TREATMENT OF NEUROPATHIC PAIN USING 1-O-HEXYL-2,3,5- TRIMETHYLHYDROQUINONE (HTHQ)

by

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ABSTRACT

TREATMENT OF NEUROPATHIC PAIN USING 1-O-HEXYL-2,3,5-TRIMETHYLHYDROQUINONE (HTHQ)

By: ELIZABETH OMOLARA SHOAGA

Under the direction of DR. HAN-RONG WENG, Ph.D., M.D.

Neuropathic pain is caused by a primary lesion or injury to the nervous system and can be spontaneous, with no obvious peripheral stimulus. Pain results from the complex interplay between signaling systems and individual perception. Neuropathic and chronic pain effect more than 100 million Americans while costing the country billions of dollars annually to treat and manage. The purpose of this research study was to discover if preemptive or post-injury treatment with 1-O-Hexyl-2,3,5- trimethylhydroquinone (HTHQ), a hydroquinone monoalkyl ether known for its antioxidative abilities, effectively alleviates neuropathic pain induced by partial sciatic nerve ligation in rats.

In order to test the effectiveness of HTHQ in reducing neuropathic pain, we began by taking preliminary baseline behavior assessments to test animal pain response prior to injury. Some animals were treated with HTHQ for three consecutive days prior to the performance of partial sciatic nerve ligation (pSNL) surgery, while some were treated each day beginning four days after the induction of injury. Animal behavior was observed again for 10 days after nerve injury and drug treatment. The levels of antioxidant and pro-inflammatory proteins were analyzed using the western blot technique in order to determine the effectiveness of HTHQ at treating neuropathic pain. Preliminary findings suggests that treatment with HTHQ three days

prior to nerve ligation surgery showed an increase in antioxidant catalase, an enzyme responsible for converting hydrogen peroxide to water and oxygen, in the spinal cords of injured rats compared to those that were not treated 10-days after nerve injury. Our findings also demonstrate that rats treated with HTHQ three days prior to nerve injury showed an increase in antioxidative protein SOD2 but also decreases the expression of pro-inflammatory protein IL-1ß compared to vehicle treated neuropathic rats. There were no therapeutic changes seen in rats treated with HTHQ four days after pSNL surgery.

CHAPTER 1

INTRODUCTION

What is Pain?

Pain has been defined by the International Association for the study of Pain as "an unpleasant sensory or emotional experience associated with actual or potential tissue damage." (Steeds, 2016). Neuropathic pain is a result of nerve damage. The measurement of pain can be extremely difficult and complex due to the fact that the perception of pain is largely subjective. Due to this subjectivity, pain has been separated from the concept of nociception. Nociception is the "neural process involving the transduction and transmission of a noxious stimulus to the brain via a pain pathway" (Steeds, 2016). Tissues contain free nerve ending that respond only to painful stimuli called nociceptors. Nociceptors are located at the free nerve endings of the primary afferent neuron and convert painful stimulus into an electrical signal that can then be transmitted by axons from the periphery to central nervous system. Nociceptors can be found all over the body and different types of nociceptors can be activated in response to varying types of stimuli including sharp pain (mechanical nociceptors), slow, burning pain (thermal nociceptors), or mechanical, thermal, and chemical stimuli (polymodal nociceptors). There are two key nerve fibers, A∂ and C fibers that interact with second-order neurons in the dorsal horn of the spinal cord. A∂ primarily transmit signals from mechanical and thermal nociceptors to the dorsal horn of the spinal cord. A∂ are small and myelinated, allowing them to conduct signals quickly and provide specific localization of pain (Dafny, 2020). C fibers are unmyelinated and conduct signals much more slowly. Polymodal nociceptors use C fibers to transmit signals to the dorsal horn. There are ascending and descending tracts of pain. The ascending tracts of pain

communicate with the brain, where pain signals get processed and sent to the cortex. The descending tracts of pain work to control pain.

Inflammatory Pathway

The pain pathway consists of three main processes: transduction, transmission, and modulation. The pain mechanism begins with some sort of noxious stimulus that results from injury or trauma that has taken place. The noxious stimulus is transformed into electrical signals to be recognized by neurons in the dorsal horn of the spinal cord. These electrical signals are then transduced as chemical events at synapses. Following transduction, the neurons on the dorsal horn transmit this noxious information to the thalamus until it reaches the somatosensory cortex through the spinothalamic tract. Once the signal has reached the somatosensory cortex it is able to provide information about the intensity and location of the painful stimulus. It is important to note that the spinothalamic tract consists of the lateral and anterior spinothalamic tracts, each with their own functions. The lateral spinothalamic tract functions to transmit painful and thermal stimuli, while the anterior spinothalamic tract transmits information about crude touch and firm pressure to the thalamus.

Nerve injury and inflammation in the peripheral nervous system results in the activation of numerous signaling pathways in the neurons of the dorsal horn that eventually activate mitogen-activated protein kinases (MAPKs) including ERK and p38 MAPK in astrocytes and microglia. This MAPK mediated activation of glial cells leads to their production of pro-inflammatory mediators, such as IL-1ß and TNF-α, that induce hypersensitivity as well as mechanical allodynia and thermal hyperalgesia (C.Y. Chiang, 2012).

Key Players of Neuroinflammation

The nervous system contains several types of cells with differing functions, with some being activated specifically during the inflammatory process. There are two types of glial cells: astrocytes and microglia, both of which are activated following injury to a peripheral nerve, leading to the release of pro-inflammatory cytokines. These glial cells in the spinal dorsal horn are activated during the neuropathic state and contain certain cell markers and inflammatory proteins that allow for the analysis of neuroinflammation. Neuroinflammation is vital in the development of chronic, neuropathic pain as it leads to the upregulation important pro-inflammatory cytokines mentioned before, such as IL-1ß and TNF-α. It is important to note that neuroinflammation to a certain extent can be beneficial as it can allow for the repolarization of macrophages, promoting recovery and regrowth of axons (DiSabato et al., 2016). Neuroinflammation can also provide the body with a means of immune pre-conditioning allowing for the training of the immune system towards a more neuroprotective version of itself (DiSabato et al., 2016). Neuroinflammation is problematic when it chronically and uncontrollably causes the production of cytokines, chemokines, and reactive oxygen species.

Microglia, which can be identified by Iba1 antibodies, are the resident macrophage cells of the central nervous system and act first in immune defenses. Microglia act as scavengers of the central nervous system by finding and decomposing plaques, damaged neurons, or other infectious materials (Gehrmann et al., 1995). Activation of microglia by ATP, colony-stimulating factor-1 (CSF-1), chemokines, and proteases leads to a positive feedback loop that cause a neuropathic pain state. After activation, microglia release pro-inflammatory cytokines and chemokines, such as TNF-α and IL-1ß, causing a positive excitatory feedback loop that also decreases the release of anti-inflammatory elements (C. Y. Chiang, 2012). This imbalance

between excitatory and inhibitory signaling ultimately lead to the neuropathic state.

Astrocytes which express GFAP protein are activated following nerve injury and remain activated for the duration of chronic pain (C.Y Chiang, 2012). Activation of astrocytes results in an increased release for pro-inflammatory cytokines that triggers an increase of excitatory synaptic transmission and decrease inhibitory synaptic transmission causing the imbalance between excitatory and inhibitory signaling, the causative factor of neuropathic pain. Activation of astrocytes leads to an increased release of cytokine IL-1ß which can then activate transcription factors NFKB, which then cause increased transcription of several inflammatory mediators.

Protein kinases have been identified as playing a role in the sensitization of the central nervous system following a noxious stimulus. Specifically, mitogen-activated protein kinases (MAPKs) that consist of extracellular signal-regulated kinases (ERK) are activated by nociceptive or noxious activity and inflammatory mediators within primary sensory and dorsal horn neurons (Ji et al., 2007). MAPKs are responsible for the initial and prolonged prevalence of neuropathic pain by posttranslational, translational, and transcriptional regulation. MAPKs are also activated in microglia and astrocytes following injury, again resulting in the upregulation of the synthesis of pro-inflammatory substances (Ji et al., 2007). Immediately after injury, neuronal phosphorylated ERK (p-ERK) is present for several hours, followed by microglial p-ERK that is present during early phase nerve injury and slowly decreases, and finally astrocytic p-ERK is upregulated in late phase nerve injury (Zhuang et al., 2005). These data suggest that p-ERK is critical in not only the initiation, but also the maintenance of neuropathic pain.

Reactive Oxygen Species and Neuropathic Pain

Reactive oxygen species (ROS) are radical and non-radical oxygen species produced by the partial reduction of oxygen. Examples of reactive oxygen species include superoxide anion,

hydrogen peroxide, and hydroxyl radical (Ray et al., 2012). ROS are created during mitochondrial oxidative metabolism and following an intracellular increase in cytokines. An increase in ROS or a decrease in cellular antioxidant regulation can result in oxidative stress that can lead to ROS-mediated nucleic acid, protein, or lipid damage. Oxidative stress has been implicated in the development and progression of carcinogenesis, neurodegeneration, atherosclerosis, aging, and the genesis of pathological pain (Ray et al., 2012). Neuroinflammation from oxidative stress results when there is an increase in reactive oxygen species and a decrease in antioxidant free radical scavenging ability (Solleiro-Villavincencio & Rivas-Arancibia, 2018). Under normal conditions, an increase in free radicals or reaction oxygen species would be counteracted by antioxidant defense systems. The interaction between these two systems work to maintain the oxidation-reduction balance play a role in cellular metabolism and homeostasis (Solleiro-Villavincencio & Rivas-Arancibia, 2018). When redox homeostasis is lost, the regulation signal transduction by cells is impossible. This loss in redox homeostasis causes an upregulation of phosphorylation pathways and downregulation of the release of dephosphorylation enzymes (Solleiro-Villavincencio & Rivas-Arancibia, 2018). During redox imbalance, the signaling pathways responsible for the modulation of immune systems becomes altered, leading to the malfunctions of the immune response, thus promoting an increase of pro-inflammatory responses. Reactive oxygen species can alter the signaling systems that result in the activation of glial cells, microglia and astrocytes (Solleiro-Villavincencio & Rivas-Arancibia, 2018). High concentrations of reactive oxygen species can also promote an increased secretion of pro-inflammatory cytokines such as TNF-α and IL-1ß, which can then provoke the creation of reactive species in cells like vascular smooth muscle cells, tubular cells, fibroblasts, and endothelial cells.

Antioxidative Agents: Superoxide dismutase 2, Catalase, and Glutathione

Antioxidant levels in proteins can be measured using the western blot technique and primary antibodies targeted to detect level of antioxidants in the sample tissue. One important antioxidant is superoxide dismutase 2 (SOD2), an enzyme encoded by the SOD2 gene on chromosome 6 in humans. Superoxide dismutase 2 works as an antioxidative agent by converting unstable reactive oxygen species, superoxide ($O2^{-}$), to a stable hydrogen peroxide and oxygen (National Center for Biotechnology Information, 2021). SOD2 can be found in the matrix of the mitochondria. SOD2 has a critical function because it converts the negative superoxide ion, that is unable to easily permeate membranes into hydrogen peroxide that can easily and quickly diffuse across membranes. It is important for organisms to maintain a high concentration of SOD2 in order to minimize the level of harmful reactive oxygen species in the cell.

Catalase is another antioxidative enzyme found in almost all living organisms. Within the cell, catalase can most often be found inside of the peroxisomes. Catalase's most notable function is the catalyzation of the reaction that decomposes reactive oxygen species, hydrogen peroxide to water and oxygen (Goodsell, 2004). Catalase's ability to convert hydrogen peroxide to water and oxygen make it one of the most essential enzymes for protecting the cell from oxidative damage caused by an excess amount of reactive oxygen species. Catalase enzymes are some of the most efficient found in cells, with an extremely high turnover rate allowing one molecule of catalase to decompose millions of hydrogen peroxide molecules per second (Goodsell, 2004). The structure of catalase is described as four identical subunits, each with their own interior active site. Catalase uses an iron ion, held in the middle of heme group, to assist in the speed of reactions.

Glutathione (GSH) is an antioxidant found in animals, plants, and fungi and functions to protect the cell from damage by converting detrimental reactive oxygen species to more stable, less harmful components. Glutathione is a tripeptide, composed of cysteine, glycine, and glutamic acid with gamma peptide linkage between the carboxyl group of the glutamate side chain and cysteine (Pompella et al., 2003). Glutathione exists in two forms, the reduced from, GSH and the oxidized from, GSSG. The ratio of GSH to GSSG is important way of measuring oxidative stress, and increased GSSG:GSH ratio can be indicative of increase oxidative stress (Pastore et al., 2001). Glutathione is most notably present in the cytosol of the cell. Glutathione works to protect the cell from reactive oxygen species neutralizing oxygen ions, hydroxyl radicals, and superoxide radical (Pizzorno, 2014). It is critical to have antioxidative molecules like SOD2, Catalase, and GSH as an accumulation of reactive oxygen species can lead to oxidative stress, thus inducing neuroinflammation.

Importance of Pain Management

Neuropathic pain is a chronic condition that results from a lesion that directly effects the somatosensory system and effects a significant portion of people in the United States and around the world (Nishikawa & Nomoto, 2017). Data indicates that 9.8% of Americans have been clinically diagnosed with neuropathic pain with 12.4% self-report suffering from neuropathic pain (Brooks & Kessler, 2017). An accurate representation of the percentage of the population effected by neuropathic pain is difficult to determine due differing methods of diagnosis. Chronic pain dramatically decreases one's quality of life and can often make it difficult to carry out daily activities. It is also important to note that neuropathic pain not only effects patients physically, but psychologically and emotionally as well, with patients diagnosed with neuropathic pain having higher rates of anxiety and depression (Cruccu, & Truini, 2017). Chronic pain effects the

lives of those with the condition, but it also negatively impact the healthcare system. The current treatments for chronic pain can be costly and also lead to other detrimental side effects such as addiction, hypertension, and liver diseases. Although there are treatments, not all patient experience relief of their pain and proper management remains an obstacle.

The most commonly used first line methods for treating neuropathic pain are antidepressant drugs. Tricyclic antidepressants are used to treat neuropathic pain as they are successful at inhibiting norepinephrine reuptake in the spinal dorsal synapses (Brooks & Kessler, 2017). Unfortunately, use of tricyclic antidepressants can lead to cardiotoxicity and other adverse effects, limiting their use in many countries. Serotonin norepinephrine reuptake inhibitors (SNRIs) are also class of antidepressants prescribed to treat neuropathic pain. SNRIs inhibit the reuptake of serotonin and norepinephrine by blocking their presynaptic transport proteins (Brooks & Kessler, 2017). Calcium channel Î±$_2$ -Î´ subunit ligands are used as therapeutic agents for neuropathic pain as they decrease the sensitization of the central nervous system and nociceptive transmission (Colloca et al., 2017). There are several adverse effects associated with calcium channel Î±$_2$ -Î´ subunit ligands, including dizziness, weight gain, fatigue, ataxia, and increased suicidal thoughts (Pop-Busui et al., 2017). Additionally, there are topical treatments for neuropathic pain; however, these are used much less often as they are less effective for more severe symptoms of neuropathic pain. Capsaicin and lidocaine patches are topical treatments more effective at treating peripheral pain by working locally to decrease ectopic nerve release by blocking sodium channels (Pop-Busui et al., 2017). Opioids are also a treatment option for neuropathic pain but are often avoided due to apprehensions over their misuse and morbidity. Opioids, like several other treatments, reduce the reuptake of norepinephrine and serotonin

(Brooks & Kessler, 2017). These current drugs either lack potency or safety feature, making development of safe and potent analgesics is highly demanded.

1-O-Hexyl-2,3,5- trimethylhydroquinone (HTHQ)

1-O-Hexyl-2,3,5- trimethylhydroquinone (HTHQ) is a lipophilic phenolic agent that has been found to have significant anti-inflammatory activity as well as reactive oxygen species (ROS) scavenging abilities. It has been hypothesized that HTHQ reacts directly with peroxyl radicals, like t-butyl peroxyl radicals and peroxides to convert them to more stable free radicals (Hino et al., 1998). An increase in oxidative stress is often a result of neuropathic pain and spinal cord inflammation. Nuclear factor E2-related factor (Nrf2) is a basic leucine zipper transcription factor that exhibits neuroprotective activity after injury (Tang et al., 2020). Inflammation and other conditions of oxidative stress results in the binding of Nrf2 to the antioxidant response element (ARE) sequences in order to encourage the expression of haemoxygenase-1 (HO-1), an antioxidant enzyme. HTHQ has been shown to lessen the oxidative stress that results from liver fibrosis and cerebral ischemic/reperfusion (I/R) injury using a currently unknown mechanism. HTHQ treatment also decreases oxidative stress and neuronal apoptosis in vitro as seen in PC12 cells. Nrf2 is thought to be involved in the neuroprotection of HTHQ. After knockdown of Nrf2, HTHQ was no longer effective in attenuating the adverse effects of cerebral I/R injury. Currently, not much is known about the mechanism of HTHQ as a reactive oxygen species scavenger.

Hypothesis and Specific Aims

The hypothesis for this study is that treatment with 1-O-Hexyl-2,3,5-trimethylhydroquinone (HTHQ) can prevent the development of symptoms of neuropathic pain. The specific aims of this study are to determine if treatment with HTHQ can prevent the

development of neuropathic pain symptoms using behavior testing and to determine if treatment with HTHQ effects the anti- inflammatory pathway.

CHAPTER 2

METHODOLOGY

All experiments for this study were conducted in Mercer University School of Medicine's laboratories and animal care facility. The animals used for the experiments conducted throughout this study were male Sprague-Dawley rats purchased from Charles River Laborites (Raleigh, NC) between ages 7 to 8 weeks. All rats were fed Teklad Global 2014 (Envigo; Indianapolis, IN). Rats were maintained on a 12-hour light/dark cycle and offered food and water ad libitum. The rats were housed 3 to 4 per cage and were allowed a week to acclimate to the new environment before any experiments were performed.

Neuropathic Pain Model

In order to determine the effects of neuropathic pain, this study used the partial sciatic nerve ligation (pSNL) animal model, as it was determined to be the most reliable, minimally invasive, and cost effective. The partial sciatic nerve ligation technique consistently produced thermal hyperalgesia and mechanical allodynia in the short timespan allotted for the experiments.

In preparation for the pSNL surgery, animals are first anesthetized using 3% aerosolized isoflurane (Covetrus; Portland, ME) with anesthetization continued throughout the surgery by placing the outlet tube over the mouth and nose of the animal. The left thigh was then shaved and sterilized using iodine, 70% isopropanol, and saline. Using a scalpel, a small incision was made along on the thigh from below the spine to above the knee in order to expose the biceps femoris. To expose the sciatic nerve, a small incision was made along the muscle length and then expanded using surgical scissors. Once the incision reached the bottom of the muscle and the sciatic never was visible, surgical scissors were then used to separate the sheath surrounding the nerve, by placing the tip of the scissors on either side of the nerve and spreading the scissors

until the nerve was freed. Once the sciatic nerve was freed, a pair of angled forceps were inserted underneath the nerve and a sterile thread of 3-0 silk suture (KeeboMed; Mount Prospect, IL) was passed into the angled forceps and underneath the nerve. Once underneath the nerve, the suture was tied in knot twice until the nerve was constricted to approximately half of its original diameter. The nerve is then returned to original placement inside the cavity and the wound is closed with 9mm stainless steel AutoClips (Mikron Precision Inc.; Gardena, CA). The sham control surgery followed the same steps up until the insertion of the silk suture. After the sciatic nerve was exposed and freed from its sheath, the wound was closed using the 9mm stainless steel AutoClips.

Behavior Testing

To ensure that the pSNL surgery was performed correctly in order to produce neuropathic pain through the development of mechanical allodynia and thermal hyperalgesia behavior tests were conducted. Behavior tests for both mechanical and thermal sensitivity were performed as a way to quantify the effectiveness of the pSNL surgery. Behavior tests are conducted prior to pSNL surgery in order to provide a baseline for the animal's mechanical and thermal pain thresholds. Both mechanical and thermal behavior testing were conducted again starting two days after the pSNL surgery and then every other day following for 10 days.

To measure mechanical allodynia, rats were placed in a plexiglass box atop a wire mesh surface. The rats were allowed to acclimate to this environment for 30 minutes before testing to minimize animal stress and potential errors in measurements. A variety of von Frey monofilaments (DanMic Global; Campbell, CA), between 4g to 26g bending forcer were used to stimulate the midplantar area of the left hind paw. Pressure was applied to the hind paw of each animal in ascending bending forces five times each for approximately one second with 3 minutes

between each application. The pain threshold was determined if the rat produced a painful paw withdrawal of 50% or more of the stimulations, these thresholds were then averaged with all animals in the same group. After five applications and before applying a von Frey filament with a greater bending force, the rats were given a five-minute rest period.

To measure thermal hyperalgesia, rats were placed in a plexiglass box atop a preheated (32°C) glass surface. The rats were allowed to acclimate to this environment for 30 minutes before testing to minimize animal stress and potential errors in measurements. A radiant heat source (IITC Life Sciences; Woodland Hills, CA) was directed at the midplantar area of the left hind paw. The withdrawal latency was established as the period from the start of the application of the radiant heat to the time the animal withdrew its paw. Three readings were taken for each animal with a resting period of 3 minutes taken between each reading. These readings were averaged for each individual animal, these averages were then averaged across all animals in the same group. The rat's paws were exposed to the radiant heat source no longer than 20 seconds as to avoid damage to the animal that would later skew data.

Preparation and Administration of 1-O-Hexyl-2,3,5- trimethylhydroquinone (HTHQ)

The goal of this study was to determine if the administration of 1-O-Hexyl-2,3,5-trimethylhydroquinone (HTHQ) could treat neuropathic pain and return the pain threshold of pSNL rats to those observed prior to nerve injury.

HTHQ was purchased from MedChem Express in a powder form. Before preparing the HTHQ solution for administration, the necessary amount was determined in order to give a 200mg/kg oral dose to each rat. The appropriate amount of HTHQ in milligrams was obtain and then made into solution using 0.9% sodium chloride and dimethyl sulfoxide. The solution was then sonicated to ensure that the solution could pass through the oral gavage. Once the solution

was made, the drug solution was administered either everyday for 3 days prior to pSNL surgery or 4 days following pSNL surgery, and then every day after until 10 days after surgery. The HTHQ drug solution was given to designated rats using an oral gavage. If behavior testing was to be performed on the same day as drug administration, the behavior testing was conducted before drug administration to prevent potential errors in measurements. If rats were in the control/vehicle group they were administered 0.9% sodium chloride via oral gavage.

Protein Measurements of Antioxidant and Inflammatory Molecular Markers

Protein measurement was an important tool used in conjunction with behavior test results in order to determine the effectiveness of treatment and pSNL surgery. Protein levels between pSNL and sham models were compared and correlated with behavior test results, with increased levels of inflammatory markers corresponding to decreased pain thresholds from nerve ligated rats. In addition to widely used inflammatory markers, the levels of antioxidant activity was also measured, with its expression expected to be decreased in pSNL animals. In order to measure protein levels, the western blot technique was used.

On the tenth day following the pSNL surgery, rats were continuously anesthetized using 3-5% aerosolized isoflurane. The lumbar enlargement of the spinal cord was removed from the spinal column and then dissected to obtain the left dorsal quarter of the spinal cord. The spinal cord tissue was then placed on ice until it was either homogenized immediately or stored in a -80°C freezer. After homogenization, the protein concentration was determined then adjusted to load 30 μg of protein per well. SDS-PAGE gels were prepared at either 13% or 15% depending on the molecular weight of the protein of interest. Gels were run using a gel apparatus set at 0.01 amps and set to run until the loading buffer ran out of the gel. Gels were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad; Hercules, CA) overnight at 30 volts in a 4°C refrigerator.

PVDF membranes were cut based on the molecular weight of the target of interest. The cut membranes were then incubated in blocking solution made from 5% non-fat dried milk at 1x phosphate buffered saline/tween (PBST) for two hours. The membranes were then removed from blocking solution and washed three times for 5 minutes each with 1x PBST before being incubated with primary antibodies detailed in *Table 1* either for 2 hours at room temperature, or overnight at -25°C. The membranes were washed once again three times for 10 minutes each with 1x PBST before being incubated with the appropriate secondary antibodies (*Table 1*) for one hour. After the secondary antibodies were removed, membranes were washed for a final time three times for 10 minutes each. Membranes were then developed using Pierce Enhance Chemiluminescence Western Blotting Substrate (Thermo Scientific; Rockford, IL), imaged using Bio-Rad ChemiDoc (Bio-Rad; Hercules, CA), and analyzed using Bio-Rad Image Lab software and ImageJ.

Antibodies Used for Western Blot Analysis

1° Antibody	1° Antibody Concentration	2° Antibody	2° Antibody Concentration
Mouse anti-GAPDH (Proteintech; Rosemont, IL)	(1 : 5000)	Goat anti-Mouse-IgG, horseradish peroxidase conjugated (Thermo Fisher Scientific; Waltham, MA)	(1 : 10000)
Mouse anti-IL-1ß (Santa Cruz Biotechnology; Dallas, TX)	(1: 500)		(1:1000)
Mouse anti-Beta Actin (Proteintech; Rosemont, IL)	(1 : 5000)		(1: 10000)
Rabbit anti-Catalase (Novus Biologicals; Centennial, CO)	(1 : 2000)	Goat anti-Rabbit-IgG, horseradish peroxidase conjugated (Thermo Fisher Scientific; Waltham, MA)	(1 : 4000)
Rabbit anti-p16 INK4A (Cell Signaling Technology; Danvers, MA)	(1 : 500)		(1 : 1000)
Rabbit anti-p-p44/42 MAPK (Cell Signaling Technology; Danvers, MA)	(1 : 500)		(1 : 1000)
Rabbit anti-SOD2 (Novus Biologicals; Centennial, CO)	(1 : 500)		(1 : 1000)

Table 1. All antibodies were diluted to working concentration with 1x PBST. Original image.

CHAPTER 3

RESULTS

Protein Content of Antioxidant and Inflammatory Molecular Markers

In order to test the hypothesis of the present study: treatment with 1-O-Hexyl-2,3,5-trimethylhydroquinone (HTHQ) will work to alleviate symptoms of neuropathic pain, the expression of markers for antioxidant levels such as Catalase and Superoxidase dismutase 2 (SOD2) were assessed. To test the success of the partial sciatic nerve ligation surgery as well as the effectiveness of HTHQ on treating inflammation, the expression of inflammatory markers such as IL-1B and p-ERK were also examined.

Analysis of western blot data indicates that expression of all inflammatory markers tested increased after nerve injury, indicating an effective partial sciatic nerve ligation. For animals treated with vehicle solution, HTHQ prior to nerve injury and those treated 4 days after surgery, levels of inflammatory markers increased following pSNL surgery (*Figure 1*). Both molecular markers were normalized to either GAPDH or Beta-actin. The results of western blot analysis are displayed as the mean of two rats per group with error bars representing the standard error from the mean.

Effects of HTHQ on Antioxidant Molecular Markers

HTHQ's effectiveness following oral administration at treating symptoms of neuropathic pain in an animal model following partial sciatic nerve ligation has not yet been documented. To test its effectiveness, HTHQ oral administration began either three days prior to pSNL surgery or four days after. In rats treated with HTHQ four days after surgery, levels of the Catalase indicate the successful treatment of pain, while levels of SOD2 showed no significant changes. However,

in rat groups treated with HTHQ preemptively showed promising effects in the protein expression of both Catalase and SOD2.

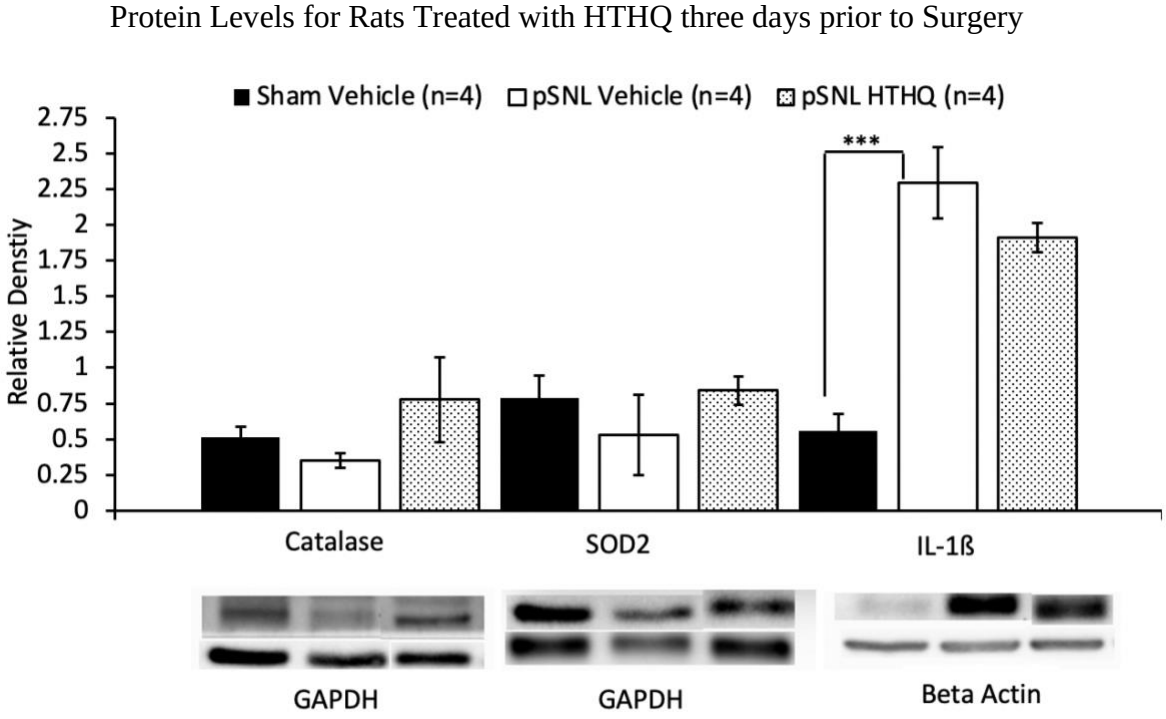

Figure 1. Protein levels of the antioxidant markers Catalase, SOD2, and inflammatory marker IL-1ß in rats treated with HTHQ or vehicle three days prior to pSNL surgery and then daily until 10 days after the surgery. Although there is a tendency that HTHQ reversed the decreased expression of antioxidant markers and increased the expression of pro-inflammatory markers following nerve injury, there is no statistical significance seen in antioxidant markers due to an insufficient number of experiments. Statistical significance between pSNL and sham for each protein is labeled with *. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$. Original image.

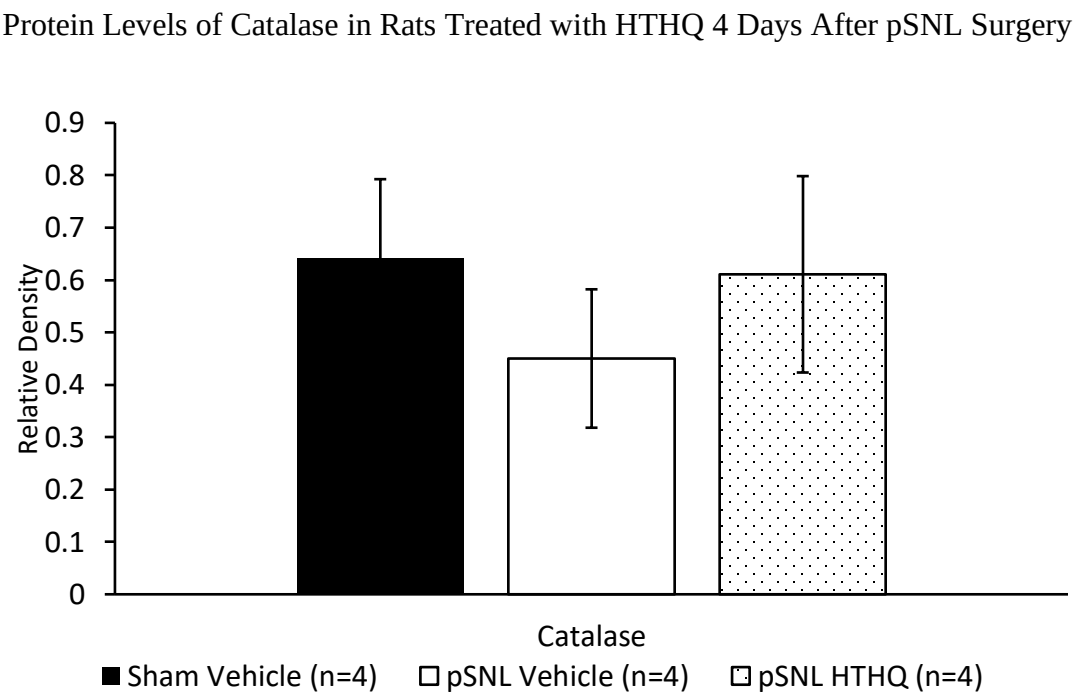

Figure 2. Protein levels of Catalase in rats treated with HTHQ 4 days after pSNL surgery. Rats in the pSNL + Vehicle group displayed decreased levels of antioxidant. Rats in pSNL + HTHQ groups display levels of antioxidant markers that are greater than pSNL + Vehicle. There was no indication of statistical significance between any of the groups. Original image.

Behavior Data

Behavior data indicated that treatment with HTHQ both before and after nerve ligation has a therapeutic effect on both mechanical allodynia and thermal hyperalgesia in nerve ligated rats. The behavior data for mechanical and thermal data for treatment before and after nerve ligation was normalized to 100 in order to more easily analyze the data.

Mechanical behavior data for rats treated with HTHQ three days before (*Figures 3 and 4*) and four days following pSNL (*Figures 5 and 6*) showed an initial decrease in withdrawal latency followed by gradual increase in withdrawal threshold, the maximum being reached 6 days after injury. Two days following nerve ligation surgery is when the most significant

difference is seen between the baseline for pSNL+ HTHQ and pSNL+ Vehicle for animals treated preemptively with HTHQ. The most significant difference between pSNL+ HTHQ and pSNL+ Vehicle groups is seen 6 days after surgery for both treatment timepoints. Rats treated four days after pSNL surgery showed the most significant difference between pSNL+ HTHQ and pSNL+ Vehicle groups 8 days after surgery. The greatest deviation from baseline occurred in the pSNL+ HTHQ and pSNL+ Vehicle groups took place 6 days post-surgery in those treated 4 days after surgery. For groups treated preemptively, the greatest deviation in thermal withdrawal latency from baseline and between pSNL+ HTHQ and pSNL+ Vehicle groups took place 2 days following injury.

Mechanical behavior tests for rats that were treated with HTHQ three days prior to pSNL surgery

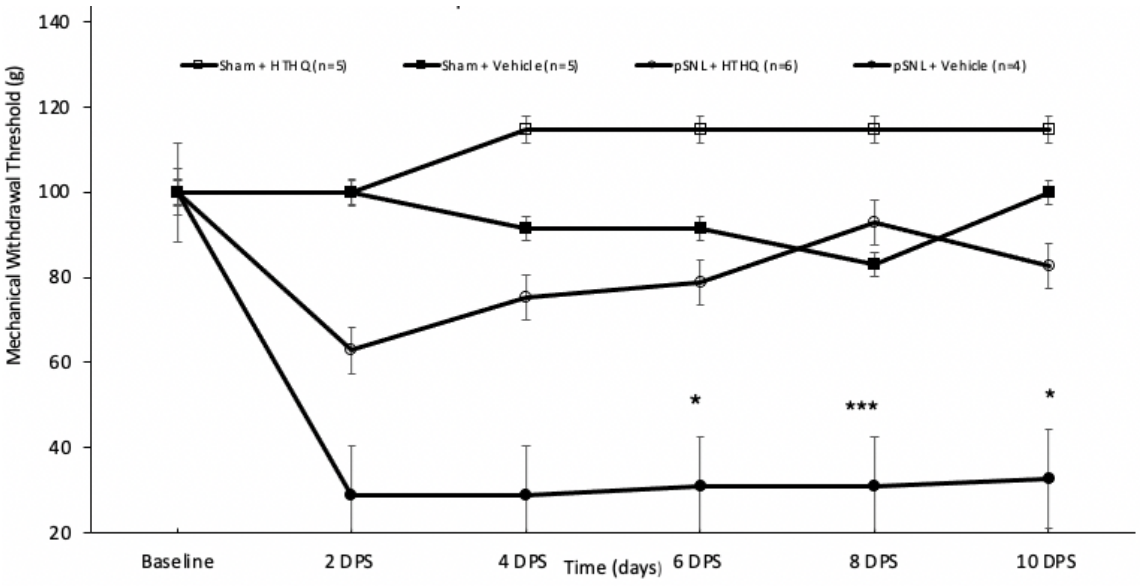

Figure 3. Mechanical behavior tests for rats that were treated with HTHQ three days prior to pSNL surgery. These values have been normalized to 100 in order to more easily analyze the data. The maximum effect of HTHQ treatment occurred eight days after pSNL surgery. Statistical significance between pSNL + Vehicle and pSNL + HTHQ is labeled with *. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$. Original image.

Thermal behavior tests for rats that were treated with HTHQ three days prior to pSNL surgery.

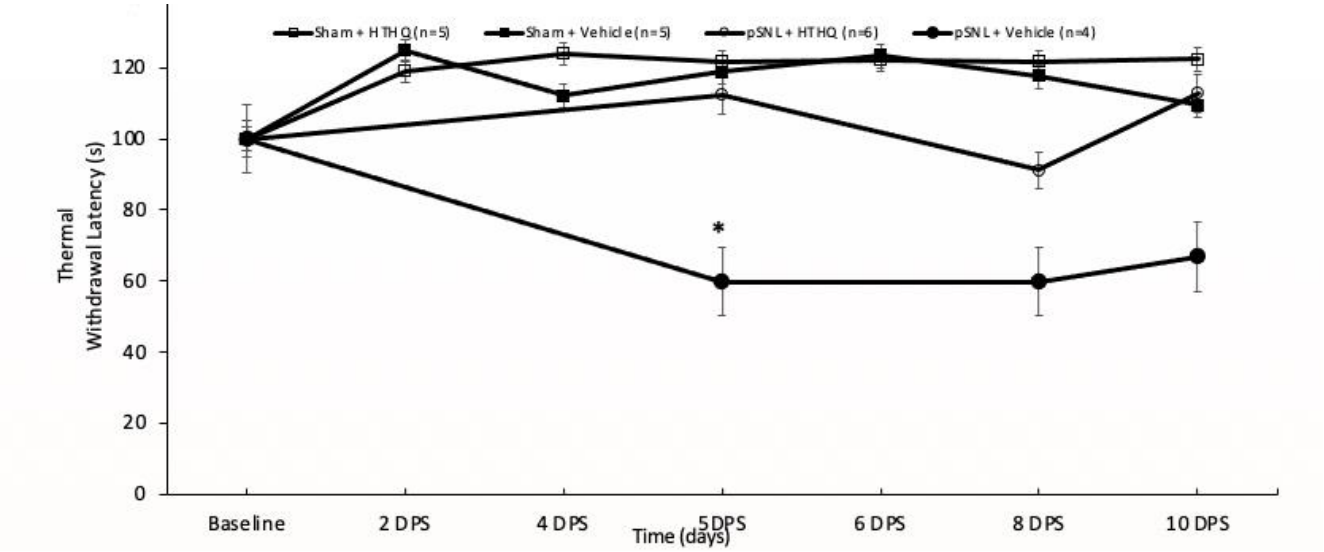

Figure 4. Thermal behavior tests for rats that were treated with HTHQ three days prior to pSNL surgery. These values have been normalized to 100 in order to more easily analyze the data. The maximum effect of HTHQ treatment occurred five days after pSNL surgery. Statistical significance between pSNL + Vehicle and pSNL + HTHQ is labeled with *. One symbol: $p < 0.05$; two symbols: $p < 0.005$; three symbols: $p < 0.001$. Original image.

Mechanical behavior tests for rats that were treated with HTHQ 4 days after pSNL surgery

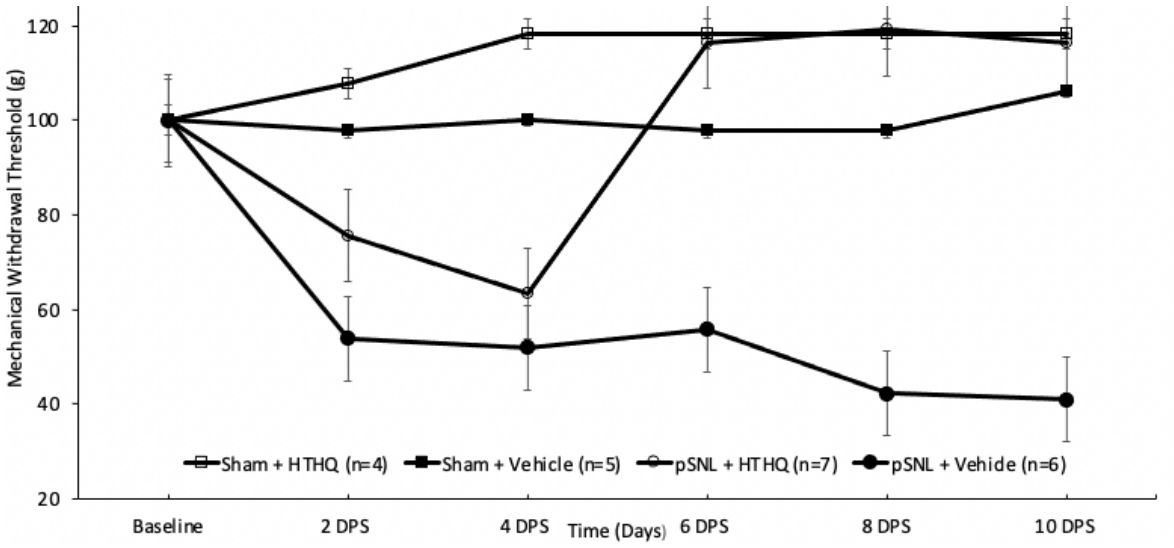

Figure 5. Mechanical behavior tests for rats that were treated with HTHQ 4 days after pSNL surgery. These values have been normalized to 100 in order to more easily analyze the data. Original image.

Thermal behavior tests for rats that were treated HTHQ 4 days after pSNL surgery

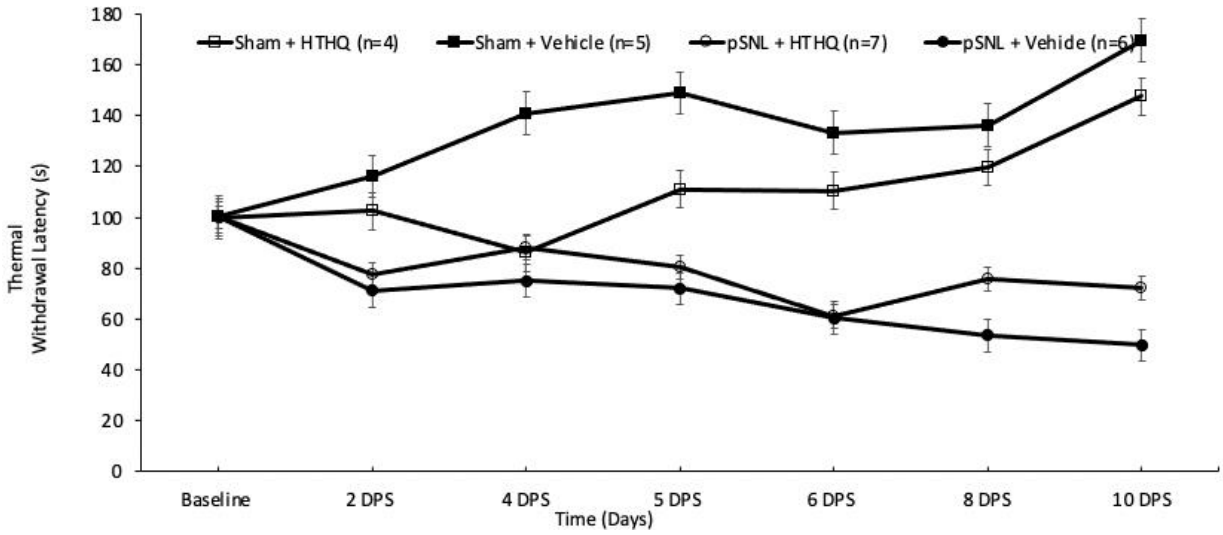

Figure 6. Thermal behavior tests for rats that were treated HTHQ 4 days after pSNL surgery. These values have been normalized to 100 in order to more easily analyze the data. Original image.

CHAPTER 4

DISCUSSION

The purpose of the present study was to determine if HTHQ was an effective treatment, when administered orally, at alleviating symptoms of neuropathic pain, such as mechanical allodynia and thermal hyperalgesia. This study is dependent on our ability to successfully perform the partial sciatic nerve ligation surgery in the rat animal model, measure behavior, administer HTHQ, remove the correct section of the spinal cord, and determine the expression of the proteins of interest.

Western blot analysis suggests that in groups of animals treated with HTHQ three days prior to pSNL, and continuously for 10 days, HTHQ effectively increased the expression of Catalase following nerve injury compared to those injured who received the saline vehicle. In animals that received saline, levels of Catalase were higher in sham rats compared to injured rats. SOD2, another antioxidant molecular marker used in this study, also followed the same trend in preemptively treated animals. When analyzing inflammatory markers (p-p38, p-NFKB, TNF-α, and IL-1β), pSNL + Vehicle samples displayed the expected increased marker expression compared to Sham + Vehicle samples, however there was an insufficient amount of data collected to make accurate conclusions. These findings indicate that the partial sciatic nerve ligation was successful at inducing neuropathic pain in rats. IL-1β increased in pSNL + HTHQ groups but not to the same degree that the marker increased in pSNL + Vehicle groups, indicating that HTHQ has a minimal, but notable, therapeutic effect on nerve injured rats.

After analysis of the western blot data from rats that were treated with either saline vehicle or HTHQ four days after pSNL surgery it was found that catalase in the pSNL + HTHQ levels were similar to those for Sham + Vehicle, indicating that HTHQ aided the return of

antioxidant levels to baseline following treatment. Similar to rats treated with HTHQ three days prior to nerve injury, catalase levels in rats treated four days after surgery exhibited decreased levels in pSNL + Vehicle rats compared to Sham + Vehicle rats. In rats treated four days after surgery, the catalase levels of pSNL + HTHQ rats returned to the same level as Sham + Vehicle rats while the level of catalase in pSNL + HTHQ rats exceed that of Sham + Vehicle rats treated preemptively. Expression of the other inflammatory markers were variable in the pSNL + HTHQ group.

Based on the preemptive mechanical behavior data, HTHQ improved the withdrawal threshold to nearly baseline level 10 days after nerve ligation surgery. When comparing the pSNL + Vehicle group to pSNL + HTHQ group statistical significance can be seen starting at the 6 days post-surgery timepoint with the most notable difference occurring 8 days after surgery. The only point of statistical significance in thermal behavior for preemptively treated rats was seen 5 days after surgery. The increase in mechanical withdrawal threshold following nerve ligation and daily treatment with HTHQ indicates that preemptive drug treatment therapeutic results. Following the analysis of mechanical and thermal behavior data for rats treated with HTHQ four days after pSNL surgery, there is trend indicating that following treatment the withdrawal threshold significantly increased in the days following treatment. Unfortunately, no statistical significance is apparent possibly due to insufficient number of animals in each experimental group.

CHAPTER 5

CONCLUSIONS

Treatment with 1-O-Hexyl-2,3,5- trimethylhydroquinone (HTHQ) three days prior to nerve ligation surgery showed an increase in antioxidant markers for SOD2 and catalase compared treated with vehicle. This study also shows that when rats were treated three days prior to nerve injury that the levels of pro-inflammatory protein IL-1ß decreased in injured rats that received treatment compared to those that were injured and received saline. Unfortunately, not enough data was able to be obtained to conclude if treatment with HTHQ four days after nerve ligation was effective in treating neuropathic pain. Although there is not enough data at this time to determine the effectiveness of HTHQ treatment with other antioxidant or pro-inflammatory markers, the expected trends in western blot data were seen between vehicle treated sham and nerve ligated animals.

CHAPTER 6

FUTURE DIRECTIONS

This study was able to answer the question of whether or not treatment with HTHQ has therapeutic effects on neuropathic pain. The initial findings indicate that HTHQ is somewhat effective at relieving symptoms of neuropathic pain, however, there are still several factors left to explore.

The current western blot data is incomplete due to insufficient experiments. Ideally, in the future, a larger sample size including more surgeries, drug administrations, and behavioral data collections would be conducted. It is expected that with the opportunity to perform more experiments, statistical significance would be more evident. Although we have found trends that indicate the effectiveness of HTHQ when administered preemptively, it would be interesting to perform more experiments to ensure that treatment after surgery is actually effective. It would also worth exploring to see how HTHQ treatment effected astrocytes, neurons, and microglia specifically through immunohistochemistry experiments.

While the oral administration of HTHQ was effective at treating symptoms of mechanical allodynia and thermal hyperalgesia, it would be intriguing to explore other methods of administration. An alternative method of administration to investigate would be spinal administration using intrathecal catheters. It would also be insightful to discover how different concentrations of HTHQ treat neuropathic pain.

Additionally, it would be ideal to perform experiments to completely understand the pathway in which HTHQ works to treat neuropathic pain. It would also be interesting to further explore the role that Nuclear factor E2-related factor (Nrf2) plays in HTHQ neuropathic pain treatment.

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APPENDICES

MERCER IACUC APPROVAL

MERCER UNIVERSITY
Institutional Animal Care and Use Committee

29-Oct-2020

Dr. Han-Rong Weng
1501 Mercer University Drive
School of Medicine - Macon
Macon, GA 31207

IACUC Ref. No. A1810020, Molecular and synaptic mechanisms underlying pathological pain induced by systemic lupus erythematosus (SLE)

Dear Dr. Weng:

Mercer University's Institutional Animal Care and Use Committee (IACUC) reviewed your application for a modification to the Approved Study Protocol (ASP) identified above and approved the modification on 29-Oct-2020.

Changes Approved:
Increase number of animals and add a procedure

PHS Guidance NOT-OD-14-063: Significant Changes to Animal Activities Previously Approved by IACUC; 4) A significant change that may be handled administratively without IACUC-approved policies, consultations, or notifications include: d) change in personnel, other than the PI. (There must be an administrative review to ensure that all such personnel are appropriately identified, adequately trained and qualified, enrolled in occupational health and safety programs, and meet other criteria as required by the IACUC.)

Attached is a complete copy of your modification application marked *approved*. Please ensure that an *approved copy* is located with your ASP where you conduct this study, and that the animal facility director, where you house your animals, has on file an *approved copy*.

Please note that the modification *does not affect the three-year period* of approval for the approved study protocol.

The IACUC must review and approve in advance any additional changes to your protocol. *Significant Changes* in Approved Study Protocols (ASP) require the submittal of a *Request for Modification Form* and must be reviewed and approved in advance of implementation by the IACUC. The NIH interprets significant changes to signify those that have the potential to impact substantially and directly on the health and well-being of the experimental animals.

Examples of Significant changes, as defined by PHS Policy issued by OLAW (NOT-OD-14-126) include:

1. A change in the overall aims or objectives of the study;
2. A change which may involve an increase in the levels of pain, distress, discomfort and/or invasivenesss;
3. A change from non-surgery to surgery, from minor to major surgery, from non-survival to survival surgery, or from single to multiple survival surgery;
4. An increase (greater than 5%) in the approximate number of anima ls used;
5. A change in the genus or species of animals used;
6. A change in the principal investigator for an ASP; or
7. The addition of the use of hazardous agents in animal procedures.

The IACUC Web site contains all application forms: orc.mercer.edu/iacuc/

Respectfully,

Roger Broderson, DVM, Chairman
Institutional Animal Care and Use Committee

JRB/AW
Rev. 09/19/2016