THE MECHANISMS OF NEUROTOXICITY INDUCED BY A *STACHYBOTRYS CHARTARUM* TRICHOTHECENE MYCOTOXIN IN AN *IN VITRO* MODEL

by

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A DISSERTATION

IN

MICROBIOLOGY AND IMMUNOLOGY

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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ACKNOWLEDGMENTS

It is difficult to know what milestones may come our way as we move through life. I certainly could not have predicted the avenues of my education that I have ventured through during my studies at Texas Tech University Health Sciences Center. I have always appreciated the value of education, not only for the opportunities in life that it opens, but for the extension of knowledge it provides from one person to another. As many a person has said, there is no greater gift than the gift of knowledge. Many people have demonstrated the beauty behind these words throughout my life.

I consider myself fortunate to be able to recognize my mentor Dr. David C. Straus as someone who has enriched my education by allowing me the freedoms to explore the creativity and splendor behind research and science. I am truly grateful for his idealism, enthusiasm, patience, and kindness as an educator. Above all else, I am thankful for his genuine interest in my dreams and his motivation throughout the years to help me fulfill them.

During the course of my studies at TTUHSC I have had the opportunity to work with many talented people who have enriched my education. I am extremely thankful to Dr. Jan S. Simoni and Grace Simoni who have shared their time, energy, and resources throughout the course of my studies. I would not have been able to conduct these studies without their generosity and enthusiasm towards this work. I will always be grateful to Dr. Simoni for taking the time to teach me and to share his knowledge with me, to further my own. I would also like to thank Dr. David R. Douglas, for his generous assistance in this work, and also without whom these studies would not have been possible.

I would like to thank the members of my advisory committee, Drs. Robert K. Bright, Abdul N. Hamood, Catherine S. McVay, and Jean C. Strahlendorf for their interest in my research and their considerate contributions towards the development of this study.

I would like to thank the members of my laboratory from throughout the years who have come and gone and many who have become my friends including, William C. Wong, Matthew R. Fogle, R. Colby Layton, Toby W. Stunkard, Trevor L. Brasel, and in memory Dr. J. Danny Cooley. One of the greatest assets I've obtained during my time in graduate school is the friendships that I have made with my fellow graduate students. I would like to thank Brendan J. Headd for being my best friend and confidant all these years at Tech, and now from New Mexico. I would also like to thank Jennifer M. Gaines and Michael D. Larrañaga for their genuine friendships, support, and for always being a-phone-call-away.

I would also like to thank the research staff and faculty of our laboratory that has assisted me through the course of my studies, Drs. Stephen C. Wilson, Chunfa Wu, and Cynthia S. Jumper. I would like to acknowledge the faculty and staff of the Department of Microbiology and Immunology at TTUHSC for their leadership and assistance in the development of my education.

Finally, I would like to thank my parents, Don and Deepani Karunasena, who define compassion, contribution, and sacrifice in my life. It is difficult to know why we choose the paths in life that we do. I chose to study science because of the good that can be created from it. I realize now, that it is the way of life that my parents have always shown me that made this choice possible. I am eternally grateful to them for demonstrating the lessons in life that have made these opportunities a reality. They are everything that is generous and good in my world.

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ABSTRACT

Sick-building syndrome (SBS) is a phenomenon in which individuals in buildings with poor indoor air quality (IAQ) experience health problems associated with the environment of the building. Fungal contamination in buildings due to species such as *Stachybotrys chartarum* and *Penicillium chrysogenum* has been correlated to poor IAQ. Symptoms experienced by individuals exposed to mycotoxins produced by *Stachybotrys* species include, headaches, fatigue, nausea, vomiting, bleeding from mucosal membranes, depression, sleep disturbances, anxiety, vertigo, memory-loss and seizures. Although these symptoms have been observed in individuals exposed to *Stachybotrys* sp. mycotoxins, the mechanisms by which these compounds may contribute to neurotoxicity are unknown. In this study, a series of experiments were conducted on human brain-capillary endothelial cells (HBCEC), astrocytes, and progenitor neuronal cells. The purpose of this study was to evaluate the effects induced by satratoxin H on neural tissues; this includes the HBCEC which forms the blood-brain barrier, followed by the astrocytes which act as immune cells, and the neurons. These cell lines were exposed to satratoxin H at concentrations ranging from 1ng/ml to 5000ng/ml. These data were compared to controls; cells exposed to known inflammatory compounds such as lipopolysaccharide (LPS), cells exposed to oxidative stress induced by hydrogen peroxide (H_20_2), and to both LPS and H_20_2 with satratoxin H. Immunofluorescent examination was used to evaluate apoptosis events, and the expression of cellular receptors including. Supernatants and cellular extracts were examined for inflammatory agents as well as compounds associated with apoptosis. The results of these studies demonstrated that at satratoxin H concentrations (1ng/ml- 10ng/ml),

results were similar to control cells, while cells exposed to moderate concentrations of 100ng/ml-1000ng/ml of satratoxin H alone or with LPS or $H_20_{2,}$ demonstrated high expression of inflammatory and apoptotic events. These experiments demonstrate that the macrocyclic trichothecenes produced by *Stachybotrys chartarum* are able to induce apoptotic and inflammatory cascades in endothelial cells, astrocytes, and neurons. These studies suggest that exposure to low to moderate doses of satratoxin could activate cellular pathways that induce a series of events leading to neurological tissue damage, which may induce the symptoms observed in individuals exposed to *Stachybotrys chartarum*.

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CHAPTER I

INTRODUCTION

Sick-Building Syndrome (SBS) has become increasingly recognized by the scientific community to be a major cause of illness in people working in buildings with recycled air. The concept of SBS was first recognized in association with poor indoor air quality (IAQ) in 1982 [1]. The definition of SBS is continuously evolving, and as such, there is no one definition for the conditions associated with it [2]. The current definition of “sick”-building syndrome describes individuals, who experience symptoms such as allergic rhinitis, headaches, lethargy, malaise, and asthma-like symptoms in a particular indoor environment, but do not experience these same symptoms when they leave this environment [3, 4]. In 1981, the National Academy of Sciences recognized several causes associated with SBS including chemicals such as nitrogen and sulfur dioxides, hydrocarbons, and potentially carcinogenic agents such as asbestos, radon, formaldehyde, tobacco smoke, or volatile organic compounds (VOCs) from new building materials [5,6].

Studies by the scientific community involving SBS have demonstrated a correlation between fungal contaminations in buildings with poor IAQ [3,4]. The first study to evaluate the variations in fungal spore concentrations between the outdoor and indoor air was conducted in Japan in 1904 [6,7]. Following studies accumulated through the early 1900s and in the 1930s, the association between

inhaling fungal particulates and the induction of allergic symptoms was established [6, 8].

In more recent times, fungal contamination has become a recognized problem in industrialized nations. Following the oil embargo by the Organization of Petroleum Exporting Countries (OPEC) in the early 1970s, buildings were designed with recycled ventilation systems to reduce the energy consumed in cooling and heating [3]. Large occupant buildings were designed with windows that could not be opened in order to control the fluctuations of temperature, and to conserve the amount of energy consumed in cooling or heating in order to maintain an ambient environment [3]. Also, there was a change in the types of building material used to construct buildings. More inexpensive and lighter weight materials were developed to reduce the consumption of fuel needed to heat or cool homes and office buildings [3]. These materials include cellulose-based ceiling tiles, which are mostly made of recycled paper products and are inexpensive to produce.

Buildings that have experienced water damage through pipe leaks, sewage leaks, and floods have been found to support fungal growth on cellulose based building materials [3, 4, 9-13]. Such building materials include ceiling tile, drywall, wallpaper, and other cellulose-based products [3, 4, 10]. *Stachybotrys* species are some of many fungi that are able to utilize the organic resources in building materials as a food supply; other fungi that are recognized as organisms

contaminating water-damaged buildings contaminants include *Cladosporium* species, *Penicillium* species, *Alternaria* species, and *Aspergillus* species [9-15].

Studies have reported that individuals spend nearly 90% of their time in the indoor environment [16]. This increases the exposure time to pollutants that may be present in the indoor environment [16]. Nearly 30% of all buildings in developed nations are believed to have poor IAQ [17]. In addition to large occupancy buildings, 20-50% of modern homes are considered to have dampness and fungal contamination [19-22]. The Bureaus of National Affairs in 1992 identified that these conditions can lead to occupational concerns, as 30-70 million individuals are affected by SBS conditions.

This “tight” control of air systems in buildings has led to variations in the fungal spore concentrations in indoor air versus outside air [3]. Moisture problems have been encountered with increasing frequency both in family housing and in the workplace in the U.S. and Europe [23]. Studies conducted by McGrath et al., demonstrated that *Penicillium chrysogenum* spore concentrations fluctuated throughout the day in the outdoor air, but in “sick” buildings the concentration of airborne spores did not vary [24]. In order to conserve energy costs, many large occupancy buildings, such as public schools and office buildings are known to turn off their air-conditioning and heating systems during the weekends or holidays [3]. This can often lead to higher humidity levels in the air and the formation of condensation around cooling pipes. When this condensation drips continuously over a period of time onto a surface such as

cellulose-based materials, where fungal spores produced by *Stachybotrys*, *Memnoniella*, *Penicillium*, *Alternaria*, or *Cladosporium* are present, these spores are able to germinate and grow [1, 2, 26-29]. If the water damage to the cellulose-based ceiling tile continues to occur, fungi are able to continue proliferation on the building material [9, 11, 25]. In addition, to cellulose based building materials, inadequately maintained heating, ventilation and air conditioning (HVAC) systems are a major contributor to SBS conditions and fungal growth.

Many of the above fungi produce mycotoxins that are known to be harmful agents toward human health [3, 4, 9, 10-15, 26-31]. The discovery of the harmful effects of mycotoxins is not recent. Certain types of cereal grains are prone to *Stachybotrys* fungal contamination. During World War II, *Stachybotrys* was a major contaminant of agricultural crops such as wheat and hay in Eastern Europe [10, 13, 14, 25, 26]. The ingestion of *Stachybotrys* mycotoxins found in breads and cereals led to the death of thousands [9, 11, 13, 14]. Alimentary toxic aleukia (ATA), and septic angina, were diagnosed in humans and animals who had ingested grain foods such as millet, rye, and wheat contaminated with T-2 toxin, a trichothecene produced by *Fusarium* species [30, 31]. Symptoms from exposure to these mycotoxins included inflammation of the skin, diarrhea, hemorrhage, emesis, decrease in hematopoietic activity, nervous system abnormalities, and feed refusal by animals [31]. Some fungal species are known to produce various mycotoxins that have been utilized in biological warfare. A

trichothecene known as T-2 toxin, which is produced by *Fusarium*, was believed to have been sprayed on agricultural crops in Southeast Asia during the early 1940s [31]. Ingestion and inhalation of these fungal toxins have been shown to produce deleterious effects in humans and animals. Thus, these mycotoxin-producing fungi not only present health hazards to humans but also to our domestic animals [4, 9-11, 13, 14, 25-28, 30, 31]. Therefore, the growth of these mycotoxin producing fungi on water-damaged building materials is of great concern in SBS.

Stachybotrys species are thought to play an important role in SBS. This organism is known to be ubiquitous in the environment and can be found on plants and in soil samples. It is a saprophytic organism and thus can utilize decomposing organic material as a food source [11]. The ideal growth environment for *Stachybotrys* is at room temperature (25°C), low nitrogen, high cellulose, dark, and a wet environment [4,9-11]. The mycotoxins produced by these organisms are chemical metabolites synthesized through secondary metabolic pathways [4,9-11,25-33]. They are neither protein nor lipid in their chemical composition [31-33]. Some of the mycotoxins produced by these fungi are the most potent biological toxins produced by a living organism. There are several major toxins produced by *Stachybotrys.* Some strains of *Stachybotrys* produce toxins known as sesquiterpenes; these include trichothecene mycotoxins which are potent protein synthesis inhibitors [31-33]. With regard to protein synthesis inhibition, trichothecenes can be divided into two groups;

initiation inhibition, and elongation/ termination inhibition [32, 33]. However, all trichothecenes are able to inhibit the initiation of protein synthesis [32, 33]. Inhibition of protein synthesis occurs when trichothecenes bind to the 60S ribosomal subunit of a eukaryotic ribosomal complex to prevent peptidyl transferase activity [32]. Studies have demonstrated that trichothecenes compete for the same binding site on a ribosome [32]. Organisms that are resistant to trichothecenes include mutant strains of the yeast *Saccharomyces cerevisiae* where changes in the 60S ribosomal component prevent these mycotoxins from inhibiting protein translation [32]. Other mutant strains of yeast are able to reduce the permeability of trichothecenes across the cellular membrane [32].

The basic chemical structure of a trichothecene is a cyclopentane ring, cyclohexane, and a six-membered oxyrane ring with four methyl groups [31]. In addition to the ring structure of the trichothecene skeleton, an epoxide group is found on C-12, 13 and a double bond exists between C-9, 10 [31]. The C-12, 13 epoxide group is necessary for protein translation inhibition [31, 32]. Substitutions at specific R-groups in the trichothecene skeleton can enhance toxicity and specificity of protein synthesis inhibition [32]. Substitutions in the R-2 position allow trichothecenes to inhibit protein elongation and termination, whereas, changes in the R-1 and R-3 position inhibit protein synthesis at the initiation step [32]. Studies have demonstrated that the most toxic trichothecenes are able to inhibit protein synthesis initiation [32]. Trichothecene mycotoxins are divided into several subcategories. One division is between macrocyclic and simple

trichothecenes. The four divisions for trichothecenes are groups A,B, C, and D. Group A trichothecenes have a hydroxyl or esterified hydroxyl group in the 3,4,7,8,or 15 position [32]. Group B trichothecenes have a carbonyl group at the carbon bond in the 8 position and other functional groups found in group A [32]. Group C trichothecenes have an epoxide group at carbon 7 or 8 [32]. The last category, Group D trichothecenes consist of the macrocyclic trichothecenes which have a ring structure attached to C-4, 5 which spans to the C15 position [32]. Macrocyclic trichothecenes consist of the basic trichothecene, verrucarol, but are lactone di- and tri-esters of this simple compound [30,32]. The additional ring in macrocyclic trichothecenes can vary in length and functional groups. These variations can alter the degree of cellular toxicity induced by these mycotoxins [32]. The macrocyclic trichothecenes produced by *Stachybotrys* include, satratoxin G, H, and F. Isoforms of these compounds are also observed. Satratoxin G and H are the most abundantly produced macrocyclic trichothecenes [27,31,32]. Satratoxin G is considered the most toxic of the macrocyclic family [27].

The mycotoxins produced by *Stachybotrys* are known to be carried on the surface of fungal spores, mycelial fragments, and other particulate [29,34]. Fungal spores are produced by the organism when nutrients in the environment are available. Fungal species such as *Stachybotrys* are known to produce wet spores during development as opposed to dry spores [29,34]. Dry spores are produced by fungal organisms like *Penicillium* species. These spores are easily

dispersed into the environment. However, wet spores like those produced by *Stachybotrys* species, are held in a cluster by a polysaccharide coating secreted by the organism onto the surface of the fungal growth [35]. This coating, also referred to as polysaccharide “slime” or matrix, is produced by organisms to prevent water loss through evaporation [35]. While the fungal growth is coated with the matrix, secondary metabolites excreted by the organism (such as mycotoxins) are captured indirectly in the slime and coat the surface of fungal spores and mycelia [37]. Studies conducted by our laboratory have demonstrated that removal of the polysaccharide matrix from the surface of spores decreases the toxicity of S*tachybotrys* spores in a eukaryotic protein translation assay [36]. These and other studies have demonstrated that the spores produced by these organisms act as a vehicle for mycotoxin distribution and human exposure.

Previous studies related to the evaluation of mycotoxins such as trichothecenes produced by *Stachybotrys*, *Fusarium*, *Trichoderma*, and *Memnoniella* have demonstrated greater toxic potential via inhalation, compared to intravenous, intragastric, subcutaneous, or intraperitoneal exposure routes [37,38]. Toxicological studies have demonstrated that inhalation versus other routes of entry with trichothecene exposure tends to have greater toxicity at lower doses [31]. The lungs are a heavily vascularized organ of the body, and upon inhalation of any toxic compounds, these agents are able to enter directly into the vascular system [39]. Current studies on the pathological effects of fungi involved with SBS have focused on the damage induced by exposure to conidia.

In fact, recent studies have demonstrated that inhalation of *Stachybotrys* conidia leads to inflammation and scarring of lung tissue [40,41]. In addition, these studies have demonstrated changes in the mucosal lining of the lung tissue and the composition of the mucus and lung surfactant phospholipids [40, 41]. The results of these studies suggest pathological effects induced by fungal conidia and the mycotoxins present on the conidial surface [40].

The presence of fungal conidia in buildings has been linked to asthma, a disease with prevalence in industrialized nations [3, 4]. *Stachybotrys chartarum* spores, and *Penicillium chrysogenum* conidia and protease production have been associated with inducing asthma-like events in a murine model [3, 4, 42]. The inhalation route has demonstrated greater toxicity with fungal mycotoxins, allergens, gases, and fine particulate material due in part to the highly dynamic cell population within it.

Stachybotrys spores are ellipsoidal, and unicellular with dimensions of 7-12 x 4-6 μm [29]. Studies conducted on particulate matter toxicity demonstrate that materials greater than 10μm in diameter are able to reach the proximal airway regions of the lung, but are removed by the ciliary action of lung cells [43]. When small particles less than 2.5 μm enter the lungs, there is an 85% total lung deposition [43]. In addition to greater deposition, particles that are 2.5 μm in diameter and smaller have a greater duration period in the lung tissue (77% retention) compared to particles that are 8.2 μm and greater (15% retention) [42]. These small particles are able to reach the regions of the lung critical for oxygen

absorption and are phagocytized by macrophages [43]. In vivo studies have shown that alveolar macrophages are quickly burdened by the accumulation of small particles, leading to cell membrane damage and depressed phagocytic activity [43]. These events may lead to further damage of the ciliated epithelial cell lining, and an increase in inflammatory agents, such as IL-1, IL-6, and TNF-α [43]. Other studies have also illustrated that 6% occupancy of the macrophage volume can lead to impaired ability in particle clearance, and 60% occupancy leads to complete inhibition of cell activity [43]. These studies demonstrated the devastating effects induced by the presence of foreign substances inhaled into the lungs.

However, the evaluation of homes and buildings with complaints regarding SBS often do not show large quantities of *Stachybotrys* species spores in the air [4]. Individuals in these buildings may demonstrate physiological symptoms related to *Stachybotrys* mycotoxin exposure, while conidial counts are minimal in comparison to the degree of fungal contamination found behind walls. This has led to many studies to determine whether particles smaller than spores could be aerosolized and carry the mycotoxins produced by *Stachybotrys*, thus exposing building occupants to the harmful compounds [44]. Research has demonstrated that there are three-hundred times more fungal fragments than conidia that are aerosolized [45]. Experiments conducted in our laboratory, based on an enzyme-linked immunosorbent assay (ELISA) specific for trichothecene mycotoxins, have demonstrated that particles smaller than 5μm

carried detectable concentrations of trichothecenes [46]. These studies suggested the existence of potentially respirable particulate smaller than spores which could carry trichothecene mycotoxins, thus exposing building occupants to harmful health conditions.

Many building occupants demonstrate prolonged and frequent upper and lower respiratory ailments in SBS conditions. In vivo studies in mouse models have demonstrated increased inflammation and scarring in lung tissue from *Stachybotrys* conidial exposure [40]. A report documented *Stachybotrys* conidia found in the bronchoalveolar lavage (BAL) of a child with pulmonary hemorrhage [47]. In addition to the physiological effects induced by inhaled mycotoxins, the immunological events surrounding mycotoxin exposure has been progressively evaluated. Epidemiological studies conducted at office buildings contaminated with *S. chartarum* have demonstrated abnormal respiratory activity, elevated white blood cell (WBC) counts, and central nervous system (CNS) disorders [48]. In addition, this study also observed abnormalities in both the cellular and humoral branches of the immune system [48]. The researchers in this study were able to identify an association with prolonged and increased exposure with mitogen expression levels [48]. The closer to the area of fungal contamination the workers were, the greater their mitogen proliferation and natural killer cell (NK) counts [48].

The accumulation of data with regard to immunological and cellular damage due to mycotoxins has increased since the previous decade. Studies conducted with *Aspergillus fumigatus* mycotoxins demonstrated interference with complement association for opsonization of conidia, thus preventing phagocytosis [49]. Ochratoxin A has been implicated as a potential cause of testicular cancer [50]. Stachylysin is a recently identified compound produced by *Stachybotrys* that has been implicated in inducing hemorrhaging in people exposed to *S.chartarum* [51]. This compound was suggested as being able to produce vascular leakage [51]. Early studies performed with the compound nivalenol, a trichothecene produced by *Fusarium nivale*, demonstrated the ability of this compound to inhibit protein synthesis [52]. T-2 toxin, one of the most thoroughly studied trichothecenes, has been shown to induce many forms of cellular damage in different types of cells. Studies with T-2 toxin have demonstrated the ability of this agent to damage erythrocyte cell membranes [53]. This trichothecene is able to disrupt the phospholipid bilayer of red blood cells (RBC) leading to structural changes in the outer membrane [53]. When exposed to T-2 toxin, RBCs change from a biconcave shape to echinocyte [53]. Additionally, research has demonstrated the ability of two trichothecenes, T-2 toxin and deoxynivalenol (DON), to disrupt hematopoiesis, which is the process by which progenitor cells in the bone marrow give rise to new RBCs, WBCs, and platelets. Studies have demonstrated the ability of these trichothecenes to be cytotoxic at low doses to human white blood cell progenitors (CFU-GM), red

blood cell progenitors (BFU-E), and platelet progenitors (CFU-MK) [53]. This evidence further demonstrated the ability of trichothecenes to effect progenitor cells versus mature cells [53]. People who have experienced trichothecene intoxication were found to have developed neutropenia, thrombocytopenia, and coagulation disorders which corresponded to damage with progenitor cells [53]. In addition, the decrease in WBC progenitors could lead to a depression in the immune system, which could contribute to the increase in lung infections seen in individuals in SBS conditions.

In vitro studies conducted with several trichothecenes have demonstrated the ability of these agents to induce inflammatory and apoptotic pathways. Isolates of *Stachybotrys* species collected from water-damaged buildings in Finland demonstrated a strong inflammatory response when exposed to murine RAW 264.7 macrophages [54]. Eleven of the isolates produced trichothecenes and the other 9 produced atranones [54]. Atranones are compounds produced by *Stachybotrys* species that do not produce macrocyclic trichothecenes [54]. Atranones are produced by the same biochemical pathway as trichothecenes, but their synthesis is stopped at the trichodermin/trichodermol stage, which renders these compounds less toxic [54]. Strains of *Stachybotrys* that produce atranones are found to be less toxic than trichothecene producing strains [54]. Macrophages exposed to the satratoxin producing strains demonstrated 55-85% lethality levels [54]. Low doses of iso-satratoxin F, veruccarin J, and atranone B and D induced the production of inflammatory compounds, such as reactive

oxygen species (ROS), nitric oxide (NO), cytokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) [54]. Similar parameters have been evaluated in human cell lines, specifically human 28SC macrophages and human A549 lung epithelial cells [55]. Results demonstrated increases in IL-6 production by human lung epithelial cells; however other inflammatory agents did not increase to the extent that was observed in murine RAW 264.7 macrophages [55].

Several in vitro cell line studies have been conducted with trichothecenes to elucidate key differences in cell activity upon exposure to low versus high doses of toxin. Experiments using DON (vomitoxin), satratoxins G, H, F, and other macrocyclic trichothecenes were examined on a murine T-cell line, EL-4 thymoma cells, and a human macrophage model (U-937 cells) [56,57]. Results demonstrated that at satratoxin dosages of 5-50ng/ml, production of inflammatory agents was significantly less than in cells exposed to 0.05ng/ml-0.50ng/ml [56]. These studies demonstrated that a low dose of an agent that is a known protein synthesis inhibitor is able to trigger the activation of cytokine pathways to induce an inflammatory response [56, 57]. In addition, evidence exists which shows DON inhibiting the synthesis of IκB-α, thus allowing NF-κB to remain active, which increases the transcription of pro-inflammatory agents [57]. The ability of trichothecenes at low doses to trigger inflammatory events and protein synthesis, also introduces the possibility of these compounds activating apoptotic pathways. TNF- α is a pro-inflammatory cytokine, however, there are two receptors for this compound- TNF-receptor one (TNF-RI) and TNF-receptor

two (TNF-RII). TNF-RI is also known as the death receptor, because apoptotic events are triggered upon its activation, [58]. Recent studies conducted with DON have demonstrated the ability of this trichothecene to induce apoptosis in T-helper leukocytes exposed to TNF- α and anti-Fas ligand antibody [59]. Further studies confirmed that since these compounds are protein synthesis inhibitors, they were able to activate a ribotoxic stress response [59]. This response led to the activation of c-Jun N-terminal kinase (JNK)/p38, a mitogen-activated protein kinase (MAP kinase) [59,60]. The results of these studies demonstrated that trichothecenes capable of greater protein synthesis inhibition were able to activate the JNK/p38 pathway and apoptosis in a linear fashion [60]. In addition to the activation of JNK/p38, trichothecenes (including satratoxins) have been found to activate other MAP kinases, like stress-activated protein kinase (SAPK) and deactivate extracellular signal-regulated protein kinase (ERK), which enhances the apoptotic response [60].

With the accumulation of research on the immunological effects of satratoxins and other trichothecenes, interest in the neurological effects induced by *S. chartarum* compounds is gaining momentum. Clinical studies regarding the neurological effects of trichothecenes or other mycotoxins associated with SBS are limited [61]. Evaluating occupants from buildings contaminated with fungi is difficult because there are no standards to evaluate psychological or neuropsychological impairments evidenced in these individuals [61]. In addition, with litigation against builders and home insurance companies, individuals

pursuing these avenues are viewed as having questionable ailments, which increases the difficulty the scientific community has had in establishing legitimate psychological analysis as evidence of neurological damage due to mycotoxin exposure [61]. However, this body of evidence is increasing. Recent studies have demonstrated mycotoxicosis due to trichothecenes, by measuring the presence of trichothecenes in patient urine samples [62]. Samples were collected from individuals who displayed symptoms of trichothecene exposure, such as burning eyes, headaches, nausea, diarrhea, respiratory problems, and skin rashes [62]. The samples were further analyzed for the presence of trichothecenes through thin-layer chromatography (TLC) [62]. The samples were purified for trichothecenes and following Koch's postulates, the samples were injected into weaning Sprague-Dawley rats [62]. The symptoms and pathological effects observed in the individuals exposed to trichothecenes were recreated in rat studies [62]. Evidence of mycotoxicosis was the presence of tissue damage in the lungs and central nervous system [62]. These experiments confirmed Koch's postulates, by demonstrating the ability to identify and isolate the toxic substance [62].

Studies related to trichothecenes inflicting brain damage leading to cognitive effects in humans have been advancing. Analyses of adolescent students exposed to organisms associated with SBS were found to display the symptoms connected with airborne mycotoxin exposure [63]. These students were found to have decreased hearing ability based on brainstem auditory

evoked response (BAER) techniques [63]. These individuals that were confirmed to have hearing-loss were diagnosed with acoustic mycotic neuroma [63]. Further evidence of cognitive effects were seen when 20 patients with known exposure to *S. chartarum* were analyzed for the presence of cognitive damage [64]. Problems with cognition were defined as deficits in verbal memory, verbal learning, attention/concentration, and set shifting [64]. All of the examinees were given a series of standardized adult intelligence tests and screened for specific symptoms associated with somatic, cognitive and behavioral symptoms [64]. These studies evidenced cognitive damage in people exposed to *S.chartarum* [64]. In addition, the observed conditions of these individuals was considered equivalent to minor traumatic brain injury [64]. In addition to evaluating patients for cognitive and physiological effects induced by trichothecene exposure, autoantibodies against neural antigens have been evaluated in patients exposed to SBS conditions [65]. These studies demonstrated that patients in SBS conditions had elevated antibody levels of IgA, IgM, and IgG to neural specific antigens [65]. Serum samples collected from these individuals were evaluated using an ELISA method for antibodies, against myelin basic protein, myelin associated glycoprotein, ganglioside GM_1, sulfatide, myelin oligodendrocyte glycoprotein, chondroitin sulfate, tubulin, and neurofilament [65]. Compared to 500 healthy individuals, all of the groups analyzed with ailments associated with mycotoxicosis had elevated neural antibodies [65]. These studies demonstrated qualitative and quantitative analysis of subjects exposed to SBS conditions with

symptoms of neurological effects. However, the mechanism of this damage has not yet been identified.

The purpose of this research was to identify the mechanisms that induce neurological damage at a cellular level. Through the course of this study, immunological and apoptotic events were evaluated to identify the cellular events that were disrupted due to the presence of satratoxin H. The objective of this study was to utilize three human cell lines in an in vitro study to examine the toxic effects of satratoxin H at low doses versus high doses. Once a low dose was established, these concentrations were evaluated for further examination of cellular events. The cells utilized in this study form the blood-brain barrier (BBB) and compose the fundamental neural tissues. Human brain capillary endothelial cells (which form the BBB), astrocytes (which act as macrophages in neural tissues) , and neural progenitor cells (neurons) were employed in this study.

REFERENCES

1. Finningan, M. S., Pickering, C.A.C., Burge, P.S. 1984. The sick building syndrome: prevalence studies. Br Med J 289: 1573-1575.

2. Hodgson, M. 1992. Field studies on the sick building syndrome. Ann NY Acad Sci 641: 21-36.

3. Cooley, J.D., Wong, W.C., Jumper, C.A., Straus, D.C.1998. Correlation between the prevalence of certain fungi and sick building syndrome. Occup Environ Med. 55:579-584.

4. Cooley, J.D., 1999. The role of *Penicillium chrysogenum* conidia in sick building syndrome and an asthma-like animal model. A Dissertation in Medical Microbiology. Texas Tech University Health Sciences Center.1-18.

5. Sterling, T. D., Arundel, A. 1984. Possible carcinogenic components of indoor air quality, combustions by-products, formaldehyde, mineral fibers, radiation, and tobacco smoke. J Environ Sci Hlth. 62: 185-230.

6. Larrañaga, M.D. 2004. The capability of a solid sorbent desiccant unit at removing selected indoor air quality-related microorganisms from the air. A Dissertation in Industrial Engineering. Texas Tech University. 1-26.

7. Sterling, T. D., Kobayashi, D.M. 1977. Exposure to pollutants in enclosed living spaces. Environ Res.13: 1-35.

8. Saito, K. 1904. Untersuchungen über die atmospharischen Pilzkeime. I. Mittheilung. J Coll Sci Tokyo. 18: 58.

9. Bernton, H. S. 1930. Asthma due to a Mold - *Aspergillus fumigatus*. JAMA. 95: 189-192.

10. Croft, W.A., Jarvis, B.B., Yatawara, C.S.1986. Airborne outbreak of trichothecene toxicosis. Atmos Environ. 20:549-553.

11. Fung, F., Clark, R., Williams, S. 1998. *Stachybotrys*, a mycotoxin-producing fungus of increasing toxicological importance. J Toxicol Clin Toxicol. 36: 79-86.

12. Harrach, B., Mirocha, C.J., Pathre, S.V., Palyusik, M.1981. Macrocyclic trichothecene toxins produced by a strain of *Stachybotrys atra* from Hungary. Appl Environ Microbiol. 41: 1428-1432.

13. Harrach, B., Bata, A. 1982. Macrocyclic trichothecene *Stachybotrys* toxins isolated from field samples. *In* Proceedings of the 5th International IUPAC Symposium on Mycotoxins and Phycotoxins. Verein Osterrischischer Chemiker, Vienna.88-92.

14. Harrach, B., Bata, A., Bajamocy, E., Benko, M. 1983. Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotrytoxicosis. Appl Environ Microbiol. 45: 1419-1422.

15. Harrach, B., Ujszaszi K., Tamas, A.K., Lasztity, R. 1985. Macrocyclic trichothecene toxins produced by *Stachybotrys atra* strains isolated in middle Europe. Appl Environ Microbiol. 49: 678-681.

16. Teichman, K. Y. 1995. Indoor Air Quality: Research Needs. Occup Med. 10: 217-227.

17. Smith, R. C. 1990. Controlling sick building syndrome. J Environ Hlth. 53: 22-23.

18. Dales, R., Zwanenburg, H., Burnett, R., Franklin, C. 1991. Respiratory health effects of home dampness and molds among Canadian children. Amer J Epidemiol. 134: 196-203.

19. Brunekreef, B., Dockery, D.W., Speizer, F.E., Ware, J.H., Spengler, J.D., Ferris, B.G. 1989. Home dampness and respiratory morbidity in children. Amer Rev Respir Dis. 140: 1363-1367.

20. Jaakkola, J. J. K., Jaakkola, N., Ruotsalainen, R. 1993. Home dampness and molds as determinants of respiratory symptoms and asthma in pre-school children. J Exper Anal Environ Epidemiol. 3: 129-142

21. Spengler, J., Neas, L., Nakai, S. 1994. Respiratory symptoms and housing characteristics. Indoor Air. 4: 72-82.

22. Verhoeff, A. P., Van Strien, R.T., Van Wijnen, J.H., Brunekreef, B., 1995. Damp housing and childhood respiratory symptoms: the role of sensitization to dust mites and molds. Amer J Epidemiol. 141: 103-110

23. Reijula, K. 1996. Buildings with moisture problems - a new challenge to occupational health care. Scand J Work Environ Hlth. 22: 1-3.

24. McGrath, J.J., Wong C.W., Cooley, J.D. Straus, D.C. 1999. Continually measured fungal profiles in sick building syndrome. Curr Microbiol. 38: 33-36.

25. Jarvis, B.B., Lee, Y., Comezoglu, S.N., Yatawara, C.S. 1986.Trichothecenes produced by *Stachybotrys atra* from Eastern Europe. Appl Environ Microbiol. 51: 915-918.

26. Jarvis, B.B.1991. Macrocyclic trichothecenes. Sharma R.P., Salunkhe, D.K. (eds). "Mycotoxins and Phytoalexins in Human and Animal Health." Boca Raton, Florida: CRC Press, 361-472.

27. Jarvis, B.B., Salemme, J., and Morais, A. 1995. *Stachybotrys* toxins. Natural Toxins. 3: 10-16.

28. Jarvis, B.B., Sorenson, W.G., Hintikka, E.L., Nikulin, M., Zhou, Y., Jiang, J., Wang, S., Hinkley, S., Etzel, R.A., Dearborn, D. 1998. Study of toxin production by isolates of *Stachybotrys chartarum* and *Memnoniella echinata* isolated during a study of pulmonary hemosiderosis in infants. Appl Environ Microbiol. 64: 3620-3625.

29. Jong, S.C., David, E.E. 1976. Contribution to the knowledge of *Stachybotrys* and *Memnoniella* in culture. Mycotaxon. 3: 409-485.

30. Nikulin, M., Pasenen, A.L., Berg, S., Hintikka, E.L. 1994. *Stachybotrys atra* growth and toxin production in some building materials and fodder under different relative humidities. Appl Environ Microbiol. 60: 3421-3424.

31. Sharma, R.P. and Kim, Y.W. 1991. Macrocyclic trichothecenes. Sharma R.P., Salunkhe, D.K. (eds). "Mycotoxins and Phytoalexins in Human and Animal Health." Boca Raton, Florida: CRC Press, 339-359.

32. Beasley, R.V.1989. Trichothecene: Mycotoxicosis: Pathophysiologic Effect. Boca Raton, Florida: CRC Press.1-171.

33. Mycotoxins: Risks in Plant, Animal, and Human Systems. 2003. Council for Agricultural Science and Technology. Ames Iowa. 20-147.

34. http://www.scisoc.org/feature/stachybotrys

35. Sorenson, W.G., Frazer, D.G., Jarvis, B.B., Simpson, J., Robinson, V.A. 1987. Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra*. Appl Environ Microbiol. 53: 1370-1375.

36. Karunasena, E., Cooley, J.D., Douglas, D.R., Straus, D.C. 2004. Protein translation inhibition by *Stachybotrys chartarum* conidia with and without the mycotoxin containing polysaccharide matrix. Mycopathologia. 158:87-97.

37. Wannemacher, R.W. and Wiener S.L. 1997. Trichothecene mycotoxins. R. Zajtchuk and R.F. Bellamy (ed.), *In*, Textbook of military medicine, part I. Warfare, weaponry, and the casualty, medical aspects of chemical and biological warfare. Office of the Surgeon General, Washington, D.C.

38. Wilkins K, Nielsen KF, Din S.U. 2003. Patterns of volatile metabolites and nonvolatile trichothecenes produced by isolates of *Stachybotrys*, *Fusarium*, *Trichoderma*, *Trichothecium*, and *Memoniella*. Environ Sci Pollut Res Int. 10: 162-167.

39. Klaasen, C.D. 1991. Casarett & Doull's The Basic Science of Poisons. 5th ed. McGraw Hill Publications.

40. Viana M.E., Coates N.H., Gavett S.H., Selgrade M.K., Vesper S.J., Ward M.D.W. 2002. An extract of *Stachybotrys chartarum* causes allergic asthma-like responses in a BALB/c mouse model. Toxicol Sci. 70: 98-109.

41. Walker C, Bauer W, Braun R.K., Menz G., Braun P, Schwarz F, Hansel T.T., Villiger B. 1994. Activated T cells and cytokines in bronchoalveolar lavage from patients with various lung diseases associated with eosinophilia. Am J Respir Crit Care Med. 150: 1038-1048.

42. Schwab C.J., Cooley J.D., Jumper C.J., Graham S.C., Straus D.C. 2004. Allergic inflammation induced by a *Penicillium chrysogenum* conidia-associated allergen extract in a murine model. Allergy. 59:758-65.

43. Salvi S, Holgate S.T. 1999. Mechanisms of particulate matter toxicity. Clin Exp Allergy. 29: 1187-1194.

44. Pasanen, A.L., Nikulin, M., Tuomainen, M., Berg, S., Parikka, P., Hintikka, E.L. 1993. Laboratory experiments on membrane filter sampling of airborne mycotoxins produced by *Stachybotrys atra* Corda. Atmos Environ. 27A : 9-13.

45. Gorny, R.L., Reponen, T., Willeke, D., Schmechel, E., Robine, M., Grinshpun, S.A. 2002. Fungal fragments as indoor air biocontaminants. Appl Environ Microbiol. 68: 3522-3531.

46. Brasel, T.L., Douglas, D.R., Wilson S.C., Straus, D.C.2005. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins on particulates smaller than conidia. Appl Environ Microbiol. 71:114-122.

47. Elidemir, O. Colasurdo, G.N., Rossmran, S.N., Fan, L.L. 1999. Isolation of *Stachybotrys* from the lung of a child with pulmonary hemosiderosis. Pediatrics. 104: 964-966.

48. Johanning, E., Biagini, R., Hull, D., Morey, P. Jarvis, B.B., Landsbergis, P. 1996. Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment. Int Arch Occup Environ Health. 68: 207-218.

49. Tsai, H.F., Washburn, G.R., Chang, Y.C., Kwon-Chung, K.J. 1997. *Aspergillus fumigatus* arp1 modulates conidial pigmentation and complement deposition. Molec Microbiol. 26: 175-183.

50. http://www.themoldsource.com

51. Vesper, J., Vesper, M.J. 2002. Stachylysin may be a cause of hemorrhaging in humans exposed to *Stachybotrys chartarum*. Infect Immun. 70: 2065-2069.

52. Ueno, Y., Hosoya, M., Morita, Y., Ueno, I., Tatsuno, T. 1968. Inhibition of the protein synthesis in rabbit reticulocyte by nivalenol, a toxic principle isolated from *Fusarium nivale*- growing rice. T J Biochem. 64: 479-485.

53. Froquet, R., Arnold, F., Batina, P., Parent-Massin, D. 2003. Do trichothecenes reduce viability of circulating blood cells and modify haemostasis parameters? Mycopathologia. 156: 349-356.

54. Nielsen, K.F., Huttunen, K., Hyvarinen, A., Andersen, B., Jarvis, B.B., Hirovonen, M. 2001. Metabolite profiles of *Stachybotrys* isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages. Mycopathologia. 154: 201-205.

55. Huttunen, K., Hyvarinen, A., Nevalainen, A., Komulainen, H., Hirvonen, M. 2003. Production of proinflammatory mediators by indoor air bacteria and fungal spores in mouse and human cell lines. Environ Hlth Perspec. 111: 1-18.

56. Lee, M.G., Li, S., Jarvis, B.B., Pestka, J.J. 1999. Effects of satratoxins and other macrocyclic trichothecenes on IL-2 production and viability of EL-4 thymoma cells. J Toxicol Environ Hlth. 57: 459-474.

57. Konishi-Sugita, Y and Pestka, J.J. 2001. Differential upregulation of TNF-alpha, IL-6, and IL-8 production by deoxynivalenol (vomitoxin) and other 8-ketotrichothecenes in a human macrophage model. J Toxicol Environ Hlth. 64: 619-36.

58. Beck, S.C., Meyes, T.F. 2000. IgA1 protease from *Neisseria gonorrhoeae* inhibits TNF-alpha mediated apoptosis of human monocytic cells. FEBS. 472: 287-292.

59. Shifrin, V.I., Anderson, P. 1999. Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J Biol Chem. 274: 13985-13992.

60. Yang, G.H., Jarvis, B.B., Chung, Y., Pestka, J.J. 2000. Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. Toxicol Appl Pharmacol. 164: 149-160.

61. Lees-Haley, P.R. 2003. Toxic mold and mycotoxins in neurotoxicity cases: *Stachybotrys, Fusarium, Trichoderma, Aspergillus, Penicillium, Cladosporium, Alternaria*, Trichothecenes. Psychol Rep. 93: 561-584.

62. Croft, W.A., Jastromski, B.M., Croft, A.L., Peters, H.A. 2002. Clinical confirmation of trichothecene mycotoxicosis in patient urine. J Environ Biol. 23: 301-320.

63. Anyanwu, E., Campbell, A.W., High, W. 2002. Brainstem auditory evoked response in adolescents with acoustic mycotic neuroma due to environmental exposure to toxic molds. Int J Adolesc Med Hlth. 14: 67-76.

64. Gordon, W.A., Johanning, E., Haddad, L. 1999. Cognitive impairment associated with exposure to toxigenic fungi. Eckardt Johanning (Ed.). *In* Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention, and Control.

65. Cambell, A.W., Thrasher, J.D., Madison, R., Vojdani, A., Gray, M.R., Johnson, A. 2002. Neural antibodies and neurophysiologic abnormalities in patients exposed to molds in water-damaged buildings. Arch Environ Health. 58:464-74.

CHAPTER II

THE ISOLATION, PURIFICATION AND ANALYSIS OF SATRATOXIN H FROM *STACHYBOTRYS CHARTARUM*

Previous methodologies for the isolation and purification of mycotoxins were established for filamentous fungi that were contaminants of agricultural crops. The method currently used for maximum sporulation and mycotoxin production employs fungal growth on rice cultures [1]. This process was described for the production of aflatoxins from *Aspergillus flavus* in 1966, and continues to be used as an established method for mycotoxin collection [1].

The most toxic mycotoxins produced by *S. chartarum* belong to the trichothecene family. Specifically, satratoxins G and H are the most abundant and toxic of the trichothecenes produced by *Stachybotrys* species [2]. Satratoxin G has a molecular mass of 544.23 and the chemical formula is $C_{29}H_{36}O_{10}$ [3]. Satratoxin H has a similar molecular mass of 528.24 and a chemical formula of $C_{29}H_{36}O_9$ [3]. Both satratoxins have similar LD_{50}'s in mice ranging between 1mg/kg to 1.23mg/kg through an intraperitoneal route [3]. Smaller doses of these mycotoxins are required for inhalation toxicity. The LD_{50} for T-2 toxin, a trichothecene produced by *Fusarium* species, is 0.6mg/kg via inhalation versus 1.3-2.6 mg/kg through intraperitoneal injection [4].

The aim of this portion of the project was to isolate satratoxin H and determine its purity prior to using it in cell culture assays. Chromatographic methods were used to isolate and purify satratoxin H, followed by specific assays to determine the levels of endotoxin that may be present in the purified satratoxin H samples.

MATERIALS AND METHODS

Sixteen 2L sterile, autoclaved Erlenmeyer flasks (Pyrex, Fisher Scientific, Houston, TX) were filled with 250g of Uncle Ben's long grain enriched parboiled rice and 125mL of sterile pyrogen-free water. The flasks were autoclaved for 15 minutes at 120˚C at 15psi and allowed to cool. Each flask was inoculated with 50ml of *Stachybotrys chartarum* growth from 14-day cultures grown on potato-dextrose agar plates (PDA). To produce maximum sporulation and mycotoxin production, a strain known to be a toxin producer was graciously provided by Dr. Bruce Jarvis at the University of Maryland, (strain number 29-16-6). After each rice culture was inoculated with 50ml of *S. chartarum* growth, the flasks were covered with sterile gauze and foil. The flasks were incubated for six weeks in a disinfected, negative-pressure chamber with high efficiency particle arrestor (HEPA) filtered air circulating through the chamber. The rice cultures were allowed to incubate at room temperature (RT), 25˚C, with a relative humidity (RH) level in the chamber of 43% with periodic shaking every 2 days to prevent the rice from clumping.

During the course of fungal growth on the rice cultures, the rice kernels developed a confluent black color from *S. chartarum* growth. After the six-week growth period, each culture was washed and agitated in a 1:1 chloroform: methanol (Fisher Scientific, Houston, TX) solution at 4˚C overnight with constant shaking at 3000 rpm. The black chloroform: methanol solution recovered from the washing process was collected into holding containers. Each rice culture was washed a second time with 150ml of methanol following the same procedure previously described.

A total of 10 liters of harvested solution was collected. The solution was vacuum filtered through a Whatmann's number 1 filter to remove large debris collected from the rice cultures. Next, aliquots of the harvested solution were vacuum filtered through a 0.45μm filter (Millipore, Fisher Scientific). Approximately, 500ml of solution could be filtered through one 0.45μm filter. Following this process, the 8L solution was further vacuum filtered through 0.22 μm filters (Millipore, Fisher Scientific). However, due to the degree of debris still in solution, the 8L of extract was again filtered through 0.45μm filters. After this filtration step, the solution could be filtered more easily through 0.22 μm filters. At this stage of the filtration process, a large amount of lipids would collect on the surface of the filters, only allowing 150ml of solution to be collected from one filter.

After the filtration process, the solution was rotary evaporated to remove the solvent and collect crude toxin extract. The boiling point (BP) of chloroform is between 61˚C and 62˚C and the BP for methanol is 64.7˚C. The rotary evaporator (Büchi Model, Brinkman Instruments, Westbury, NY) was kept under vacuum pressure with cool water (2.4˚C – 4.0˚C) flowing through the condenser. Approximately, 210 ml of the filtered solution was evaporated and a golden-brown solution remaining in the flask was suspended in methanol and stored in sterile 50 ml centrifuge tubes (Falcon, Fisher Scientific, Houston, TX).

The crude extracts were purified by column chromatography methods as follow. Samples of the crude extract were dried overnight on 5g of Florisil (100-200 mesh) (Fisher Scientific, Fair Lawn, NJ). Purified extracts were collected by solid-phase extraction methods. The solid phase was re-purified with high performance liquid chromatography (HPLC). HPLC analysis was performed using an 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with an ultraviolet-visible (UV/Vis) diode array detector [5]. A 400 (250 + 150) mm Eclipse C8 analytical column plus a 12.5 mm guard column set at 40˚C was used in the system [5]. The flow rate was set at 1.0ml/ min [5]. Crude toxin samples in methanol were run in a mobile phase in which the gradient changed from 35% acetonitirile in water to 80% in 18 minutes [5]. Samples were detected at 260nm and analyzed using Chemstation software (Agilent Technologies, Palo Alto, CA). Standards of satratoxin H were kindly donated by B.B. Jarvis. Satratoxin H samples collected through HPLC methods in our

laboratory were compared with B.B. Jarvis' sample and further verified with UV spectra analysis compared to previous descriptions in the literature for satratoxin H [2,3,4,5]. In addition, to further verify that the purified samples collected were satratoxin H, samples were analyzed with columns of different polarity to verify individual peaks.

Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, is a common contaminant in the environment. LPS, also known as endotoxin, is a pyrogenic agent that is able to activate a strong inflammatory response. LPS is able to withstand sterilization procedures such as autoclaving, but can be destroyed on glassware exposed to a minimum of 180˚C for 4 hours. To prevent LPS induced activation of cell cultures, samples of satratoxin H were tested for contamination levels. A QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) Assay (Cambrex- Biowhitakker, Walkersville, MA) was used to determine LPS concentrations. The QCL-1000 LAL kit is a quantitative assay and in the presence of endotoxin, a proenzyme in the assay is converted to its active form, a coagulase enzyme. The coagulase enzyme cleaves a colorless substrate [para-nitroaniline (pNA)] into a peptide and pNA. The pNA forms a yellow color that can be read photometrically at 405-410nm after a stopping reagent is added. The concentration of endotoxin in a sample was calculated from a standard curve developed with an *Escherichia coli* LPS sample provided in the kit.

Trichothecenes are known to be able to withstand temperatures between 500˚F- 900˚F [4]. Thus, known concentrations of satratoxin H were placed in 2ml glass vials (Agilent Technologies, Palo Alto, CA) and heated in a gravity oven (Gravity Oven Lindberg/Blue M, Asheville, NC) at 180˚C for 4 hours. These heated satratoxin H samples were suspended in pyrogen-free water, supplied in the QCL-1000 kit to a concentration of 1mg/ml and tested through HPLC analysis which demonstrated that the satratoxin H was not present in the vials. This showed that the compound was unable to withstand prolonged heat exposure at 180˚C for 4 hours. Following these results, careful procedures were undertaken to transfer purified samples of satratoxin H from the HPLC into baked, sterile glassware. These samples of satratoxin H were tested for endotoxin level using the QCL-1000 assay. The standard level of endotoxin contamination below which an inflammatory response does not occur is 250pg/ml (0.25 EU/ml).

RESULTS & DISCUSSION

The results from the QCL-1000 assay demonstrated that the purified satratoxin H samples had a mean endotoxin concentration of 0.075 EU/ml (± 0.011 S.E.). The results, demonstrated that using pyrogen-free glassware and other precautions in the process of satratoxin H purification removed the possibility of LPS contamination, and confirmed that HPLC methods did not contribute endotoxin to the mycotoxin samples. Since the concentration of endotoxin in satratoxin H samples was below the acceptable level of

contamination, LPS contamination was not a contributing factor in our experimental results.

Figure 1 demonstrates the presence of satratoxin H from harvested samples of *S. chartarum* compared to known sample from B.B. Jarvis. Panel A represents the chromatogram of the known satratoxin H sample, and panel B demonstrates the satratoxin H in crude samples of *S. chartarum* harvested in the laboratory. To confirm the toxicity of the purified samples of satratoxin H, a rabbit reticulocyte lysate protein translation inhibition assay was used [5]. The results of this experiment confirmed that there was significant inhibition of protein translation by *S. chartarum* samples containing satratoxin H [5].

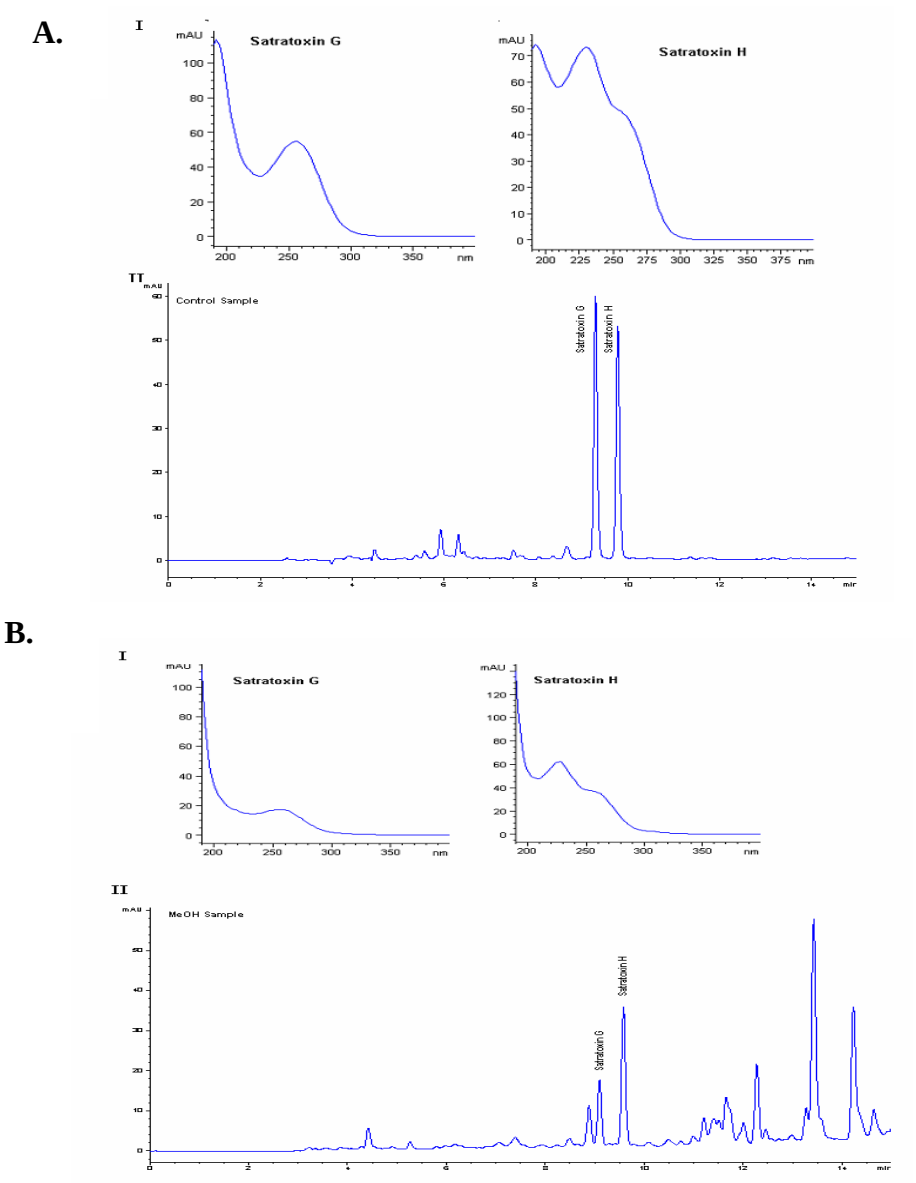


Figure 1. HPLC chromatogram of satratoxins H and G. A is known sample of satratoxins H and G. B is our laboratory harvested sample of *S. chartarum* with satratoxins H and G. Panel I represents the UV spectra of peaks identified as satratoxins G and H. Panel II is the chromatogram of each sample with the location of peaks identified as satratoxin G and H. The separation was conducted at a flow of 1.0ml/min, λ 230-320nm, and absorbance is in units of milli-Absorbance Units (mAU).

REFERENCES

1. Shotwell O.L., Hesseltine C.W., Stubblefield R.D., Sorenson W.G. 1966. Production of aflatoxin on rice. Appl Microbiol. 14: 425-427.

2. Jarvis, B.B., Salemme, J., Morais, A. 1995. *Stachybotrys* toxins. Natural Toxins. 3: 10-16.

3. Hinkley S.F., Jarvis B.B. 2000. Chromatographic methods for *Stachybotrys* toxins. Methods in Molecular Biology. 157:173-194.

4. Wannemacher, R.W., Wiener S.L. 1997. *In*, Trichothecene mycotoxins. R. Zajtchuk and R.F. Bellamy (ed.), Textbook of military medicine, part I. Warfare, weaponry, and the casualty, medical aspects of chemical and biological warfare. Office of the Surgeon General, Washington, D.C.

5. Karunasena, E., Cooley, J.D., Douglas, D.R., Straus, D.C. 2004. Protein translation inhibition by *Stachybotrys chartarum* conidia with and without the mycotoxin containing polysaccharide matrix. Mycopathologia. 158:87-97.

CHAPTER III

THE TOXOLOGICAL EFFECTS OF SATRATOXIN H ON HUMAN BRAIN CAPILLARY ENDOTHELIAL CELLS (HBCEC)

Brain capillary endothelial cells are one of the tissues that form the blood brain barrier (BBB) [1]. The German microbiologist Ehrlich was the first to identify its existence more than 100 years ago [1]. The blood brain barrier consists of tissues that prevent microorganisms and toxic chemical compounds from entering into the brain tissue. The only substances able to readily diffuse across the barrier are small and lipophilic compounds [1]. Large compounds, such as glucose and hydrophilic elements pass through active transport membrane proteins [1]. The fundamental elements of the BBB are the adherens and tight junctions which hold endothelial cells (ECs) together; second, the P-glycoprotein receptors that eject materials from the central nervous system (CNS); and third, the process of transcytosis which limits the influx of materials into the CNS [1]. The final component is the astrocyte processes that cover the ECs [1].

Some of the receptors that bind molecules for transport are transferrin and P-glycoprotein [1]. These membrane transport systems are a component of the limited transfer of materials across the BBB [1]. Other distinguishing features of the BBB are the limited number of endocytic vesicles, which limit transcellular

flux [1]. Tight junctions between the cells maintain a low level of paracellular flux [1]. It are these two features that distinguish ECs of the BBB compared to other EC of different tissues [1]. The tight junctions between cells are formed by specific proteins. One group is termed cadherin, another is occludin [1]. Cadherin forms a single-pass transmembrane glycoprotein and is considered a major cell-to-cell adhesion molecule [1]. Occludin is a four-pass transmembrane that is similar to connexin, a protein commonly found in gap junctions [1]. In the event of EC damage, the junctions are compromised and the tightness of the BBB is lost [1]. Thus, the junctions play a critical role in the infrastructure of the BBB [1].

Studies conducted on gap-junctional intracellular communication in Chinese hamster ovary (CHO) V79 cells after exposure to members of the trichothecene family, (T-2 toxin and vomitoxin) demonstrated the ability of these toxins to decrease metabolic cooperation associated with gap-junctional intracellular communication [2]. Gap-junctional communication allows for metabolic cooperation between cells which regulate cell proliferation, differentiation, and development [2]. When this form of cellular communication decreases, tumors are able to form [2]. These studies suggested that low doses of trichothecenes maybe tumorgenic [2]. These studies further demonstrated the function of junctions between cells and the potential devastation trichothecene toxins may induce in the integrity of cell communication and development [2]. It further potentates the devastating effects that may result to junctions associated with the integrity of the BBB [2].

In the event that the BBB is damaged, inflammatory responses can contribute to CNS injury. The accumulation of evidence that demonstrates the negative effects due to inflammation on the CNS continues to expand. Studies have demonstrated that regardless of the causative agent or event, such as infection, trauma, ischemia, necrosis, or hemorrhage, the inflammatory response generates further damage [3]. There are four major events that are used to describe inflammation as stated by Celsus: pain, tumor, rubor, and heat [3]. The events that lead to this pathology are the increase in blood flow to the site of injury, followed by increased capillary permeability of ECs to allow the release of secretory mediators that recruit immune cells to enter damaged tissues [3]. Cells that arrive at the site include neutrophils, macrophages, mast cells, lymphocytes, platelets, dendritic cells, and fibroblasts [3]. Chemokines released by endothelial cells and the expression of adhesion molecule receptors on the surface, allow endothelial cells to interact with leukocytes [3]. Leukocytes are able to pass through the endothelial cell layer to the site of inflammation. The process of inflammation is intended to repair injured tissues; however this mechanism tends to induce greater damage to the CNS upon activation [3]. Diseases such as multiple sclerosis, Alzheimer's, rheumatoid arthritis, and systemic lupus erythematosus (SLE) all demonstrate events of inflammation that lead to damage in the CNS [3]. Cytokines released by immune cells, such as tumor necrosis factor- alpha (TNF-α), are suggested to have an effect on the endothelial cells of the BBB, leading to the rupture of the BBB in inflammation associated diseases

[3,4]. TNF- α is able to induce the expression of adhesion molecule receptors on ECs, which allow the binding and passage of lymphocytes and leukocytes [3,4]. In addition to the passage of T cells, studies have demonstrated that B cells, dendritic cells, natural killer (NK) cells, mast cells, and macrophage or monocytes also pass through the BBB [3,4]. TNF- α is also able to activate the production of nitric oxide in macrophage which leads to greater permeability across the BBB [3, 4]. In addition to TNF-α, interleukin-6 (IL-6) is also associated with proinflammatory events, and is produced in conjunction with TNF-α and IL-1 [3]. Diseases associated with inflammation and CNS damage have demonstrated the expression of IL-6 in the brain [3, 4]. These events led to studies which demonstrated that the high expression of IL-6 in the CNS of mice resulted in ataxia, tremor, motor impairment and seizures [3, 4]. Similar events are seen in individuals exposed to trichothecene mycotoxins in SBS conditions [5]. Although the function of inflammation is to repair damaged tissues, the effects in the CNS tend towards damage versus repair [2, 3, 4]. The events described may be the reason why inflammatory events in the CNS lead to toxic effects in the ECs and damage to the BBB [2, 3, 4].

Studies conducted with trichothecenes have demonstrated disruption of biochemical pathways in immune cells and other cell types that evidence events of inflammation and apoptosis. Studies conducted with T-2 toxin on L-6 myoblasts demonstrated the ability of T-2 toxin to disrupt glucose transport, amino acid uptake, and calcium efflux decreases across the cellular membrane

[6]. This study suggested the ability of T-2 toxin to disrupt cell membrane integrity and activity [6]. The authors further suggested the ability of T-2 toxin to disable the activity of mitochondrial and nuclear membranes in cells, which could lead to apoptotic events [6].

Studies have also demonstrated the ability of cells to induce mitochondrial damage and lipid peroxidation, which activates apoptosis pathways [7,8]. Studies conducted by Pace et. al., have demonstrated the ability of T-2 toxin to inhibit mitochondrial protein synthesis [8]. Mitochondria, like prokaryotes, contain 70S ribosome compared to eukaryotes, which have 80S ribosomes [8]. Trichothecenes are known to inhibit eukaryotic protein synthesis; however, these studies demonstrate the ability of T-2 toxin to inhibit the electron transport chain (ETC) in mitochondria and non-specific inhibition of protein synthesis [8]. If mitochondrial protein synthesis and ETC activity are inhibited in a dose-dependent manner by T-2 toxin, this would suggest that apoptotic events may be activated due to mitochondrial damage by trichothecenes [8].

Further evidence of apoptotic events induced by trichothecene exposure has been elucidated. Experiments conducted with different trichothecenes have demonstrated variations in apoptotic induction. These results showed that structural nuances between trichothecenes can determine the degree of c-Jun N-terminal kinase (JNK/p38) activation [9]. JNK-p38 is expressed as a ribotoxic stress response that signals apoptotic pathways [9]. These events imply the ability of trichothecenes to activate rapid apoptosis by inhibiting protein

translation and activating JNK/p38 [9]. Studies conducted by Pestka et. al., have demonstrated the ability of trichothecenes and specifically satratoxins to induce apoptosis in leukocytes both in vitro and in vivo [9]. Further studies conducted by this group have demonstrated the ability of satratoxins to activate JNK/p38 and other kinases associated with apoptosis [9]. These kinases include several groups of mitogen activated protein kinases (MAPKs), such as extracellular signal-regulated protein kinase (ERK), p38, and stress associated protein kinase/ c-Jun N-terminal kinase (SAPK/JNK) at low doses in murine macrophages [9]. Additional work conducted by other researchers has shown apoptotic events in lymphoid organs of mice exposed to trichothecenes [10]. These studies demonstrated in vivo activity that correlates to events observed in in vitro analysis. In addition, this research demonstrates the ability of trichothecenes to induce pathological events that may contribute to leukopenia and other immunosuppressive events in individuals exposed to SBS conditions [10]. Recent reports have demonstrated that *S. chartarum* spores in the presence of *Streptomyces californicus* are able to induce *S. californicus* to produce biological compounds that arrest cell cycle activity [11, 12]. *S. chartarum* alone was found to induce apoptosis in macrophages, but unable to induce cell cycle arrest. *S. californicus* was unable to inhibit cell cycle activity without the presence of *S. chartarum* spores [11, 12]. These events demonstrate additive and synergistic toxicity induced by *S.chartarum* [11, 12].

Previous in vivo studies conducted with T-2 toxin have demonstrated the ability of trichothecenes to induce neurotoxic events when toxin was injected into brain tissue directly [13, 14]. These studies suggested the ability of trichothecenes to produce cytotoxic events, inflammation, and apoptosis, which illustrate the potential immunopathological events that have been witnessed in neurophysiological studies conducted with trichothecenes in murine and rat models. T-2 toxin has been closely evaluated for its effects in livestock and poultry [15, 16, 17, 18]. T-2 toxin is a common contaminant of feed for animals, and is associated with lethargy, ataxia, emesis, and feed refusal in animals and humans [15, 16, 17, 18]. There is evidence of T-2 toxin transforming neurotransmitter balance [17]. Increases in neurotransmitter concentrations, such as catecholamines and serotonin are associated with loss of appetite [17]. These neurotransmitters are synthesized from specific amino acids, and the transfer of these amino acids across the blood-brain barrier is highly regulated by transport proteins [17]. When the BBB is compromised, studies have demonstrated changes in the passage of these amino acids into the CNS, leading to changes in neurotransmitter concentrations [17]. These studies have demonstrated that the ingestion of T-2 toxin leads to changes in amino acid permeability across the BBB, which could lead to the neurological effects observed in animals exposed to trichothecene mycotoxins [17]. Other animal studies conducted with trichothecenes have demonstrated the ability of these agents to cause neurological events regardless of the administration route [13].

Experimental evidence indicated that T-2 toxin administered intracerebrally (i.c.) or subcutaneously (s.c.) resulted in similar events [13]. Rats exposed via either route demonstrated depression of respiration and muscle paralysis, followed by convulsions which led to death [13]. These results demonstrated that regardless of the route of exposure, systemic distribution of trichothecenes can reach the brain, resulting in neurological events [13]. Further evidence showed that low levels of T-2 toxin were responsible for the changes in the metabolism of brain biogenic monoamines, compared to lethal doses [13]. Previous analyses have demonstrated that the disruption of monoamine metabolism could alter food intake by altering hormone secretion, peristaltic contractions, or thermal regulation.

The above studies provided evidence which suggested low levels of trichothecenes were able to injure CNS activity and disrupt the integrity of the BBB. There is also evidence that suggested the stimulation of endothelial cells by trichothecenes led to pro-inflammatory activity. This continuous stimulation of pro-inflammatory events appeared to further aggravate the CNS in addition to the BBB.

In the present experiments, human vascular endothelial cells were exposed to satratoxin H, LPS, and oxidative stress conditions to evaluate the cellular pathways that were activated. Cells were also evaluated for additive effects, due to exposure from satratoxin H and LPS, satratoxin H and H_20_2. The purpose of these experiments was to determine what effects low doses of a

trichothecene mycotoxin from *S.chartarum* would induce in cells that compose the BBB. The objective was to utilize HBCEC as an in vitro model to determine the mechanism of toxicity produced by exposure to satratoxin H.

MATERIALS AND METHODS

A human brain capillary endothelial cell line (HBCEC) (Cambrex-Biowhittaker, Walkersville, MD), cell culture medium, and growth factors were purchased from Cambrex-Biowhittaker (Walkersville, Maryland), Clonetics Division for these experiments. Five $x10^5$ cells were cultured in 25 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). Cells were maintained in 5 ml of endothelial cell growth medium (EGM-2MV) with the following growth factors: 2% (vol/vol) fetal bovine serum (FBS), human recombinant growth factor (10ng/ml), EC growth supplement (12 µg/ml), hydrocortisone (1.0µg/ml), and gentamicin (50 µg/ml) incubated with 5% CO_2 at 37˚C. The confluent cells were sub-cultured on unsiliconized glass cover slips (25mm x 25mm) [19]. These glass cover slips were treated with a series of ethanol and water washes, to remove the silicon coating, according to procedures published by Simoni et. al., [19- 22]. Two coverslips were seeded per well in a 6-well plate (Corning Inc.). In addition to coverslips, the cells were subcultured in a series of 24-well plates until confluent (Corning Inc.). Experiments were conducted on cells from either a fourth or fifth passage. Cell passages were performed using the trypsin method recommended by Cambrex-Biowhittaker, Walkersville, MD. The cell lines were

tested by the supplier for human immunodeficiency virus (HIV), hepatitis, *Mycoplasma*, bacteria, yeast, fungi, and smooth muscle α-actin expression. The results of these tests were presented in Clonetics Certificate of Analysis.

Prior to experimentation, satratoxin H samples were tested for endotoxin contamination utilizing the QCL-1000 assay (Cambrex-Biowhittaker, Inc., Walkersville, MD). If endotoxin removal was required, an affinity chromatography method was utilized according to the manufacturer protocols for Detoxi-Gel Endotoxin Removal Gel AffinityPak Prepacked Columns (Pierce, Rockford, IL), followed by further evaluation using the QCL-1000 assay.

Previous in vitro studies have demonstrated that concentrations of 10ng/ml of macrocyclic trichothecenes were able to induce significant increases in cytokine synthesis and other cellular processes [24]. In the experiments here, 1, 10, 100, 1000, and 5,000 ng/ml concentrations of satratoxin H were evaluated. Prior to the addition of the compounds, the media in the 6 and 12-well plates were replaced with fresh medium. The samples were then suspended in pyrogen-free, sterile water (Cambrex-Biowhittaker, Inc., Walkersville, MD) and incubated with satratoxin H for 18 hours. Control cells received an equal volume of water. A second subset of cells were exposed to the concentrations of satratoxin H previously mentioned with the addition of 250μM H_2O_2 or 50 EU/ml LPS to induce an inflammatory response.

A general cytotoxicity assay was conducted to determine which sample groups were able to produce cytotoxic effects in cells. Lactate dehydrogenase

(LDH) concentrations were used to determine the cytotoxic effects of satratoxin H on HBCEC. A commercial assay, LDH (DG-1340-UV) concentration assay (Sigma Diagnostics), was utilized to determine the concentration of LDH released by cells into the culture medium after an 18h incubation period. The procedures described in the assay were followed to determine the concentration of LDH released by cells due to cellular damage. Samples were read spectrophotometrically at 340nm. This assay reflects cellular damage induced by satratoxin H.

A series of immunofluorescent studies were conducted to evaluate inflammatory events in EC due to satratoxin H exposure. Cells grown on coverslips as described previously were washed with cold PBS twice, followed by exposure to 3% formaldehyde in PBS for 20 minutes at RT [20]. The cells were exposed to cold methanol, followed by a series of 4 washings with PBS for a 30 minute period to permeabilize the cell membrane [20]. These cells were then blocked against nonspecific antibody binding with 1% bovine serum albumin (BSA) (Sigma Chemical Co.). Cells were exposed to monoclonal antibody against human ICAM-1, VCAM-1, and P/E selectin for 1 hour at 37˚C (R&D Systems). The wells were washed 5 times with PBS, followed by a 1 hour incubation with secondary antibody anti-mouse, fluorescein isothiocyanate (FITC) conjugated F (ab') $_2$ (Boehringer Mannheim, Co., Biochemical Products, Indianapolis, IN). Following a 30 minute incubation period, coverslips were mounted onto slides, and later evaluated microscopically at 60x under oil-

emersion. This procedure demonstrated if satratoxin H was able to induce the expression of adhesion molecule receptors. To quantitate the degree of fluorescence, photographs of cells were scanned and evaluated with Scan Analysis Software (Biosoft, Cambridge, UK). This procedure measured the density of fluorescence in each picture and reported the results in units of luminescence. The total area under a peak was used to determine the degree of fluorescence per picture evaluated. Photographs from duplicate coverslips under the same experimental conditions conducted in triplicate for each of the adhesion molecule receptors were evaluated.

In the event that inflammatory pathways are activated, the permeability of the BBB can increase to traffic cells across the BBB. In addition, substances that are toxic to HBCEC may increase the permeability across the BBB due to programmed cell death. During these events cell shrinkage can occur which can lead to increased permeability across the BBB. To evaluate whether satratoxin H is able to increase permeability across the BBB, the passage of radio-labeled ^{125}I- albumin (Diagnostic Products Corp., Los Angeles, CA) was evaluated. Cells were cultured in 75 cm^2 tissue culture flasks with EGM-2MV (Cambrex-Biowhittaker, Walkersville, MD), in humidified conditions with 5% CO_2, until they reached confluence, and were subcultured in 24 well plates tissue culture (TC) treated, 0.4μm polycarbonate membrane cell culture devices (Whatman Inc., Clifton, NJ). A monolayer of cells grown on 0.4μm polycarbonate membranes in 24 well plates were exposed to ^{125}I- albumin and monitored every 15min for the

diffusion rate of albumin across the monolayer. Radioactivity was measured using a scintillation machine as counts per unit (CPU).

Endothelial cells on unsiliconized glass coverslips were evaluated after exposure to satratoxin H and control conditions for apoptosis utilizing Apoptosis Detection Kit Annexin V-FITC (APO-AP) (Sigma, St. Louis, MO). Early apoptotic events were determined by monoclonal antibody (anti-PS fluorescein conjugate) binding to phosphatidylserine (PS) translocated from the inner cell membrane to the outer membrane. Late apoptotic events were observed by the translocation of propidium-iodide (PI) through compromised cellular and nuclear membranes. PI is able to bind to DNA fragments upon translocation to the nucleus. The detection of immunofluorescent staining was evaluated microscopically (Olympus-IX71 Confocal Microscope, Leeds Inst. Inc., Irving, TX). The software program used to view fluorescence was Metamorph® (Universal Imaging Corp., Dowington, PA). These assays demonstrated the concentration of satratoxin H able to induce apoptosis in HBCEC.

To quantitate the degree of apoptosis in cells, an ELISA method was used to determine the levels of cytochrome C from cellular extracts. Cytochrome C is maintained in the mitochondria of healthy cells. In the event of programmed cell death, cytochrome C is released from the mitochondria into the cytosol where it activates pathways associated with apoptosis. The ELISA for the evaluation of cytochrome C was conducted according to protocols described in the manual provided by R&D Systems.

During an inflammatory response, and early apoptotic events cells are under oxidative stress, which leads to the production of lipid radicals and lipid peroxidation. Previous studies have demonstrated that lipid radicals are able to inhibit anti-apoptotic genes which allow a cell to enter into apoptosis. In these experiments, the presence of oxidative stress was evaluated by measuring lipid hyperperoxides (LOOH) and reduced glutathione (GSH) levels according to established methods [21, 22].

Established methods were utilized to measure oxidative stress conditions to determine the concentration of GSH [21]. In a normal cell, GSH is unable to cross the nuclear membrane, but during oxidative stress the oxidized form of GSH, oxidized glutathione (GSSG), is able to cross this structure. GSSG is able to decrease the binding of p60/p65 complex of NF- κB to DNA, which reduces the pro-inflammatory cascade that is activated by NF- κB. The intracellular GSH levels were measured from cell homogenates [21]. The reaction of GSH with 5, 5' dithiobis and sulfhydryl compounds leads to a color change, producing a yellow pigment. The samples were read spectrophotometrically at 412nm (Sigma Chemical Co., St Louis, MO). Results were compared to a standard curve using GSH in nanomoles per milligram of protein. Protein concentrations for the cell homogenate fractions were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Lipid hydroperoxides (LOOH) were evaluated in cell homogenate fractions using previously established methods [21, 22]. This assay is able to directly measure the LOOH concentration Samples were mixed with SDS-acetate buffer, pH3.5, and aqueous solution of thiobarbituric acid. The reaction mixture was heated at 95˚C for 90 minutes. After cooling, the red-colored complex was extracted with n-butanol-pyridine [21, 22]. The data were measured spectrophotometrically at 532 nm, and compared to a standard curve [21, 22]. Results were expressed in nanomoles of LOOH per microgram of cells [21, 22].

Statistical analysis ($\alpha = 0.05$) was performed using Sigma Stat, a statistical software program designed by Jandel (SPSS), to analyze the data using one-way analysis of variance (ANOVA) of each experimental group with the controls. Normality of the data was determined using a Kolmogorov-Smirnov normality test. If data did not meet normality, a non-parametric Kruskal-Wallis ANOVA would have been applied. If data were normal, and ANOVA demonstrated significance, a post hoc test, Tukey test (a modified t test), was used to make multiple comparisons to determine which experimental groups demonstrated significance compared to the controls and between experimental groups. Results were graphed using Sigma Plot, a graphical program designed by Jandel (SPSS).

RESULTS

In these experiments HBCEC were evaluated for the expression of inflammatory and apoptotic events from the exposure of satratoxin H, LPS, and H_20_2. Negative controls were exposed to sterile, pyrogen-free water, and positive controls were exposed to 50EU/ml LPS and 250μM H_20_2 in a volume of 20μl. These cells were incubated for a period of 18h at 37˚C with 5% CO_2 in 6 well plates and unsiliconized coverslips. The evaluation of LDH levels demonstrated significant ($P<0.05$) cytotoxic events in cells exposed to 1000ng/ml SH and additive conditions (10ng/ml + LPS and 10ng/ml + H_2O_2). These results can be seen in Figure 2.

Cells were later evaluated for the expression of adhesion molecule receptors (ICAM, VCAM, P/E selectin) expressed in the event of inflammation. Figures 3, 5, and 7 demonstrate immunofluorescent results from the expression of ICAM, VCAM and P/E selectin. Cells that expressed the adhesion molecules receptors on the surface expressed were bound by Ab conjugated to the green fluorescent stain FITC. A live video-camera attached to the microscope was used to photograph three sections of each slide. A total of 6 slides per an experimental group were evaluated. Pictures of the cells were further evaluated to quantitate the density of fluorescence using Scan Analysis Software (BioSoft, Cambridge, UK). Figures 4, 6, and 8 demonstrate the degree of fluorescence produced by cells. The total area beneath a peak was used to determine the intensity of

receptor expression for each sample group. Figure 4 demonstrates a significant increase ($P< 0.05$) in the expression of ICAM on cells exposed to satratoxin H 100ng/ml, 1000ng/ml, and 10ng/ml + H_20_2. VCAM expression was significantly greater ($P<0.05$) than control cells when exposed to 100ng/ml SH, LPS, H_20_2, 10ng/ml + LPS, and 10ng/ml + H_20_2. A significant expression ($P< 0.05$) of P/E selectin was detected in HBCEC cells exposed to 100ng/ml, 1000ng/ml, LPS, H202, 10ng/ml + LPS, and 10ng/ml + H_20_2.

Further evidence of damage to the BBB, is EC cell shrinkage which leads to greater permeability across the BBB. To evaluate whether satratoxin H is able to induce HBCEC shrinkage, a monolayer of cells grown on 0.4μm polycarbonate membranes in 24 well plates were exposed to ^{125}I- albumin and monitored every 15min for the diffusion rate of albumin across the monolayer. There was a significantly greater rate ($P< 0.05$) of diffusion across cells exposed to 100ng, and 1000ng/ml of SH. A significant ($P< 0.05$) additive effect was observed in cells exposed to 10ng/ml + LPS and 10ng/ml + H_20_2. These results are seen in Figure 9.

Apoptotic events were observed in addition to inflammation. An annexin V apoptotic detection assay (Sigma, St. Louis, MO) was utilized to evaluate apoptosis. In the event of early apoptosis, phosphatidylserine (PS) expressed on the inner cell membrane is flipped to the outer surface of the cell membrane as an indication of apoptosis. In the detection assay, secondary Ab conjugated to FITC (green) binds to PE on the surface of cells in the event of apoptosis. Late

stages of apoptosis consist of chromatin fragmentation and permeability of the nuclear membrane. In the event of late stages of apoptosis, propidium iodide (red) binds to damage chromatin material. These events are observed in Figure 10. Compared to the negative control cells that received water, cells exposed to 100ng/ml SH, and 1000ng/ml SH, and LPS demonstrated early and late stages of apoptosis, whereas the control cells did not have a red stain in the nucleus of the cell. To further evaluate apoptosis, cytochrome C levels from cell extracts were evaluated using an ELISA method. These results demonstrated that a significantly increased amount ($P< 0.05$) of cytochrome C was released from cells exposed to 10ng/ml, 100ng/ml, LPS, 10ng/ml + LPS, and 10ng/ml + H_20_2. These results can be seen in figure 11.

An additional indicator of apoptosis is oxidative stress. In the event of oxidative stress, glutathione (GSH) acts as a reducing agent against reactive oxygen species (ROS) such as lipid radicals and peroxides. However, if GSH levels in a cell are insufficient to compensate for the degree of oxidative stress, both apoptotic and inflammatory pathways are further activated. To determine whether mycotoxins increased oxidative stress levels in HBCEC, a quantitative method was used to determine the levels of GSH present in cell extracts exposed to various experimental conditions. The results demonstrated a significant decrease ($P> 0.05$) in the concentration of GSH (µg/ml) in cells exposed to 100ng/ml SH, 1000ng/ml SH, LPS, H_20_2, 10ng/ml + LPS, 10ng/ml + H_20_2. These results are seen in Figure 12. The production of lipid peroxidation,

further demonstrates the degree of oxidative stress induced on HBCECs. Results from the thiobarbituric acid assay (T-BARS) demonstrated that there was a significant increase ($P>0.05$) in lipid peroxidation when cells were exposed LPS, H_2O_2, moderate concentrations of SH (100ng/ml and 1000ng/ml), and additive conditions (10ng/ml + LPS and 10ng/ml + H_2O_2). These results can be seen in Figure 13.

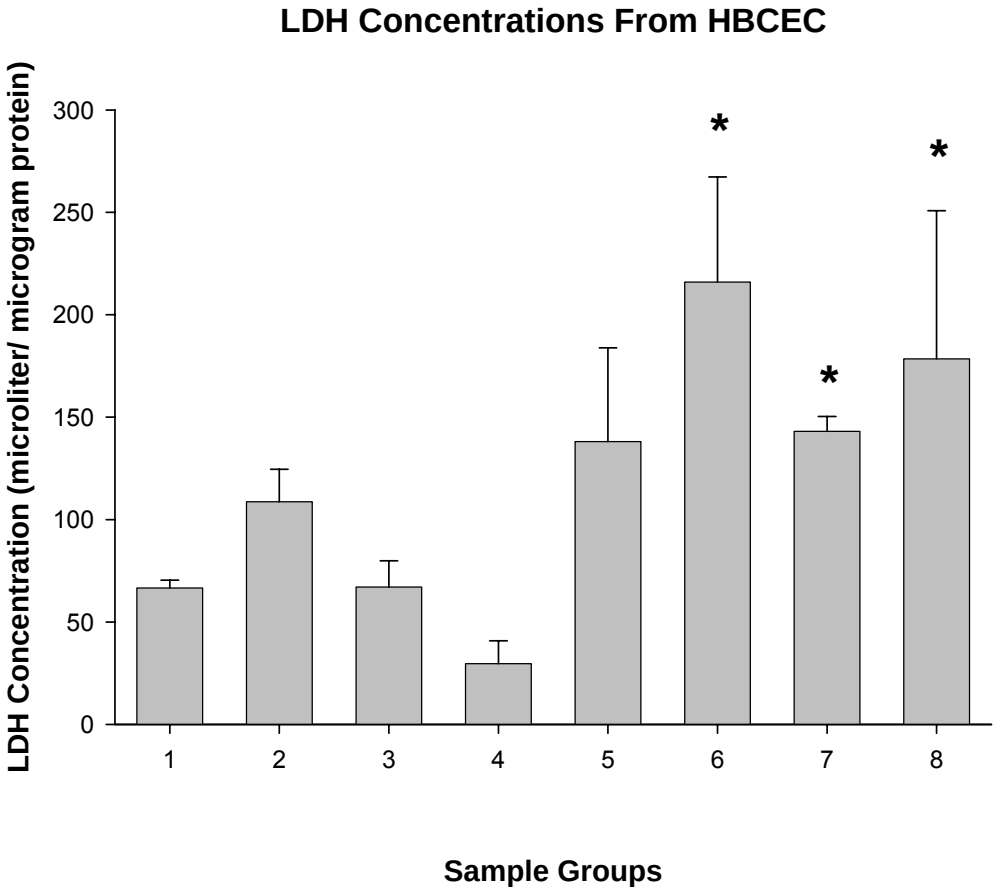


Figure 2. LDH assay for HBCEC. The degree of cytotoxicity induced in cells was quantitated by measuring the levels of LDH in supernatants. 1. Control (H_2O) 2. LPS (50 EU/ml) 3. H_2O_2 (250µM) 4. 10ng/ml SH 5. 100ng/ml SH 6. 1000ng/ml SH. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 340nm. (*) indicates a significant difference ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compare the experimental groups with the control.

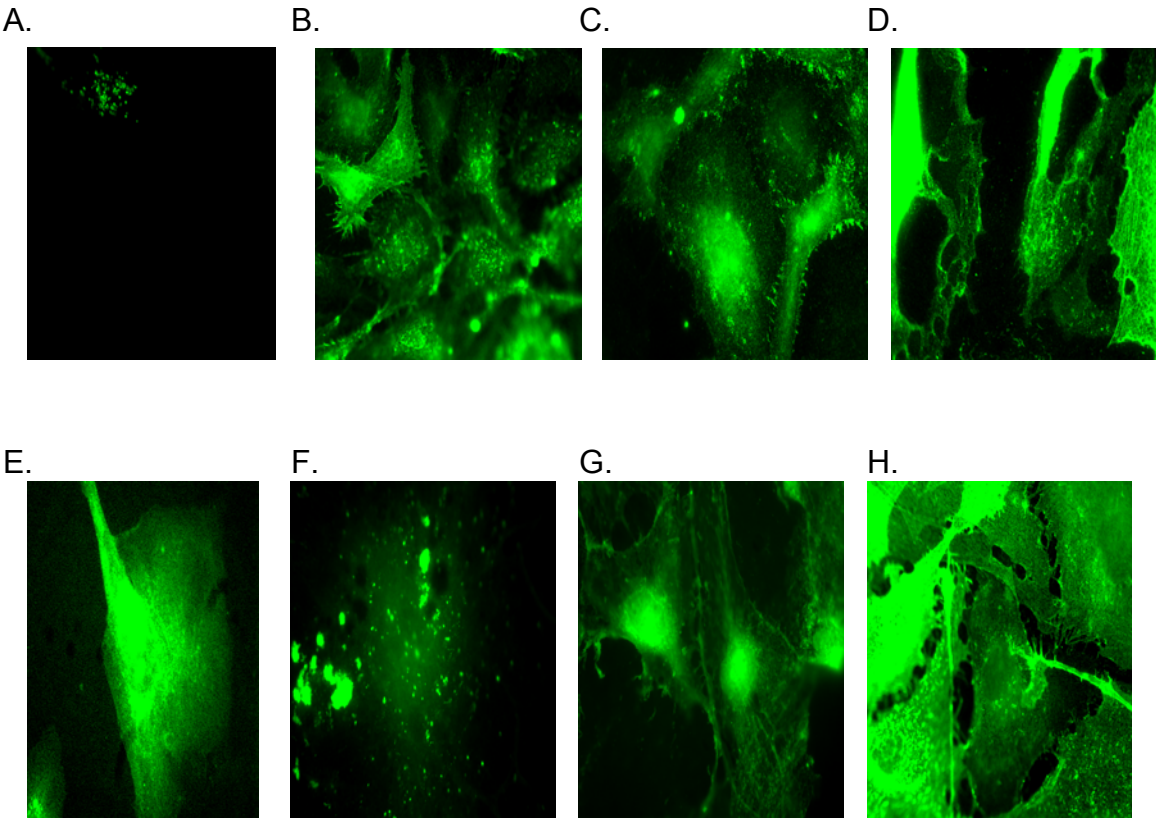


Figure 3. ICAM expression on HBCEC. The expression of ICAM results in the immunofluorescence of FITC-conjugated Ab to ICAM receptors. A. Control cells (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml . F. 250μM H_2O_2. G. 10ng/ml + LPS H. 10ng/ml + H_2O_2.

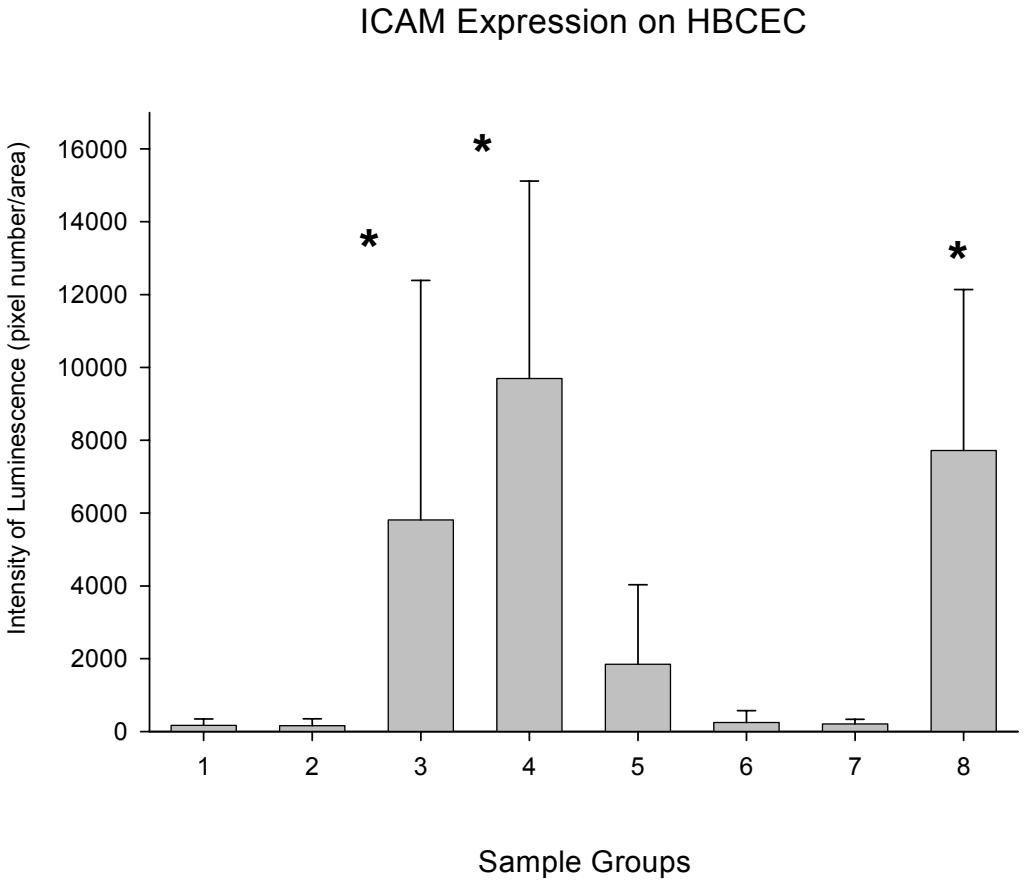


Figure 4. Densitometric evaluation of ICAM expression in HBCEC. The expression of ICAM results in the immunofluorescence of FITC-conjugated Ab to ICAM receptors. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 10ng/ml + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant increase ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compare the experimental groups with the control.

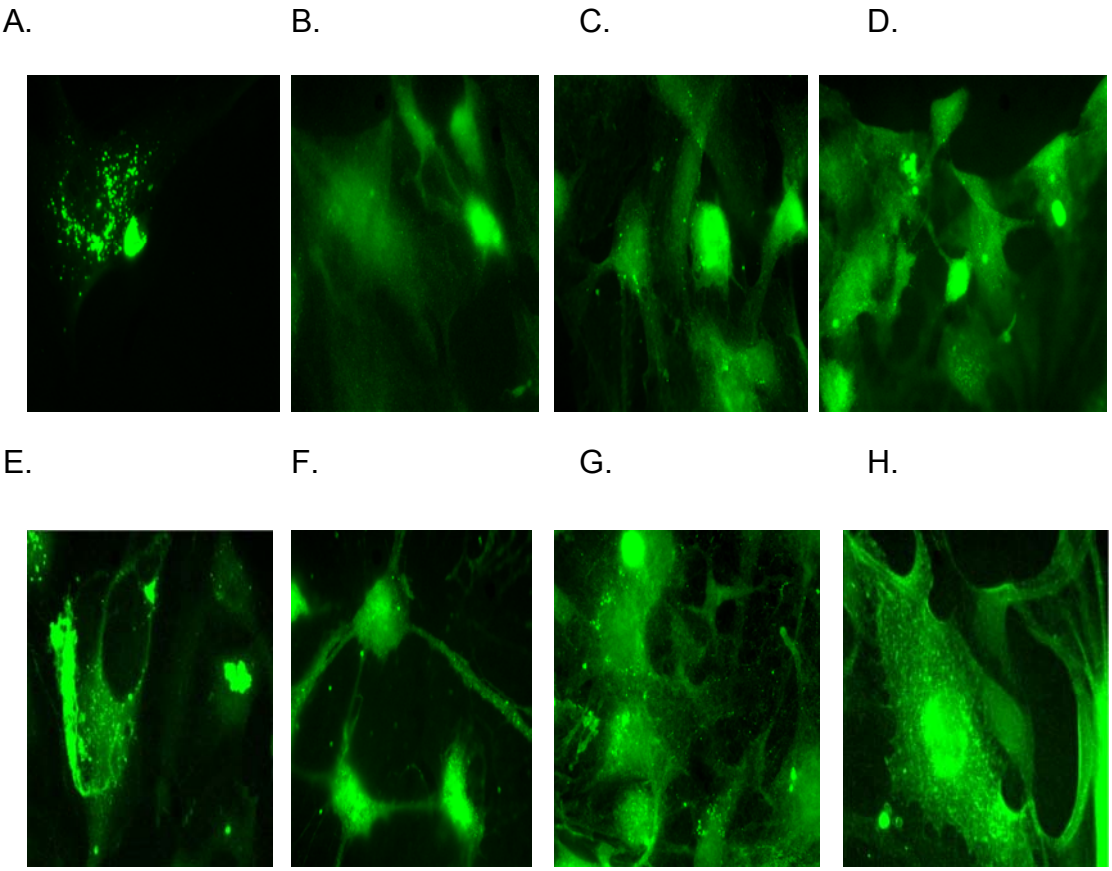


Figure 5. VCAM expression on HBCEC. The expression of VCAM results in the immunofluorescence of FITC-conjugated Ab to VCAM receptors. A. Control cells (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml . F. 250μM H_2O_2. G. 10ng/ml + LPS H. 10ng/ml SH + H_2O_2.

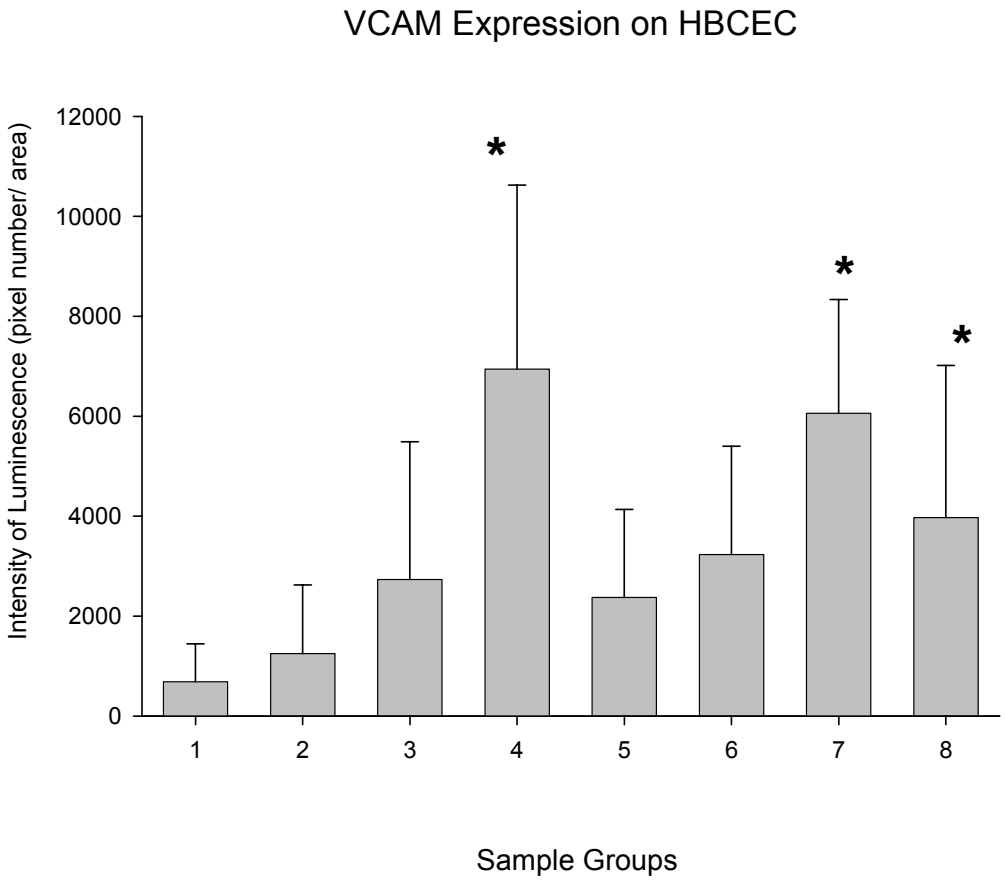


Figure 6. Densitometric evaluation of VCAM expression in HBCEC. The expression of VCAM results in the immunofluorescence of FITC-conjugated Ab to VCAM receptors. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant increase (P< 0.5) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compare the experimental groups with the control.

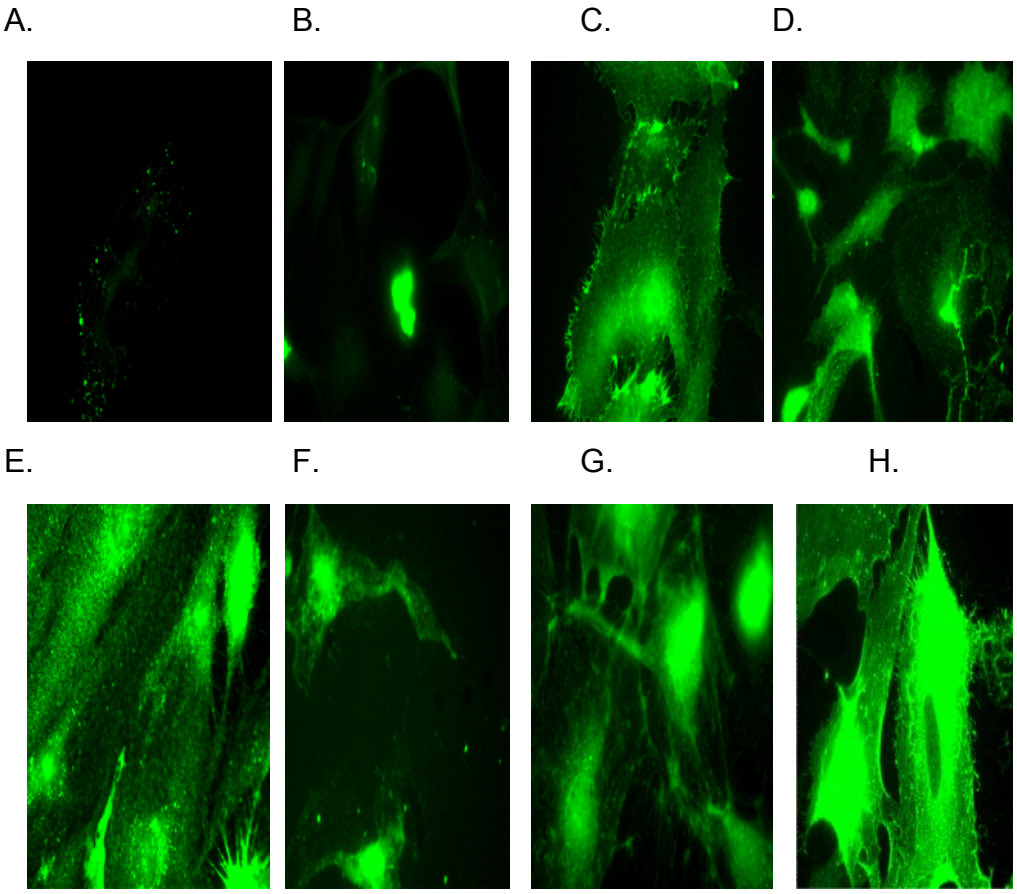


Figure 7. P/E Selectin expression on HBCEC. The expression of P/E Selectin results in the immunofluorescence of FITC-conjugated Ab to P/E selectin receptors. A. Control cells (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml . F. 250μM H_2O_2. G. 10ng/ml SH + LPS H. 10ng/ml SH + H_2O_2.

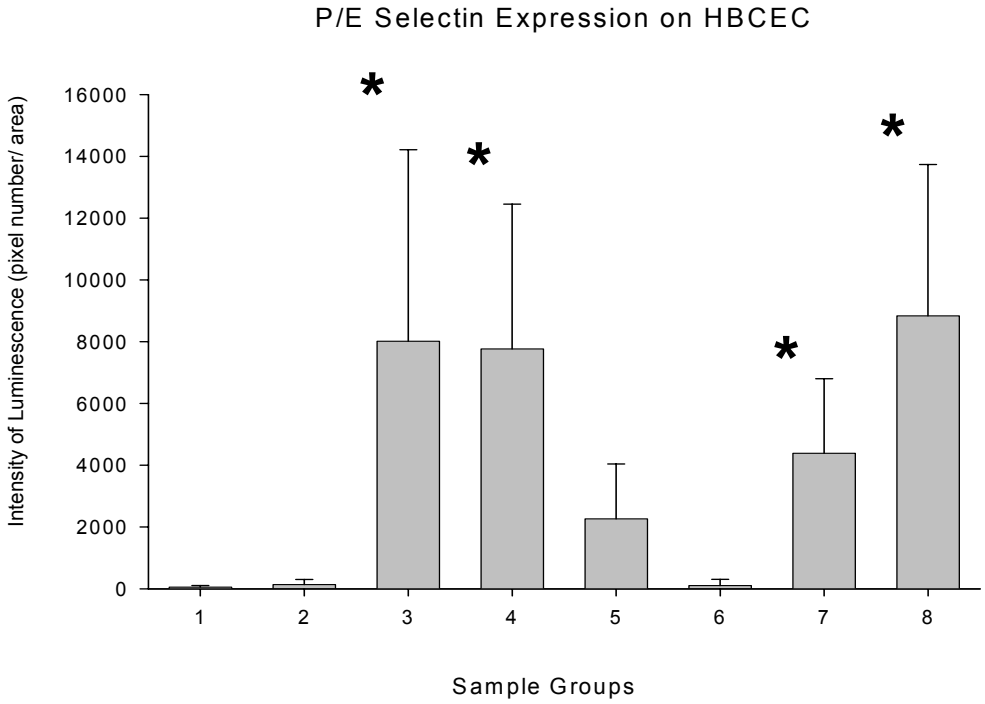


Figure 8. Densitometric evaluation of P/E Selectin expression in HBCEC. The expression of P/E results in the immunofluorescence of FITC-conjugated Ab to P/E receptors. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant increase (P< 0.5) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compare the experimental groups with the control.

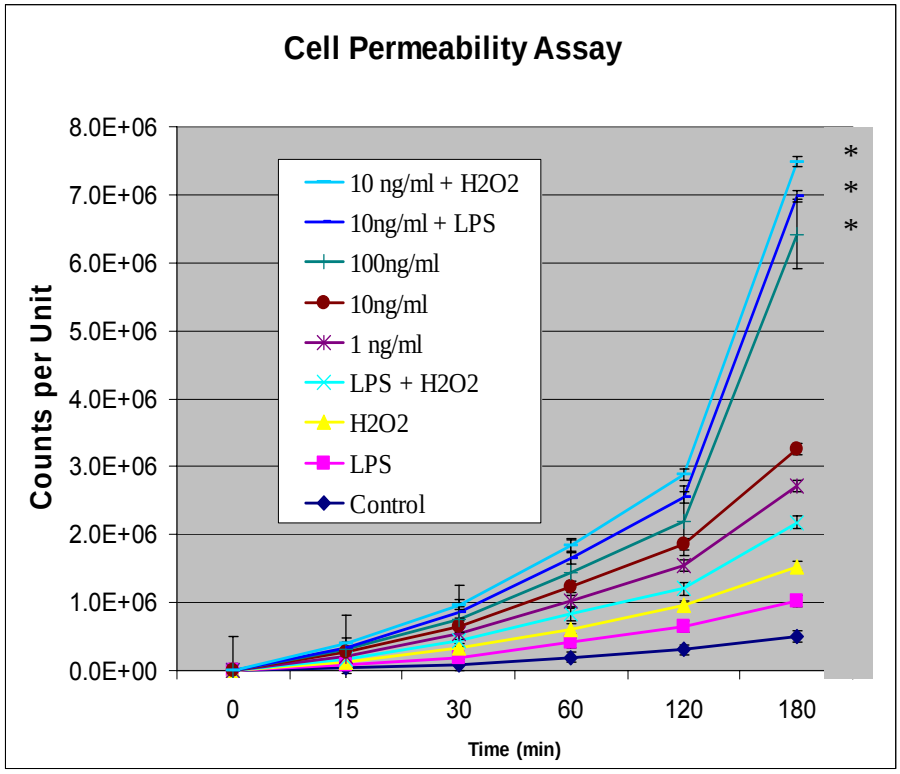
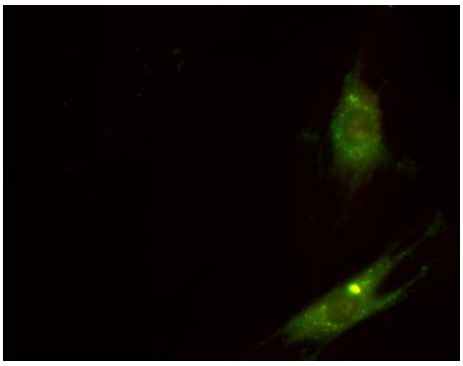


Figure 9. HBCEC evaluation for cell shrinkage and permeability. Radioactive-albumin (^{125}I) was incubated with cells exposed to control conditions (pyrogen-free water), LPS, H_20_2, and satratoxin H. Supernatants were collected every 15 minutes for 3h. Radioactivity was measured as CPU. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compare the experimental groups with the control.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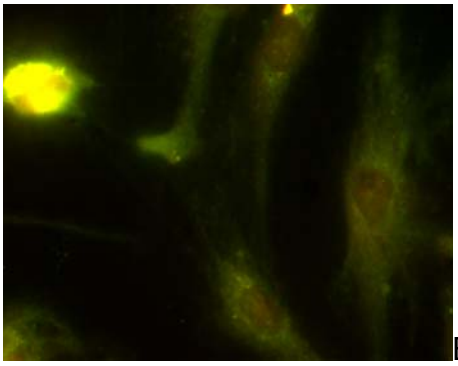
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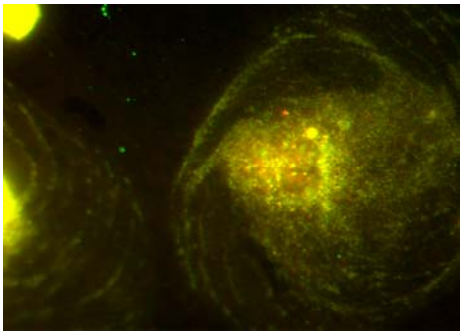
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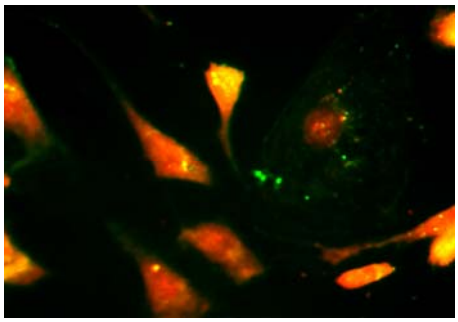
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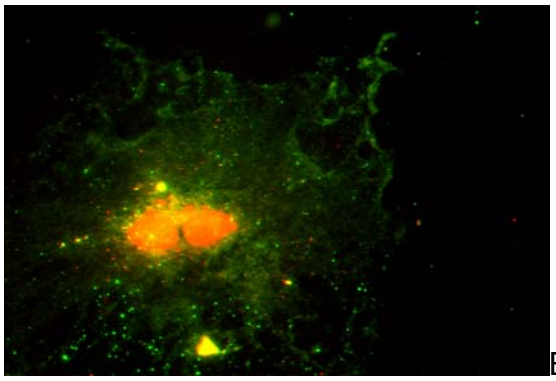

E.

Figure 10. Evaluation of HBCEC for apoptosis. Annexin V-FITC staining of PS (green) and PI staining of DNA (red). A. Control (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. LPS 50 EU/ml .

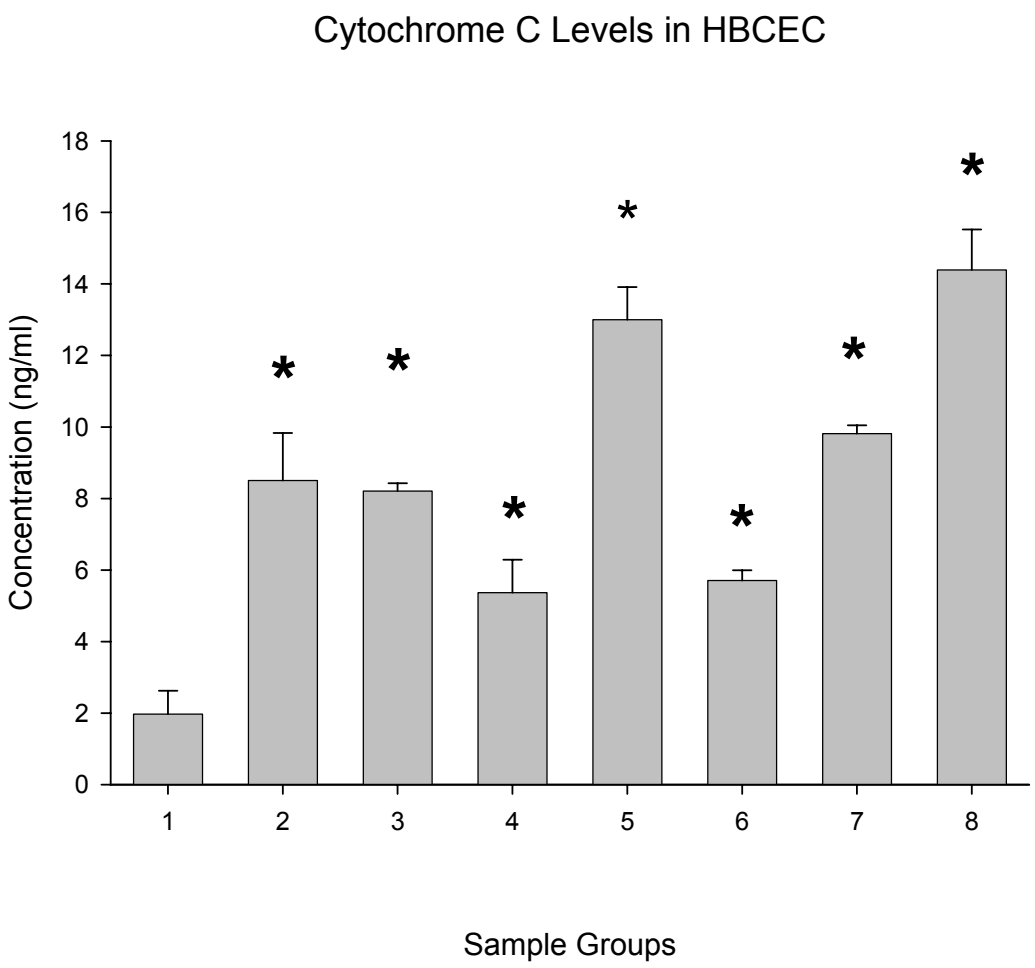


Figure 11. Cytochrome C concentration from HBCEC. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant increase (P< 0.5) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compared the experimental groups with the control.

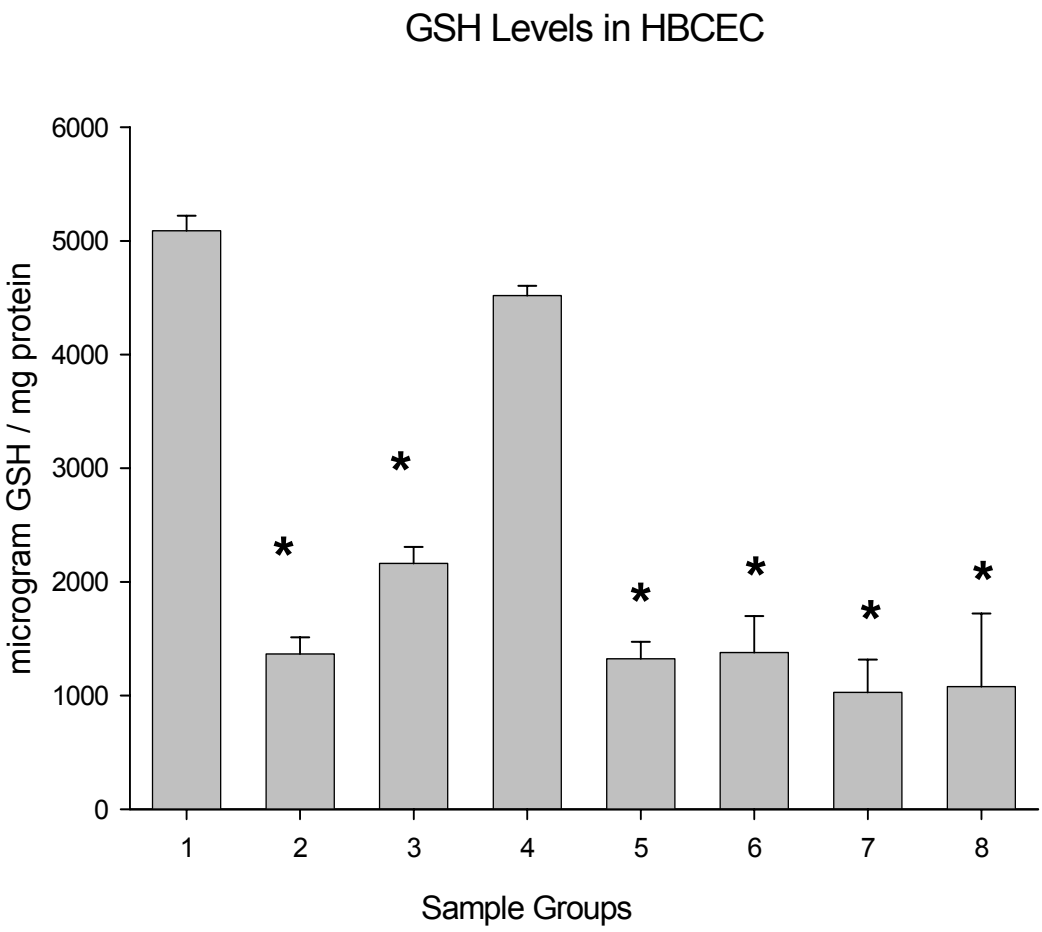


Figure 12. GSH concentrations from HBCEC. The expression of ICAM results in the immunofluorescence of FITC-conjugated Ab to ICAM receptors. 1. Control cells (H_20). 2. 50 EU/ml LPS. 3. 250μM H_2O_2. 4. 10ng/ml satratoxin H (SH). 5. 100ng/ml SH. 6. 1000ng/ml SH. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant increase (P< 0.5) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compared the experimental groups with the control.

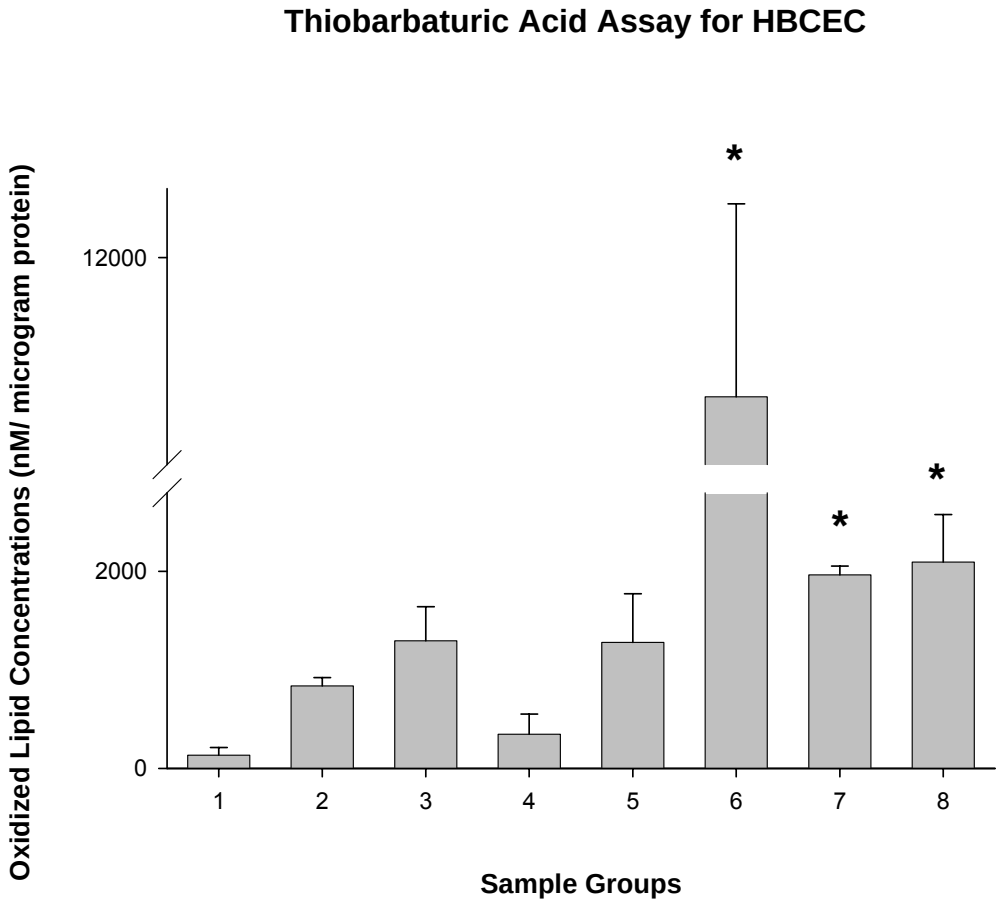


Figure 13. T-BARS assay for HBCEC. The degree of lipid oxidation induced in cells was quantitated by measuring the levels of lipid radicals from samples. 1. Control (H_2O) 2. LPS (50 EU/ml) 3. H_2O_2 (250μM). 4. 10ng/ml SH 5. 100ng/ml SH 6. 1000ng/ml SH. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 532nm. (*) indicates a significant difference ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

CONCLUSIONS

Results from the adhesion molecule receptor expression on HBCEC demonstrate that satratoxin H levels of 100ng/ml and 1000ng/ml are able to induce inflammatory pathway activation alone. Additive effects are demonstrated with very low concentrations of SH, such as 10ng/ml in the presence of inflammatory agents such as LPS and H_20_2. Similar concentrations of the mycotoxin are able to induce apoptotic pathways leading to the activation of early stages of apoptosis in the presence of 100ng/ml SH, however evidence of late stages of apoptosis are observed with 1000ng/ml and 10ng/ml + LPS or 10ng/ml + H_20_2. These results demonstrate the ability of satratoxins to induce apoptotic pathways at the same concentrations that inflammatory pathways are being activated. This suggests that low levels of inflammation and apoptotic events can be induced in the presence of moderate levels of SH, and low levels of SH are able to induce similar events in the presence of other inflammatory agents and oxidative stress conditions, as demonstrated by the levels of GSH and cytochrome C in cell extracts. In addition, the ability of the mycotoxins to induce cell shrinkage at moderate to low levels of SH demonstrate the potential ability of these agents to compromise the integrity of the BBB which could lead to further neurological damage from mycotoxins or other harmful agents. The presence of lipid peroxidation in cells exposed to moderate concentrations of SH and additive conditions, further demonstrates the ability of the mycotoxins to amplify cellular

damage through the indirect production of lipid radicals and other ROS. The results further suggest that low to moderate levels of SH are able to induce inflammatory and apoptotic pathways that amplify the cellular damage by the continuous activation of these biological pathways.

REFERENCES

1. Rubin L.L., Staddon J.M. 1999. The cell-biology of the blood-brain barrier. Annu Rev Neurosci. 22:11-28.

2. Jone C., Erickson, L., Trosko J.E., Chang C.C. 1987. Effect of biological toxins on gap-junctional intercellular communications in Chinese hamster V79 cells. Cell Biol Toxicol. 3:1-15.

3. Campbell I.L. 1995. Neuropathogenic actions of cytokines assessed in transgenic mice. Int J Devl Neuroscience. 13: 275-284.

4. Chavarria A., Varela J.A. 2004. Is damage in central nervous system due to inflammation?. Autoimmun Rev. 3: 251-260.

5. Johanning, E., Biagini, R., Hull, D., Morey, P. Jarvis, B.B., and Landsbergis, P. 1996. Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment. Int Arch Occup Environ Health. 68: 207-218.

6. Bunner D.L., Morris E.R. 1988. Alteration of multiple cell membrane functions in L-6 myoblasts by T-2 toxin: An important mechanism of action. Toxicol Appl Pharmacol. 92: 113-121.

7. Rizzo A.F., Atroshi F., Ahotupa M., Sankari S., Elovaara E. 1994. Protective effects of antioxidants against free radical-mediated lipid peroxidation induced by DON or T-2 toxin. J Vet Med. 41: 81-90.

8. Pace J.G. 1988. T-2 mycotoxin inhibits mitochondrial protein synthesis. Toxicon. 26: 77-85.

9. Shifrin V.I., Anderson P. 1999. Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. J Biol Chem. 274: 13985-13992.

10. Okumura H., Yoshino N., Sugiura Y., Sugamata M., Hintikka E.L., Jarvis B., Ueno Y. 1999. Trichothecenes as a potent inducer of apoptosis. *In*, Bioaerosols, fungi and mycotoxins: Health effects, assessment, prevention, and control. (Ed.) Eckardt Johanning. 221-231.

11. Huttunen K., Pelkonen J., Nielsen K.F., Nuutinen U., Jussila J., Hirvonen M.R. 2004. Synergistic interaction in simultaneous exposure to *Streptomyces californicus* and *Stachybotrys chartarum*. Environ Hlth Persp. 112: 659-665.

12. Penttinen P., Pelkonen J., Huttunen K., Toivola M., Hirvonen M.J. 2005. Interactions between *Streptomyces californicus* and *Stachybotrys chartarum* can induce apoptosis and cell cycle arrest in mouse RAW264.7 macrophages. Toxicol Appl Pharm. 202: 278-288.

13. Bergmann F, Yagen B, Jarvis B.B. 1992. The toxicity of macrocyclic trichothecenes administered directly into the rat brain. Toxicon. 30: 1291-1294.

14. Sirkka U, Nieminen S.A., Ylitalo P. 1992. Acute neurobehavioral toxicity of trichothecene T-2 toxin in the rat. Pharmacol Toxicol.70: 111-114.

15. Wang J, Fitzpatrick D.W., Wilson J.R. 1998. Effects of the trichothecene mycotoxin T-2 toxin on neurotransmitters and metabolites in discrete areas of the rat brain. Food Chem Toxicol. 36: 947-953.

16. Richard J.L., Cysewski S.J., Pier A.C., Booth G.D. 1978. Comparison of effects of dietary T-2 toxin on growth, immunogenic organs, antibody formation, and pathologic changes in turkeys and chickens. Am J Vet Res. 39: 1674-1679.

17. Cavan K.R., MacDonald E.J., Smith T.K. 1988. Potential for dietary amino acid precursors of neurotransmitters to overcome neurochemical changes in acute T-2 toxicosis in rats. J Nutrition. 118: 901-907.

18. Wang J, Fitzpatrick D.W., Wilson J.R. 1993. Effects of dietary T-2 toxin on biogenic monoamines in discrete areas of the rat brain. Food Chem Toxicol. 31: 191- 197.

19. Simoni J, Simoni G, Lox C.D., Prien S.D., Shires G.T. 1995. Evidence for the direct inhibition of endothelin-1 secretion by hemoglobin in human endothelial cells. ASAIO. 41: 641-51.

20. Simoni J, Simoni G, Martinez-Zaguilan R., Wesson D.E., Lox C.D., Prien S.D., Kumar R.V. 1998. Improved blood substitute: Evaluation of its effects on human endothelial cells. ASAIO. 44: 356-67.

21. Simoni J, Simoni G, Wesson D., Griswold J.A., Feola M. 2000. A novel hemoglobin-adenosine-glutathione based blood substitute: evaluation of its effects on human blood ex vivo. ASAIO.46: 679-92.

22. Simoni J, Simoni G, Garcia E.L, Prien S.D., Tran R.M., Feola M, Shires G.T. 1995. Protective effect of selenium on hemoglobin mediated lipid peroxidation in vivo. Artif Cells Blood Substit Immobil Biotechnol. 23: 469-86.

CHAPTER IV

THE TOXOLOGICAL EFFECTS OF SATRATOXIN H ON HUMAN ASTROCYTES

Astrocytes are the immune cells in neurological tissues and are therefore, able to produce cytokines [1]. They are dispersed in the CNS and network with endothelial cells, neurons, and other glial cells [1]. These cells are expressed late in the gestation period [1]. They are able to produce many types of responses due to changes in the environment [1]. Many of these responses lead to the production of cytokines and inducible nitric oxide synthase (iNOS) if the CNS is insulted [1].

Astrocytes play a critical role in the development of the BBB and this structure is not limited to the development of endothelial cells [2,3]. Research has demonstrated that the interaction of astrocytes with ECs determines the integrity of the BBB. The astrocytes are the closest cells to the ECs of the BBB. The astrocyte endfeet interact with the ECs, and may be responsible for inducing ECs to develop a brain phenotype [2, 3]. Some researchers have suggested that for optimal BBB development, astrocytes interaction is necessary [3]. ECs cultured in media pretreated with astrocyte-conditioned media increased the degree of tight junctions between ECs compared to cells that were only cultured in EC media [3].

Astrocytes produce soluble factors that are protective to ECs. Tissue growth factor- beta (TGF-β) produced by astrocytes is able to reduce tissue plasminogen activator (tPA) and anticoagulant factors [3]. The reduction in these factors protects the brain from intracerebral bleeding [3]. The production of TGF-β by astrocytes also leads to morphological and organizational events in ECs that form the BBB [3]. In the presence of TGF- β, ECs organize into capillary-like structures (CLS) [3]. In addition to protecting the integrity of the BBB, astrocytes produce factors such as glutamate, aspartate, taurine, adenosine tri-phosphate (ATP), endothelin-1, nitric oxide (NO), MIP-2, TNF-α, and IL-β that increase the permeability of the BBB [3]. Knowledge of the pathways activated by astrocytes has been elucidated by studies conducted on hypoxia-ischemia events in the brain. The effects of hypoxic conditions on the brain have been extensively evaluated, and demonstrated that pro-inflammatory pathways are activated during these conditions [3]. In human astrocytes exposed to ischemic conditions in vitro, inflammatory mediators lead to an increase in the production of IL-8, ICAM, E-selectin, IL-1β, TNF-α, and MCP-1 in endothelial cells [3]. These events lead to the recruitment of leukocytes and neutrophils which lead to the disruption of tight junctions and BBB compromise [3].

Studies have illustrated the ability of satratoxins and other trichothecenes to increase the production of TNF-α, IL-6, IL-8, IL-2, IL-5, IL-1β, and Fas ligand expression, which as previously described are able to activate inflammation [4]. This research was conducted in EL-4 thymoma cells which are a T-cell model [4].

These studies have also demonstrated that trichothecenes exposed to cells in the presence of LPS lead to more toxic events in tissue injury [5]. In addition, results have demonstrated that protein synthesis inhibitors like trichothecenes disrupt the homeostasis of immune responses, leading to amplified cytokine expression [6-10]. In normal conditions, cytokine responses are down-regulated to decrease tissue injury in the event of constant environmental stimuli [6-10].

The communication between astrocytes and ECs or neurons is a complex system that involves chemical and electrical stimulation. Astrocytes are viewed as being able to detect neural activity. The strength of a neural response can be evaluated based on the increase in Ca^{2+} concentration oscillations produced by synaptic impulses received from neurons [11, 12]. This method allows neurons to propagate information to astrocytes and ECs that are distant from the site of impulse [11, 12]. Neurotransmitters are another method utilized in neuron-astrocyte communication. An essential component of astrocytes is in assisting blood flow through cerebral blood vessels. Neurotransmitters that participate in regulating blood flow are atrial natriuretic peptide (ANP), nitric oxide (NO), and prostanoids [11, 12]. The release of prostanoids and ATP allow astrocytes to activate other glial cells and the secretion of cytokines [11, 12]. These cytokines are able to amplify the signal to further propagate astrocyte activity [11, 12]. The gap junctions between ECs are also a method of communication used by astrocytes to propagate events to endothelial cells. Channels present between cells allow for the passage of high molecular weight compounds, and voltage

dependent compounds. Astrocytes contain these channels between them, and allow the passage of ATP and glutamate [11, 12]. Glutamate is one the most abundant excitatory neurotransmitters in the CNS [11, 12]. Damage to the CNS can lead to amplification of these signals and further production of cytokines and other immune signals.

MATERIALS AND METHODS

A human glial astrocyte cell line (AC) (Cambrex-Biowhittaker, Walkersville, MD), cell culture medium, and growth factors were purchased from Cambrex-Biowhittaker (Walkersville, Maryland), Clonetics Division to be used in these experiments. Five $x10^5$ cells were cultured in 25 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). Cells were maintained in 5ml of EBM medium (CC-3121 Cambrex-Biowhittaker, Walkersville MD) with other components provided by the suppliers as a part of the AGM Bullet Kit (CC-3186, Cambrex-Biowhittaker, Walkersville, MD) and incubated with 5% CO_2 at 37˚C. The confluent cells were sub-cultured on unsiliconized glass cover slips (25mm x 25mm) [13]. These glass cover slips were treated with a series of ethanol and water washes, to remove the silicon coating [13-16]. Two coverslips were seeded per well in a 6-well plate (Corning Inc.). In addition to coverslips, the cells were subcultured in a series of 12-well plates until confluent (Corning Inc.). Experiments were conducted on cells from either a fourth or fifth passage. Cell

passages were performed using the trypsin method recommended by Cambrex-Biowhittaker, Walkersville, MD. The cell lines were tested by the supplier for human immunodeficiency virus (HIV), hepatitis, *Mycoplasma*, bacteria, yeast, fungi, and smooth muscle α-actin expression. The results of these tests are presented in Clonetics Certificate of Analysis.

Prior to experimentation, satratoxin H samples were tested for endotoxin contamination utilizing the QCL-1000 assay (Cambrex-Biowhittaker, Inc., Walkersville, MD). If endotoxin removal was required, an affinity chromatography method was utilized according to the manufacturer protocols for Detoxi-Gel Endotoxin Removal Gel AffinityPak Prepacked Columns (Pierce, Rockford, IL), followed by further evaluation using the QCL-1000.

In the experiments conducted here 1, 10, 100, 1000, and 5,000 ng/ml concentrations of satratoxin H were evaluated. Prior to the addition of the compounds, the media in the 6 and 12-well plates were replaced with fresh medium. The samples were then suspended in pyrogen-free, sterile saline solution (Cambrex-Biowhittaker, Inc., Walkersville, MD) and incubated with satratoxin H for 18 hours. Control cells received an equal volume of water. A second subset of cells were exposed to the concentrations of satratoxin H previously mentioned with the addition of 250μM H_20_2 and 50 EU/ml LPS to induce an inflammatory response.

Lactate dehydrogenase (LDH) concentrations were used to determine the cytotoxic effects of satratoxin H on human astrocytes. A commercial assay, LDH (DG-1340-UV) concentration assay (Sigma Diagnostics), was utilized to determine the concentration of LDH released by cells into the culture medium after an 18h incubation period. The procedures described in the assay were followed to determine the concentration of LDH released by cells due to cellular damage. Samples were read spectrophotometrically at 340nm. This assay reflects cellular damage induced by satratoxin H.

To evaluate adhesion molecule receptor expression in the event of inflammatory pathway activation, immunofluorescent staining of ICAM, VCAM, and P/E selectin were conducted. Cells grown on coverslips were washed with cold PBS twice, followed by exposure to 3% formaldehyde in PBS for 20 minutes at RT [14]. The cells were exposed to cold methanol, followed by a series of 4 washings with PBS for a 30 minute period to permeabilize the cell membrane [16]. These cells were then blocked against nonspecific antibody binding with 1% bovine serum albumin (BSA) (Sigma Chemical Co.). Cells were exposed to monoclonal antibody against human ICAM-1, VCAM-1, and P/E selectin for 1hour at 37˚C (R&D Systems). The wells were washed 5 times with PBS, followed by a 1 hour incubation with secondary antibody anti-mouse, fluorescein isothiocyanate (FITC) conjugated F (ab') $_2$ (Boehringer Mannheim, Co., Biochemical Products, Indianapolis, IN). Following a 30 minute incubation period, coverslips were mounted onto slides, as previously described and

evaluated microscopically at 60x with oil-emersion. This procedure demonstrated if satratoxin H was able to induce the expression of adhesion molecule receptors. Pictures of the cells were further evaluated to quantitate the density of fluorescence using Scan Analysis Software (BioSoft, Cambridge, UK). The total area beneath a peak was used to determine the intensity of receptor expression for each sample group.

Upon exposure to low levels of a toxic compound or infectious agents, cells are able to produce low levels of inflammation and apoptotic events simultaneously. The cellular receptors for inflammation can lead to the activation of apoptotic pathways as well. NF- κB is a transcription factor that is associated with inflammation, however it is also involved with the regulation of apoptotic events in a cell. Since, astrocytes behave as macrophages in neural tissues, the activation of NF- κB was evaluated. This transcription factor is able to increase the transcription of pro-inflammatory agents, such as cytokines and chemokines in the event of inflammation. To evaluate the expression of NF- κB in astrocytes, cell extracts from astrocytes exposed to previously described experimental conditions were measured using an ELISA method. The methods used to conduct this assay were performed according to the procedures outlined by the manufacturers of the ELISA kit (R&D Systems Inc.). The nuclear translocation and DNA binding of NF- κB were assayed in nuclear cell extracts using a TransAM™ NF- κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA). This method is the first ELISA-based kit to detect and quantify NF- κB

activation, that contains a 96-well plate on which there is an immobilized oligonucleotide containing the NF- κB consensus site (5'- GGGACTTTCC-3'). The whole-cell extracts were obtained from living cells using complete lysis buffer that contained dithothreitol (DTT) and a protease inhibitor cocktail supplied by the manufacturer. The complete binding buffer was supplemented with DTT and herring sperm DNA. Results were expressed in optical density (OD) at 450nm per 2.5μg of whole cell extract. The HeLa whole-cell extracts, provided by the manufacturer, were used as a positive control for NF-κB activation and DNA binding. This colorimetric assay provided quantitative evidence of inflammatory pathway activation in astrocytes.

Human astrocyte cells on unsiliconized glass coverslips were evaluated after exposure to satratoxin H and control conditions for apoptosis utilizing Apoptosis Detection Kit Annexin V-FITC (APO-AP) (Sigma, St. Louis, MO). Early apoptotic events were determined by monoclonal antibody (anti-PS fluorescein conjugate) binding to phosphatidylserine (PS) translocated from the inner cell membrane to the outer membrane. Late apoptotic events were observed by the translocation of propidium-iodide (PI) through compromised cellular and nuclear membranes. PI is able to bind to DNA fragments upon translocation to the nucleus. The detection of immunofluorescent staining was evaluated microscopically (Olympus-IX71 Confocal Microscope, Leeds Inst. Inc., Irving, TX). The software program used to view fluorescence was Metamorph®

(Universal Imaging Corp., Dowington, PA). These assays demonstrated the concentration of satratoxin H able to induce apoptosis in HBCEC.

To quantitate the degree of apoptosis in cells, an ELISA method was used to determine the levels of cytochrome C from cellular extracts. Cytochrome C is maintained in the mitochondria of healthy cells. In the event of programmed cell death, cytochrome C is released from the mitochondria into the cytosol where it activates pathways associated with apoptosis. The ELISA for the evaluation of cytochrome C was conducted according to protocols described in the manual provided by R&D Systems Inc.

During an inflammatory response and early apoptotic events, cells are under oxidative stress, which leads to the production of lipid radicals and lipid peroxidation. Previous studies have demonstrated that lipid radicals are able to inhibit anti-apoptotic genes which allow a cell to enter into apoptosis. In these experiments, the presence of oxidative stress was evaluated by measuring lipid hyperperoxides (LOOH) and reduced glutathione (GSH) levels according to established methods [13-16].

In a normal cell GSH is unable to cross the nuclear membrane, but during oxidative stress the oxidized form of GSH, oxidized glutathione (GSSG), is able to cross this structure. GSSG is able to decrease the binding of p60/p65 complex of NF- κB to DNA, which reduces the pro-inflammatory cascade that is activated by NF- κB. The intracellular GSH levels were measured from cell homogenates [15]. The reaction of GSH with 5, 5’ dithiobis and sulfhydryl compounds leads to

a color change, producing a yellow pigment. The samples were read spectrophotometrically at 412nm (Sigma Chemical Co., St Louis, MO). Results were compared to a standard curve using GSH in nanomoles per milligram of protein. Protein concentrations for the cell homogenate fractions were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Lipid hydroperoxides (LOOH) were evaluated in cell homogenate fractions with a previously established method [13-16]. This assay is able to directly measure the LOOH concentration Samples were mixed with SDS-acetate buffer, pH3.5, and aqueous solution of thiobarbituric acid. The reaction mixture was heated at 95˚C for 90 minutes. After cooling, the red-colored complex was extracted with n-butanol-pyridine [13-16]. The data were measured spectrophotometrically at 532 nm, and compared to a standard curve [13-16]. Results were expressed in nanomoles of LOOH per microgram of cells [13-16].

Statistical analysis ($\alpha = 0.05$) was performed using Sigma Stat, a statistical software program designed by Jandel (SPSS), to analyze the data using one-way analysis of variance (ANOVA) of each experimental group with the controls. Normality of the data was determined using a Kolmogorov-Smirnov normality test. If data did not meet normality, a non-parametric Kruskal-Wallis ANOVA would have been applied. If data were normal, and ANOVA demonstrated significance, a post hoc test, Tukey test (a modified t test), was used to make multiple comparisons to determine which experimental groups demonstrated significance compared to the controls and between experimental groups.

Results were graphed using Sigma Plot, a graphical program designed by Jandel (SPSS).

RESULTS

In these experiments astrocytes were evaluated for the expression of inflammatory and apoptotic events from the exposure of satratoxin H, LPS, and H_20_2. Negative controls were exposed to sterile, pyrogen-free water, and positive controls were exposed to 50EU/ml LPS and 250μM H_20_2 in a volume of 20μl. These cells were incubated for a period of 18h at 37˚C with 5% CO_2 in 6 well plates and unsiliconized coverslips. Cytotoxicity results were determined by LDH levels measured from cell media. The results demonstrate significant ($P<0.05$) cytotoxicity in cell exposed to 1000ng/ml SH and additive conditions (10ng/ml SH + LPS, and 10ng/ml + H_20_2). These results are shown in figure 14.

Cells were later evaluated for the expression of adhesion molecule receptors (ICAM, VCAM, P/E selectin) expressed in the event of inflammation. Figures 15, 17, and 19 demonstrate immunofluorescent results from the expression of ICAM, VCAM and P/E selectin. Cells that expressed the adhesion molecules receptors on the surface were bound by Ab conjugated to the green fluorescent stain FITC. A live video-camera attached to the microscope was used to photograph three sections of each slide. A total of 6 slides per an experimental group were evaluated. Pictures of the cells were further evaluated to quantitate the density of fluorescence using Scan Analysis Software (BioSoft, Cambridge, UK). Figures 16, 18, and 20 show levels of fluorescence produced by cells. The

total area beneath a peak was used to determine the intensity of receptor expression for each sample group. These results demonstrate that there is a significant increase ($P<0.05$) in the expression of ICAM, VCAM, and P/E selectin under additive conditions (100ng/ml SH + LPS and 100ng/ml SH + H_20_2).

The role of astrocytes in neural tissue is to produce protective inflammatory compounds in the event of disease. Immune cells are able to specifically amplify immune responses by the activation of transcription factors such as NF-κB. The activation of NF-κB leads to the nuclear translocation of the p60/p65 subunit. The binding of the subunit to DNA leads to the transcription of cytokines and chemokines that further amplify the inflammatory process. Using an ELISA method, the concentration of NF-κB was quantitated as a measure of inflammation induced by SH. Results demonstrate a significant increase ($P<0.05$) in the expression of NF-κB in cells exposed to 100ng/ml SH, 1000ng/ml SH, LPS, 100ng/ml SH + LPS, and 100ng/ml + H_20_2. These results can be seen in figure 21.

Apoptotic events were observed in addition to inflammation. An annexin V apoptotic detection assay (Sigma Aldrich) was utilized to evaluate apoptosis. In the event of early apoptosis, phosphatidylserine (PS) expressed on the inner cell membrane is flipped to the outer surface of the cell membrane as an indication of apoptosis. In the detection assay, secondary Ab conjugated to FITC (green) binds to PE on the surface of cells in the event of apoptosis. Late stages of apoptosis consist of chromatin fragmentation and permeability of the nuclear

membrane. In the event of late stages of apoptosis, propidium iodide (red) binds to damaged chromatin material. These events can be seen in figure 22. Compared to the negative control cells that received water, cells exposed to 100ng/ml SH, and 1000ng/ml SH, and LPS demonstrated early and late stages of apoptosis, whereas the control cells did not have a red stain in the nucleus of the cell. To further evaluate apoptosis, cytochrome C levels from cell extracts were evaluated using an ELISA method. These results demonstrated that a significant increase ($P< 0.05$) in the amount of cytochrome C released from cells occurred when they were exposed to 100ng/ml SH, 1000ng/ml SH, LPS, 10ng/ml + LPS, and 10ng/ml + H_20_2. Figure 23 shows these results.

An additional indicator of apoptosis is oxidative stress. In the event of oxidative stress, glutathione (GSH) acts as a reducing agent against reactive oxygen species (ROS) such as lipid radicals and peroxides. However, if GSH levels in a cell are insufficient to compensate for the degree of oxidative stress, both apoptotic and inflammatory pathways are further activated. To determine whether mycotoxins increased oxidative stress levels in astrocytes, an ELISA method was used to quantitate the levels of GSH present in cell extracts exposed to various experimental conditions. The results demonstrated a significant decrease ($P> 0.05$) in the concentration of GSH (µg/ml) occurred in cells exposed to 10ng/ml -1000ng/ml, LPS, H_20_2, 100ng/ml + LPS, 100ng/ml + H_20_2 (Figure 24).

The presence of ROS, such as lipid peroxides was determined by a T-BARS assay to quantitate the degree of lipid peroxidation due to the experimental conditions evaluated. These results demonstrated that, at moderate concentrations of SH (100ng/ml and 1000ng/ml), lipid peroxidation was significantly higher (P> 0.05) compared to control conditions. Additive conditions, such as 100ng/ml SH + LPS and 100ng/ml SH + H_20_2 produced significant (P>0.05) levels of lipid radicals. These results can be seen in Figure 25.

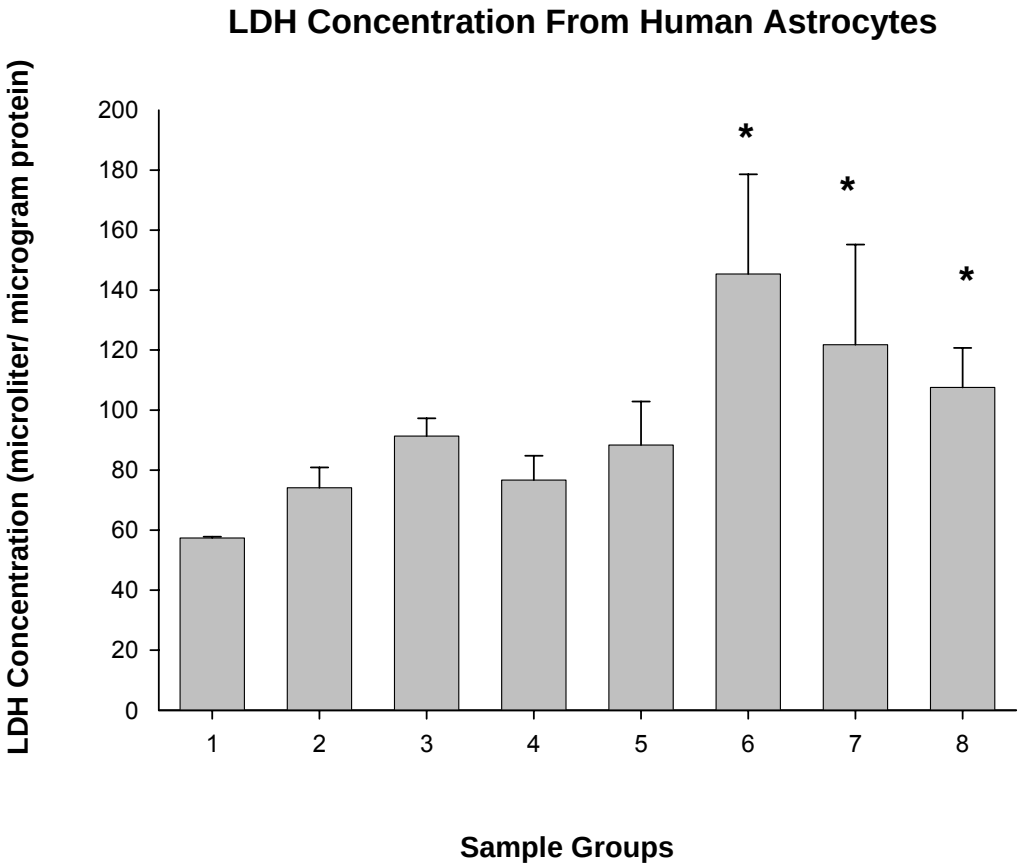


Figure 14. LDH assay for human astrocytes. The degree of cytotoxicity induced in cells was quantitated by measuring the levels of LDH released from cells into the supernatant. 1. Control (H_2O) 2. LPS (50 EU/ml) 3. H_2O_2 (250μM). 4. 10ng/ml SH 5. 100ng/ml SH 6. 1000ng/ml SH. 7. 100ng/ml SH + LPS 8. 100ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 340nm. (*) indicates a significant difference ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

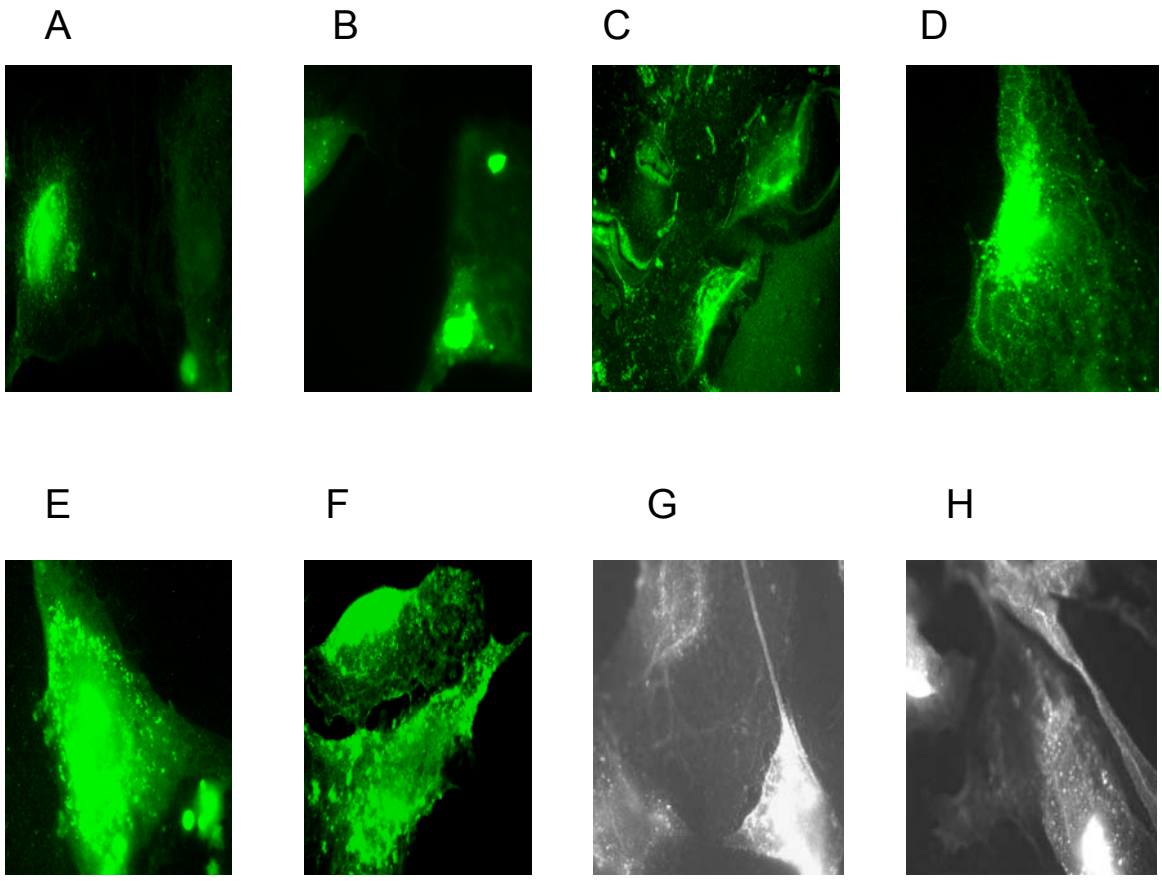


Figure 15. ICAM expression in human astrocytes. The expression of ICAM results in the immunofluorescence of FITC-conjugated Ab to ICAM receptors. A. Control cells (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml . F. 250μM H_2O_2. G. 100ng/ml SH + LPS H. 100ng/ml SH + H_2O_2.

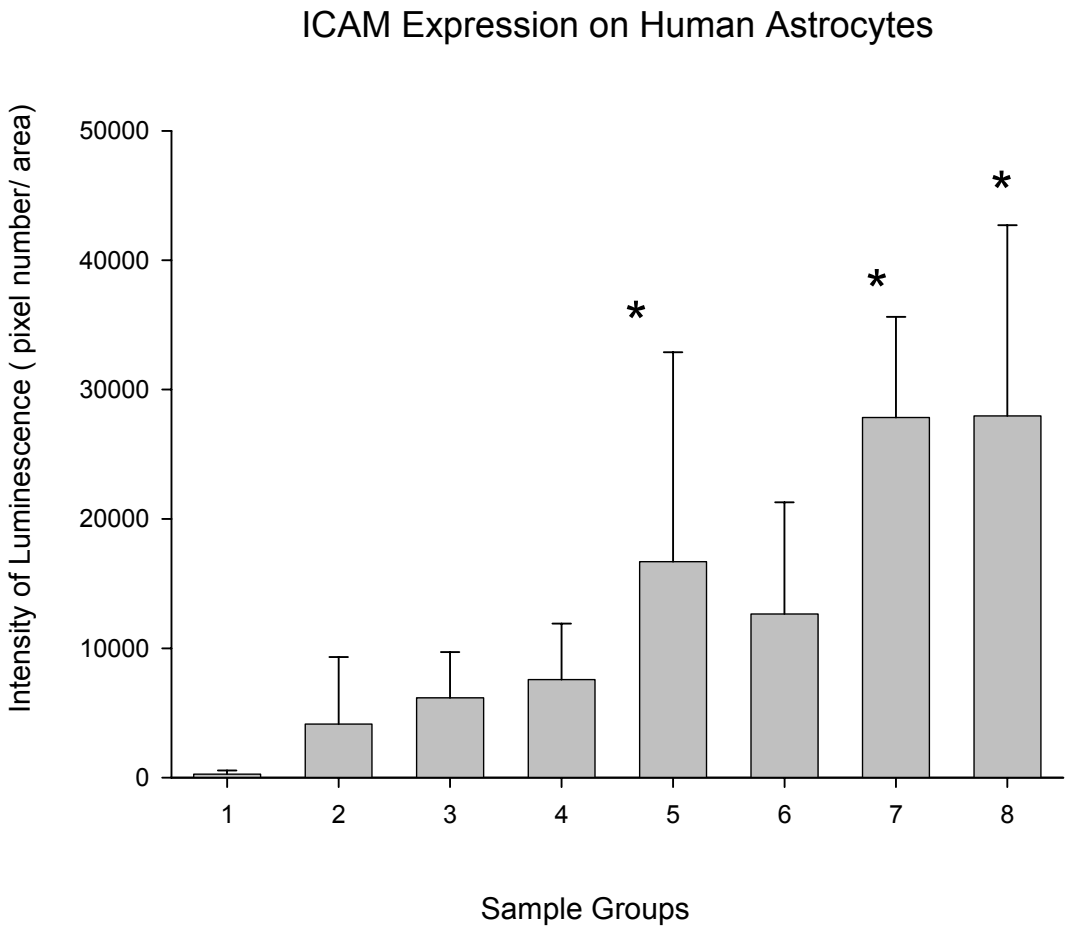


Figure 16. Densitometric evaluation of ICAM expression by human astrocytes. The expression of ICAM results in the immunofluorescence of FITC-conjugated Ab to ICAM receptors. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 100ng/ml SH + LPS 8. 100ng/ml SH + H_2O_2. An (*) indicates a significant increase ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

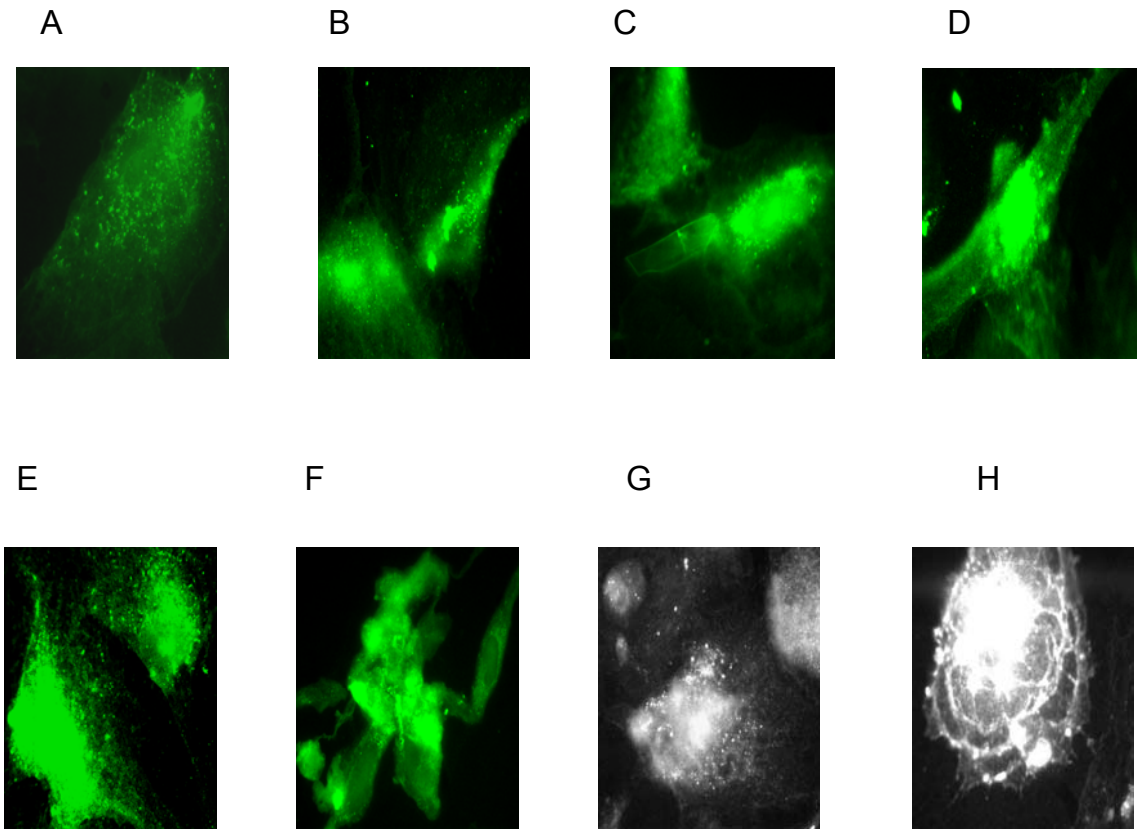


Figure 17. VCAM expression in human astrocytes. The expression of VCAM results in the immunofluorescence of FITC-conjugated Ab to VCAM receptors. A. Control cells (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml LPS. F. 250μM H_2O_2. G. 100ng/ml SH + LPS H. 100ng/ml SH + H_2O_2.

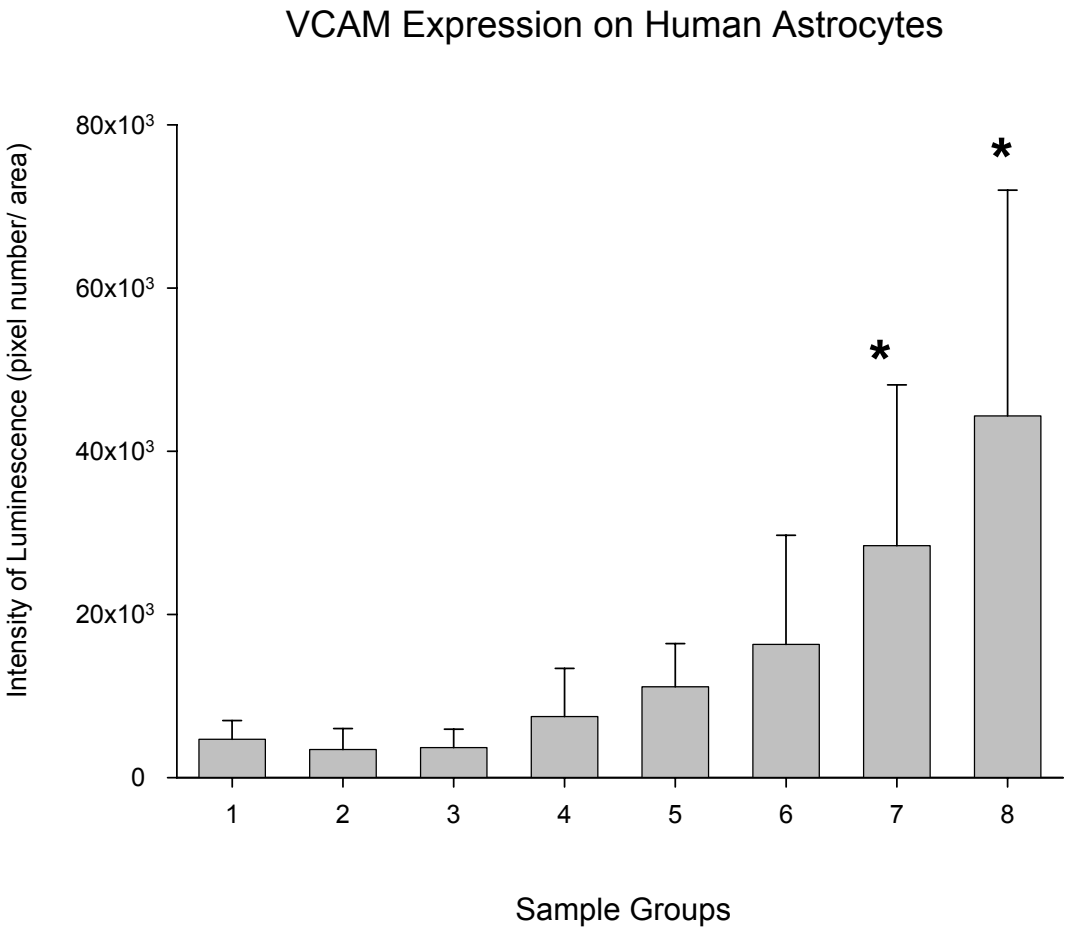


Figure 18. Densitometric evaluation of VCAM expression in human astrocytes. The expression of VCAM results in the immunofluorescence of FITC-conjugated Ab to VCAM receptors. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 100ng/ml SH + LPS 8. 100ng/ml SH + H_2O_2. An (*) indicates a significant increase ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

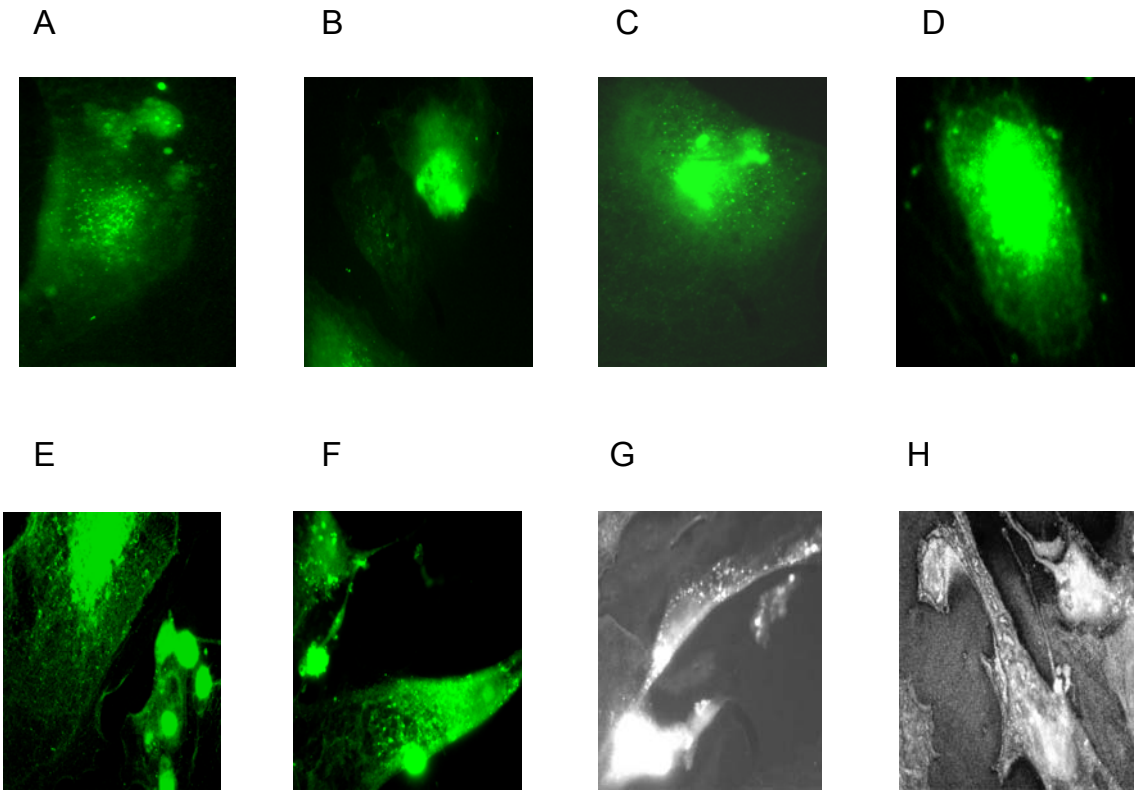


Figure 19. P/E Selectin expression on human astrocytes. The expression of P/E results in the immunofluorescence of FITC-conjugated Ab to P/E Selectin receptors. A. Control cells (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml LPS . F. 250μM H_2O_2. G. 100ng/ml SH + LPS. H. 100ng/ml SH + H_2O_2.

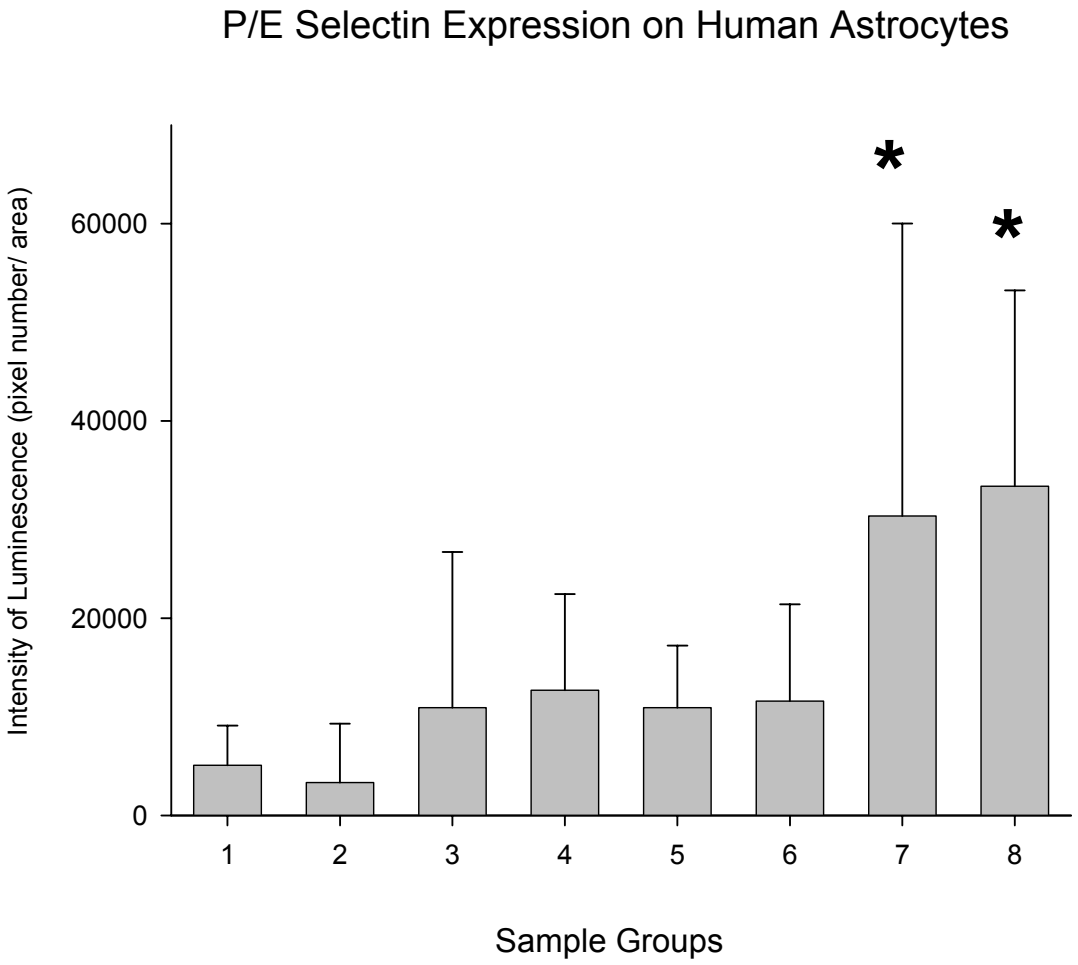
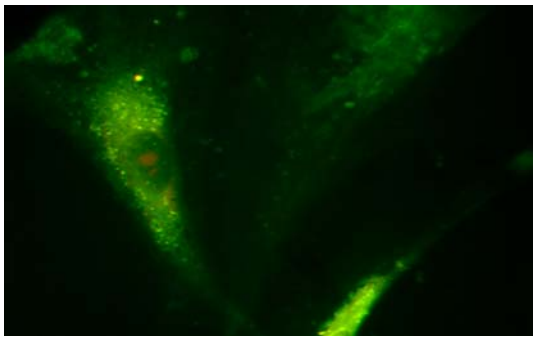


Figure 20. Densitometric evaluation of P/E Selectin expression in human astrocytes. The expression of P/E Selectin results in the immunofluorescence of FITC-conjugated Ab to P/E Selectin receptors. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 100ng/ml SH + LPS 8. 100ng/ml SH+ H_2O_2. An (*) indicates a significant increase ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

A



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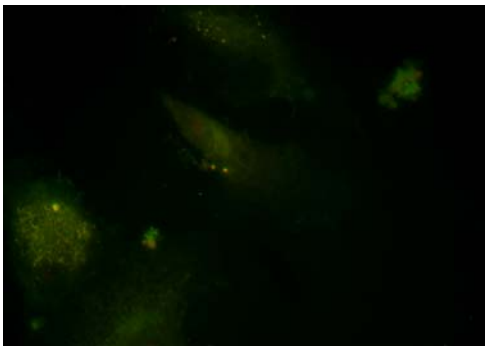

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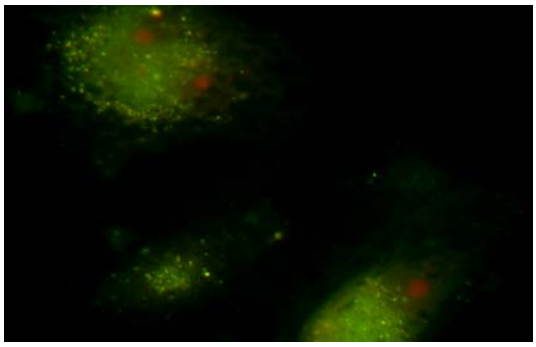

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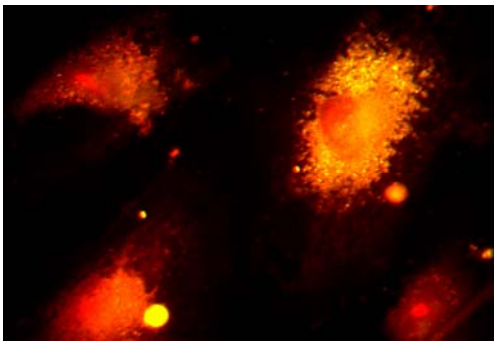

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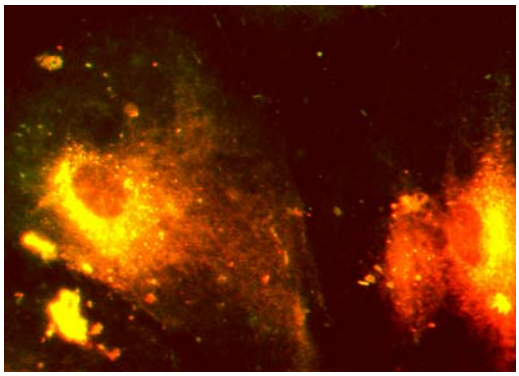

Figure 21. Evaluation of human astrocytes for apoptosis. Annexin V-FITC staining of PS (green) and PI staining of DNA (red). A. Control (H_20). B. 10ng/ml satratoxin H (SH). C. 100ng/ml SH. D. 1000ng/ml SH. E. 50 EU/ml LPS.

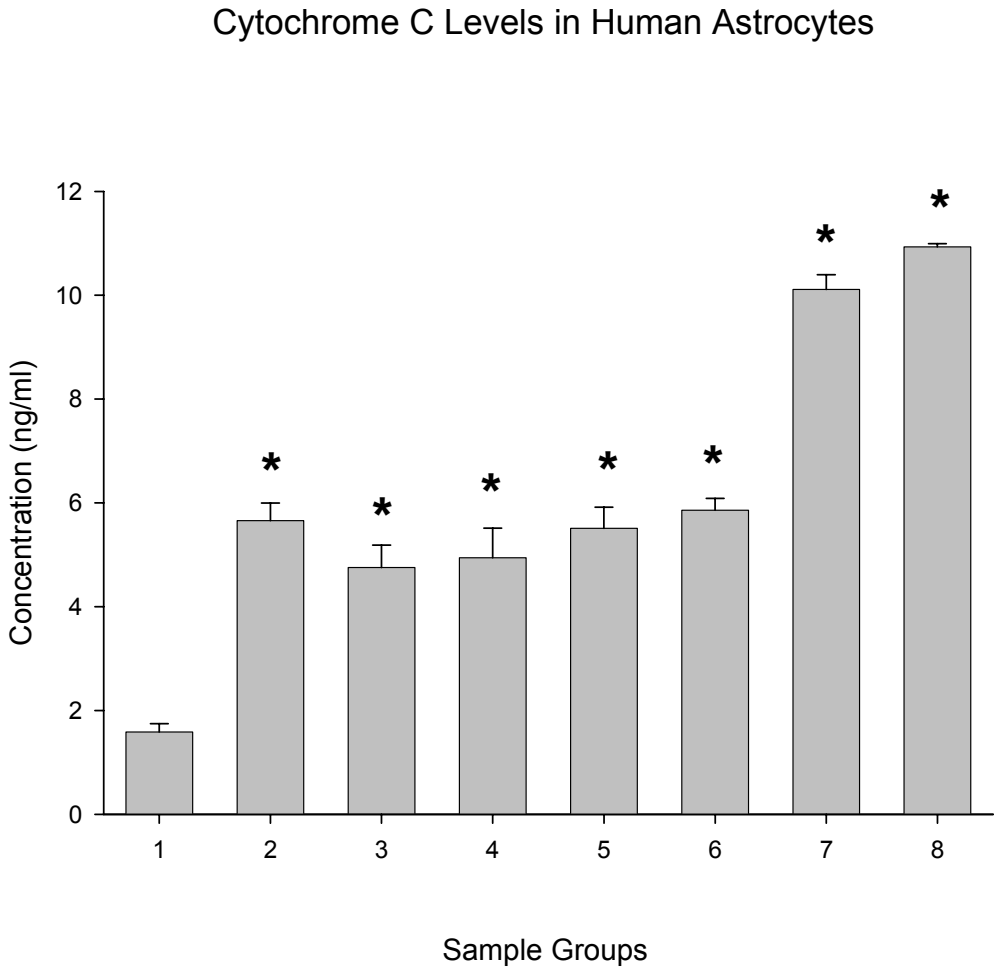

Figure 22. Cytochrome C concentrations from human astrocytes. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml LPS. 6. 250μM H_2O_2. 7. 100ng/ml SH + LPS 8. 100ng/ml SH+ H_2O_2. An (*) indicates a significant increase ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

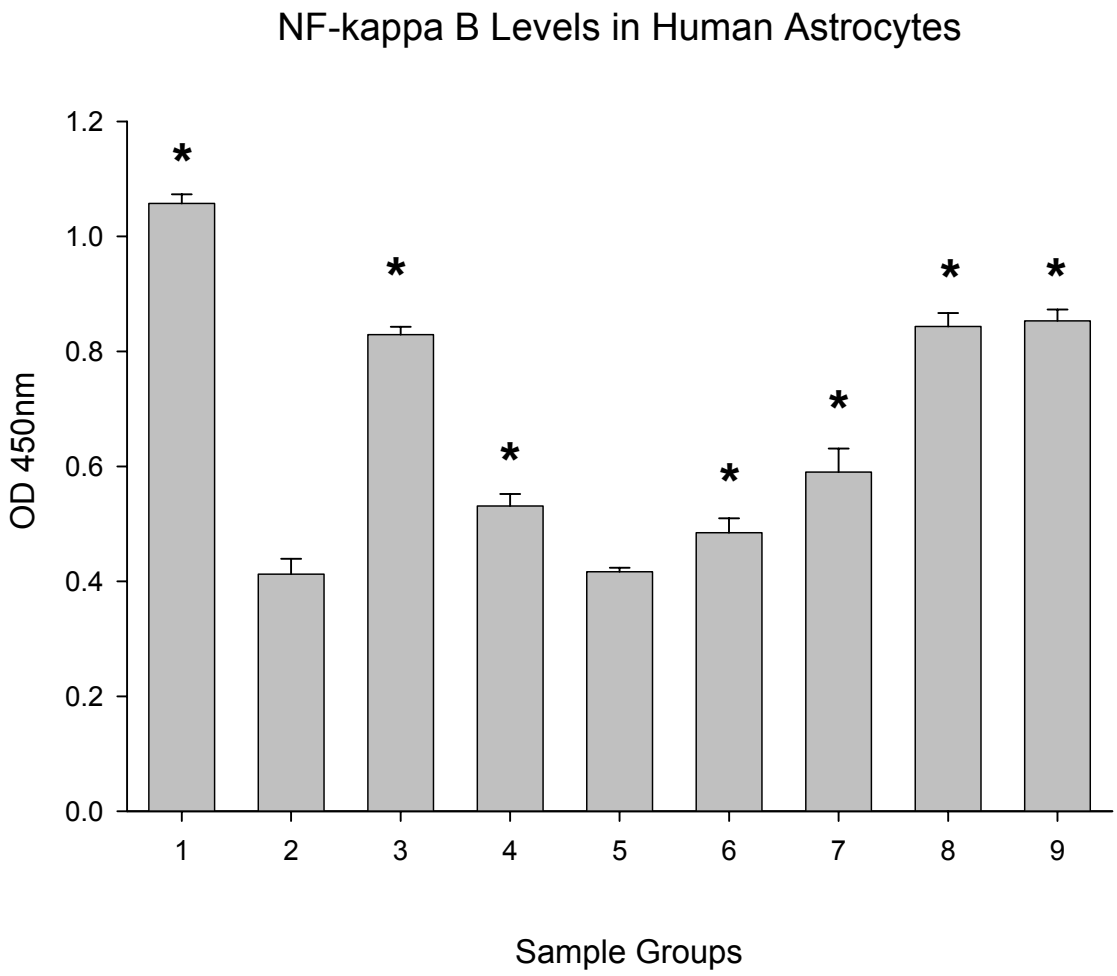

Figure 23. NF-κB concentrations from human astrocytes. 1. Positive control (HeLa cell extract) 2.Control cells (H_20). 3. 50 EU/ml LPS. 4. 250μM H_2O_2. 5. 10ng/ml satratoxin H (SH). 6. 100ng/ml SH. 7. 1000ng/ml SH. 8. 100ng/ml SH + LPS 9.100ng/ml SH + H_2O_2. An (*) indicates a significant increase (P< 0.5) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compared the experimental groups with the control.

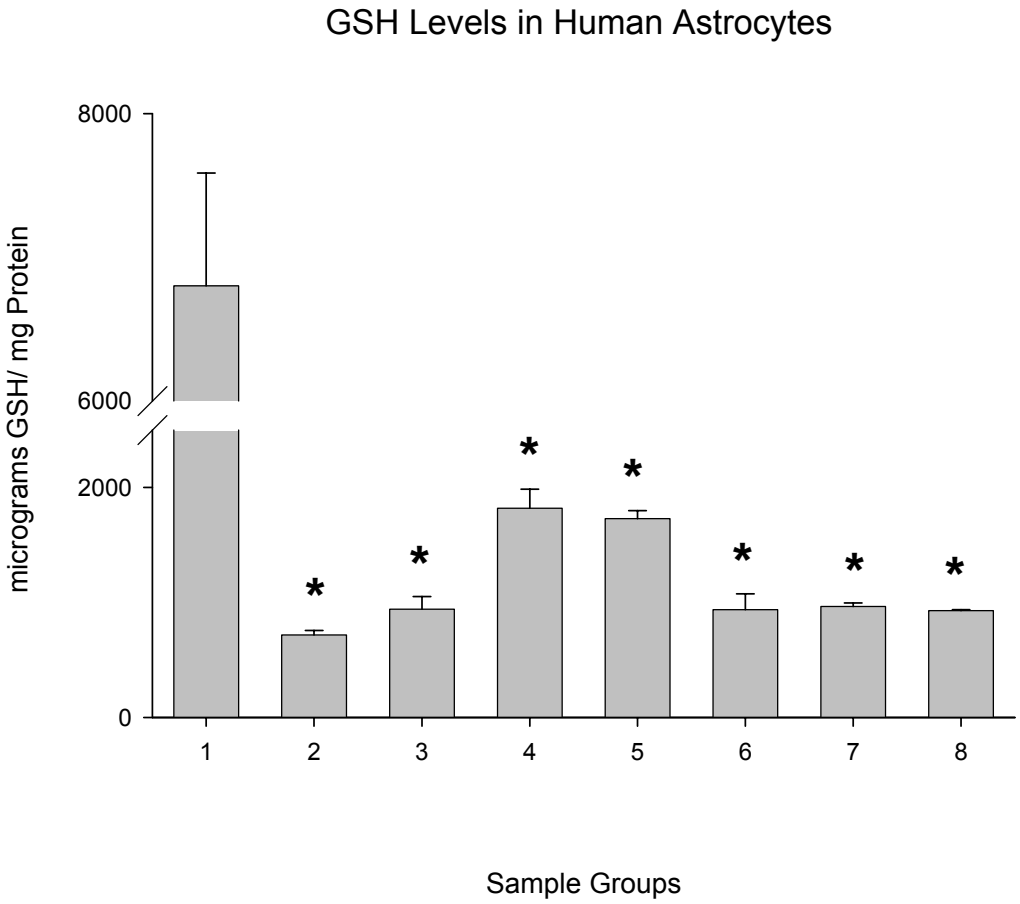

Figure 24. GSH concentrations from human astrocytes. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml LPS. 6. 250μM H_2O_2. 7. 100ng/ml SH + LPS 8. 100ng/ml SH + H_2O_2. An (*) indicates a significant decrease (P> 0.05) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compared the experimental groups with the control.

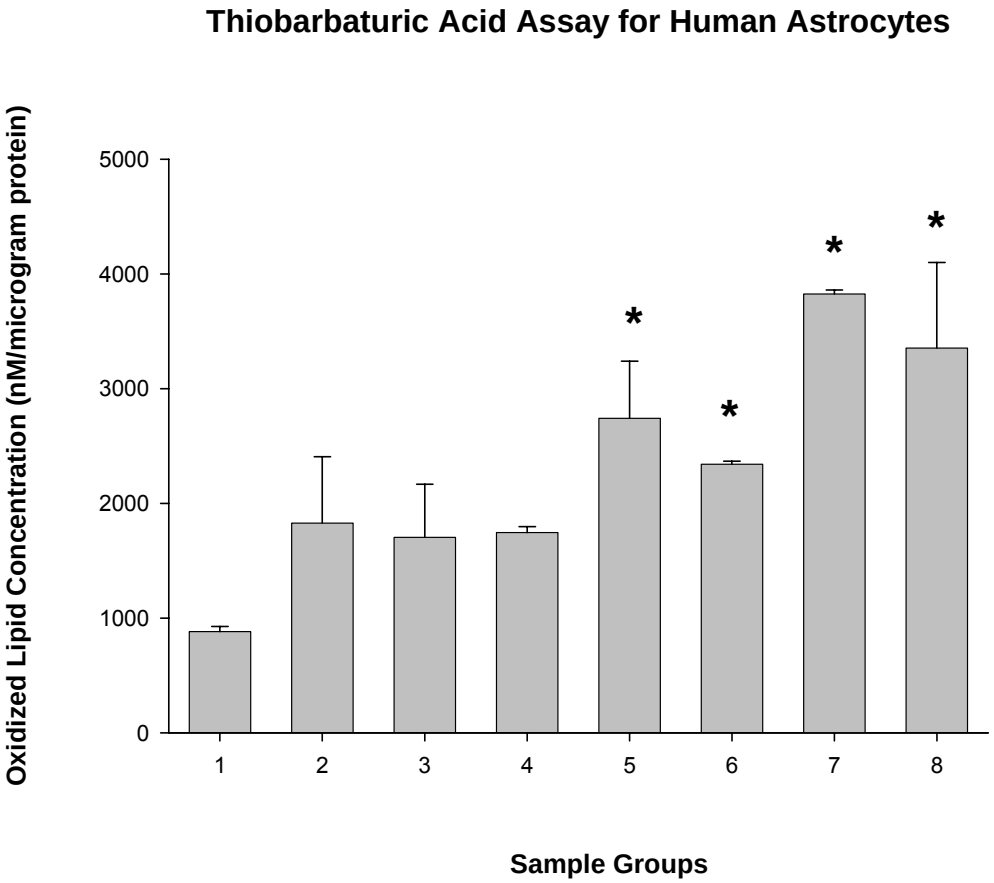

Figure 25. T-BARS assay for human astrocytes. The degree of lipid oxidation induced in cells was quantitated by measuring the levels of lipid radicals from samples. 1. Control (H_2O) 2. LPS (50 EU/ml) 3. H_2O_2 (250μM). 4. 10ng/ml SH 5. 100ng/ml SH 6. 1000ng/ml SH. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 532nm. (*) indicates a significant difference ($P < 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

CONCLUSIONS

These studies demonstrate that mycotoxins at moderate concentrations and under additive conditions are able to produce cytotoxic events. These results demonstrate that direct exposure of astrocytes to satratoxin H at low to moderate concentrations alone do not produce a strong inflammatory response as evidenced by no significant increase in ICAM, VCAM, and P/E selectin as well as NF-κB expression. However, these results do demonstrate an additive effect in the expression of inflammatory events with a moderate dose (100ng/ml) of satratoxin H in the presence of other inflammatory compounds (LPS) or under oxidative stress conditions.

Early and late apoptotic events are evidenced with moderate levels of satratoxin H (100ng/ml and 1000ng/ml), as demonstrated by the Annexin V assay for apoptosis. In addition, the evaluation of cytochrome C and GSH levels demonstrate that moderate concentrations of SH alone and under additive conditions of pro-inflammatory compounds and oxidative stress, produce significantly higher levels ($P>0.05$) of apoptosis in astrocytes.

These results demonstrate that moderate levels of mycotoxins are able to activate inflammatory pathways in astrocytes under additive conditions, which could lead to increased expression of inflammatory compounds by astrocytes. This would increase the production of ROS, such as lipid radicals through lipid peroxidation, under oxidative stress conditions, which would lead to cell damage.

Further evaluation of these data also demonstrates that astrocytes significantly increase (P> 0.05) the expression of apoptotic events, alone and under additive conditions. The production of ROS can further cause cell damage under conditions of continuous inflammatory and apoptotic pathway activation.

These results further suggest that the production of pro-inflammatory and apoptotic compounds by astrocytes, when released in the environment of neural tissues, could activate HBCEC and neurons leading to inflammation and apoptosis. These studies propose that under SBS conditions, individuals exposed to satratoxin H and microbial organisms in the environment over a prolonged period could have increased sensitivity to these agents, leading to neural damage.

REFERENCES

1. Campbell I.L. 1995. Neuropathogenic actions of cytokines assessed in transgenic mice. Int J Devl Neurosci. 13: 275-284.

2. Rubin L.L., Staddon J.M. 1999. The cell-biology of the blood-brain barrier. Annu Rev Neurosci. 22:11-28.

3. Ballabh P., Braun A., Nedergaard M. 2004. The blood-brain barrier: an overview structure, regulation, and clinical implications. J Neurobio Dis. 16: 1-13.

4. Dong W., Azcona-Olivera J.I., Brooks K.H., Linz J.E., Pestka J.J. 1994. Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. Tox Appl Pharm. 127: 282-290.

5. Marin M.L., Murtha J., Dong W., Pestka J.J. 1996. Effects of mycotoxins on cytokine production and proliferation in EL-4 thymoma cells. J Tox Environ Hlth. 48: 379-396.

6. Lee M.G., Li S., Jarvis B.B., Pestka J.J. 1999. Effects of satratoxins and other macrocyclic trichothecenes on IL-2 production and viability of EL-4 thymoma cells. J Tox Environ Hlth. 57: 459-474.

7. Yang G.H, Jarvis B.B., Chung Y.J., Pestka J.J. 2000. Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. Tox Appl Pharm. 164: 149-160.

8. Sugita-Konishi Y, Pestka J.J. 2001. Differential upregulation of TNF-alpha, IL-6, and IL-8 production by deoxynivalenol (vomitoxin) and other 8-ketotrichothecenes in a human macrophage model. J Toxicol Environ Hlth. 64: 619-636.

9. Uzarski R.L., Zahidul I, Pestka J.J. 2003. Potentiation of trichothecene-induced leukocyte cytotoxicity and apoptosis by TNF-α and Fas activation. J Toxicol Environ Hlth.146: 105-119.

10. Zhou H.R., Harkema J.R., Yan D., Pestka J.J. 1999. Amplified proinflammatory cytokine expression and toxicity in mice coexposed to lipopolysaccharide and the trichothecene vomitoxin (deoxynivalenol). J. Toxicol Environ Hlth. 56: 115-136.

11. Fellin T., Carmignolo G. 2004. Neurone-to-astrocyte signaling in the brain represents a distinct multifunctional unit. J Physiol. 559: 2-15.

12. Morest D.K., Silver J. 2003. Precursor of neurons, neuroglia, and ependymal cells in the CNS: What are they? Where are they from? How do they get where they are going? Glia. 43: 6-18.

13. Simoni J, Simoni G, Lox C.D., Prien S.D., Shires G.T. 1995. Evidence for the direct inhibition of endothelin-1 secretion by hemoglobin in human endothelial cells. ASAIO. 41: 641-51.

14. Simoni J, Simoni G, Martinez-Zaguilan R., Wesson D.E., Lox C.D., Prien S.D., Kumar R.V. 1998. Improved blood substitute: Evaluation of its effects on human endothelial cells. ASAIO. 44: 356-67.

15. Simoni J, Simoni G, Wesson D., Griswold J.A., Feola M. 2000. A novel hemoglobin-adenosine-glutathione based blood substitute: evaluation of its effects on human blood ex vivo. ASAIO.46: 679-92.

16. Simoni J, Simoni G, Garcia E.L, Prien S.D., Tran R.M., Feola M, Shires G.T. 1995. Protective effect of selenium on hemoglobin mediated lipid peroxidation in vivo. Artif Cells Blood Substit Immobil Biotechnol. 23: 469-86.

CHAPTER V

THE TOXOLOGICAL EFFECTS OF SATRATOXIN H ON HUMAN PROGENITOR NEURAL CELLS

Neurons develop from the same cells as neuroglia in the CNS [1]. However, neurons and glial cells are distinguished by their physiology [1]. Neurons have polarized extensions, such as the axon and dendrites [1]. In addition these cells are able to propagate an action potential, corresponding with other neurons with the use of neurotransmitters at synaptic junctions [1]. Glial cells and ependymal cells of the CNS do not have axons or dendrites [1]. During neuroinflammation, neurons tend to be damaged by immune responses and studies have demonstrated that the reaction of astrocytes and endothelial cells amplify CNS damage [2].

Neural damage can lead to permanent physiological effects. Studies conducted on students who were exposed to poor IAQ due to fungal contamination, demonstrated acoustic mycotic neuroma. Initial symptoms associated with this condition included sensorineural hearing loss, tinnitus, and unsteadiness [3]. This condition is associated with hearing loss and tumor development in nerve tissues associated with hearing and this tumorous tissue must be removed through surgical techniques [3]. This syndrome is normally seen in the elderly population, however in one study, adolescent students were

found to have high levels of this condition [3]. In addition, these individuals had other symptoms associated with neurological damage from SBS conditions, such as headaches, memory loss, and lack of concentration, fatigue, sleep disturbance, facial swelling, rashes, nosebleeds, diarrhea, abdominal pains and respiratory problems [4].

The purpose in evaluating neural cells was to determine if satratoxin H would produce toxic events in these cells. Neural cells are unable to repair extensive cellular damage in the event of cytotoxic events that induce apoptosis or severe inflammatory events. Thus, the objective of these experiments was to evaluate, if in the event that macrocyclic trichothecenes such as satratoxin H were able to enter neural cells, the effects these toxins would have on cellular homeostasis. The purpose of these experiments was to determine if neural damage could be induced by macrocyclic trichothecenes at the low-doses that may be present in SBS conditions.

MATERIALS AND METHODS

Neurons were obtained from human neural progenitor cells (Clonetics Co., San Diego, CA) cultured on polyethylenenimine coated plates, using NPMM™ BulletKit® medium (Cambrex-Biowhittaker, Walkersville, MD). Cells were sub-cultured in a series of 12-well plates until confluent in a humidified atmosphere of 5% CO_2, until they reached confluence.

Experiments were conducted on cells from either a fourth or fifth passage. The cell lines were tested by the supplier for human immunodeficiency virus (HIV), hepatitis, *Mycoplasma*, bacteria, yeast, fungi, and smooth muscle α-actin expression. The results of these tests were presented in Clonetics Certificate of Analysis.

Prior to experimentation, satratoxin H samples were tested for endotoxin contamination utilizing the QCL-1000 assay (Cambrex-Biowhittaker, Inc., Walkersville, MD). If endotoxin removal was required, an affinity chromatography method was utilized according to the manufacturer protocols for Detoxi-Gel Endotoxin Removal Gel AffinityPak Prepacked Columns (Pierce, Rockford, IL), followed by further evaluation using the QCL-1000.

In the experiments conducted here 1, 10, 100, 1000, and 5,000 ng/ml concentrations of satratoxin H were evaluated. Prior to the addition of the compounds, the media in 24-well plates were replaced with fresh medium. The samples were then suspended in pyrogen-free, sterile water (Cambrex-Biowhittaker, Inc., Walkersville, MD) and incubated with satratoxin H for 18 hours. Control cells received an equal volume of water. A second subset of cells were exposed to the concentrations of satratoxin H previously mentioned with the addition of 250μM H_2O_2 and 50 EU/ml LPS to induce an inflammatory response.

Lactate dehydrogenase (LDH) concentrations were used to determine the cytotoxic effects of satratoxin H on human neural cells. A commercial assay, LDH (DG-1340-UV) concentration assay (Sigma Diagnostics), was utilized to

determine the concentration of LDH released by cells into the culture medium after an 18h incubation period. The procedures described in the assay were followed to determine the concentration of LDH released by cells due to cellular damage. Samples were read spectrophotometrically at 340nm. This assay reflects cellular damage induced by satratoxin H.

To quantitate the degree of apoptosis in cells, an ELISA method was used to determine the levels of cytochrome C from cellular extracts. Cytochrome C is maintained in the mitochondria of healthy cells. In the event of programmed cell death, cytochrome C is released from the mitochondria into the cytosol where it activates pathways associated with apoptosis. The ELISA for the evaluation of cytochrome C was conducted according to protocols described in the manual provided by R&D Systems Inc.

In a normal cell GSH is unable to cross the nuclear membrane, but during oxidative stress the oxidized form of GSH, oxidized glutathione (GSSG), is able to cross this structure. GSSG is able to decrease the binding of p60/p65 complex of NF- κB to DNA, which reduces the pro-inflammatory cascade that is activated by NF- κB. The intracellular GSH levels were measured from cell homogenates [5]. The reaction of GSH with 5, 5' dithiobis and sulfhydryl compounds leads to a color change, producing a yellow pigment. The samples were read spectrophotometrically at 412nm (Sigma Chemical Co., St Louis, MO). Results were compared to a standard curve using GSH in nanomoles per milligram of

protein. Protein concentrations for the cell homogenate fractions were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

During an inflammatory response, cells are under oxidative stress, which leads to the production of lipid radicals and lipid peroxidation. Previous studies have demonstrated that lipid radicals are able to inhibit anti-apoptotic genes which allow a cell to enter into apoptosis. In these experiments, the presence of oxidative stress was evaluated by measuring lipid hyperperoxides (LOOH) and reduced glutathione (GSH) levels according to established methods [5-8].

LOOH were evaluated in cell homogenate fractions with a thiobarbaturic acid assay (T-BARS). This assay is able to directly measure the LOOH concentration. Samples were mixed with SDS-acetate buffer, pH3.5, and aqueous solution of thiobarbituric acid. The reaction mixture was heated at 95˚C for 90 minutes [5-8]. After cooling, the red-colored complex was extracted with n-butanol-pyridine. The data were measured spectrophotometrically at 500nm, and compared to a standard curve [5-8]. Results were expressed in nanomoles of LOOH per microgram protein [5-8].

Statistical analysis ($\alpha = 0.05$) was performed using Sigma Stat, a statistical software program designed by Jandel (SPSS), to analyze the data using one-way analysis of variance (ANOVA) of each experimental group with the controls. Normality of the data was determined using a Kolmogorov-Smirnov normality test. If data did not meet normality, a non-parametric Kruskal-Wallis ANOVA would have been applied. If data were normal, and ANOVA demonstrated

significance, a post hoc test, Tukey test (a modified t test), was used to make multiple comparisons to determine which experimental groups demonstrated significance compared to the controls and between experimental groups. Results were graphed using Sigma Plot, a graphical program designed by Jandel (SPSS).

RESULTS

Results from the LDH assay demonstrate a significant increase ($P<0.05$) in the release of LDH by neurons exposed to all of the experimental conditions except LPS, H_2O_2, and 10ng/ml SH. Moderate concentrations of SH and additive conditions demonstrated cytotoxic events as measured by the concentration of LDH released into the media compared to control conditions. These results can be seen in Figure 26.

Cytochrome C levels from cell extracts were evaluated using an ELISA method. These results demonstrated that a significant amount ($P< 0.05$) of cytochrome C was released from cells exposed to all of the experimental conditions compared to control conditions (H_20). These data are presented in Figure 27.

An additional indicator of apoptosis is oxidative stress. In the event of oxidative stress, glutathione (GSH) acts as a reducing agent against reactive oxygen species (ROS) such as lipid radicals and peroxides. However, if GSH levels in a cell are insufficient to compensate for the degree of oxidative stress, both apoptotic and inflammatory pathways are further activated. To determine whether mycotoxins increased oxidative stress levels in astrocytes, an ELISA

method was used to quantitate the levels of GSH present in cell extracts exposed to various experimental conditions. These data demonstrated that compared to control conditions there was significantly less ($P>0.05$) GSH from cell extracts exposed to the experimental conditions previously described. These data are presented in Figure 28.

During the event of oxidative stress, ROS such as lipid radicals are produced due to lipid peroxidation. To quantitate the degree of lipid peroxidation, a T-BARS assay was conducted to measure the concentration produced by cells exposed to the previously described experimental conditions. The data demonstrated that neurons exposed to all of the experimental conditions produced significant levels ($P<0.05$) of lipid peroxidation compared to the control conditions. These results can be seen in Figure 29.

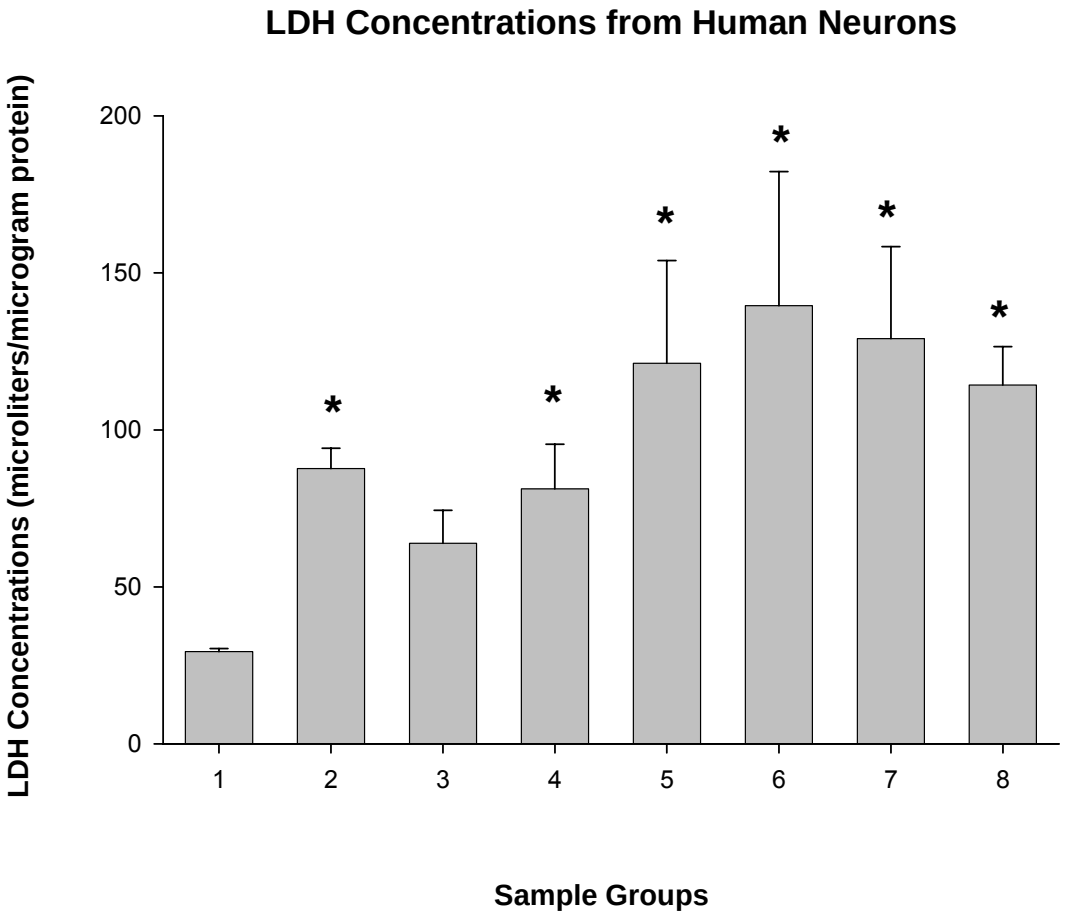

Figure 26. LDH assay for human neurons. The degree of cytotoxicity induced in cells was quantitated by measuring the levels of LDH released from cells into the supernatant. 1. Control (H_2O) 2. LPS (50 EU/ml) 3. H_2O_2 (250μM). 4. 10ng/ml SH 5. 100ng/ml SH 6. 1000ng/ml SH. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 340nm. (*) indicates a significant difference ($P < 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

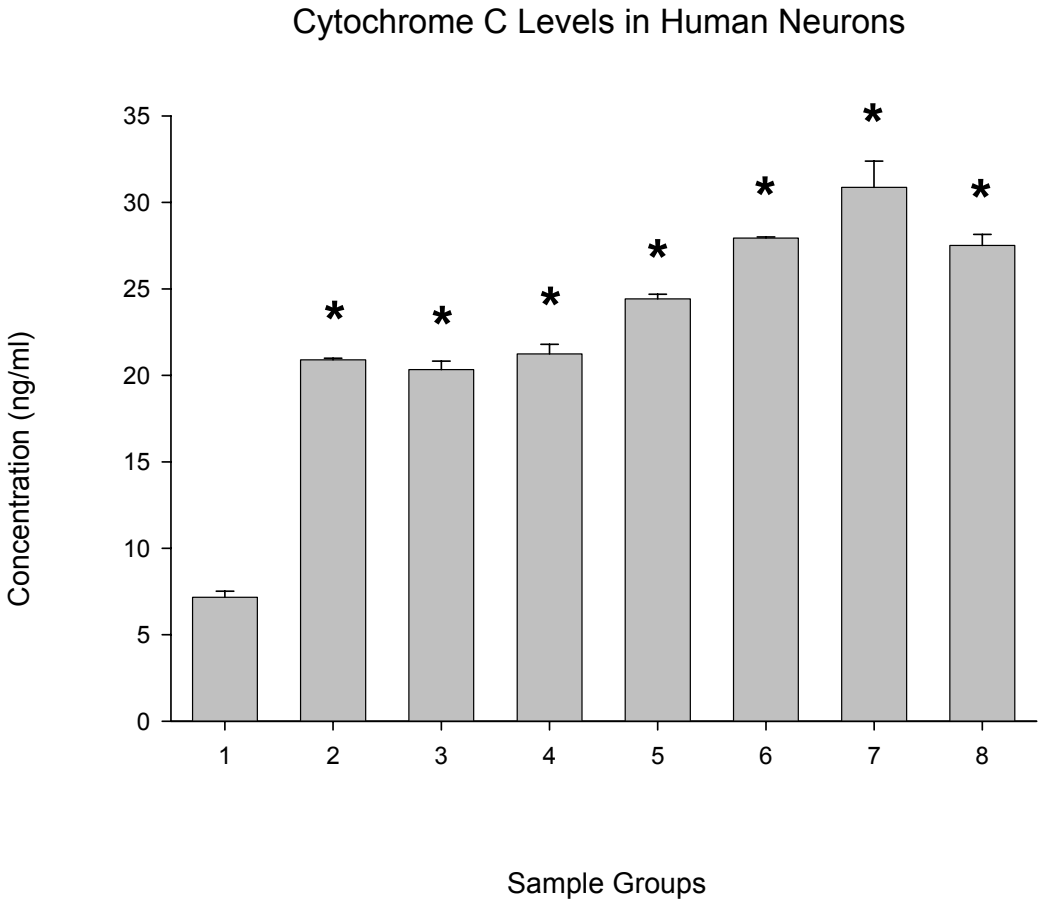

Figure 27. Cytochrome C concentration from human neurons. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250µM H_2O_2. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant increase ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

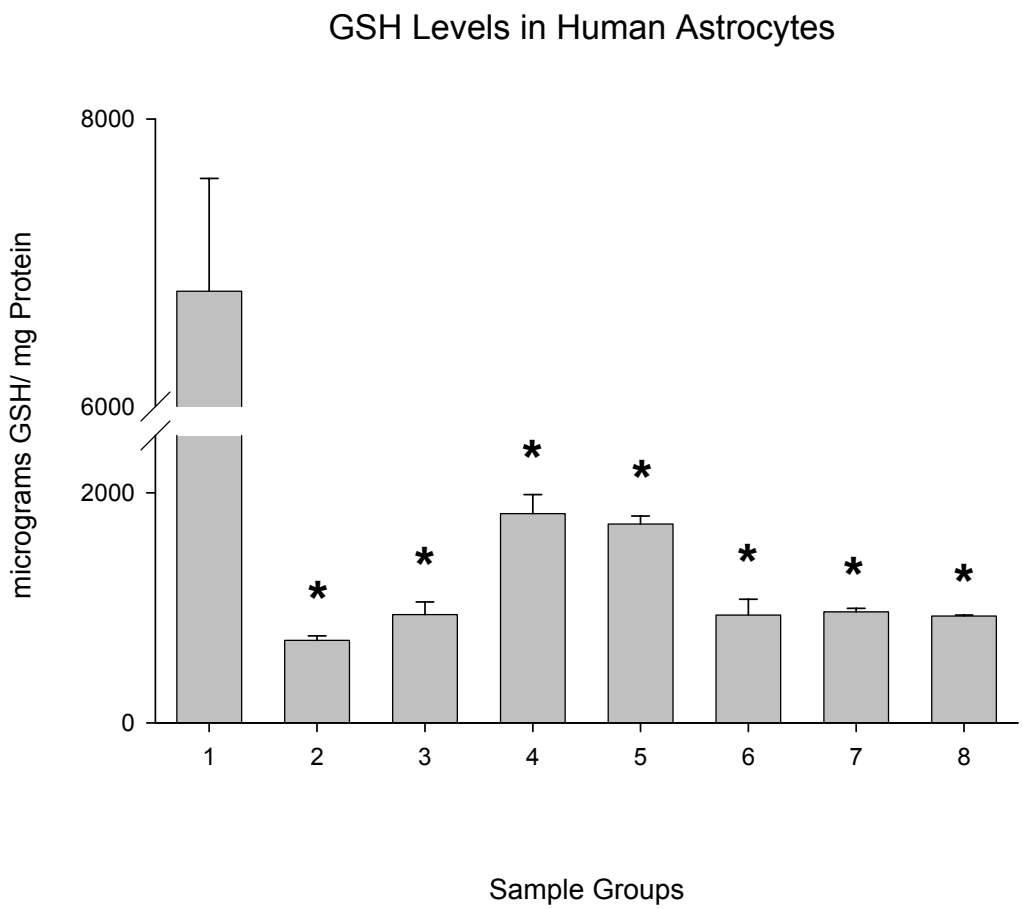

Figure 28. GSH concentrations from human neurons. 1. Control cells (H_20). 2. 10ng/ml satratoxin H (SH). 3. 100ng/ml SH. 4. 1000ng/ml SH. 5. 50 EU/ml. 6. 250μM H_2O_2. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. An (*) indicates a significant decrease (P> 0.05) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized (P<0.05) to compared the experimental groups with the control.

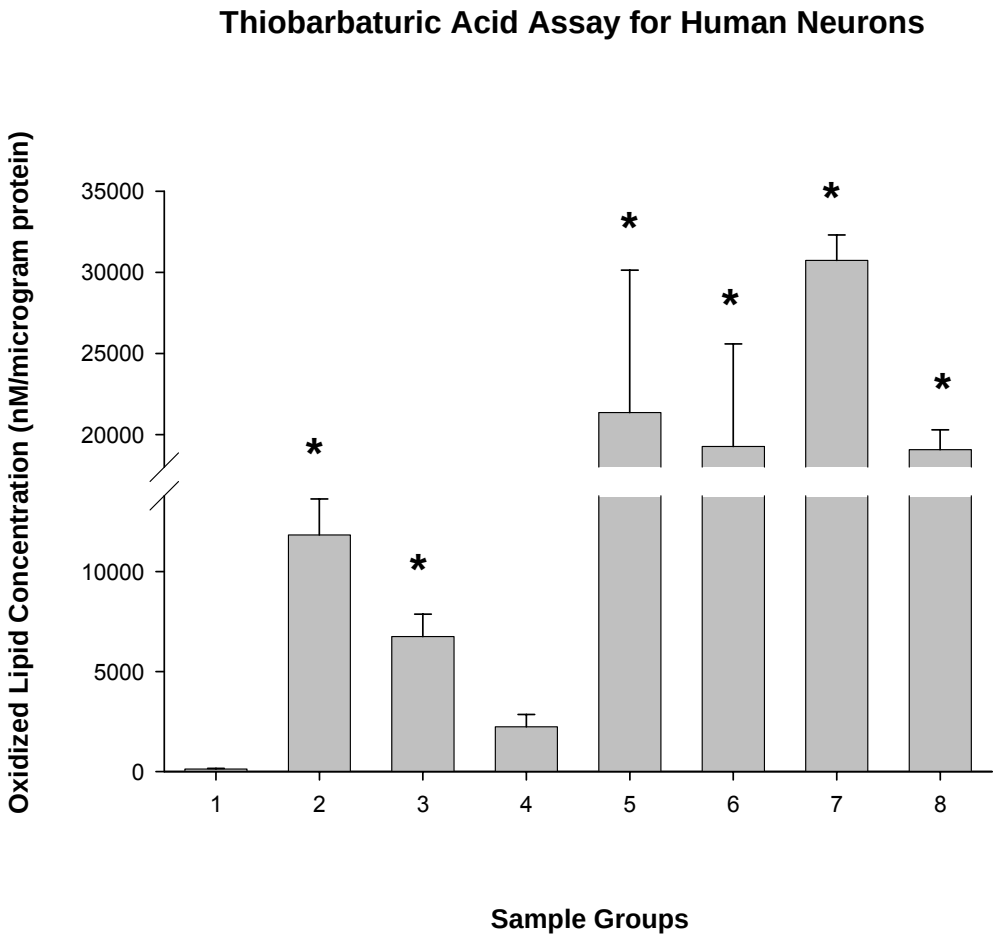

Figure 29. T-BARS assay for human neurons. The degree of lipid oxidation induced in cells was quantitated by measuring the levels of lipid radicals from samples. 1. Control (H_2O) 2. LPS (50 EU/ml) 3. H_2O_2 (250μM). 4. 10ng/ml SH 5. 100ng/ml SH 6. 1000ng/ml SH. 7. 10ng/ml SH + LPS 8. 10ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 532nm. (*) indicates a significant difference ($P< 0.05$) compared to the control. An all pairwise mutli-comprison procedure (Tukey test) was utilized ($P<0.05$) to compared the experimental groups with the control.

CONCLUSIONS

These results demonstrate that regardless of whether the neurons were exposed to satratoxin H alone or under additive effects, the sensitivity of the neurons to these compounds is high. These studies demonstrate that direct contact of neurons with satratoxin H leads to apoptotic events that produce cell death. These data suggest that under SBS conditions, low to moderate concentrations of satratoxins may produce neurological damage via programmed cell death. The data further suggest that satratoxins alone, and additive conditions of low doses of satratoxin H under oxidative stress conditions or inflammatory agents, can lead to permanent neurological damage.

REFERENCES

1. Morest D.K., Silver J. 2003. Precursors of neurons, neuroglia, and ependymal cells in the CNS: What are they? Where are they from? How do they get where they are going? Glia. 43: 6-18.

2. Chavarria A., Alcocer-Varela J. 2004. Is damage in central nervous system due to inflammation? Autoimmun Rev. 3: 251-260.

3. Ceccatelli S., Tamm C., Sleeper E., Orrenius S. 2004. Neural stem cells and cell death. Tox Letters. 149: 59-66.

4. Anyanwu E., Campbell A.W., High W. 2002. Brainstem auditory evoked response in adolescents with acoustic mycotic neuroma due to environmental exposure to toxic molds. Int J Adolesc Med Hlth. 14: 67-76.

5. Simoni J, Simoni G, Lox C.D., Prien S.D., Shires G.T. 1995. Evidence for the direct inhibition of endothelin-1 secretion by hemoglobin in human endothelial cells. ASAIO. 41 (3): 641-51.

6. Simoni J, Simoni G, Martinez-Zaguilan R., Wesson D.E., Lox C.D., Prien S.D., Kumar R.V. 1998. Improved blood substitute: Evaluation of its effects on human endothelial cells. ASAIO. 44: 356-67.

7. Simoni J, Simoni G, Wesson D., Griswold J.A., and Feola M. 2000. A novel hemoglobin-adenosine-glutathione based blood substitute: evaluation of its effects on human blood ex vivo. ASAIO.46: 679-92.

8. Simoni J, Simoni G, Garcia E.L, Prien S.D., Tran R.M., Feola M, Shires G.T. 1995. Protective effect of selenium on hemoglobin mediated lipid peroxidation in vivo. Artif Cells Blood Substit Immobil Biotechnol. 23 : 469-86.

CHAPTER VI

OVERALL CONCLUSIONS

Several conclusions can be drawn from the overall study of each of these cell lines. The experiments conducted on the HBCEC demonstrate that in the event of exposure to trichothecene mycotoxins such as satratoxin H, low to moderate doses of these compounds are able to activate immunological pathways in these cells. Under additive events, these compounds can further stimulate the production of significant pro-inflammatory pathways and apoptotic pathways at low levels on a continuous basis. The activation of these pathways on a constant basis can shift the homeostasis of these cells, leading to changes in the integrity of the BBB. These results have demonstrated that low to moderate levels of satratoxin can cause cell shrinkage in HBCECs leading to the compromise of the BBB. The loss of activity of the BBB due to satratoxin exposure could potentially amplify the damage to the neural tissue in multiple ways. The BBB prevents other toxic substances and microorganisms from invading the neural tissues. If an individual is exposed to low to moderate levels of satratoxin on a regular basis under SBS conditions, this could increase the potential risk of neurological damage due to the constant activation of cellular pathways in the body, however other harmful agents would also be able to pass through a damaged BBB.

In the event that HBCECs are damaged by trichothecenes such as satratoxin H, these mycotoxins and other agents would then come in contact with astrocytes. Since astrocytes behave as macrophages, the production of reactive oxygen species, cytokines, chemokines, and other pro-inflammatory agents released into the environment of these tissues due to the additive effects of moderate doses of satratoxin in the presence of other agents or oxidative stress, could overwhelm the astrocytes themselves leading to programmed cell death. In addition, the release of these agents into the neural tissues could further activate HBCECs and neurons leading these cells to enter programmed cell death or produce increase inflammatory events. This could lead to devastating effects especially in neurons, since astrocytes initiate protective mechanisms to prevent neurons from going into apoptosis. However, under SBS conditions, if individuals were constantly being exposed to satratoxins that overwhelmed the astrocytes, the protective compounds produced by astrocytes for neurons, such as erythropoietin, which prevents neurons from going into apoptosis, would be compromised. These events could then lead to programmed cell death in neurons. In the event of programmed cell death in neurons, these cells are not regenerated in adult neural tissues, which could lead to the permanent neurological damage witnessed in individuals exposed to *S. chartarum* in SBS conditions.

This study demonstrates that tissue damage can occur from satratoxin H exposure to neurological tissues. This study suggests that it is the constant activation of inflammatory and apoptotic pathways at low levels in these tissues, that amplifies the devastation in these tissues, and leads to neurological damage due to indirect events triggered by the presence of trichothecenes.