THERAPEUTIC STRATEGIES USING SULINDAC AND G-CSF GENE THERAPY FOR NEUROLOGICAL DISEASE

by

Belinda Chen

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Howard Prentice, Department of Biomedical Science and has been approved by all members of the supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

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Alzheimer's disease is a neurodegenerative disease that causes cognitive dysfunction and leads to progressive memory loss and behavioral impairment. About 60% to 80% of dementia cases are attributed to Alzheimer's disease and currently afflict about 50 million people worldwide. Although it primarily affects people over the age of 65, a person's risk for developing Alzheimer's disease earlier can depend on factors such as a family history (genetic inheritance) or experiencing an ischemic stroke event. Current treatments for Alzheimer's disease include behavioral therapy and drug treatment that can lessen the severity of symptoms but cannot stop progression indefinitely. Sulindac is a non-steroidal anti-inflammatory drug that, by a mechanism independent of its anti-inflammatory properties, has shown to express a preconditioning response to protect from oxidative damage. Granulocyte colony stimulating factor is a hematopoietic glycoprotein that can stimulate the production of granulocytes and stem cells that has proven to provide

neuroprotection in models of ischemic stroke via mechanisms including anti-apoptosis and anti-inflammation. In this *in vitro* study, the potential neuroprotective effects of Sulindac is measured against the effects of oxidative stress when subjected to hypoxia and reperfusion. Regarding un-transfected SHSY-5Y cells, hypoxia was demonstrated to lower cell viability starting at a period of 12 hours. It was found that a low concentration of Sulindac (200 uM) was effective in protecting SHSY-5Y cells against oxidative stress and overall lowering the rate of cell death in the event of hypoxic and reperfusion injury. When SHSY-5Y cells were transfected with Swedish APP mutation, cell viability was also markedly decreased in hypoxic conditions. However when treated with a concentration of 600 uM of Sulindac, cell viability levels were near matched with its normoxic counterparts.

In an *in vivo* study, Granulocyte colony stimulating factor gene therapy was assessed as a potential therapeutic treatment for Alzheimer's disease. Locomotive activity was measured using a force plate actometer while MWM was used to observe spatial learning and memory. Post treatment, mice that were treated with hG-CSF gene therapy showed higher levels of motor function than the control drug counterparts. The mice given the gene therapy drug also demonstrated decreased movement deficits in comparison to the wild type mice. In MWM, the studies conducted were to establish a baseline of behavior pre drug treatment. Preliminary results reveal that Alzheimer's mice showed improvement in motor function and an upward trend in short term memory retention during a training period. However, during the probe trial, the mice showed a reduced level of spatial learning ability compared to the wild type counterparts.

DEDICATION

I would like to dedicate this thesis manuscript to my parents Jie Liang and Zhenquan Chen. Thank you for the unconditional love and unwavering support in my desire to follow my educational goals. In addition, I want to dedicate this work to my older sister Deborah Chen. I could not have achieved the success that I have without your wisdom and advice.

THERAPEUTIC STRATEGIES USING SULINDAC AND G-CSF GENE THERAPY FOR NEUROLOGICAL DISEASE

LIST OF FIGURES

1. INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease that currently affects an estimated 50 million people worldwide, expected to rise to 113 million by 2050 [1]. Afflicted patients experience significant cognitive impairment and negative behavioral changes. Cognitive impairment most commonly manifests itself as a decrease of memory while behavioral changes include increased episodes of aggression or general anxiety. Dementia is one of the most commonly displayed clinical symptoms of AD. Risk factors of AD include age progression, familial genetic factors (Swedish APP, PSEN1 and PSEN2), head injury, and vascular disease [2]. Pathologically, the two hallmarks of AD are the formation of alpha *beta* 42 (Aβ-42) plaques and neurofibrillary tangles (NFTs) composed of tau protein. [3]

Aβ-42 plaques are formed when APP is imprecisely cleaved via β and γ secretases. Normal cleavage involves APP being cleaved by β and then γ secretases to form the polymer Aβ-40, which accounts for about 80 to 90% of Aβ species. The other 10% formed is Aβ-42, the properties of which are hydrophobic and insoluble. [4] Increased production of Aβ-42 has been linked to the development of early onset AD. [5] The loss of cytoskeletal microtubules and tubulin associated proteins play a prominent role in the creation of NFTs. The presence of NFT afflicted neurons have demonstrated abnormal tau hyperphosphorylation, in compared to patients not afflicted with dementia.[6]

In addition, there is a growing evidential link of oxidative stress in AD. [7] Oxidative stress occurs when there is an imbalance between the amount of reactive oxygen species (ROS) and antioxidant complexes and are prominently featured traits in progression of neurodegeneration and cognitive decline associated in AD [8]. ROS form when oxygen is partially reduced, either from mitochondrial oxidative phosphorylation or exchanges with exogenous sources.

The product consists of radical and non-radical oxygen species, e.g. superoxide anion, hydrogen peroxide, and hydroxyl radicals [9]. They are an obligatory part of homeostatic biological function that regulates protein structure and function; this results in the activation of signal transduction pathways leading to the regulation of gene expression [10]. Specifically, they are able to prompt the receptor tyrosine kinases to activate downstream signal transduction pathways such as mitogen-activated protein (MAP) the same way they would be if stimulated by ligand activated traditional growth factors [11]. They are also produced as a result of receptor activated signaling cascades such as platelet-derived growth factor (PDGF) and epidermal growth factor EGF [12].

Patients who experience mild cognitive impairment (MCI) are in the beginning stages of AD development. [13]. This is seen as an interim phase in which afflicted patients exhibit no notable growth of Aβ plaques or NFTs compared to their non afflicted age control peers [14]. There has been evidence that the presence of increased levels of oxidative damage are one of the earliest signs of MCI stage AD [15]. Lipid peroxidation is found to be intensely multiplied in AD [16]. ROS can react with molecules such as lipids to undergo lipid peroxidation; which produces oxidative stress compounds such as 4-hydroxy-2,3,-nonenal (HNE) [17]. HNE's are one of the most exceedingly volatile free

radicals and 4-HNE in particular is able to reshape the conformation of tau protein to contribute to the development of NFTs [18]. The ability of HNE's to adapt proteins can result in the dysfunction of intracellular calcium signaling, which results in a cascade mechanism that leads to cellular death [19]. Malondialdehyde (MDA) is a marker of lipid peroxidation and the presence in serum levels in MCI stage AD patients is used to measure the accumulation level of free radical and subsequently oxidative damage [20]. It has been shown in these patients that MDA levels are elevated and levels of ROS neutralizing antioxidants superoxide dismutase (SOD) and glutathione peroxidase (GPX) are decreased compared to their healthy age counterpart controls [21]. This also proves a direct link of oxidative stress contributing to the pathogenesis of AD. In addition, oxidative stress via nitration of tau protein could prompt a conformational change in a way that benefits the fabrication of fibrils [22]. The binding of metal ions such as copper and iron to produce ROS that are bound to Aβ will form a particularly reactive hydroxyl radical. This ROS production will have a significant consequential damaging effect on Aβ peptide itself and establishes a direct link of the role of oxidative stress to AD [23].

Hypoxia occurs when there are lower oxygen levels below the norm for body function and is known to be a risk factor for AD; the drastic slowing of cerebral blood flow to the afflicted area can cause neuronal cell death and a defect to memory [24]. Approximately 30% of patients who have suffered a stroke event develop cognitive dysfunction and a decline in memory within three years [25]. Hypoxia inducible factor (HIF) is responsible for distinguishing oxygen levels within cells and deploying the appropriate cellular response to oxygen level fluctuations. In hypoxic conditions the process of aerobic oxidative respiration declines and directly impacts mitochondrial

electron transport rate [26]. This sets off a chain reaction in which ROS and nitric oxide (NO) formation synthase is escalated, and HIF is activated in response [27]. Hypoxia has also been linked to endoplasmic reticulum (ER) stress, which refers to an event that has the ability to disrupt and alter its protein folding capability [28]. Hypoxia has also been connected to a rise in production of Aβ plaques [29]. The enzyme BACE11 has been proven that it is a requisite part of β amyloid creation, knockout gene mice that were simultaneously hemizygous for an APP transgene were free of Aβ and associated C-terminal fragments (CTFs) [30]. BACE11 contains a gene promoter that is hypoxia sensitive. When exposed to hypoxic conditions, increasing upregulation of β secretase cleavage directly enhances APP and Aβ protein production [31]. The increase in gene transcription and expression can be observed in both *in vitro* and *in vivo* models. It drastically increased the number of Aβ plaque deposits and is part of the basis of memory deficiency in Swedish mutant APP mice [32].

1.2 Alzheimer's disease treatment

Currently there is no cure for AD, but there are treatments available that can slow the progression of symptoms. There are two main treatment strategies that are deployed to AD, behavioral and pharmacological. Behavioral approaches include cognitive therapy, consistent established routines, and practice of simple communication. Pharmacological treatments consist of acetylcholinesterase inhibitors (AChEis) and partial *N*-methyl-*D*-aspartate (NMDA) antagonists [33]. Acetylcholine (ACh) features a prominent role in normal brain functions such as memory, attention and processing sensory information [34]. ACh is a product of synthesis that occurs between choline and acetyl-coenzyme A via the ChAT enzyme. Synthesis occurs in the cytoplasm of

cholinergic neurons and vesicular acetylcholine transporter. (VAChT) is responsible for transferring ACh via synaptic vesicle. [35].

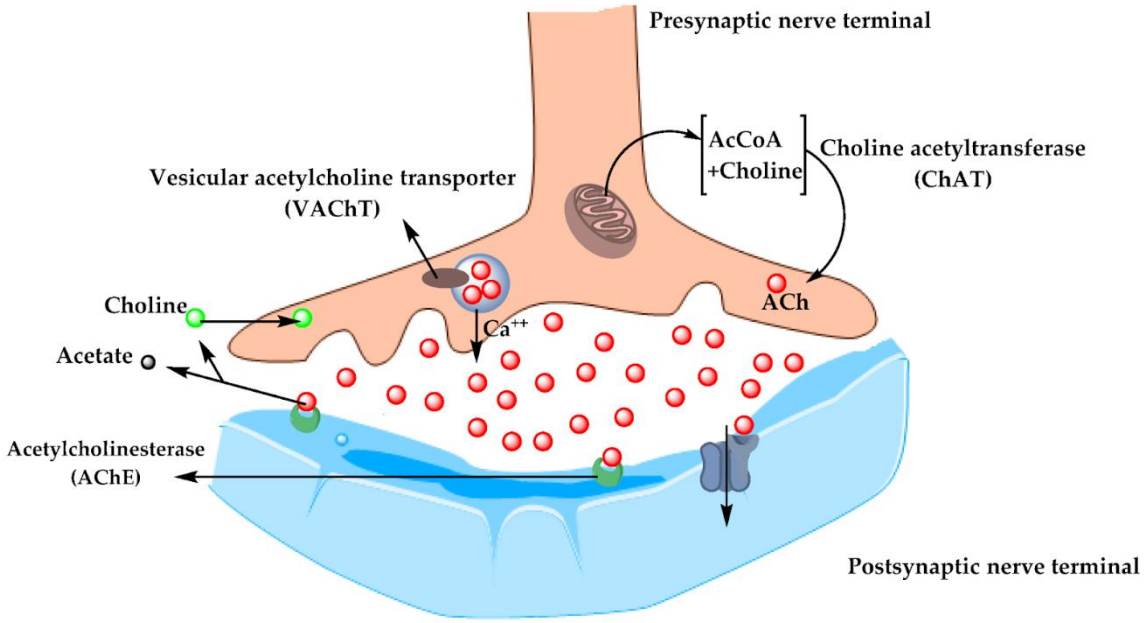


Figure 1: Creation and transport of acetylcholine in pre and postsynaptic nerve terminals.

The trajectory of the synthesis and transport of acetylcholine starting from the presynaptic nerve terminal and ending at the postsynaptic nerve terminal.

(*Breijyeh et al., 2020*)

Beta amyloid plays a part in reducing the uptake of choline and an increased discharge of ACh. Patients affected with AD experience a decline in cholinergic neurons, directly impacting cognitive and memory function. AChEis increases cholinergic neurotransmission by suppressing the enzyme acetylcholinerase at the synaptic cleft. The increase of ACh will enhance cholingeric transmission and therefore temporarily halt loss of neuronal function.

Partial NMDA antagonists are another therapeutic agent that has proved clinically effective in reducing AD symptoms for patients whose AD progression is moderate to severe. The NMDA receptor is a glutamate responsive gated channel with a high calcium (Ca^{2+}) permeability. Glutamate is the predominant excitatory neurotransmitter that controls the quick rate of neuronal communication at the points of excitatory synapse. Synaptic dysfunction occurs when normal Ca^{2+} processing is disrupted and the NMDA receptor is overstimulated. [36]. Overstimulation can cause an excess of glutamate to be present, leading to glutamatergic signaling, resulting in acute excitotoxicity. This excitotoxicity leads to loss of normal synaptic function and contributes to neuronal cell death. Clinically, it results in a decline in cognition and processing of memory. Memantine is a partial NMDA antagonist that preferentially binds to available NMDA receptors that results in blocking NMDA mediated ion flow. [37]

1.3 Ischemic stroke

Globally, incidents of stroke are the second and third leading cause of mortality and disability, respectively. Of these incidents of stroke, 68% are ischemic and the other 32% are hemorrhagic. [38] Ischemic stroke occurs where there is a blockage that forms in a cerebral artery reducing blood flow. This results in a cascade effect in which there is a lack of flow of oxygen and glucose. [39]. When this ischemic cascade is set in motion, the production of adenosine triphosphate (ATP) is halted due to the lack of oxygen, and the consumption of previously stored cellular energy adapted by mitochondria will cease. The membrane ion pump will malfunction causing an influx of water due to the disrupted balance in the levels of sodium, potassium, chloride and calcium ions. This event, coupled with the release of an excess of glutamate, will lead to neuronal cell death. In the

event that blood flow is restored, further injury can occur due to the creation of ROS during the reperfusion process [40]. Reactive oxygen species are a major contributor to tissue damage during reperfusion injury. When ATP is broken down during an ischemic episode, hypoxanthine is produced, and when oxygen flow is restored, xanthine oxidase will degrade the hypoxanthine to form uric acid and release the superoxide anion O_2. Once produced, O_2^- will be transformed into hydrogen peroxide and a hydroxyl radical. In response to the free radical production, a sustained production and release of proinflammatory cytokines [41].

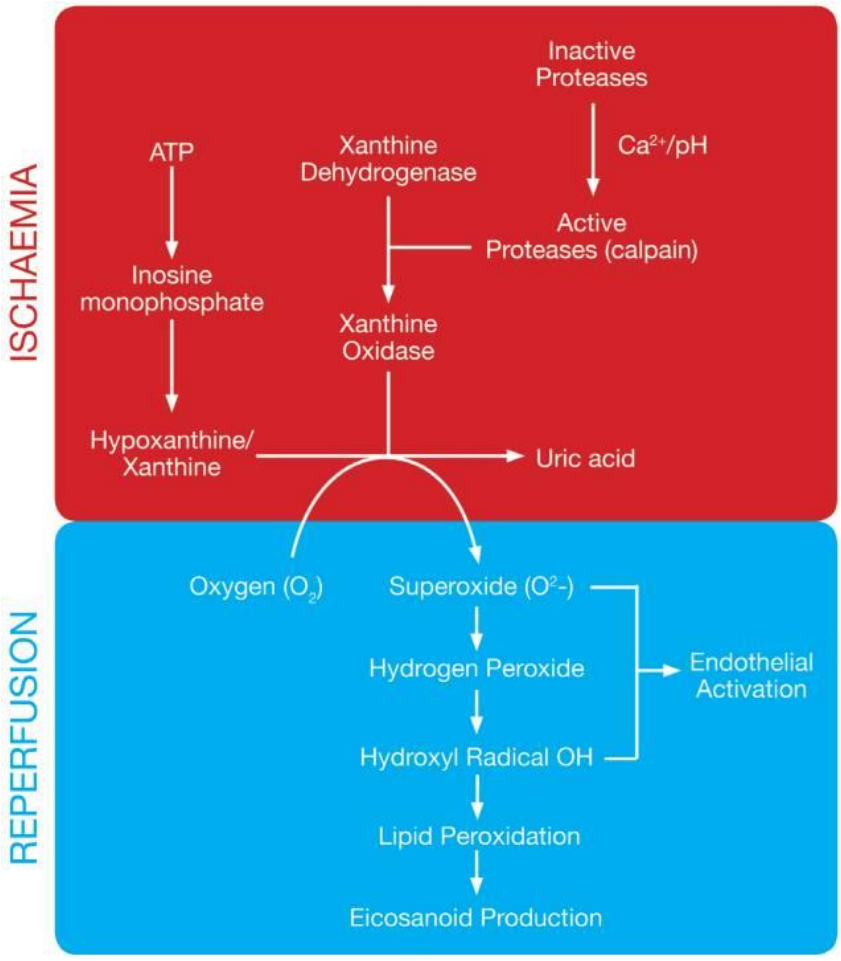


Figure 2: Reperfusion process: Creation of reactive oxygen species

Beginning with loss of ATP production, balance is attempted to be maintained with potassium coming in and calcium being released from the mitochondria into the cytoplasm and surrounding extracellular areas.

(*Cowled P. et al 2011*)

1.4 Treatment of Ischemic Stroke

The current gold standard of treatment, tissue plasminogen activator (tPA) which must be administered within 4 and a half hours of the initial stroke event. [42] tPA's amino acid chain can degrade fibrin that is plasminogen dependent, dissolving blood clots and opening the pathway for reperfusion [43]. It has also been shown to exert a neuroprotective effect against zinc mediated cell death as evidenced by the activation of mTOR and JAK/STAT pathways, the presence representing neuroprotection [44]. While considered highly effective as a first line stroke treatment, the timing of which the drug needs to be administered is one of its major disadvantages. tPA also has shown to have various side effects, including hemorrhage and neurotoxicity [45]. The use of tPA can cause glutamate toxicity as it can have an inhibitory effect on N-methyl-*D*-aspartate receptors (NMDAR), causing buildup and ultimately neuronal cell death. An upcoming alternative treatment can be found in the form of Tenecteplase which is created to have a more specificity than fibrin and possesses a longer half life than tPA [46]. Its administration window is longer than tPA and showed significant neurological function improvement in clinical studies [47].

1.5 Sulindac

Sulindac is a non-steroidal anti-inflammatory drug (NSAID) that acts as a nonspecific COX 1 AND COX-2 inhibitor [48]. When metabolized, it is processed as a sulfide derivative that hinders prostaglandin synthesis [49]. Its COX inhibitory activity

made it an ideal cancer drug, and it is effective in inhibiting tumor cell growth and prompting apoptosis [50]. Recent studies have indicated that aside from its NSAID properties, Sulindac was able to initiate a preconditioning response which is independent of the drug's NSAID function. Sulindac was found to protect normal cells from oxidative damage [51] and was able to selectively target and terminate human lung and colon cancer cell lines by sensitizing them to agents that influence mitochondrial respiration [52]. Its preconditioning response has extended to ischemic protection of the heart; it has been shown that when rat cardiac myocytes were exposed to hypoxia and reoxygenation, cardiac tissue was protected from oxidative damage by low concentration of Sulindac [53].

1.6 Granulocyte colony stimulating factor (G-CSF)

Granulocyte Colony Stimulating Factor (G-CSF) is a neuronal hematopoietic glycoprotein that is able to invigorate bone marrow to produce granulocytes and stem cells that are subsequently released into the bloodstream. It has been shown to display anti apoptotic, anti inflammation, and neuroprotective effects by mobilizing microglia from bone marrow cells [54]. They can be deployed against inflammatory mediators including endothelial growth factor (VEGF), necrosis factor alpha (TNF-α), and interleukins β (IL-1β and 17 (IL-17) [55]. G-CSF is currently most widely used as a treatment for use in cancer patients relating to chemotherapy related neutropenia and infections [56]. Recently, G-CSF gene therapy has been studied as a novel therapy for ischemic stroke in a mouse model [57]. It was shown that G-CSF bound to its respective receptors and when activated, upregulated Akt phosphorylation (P-Akt) which subsequently reduced levels of ER stress and apoptosis protein expression. Anti- apoptotic proteins Bcl-2 were observed to be upregulated while pro-apoptotic proteins such as Bax and Bak were downregulated [58].

1.7 SH-SY5Y cells

SHSY-5Y cells are a cell line that is derived from the SK-N-SH neuroblastoma cells that were originally acquired from a bone marrow biopsy. The SK-N-SH line were observed to possess a neuronal like phenotype [59] The original cell line of SK-N-SH were then subcloned to produce SH-SY, SH-SY5, and then today's standard of SH-SY5Y. The SH-SY5Y cell line can be utilized in both undifferentiated and differentiated form. In undifferentiated form, SH-SY5Y cells morphologically display a neuroblast-like cell body in a non-polarized form [60]. The primary advantage of use of undifferentiated cells is that they can consistently multiply and contain premature neuronal markers. [61] Differentiated forms of SH-SY5Y cells contain more mature neuronal-like projections and can generate an increase of neuron specific enolase (NSE) activity, the predominant enolase-isoenzyme that is found in neuronal tissue. After full differentiation, it has been seen that the inherent cell properties have been altered and affects cell survival and its associated signaling pathway. However, when the differentiation is performed, the cell generation cycle ceases, and a reliance on a brain derived neurotrophic factor (BDNF) is required for cell survival. [62]

1.8 Research aim

Hypoxia is known to cause oxidative stress that contributes to the formation of NFTs and Aβ plaques, hallmarks of AD pathology. The creation of reactive oxygen species (ROS) has been connected to (ER) stress in models of ischemic stroke. As previously mentioned, about 30% of patients who have suffered from a stroke event will develop symptoms of cognitive dysfunction within the next few years. Oxidative stress damage from ROS formation is seen in patients in the MCI stage of AD.

It has been shown that Sulindac and Sulindac derivative MCI 100 is able to protect retinal pigmented epithelial (RPE) cells against oxidative stress [63]. It has also been demonstrated to protect cardiac tissue from oxidative stress in events of hypoxia/reperfusion via preconditioning mechanism [52]. However, the potential neuroprotective effects of Sulindac as an AD treatment against oxidative stress is currently unknown. In this *in vitro* model study, the objective is to observe the effects of Sulindac as a neuroprotective agent against oxidative damage in events of hypoxia/reoxygenation.

G-CSF has demonstrated its ability to support neurogenesis and provide neuroprotection against ischemic event induced ER stress. Although proven effective in a neuronal ischemic stroke model, it has yet been tested in an AD model. In this study in an *in vivo* model, it is theorized that G-CSF gene therapy may be an effective treatment for AD.

2. MATERIALS AND METHODS

2.1 Materials

SHSY-5Y cells were obtained from ATCC (Manassas, VA, USA). DMEM/F-12 culture media and supplementing culture and viability material fetal bovine serum (FBS), glutamate, penicillin/streptomycin (P/S), and trypan blue were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). MTS tetrazolium assay was obtained from Sigma-Aldrich (St. Louis, Missouri).

2.2 *In vitro* study

In vitro study used the cell line SHSY-5Y to determine the efficacy of neuroprotection by Sulindac at varying concentrations. SHSY-5Y cells either transfected or non-transfected with Swedish APP mutation were subjected to hypoxic and normoxic conditions to simulate oxidative stress and the creation of ROS. Resulting cell viability was determined by either trypan blue or MTS tetrazolium assay.

2.2.1 SHSY-5Y cell culture

SHSY-5Y cells were cultured in DMEM-F12 media containing 20% (v/v) FBS, 1% (v/v) glutamate, 1% (v/v) P/S solution. Cells were maintained in an incubator set at 37 ℃ and 5% CO_2 conditions. Cells were cultured until 70% confluency in which they were subsequently subcultured at a 1:3 ratio until additional plates reached 70% confluency.

2.2.2 SHSY-5Y cell transfection with Swedish APP mutation

SHSY-5Y cells were plated on a 10 cm polystyrene tissue culture plate and grown to 70% confluency. The cells were then transfected with the Swedish APP genetic mutation using a pCAX-APP plasmid via lipofectamine. Following initial transfection, the cells were incubated at 37 ℃ and 5% CO_2 in an incubator for 24 hours to ensure completion of the transfection process. The cells were then cultured for six, 12, and 24 hours.

2.2.3 Sulindac treatment

SHSY-5Y cells were seeded at a density of 10,000 cells per well in a 96 well plate. 24 hours after seeding, cell media was replaced with media containing 100 uM, 200 uM, or 400 uM of Sulindac.

2.2.4 Hypoxia and normoxia

SHSY-5Y cells were seeded at a concentration of 10,000 cells per well in a 96 well plate. The cell media contained 100 uM, 200 uM, or 400 uM of Sulindac. The plates were then placed into a hypoxia chamber with an oxygen level of 1% with a CO_2 level of 5%. The plates remained in the hypoxia chamber for a period of 12 or 24 hours. The respective plates were then placed in an incubator with conditions set at 37 ℃ and 5% CO_2 for 12 or 24 hours to further induce oxidative stress. The total incubation time for the plates were 24 and 48 hours respectively.

2.2.5 Measuring cell viability by trypan blue

A sample of 10 uL of SHSY-5Y cells diluted with a 1:1 ratio of trypan blue were loaded into a haemocytometer and live cell viability was determined by a TC20 Automated Cell Counter (Bio Rad; Hercules, CA, USA).

2.2.6 Measuring cell viability by MTS Tetrazolium assay

After incubation of the 96 well plates containing SHSY-5Y cells media and Sulindac, 20 uL of MTS reagent was added to each respective well. After one hour of incubation at 37 ℃ and 5% CO_2, the plate was removed and absorbance was read via CLARIOstar® Plus microplate reader (BMG LABTECH; Ortenberg, Germany).

2.3 *In vivo* study

The animal model utilized is the 3xTg AD mouse model (Jackson Laboratory; Bar Harbor, Maine, USA). All 3xTg mice have inherited genetic mutations (APPSwe/TauP301L/PSen1) which are all hallmarks of AD. The mice were 3 months of age. The wild type (WT) mice strain is C57BL6. Animal studies reported in this investigation were approved by the Institutional Animal Care and Use Committee at Florida Atlantic University in accordance with federal guidelines.

2.3.1 *In vivo* study

The mice used in this study were B6;129-Tg (APPSwe, tauP301L) 1Lfa $Psen1^{tm1Mpm}$ mice (3xTg-AD) and B6129SF2J wild-type controls (WT) (Jackson Laboratory, Bar Harbor, ME, USA). Mice were randomly assigned to either one of two groups; transgenic disease control or the treatment group. For this study, G-CSF gene therapy was chosen as the therapeutic treatment due to previously reported success in a mouse stroke model [56]. Mice were given a single dose of AAV-CMV-hG-CSF (Treatment group, 1x10^14 pfu, 1.5uL) or AAV-CMV-GFP (disease control group, 1x10^14 pfu,1.5uL) in the form of drops in the left eye sac. Behavioral analysis was then conducted on day 28 to assess the effects of the drug treatment.

2.3.2 Morris Water Maze

The Morris Water Maze (MWM) is a test that assesses spatial learning and memory in rodents. Mice are placed in a pool of water and are tasked with finding the location of a hidden platform placed one cm below the surface of the water via four external spatial cues of four geometric shapes. The water was rendered opaque by white tempera paint and the apparatus was surrounded by black curtains with four evenly spaced light sources placed within. During each trial, the mouse is allocated 120 seconds to locate the hidden platform. The MWM trial consists of two parts. The first part is the training phase, where the mice were given time to familiarize themselves with the surrounding environment and locate the hidden platform. The second part is the probe trial, in which the hidden platform is removed and the spatial learning of the mice is observed. The mice were trained over a period of four days to discover the hidden platform and acclimate to the aquatic environment. After the training period a probe trial was conducted which lasted for 60 seconds, afterwards the mice were removed.

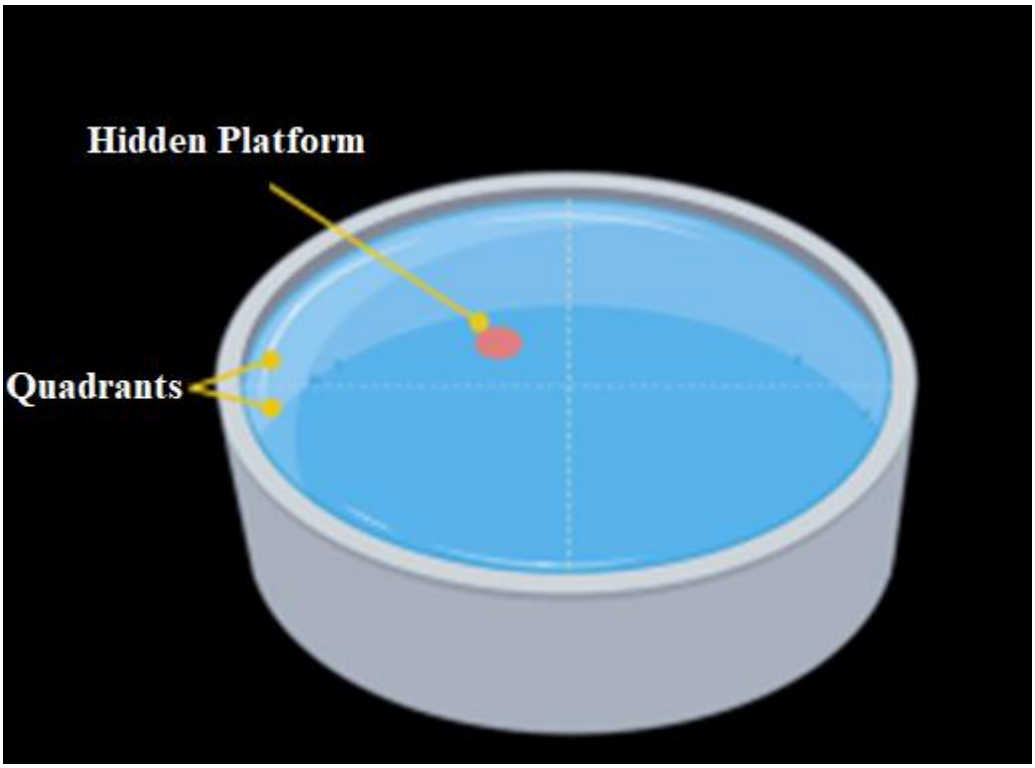


Figure 3. Configuration of Morris Water Maze.

The tub is divided into four different quadrants, with the hidden platform placed in one chosen quadrant. The platform is placed 1 cm below the water level and the pool is rendered opaque by non-toxic white tempera paint.

2.3.3 Locomotion test

Locomotion activity was measured by force-plate actometer, (Bioanalytical Systems Inc.; West Lafayette, IN, USA). The force sensitive plate measuring 424 cm x 24 cm was surrounded by a clear polycarbonate cage (15 cm tall) and top containing holes for ventilation. Attached to the plate was a sound attenuation chamber that was connected to an analysis system which monitored the animal's activity. The experiment was conducted at two different intervals of time. The first took place before

administration of the G-CSF gene therapy treatment while the second took place 28 days after treatment administration.

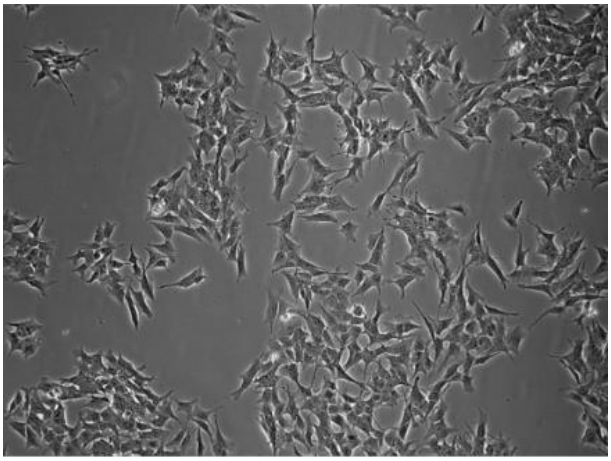
2.4 Data analysis

Data was analyzed and results were created using GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistical analysis for *in vivo* study was performed via two-way ANOVA. *In vitro* studies utilized four independent samples for each experiment..

3. RESULTS

3.1.1 Viability of SHSY-5Y cells in a hypoxic environment

It has been demonstrated that following a cerebral ischemic event, hypoxic circumstances may play a role in increasing the occurrence of AD. [64] The effect of hypoxia and reoxygenation on SHSY-5Y cells had to be determined in order to be able to assess whether SHSY-5Y cells would be protected by Sulindac. Cells were plated in concentrations of 5,000, 10,000, 15,000 and 20,000 cells per well. Cell viability was then determined via MTS assay. Over the total period of the first 12 hours, the results display virtually no decrease in cell viability at cells that were seeded at 5,000 and 15,000 cells per well. A small decrease in cell viability was observed in a 24 hour hypoxic period for all seeded cell densities, but a significant decline in cell viability when seeded at a capacity of 10,000 cells per well from the period of six, 12, and 24 hours.



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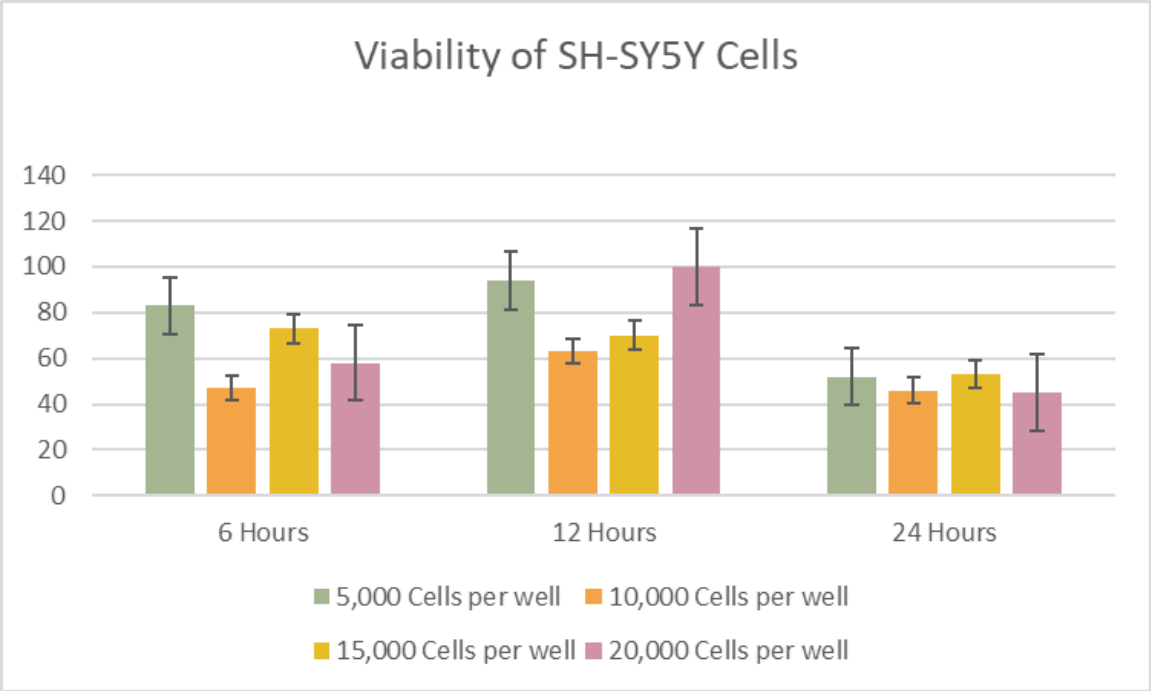


B

Figure 4. SHSY-5Y Cell Viability in Hypoxia

(A) Image of SHSY-5Y Cells In Culture. (B) Cell viability analysis of SHSY-5Y cells subjected to 6, 12, and 24 hours of hypoxia. Each experimental data bar represents the mean of four individual samples.

3.1.2 APP transfection of SHSY-5Y cell viability

SHSY-5Y Cells were transfected with or without the Swedish APP mutation via pCAX-APP (Swe/Ind) plasmid. After 24 hours, cell viability was measured as zero hour time point and the cells were then placed in hypoxic and normoxic conditions for a period of six, 12, and 24 hours. The resulting cell viability was measured via trypan blue. At the zero hour time point, cell viability was virtually equal. Untransfected cells had similar viability in both hypoxic and normoxic conditions at the six, 12, and 24 hour time points. Regarding the APP transfected cells, the samples that were placed in a hypoxic

environment had notably lesser cell viability compared to its counterparts in normoxic conditions (Figure 5).

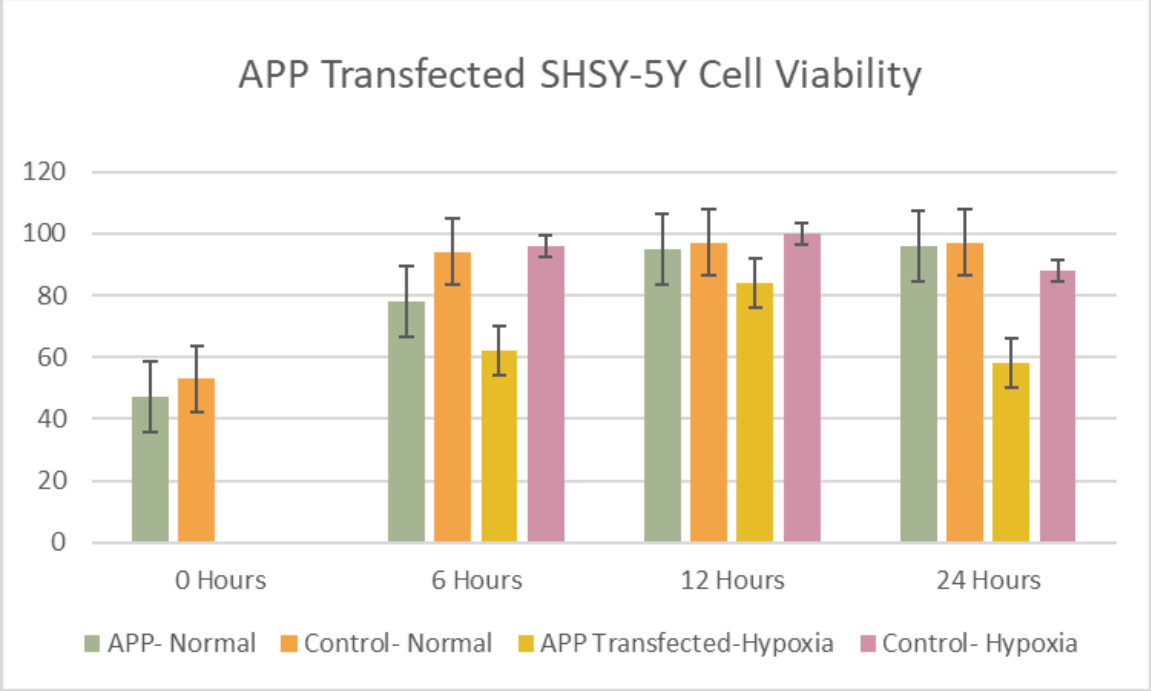


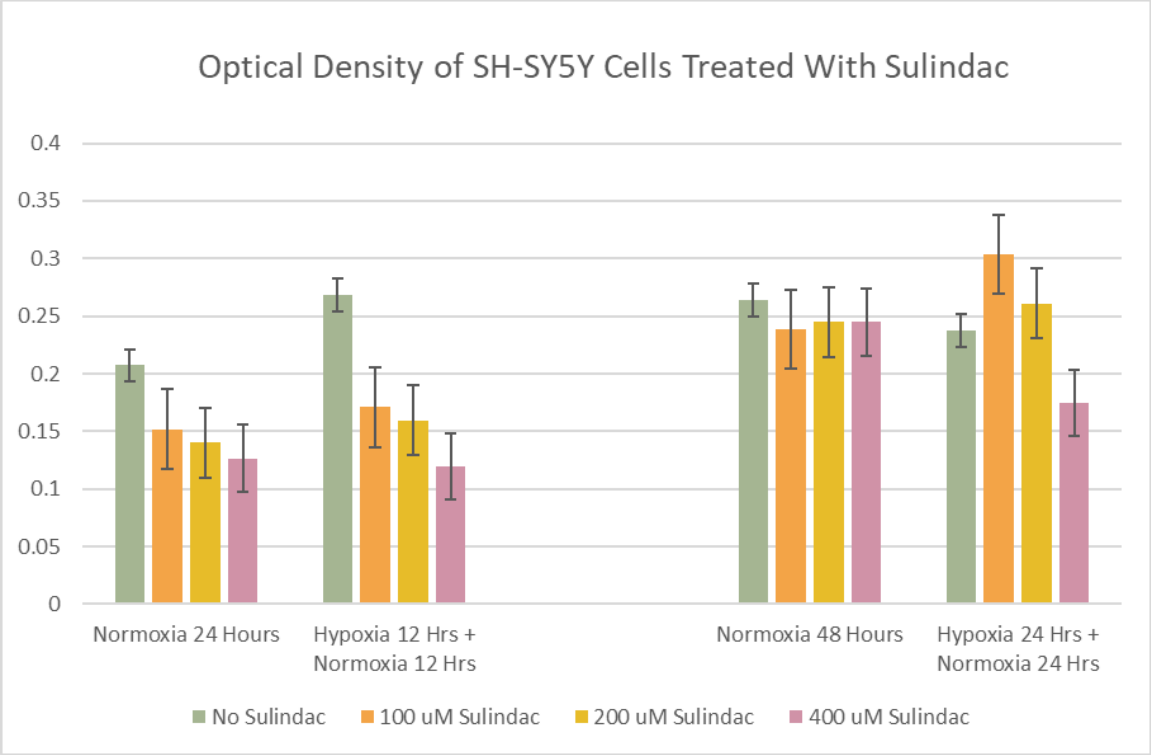
Figure 5. APP Transfected SHSY-5Y Cell Viability In Hypoxic and Normoxic Environments.

Cell viability is displayed in percentage maximum (control hypoxia 12 hours) and was measured via trypan blue. Each experimental data bar represents the mean of four individual samples.

3.1.3 Viability of SHSY-5Y cells treated with Sulindac

SHSY-5Y Cells were seeded in a 96 well plate and after a 24 hour period, the culture media was replaced with culture media with Sulindac added in concentrations of 100, 200, and 400 uM. The plates were then placed in both normoxic and hypoxic environments to induce oxidative stress and observe viability when treated with Sulindac. When observing optical density, cells that were treated with 400 uM of Sulindac were

observed to have inflicted cytotoxic effects in addition to reperfusion injury. 100 and 200 uM of Sulindac displayed similar protective effects. However when observing percent cell viability, 200 uM of Sulindac was demonstrated to offer the highest protection across the 24 and 48 hour time period (Figure 6).



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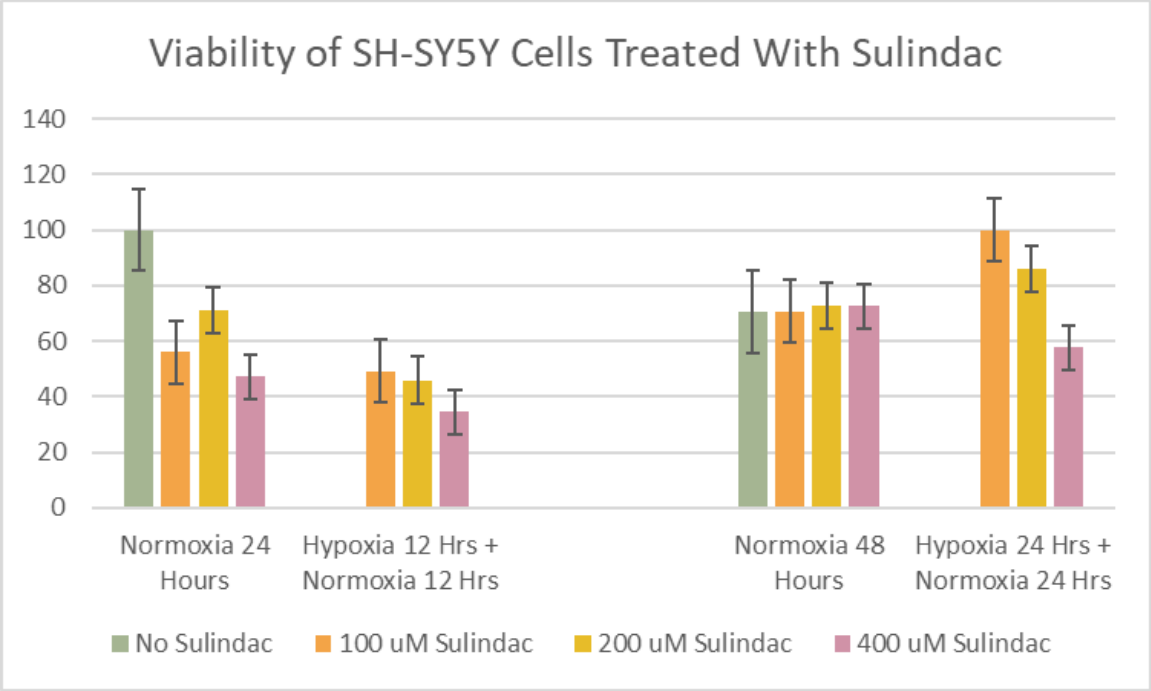

Figure 6. Optical Density of MTS Assay and Percent Viability of Sulindac Treated SHSY-5Y Viability Measured In Hypoxic and Normoxic Environments.

Samples were treated with Sulindac and subjected to hypoxia and reoxygenation to induce oxidative stress. Each experimental data bar represents the mean of four individual samples.

3.1.4 SHSY-5Y cell transfection and protein expression

SHSY-5Y cells were treated with 200 uM, 400 uM, or 600 uM of Sulindac and placed in a hypoxic environment for 24. Hours. Cell viability was measured via MTS tetrazolium assay. The samples were replicated in triplicate and the mean cell viability was recorded. Results show that at 600 uM, cell viability was the highest, followed by 200 uM and 400 uM. 200 uM of Sulindac is closest to cell viability at the zero hour time point, which represents the amount of cells pre hypoxia chamber placement.

10,000 SHSY-5Y cells were plated and subsequently transfected with APP using a pCAX-APP (Swe/Ind) plasmid,The cells were then treated with 200 uM, 400 uM, or 600 uM of Sulindac and placed in a hypoxic environment for 24 hours. The samples were replicated in triplicate and the mean cell viability was recorded. Cell viability was then measured via MTS tetrazolium assay. Results show at 200 uM of Sulindac, cell viability was almost at viability level of the zero hour. Increasing concentrations of Sulindac at 400 uM and 600 uM showed minimal increases in cell viability.

3.2 Locomotive activity

A force plate actometer (FPA) was used to measure locomotion activity. When an animal moves on the enclosed plate, its movements such as position and distance traveled are sensed and tracked by the FPA.

The mouse model used were 3xTg AD mice. TgAD mice all have genetic mutations (APPSwe, TauP301, PSen1) and exhibit both Aβ plaque and NFT pathology that is associated with AD. There were a total of eight, three months old male and female mice. The mice were given a 28-day treatment of G-CSF via eye drop delivery. Five of the mice were C57BL6 WT mice, which are the parental generation of the 3xTg mice. Three of the 3xTg AD mice received the disease control of green fluorescent protein (GFP) via AAV-CMV-GFP and the other three 3xTg AD mice received the treatment of human G-CSF via AAV-CMV-hCSF. Locomotion assay was conducted to establish a baseline of motor activity in 3xTgAD mice and the effect of hG-CSF gene therapy to be compared to its respective WT mice. The transgenic mice control that were given the vector carrier of GFP showed a reduced level of total locomotor activity while the mice given hG-CSF gene therapy showed a raised level of motor function. These are

preliminary studies looking at the baseline trend indicative of the difference between vectors and are currently ongoing. Additional analysis of locomotor activity within the five-minute intervals revealed that all three groups showed the highest level activity in the beginning, and that the level of activity decreased as a function of time. The GFP vector control group displayed the highest reduction and G-CSF gene therapy treatment was shown to reverse some of the observed deficits (Figure 8). The distance traveled steadily decreases over the period of 30 minutes, with the hG-CSF mice showing a higher level of activity compared to the mice that were treated with GFP.

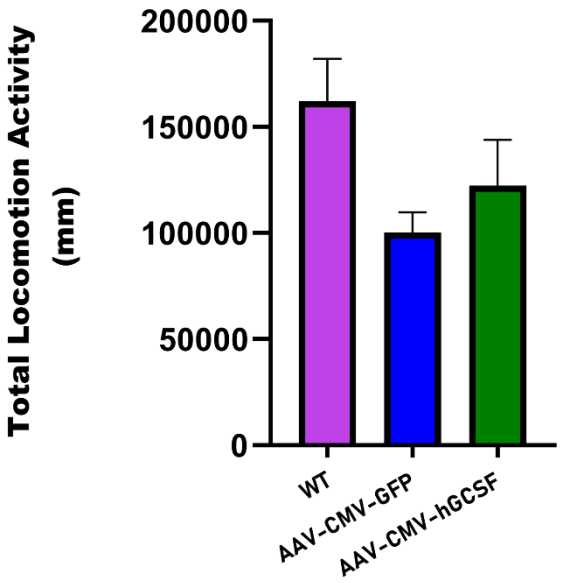

Figure 7. Total locomotion activity exhibited by 3xTgAD mice after G-CSF gene therapy administration.

Total distance traveled by Wildtype (WT) (n=5), disease control (AAV-CMV-GFP) (n=3) and treatment group (AAV-CMV-hG-CSF) (n=3) mice in a 30-minute FPA test 28 days after drug administration.

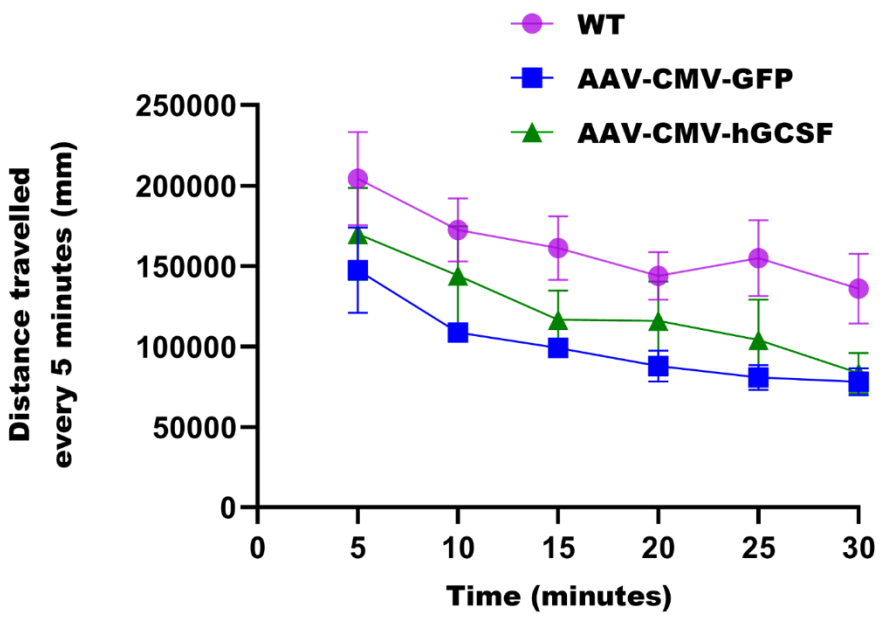

Figure 8. Locomotion activity of mice as a function of time.

Total distance traveled by (WT) (n=5), disease control (AAV-CMV-GFP) (n=3) and treatment group (AAV-CMV-hG-CSF) (n=3) mice in a 30-minute FPA test 28 days after administration of drug treatment. Each time point represents the total distance traveled within a five-minute interval.

3.2.1 Morris Water Maze

The Morris water maze (MWM) was used to measure spatial learning and memory. Five WT and five 3xTgAd mice were used in this preliminary study to establish a baseline behavior of AD mice comparative to their respective WT control. In the course of the hidden platform training phase, mice were given 120 seconds to find the hidden platform, hidden 1 cm below the surface of the water that was colored by non-toxic white tempera paint. If the mouse did not locate the platform within that time frame, they were guided to the platform and allowed 15 seconds to acclimate to its presence. During the

process of hidden platform training, the preliminary results showed that both groups of mice spent longer periods of time in the training quadrant as a function of training days. However, WT mice showed significantly better short term memory compared to 3xTgAD mice (Figure 9) as previously reported [65]. The rate of platform location is acutely diminished in 3xTgAD mice compared to its WT counterparts. The 3xTgAD and WT mice showed improvement in the occupancy of the target quadrant from the training period to the probe trial. However, comparative to the WT and AD mice, the 3xTgAD mice showed an overall reduced level of short term memory retention (Figure 10). On day one and day four of the training period, WT control and AD mice spend similar amounts of time in the hidden platform quadrant. However, by day four, there is a significant increase in the time that the WT mice spend in the quadrant containing the hidden platform. While there is a non-significant difference between the AD mice as a function of time, there is an upward trend shown in the percentage of memory retention as a function of training days. While both control and AD mice showed improvement in locating the platform in the correct quadrant during the training period, AD mice displayed a lower level of spatial learning ability compared to their peers. During the 60 second probe trial, AD mice showed a reduced capability to retain short term memory, and this capability did not improve compared to the WT mice as they underwent the training period.

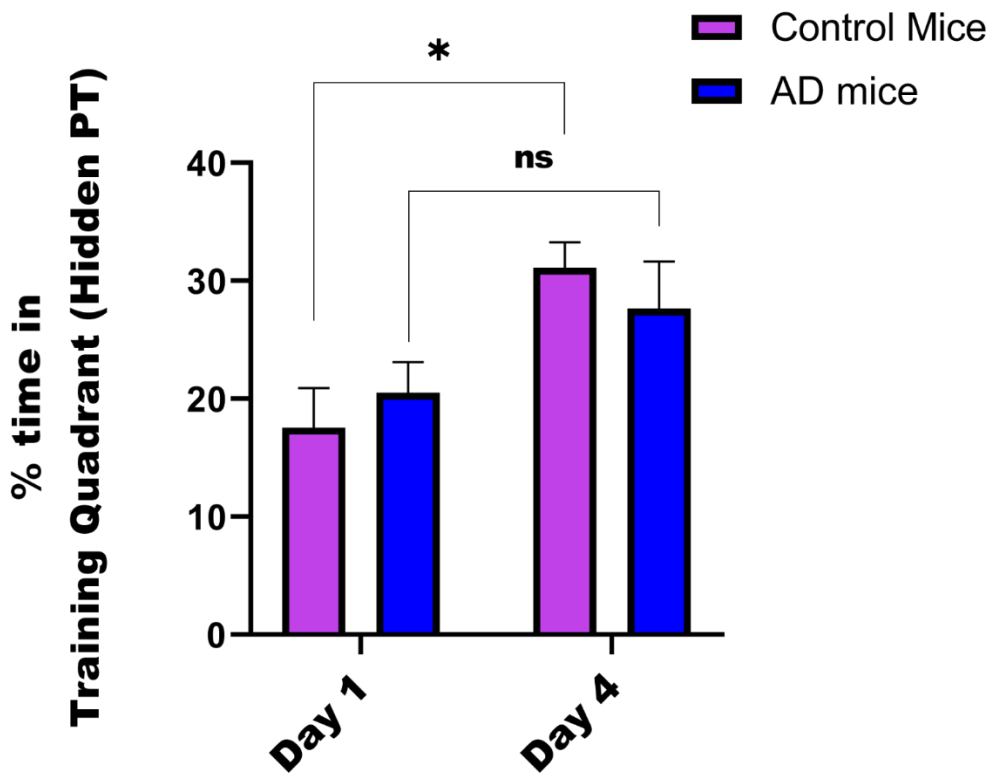

Figure 9. Morris Water Maze Hidden Platform Training

Mice were given training over a four day period, the graph represents the day and average percent of time the mice (n=10) had spent in the quadrant where the hidden platform was located. Data analysis was performed using two-way ANOVA and represented as a Mean ± SEM.

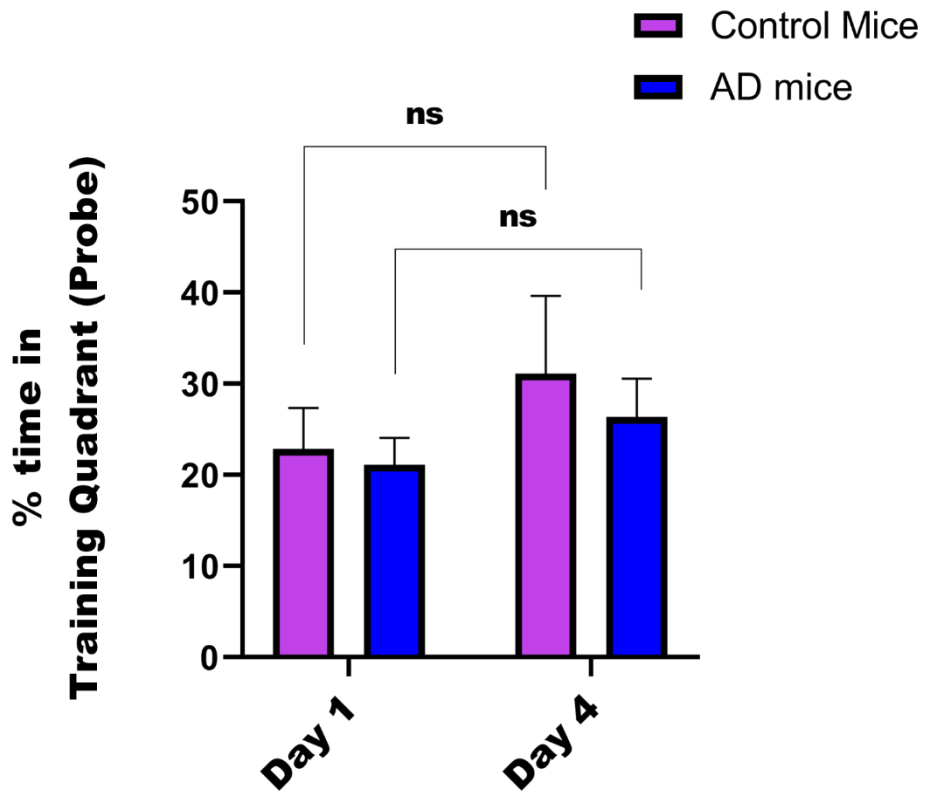

Figure 10. Morris Water Maze Probe Trial

With the hidden platform removed from the pool, the percentage of time the mouse (n=10) spends in the quadrant that used to contain the platform.

4. DISCUSSION

It has been confirmed that there is a link between ischemic stroke and reperfusion injury, which are risk factors in the development of AD [25]. The mechanisms behind the hypoxia/reperfusion injury process of ischemic stroke [39] also contribute to the pathogenesis in AD [64]. It is evident that hypoxia plays a role in the creation of both NFTs (via hyperphosphorylation of tau protein) [66] and Aβ plaques (upregulation of APP) [67]. Besides cognitive therapy, there are currently only two mainstream pharmacological treatments for AD; partial NMDA antagonists and AChEis [33]. As there is a connection between ischemic stroke and AD, it can be postulated that novel treatments that have been proven effective in the former may produce a similar effectiveness for the latter. Sulindac has been proven effective in protecting cells from oxidative damage in RPE cells [50] while G-CSF has been determined to be an effective therapeutic in a model of stroke [56].

In this study, Sulindac and G-CSF gene therapy were tested as potential therapeutics for AD. In the *in vitro* study, SHSY-5Y cells were treated with Sulindac to determine whether it would offer neuroprotective effects against oxidative stress triggered by a hypoxic environment. In an initial pre-treatment study, SHSY-5Y cells were plated in varying densities (5,000, 10,000, 15,000 and 20,00 cells per well) and subjected to hypoxic conditions for six, 12, and 24 hours. There was a general decrease in viability across all concentrations, however it was found that at 10,000 cells per well viability had the most significant decrease (Figure 4B). It has been previously revealed

that oxidative stress can upregulate APP expression [68] and as a result can cause neuronal cell death [69]. In our study untransfected SHSY-5Y cells were compared to their transfected counterparts in both hypoxic and normoxic conditions. SHSY-5Y cells that were transfected with the Swedish APP genetic mutation and placed in a hypoxic environment had distinctly lower viability compared to its counterparts in normoxic conditions. The lowest point of viability was seen with the sample of APP transfected hypoxia at the 24 hour time point (Figure 5).

Sulindac was applied as a therapeutic agent, to protect cells from oxidative stress and apoptosis. When observing optical density, non-transfected SHSY-5Y cells that were treated with 400 uM of Sulindac were observed to offer little protection against cell death (Figure 6B). Concentrations of 100 uM and 200 uM of Sulindac displayed similar protective effects, though when observing percent cell viability more broadly, 200 uM of Sulindac was demonstrated to offer the highest protection across the 24 and 48 hour time period. However when observing SHSY-5Y cells that were transfected with the Swedish APP mutation, 600 uM of Sulindac offered the highest amount of protection as demonstrated with retaining the highest cell viability rate out of all concentrations measured. The difference in effective concentrations comparing transfected and non-transfected SHSY-5Y cells could show a tailoring of treatment depending on the stages of AD progression.

G-CSF gene therapy was shown to be an effective therapeutic for ischemic stroke in a mouse model as pro-apoptotic proteins were reduced and subdued endoplasmic and mitochondrial stress [58]. This study focused on the application of G-CSF gene therapy

as a treatment for AD and how it may offer improvement in cognitive dysfunction. The behavior tests used in this study was locomotive activity measured via FPA and MMW. Locomotive activity was measured after the mice were given a 28 day treatment of human G-CSF via AAV-CMV-hCSF. Results showed the mice given hG-CSF gene therapy showed a raised level of motor function compared to its counterparts that were given a GFP vector (Figure 7). The distance that the mice in all groups traveled showed a high level of activity in the beginning, but steadily dropped over the 30 minute testing period (Figure 8). Of all tested groups, the hG-CSF treated mice showed higher overall activity than their GFP treated counterparts. These are preliminary studies looking at the baseline trend indicative of the difference between vectors and are currently ongoing.

The MWM tested spatial learning and memory in AD mice that were given the hG-CSF drug treatment or control (GFP).On day one and day four of the training period, both groups spent about the same amount of time in the quadrant containing the hidden platform. During the hidden platform training phase, both groups of mice were observed to spend longer periods of time in the training quadrant (Figure 9). However, the rate of locating the hidden platform was seen to be severely reduced in the 3xTgAD mice in [comparison to its WT counterparts. While there is a general improvement in platform localization (Figure 10), as seen in the probe trial, AD mice also displayed a lower capacity in spatial learning ability compared and did not improve compared to their WT peers even after the training period. These studies serve as a baseline for spatial learning and memory pre drug treatment to establish a baseline for future comparison.

Future directions pertaining to *in vitro* studies will include Western blot analysis of SH-SY5Y cells regarding survival and apoptosis biomarkers. Induced pluripotent stem cells

(IPSC) will be transfected with Swedish APP mutation that will be used as a future model of AD. G-CSF gene therapy will stimulate the stem cell mobilization for repair. Subsequent studies will then feature a combination treatment therapy of G-CSF and Sulindac in an *in vitro* and *in vivo* mice behavior model. As Sulindac is known for its ability to protect cells oxidative damage [63], combining Sulindac with G-CSF gene therapy may prevent oxidative damage in hypoxic conditions as the neuroprotective pathways have prolonged effectiveness due to the resulting high G-CSF protein bioavailability.

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