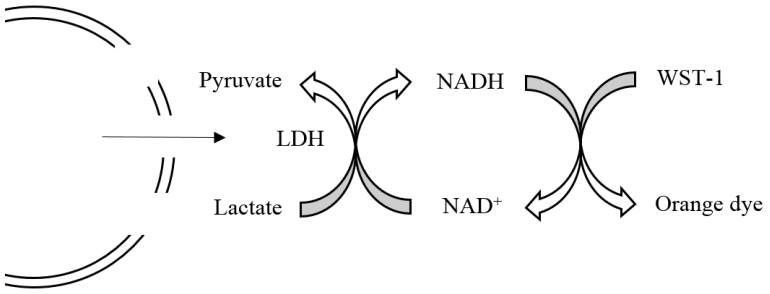

Figure 3 – ***Schematic representation of LDH release and subsequent reactions***

LDH Cytotoxicity Assay Kit II was obtained from Abcam®. For high control, 10 µL of Cell Lysis Solution was added per 100 µL of cells. Low control cells were untreated cells. 10µl of cell media is transferred into a 96-well plate. 100ul of LDH reaction mix is added and allowed to react for 30 minutes at room temperature. LDH is measured using VersaMaxMicroplate readerat 450nm.

2.9.1. LDH Cytotoxicity Calculation Method 1

The following formula was provided by Abcam®:

$$Cytotoxicity\ (\%) = \frac{(Test\ Sample - Low\ Control)}{(High\ Control - Low\ Control)} x\ 100\%$$

Test sample is the absorbance level for each SFN dosages, low control represents the untreated cells, and high control represents the cells treated with cell lysis solution. However, the formula does not allow for comparison between 0 µM and other SFN dosages. The "low control" that is referred by Abcam®'s formula is the 0 µM group in this study. Thus, because 0 µM is used as the "low control", comparisons can be only made between 0.5, 1, 2, 2.5, 5, 10, 25, and 50 µM. For

statistical comparison between SFN dosages to 0 µM, cytotoxicity calculation was modified to subtract the background LDH levels from just the cell media.

2.9.2. LDH Calculation Method 2

Cytotoxicity calculation was adjusted to allow for comparison between untreated to treated cells.

$$Cytotoxicity\ (\%) = \frac{[(Test\ Sample - Background)]}{[(High\ Control - Background)]} x\ 100\%$$

Using the new LDH cytotoxicity formula, calculations were redone, which allowed for one-way ANOVA analysis of all the dosages.

2.10 MTT Cell Viability Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) penetrates viable eukaryotic cells to reduce tetrazolium into the colored formazan product. Unviable cells are unable to convert tetrazolium into formazan. The intensity of signal is proportional to the number of viable cells present (Assay Guidance Manual, Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004).

Cells are plated on 96-well plates. After exposing them to experimental conditions, 12nM MTT (Sigma) was added per well and incubated for 4 hours at 37°C. After incubation, 50 µL of DMSO was added to dissolve formazan. The sample was read at 540 nm on JennsMTT protocol available on the PerkinElmer software usingFLUOstar OMEGA Multi-mode microplate reader (BMG Labtech).

2.11 Fam-DEVD-fmk

Carboxyfluorescein Fluorescent Labelled Inhibitor of Caspases (FAM FLICA^{TM}) detects active caspase 3 and 7. The fluorescent inhibitor FAM-DEVD-FMK labels caspases that can be

analyzed using a fluorescent plate reader. Green fluorescent probes carboxyfluorescein (FAM) and fluoromethylketone (FMK) binds and interacts with amino sequences of aspartic acid-glutamic acid-valine-aspartic acid (DEVD) of the caspases.

Cells were stained with FAM FLICA^{TM} Caspase-3 & 7 Assay Kit (ImmunoChemistry Technologies LLC). After insult and recovery, cells are harvested and centrifuged at 200 g for 5 minutes with the media. 30X FLICA is added at 1:30 and incubated for 60 minutes at 37°C. Cells are washed with 1X Apoptosis Wash Buffer, centrifuged, and re-suspended after the removal of supernatant. After repeating the previous step, cells are incubated for 10 minutes at 37°C. Cells are centrifuged again at 200 g and re-suspended in 500 µL of PBS. Cell density is adjusted to be > 3 x 10^6 cells/mL. Three 100 µL/well of the solution is transferred into a 96 well black microtiter plate. Endpoint read was performed with the excitation wavelength at 488 nm and emission wavelength at 530 nm with a VersaMax Microplate reader.

2.12 DNA quantification

Nucleic acids absorb ultraviolet light, which can be measured using a spectrophotometer at 260 nm. Higher the absorbance from the sample corresponds to higher concentration of nucleic acids that are present. After SFN treatment during OGD and recovery, amount of DNA is quantified using a VersaMax Microplate reader at 260 nm.

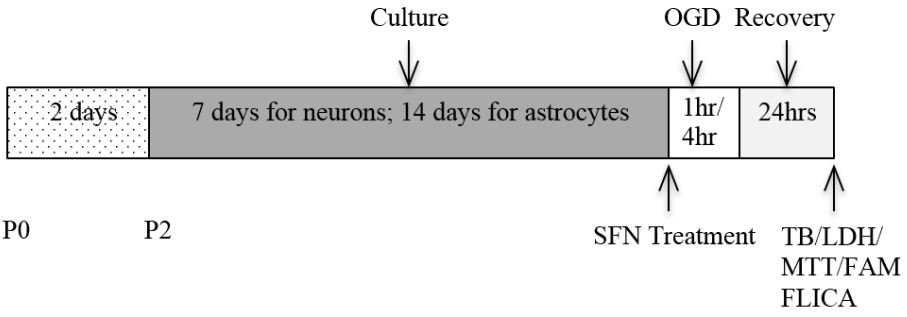

Figure 4 – ***Schematic representation of experimental time line***

2.13 Statistical Analysis

GraphPad Prism 5 and 6 computer software was used. Data were from 3 to 6 experiments with triplicates per experiment. Values were expressed as means ± SE. Statistical significance was analyzed using Ordinary one-way ANOVA with Tukey's multiple comparisons test. Significance is indicated by * ($p<0.05$), ** ($p<0.01$), or *** ($p<0.001$).

Chapter 3

Results

3.0 Results

3.1 Neuronal and Astrocyte Cultures

Western blot analysis showed that neuronal cell cultures were successfully isolated without astrocytes contamination (Figure 5A). Neuronal cells did not react with the astrocyte marker, anti-Glial Fibrillary Acidic Protein (GFAP) antibody. On the other hand, astrocytes cell cultures appeared to be contaminated with neuronal cells. Staining of astrocyte cultures using neuronal cell markers, anti-Neuronal Specific Enolase (NSE) and anti-β-tubulin III, demonstrated that astrocytes cultures reacted positively with neuronal antibodies (Figure 5B and 5C). To eliminate growth of neurons in astrocyte cultures, we used an alternate isolation technique with a different growth media to enhance astrocyte growth and supress neuronal growth. Initially, astrocytes were isolated using a glucose containing minimal essential growth medium (MEM). However, both neurons and astrocytes are able to utilize glucose as a source of energy, which enable the neurons to grow in the glucose containing astrocyte media leading to neuronal contamination of the astrocyte culture. In the alternate method of astrocyte isolation we used glucose-free growth media supplemented with sorbitol. Astrocytes are able to utilize different types of glucose sources including monosaccharide glucose, mannose, fructose, and sorbitol. In primary cultures, only astrocytes can survive in glucose-free medium supplement with sorbitol [98]. Neurons and other glial cells cannot be cultured in sorbitol without glucose [99]. Western blot analysis showed that astrocyte cultures were now free of contaminating neuronal cells (Figure 5D).

Astrocyte Growth Media Serum Selection

While switching to the use of glucose-free media for astrocyte culturing gave us non-contaminated astrocyte cultures, the isolated astrocytes did not have the correct morphological shape (Figure 6A). Our original protocol suggested the use of rat serum. As an alternative method for the isolation of healthy astrocyte cultures of correct morphology, we changed to

using fetal bovine serum (FBS) (Figure 6B). Using 10% FBS in the glucose-free media supplemented with sorbitol, we were successful in isolating and culturing contamination free astrocytes of correct morphology.

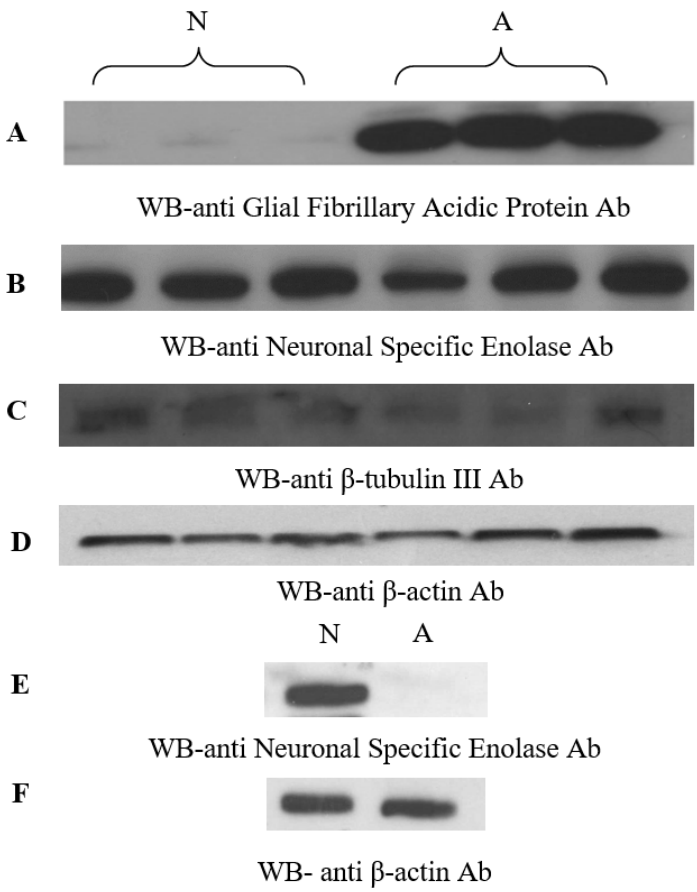

Figure 5 – ***Western blot analysis of cortical neuronal and astrocyte cultures.*** Neurons were cultured for 1 week and astrocytes for 12 days prior to lysis and Western blot analysis of cell specific markers. β-actin was used as a loading control. Results are from 3 separate cultures. **A)** Neuronal and astrocyte cultures were probed with anti-glial fibrillary acidic protein (GFAP) for astrocyte detection. Only the astrocyte cultures reacted with the GFAP antibody. There was no GFAP staining in the neuronal cultures, indicating they were not contaminated with astrocytes. **B & C)** Neuronal cultures reacted positively with neuronal markers, anti-Neuronal Specific Enolase (NSE) and anti-β-tubulin III antibodies. However, the astrocyte cultures grown in glucose-containing media also reacted positively with anti-NSE and anti-β-tubulin indicating contaminating neurons in the astrocyte cultures. **D)** Astrocyte cultures were free of neuronal cells, and did not react with anti-NSE antibody.

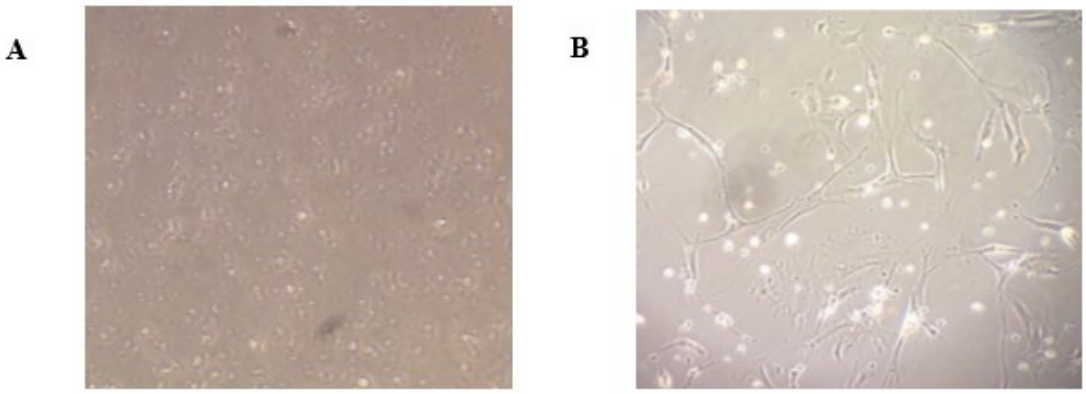

Figure 6 – ***Isolation of astrocytes with correct morphology requires fetal bovine serum (FBS) in the growth media.*** Astrocytes are cultured from cortices of P2 Long Evan rats in glucose free growth medium supplemented with sorbitol. **A)** The astrocyte growth medium contained 10% rat serum. After 1 week in culture, astrocytes appeared to be round and were growth restricted. **B)** When astrocytes were cultured for 1 week in medium supplemented with 10% FBS, astrocytes were of correct morphology and exhibited normal growth and proliferation.

3.2 Determination of optimal times for Oxygen Glucose Deprivation (OGD) and treatment with Sulforaphane (SFN).

3.2.1 LD50: OGD

Our next aim was to determine the LD 50 for oxygen glucose deprivation (OGD) of neurons and astrocytes. LD50 (Lethal dose 50 %) refers to the time point when 50% of cells die from the treatment under consideration, in this case OGD. LD 50 was determined using trypan blue exclusion assay of cells (neurons or astrocytes) exposed to various times of OGD (1, 4, 8, 12, 18, or 24 hours) followed by 24 hours of recovery using normoxic glucose containing growth media. Our results indicate that LD50 for neurons was achieved with 1 hour of OGD and 24 hours of recovery, while LD50 for astrocytes was observed after 4 hours OGD and 24 hours of recovery (Figure 7B and 7C). The greater resistance of the astrocytes to death by OGD is in accordance their ability express ARE-linked genes better than neurons [40].

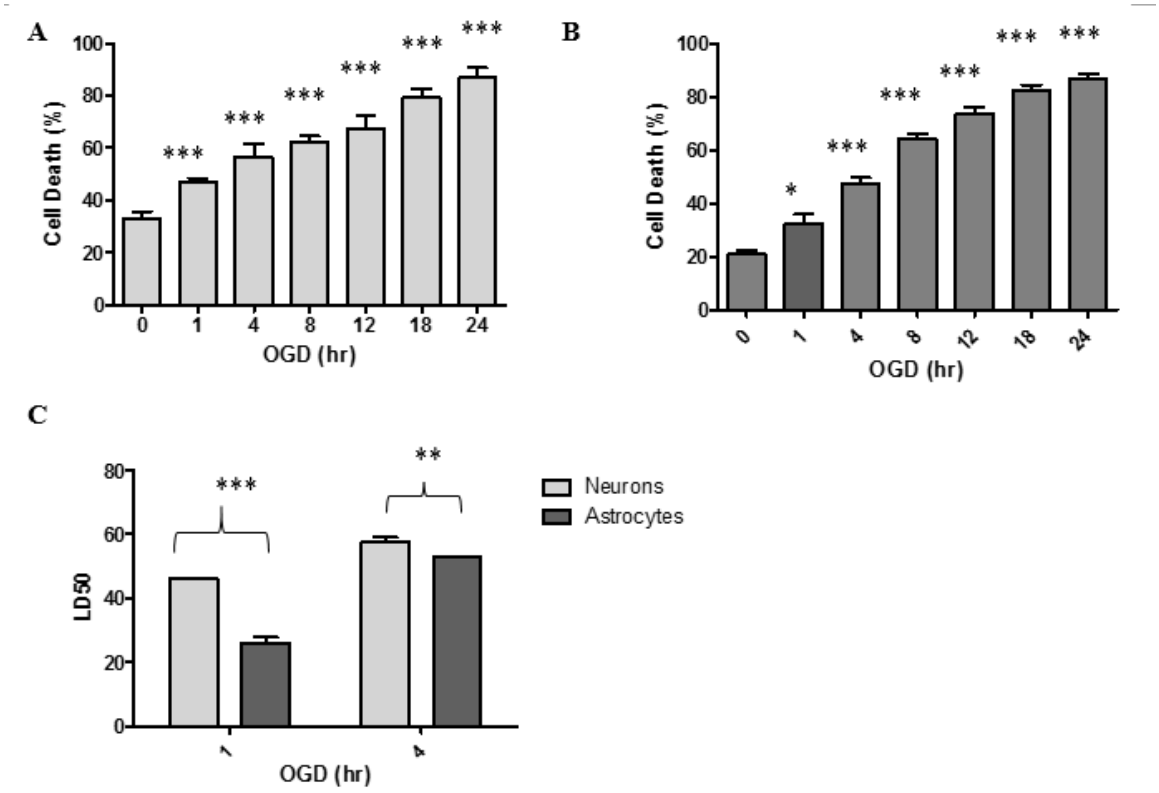

Figure 7 – ***Effects of OGD on neuronal and astrocyte cell death.*** After culturing neurons for 1 week and astrocytes for 12 days, respectively, each cell type was exposed to OGD. Cell death was evaluated using trypan blue exclusion assay to evaluate cell viability. Neurons and astrocytes were exposed to 1, 4, 8, 12, 18, or 24 hours of OGD with 24 hours of recovery. **A)** LD50 for neurons was achieved with 1 hour of OGD and 24 hours of recovery and **B)** LD50 for astrocytes was achieved with 4 hours of OGD and 24 hours of recovery. **C)** 1 and 4 hours of OGD with 24 hours of recovery was compared between neurons and astrocytes. At both 1hour and 4-hour time points of OGD astrocytes exhibited significantly less cell death compared to neurons. * indicates significance in comparison to 0 μM of SFN *$p<0.05$. **$p<0.01$. ***$p<0.001$.

3.2.2 SFN treatment during OGD only vs. OGD and recovery

Having determined the LD50 for neurons and astrocytes during OGD and OGD/ recovery, our next aim was to determine the ideal time for treatment with SFN. We compared the efficacy of SFN to limit or prevent changes in cell death due to OGD only, or during OGD followed by recovery with oxygen and glucose containing media. Cell viability was assessed with trypan blue exclusion assay. Figures 8A and 8B demonstrate that regardless of whether SFN was used during OGD or OGD/recovery, neuronal protection from cell death was evident and significant at a SFN dosage of 2.5 μM. Protection of neurons from cell death by 2.5 μM SFN was statistically comparable under both treatment regimens. In addition, based on this finding, all the following experiments were carried out with SFN added during OGD and recovery period.

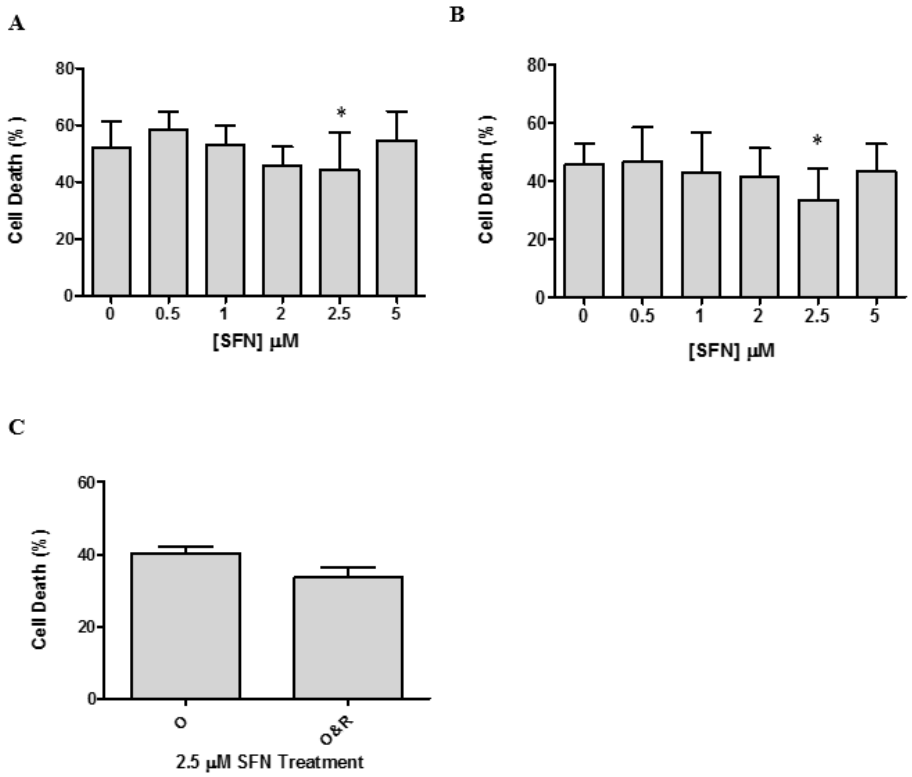

Figure 8 – *SFN treatment during OGD only vs OGD and recovery.* Neuronal cells after 1 week in culture are exposed to 1 hour of OGD and 24 hours of recovery. **A)** 0.5, 1, 2, 2.5, and 5µM of SFN was added to neurons only during OGD into the degassed glucose free DMEM. During recovery, the degassed glucose free media was replaced with regular neuronal growth media without SFN. **B)** SFN was added during OGD in the glucose free DMEM and also during recovery in with oxygen and glucose containing growth media. 2.5 µM of SFN was found to significantly decrease cell death under both treatment regimens. **C)** Cell death of neurons treated with 2.5 µM during OGD only was not statistically different from treatment during OGD and reperfusion. * indicates significance in comparison to 0 µM of SFN. *$p<0.05$.

3.3 Assessment of SFN's effects on Normoxic Neurons and Astrocytes

Having successfully completed culturing of neurons and astrocytes, determination of time course for LD50 and recovery, we next wanted to determine the effect of SFN's on cell cultures under normoxic condition of 37 °C and 95% O_2/5% CO_2. This would highlight potential effects of SFN under conditions of normal pregnancy. Assessments were done using four different assays: trypan blue viability assay, LDH cytotoxicity assay, MTT viability assay, and FAM FLICA caspase 3 & 7 apoptosis marker assay.

Trypan blue exclusion assay is one of the most frequently used methods for assessing cell death. It allows dead/damaged cells to be distinguished from viable cells as dead/damaged cells are stained dark blue [100]. Similarly, the integrity of the cell membrane is measured through the amount of LDH released into the media. To observe the effects of SFN on cell viability, MTT assay was utilized. MTT determines cell viability by measuring its ability to convert tetrazolium salts into formazan crystals. The crystals are dissolved using DMSO, and the resulting color change can be quantified [101]. The amount of DNA was quantified for neurons astrocytes to determine whether SFN increased cell viability by increasing the number of astrocytes through proliferation or decreasing number of cell death. In addition to analyzing necrosis, overall cell death, and viability, FAM FLICA™ caspase 3 and 7 assay was used to study putative apoptosis in neurons and astrocytes.

3.3.1 Effects of SFN on Neurons during Normoxic Condition

Trypan blue viability assay

Dosages of 0.5, 1, 2, 2.5, 5, 10 and 25 μM of SFN did not have any effect on cell death measured by Trypan blue exclusion assay of normoxic neurons. However, 50 μM SFN did cause

significant increase in normoxic neuronal cell death. Significant decrease in overall cell death was observed with 10 μM (Figure 9A).

MTT viability assay

Neuronal viability under normoxic conditions did not appear to be affected by SFN (Figure 9B). A slight decrease in viability was observed with 50 μM but this was not significant.

LDH cytotoxicity assay

No cytotoxic effects were observed at 0.5, 1, 2, 2.5, 5, 10 and 25 μM of SFN treatment of normoxic neurons. The only exception was 50 μM SFN where we observed a significant increase in LDH cytotoxicity (Figure 9C).

DNA quantification

There was no alteration on DNA content with SFN treatment indicating that SFN did not affect proliferation of cortical neurons (Figure 9D).

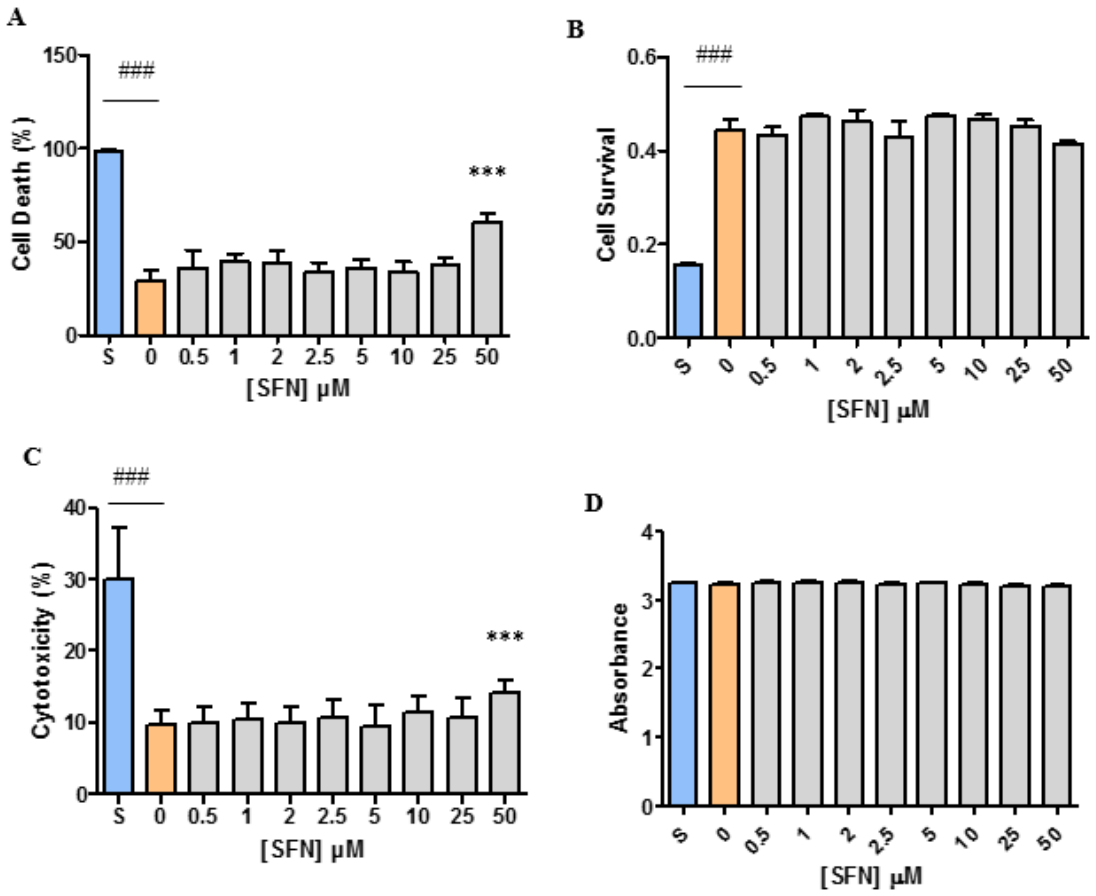

Figure 9 – ***Effects of SFN treatment on neurons in normoxic conditions.*** After 1 week in culture, assays are performed without exposure to OGD. Neurons treated with 100 nM of staurosporine served as a control. **A)** Neuronal cell death was assed using trypan blue exclusion assay. **B)** Cell viability was assessed using MTT. **C)** LDH released into the media is represented as cytotoxicity index. **D)** DNA amount was quantified at 260 nm. S: culture treated with 100 nM of staurosporine to induce massive cell death. * indicates significance in comparison to 0 µM of SFN, while # indicates significance between S and 0 µM of SFN. *p<0.05. **p<0.01. ***p<0.0001. ###<0.0001.

3.3.2 Effects of SFN on Astrocytes during Normoxic Condition

Trypan blue viability assay

Non-significant decrease in cell death was seen with 0.5, 1, 2, and 5 μM of SFN compared to control (0 μM SFN). At 25 and 50 μM, cell death was significantly increased (Figure 10A).

MTT viability assay

0.5, 1, 2, and 2.5 μM SFN was seen to significantly increase cell viability of astrocytes. Dosages of 0.5 to 2.5 μM increased absorbance by almost 2 fold compared to untreated astrocytes (0 μM). However, significant reduction in cell viability was observed at 50 μM (Figure 10B).

LDH cytotoxicity assay

Dosages of 0.5, 1, 2, 2.5, 5, and 10 μM were shown to have a significant beneficial effect upon cell death of normoxic neurons as measured by LDH secretion which was decreased significantly upon treatment with these doses of SFN. However, at 25 and 50 μM of SFN, LDH cytotoxicity significantly increased compared to the control (Figure 10C).

DNA quantification

Similarly to neurons, SFN did not alter DNA content in astrocytes indicating that SFN does not affect astrocyte proliferation in normoxic conditions (Figure 10D).

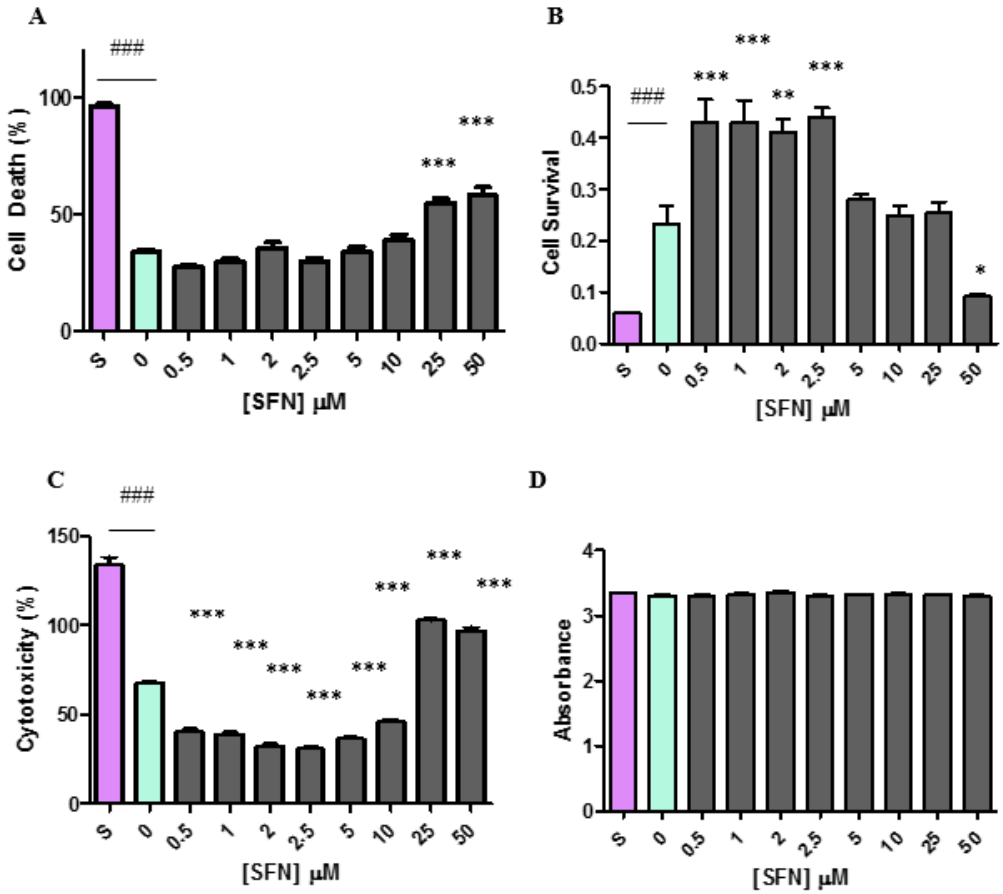

Figure 10 – ***Effects of SFN treatment on astrocytes in normoxic conditions.*** After 12 days in culture, assays are performed without exposure to OGD. Astrocytes treated with 100 nM of staurosporine and normoxic astrocytes served as controls. **A)** Astrocyte cell death was assed using trypan blue exclusion assay. **B)** Cell viability was assessed using MTT. **C)** LDH released into the media was converted into cytotoxicity. **D)** DNA amount was quantified at 260 nm. S: culture treated with 100 nM of staurosporine to induce massive cell death. * indicates significance in comparison to 0 µM of SFN, while # indicates significance between S and 0 µM of SFN. *p<0.05. **p<0.01. ***p<0.0001. ###<0.0001.

3.3.3 Effects of SFN on Apoptosis of Normoxic Neurons and Astrocytes

Amount of apoptosis activity was evaluated to determine whether SFN affected neurons and astrocytes under normoxic conditions. Cultures treated with staurosporine were used as positive controls of massive cell death.

Neurons

Under normoxic conditions, SFN appeared to increase apoptotic activity. Significant increase in active caspase 3 and 7 fluorescence was observed at 2.5 and 50 μM (Figure 11A).

Astrocytes

SFN appeared to increase apoptosis in normoxic astrocytes as well. There was a significant increase in fluorescence levels with 1 and 10 μM of SFN (Figure 11B).

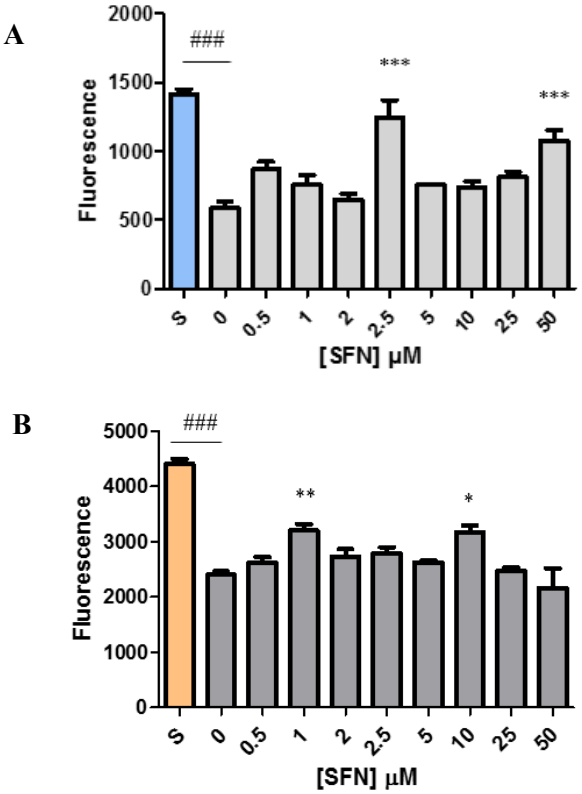

Figure 11 – ***Quantification of caspase 3 and 7 fluorescence of normoxic neurons and astrocytes.*** **A)** After 7 days in culture, neurons were exposed 25 hours of SFN. Using FAM-DEVD-FMK, caspase 3 and 7 activity was quantified. **B)** After 12 days in culture, astrocytes were exposed to 28 hours of SFN. Caspase 3 and 7 activity was measured using FAM-DEVD-FMK.S: culture treated with 100 nM of staurosporine to induce massive apoptosis. *$p<0.05$. **$p<0.01$. ***$p<0.001$.

3.4 Assessment of SFN's effects on Hypoxic-Ischemic Neurons and Astrocytes

Having successfully completed culturing of neurons and astrocytes, determination of time course for LD50 and recovery, and determining the effects of SFN in the normoxic condition, we next wanted to determine the effect of SFN's on cell cultures under the OGD condition. This assessment was done using four different assays: LDH cytotoxicity assay, trypan blue viability assay, MTT viability assay, and FAM FLICA caspase 3 & 7 apoptosis marker assay.

3.4.1 Effects of SFN on Neurons in OGD condition

Trypan blue

Trypan blue staining of neurons was quantified in the presence or absence of various doses of SFN as shown in Figure 11A. We observed a significant reduction in cell death in the presence of 10 μM of SFN compared to untreated control (0 μM SFN). Interestingly, cell death increased in the presence of 50 μM SFN (Figure 12A).

MTT viability assay

MTT cell viability assay of neurons was quantified in the presence or absence of various doses of SFN as shown in Figure 11B. Treatment with 0.5 to 10μM of SFN was seen to increase cell viability significantly in neurons (Figure 12B). Although there was an increase with 25 μM, it was not significant. At 50 μM, cell viability was significantly reduced.

LDH cytotoxicity assay

LDH release by neurons was quantified in the presence or absence of various doses of SFN as shown in Figure 10C. SFN dosages of 2, 2.5, 5, and 10 μM were shown to reduce cytotoxicity compared to no SFN treatment (0 μM SFN) (Figure 12C).

DNA quantification

There was no alteration on DNA content with SFN treatment indicating that SFN did not affect proliferation of cortical neurons in the OGD condition (Figure 12D).

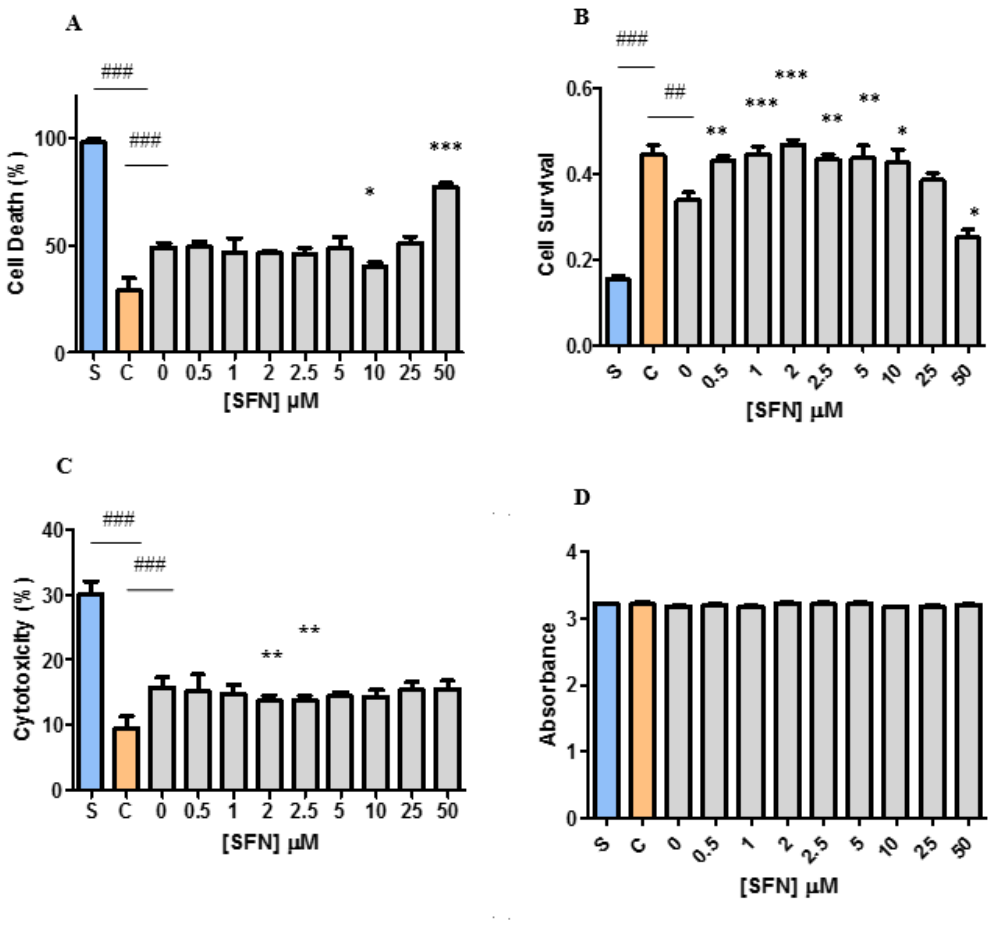

Figure 12 – E*ffects of SFN treatment on neurons exposed to OGD.* After 1 week in culture, neuronal cells are exposed to 1 hour of OGD and 24 hours of recovery with oxygen and glucose containing media. Neurons treated with 100 nM of staurosporine and normoxic neurons served as controls. **A)** Neuronal cell death was assed using trypan blue exclusion assay. **B)** Neuronal cell viability was assessed using MTT. **C)** LDH released into the media was converted into cytotoxicity index. **D)** DNA amount was quantified at 260 nm. S: culture treated with 100 nM of staurosporine to induce massive apoptosis. C: culture that is not exposed to any OGD or SFN. * indicates significance in comparison to 0 µM of SFN, while # indicates significance between S and C, and C and 0 µM of SFN. *p<0.05. **p<0.01. ***p<0.0001. ###<0.0001.

3.4.2 Effects of SFN on Astrocytes in OGD condition

Trypan blue viability assay

Trypan blue staining of astrocytes was quantified in the presence or absence of various doses of SFN as shown in Figure 13A. Non-significant decreases in overall cell death were observed with doses of 0.5 to 10μM of SFN. Similar to neurons, at 50 μM, cell death of astrocytes was increased compared to control (0 μM SFN) (Figure 13A).

MTT viability assay

MTT cell viability assay of astrocytes was quantified in the presence or absence of various doses of SFN as shown in Figure 12B. Treatment with 0.5 to 2.5 μM of SFN also increased cell viability of astrocytes. At doses greater than 5 μM, cellular viability appeared to show a trend towards decreasing. However, cell viability was decreased significantly only at 50 μM (Figure 13B).

LDH cytotoxicity assay

LDH release by astrocytes was quantified in the presence or absence of various doses of SFN as shown in Figure 10D. SFN doses of 0.5 1, 2, 2.5, 5, and 10 μM significantly reduced LDH cytotoxicity (Figure 13C).

DNA quantification

Astrocytes respond to ischemic injury by proliferating, and some are even capable of forming self-renewing multipotent cells [102]. However, DNA quantification revealed that SFN was not protecting astrocytes through increasing cell proliferation (Figure 13D). The constant quantified DNA absorbance suggests that increase in astrocyte viability is not a result of astrocyte proliferation, but decreased number of cell death.

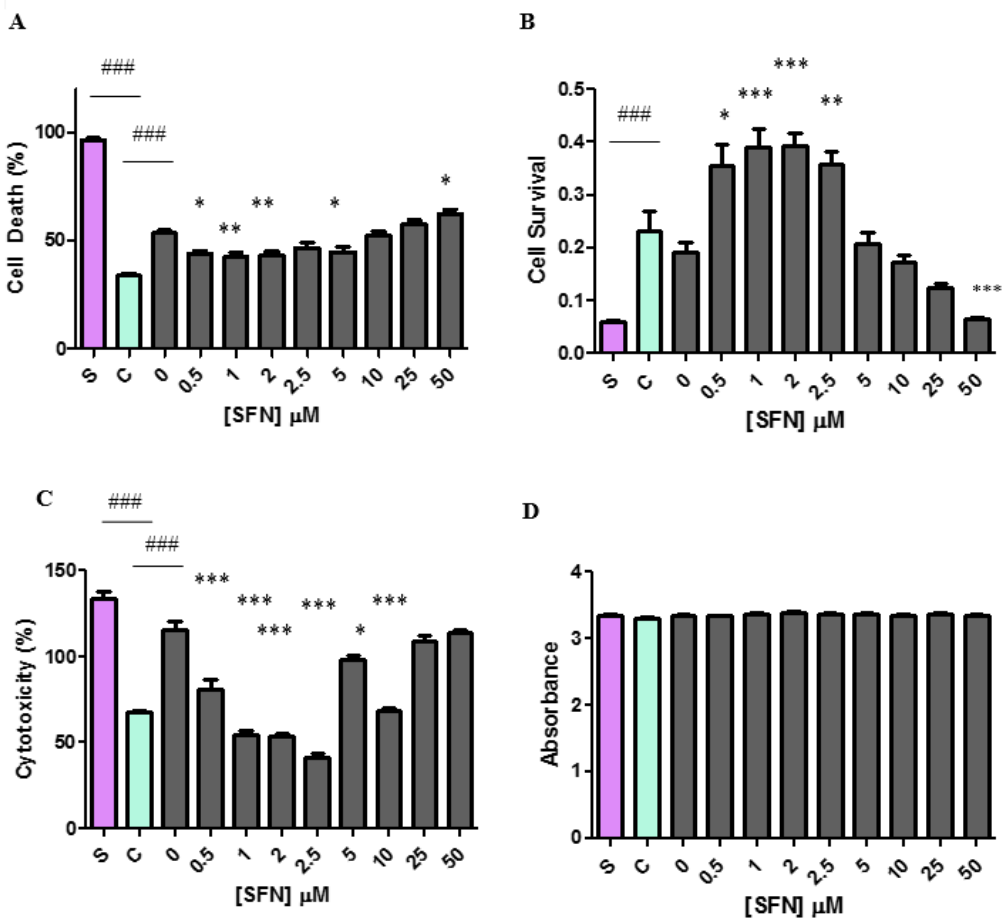

Figure 13 – E*ffects of SFN treatment on astrocytes exposed to OGD.* After 12 days in culture, astrocytes are exposed to 4 hours of OGD and 24 hours of recovery with oxygen and glucose containing media. Astrocytes treated with 100 nM of staurosporine and normoxic astrocytes served as controls. **A)** Astrocyte cell death was assed using trypan blue exclusion assay. **B)** Cell viability was assessed using MTT. **C)** LDH released into the media was converted into cytotoxicity index. **D)** DNA amount was quantified at 260 nm. S: culture treated with 100 nM of staurosporine to induce massive cell death. C: culture that is not exposed to any OGD or SFN. * indicates significance in comparison to 0 µM of SFN, while # indicates significance between S and C, and C and 0 µM of SFN. *p<0.05. **p<0.01. ***p<0.0001. ###<0.0001.

3.4.3 Effects of SFN on Apoptosis of Hypoxic-Ischemic Neurons and Astrocytes

Neurons

Results from the FAM-DEVD-FMK experiments showed that SFN did not increase caspase 3 & 7 activities, indicating that SFN itself does not induce apoptosis at any of the doses used (Figure 14A). However dosages of 1, 2, 2.5 and 50 μM appeared to decrease the amount of caspase fluorescence non-significantly indicating that SFN may actually protect neurons against normal apoptotic activity.

Astrocytes

2.5, 5, 25, and 50 μM showed significant decreased caspase 3 and 7 activity (Figure 14B). There is a downward trend of caspase activity with increasing dosages of SFN. This again indicates that SFN may protect astrocytes against apoptosis.

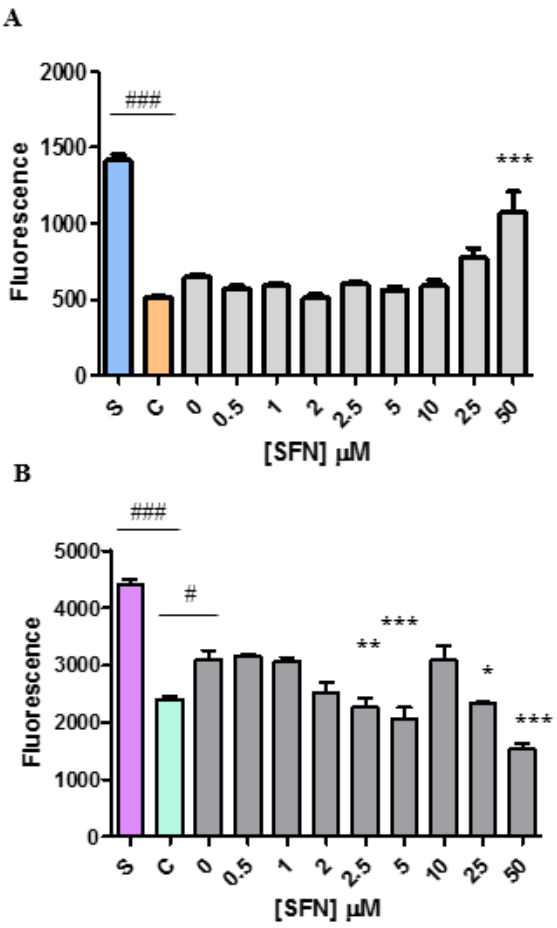

Figure 14 – ***Quantification of caspase 3 and 7 fluorescence of OGD neurons and astrocytes.*** **A)** After 7 days in culture, neurons were exposed to 1 hour of OGD then 24 hours of recovery. Using FAM-DEVD-FMK, caspase 3 and 7 activity was quantified. **B)** After 12 days in culture, astrocytes were exposed to 4 hours of OGD then 24 hours of recovery. Caspase 3 and 7 activity was measured using FAM-DEVD-FMK.S: culture treated with 100 nM of staurosporine to induce massive apoptosis. C: culture that is not exposed to any OGD or SFN. * indicates significance in comparison to 0 µM of SFN, while # indicates significance between S and C, and C and 0 µM of SFN. *p<0.05. **p<0.01. ***p<0.0001. #<0.05. ###<0.0001.

Chapter 4

Discussion

4.0 Discussion

Asphyxia, placental abruption, and prenatal stroke lead to shortage of oxygen and glucose to the fetal brain. Insufficient blood supply to the fetus increases the risk of CP. Despite numerous factors and conditions that can lead to hypoxia-ischemia in the fetal brain, the current preventative and post-partum treatments remain limited. The anti-apoptotic, anti-inflammatory effects of SFN may potentially represent preventative measures that can be used by expectant mothers to protect the fetus against the development of CP. This study first determined the optimal time for SFN treatment under conditions that simulate hypoxia-ischemia and then evaluated its effects under OGD and normoxic conditions using trypan blue exclusion assay, MTT viability assay, LDH cytotoxicity assay, and FAM-DEVD-FMK caspase 3 and 7 assay.

LD50 determination experiment revealed that neurons are more vulnerable than astrocytes to OGD. Neurons achieved LD50 with 1 hour of OGD compared to 4 hours for astrocytes, demonstrating higher sensitivity of neurons to hypoxia and glucose deprivation. The use of longer OGD times for astrocytes is consistent with other studies that model hypoxia-ischemia *in vitro* [85, 91, 103, 104]. Soane et al. (2010) exposed neurons to 1 hour of OGD, while Danilov et al. (2009) exposed astrocytes to 4 hours of OGD. Both studies determined effects of SFN in OGD induced death. Similarly, in other oxidative stress-induced studies, Kim et al. (2012) used 1 hour of OGD for neurons for examining neuroprotective effects of L-carnitine against OGD, and Zhou et al. (2015) used 4 hours of OGD to study protective effect of sulfiredoxin 1, an antioxidant protein, on astrocytes.

Astrocyte's higher resistance to models of cerebral ischemia is a result of greater glutathione (GSH) synthesis, which allows them to remain viable and functional [92]. GSH is an important cofactor in eliminating free radicals. O_2^- is converted into H_2O_2 by reacting with Cu/Zn-

superoxide dismutase (SOD). H_2O_2 is removed by GSH. GSH is oxidized into GSSG to reduce H_2O_2 into H_2O. GSSG is then reduced again back to GSH [105].

The present study found that SFN is able to protect neurons and astrocytes under OGD/ recovery conditions using Trypan Blue exclusion assay and LDH cytotoxicity assay. Both assays measure cells with permeabilized membranes. This permeabilization of the membrane is a key marker of necrosis. Necrosis is known to occur during and shortly after the oxidative insult. Taken together, these results suggest that SFN rescues neurons and astrocytes during OGD through attenuating necrosis.

The single most beneficial SFN dose for both neurons and astrocytes under OGD-Recovery condition appears to be 2.5 μM. Neurons and astrocytes exposed to OGD were observed to with significant increase in cell viability and decrease LDH release in the presence of 2.5 μM of SFN. OGD-treated astrocytes also showed significant decrease in apoptosis in the presence of 2.5 μM of SFN. In addition, normoxic astrocytes had increased viability and decrease LDH cytotoxicity. Interestingly, treatment of normoxic neurons with 2.5 μM of SFN resulted in a significant increase in apoptosis as demonstrated by the increased activity of caspase 3 & 7 in the SFN treated normoxic neurons compared to untreated. The increase in apoptosis may be associated with the fact that immature fetal brains have higher expressions of apoptotic players to counteract neuronal overproduction [26]. To this end it is reported that partial inhibition of caspase-3 protects neonatal brain against HI, while complete inhibition of caspase-3 or lack of caspase-3 is injurious due to amplification of necrosis and caspase-3-independent injury. [26].

Thus, SFN's inhibition of apoptotic pathways may actually exacerbate apoptotic cell death in neurons.

There are two main pathways of apoptosis: the extrinsic and the intrinsic pathway. The extrinsic pathway begins via activation of death receptors. Upon activation, the death domain of the receptor is able to transmit the signal from the cell membrane to the inside of the cell. Additional adaptor proteins are recruited through the death domain of the receptor. For example, once tumour necrosis factor (TNF)-α binds to TNF receptor 1, adaptor protein TRADD recruits FADD and RIP. FADD is then able to associate with procaspase-8 to form the death-inducing signaling complex (DISC). DISC is able to cause autocatalytic activation of procaspase-8.
The intrinsic pathway is triggered by intracellular signals. Various stimuli of intracellular origin induce the mitochondrial permeability transition (MPT) pores to open, which subsequently results in the release of pro-apoptotic proteins into the cytosol. Once Cytochrome c is released it activates Apaf-1 and procaspase-8 to form the apoptosome in the cytosol. There is also an aggregation of procaspase-9 which leads to caspase-9 activation [106].
The active caspase 8 from the extrinsic pathway and the active caspase 9 from the intrinsic pathway converge to activate caspases 3 and 7, which triggers morphological and biochemical changes of apoptosis [107].

We used MTT viability assay to further investigate OGD-induced cell death and the protective effects of SFN. Astrocyte viability increased under both OGD and normoxic conditions in the presence of 2.5 μM of SFN. To distinguish between increase in viability as a result of astrogliosis and decrease in astrocyte death, amount of DNA was quantified. Astrogliosis is the

increase in number of astrocytes after injury such as trauma, infection, ischemia, and stroke. The increase in viability also could be due to reduction in astrocyte death, which would not increase the number of astrocytes. The stable amount of DNA in all doses of SFN treatment suggests that SFN does not increase astrocyte proliferation. This may be beneficial in the fetal brain as inhibition of astrogliosis is reported having positive outcomes. Glial fibrillary acidic protein is an intermediate protein important in activating astrocytes and thus astrogliosis. Mice deficient in glial fibrillary acidic protein ($GFAP^{-/-}$) exposed to prenatal hypoxic-ischemic episode had better neuro-regeneration than wild type mice post day 4 after injury [108]. In addition, astrocytes are also reported to limit regeneration of synapses and axons that help brain recovery after an injury [109, 110]. The hypertrophy of astrocytic processes after injury may contribute to the inhibition of CNS regeneration. The constant amount of DNA in astrocytes with all doses of SFN demonstrates that the protective mechanism of SFN is not due to enhancing astrogliosis. It is also interesting to note that SFN is able to increase cell viability compared to the control in normoxic condition. The significant in increase in astrocyte viability suggests SFN's protective role in oxidative stress injuries.

Under normoxic conditions, up to 25 μM SFN did not have any effect on survival of neurons, while it had positive/beneficial effects on survival of astrocytes. The absence of cytotoxic effects of SFN from 0 to 25 μM suggests that SFN containing vegetable or SFN as a supplement is safe for ingestion pregnant women. However, it is important to note the significant detrimental effect of SFN at the high dose of 50 μM. During control/normoxic and OGD conditions, 50 μM of SFN appears to be cytotoxic for both neurons and astrocytes. The significant increase in cell death is demonstrated with trypan blue exclusion assay, increased LDH release, decreased cell viability,

and increase active caspase 3 and 7 activity. In fact the negative effects trend of SFN can already be observed at doses of 25 μM although it is not significant at this dose.

SFN displays a biphasic dose response characterized by neuroprotection seen at low dosages but cytotoxicity at high dosages (Figure 15). Cytotoxicity of SFN at higher dosages has been observed in other studies. In primary hippocampal neurons, 24 hours of 2 and 5 μM of SFN under normoxic condition significantly decreased cell death [85]. However, others have reported that 10 and 100 μM of SFN treatment for 48 hours under normoxic conditions significantly decreased rat striatal cell viability compared to no treatment [79]. Cytotoxic effects of SFN are also observed in astrocytes. Danilov et al. found that when astrocytes were treated with 0.1, 0.5, 1.0, 5.0, and 10 μM SFN 48 hours prior to OGD, 5.0μM significantly decreased cell death. However, 10 μM increased cell death compared to 5.0 μM when measured with propidium iodide (PI) staining [91].

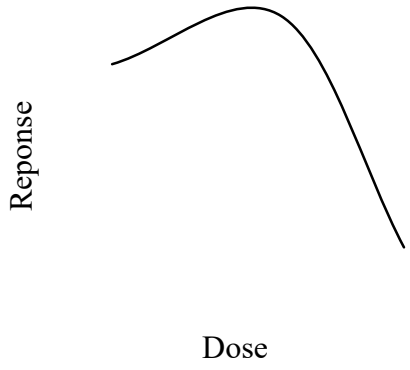

Figure 15 – ***Schematic representation of a biphasic response***

50 μM of SFN is cytotoxic was shown to be cytotoxic for both neurons and astrocytes in our study. In this context it is important to consider the pharmacokinetic properties including the absorbance and clearance rates of SFN in biological systems. In this study, neurons and

astrocytes are exposed for a finite amount of time to SFN, 25 and 28 hours, respectively. However, in in vivo situations where animals and humans, the absorbance and excretion of SFN ingest SFN need to be considered. Isothiocyanates (ITC) react with 1, 2-benzenedithiol producing 1, 3-benzodithiole-2-thione which can be quantified. Because negligible amounts of ITC itself are excreted unless humans consume cruciferous vegetables, quantification of 1, 3-benezodithiole-2-thione is a reliable way to obtain information regarding isothiocyanates and glucosinolates in vegetables. Human subjects who received 200 μmol of broccoli sprout reached a peak concentration in plasma, serum and erythrocytes after 1 hour [111]. Similarly in rats, after 0.5 mg/kg of SFN oral administration, SFN concentration was found to peak at 1 hour. Subsequently, concentration of SFN decreased steeply, by 90%, within 2 hours. Therefore, the time of exposure in vivo would be less than in vitro experiments. In addition, higher dose of SFN (50mg/kg) in rats resulted in decrease rate of absorption and bioavailability. At 0.5mg/kg of oral SFN ingestion, bioavailability was 82% while at 50mg/kg of oral SFN ingestion, bioavailability was 20% [112] . Thus, the detrimental effects of 50 μM of SFN would be less applicable in humans with less time of exposure and decreased rate of absorption and bioavailability of SFN.

In conclusion, this study demonstrated the potential use of SFN to prevent CP in children as a result of a hypoxic-ischemic episode in utero. We were able to reveal both positive and negative effects of SFN on neurons and astrocytes in normoxic and simulated hypoxic-ischemic conditions. Protective effects of SFN are mainly through the attenuation necrotic cell death, while the cytotoxic effect found at higher dose may be through potentiation of apoptosis. Although modulation of cell death through other pathways like autophagy by SFN remains to be established, the four different assays used in this study provide an overview of mechanism of

protection. Overall cell death was assessed through trypan blue exclusion assay, necrosis was assessed through MTT viability assay and LDH cytotoxicity assay, and apoptosis was assessed through FAM-DEVD-FMK caspase 3 and 7 assay.

Chapter 5

Future Directions

5.0 Future Directions

5.1 Improvements for current study

5.1.1 Additional analysis of purity of neuronal and astrocyte cell cultures

In addition to using Western blot analysis to check for contamination in cell cultures (Figure 1), we can also carry out immunocytochemical-staining analysis in future studies. Also, contamination by other types of glial cells like microglia and oligodendrocytes should be examined for both neuronal and astrocyte cultures. Glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule (IBA 1), 2'3-cyclic nucleotide 3'-phosphodiesterase (CNPase), and neuron nuclear antigen (NeuN) markers can be used for astrocytes, microglia, oligodendrocytes, and neurons, respectively. This can be used to determine a more accurate representation of the purity of our cell cultures. Our findings can also be quantified by the determination of percent contamination by each brain cell types.

5.1.2 Alternative approaches to degassing glucose free DMEM

In this study, glucose free DMEM was degassed using a vacuum for one hour at room temperature prior to its use for OGD. As an alternative, the medium can be placed in the hypoxia chamber for 24 hours prior to OGD post 1 hour of degassing as described above. This approach would allow for longer period of degassing and also for the medium to be maintained at 37 ºC. Additionally, the dissolved oxygen level in the medium can be evaluated using optical sensor. TheSensorDish reader is a device that is able to read oxygen levels in the medium from the fluorescent metal complex located on the bottom of each well that is sensitive to oxygen levels [113].

5.1.3 Alternative approaches to harvesting cells

Neurons and astrocytes are plated in wells and flasks that are coated with poly-D-lysine (PDL). The PDL coating facilitates cell attachment, growth, survival, and morphology by providing more sites for cell binding [114]. As a result, using trypsin, which is normally used to facilitate cell detachment from wells, is not as effective. In addition, trypsin can lead to cell damage as well. To harvest cells, cell scrapers were used to gently remove cells from the wells. Although this was effective, using mechanical methods could potentially have damaged the cells in the process. Since assessment of cell death was prominent importance in this study, any method that could lead to increased cell death should be avoided. Accutase™ is a possible alternative to using trypsin and cell scrapper. It is added to wells for 5 to 10 minutes at room temperature and allows cells to be detached with minimal damage.

5.1.4 Optimization of FAM FLICA caspase 3 & 7 detection

Generally, trypan blue, LDH cytotoxicity assay, and MTT assay showed congruent significant results. Although similar trends are found with FAM FLICA, the results are not significant. For example, decrease in active caspase 3 & 7 activities can be observed in neurons exposed to OGD with 1, 2, 2.5 μM of SFN (Figure 14A). However, these reductions were not significant to the 0μM. Additional optimization and experiments are required.

5.2 Prospective Studies

5.2.1 Protective effects of SFN on other glial cells

This study examines neurons and astrocytes in separate cultures. Although neurons and astrocytes comprise the majority of cell types in the brain, it is important to study the effects of OGD and/or SFN on other glial cells such as oligodendrocytes and microglia. Oligodendrocytes

may play a protective role after oxidative stress, while microglia could be both beneficial and detrimental [93, 94]. Thus, it is crucial to examine the effects of SFN during OGD in separate oligodendrocytes and microglial cell culture.

5.2.2 Examining effects of SFN on co-cultures of neurons and glial cells

Although our study shows that SFN has protective effects individually on neurons and astrocytes cultures, its effects in a system with multiple brain cell types should be examined. Exclusively targeting to protect neurons has been met with limited success in clinical trials. The future research and therapeutic interventions should target all components of neurological system [40]. Accordingly, our study would need to investigate the effects of OGD with or without SFN on co-cultures of neurons and glial cells.

Neuron & Astrocyte co-cultures

Presence of astrocytes is reported to be beneficial to neurons during oxidative stress. When neurons were exposed to OGD then recovered with astrocytes in a co-culture, there was significant reduction in cell death compared to neuronal culture only recovery. The reduction in cell death was also seen with neurons recovering in presence of astrocyte-conditioned medium [115]. During HI, neurons take up and store lactate that is released by astrocytes. Lactate is then used as a source of energy [116]. Additionally, astrocytes release GSH, which can be used by neurons to produce their own GSH [117].

Interestingly, ischemic neurons appear to affect astrocytes negatively. Neuronal cells were exposed to OGD for 3 hours prior to interaction with astrocytes. Ischemic neurons were shown to increase vascular endothelial growth factor (VEGF) production from astrocytes. VEGF induces endothelial barrier disruption, which was demonstrated by significant increase in permeability of the endothelial layer. Increased disruption to the endothelial barrier represents the damage to

BBB after ischemic stroke [118]. Due to the complex and dynamic nature to brain cell interaction, future studies of SFN in co-cultures will be beneficial.

5.2.3 The effects of SFN on Autophagy

SFN also has been shown to be involved in autophagy in addition to necrosis and apoptosis. Autophagy is a process that degrades damaged and misfolded proteins and cellular proteins through lysosomes. SFN is reported to induce autophagy by activating AMP-activated protein kinase (AMPK). AMPK activates tuberous sclerosis complex 1/2 (TSC1/2), which in turn blocks a negative regulator of autophagy, mammalian target of rapamycin (mTOR). By indirectly blocking mTOR, SFN induces autophagy. SFN increases AMPK activity by increasing phosphorylation of AMPK. SFN's ability to induce autophagy has been demonstrated by increasing levels of LC3-II and p62, proteins of autophagy, with SFN treatment. The role of autophagy is unclear as it may trigger apoptosis to worsen brain injury, but clearance cellular debris can be neuroprotective [119].

In addition to AMPK pathway, SFN may increase levels of LC3-II through extracellular signal-regulated kinase (ERK) activation in neuronal cells. When immortalized mouse cortical cells were treated with SFN, LC3-II levels rose after 24 hours. LC3-II levels increased as a result of increase in phosphorylation of a mitogen-activated protein kinase (MAPKs), ERK1/2. When mitogen protein kinase (MEK) inhibitors were used before SFN treatment, MEK inhibitors blocked the increase in LC3-II levels and phosphorylation of ERK1/2 [120]. From the results of these findings, SFN's effects on autophagic players in neuronal and/or astrocyte cultures after exposure to OGD can be studied in the future.

5.2.4 Clinical implications and conclusions

From the results of the study, SFN, which is found in broccoli and other cruciferous vegetables, seem to be protective against oxidative stress. However, the use of commercially available broccoli may not be able to provide the necessary benefits to mothers. The active enzyme in broccoli and broccoli sprouts is myrosinase. Myrosinase converts glucosinolate glucoraphanin into sulforaphane. However, during a common blanching process for frozen vegetable processing in order to increase shelf-live, 90% of the enzyme is degraded [121]. Using ingestion of fresh BrSps on the other hand shows clinical potential. 12 healthy subjects with 1-week intake of 100g/day of BrSps were shown to have decreased oxidative stress makers. Subjects had decreased amounts of phopshatidylcholinehydroperoxide (PCOOH), an indicator of lipid oxidation and organ damage, and increased amounts of $CoQ_{10}H_2$, which is an antioxidant [122]. However, because BrSps contain other isothiocyanates, direct correlation between SFN and neuroprotection cannot be assumed [77].

Chapter 6

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Appendix

A1: LDH Cytotoxicity Calculation

LDH cytotoxicity

The absorbance values obtained from a spectrometer are converted into cytotoxicity percentage using the suggested LDH cytotoxicity formula below provided by Abcam®. However, the formula does not allow for comparison between 0 µM and other SFN dosages. The "low control" that is referred by Abcam®'s formula is the 0 µM group in this study (Table1). Thus, because 0 µM is used as the "low control", comparisons can be only made between 0.5, 1, 2, 2.5, 5, 10, 25, and 50 µM (Figure A1A). For statistical comparison between SFN dosages to 0 µM, cytotoxicity calculation was modified to subtract the background LDH levels from just the cell media (Table1). Using the new LDH cytotoxicity formula, calculations were redone, which allowed for one-way ANOVA analysis of all the dosages (Figure A1B).

Abcam® LDH Cytotoxicity Calculation Formula	*Modified LDH Cytotoxicity Formula*
Cytotoxicity = (Test Sample – Low Control) / (High Control – Low Control) x 100%	Cytotoxicity = (Test Sample – Background) / (High Control – Background) x 100%

Table 1 – *LDH cytotoxicity calculation formulas*

Cultures treated with staurosporine was used as a positive control of death by apoptosis, labeled as S. LDH cytotoxicity was also compared to the normoxic condition, which also served as a control, is labeled as C (Figure A1C and A1D).

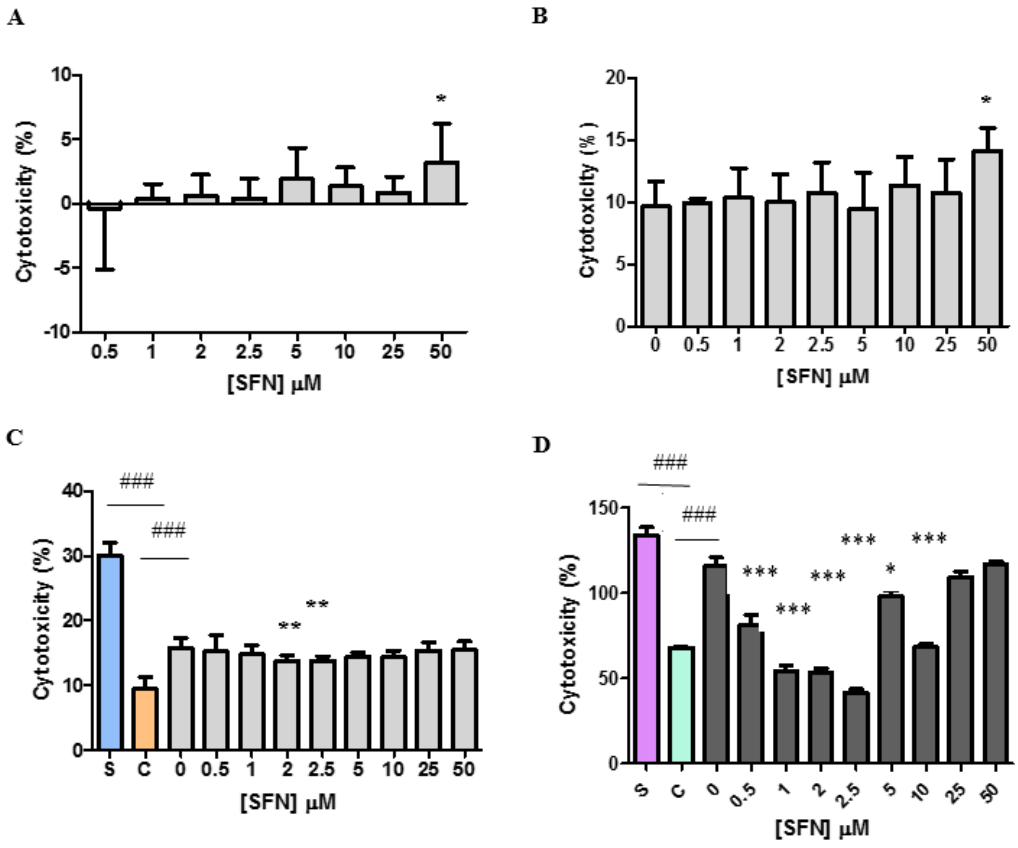
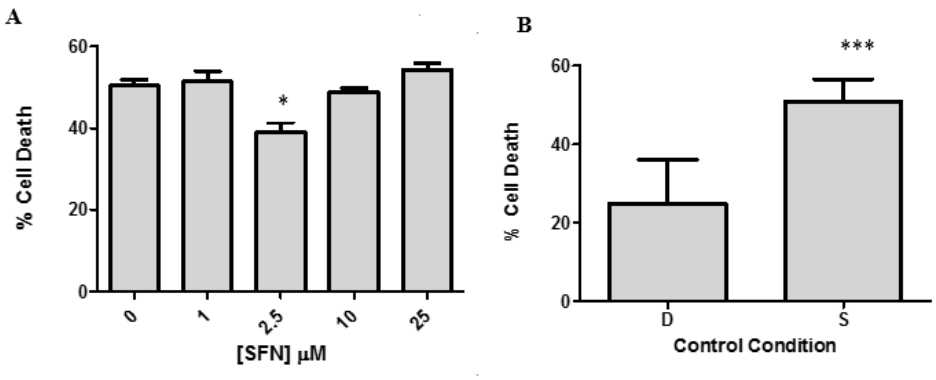


Figure A1 – *LDH cytotoxicity calculation and analysis.* The amount of LDH released into the media after 1 hour OGD and 24 hours of recovery with oxygen and glucose containing media is represented as cytotoxicity. **A)** After 1 hour of OGD and 24 hours of recovery with oxygen and glucose containing media, LDH released into the neuronal media is calculated using Abcam®'s suggested formula. **B)** LDH cytotoxicity is calculated using 0 µM as a relative comparison point using the modified formula by subtracting background LDH amount in the media. All dosages of SFN including 0 µM can be represented. **C)** After additional optimization to the LDH cytotoxicity protocol, neuronal LDH cytotoxicity is calculated. SFN dosages of 2, 2.5, 5, and 10 µM are able to reduce cytotoxicity compared to 0 µM. **D)** LDH released into the astrocyte media after 4 hours of OGD and 24 hours of recovery is quantified. 0.5 1, 2, 2.5, 5, and 10 µM of SFN significantly reduced LDH cytotoxicity. S: culture treated with 100 nM of staurosporine to induce massive apoptosis. C: culture that is not exposed to any OGD or SFN. * indicates significance in comparison to 0 µM of SFN, while # indicates significance between S and C, and C and 0 µM of SFN. *$p<0.05$. **$p<0.01$. ***$p<0.0001$. ###<0.0001.

A2: Control group requires separate incubator from the hypoxia chamber.

In our initial studies, when neuronal cells were treated with SFN under normoxic condition, we noted unusually high levels (approximately 50%) of cell death (Figure A2A). Querying this anomaly, we discovered that this might be because we were culturing our normoxic controls in the same incubator that housed the hypoxia chamber, which was inadvertently affecting the oxygen levels in the incubator. Since the hypoxia chamber is not completely sealed, there was leakage of nitrogen from this chamber to the surrounding incubator, which effectively decreased the oxygen level within the incubator as oxygen was being replaced, by nitrogen. This resulted in abnormal levels of cell death in the control group Neurons. To confirm that this was indeed the case, we compared cell death of neurons growing in the incubator containing the hypoxia chamber with cells growing in a separate incubator. Neuronal cell death was evaluated to be 20-25% when placed in a separate incubator compared to 50% for the neurons being cultured in the incubator containing the hypoxia chamber (Figure A2B).



Figures A2 – ***Optimization of control group experimental conditions*****. A)** There was 50% cell death for neurons growing under control conditions (0 µM SFN; in regular growth media) when grown inside the same incubator as an operating hypoxia chamber. **B)** The cell death of the neurons growing under control conditions (37 °C, 95% oxygen, 5% CO_2 in regular growth media with 0 µM of SFN was 20-25% when neurons were cultured in a separate incubator. D: Neurons grown in a separate chamber than that containing the hypoxia chamber; S: Neurons grown in the incubator that housed the hypoxia chamber while it is operational. *$p<0.05$. ***$p<0.001$.