Sulforaphane Protects Brain Cells from Oxygen & Glucose Deprivation

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Abstract

Perinatal brain injury results in neurodevelopmental disabilities (NDDs) that are inclusive of motor, cognitive and mental health disease. Some of these include cerebral palsy, intellectual disability, autism, attention deficit disorder and even schizophrenia. One of the most prominent etiologies is placental insufficiency which results in a hypoxic-ischemic (HI) environment in-utero. This leads to perinatal compromise, characterized by fetal growth restriction and brain injury. Since 80% of perinatal brain injuries occur during gestation, preventative approaches are needed to reduce or eliminate the potential for injury to the fetus and subsequent NDDs. Sulforaphane (SFA) derived from cruciferous vegetables such as broccoli sprouts (BrSps) is a phase-II enzyme inducer that enhances the production of antioxidants in the brain through the glutathione pathway. We have previously shown profound *in-vivo* neuro-protective effects of BrSp/SFA as a dietary supplement in pregnant rat models of both placental insufficiency and fetal inflammation. Strong evidence also points to a role for SFA as treatment for various cancers. Paradoxically, then SFA could enhance cell survival as a neuroprotectant, but with cancer, works to increase cell death. It is therefore important to determine the dosing parameters around which SFA is safe for the fetus, beneficial for brain protection, and which dosing range is toxic to cancer cells, and therefore may be very detrimental for use in the pregnant mother. We therefore explored, *in-vitro*, the dosing range of SFA for neuronal and glial protection and toxicity in normal and oxygen/glucose deprived (OGD) cell cultures.

OGD simulates, *in-vitro*, the condition experienced by the fetal brain due to placental insufficiency. We developed a cell culture model of primary cortical neuronal, astrocytes and combined brain cell co-cultures from newborn rodent brains. The cultures were exposed to an

OGD environment for various durations of time to determine the duration of OGD required to reach 50% cell death (LD50). We then evaluated the efficacy of varying doses of SFA for neuroprotective and neurotoxicity effects at LD50. Control cultures were exposed to normal media without OGD, and cytotoxicity of varying doses of SFA was also evaluated. Immunofluorescence (IF) and Western blot analysis of cell specific markers were used for culture characterization, and quantification of LD50. Efficacy effect of SFA was assessed by Live/Dead assay (IF/high content microscopy), and toxicity effect of SFA was assessed by both AlamarBlue viability and Live/Dead assay.

We determined LD50 to be 2 hours for neurons, 8 hours for astrocytes, and 10 hours for co-cultures, $p<0.0001$ compared to 0 hour OGD. The protective effect of SFA was noticeable at 2.5 μM for neurons, although not significant. There was a significant protective effect of SFA at 2.5 μM for astrocytes and co-cultures, $p<0.05$ compared to 0 μM SFA. Significant toxicity ranges were also confirmed in OGD cultures as ≥ 100 μM for astrocytes, ≥ 50 μM for co-cultures, $p<0.05$ compared to 0 μM SFA, but not toxic in neurons; and toxic in control cultures as ≥ 100 μM for neurons, and ≥ 50 μM for astrocytes and co-cultures, $p<0.01$ compared to 0 μM SFA. One Way ANOVA and Dunnett's Multiple Comparison Test were used for statistical analysis.

Our results indicate that cell death is significantly reduced in co-cultures treated with low doses of SFA exposed to OGD. Doses of SFA that were 10 times higher were toxic, not only under conditions of OGD, but in normal control cultures as well. The findings suggest that SFA shows promise as a preventative agent for fetal ischemic brain injury that could be developed as a safe innovative therapy for the prevention of childhood NDD.

Dedicated to my Mom.

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List of Abbreviations

AB	AlamarBlue
AA	Antibiotic-Antimycotic
ADHD	Attention deficit hyperactive disorder
AGS	Astrocyte growth supplement
AMPK	Adenoside monophosphate-activated protein kinase
Ara-C	Cytosine β-D-arabinofuranoside
ARE	Anti-oxidant response element
ASD	Autism spectrum disorder
BBB	Blood brain barrier
BCA	Bicinchoninic Acid Protein
BSA	Bovine Serum Albumin
BUAL	Bilateral uterine artery ligation
CAT	Catalase
Co-Culture	Combined Culture
CD	Cell Death

CD68	Cluster of Differentiation 68
CMF	Calcium and magnesium free
CP	Cerebral palsy
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNase I	Deoxyribonuclease 1
EtOH	Ethanol
FASD	Fetal Alcohol Spectrum Disorder
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
GlutaMAX I	L-alanine-L-glutamine
GPX	Glutathione peroxidase
GR	Glutathione reductase
GRA	Glucoraphanin
GSH	Reduced glutathione
GSSG	Oxidized glutathione

GST	Glutathione S-transferase
HI	Hypoxic-ischemic/Hypoxia-Ischemia
HO-1	Heme oxygenase 1
IF	Immunofluorescent/Immunofluorescence
IL	Interleukin
IUGR	Intrauterine growth restriction
KEAP1	Kelch-like ECH-associated protein
LD50	Lethal does (OGD time) to achieve 50% cell death
MIA	Maternal immune activation
NCC	Neural Crest Cell
NQO-1	NADPH quinine oxidoreductase 1
NDD	Neurodevelopmental disability
NHP	Natural health product
Nrf2	Nuclear factor E2-related factor
NSE	Neuronal Specific Enolase
OGD	Oxygen and glucose deprivation

Olig-2	Oligodendrocyte Transcription Factor 2
PAM	Pregnancy associated malaria
PBI	Perinatal brain injury
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PI	Placental insufficiency
PVDF	Polyvinylidenedifluoride
REDOX	Reduction-Oxidation
RFU	Relative fluorescent unit
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SFA	Sulforaphane
SOD	Superoxide dismutase
TBS	Tris-Base, NaCl
TBS-T	TBS and Tween

Chapter 1

Introduction

1.0 Introduction

1.1 Perinatal Brain Injury

Perinatal brain injury (PBI) refers to damage that occurs from approximately 24 weeks gestation to 1 month after birth. Eighty to ninety percent of injuries that result in cerebral palsy and other developmental disabilities occur in utero, before birth.[1] Current therapies focus on rescue rather than prevention; hypothermia is the most widely used rescue therapy accepted as a standard of care, and targets only 10-20% of newborn injuries at term. It therefore does not address the majority of PBIs.[1,2] The extent of injury depends on the underlying cause and gestational age at the time of injury.[3] There are multiple factors which contribute to PBIs such as placental insufficiency, inflammation, infection, toxicity, and genetics. This study focuses on PBI associated with placental insufficiency (PI).[4] The placenta is a vital organ in fetal development as it is responsible for transporting nutrients and oxygen to the fetus from the mother.[4] PI manifests as an inadequate transfer of oxygen and nutrients to the fetus by a reduced supply of blood; this results in a hypoxic-ischemic (HI) environment for the fetus.[5,6] PBI by HI occurs in 3 in 1000 newborns and 7 in 1000 preterm newborns.[7] The type of PBI is dependent on the gestational age of the fetus at the time of injury and duration of placental insufficiency. While a cortical gray matter injury would mainly affect neuronal and astroglial function in an infant closer to term, a white matter injury would more likely affect the oligodendroglia in the preterm infant before 34 weeks gestation. Not surprisingly, there is often an overlap of both gray and white matter damage.[8-10]

1.1.1 Placental Insufficiency

PI is the reduction in transfer of oxygen and nutrients from the mother to the fetus.[11,12] During pregnancy, glucose is the main source of fuel for the fetus; more specifically, glucose is mainly

taken up by the placenta and then distributed towards other fetal organs, mainly the brain and heart. When glucose uptake is restricted, the available glucose is mainly directed to the placenta at the expense of the heart and brain. In regards to oxygen, the 'brain sparing effect' takes place whereby there is an increase in cerebral blood flow for the fetus, resulting in an increased oxygen delivery and production of reactive oxygen species (ROSs).[11] The specific causes of PI are unknown; however, there are many factors that are associated including maternal smoking, hypertension, and previous pregnancy outcomes.[11] PI is related to a range of pregnancy related disorders including intrauterine growth restriction (IUGR), pre-eclampsia, preterm birth, and perinatal morbidity and mortality.[11,13] There are many methods as to how insufficiency is measured or determined, some of which include arterial blood flow, umbilical artery blood flow, maternal serum markers, placental morphology, and fetal growth and development.[11-13] It is important to note that PI is usually unpredictable, and although these methods can detect and monitor PI, they do not prevent or reverse adverse perinatal outcomes.[11] From these adverse outcomes, if a neonate does survive, they are at a higher risk of developing a spectrum of cognitive deficits later on in child hood and adult hood.[11]

1.1.2 Therapies for Hypoxia-Ischemia Associated Perinatal Brain Injury

Currently, therapies for HI associated PBI are limited as there is a high risk associated with research in a perinatal setting.[14] Hypothermia is a commonly used clinical therapy; it slows down brain metabolism with an intent to prevent any further fetal brain damage. This therapy is effective up to 6 hours following delivery for newborns ≥ 36 weeks gestation who have gone through asphyxia and thereby HI insult during delivery.[1,2,14-18] It is also high in cost and is therefore limited in developing countries.[16]

In addition to hypothermia, there are other therapies that have the potential to treat PBI, some are used clinically while others are in early stages of research. Melatonin, a sleep hormone, has shown anti-oxidative properties in animal and cell models by scavenging ROSs and as a mitochondrial protectant from oxidative stress.[16,17,19] Erythropoietin is a natural angiogenic hormone with anti-inflammatory and neuroprotective properties and has been observed to protect against HI and animal models by reducing astrogliosis and microglia activation.[16-18] Magnesium supplementation has been used intrapartum in cases of preterm birth, but it is not for HI specifically.[14,17,19] Topiramate, an anti-convulsant drug used to treat seizures, and magnesium have both been studied for their ability to reduce excitotoxicity in HI, but results of effectiveness are not well known.[17] Melatonin, erythropoietin, topiramate, and magnesium have also been used in combination with hypothermia to enhance it's effectiveness; clinical trials are underway to see their effects in protecting against HI injury.[16,17] A drug traditionally used to treat cancer, allopurinol, has also shown additional anti-oxidant properties and clinical trials suggest that postnatal administration may be neuroprotective in moderate HI neonates.[17] Creatine maintains cellular ATP levels and is endogenously created in the liver; it is currently being investigated in animal models as a preventative in HI associated PBI.[16,19] Resveratrol is a well studied phenol in perinatal animal models that shows anti-apoptotic effects through reducing excitotoxicity upon HI insult and by protecting mitochondria from oxidative stress.[16,19] Choline is a protein found in many foods; it is involved in multiple pathways necessary for embryonic and fetal development, however, many pregnant women do not consume enough choline from their diet.[19] Therefore, choline supplementation has been proposed as a method of perinatal neuroprotection.

In recent years stem cell therapy has come to light as a possible therapeutic option of many diseases and disorders including PBI. In particular, umbilical cord blood cells have shown

promise in animal models as they possess anti-inflammatory and anti-apoptotic properties.[16,20,21] Cytokines are a popular topic of research in PBI as they can be pro-inflammatory and detrimental to cell survival and blood brain barrier (BBB) function. Anti-cytokine antibodies are being examined as potential strategies for neuroprotection; specifically interleukin (IL)-1β and IL-6 which are pro-inflammatory during an HI insult.[15] Midkine is an anti-inflammatory cytokine present during mid-gestation and has potential in PBI treatment through enhancing its expression, as it is involved in micro and astrogliosis, neuronal and oligodendroglia maturation and cell death.[14]

Of the vast research being conducting in PBI therapeutics, the transition from discovery and translational research to clinical trials is slow because of the high risk associated with pregnancy. Majority of the clinically used therapeutics are rescue methods as opposed to preventative. Natural health products including melatonin, magnesium, creatine, resveratrol, and choline are becoming interesting topics of research as they have a lower risk associated with them. Among these natural health products as potential therapeutics in PBI, is sulforaphane (SFA) which will be discussed in detail.[19]

1.2 Neurodevelopmental Disabilities

PBI results in a spectrum of neurodevelopmental disabilities (NDDs) that have lasting effects on a child's life.[3] These disabilities occur due to an injury of the central nervous system, for a specific duration, and at a specific time of fetal development. Based on these latter factors, it can result in a host of learning and behaviour disorders, epilepsy, autism spectrum disorder (ASD), attention deficit hyperactive disorder (ADHD), and most notably, cerebral palsy (CP).[22,23] CP is a permanent, non-progressive, musculoskeletal disorder, and is a common outcome of PBI. Despite

an increasing focus on perinatal health, the incidence rate of CP has not decreased; it occurs in 2.5-4.0/1000 live term births and this rate increases to 16-22/1000 for prematurely born infants.[19] It is common for more than one NDD to be present in a child, and in addition to musculoskeletal problems, CP is often accompanied by disruptions in sensation, cognition, communication, and behaviour, including ASD and ADHD.[1] Hypothermia is an effective therapeutic strategy for brain injuries that occur at the time of birth; however, it has not been shown to decrease the overall incidence of NDDs.[3] A preventative approach is needed whereby treating the fetus via the mother prevents the occurrence or extent of PBI, and ultimately reduces the incidence of NDDs.

1.3 Brain Cells & Hypoxic-Ischemic Brain Damage

1.3.1 Brain Cell Types

Glia cells (astrocytes, oligodendrocytes, and microglia) take up a significant portion of the brain, while the remaining are neurons.[7,24] White matter areas of the brain contain oligodendrocytes and almost no neuron bodies; they do contain axons of neurons; while grey matter is mainly comprised of neuron bodies.[25] Neurons are precious cells of the brain involved in synaptic communication, and they do not proliferate on demand as glial cells can. Among many functions, glial cells control synapse formation, respond to neural activity, clear debris by phagocytosis, are metabolically coupled to neurons, and contribute to disease from aberrant behaviour.[24,26] Glial cells also provide neurons lactate as an energy source.[24] Astrocytes maintain homeostasis of the central nervous system by supporting neuronal function, glial transmission, and calcium signalling.[27] As a part of the BBB, astrocytes ensheath blood vessels and maintain tight junctions to promote the selective exclusion of substances crossing the BBB.[7,28] Oligodendrocytes are involved in ensheathing axons of neurons with a myelin membrane for optimal action potentials and synaptic transmission. Near the end of gestation and first postnatal month, white matter

maturation and myelination is most crucial and therefore any insult during this time can be detrimental, as it will hinder or delay maturation, ultimately leading to improper neuron function.[29] In a healthy brain, microglial cells are 'resting' and surveying the environment.[27]

1.3.2 Hypoxia-Ischemia & Effect of Brain Cells

PBI can be categorized into primary and secondary injuries. The primary injury is the initial destruction of brain tissue and necrosis, which then leads to the secondary injury, a cascade of molecular and biochemical events including ischemia, apoptosis, and inflammation.[27] Neuron cells are mainly effected by HI via two phases.[7] Initially neuron death can occur due to lack of overall energy from reduced adenosine triphosphate (ATP). Reduced ATP levels lead to an improper functioning ion channels and cell membrane depolarization and an accumulation of extracellular glutamate. Accumulation of intracellular lactate also results in an increase of ROS which leads to cell swelling and necrosis.[7] A secondary energy failure can occur after a recovered blood flow and results in mainly apoptosis through excitotoxicity, oxidative stress, inflammation, and other factors. This second phase of neuron cell death contributes to majority of cell death in PBI.[7] Upon damage, dying or dead cells in the brain release cellular debris which prompts astrocytes and microglia cells to activate an immune response. Activated astrocytes in response to injury is termed astrogliosis;[7] in rodent models, reactive gliosis has seen to be active up to 60 days after an injury. Another secondary mechanism, extracellular toxicity, can take place upon PBI. Neurons are vulnerable to an accumulation of glutamate in the extracellular environment; astrocytes play a role in the active re-uptake of glutamate from synapse to reduce toxic environments. In cases where astrocyte function is downregulated after an injury, this would result in an excitotoxic environment leading to neuronal degeneration. Astrocytes have also been seen to have a heightened proliferation rate post-injury as part of astrogliosis response; this can

lead to formation of glial scarring.[27] During HI, the integrity of the BBB is compromised allowing passage of unwanted molecules into the brain.[27,28] Upon injury, microglia are activated and aid in exacerbating or limiting injury through phagocytosis of cellular debris, producing pro-inflammatory cytokines and ROS scavenging receptors, and they can also contribute to breaking down the BBB.[7,27] Both astrocytes and microglia are capable of neuro-protection but also neuro-degeneration depending on their stimulus.[27] Excitotoxicity, oxidative stress, and cytokine signalling also contribute to damage of oligodendrocytes. Excitotoxicty against oligodendrocytes works also by extracellular glutamate accumulation. This leads to depolarization of mitochondria via increased intracellular calcium, resulting in an increase of ROSs causing oxidative stress. These processes are detrimental to the brain; by damaging oligodendrocytes they specifically reduce or attenuate the myelination process, thereby creating vulnerable neurons.[2,8,10,29,30]

1.4 Sulforaphane

1.4.1 Sulforaphane Derivation

Isothiocyanate, 4-methylsulfinylbutyl, is commonly known as Sulforaphane (SFA), and is derived from vegetables of the family *Cruciferae*, most prominently, broccoli sprouts of the genus *Brassica*.[31-35] It is naturally found as a R-SFA isomeric form, which is more active than the S-SFA isomer. Due to rapid interconversion between the two enantiomers in a biological setting, the racemic mixture (50% of each enantiomer) can be used experimentally, and is hereby noted as SFA.[31-34] SFA is derived from a hydrolysis reaction between glucosinolate glucoraphanin (GRA) and myrosinase which are both found as part of the broccoli sprout plant. SFA can also be formed by a similar reaction between GRA and gut microbiota.[36] GRA is stable and inert within the BrSp; it is once the BrSP tissue is injured that GRA interacts with myrosinase.[37]

1.4.2 Discovery and Importance of Sulforaphane

Although first cultivated in the 1500s, broccoli was not widely known or used until early-to-mid 1900s in North America.[37] It's impact on health was primarily investigated for the relationship between consuming broccoli and other cruciferous vegetables, against cancer. This stemmed from the observed trend that incidence of cancer was positively correlated with populations of diets high in meat, carbohydrates, and fats, and negatively correlated with diets high in vegetables. Therefore, vegan or vegetarian diets became a popular field of anti-cancer research, among other diseases and disorders.[38,39] SFA was first isolated from red cabbage in 1959 for use as an antibiotic, and prior to this, glucoraphanin was isolated from radishes for a similar purpose;[35] it was not until 1992 that Paul Talalay and colleagues isolated it from broccoli.[35,39] Shortly after, they also discovered that GRA was found in abundance in young BrSps, with SFA identified as the active component.[40] Among many attempts to create synthetic analogues of SFA, it remains the most potent natural anti-oxidant and anti-inflammatory inducer.[41-44]

The main role of glucosinolates in plants is to protect against pathogens and predators, and this is mainly attributed to isothiocyanates such as SFA which are created upon damage to the plant.[37,45] This injury and subsequent production of SFA is also what gives off the popular bitter smell and taste of broccoli.[35] Correlations between glucosinolate profiles and core physiological processes of the plant such as photosynthesis have been seen, and an abundance of isothiocyanates have also shown to decrease plant biomass; these facts suggest potential similarities in possible effects of sulforaphane in plants and mammals.[45,46]

1.4.3 Broccoli Sprouts and Sulforaphane Dose Conversion

However, since the protective concentration of BrSps compared to SFA is approximately 400 mg/kg to 1 mg/kg respectively,[37,40] it would not be ideal for a pregnant woman to eat such a large amount of BrSps daily. There is also a risk of salmonella poisoning with BrSps, making them not recommended to have during pregnancy. Since the dominant protective effect of BrSp comes from the indirect mechanism of SFA, it makes sense to maximize the value of BrSps in the form of a feasible treatment with SFA.[47] Creating a supplement with higher concentrations of SFA than what is available in BrSps, would provide an efficacious option for treatment.

1.4.4 Dose Dependency of Sulforaphane

SFA is a hormetic compound, whereby it exhibits a biphasic dose response.[48] At a high dose, SFA has been shown to be pro-apoptotic and anti-cancerous, while at a low dose it is anti-apoptotic and protective against oxidative stress and inflammatory responses.[48] The dose response profile of SFA has yet to be determined;[37] although the LD50 in mice has been observed at approximately 213 mg/kg via intraperitoneal injection, and this is about 10 fold greater than its observed effective dose.[37,49] It should be noted that like cancer, the fetus is also a rapidly dividing organism. Understanding the boundaries of therapeutic benefits and toxicity are therefore necessary to move this potential therapy forward to clinical use.[50]

1.4.5 Indirect Mechanism of Sulforaphane as a Phase II Enzyme Inducer

Placental insufficiency leads to an HI environment in utero which can result in the increase production of ROSs.[3] SFA is a phase II enzyme activator and works through an anti-oxidative mechanism via the Nrf2/ARE pathway (Figure 1). The anti-oxidant response element (ARE) is responsible for increasing anti-oxidative enzymes, such as glutathione reductase, to neutralize

reactive intermediates such as ROS.[31] Its profound effect is related to the central role of glutathione as an endogenous anti-oxidant.[51] Activation of ARE is dependent on nuclear factor E2-related factor (Nrf2) translocating to the nucleus.[31] Nrf2 is normally suppressed in the cytoplasm when bound to Kelch-like ECH-associated protein 1 (KEAP1).[31,34,52,53]

As a small lipophilic molecule, SFA can easily be up taken into a mammalian cell. Once inside, SFA is conjugated with γ-glutamylcysteineglycine via glutathione transferase (GT) and then sequentially converted into an N-acetylcysteine conjugate of SFA.[37,54-56] This final conjugate of SFA acts as an alkylating agent and electrophile to bind KEAP1 in order to release Nrf2 which can then migrate to the nucleus and activate ARE.[31,33]

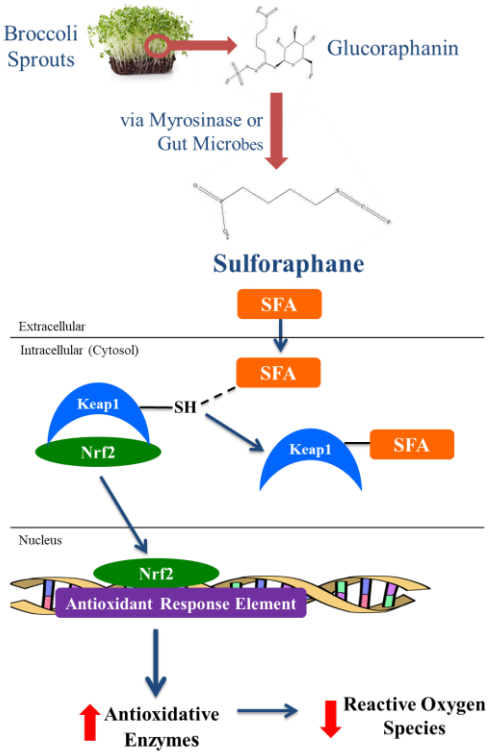

Figure 1. Indirect anti-oxidative mechanism of natural health product SFA. SFA is derived from BrSps: GRA and myrosinase present in BrSps combine to create SFA, or GRA can be converted to SFA from the gut microbiome. Intracellular: SFA releases transcription factor Nrf2 from protein Keap1. Nrf2 migrates to nucleus to transcribe antioxidant response element, increasing expression of antioxidant enzymes to neutralize reactive oxygen species.

1.4.6 Significance of Sulforaphane as an Anti-Oxidant Inducer

Anti-oxidants are a popular topic of discussion in healthy living; they are created endogenously or can be supplemented through exogenous methods. Anti-oxidant enzyme or protein production can also be induced directly or indirectly.[35,57] Direct inducers, such as Vitamin E, contribute towards reducing oxidative stress in a system primarily through scavenging ROS or by enhancing a specific anti-oxidant protein or enzyme. This effect is short termed as they are usually consumed in a RedOx reaction to perform their anti-oxidant role. Indirect inducers such as SFA have long lasting effects. SFA in particular does not go through a RedOx reaction; once it is bound to Keap1, it maintains its role in suppressing Keap1, allowing Nrf2 continue its transcriptional function.[35] Therefore, SFA acting as an indirect inducer of Phase II enzymes is quite advantageous in terms of its potential long lasting effect.

1.4.6.1 Importance of Glutathione in Oxidative Stress

SFA does not upregulate a specific anti-oxidant protein or enzyme, it enhances gene expression across the ARE. This includes glutathione peroxidase (GPX), glutathione S-transferase (GST), glutathione reductase (GR) superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), and NADPH quinine oxidoreductase 1 (NQO-1).[44,47,58] Among the enhanced enzymes, it is important to highlight GPX which is involved in regulation of reduced glutathione (GSH) and oxidized glutathione (GSSG) along with GR.[2,59] Additionally, the ARE also raises activity of L-γ-glutamyl-L-cysteine synthetase which is involved in the rate-limiting step of GSH production.[35,43,44,57,60] Therefore, SFA as a Phase II enzyme inducer will also elevate intracellular GSH levels.

GSH is extremely important in cellular RedOx homeostasis as a direct antioxidant. Along with scavenging ROSs and peroxides through GSH oxidation and reduction via GPX and GR (Figure 2), GSH is also involved in protection against lipid peroxidation, nucleotide peroxidation, and cytotoxicity of catecholamine quinones, it regenerates vitamin C and E through reducing oxidized ascorbate, and it is also involved in cellular proliferation, apoptosis and mitochondrial function.[35,57] It is evident that GSH is involved in many aspects of cell viability; therefore, SFA as an indirect inducer of the ARE and thereby GSH, is able to play a significant role in enhancing crucial cellular functions.[57]

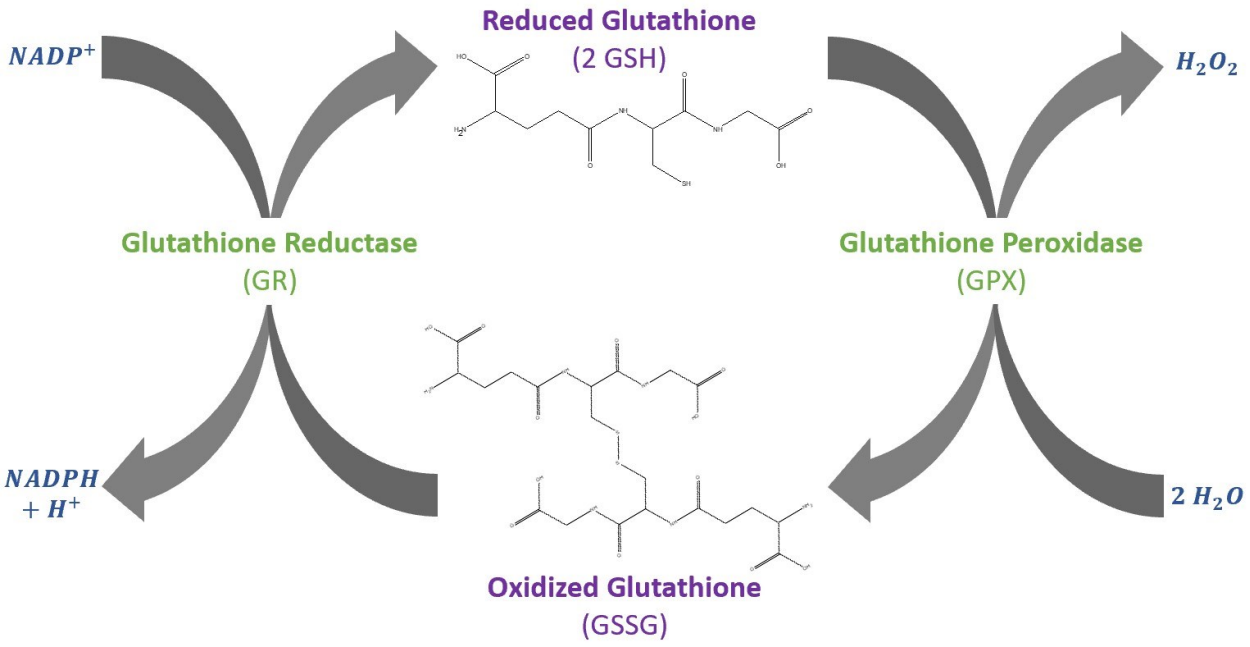

Figure 2. Glutathione as a biological redox buffer. GSH is a crucial component of intracellular RedOx homeostasis. GSH in its reduced form can be oxidized to GSSG via GPX to reduce peroxides and create water. GSH can be reformed from GSSG through a reduction via GR, and in this process $NADP^+$ is formed from NADPH and H^+.

1.4.7 Sulforaphane's Pathway to the Placenta

Mentioned previously, GRA can convert to SFA via microbes of the gut or myrosinase. When ingested as pure GRA without myrosinase, similar to cooked broccoli as myrosinase is inactive once heated, the conversion to SFA is not as effective.[54] Studies have shown that purely ingested GRA results in an excretion of less N-acetylcysteine conjugated SFA compared to ingestion of GRA and myrosinase.[54] Because of this inefficient conversion, it is thought that SFA alone as a supplement would be more effective; however, GRA is much more stable compared to SFA which is quite volatile. Nonetheless SFA is the active component and it is what is absorbed through the gut and into the blood stream.[37,54]

1.4.7.1 Absorption of Sulforaphane

As a lipophilic small molecule, SFA can passively diffuse into enterocytes of the small intestine where it is then conjugated to N-acetylcysteine, becoming a more stable molecule. This diffusion creates a favourable gradient and continues to accumulate SFA intracellularly.[56,61] SFA can then by released through the basolateral membrane of enterocytes and into the blood stream via active transporters multi-drug-resistance-associated protein 1 and P-glycoprotein.[56,61] Method of SFA uptake into other tissues is similar to absorption in the small intestine and is dependent on the availability of intracellular γ-glutamylcysteineglycine to create the stable conjugated form of SFA.[61] Studies have also shown that irrespective of route of administration, whether oral or intraperitoneal, majority of outcomes show a positive efficacy of SFA and that the difference in administration is not significant, suggesting a high bioavailability of this molecule.[37] Once in the maternal blood stream, SFA can reach the placenta among other organs of the body, and absorb into tissues and other cells by the process mentioned above.[62,63]

1.5 Sulforaphane and Glucoraphanin as a Treatment in Perinatal or Pediatric Health

Research in perinatal or pediatric health for SFA and GRA are in early stages. They can be used as a therapy during or after pregnancy. If provided as a therapy during pregnancy via the mother; once converted from GRA or when isolated, SFA is able to cross the placental blood barrier and the blood brain barrier, in order to directly impact the fetus and the brain respectively.[62,63] Alternatively, SFA or GRA can also be given to offspring following delivery. SFA has been proposed as a natural health product (NHP) that serves as an anti-oxidant and anti-inflammatory, although it has most extensively been researched for its anti-cancer properties.[33,37] Along with GRA, SFA has been also been shown to be effective as a therapeutic for neurodegenerative disorders such as Alzheimer's, Amyotrophic Lateral Sclerosis, and Parkinson's disease.[32,43,52] SFA and GRA have shown promise in *in-vitro* and *in-vivo* perinatal and pediatric models by acting primarily through the Nrf2/ARE pathway.[9,31,64]

1.5.1 *In-vitro* Studies

In many instances SFA can act through the Nrf2/ARE pathway to manage perinatal associated cellular injury due to factors including pregnancy associated malaria (PAM) and exposure to ethanol (EtOH).[9,31,64]

1.5.1.1 Protection Against Pregnancy Associated Malaria by Sulforaphane

PAM is a severe form of malaria infection which influences both the mother and infant. It leads to the recruitment of monocytes and macrophages to the placenta due to the accumulation of the parasite, *plasmodium falciparum*, in erythrocyte cells. CD36 is a scavenging receptor which may play a role in protecting hosts from inflammatory responses. Normally this receptor is controlled by Nrf2 and peroxisome proliferator-activated receptor gamma (PPARγ); however, PPARγ has

shown to be down regulated in PAM patients. During the malaria blood stage of infection, ROS are produced when hemoglobin is metabolized by *p. falciparum*. This rise in ROS activates the transcription factor Nrf2 which regulates inflammatory conditions via anti-oxidant gene HO-1; ROS are also meant to upregulate PPARγ, but this process is impaired.[64,65] Upon treating the maternal monocytes with SFA, Nrf2 transcription can further increase to manage the inflammatory response by reducing oxidative stress and by controlling CD36 by over compensating for the lack in PPARγ expression.[64,65] Therefore, increased expression of Nrf2 through SFA treatment may protect against the pathology of malaria during pregnancy.

1.5.1.2 Anti-Apoptotic Effects by Sulforaphane Following Ethanol Exposure

Excess EtOH concentrations predominantly occur in the neural crest cell (NCC) population during pregnancy. An abundance of EtOH results in oxidative stress and in histone modifications leading to epithelial-mesenchymal transition suppression, which results in apoptosis of NCCs and ultimately NDDs such as Fetal Alcohol Spectrum Disorder (FASD).[66-68] When NCCs are treated with SFA post-EtOH exposure, anti-apoptotic effects are seen through upregulation of Nrf2 resulting in enhanced SOD activity to reduce oxidative stress.[67] Although not well understood, SFA has also been seen to inhibit activity of histone deacetylase in NCCs, thereby increase DNA expression which is suppressed by EtOH exposure.[66,68] SFA treatment increased binding of acetylated histones to the Bcl-2 promoter to enhance Bcl-2 expression and diminish apoptotic effects.[68] Through a similar mechanism SFA can enhance Snail1, a factor that promotes epithelial-mesenchymal transition.[66] Through the multiple anti-apoptotic avenues seen through SFA treatment, it may be a possible therapeutic for FASD.

1.5.2 *In-vivo* Animal Studies

SFA and GRA have also been effective in animal studies, in association with the Nrf2/ARE pathway, including attenuating effects of toxic lead exposure during pregnancy and reducing schizophrenia-like abnormalities in offspring following maternal immune activation (MIA).

1.5.2.1 Sulforaphane Attenuates Oxidative Stress Following Lead Exposure

Low lead exposure of newborns during lactation can result in learning and memory decline in children through reducing the production of radical scavenging enzymes, thereby resulting in oxidative damage by accumulation of free radicals. In a study by Sun *et al.* SFA has shown to improve the spatial learning and memory ability of rats with cortical injury by attenuating the effect of oxidative stress, and decreasing blood lead levels in newborn pups.[69] Although mechanisms have not been explored well, it can be inferred that the decrease in oxidative stress by SFA may work through the Nrf2/ARE pathway, and that SFA may be a potentially therapy in treating toxic lead exposure during pregnancy.

1.5.2.2 Glucoraphanin Reduces Behavioural Abnormalities of Maternal Immune Activation

A stimulus, such as inflammation (microglial activation) or infection, can result in MIA. This can cause abnormal gene expression and increase oxidative stress in offspring, leading to possible cognitive impairments and then psychosis which could develop into schizophrenia. Dietary intake of GRA during juvenile and adolescent stages in murine rodents attenuates the increase in inflammation by converting to SFA; cognitive impairments in adulthood are also seen to reduce because of the GRA diet. It is possible that this result works through the Nrf2/ARE pathway and decreasing oxidative stress, as Nrf2 and KEAP1 proteins have actually been found to be

decreased in schizophrenia patients compared to control patients, suggesting that SFA indirectly upregulates ROS scavenging agents through activating Nrf2.[70-72]

1.5.3 *In-vivo* Human Clinical Trials

Human clinical trials in maternal and child health with SFA or GRA have focused on ASD in a pediatric population.[73,74] There has yet to be any human clinical experiments in a pregnancy or perinatal setting with SFA or GRA.

1.5.3.1 Sulforaphane and Glucoraphanin as a Therapy for Autism Spectrum Disorder

ASD is a neurodevelopmental disorder characterized by an impaired or restricted ability to communicate or interact socially, and currently there are no substantial pharmacological methods to treat the core symptoms of ASD. In a randomized control trial by Singh *et al.*, a daily SFA supplementation was given for 4 to 18 weeks in male children aged 13 to 27 years with moderate to severe ASD. A reduction in aberrant behaviour was observed; however, termination of the treatment lead to regressed improvements.[74] In another open-labeled study by Bent *et al.*, a daily GRA supplementation was given for 12 weeks in children aged 5 to 22 years with ASD. In this study an improvement in behaviour and social interactions was also observed. Additionally, urine samples were collected pre and post-intervention to analyze metabolic changes, and metabolites associated with oxidative stress were seen to change.[73] Molecular abnormalities associated with ASD surround an increase in oxidative stress and decreased anti-oxidant capacity, thus it is inferred that SFA can attenuate these effects through upregulating ROS scavengers through the Nrf2/ARE pathway, and further, it may be a possible option for early intervention or prevention of ASD.[73,74]

1.6 Sulforaphane as a Preventative Therapy in Perinatal Brain Injury

The research mentioned previously uses SFA or GRA as a reactive approach to perinatal and pediatric health; SFA as a preventative therapy has not extensively been researched. Our previous work has shown the potential for it as a neuroprotective agent in a perinatal setting.[1] Broccoli sprouts fed to pregnant rodent dams during the last trimester of pregnancy with a bilateral uterine artery ligation, showed protection of the fetal brain.[1] The aim of this project is to examine the impact of SFA on cell viability in an *in-vitro* model of perinatal brain injury. This study aims to examine whether SFA can prevent neuronal cell injury, as a potential preventative approach in PBI.

1.7 Hypothesis

i. Low dose of SFA will be protective against oxygen and glucose deprived (OGD) associated cellular damage, but have no effect on control cells

ii. High dose of SFA will be toxic to brain cells in both OGD and untreated control normal cells

1.8 Objectives

i. Develop an *in-vitro* model of PBI by exposing neurons, astrocytes, and combined cultures (co-cultures) to an OGD environment

ii. Analyze effect of SFA on neurons, astrocytes, and co-cultures exposed to OGD

iii. Analyze effect of SFA on neurons, astrocytes, and co-cultures under normal conditions.

Chapter 2

Methodology

2.0 Methodology

2.1 Animal Care and Use

All animal work was approved by the Animal Care and Use Committee at the University of Alberta (AUP00000363). Long-Evans rats were purchased from Charles River (Montreal, PQ) and were kept on a 12 hour light/dark cycle with ad libitum access to irradiated normal rat food and water. Male and female rats were bred in a 1:2 male to female ratio. Female rats used for breeding were between 9 weeks and 5 months old and were allowed no more than three parturitions. Pups were delivered vaginally and stayed with the mother for 12 to 36 hours after birth before being euthanized via decapitation. The day of birth was determined to be postnatal day 1 (PD1).

2.2 Isolation of Cortical Tissue

Rat cortical tissue was prepared from PD2 Long-Evan rats of both sexes. Brains were dissected and cortices were removed from meninges, isolated, and transferred to a Petri dish containing calcium and magnesium free (CMF) Hank's Balanced Salt Solution (HBSS) (Gibco, cat. no. 14170-112). Cortical tissues were enzymatically digested in 1mg/mL papain (Thermo Scientific, cat. no. 88285E) at 1.25 µl per 10 pups for 10 minutes at 37ºC. 30 µl of DNase I (Deoxyribonuclease 1) (Millipore Sigma, cat. no. 11284932001) per pup was added to the digestion mix for 5 minutes at the end of incubation. 200 µl of Fetal Bovine Serum (FBS) (Gibco, cat. no. 12483-020) per pup was added to stop the action of papain. Samples were centrifuged at 250g for 3 min and supernatant was aspirated. Initially, samples were centrifuged at 200g for 1 min; this did not provide a sufficient separation between the supernatant and cellular matter, as the supernatant could not be aspirated without disturbing the cellular matter; therefore, both time

and speed were increased until a sufficient separation was seen. Cortices were triturated by pipetting 10 times with a glass pasteur pipette; this was repeated two additional times for a total of three triturations.

2.2.1 Isolation of Neurons

The cell suspension was filtered through a 70 μm Nylon mesh cell strainer (ThermoFisher Scientific, cat. no. 22363547) with cell culture media containing Neurobasal-A medium (Gibco, Cat# 10888-022) supplemented with 2X B27 (Gibco, #17504-044), 1X N2 (Gibco, cat. no. 17502-048), 4X glutaMAX I (Gibco, cat. no. 35050-061), and 2X Antibiotic-Antimycotic (AA) (Gibco, cat. no. 15240-062). AA included Penicillin and Streptomycin for antibiotic properties and Amphotericin B for antifungal properties. The N2 supplement enhances growth and expression of post-mitotic neurons in primary cultures of the central nervous system; therefore, it was added to complete media to specifically enrich for neurons. Neurons were plated on poly-D-lysine (Millipore Sigma, Cat. no. P7280) coated wells at a density of $4.5x10^5$cells/well in 24-well plates and $9x10^4$cells/well in 96-well plates. This was triple the amount of cells plated for astrocytes, as neurons do not proliferate in culture. Initially, only double the amount of cells was plated, however, the cultures were not confluent at 7 days for experiments. Therefore, tripling the cell count was done to account for cell death after isolation, and this achieved confluence. 30 min after plating cells, half of the media was removed to remove any non-attached glial cells; and the amount of media removed was replenished with fresh media. Media was changed 24 hours after plating to remove cellular debris and any cells that died during extraction and isolation. Media was also changed every 2 days with the addition of 10 mM Cytosine β-D-arabinofuranoside (Ara-C) (Millipore Sigma, Cat. no. C1768). Ara-C killed any existing glial cells by preventing proliferation through inhibiting DNA replication and fragmenting DNA. Experiments started on

day 7 in culture; neurons are vulnerable compared to other cortical brain cells and therefore, experiments started earlier for neurons compared to astrocyte cultures.

2.2.2 Isolation of Astrocytes

The cell suspension was filtered through a 70 μm Nylon mesh cell strainer with cell culture media containing Astrocyte medium - animal (ScienCell, cat. no. 1831) supplemented with 2X AA, 1X Astrocyte growth supplement (AGS) (ScienCell, cat. no. 1882), and 2X FBS. The astrocyte medium provided optimal growth for normal astrocytes in vitro through a balanced nutritional environment. AGS was added specifically to enhance growth of normal rat astrocytes in vitro. Astrocytes were plated on poly-D-lysine coated wells at a density of $1.5x10^5$cells/well in 24-well plates and $3x10^4$cells/well in 96-well plates. Media was changed 24 hours after plating, and every 3 days. Experiments started on day 12 in culture.

2.2.3 Isolation of Co-Cultures

The cell suspension was filtered through a 70 μm Nylon mesh cell strainer with cell culture media containing Neurobasal-A medium, supplemented with 2X B27, 4X glutaMAX I, and 2X AA. Cells were plated on poly-D-lysine coated wells at a density of $4.5x10^5$cells/well in 24-well plates and $9x10^4$cells/well in 96-well plates. Media was changed 24 hours after plating, and every 3 days. Experiments started on day 7 in culture.

2.3 Hypoxic-Ischemic Insult

2.3.1 Oxygen and Glucose Deprivation

The oxygen sensor (Pro Ox: 110, BioSpherix) for the hypoxia chamber was set at 0% oxygen level. Oxygen in the incubator was replaced by a mixture of gas that was 95% nitrogen and 5%

carbon dioxide at 37℃. Regular growth media was removed from experimental plates and plates were washed with 1X Phosphate Buffer Saline (PBS) (HyClone, cat. no. SH3025601) (pre-warmed at 37℃) to ensure normal media was not present. Glucose-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco, cat. no. A14430-01) was warmed at 37℃ and degassed for 10 minutes using a vacuum to remove the oxygen within the media, supplemented with 1X AA, and added to the cells during the hypoxic insult. Once the oxygen was removed from both the incubator and the media, the cells were 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 levels were below 1%.

2.3.2 Control

Media was removed from control plates and plates were washed with 1X PBS (warmed at 37℃) to ensure consistent treatment between experimental and control plates. Control plates were replenished with regular media and kept in normoxic conditions at 37℃ until the last OGD time point was completed.

2.4 24 Hour Recovery

2X regular growth media (except with only 1X AA because 1X was already present in DMEM media) was added to plates from the anoxic incubator which already contained DMEM media, and then the cells were placed in a normoxic incubator at 37℃ with 5% carbon dioxide and 95% air, to recover for 24 hours. Initially, the DMEM media was removed, and then 1X of regular growth media was added; however, visually through a microscope it was seen that this caused many cells which were dead/dying to be removed when removing the DMEM media. Therefore, instead, 2X regular growth media was added to the plates in addition to the DMEM media

already present, and from the 24 hour recovery, cells were allowed to recover and reattach to the bottom of the plate.

2.5 Protein Analysis

2.5.1 Preparation of Cell Lysates

Cells from 24-well plates were washed with 1X PBS and then with 25 µl of 1X Radioimmunoprecipitation assay (RIPA) Lysis Buffer (Millipore Sigma, cat. no. 20-188) per well, which contained protease inhibitor (Millipore Sigma, cat. no. P8430) and phosphatase inhibitor (Millipore Sigma, cat. no. 524629) to lyse the cells and prevent protein degradation. Lysed cells were centrifuged at 4°C for 5 minutes at 12,000 rpm. The pellet of cellular debris was discarded, and the supernatant collected for protein quantification.

2.5.2 Immunoblotting

The Pierce™ Bicinchoninic Acid Protein (BCA) Protein Assay (Thermofisher Scientific, cat. no. 23227) was used to quantify the amount of protein in each sample. Cell lysates were diluted in ddH_2O by a factor of 10 and incubated for 30 min with BCA Reagents A and B. Protein was quantified by absorbance at 562 nm using a LUMIstar Omega microplate reader (BMG LABTECH, Germany) and compared to predetermined Bovine Serum Albumin (BSA) protein standards. Following protein quantification, equivalent amounts of protein were resolved by electrophoresis of samples on a 7.5% Tricine polyacrylmide gel. Proteins were then transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, cat. no. 1620177). After the transfer, the membranes were blocked in 5% Blocking Milk (Carnation) in TBS (Tris-Base, NaCl) and incubated with various primary antibodies at room temperature for 1 hour or overnight at 4°C. Membranes are then washed with TBS-T (TBS and Tween), then incubated with

horseradish peroxidase conjugated secondary antibodies (GE Healthcare UK Limited) at room temperature for 1 hour. Western blots were visualized using Western Lightning® Plus-ECL (PerkinElmer, LAS Inc., cat. no. NEL103001EA). Antibodies to the following proteins were used for this study; diluted in 5% Blocking Milk: Anti-Neuronal Specific Enolase (NSE, 1:2500) (Abcam, cat. No. ab53025), Anti-Glial Fibrillary Acidic Protein (GFAP, 1:5000) (Abcam, cat. no. ab7260), Anti-Cluster of Differentiation 68 (CD68, 1:3000) (Abcam, cat. no. ab31630), and Actin (1:5000) (Santa Cruz Biotechnology Inc). Anti-NSE and Anti-CD68 primary antibodies were used overnight at 4°C as 1 hour at room temperature was not enough to show a signal.

2.6 SFA Treatment

R,S-Sulforaphane, dissolved in water, is obtained from LKT Laboratories, Inc. Cells were treated with SFA at different doses (0, 1, 2, 2.5, 5, 10, 25, 50, 100, and 200 μM). SFA is added to degassed DMEM for OGD and added to regular growth media for control plates, recovery, and cytotoxicity experiments.

2.7 Live/Dead Assay

Following 24 hour recovery, Live/Dead assay (Thermofisher Scientific, cat. no. L3224) was used to determine percentage of cell death (CD) in 96-well plates; $9x10^4$cells/well for neuron and co-cultures, and $3x10^4$cells/well for astrocyte cultures. Cells were stained with green calcein-AM or red ethidium homodimer-1; greed fluorescence indicated live cells via intracellular esterase activity which fluoresced throughout the cell body and nucleus, and red indicated dead cells by loss of plasma membrane integrity which fluoresced only in the nucleus. They were left to incubate at room temperature for 10-15 minutes. Cell counting was done using a high content analysis system, MetaXpress XLS (Molecular Devices, San Jose, CA, USA), with a diameter

range of 5 to 30 µm. Live cells were counted based on FITC fluorescence at excitation/emission peak wavelengths of 495/519 nm and dead cells were counted based on TexasRed fluorescence at excitation/emission peak wavelengths of 589/615 nm. CD percentage was normalized to control plates. (See Appendix A1)

2.8 AlamarBlue Cytotoxicity Assay

The relative cytotoxicity of different concentrations of SFA was established using an AlamarBlue (AB) assay. Cellular metabolism can be spectrophotometrically measured by examining the difference between the oxidized and reduced state of the REDOX indicator resazurin. Resazurin (oxidized) is blue and non-fluorescent, whereas resorufin (reduced) is red/pink and highly fluorescent. Measuring changes in the fluorescence of the dye in intracellular environment, the number of metabolically active cells can be detected. Cells were incubated with different concentrations of SFA (0, 1, 2, 2.5, 5, 10, 25, 50, 100, and 200 µM) using untreated cells (0 µM SFA) as control cells, after 24 hours of incubation AB solution (10% [v/v] solution of AB dye) was added into 100 µl of complete media to each well. Wells containing only the AB solution/media without cells was used as the blank. Following 2 hour incubation, AB fluorescence was quantified at the respective excitation and emission wavelength of 540 and 595 nm respectively, using a LUMIstar Omega microplate reader. Viability percentage was normalized to controls using relative fluorescence units (RFUs). (See Appendix A2)

2.9 Immunofluorescence

Cells in 24-well plates were washed with 1X PBS and treated with 4% paraformaldehyde (PFA) (Thermofisher Scientific, cat. no. 15710) for 15 mins to fix cells. Cells were washed again with 1X PBS, followed by treatment with 0.2% 100X Triton (Thermofisher Scientific, Fisher

BioReagents cat. no. BP151500) in 1X PBS for 10 minutes to permeabilize cells. After washing with 1X PBS again, wells were blocked with 2% BSA (Thermofisher Scientific, Fisher BioReagents cat. no. BP1600-100) in 1X PBS at room temperature for 1 hour or overnight at 4°C, and then incubated with various primary antibodies at 4°C overnight. Wells were washed with 1X PBS, and then incubated with secondary IF antibodies at room temperature for 1 hour. Cell characterization of co-cultures was established via IF cell images visualized using a laser scanning confocal microscope (Zeiss LSM710) and quantified using a high content analysis system, MetaXpress XLS. 4′,6-diamidino-2-phenylindole (DAPI) was used as a cell staining control and total cell count. Antibodies to the following proteins were used for this study; diluted in 1X PBS: Anti-NSE (1:200), Anti-GFAP (1:1000), Anti-CD68 (1:100). Detection of fluorescence of the antibody represented the presence of that related protein, and a brighter fluorescence represented a greater quantity of protein.

2.10 Statistical Analysis

GraphPad Prism 5 computer software was used. Data were from at least 3 experiments with a minimum of triplicates per experiment. Values were expressed as mean±SEM. Statistical significance was analyzed using one-way ANOVA with Dunnett's Multiple Comparisons Test. Significance is indicated by *($p<0.05$), **($p<0.01$), ***($p<0.001$), or ****($p<0.0001$).

Chapter 3

Results

3.0 Results

3.1 Cell Culture Purity

Immunofluorescence images showed the characterization of neuronal cultures, astrocyte cultures, and co-cultures. The neuronal cultures showed enhanced staining for NSE protein (Figure 3A) and non-detectable contamination from astrocytes or microglia. The astrocyte cultures showed enhanced staining for GFAP protein (Figure 3B) and non-detectable contamination from neurons or microglia. The co-cultures (Figure 3C) showed the presence of all cell types: neurons, astrocytes, and microglia. The cell nuclei are stained with DAPI.

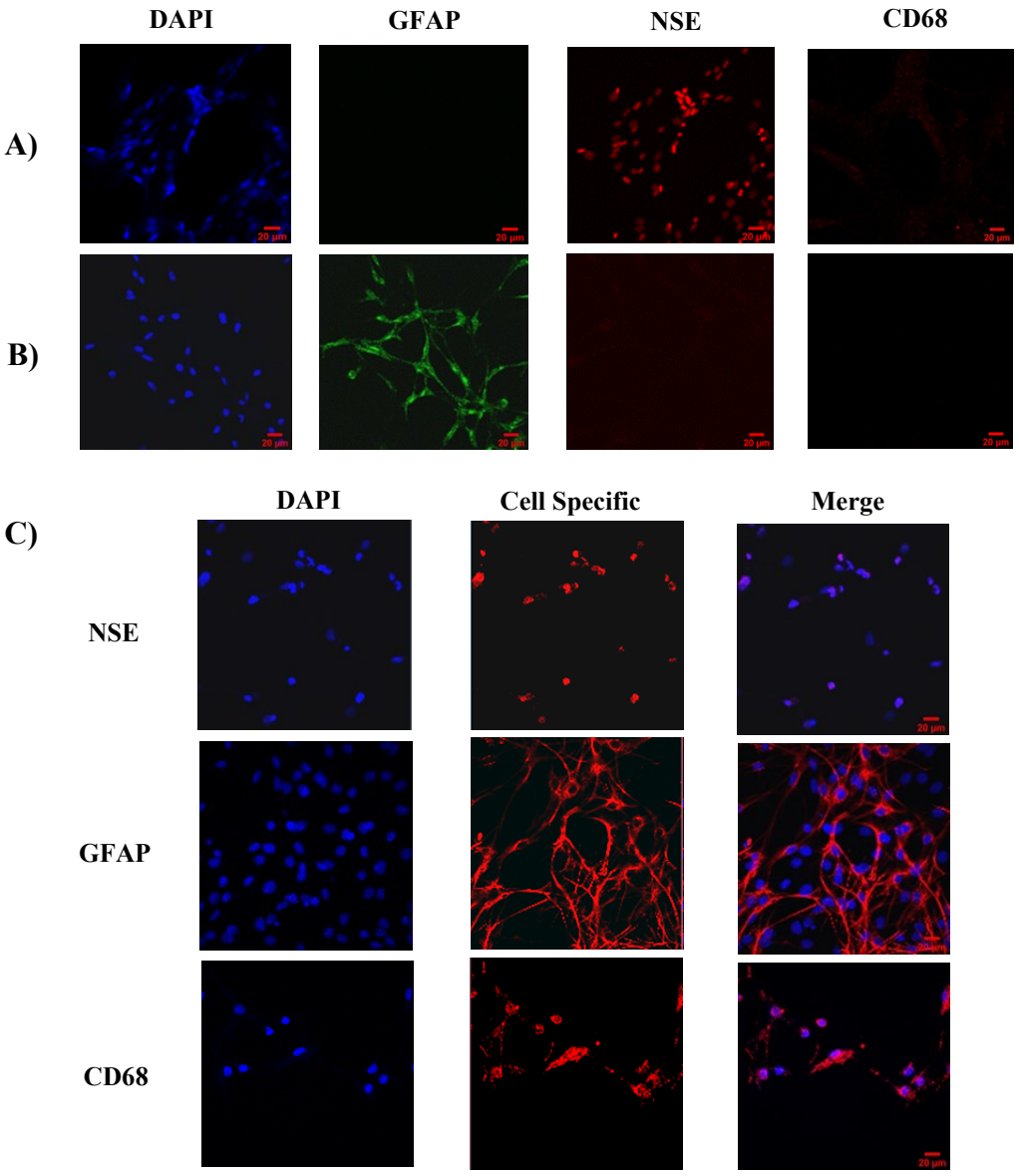

Figure 3. Cell Culture Purity. Immunofluorescence analysis to confirm the characterization of control cortical brain cell cultures. DAPI is a staining control and represents the nuclei of all cells. Cell specific markers used were: NSE for neurons, GFAP for astrocytes, and CD68 for microglia. **A)** Neuron culture **B)** Astrocyte culture, **C)** Co-culture. Scale shown is 20 µm.

3.2 LD50 Determination

LD50 was defined as the duration of OGD that achieved equal to or above 50% cell death. LD50 was determined and used as a standard for later experiments with SFA treatment. The LD50 for each cell culture was determined by a Live/Dead assay, and cell death for each OGD duration was normalized to control (0h OGD). The increase in OGD duration showed significant cell death of 36%, 53%, 59%, and 62% in neurons at 1, 2, 4, and 8 hours respectively, compared to 0 hour OGD control (Figure 4A). LD50 was determined to be 2 hours for neurons (Figure 4A). Western blot analysis of the neuronal culture for the neuronal marker NSE showed that cellular levels of NSE decreased significantly with increasing duration of OGD (Figure 4B-C). The increase in exposure to OGD produced significant cell death of 19%, 40% and 60% in astrocytes at 4, 6 and 8 hours respectively, compared to 0 hour OGD control (Figure 5A). The LD50 was determined to be 8 hours for astrocytes (Figure 5A). Western blot analysis of the astrocyte culture for the astrocyte marker GFAP shows that cellular levels of GFAP decreases significantly with increasing duration of OGD (Figure 5B-C). The increase in OGD duration displayed significant cell death of 12%, 25%, 41%, 53%, and 61% in co-cultures at 4, 6, 8, 10, and 12 hours respectively, compared to 0 hour OGD control (Figure 6A). LD50 for the co-culture of brain cells was determined to be 10 hours (Figure 6A). Figure 5B showed the relative cellular levels of the different cell markers with time of OGD, with a significant decrease in neurons being evident at 4 hours OGD. CD68, a biomarker for microglia, were either undetectable (Figure 5B, 6B) in the astrocyte and co-cultures or present in very small quantities (Figure 4B) in the neuronal culture.

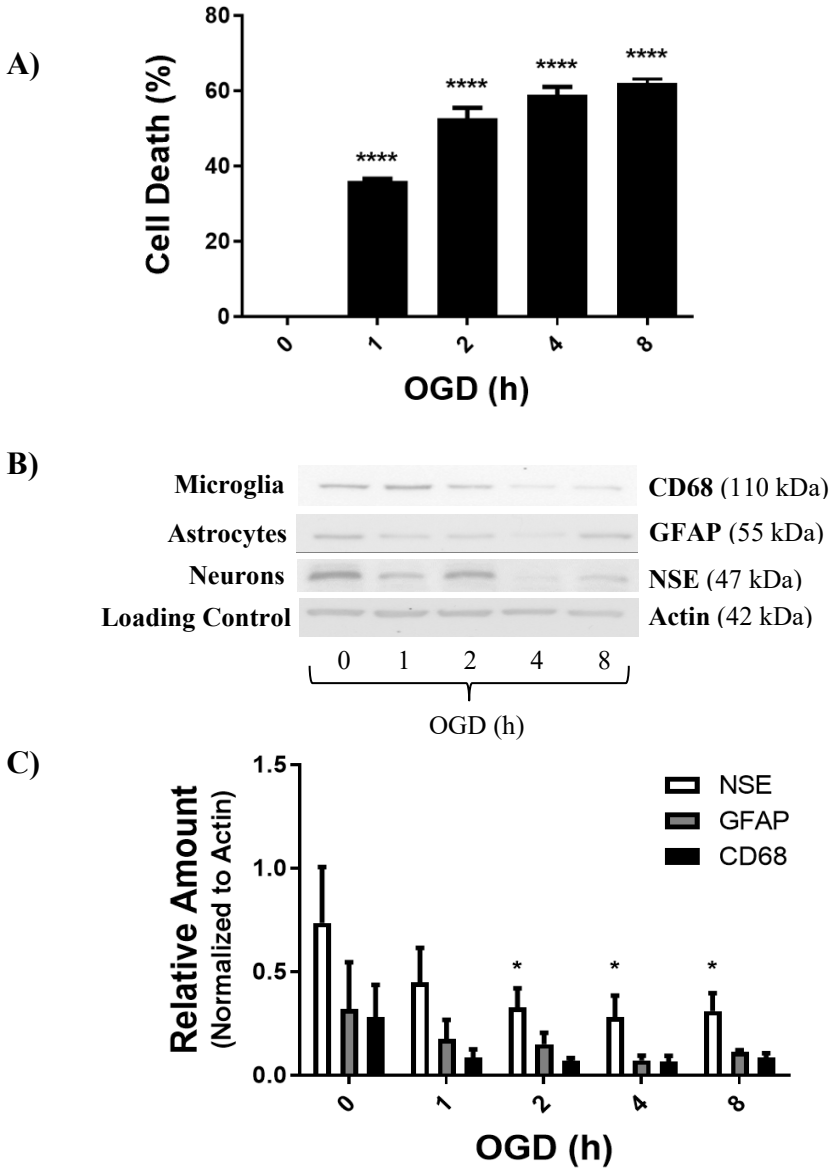

Figure 4. LD50 Determination for Neurons. LD50 for neuronal cell culture was determined using a live dead assay, and further analyzed by western blot and densitometry. **A)** Neuronal cell cultures achieved an equal to or above 50% cell death at 2 hours OGD, data was normalized to control (0h OGD). **B, C)** Actin represents the loading control. Cell specific markers used were: NSE for neurons, GFAP for astrocytes, and CD68 for microglia. Data represented as mean±SEM, n≥3. One-way ANOVA, and Dunnett's Multiple Comparison Test was completed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, compared to 0 hour OGD control.

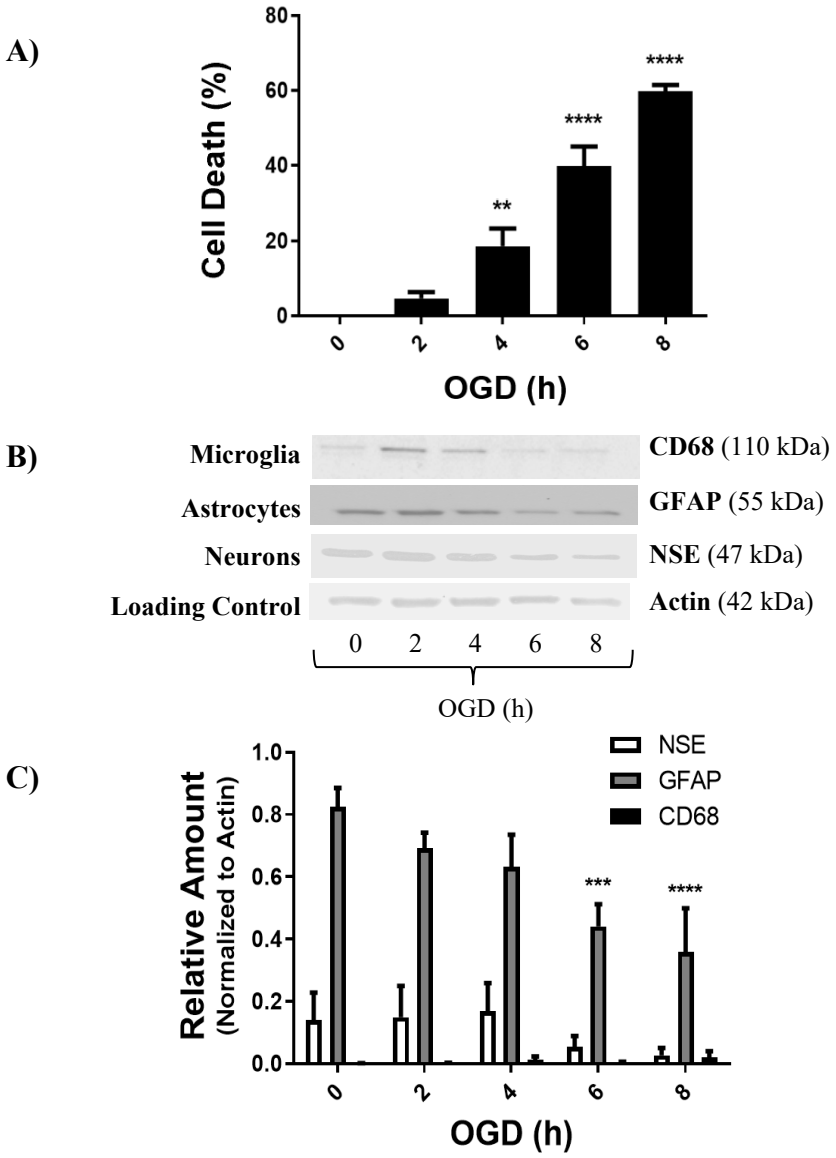

Figure 5. LD50 Determination for Astrocytes. LD50 for astrocyte cell culture was determined using a live dead assay, and further analyzed by western blot and densitometry. **A)** Astrocyte cell cultures achieved an equal to or above 50% cell death at 8 hours OGD, data was normalized to control (0h OGD). **B, C)** Actin represents the loading control. Cell specific markers used were: NSE for neurons, GFAP for astrocytes, and CD68 for microglia. Data represented as mean±SEM, n≥3. One-way ANOVA, and Dunnett's Multiple Comparison Test was completed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, compared to 0 hour OGD control.

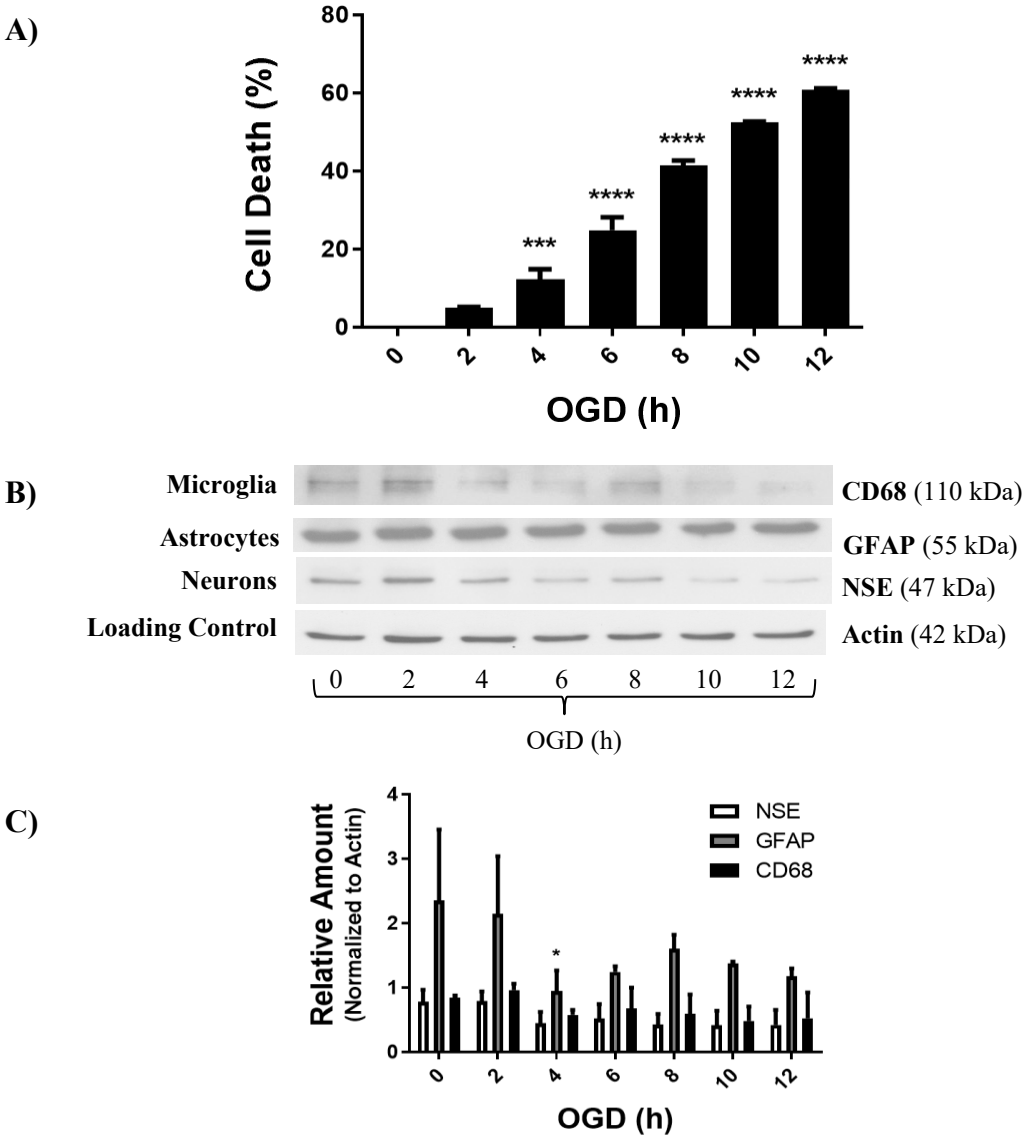

Figure 6. LD50 Determination for Co-Cultures. LD50 for co-culture was determined using a live dead assay, and further analyzed by western blot and densitometry. **A)** Co-cultures achieved an equal to or above 50% cell death at 10 hours OGD, data was normalized to control (0h OGD). **B, C)** Actin represents the loading control. Cell specific markers used were: NSE for neurons, GFAP for astrocytes, and CD68 for microglia. Data represented as mean±SEM, n≥3. One-way ANOVA, and Dunnett's Multiple Comparison Test was completed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, compared to 0 hour OGD control.

3.3 SFA Dose Response in OGD Cultures

OGD cultures at the previously determined time of LD50 (2 hours OGD for neurons, 8 hours OGD for astrocytes, and 10 hours OGD for co-cultures) were treated with SFA. The protective dose of SFA was determined as that which resulted in a significantly lower cell death due to OGD compared to control cell death (0 μM SFA). The cytotoxic dose of SFA was determined as the dose of SFA that resulted in a significantly higher cell death due to OGD compared to control cell death. Figure 7A showed that with increasing doses of SFA there was initially a decrease in cell death at 2 hours OGD treatment of neurons, most prominently at 2.5 μM SFA, by a 9% decrease in cell death, although not significant compared to control. In the presence of 2.5 μM SFA, there was a significant decrease in cell death by 24% and 39% compared to control (0 μM SFA), following 8 hours of OGD treatment in astrocytes and 10 hours OGD treatment of co-cultures respectively (Figure 7B & 7C). Therefore, compared to neurons, the difference in cell death decrease from 0 to 2.5 μM was over 2 fold greater in astrocytes and over 4 fold greater in co-cultures. Higher doses of SFA (≥ 100 μM), presented a significant toxic effect on astrocytes by a ≥ 78% increase in cell death (Figure 7B), and OGD treated co-cultures exhibited a significant toxic effect in the presence of ≥ 50 μM SFA by a ≥ 69% increase in cell death (Figure 7C).

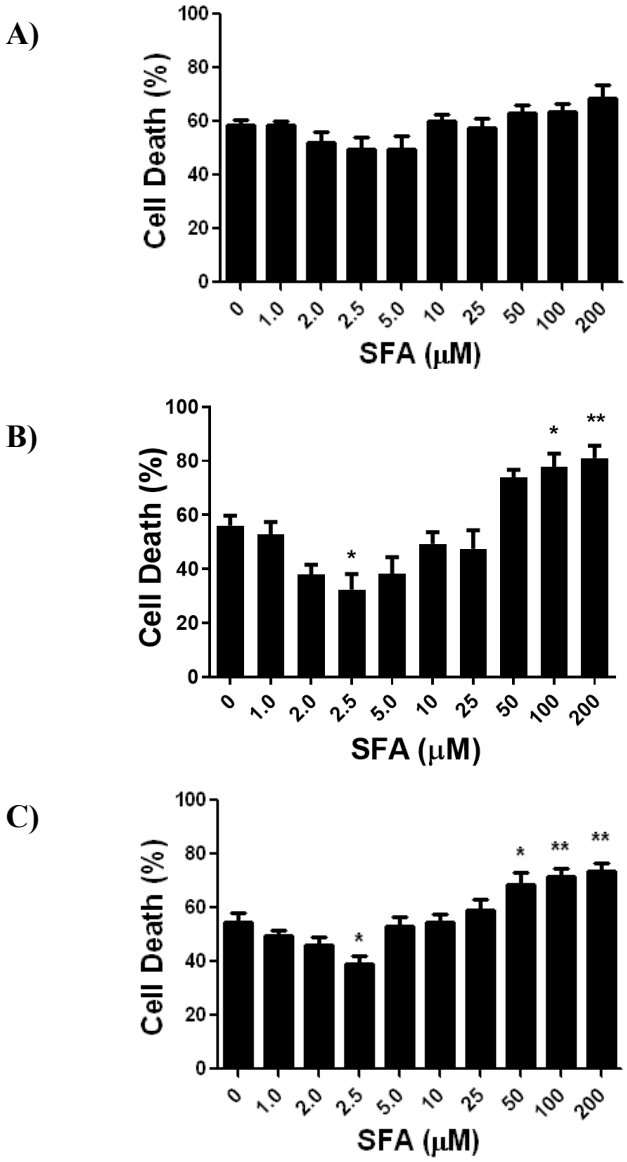

Figure 7. SFA Dose Response in OGD Cultures. SFA dose response for each brain cell-type culture in an OGD environment was determined using a live dead assay at previously determined LD50. **A)** Neuronal cultures at LD50 of 2 hours OGD, no significant changes in cell death, **B)** Astrocyte cultures at LD50 of 8 hours OGD, protection of SFA at 2.5 μM , significant toxicity of SFA ≥ 100 μM, **C)** Co-cultures at LD50 of 10 hours OGD, protection of SFA at 2.5 μM and toxicity of SFA ≥ 50 μM. Data represented as Mean±SEM, n≥3, One-way ANOVA, and Dunnett's Multiple Comparison Test was completed for all cultures; *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, compared to respective controls (0 μM SFA).

3.4 SFA Dose Response in Control Cultures

3.4.1 SFA Dose Response in Control Cultures by AlamarBlue Assay

In order to determine potential cytotoxic effects of SFA on brain cells in a normoxic/normal glucose environment, control cultures (no OGD) were treated with SFA, again at varying doses. The cytotoxic doses of SFA were determined as the dose of SFA that resulted in a significantly lower cell viability compared to 0 μM SFA. Cell viability, determined using AB assay, showed that SFA had no effect on the viability of neurons, astrocytes, or co-cultures at low concentrations (Figures 8A-C). SFA at doses ≥ 100 μM were significantly toxic to neuronal cell cultures by a ≥ 68% decrease in cell viability (Figure 8A). Both astrocyte and co-cultures showed significant toxicity at SFA doses ≥ 50 μM by a ≥ 15% and ≥ 44% decrease in cell viability respectively (Figure 8B & 8C).

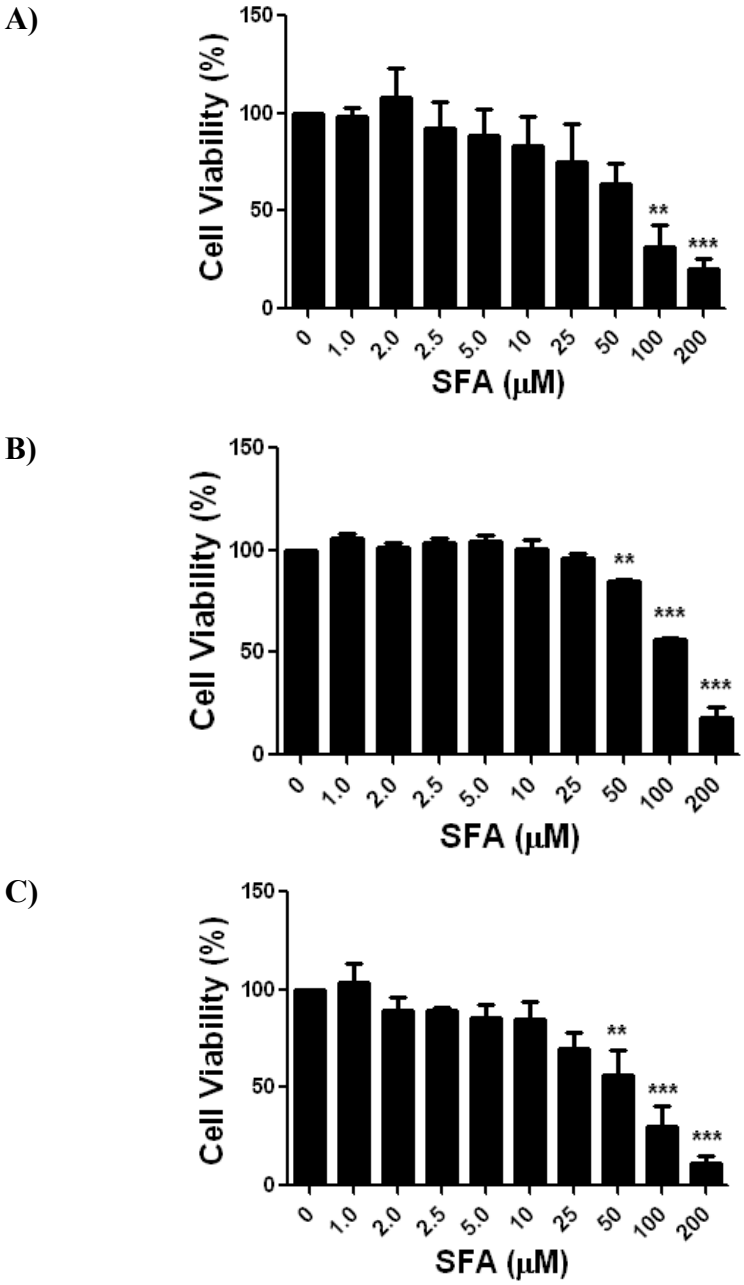

Figure 8. SFA Dose Response in Control Cultures by AlamarBlue Assay. SFA dose response for each brain cell-type control culture was determined using an AB assay. **A)** Neuronal cultures showed toxicity of SFA ≥ 100 μM, **B)** Astrocyte cultures showed toxicity of SFA ≥ 50 μM, **C)** Co-cultures showed toxicity of SFA ≥ 50 μM. Data represented as Mean±SEM, n≥3, One-way ANOVA, and Dunnette's Multiple Comparison Test was completed for all cultures; *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, compared to respective controls (0 μM SFA).

3.4.2 SFA Dose Response in Control Cultures by Live/Dead Assay

Live/Dead assay was also used to show cytotoxicity of SFA in control cultures. Cytotoxic doses of SFA were determined to be the dose of SFA that resulted in a significantly higher cell death compared to control (0 μM SFA). SFA had no effect on the viability of neurons, astrocytes, or co-cultures at lower concentrations (Figure 9A-C). SFA was not significantly toxic to neuronal cell cultures, although a gradual increase in cell death by 12% is seen as SFA dose increases from 0 to 200 μM (Figure 9A). Both astrocyte and co-cultures showed significant toxicity at SFA doses ≥ 50 μM by an increase in cell death of ≥ 35% (Figure 9B) and ≥ 200 μM by an increase in cell death by 13% (Figure 9C), respectively.

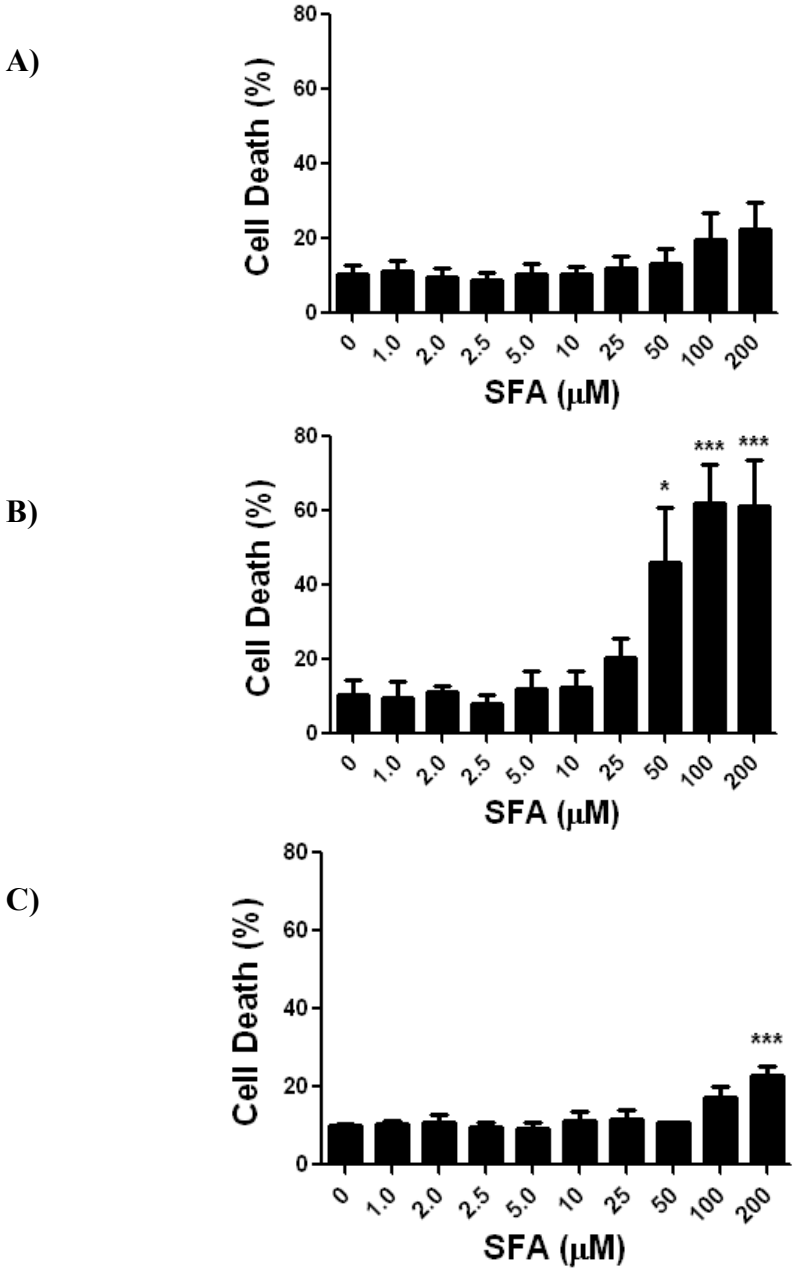

Figure 9. Dose Response in Control Cultures by Live/Dead Assay. SFA dose response for each brain cell-type control culture was determined using a Live/Dead assay. **A)** Neuronal cultures did not show significant toxicity, **B)** Astrocyte cultures showed toxicity of SFA ≥ 50 μM, **C)** Co-cultures showed toxicity of SFA ≥ 200 μM. Data represented as Mean±SEM, n≥3, One-way ANOVA, and Dunnett's Multiple Comparison Test was completed for all cultures; *p<0.05, **p<0.01, ***p<0.001, ****p<0.001, compared to respective controls (0 μM SFA).

Chapter 4

Discussion

4.0 Discussion

This study demonstrates the importance of dose dependent effects of SFA in in cortical neurons, astrocytes, and co-cultures. We show that low doses of SFA are protective by decreasing cell death in an OGD environment to PD2 cortical brain cell cultures and are safe in control cultures by showing no significant change in cell death or cell viability. On the other hand, high doses of SFA are cytotoxic under OGD and control normal oxygen and glucose conditions by increasing cell death or decreasing cell viability. Preventing normal cell death, particularly in the 3rd trimester of pregnancy in the developing fetus may be one of the keys to the prevention of neurodevelopmental disabilities, as brain cell injury can lead to impaired cell survival, migration and connectivity that results in impaired motor, sensory, cognitive, and behavioural functions. Conventional therapies such as hypothermia, used at the time of birth, have not been shown to be effective in reversing or repairing the brain damage that has already occurred. Hypothermia administered within a few hours after birth is only effective for injuries that occur during delivery; it successfully slows down brain metabolism and reduces mortality, but has not been definitively shown to reduce incidence or severity of NDDs such as CP.[3] Hypothermia treatment does not salvage brain cells that are already injured from an insult.[1] Moreover, it will only treat a small percentage of injuries around the time of birth (10-20%). Further, when used as a therapeutic after injury, there has been contradictory evidence of SFA being effective in animal models.[75] It is important to note that our previous studies aimed to determine whether SFA could be used as a prophylactic preventative regimen by pregnant women to target and reduce oxidative stress-induced injury to fetal brain cells before it leads to cell death. The current study aims to determine the range of SFA dosing that could reduce or prevent brain cell death in an *in-vitro*

model of PBI. This is one step in understanding SFA safety and toxicity with respect to the fetal brain.

Although this study focuses primarily on the effect of SFA on cortical brain cells, perinatal brain injury can take place, often simultaneously, in both gray and white matter.[8-10] A common cause for perinatal brain injury is HI induced rise in reactive oxygen species which lead to cell death through activation of apoptotic receptors or proteins, and mitochondrial dysfunction. ROS, produced by microglia and leukocytes, are a defense mechanism activated upon injury, which can cause death or damage of surrounding brain cells including neurons, astrocytes, and oligodendrocytes.[47,59]

Gray matter injury is mainly due to cell death or functional compromise of neurons of the cortex, thalamus, and hippocampus; this is accompanied by damage to astrocytes.[9,10] Astrocytes play a dominant role in the protection of neurons by increasing anti-oxidative enzymes through the Nrf2/ARE pathway which can neutralize ROS to prevent neuronal cell damage.[59,76,77] Neurons also use this same protective mechanism, though it is less effective than in astrocytes as anti-oxidant up-regulation in neurons is less efficient compared to astrocytes, and they have a low resting glutathione level, which is central for redox homeostasis of the cell.[2,47,59] This paradigm is supported by our data which showed that the protective effect of SFA on OGD-treated neurons (Figure 7A), was not as effective compared to the protective effect of SFA on OGD astrocytes (Figure 7B); the difference in SFA protection (decrease in cell death) from 0 to 2.5 μM was over 2 fold greater in astrocytes compared to neurons. However, the most significant protective effect of SFA, over 4 fold greater compared to neurons, was observed in the co-cultures (Figure 7C), which is the brain cell culture most representative of brain tissue. We postulate that the high

protective efficacy of SFA in the co-cultures is due to anti-oxidative enzymes released by astrocytes through the activation of the Nrf2/ARE pathway, upon OGD insult, effectively neutralizing ROS and preventing cell damage in neurons. Although we did not investigate this mechanism in this project, the protective effect of the Nrf2/ARE pathway induced by SFA is extensively researched in literature.[31,33] In addition to this protective pathway, other studies have also identified that SFA's prominent protective effect works through astrocytes which then protect neurons sequentially.[59,76,77] From the extensive literature and our results, we can infer that the SFA induces an anti-oxidant response indirectly through the Nrf2/ARE pathway in astrocytes, to consequently protect neurons from oxidate stress. This mechanism will be investigated in the future to further understand how SFA protects brain cells in an OGD environment.

White matter injuries result in an aberrant maturation process of oligodendrocytes.[8,9,78] The primary function of oligodendrocytes includes creating myelin which ensheaths neuronal axons in the central nervous system.[8,78] Deficiencies in oligodendrocyte maturation ensues a decrease in the differentiation of oligodendrocyte pre-cursors to mature myelin producing cells which can lead to an impaired action potential and synaptic transmission in neurons, resulting in neurological impairments.[8,78] In this study P2 day old Long-Evan rats were used as their brain at this stage is representative of a fetus or pre-term human infant at approximately 24 weeks gestation. [2,8] In this stage of development, white matter is mainly composed of oligodendrocyte pre-cursor and immature oligodendrocyte cells which are highly susceptive to an HI environment, likely leading to a delay or hindrance in the production of mature oligodendrocytes.[2,8,10,30] Studies have also found that HI leads to a decrease in oligodendrocyte maturation rather than a loss or cell death of oligodendrocytes.[10]

The Nrf2/ARE mechanism and its activation by SFA have been well established. The significance of using SFA as an inducer of the Nrf2/ARE pathway is that it upregulates many phase II anti-oxidant enzymes including GPX, GST, HO-1, NQO-1.[47,58] Phase II anti-oxidant enzymes directly remove ROS and reduce oxidative stress.[47] An important anti-oxidant enzyme, indirectly enhanced by SFA, is GPX, a key component of the glutathione dependent anti-oxidant system, vitally important in maintaining endogenous redox homeostasis.[2,30] GPX is involved in the conversion of GSH to GSSG by an oxidation process which also includes neutralizing ROS.[2,59] However, the immature brain has low levels of GPX and therefore a reduced endogenous ability to scavenge ROS.[30] Our study shows that SFA efficiently and significantly prevents cell death in an HI environment. Since SFA can cross the blood-brain and placental barrier, it is possible that SFA may prevent cell death in the fetal brain under oxidative stress through upregulation of oxidative enzyme GPX via activation of the Nrf2/ARE pathway.[30,47] However, this interaction needs to be further studied as SFA may not interact with GPX in all oxidative stress circumstances or cells.

Our study shows that SFA is safe and protective of brain cells in cultures at low doses (≤ 2.5 μM) by either having no effect or no significant decrease in cell death compared to control cultures, while it is toxic at high doses by increasing cell death. SFA has previously been shown to be a hormetic compound which exhibits a biphasic dose response. While the protective effect of SFA has been shown to work prominently through the Nrf2/ARE pathway to upregulate anti-oxidant enzymes, the toxic effect of SFA is impacted by various other mechanisms that result in cell death.[79-81] We have also shown that ≥ 50 μM SFA dose has been toxic in both OGD (Figure 7) and control cell cultures (Figure 8, 9B-C), and these mechanisms of toxicity can include inhibiting histone deacetylase,[80,81] and inhibiting upregulation of tumor suppressor proteins.[79]

Our observation that a low dose of 2.5 μM SFA is protective in brain cell cultures exposed to OGD (Figure 7) is supported by other studies that demonstrate a protective effect of SFA at doses ≤ 2.5 μM.[52,66,67,82] Kraft et al. showed that pre-treatment of cortical astrocytes and neuronal cells for 48 hours with 2.5 μM SFA resulted in an increase of ARE dependent genes including total GSH levels, upon H_2O_2 induced oxidative stress.[52] In addition, 1 μM SFA was reported to upregulated Nrf2 in neural crest cells and ameliorate the effects of ethanol induced apoptosis in animal models of fetal alcohol syndrome disorder.[66,67,82]

Most literature suggests that the protective pathway of SFA is through the ARE/Nrf2 pathway and that higher dose of SFA induces apoptosis and those apoptotic pathways vary.[79,83] The Nrf2 pathway has also been reported in SFA studies that use a higher dose. Cho et al. showed that a 10uM concentration of SFA inhibited downregulation of tumor suppressor proteins in human hepatoma liver cancer cells (HepG2).[79] This inhibition led to cell survival and silencing of the tumor suppressor protein was also linked to diminished activity of Nrf2. Therefore, SFA activation of Nrf2 may have been linked through tumor suppressor protein. Since 10 μM of SFA lead to few cytotoxic effects in the HepG2 cells, the authors inferred that the protective ability of the compound was through anti-proliferative and anti-inflammatory effects of SFA.[79] In a human bronchial epithelial (BEAS-2BR) cell line, SFA was shown to both increase and decrease Nrf2 in different conditions. Normally during the process of metal carcinogenesis Nrf2 is upregulated to decrease ROS: in stage one to protect cells from malignant transformation by cadmium, and in stage two to attain apoptotic resistance. With the addition of 10uM of SFA, Wang et al. showed that Nrf2 was activated in stage one (before transformation, normal cells) and reduced ROS, but was decreased in stage two (after transformation, cadmium-transformed cells), thereby restoring autophagy competence of cells and decreasing cell survival.[83]

In this project, two avenues of determining SFA dose response in control cultures were used, AB assay and Live/Dead assay. Initially, AB was used because it is more cost effective and has a simple methodology as it requires fewer steps in protocol, compared to the Live/Dead assay. The Live/Dead assay was performed later to confirm the results from the AB assay and to relate the results of experiments examining the SFA dose response in control cultures to the SFA dose response in OGD cultures. The AB results (Figure 8) showed that low doses of SFA had no significant decrease in cell viability at low doses ($\leq$ 2.5 μM) in control cultures, and when increased to higher doses, SFA becomes cytotoxic by decreasing cell viability significantly; SFA $\geq$ 100 μM for neurons (Figure 8A), and SFA $\geq$ 50 μM for astrocytes and co-cultures (Figure 8B-C). The Live/Dead results (Figure 9) also showed that low doses of SFA are safe in all control cultures as there was no significant decrease in cell death. However, when increased to higher doses, SFA is not significantly toxic to neurons (Figure 9A), while it is toxic at SFA $\geq$ 50 μM for astrocytes (Figure 9B), and at SFA $\geq$ 200 μM for co-cultures by significantly increasing cell death (Figure 9C). The high dose response of the AB results is not consistent with the high dose response of SFA seen in the OGD cultures which was analyzed using the Live/Dead assay (Figure 7). The Live/Dead assay results showed no significant cytotoxicity in neuron cultures (Figure 7A), and a significant cytotoxic effect at SFA $\geq$ 100 μM for astrocytes (Figure 7B) and at SFA $\geq$ 50 μM for co-cultures (Figure 7C). The AB results show control neuron cultures to be vulnerable to SFA at high doses, while the Live/Dead assay results show that OGD neuron cultures, which are already stressed due to lack of nutrients, to not be significantly killed by exposure to SFA. However, the results of control neuron cultures analyzed by the Live/Dead assay in Figure 9A do reflect the cell death results of the neuron cultures exposed to OGD in Figure 7A. This is supported by literature which explains that the SFA mechanism is primarily

active through astrocytes, and therefore, they may not affect an isolated neuronal culture as they would an astrocyte culture.[79-81]

The discrepancy in the results of the dose response of control cultures between AB and Live/Dead assay can be explained by the differences in these methodologies. AB assay indicates cell health by measuring the reducing power of the cell; therefore, the more metabolically active the cell, the greater the red fluorescence that can be detected. This would suggest that low metabolically active cells may not fluoresce, although they may still be alive. In our results this would indicate that the cytotoxicity levels seen may be greater than true levels if we are seeing no or less fluorescence from both dead cells and cells that are dying but still alive, and therefore have the potential to continue living. In the Live/Dead assay, ethidium homodimer-1 enters cells which are dead due to a loss of plasma membrane integrity. If the Live/Dead assay is representing cells which are dead while the AB assay is representing low metabolically active cells, then it can be concluded that the cytotoxicity levels in terms of cell death specifically, are more accurate using the Live/Dead analysis in comparison to the AB. This is an important distinction as although SFA may not be killing as many cells at very high doses, it may still be making them more vulnerable to stress by compromising their cellular functions.

From the literature it is evident that there may be a shift in SFA mechanism from protective to toxic in a dose dependant manner. At very low doses near 2.5 μM, SFA is cytoprotective as shown in this study and previously reported in the literature.[52,66,67,82] At high doses, >2.5 μM, SFA is cytotoxic. It seems that doses within this range may be protective and/or toxic based on culture conditions, creating a shift in metabolic processes. A significant governing body of energy homeostasis in a cell is the adenoside monophosphate-activated protein kinase (AMPK)

pathway which may play a role in the protective and toxic effects of SFA.[84,85] Many studies have shown a relationship between AMPK and SFA and pro-apoptotic or anti-apoptotic effects.[84,85] SFA has been used to activate AMPK in pancreatic cancer cells in a hypoglycemic environment to inhibit proliferation and promote apoptosis.[84] Alternatively, SFA has been shown to protect neuronal cells from prion protein induced apoptosis by increasing AMPK phosphorylation.[85] Therefore, the difference in SFA's role as a pro-apoptotic or anti-apoptotic agent is evident and likely associated with its biphasic dose response property; this can be further explored in future studies.

4.1 Conclusion

This research is significant in advancing the field of prevention in PBI. Currently, therapies such as hypothermia take a reactive approach to PBI; this study provides a novel preventative approach to PBI that may address or reduce damage that occurs in utero.[1,2] SFA has been extensively studied in the literature, specifically in a neurology setting. However, here we looked at SFA's effect on each brain cell type in addition to a co-culture, in an *in vitro* perinatal setting. This is important as now we can further infer SFA's specific interactions with each brain cell type, and how these interactions may play a role in protection of the fetal brain. A vital aspect of this research is the range of SFA dosing that was considered from 0 to 200 μM. Advancing research in a pregnancy setting can be intimidating because of the spectrum of possible impairments that can affect both the mother and fetus. Therefore, this study ensured to look at a range of dosing to determine safety and toxicity parameters of SFA *in vitro*.

In conclusion, we have determined that low doses of SFA in neuronal, astrocyte, and co-cultures is neuroprotective in an OGD environment, and safe in control cultures. High doses of SFA are toxic in all cultures in both OGD and control environments. It is important to note the difference between effective low protective dosing and high toxic dosing of SFA are at least a 10-fold difference which alludes to the safety in using SFA as a preventative approach in a perinatal setting. Next steps will involve investigating effects of SFA during pregnancy on the potential for teratogenicity or organ damage in preclinical animal models; a step required to determine a safe and effective dose that could help to inform human clinical trials.

Chapter 5

Future Directions

5.0 Future Directions

5.1 Limitations & Improvements for Current Study

5.1.1 Choice of Animal and Brain Maturity

P2 Long-Evan rats were used in this research to extract and isolate brain cells from the cortex. Long-Evans rats are used across a spectrum of experiments in our lab for both *in-vitro* and *in-vivo* work. They are especially useful for behavioural studies, and since behavioural studies will be a future direction of this project, the Long-Evans rat was also used for this study.[86-89] The rat animal was also chosen based on its cost effectiveness, reliability, properties of the placenta (more relevant when translating to *in-vivo* studies). Rodents are cheaper compared to other experimental animals such as the rabbit or pig and have been widely used to study models of human pregnancy. They are also reliable in regards to their parturition successes; their births are more successful than other small animals such as the guinea pig, and they are able to deliver multiple pups (10-20) over one parturition period.[86-90]

Although rodents are cost effective and reliable, they have a non-precocial development compared to humans which are precocial. This means that the rodent brain overall develops later than the human brain which develops earlier. Specifically, white matter in the rodent develops after birth, which in humans it begins to develop prior to birth.[91,92] The rodent placenta is also structurally similar to the human. Other animals such as the guinea pig and rabbit develop perinatally similar to humans. Also, the guinea pig placenta is more functionally similar to humans, and there is more congruency in regulation of genes and proteins of the placenta, between humans and guinea pigs, compared to mural rodents, and this may come into consideration when planning future studies.[91-93] Additionally, it has also been shown that the

larger the brain, the greater the glia-to-neuron ration. Therefore, with the obvious difference in brain size between rats and humans, it can be suggested that the glia-to-neuron ratio is quite different, and the co-culture in this project may not be representative of the human brain cell composition.[24] Therefore, the results from this research cannot be directly translated to humans. Further exploration with SFA, *in-vivo*, across different animal species needs to be taken into consideration before clinical experiments can take place.

5.1.2 Representative Analysis of Cell Culture Model

In-vitro cell culture studies do not exactly reflect an *in-vivo* full tissue environment. Firstly, only the cortex which consists of mainly neurons and astrocytes, was extracted from the P2 rat brains in this study, and this is because of the rat brain development timeline; the P2 rat brain is similar to the brain of a human fetus in the 3rd trimester of pregnancy. Oligodendrocytes, which are not a major part of the cortex, begin to mature after birth in rats. Therefore, in most literature, oligodendrocytes are examined at P7 or later when using rats as an animal model. The purpose of isolating a neuron-only and astrocyte-only culture was to determine the effect of SFA on each brain cell type. However, these are not representative of the environment of brain cells *in-vivo.* The co-cultures were done in order to best assimilate the combination of cells found in the brain, as well as the extracellular environment of the cells.[94] We did not recapitulate the exact proportion of neurons, astrocytes, and microglia, in the co-cultures, as there was variability between each cortex extraction and each count of cells that was plated. Although we did normalize our cultures using control values, we were not able to present a realistic proportion of the brain cells. Additionally, the LD50 and effect of SFA may have been different for the neurons and astrocytes if tested under co-culture conditions. The cell death of neurons and astrocytes could have been analyzed through more specific methods, within a co-culture environment.

Using cell specific markers in combination with live and dead cell markers, would have given a representation of how OGD and SFA affected neuron and astrocyte cells in a co-culture environment, which is a more accurate reflection of an *in-vivo* model. For example, within a co-culture, counting overlapping signals of a neuron and live cell would provide all live neurons, and a neuron and a dead cell would provide all dead neurons, giving you the cell death percentage of neurons, within a co-culture environment. This method would give cell death values of each cell type within the co-culture and can be done in the future with availability of more resources and time.

5.1.3 Oxygen Deprivation Consistency

In this study, cells undergoing OGD were placed in an anoxic incubator. When determining the LD50 for each culture, multiple plates reflecting multiple time points were used. Because of the nature of the experiment, the anoxic chamber was opened and closed each time a time point needed to be analyzed. Although the oxygen sensor for the anoxic chamber was consistently at 0%, unless the chamber was opened, then it would rise to at most 1%, this is still a fluctuation in oxygen deprivation and may have altered the LD50 results by having less cells die than if the oxygen sensor stayed at 0% consistently. Despite this limitation, each experiment was done the exact same way, and this fluctuation would have been consistent between experiments. Additionally, in terms of the clinical relevance of this method, placental insufficiency does restrict the amount of oxygen that is delivered to the fetus, but it is not necessarily always 0%; it is inconsistent and often unpredictable and spontaneous. In that way, these slight variations in oxygen availability might be more similar to what the fetal brain might experience with placental insufficiency. This could be an area for further investigation.

5.1.4 Cell Death and Viability Analysis

This study analyzed cell death and cell viability by the Live/Dead assay and the AB assay, respectively. We were able to determine the overall effect of SFA on metabolic activity or cell membrane integrity with these assays; however, it was not enough to determine mechanisms of cell death or damage. Determining specific harmful effects of SFA would be helpful going forward in this project. In the future, other analyses including measuring apoptotic and necrotic markers will give a more accurate representation of possible mechanisms of cell death during OGD and during SFA treatment at high doses.

5.2 Prospective Studies

5.2.1 Mechanistic Effects of SFA/Nrf2

The Nrf2/SFA mechanism has been extensively research and is available in literature; in this study we extrapolated knowledge from the literature to explain effects of SFA in our neuron, astrocyte, and co-cultures.[31,33,34,51-53] Future experiments will include determining mechanistic effects between SFA, Nrf2, ARE, cytokines, ROSs, and ROS scavenging agents such as GPX, through protein identification, quantification, and enzyme assays. These experiments will be completed in vitro using mammalian cultures from this study; and mechanistic effects in each cell type along with co-cultures from this project will be investigated.

5.2.2 Effect of SFA on Oligodendrocytes

This study analyzed the effect of SFA on neurons and astrocytes. Another important brain cell to be considered is oligodendrocytes. As oligodendrocytes are important in the protection of function of neurons, it would be interesting the see the effect of SFA on these cells. White matter damage, where oligodendrocytes are most prominent, is also a significant aspect of many NDDs such as CP.[2,8,10,30] From the literature, oligodendrocytes have shown to act differently than astrocytes and neurons in an HI environment; rather than dying, oligodendrocytes hinder their

maturation process, thereby making neurons vulnerable without sufficient myelination. Future studies can analyze the specifics of the oligodendrocyte maturation process, how it is hindered through HI exposure, and determine if SFA can improve maturation in an OGD environment.

5.2.3 Determining a Safe Dosing Range of SFA in Animals

In this study, a safe dosing range of SFA was determined *in-vitro*. Next steps should include determining a safe dosing range *in-vitro* using an animal model. The rodent model can be used as it is cost effective, reliable, can have multiple offspring, and has a placental which is structurally similar to that of a human, Other animal models can be considered such as the guinea pig which although does not have a high parturition success and therefore are difficult to work with experimentally, their placental function in addition to structure is more similar to humans. The rabbit can also be considered, as like the guinea pig, they have a precocial development similar to the human.[91-93] Determining a safe dosing range in animals would include determining an oral dose of SFA that corresponds to a safe intravenous concentration of SFA in pregnant moms of the chosen animal model. Additionally, similarities of the gut and microbiome of the animal should also be taken into consideration as this may affect the absorption of SFA into the blood stream. The rat microbiome signature is quite different than the human. However, the human population itself also does not have a consistent microbiome as it changes based on diets and environments.[90,95] Absorption rate and efficacy should be considered when transitioning from animal to human studies as this would effect the potency and efficacy of the SFA dose used; future studies of SFA absorption in rodent compared to human enterocytes can be done to better understand SFA's absorptive capacity in different animal models. This experiment would be most relevant to the clinical application of this study, since it is likely that SFA could be an oral supplement for pregnant women.

5.2.4 Animal Toxicology Testing

This study tested the toxicity of SFA *in vitro*; to advance this project towards clinical trials, toxicity should also be tested *in vivo* using an animal model to determine dosing limits for a safe range of SFA. Although this research is primarily targeting the brain, SFA will reach other parts of the body, and ensuring an overall safe dose is crucial. We are proposing that SFA be used as a supplement during pregnancy, this would mean that it could interact with fetus' and pregnant moms during the first, second, and/or third trimester. Toxicology testing of the animal model would be completed at each trimester of pregnancy, as well as after birth, for both the mother and fetus. There are many aspects of toxicology to consider including: blood samples, neuropathological lesions, behavioural abnormalities, fetal limb and tail malformations, number of total offspring from parturition, and number of live and dead offspring, and histopathology of tissues for various organs such as the heart, brain, liver, and kidneys.[96] There is a considerable amount of planning and resources that go into animal toxicology testing; therefore, it was not completed in this project, but will be completed in the future.

5.2.5 Examining the Effect of BrSp Compared to SFA in an Animal Model

Determining the effect of SFA compared to BrSp can be done in an animal model as a step towards clinical testing. Using comparable doses of BrSp and SFA and examining the differences in plausible protectiveness of the fetal brain during an HI pregnancy (IUGR). This will further test the safety of SFA in an animal model and will determine the difference in potency between BrSp and SFA in neuroprotection. The protective effect and safety of SFA should also be tested in more than one animal model to ensure consistency across species.

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Appendix

A1: Live/Dead Assay Cell Death Calculations

The CD percentage obtained from the Live/Dead assay for each plate examined, was normalized using respective control values for each sample. This was done for both determining the LD50 of each brain cell culture and when examining effects of SFA in control cultures using the Live/Dead assay. The following calculation was used to determine the normalized value of CD percentage for each sample:

$$Normalized\ sample\ CD\ (\%) = 100 \times \frac{sample\ CD - control\ CD}{100 - control\ CD}$$

A2: AlamarBlue Assay Cell Viability Calculations

The RFU obtained from the AB assay for each plate examined, was normalized using respective control values for each sample. This was done when examining effects of SFA in control cultures using the AB assay. The following calculation was used to determine the normalized value of RFU percentage for each sample:

$$Normalized\ sample\ RFU\ (\%) = (sample\ RFU - blank) \times \frac{100}{untreated\ cells\ RFU - blank}$$