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Modeling Brain Injury: Expression of Glial Fibrillary Acidic Protein in Stretch-Injured Astrocytes

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Abstract

Traumatic brain injury (TBI) is the leading cause of death and disability in individuals under 45. Individuals who survive TBI may be disabled for the rest of their lives and suffer from cognitive, physical, social and financial problems. Following TBI it has been shown that astrocytic gene expression of many proteins, including glial fibrillary acidic protein (GFAP), increases. These "reactive" astrocytes are thought to have both beneficial and detrimental effects on neuronal survival and function. The current study uses an *in vitro* model of injury which grows astrocytes on deformable silastic membranes to allow for dynamic stretch of cultured cells and tries to mimic the stress and strain on brain tissue that is seen following human TBI. The current study of reactive gliosis is two-fold: 1) a stretch injury response curve (3, 4, 5, 6, and 7mm at 24 and 48 hr after injury) and 2) a stretch injury time course (0.25, 2, 6, 24 and 48 hr after injury at 6mm). Glial fibrillary acidic protein expression, a marker for reactive gliosis, was measured by Western blotting and immunofluorescence techniques. Immunofluorescence showed that GFAP expression increased and astrocytes became more reactive as the magnitude of stretch injury increased, and that cell morphology and GFAP expression varied at different times after injury. Western blotting was inconclusive as it was difficult to discern differences in GFAP expression between samples. It can be concluded that the stretch-injury model is a useful way to injure astrocytes and can be used in the future to mimic TBI in the "dish."

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability for individuals under the age of 45 years. It is estimated that every year in the United States at least 1.4 million people sustain a TBI, and 50,000 Americans die from the condition ("Facts about," 2006). The major causes of TBI are vehicular and bicycle accidents, falls, violence, and recreational activities (Kreutzer et al., 1997). One of the highest risk factors for having a TBI is having had a previous injury because people with TBI may have problems controlling impulses and in thinking abilities which can lead to unsafe behaviors (Christensen, 2001a). According to Christensen (2001a), children from families with low socioeconomic backgrounds are at a greater risk for TBI, most likely due to a lack of access to preventative measures such as bicycle helmets and a shortage of safe play areas. Individuals who use alcohol and/or drugs and those with attention deficit disorders are also at a greater risk. It has also been found that boys are twice as likely as girls to sustain a TBI. There are two age groups that have the highest incidence for sustaining TBI: children/young adults and the elderly (Christensen, 2001a). The major cause for TBI within these age groups is falling. According to Kreutzer et al. (1997), within the young age group those most greatly affected are aged 15 to 24 and then aged 5 to 14. In 1993, 39% of all people treated at major trauma centers for brain injury were between the ages of 16 and 25 (Kreutzer et al., 1997).

Individuals who survive TBI may be disabled for the rest of their lives and suffer from low work or school productivity, social problems, and financial strains. ("Facts about," 2006) According to Kreutzer et al. (1997), "Estimates of unemployment following severe injury range as high as 70% within the first 7 years." Family adjustment problems are also common as the injured individual may not act like the same person as before and may now have special needs.

This can lead to the problems of frustration, depression, and social isolation in both the victim and caretakers. It has also been discovered that alcohol use is a "common factor among persons with brain injury, relevant to both cause of injury and post-injury adjustment" (Kreutzer, 1997).

"The most common, persistent, and problematic issues occurring after TBI are learning and behavioral problems directly related to the brain injury" (Christensen, 2001b). These problems are due to neurological complications. The first of these is post-concussion syndrome which is characterized by headache, dizziness, memory loss, frequent mood changes, sound sensitivity, difficulty concentrating, disinhibition, depression, and fatigue (Christensen, 2001b). Most of these symptoms subside or improve, but others may linger. Headaches are another problem and include migraines, tension headaches, and head and neck pain associated with whiplash from the injury. Seizures are not uncommon after TBI, but persistent seizures are uncommon and can develop into posttraumatic epilepsy (Christensen, 2001b). According to Christensen (2001b), the risk for developing posttraumatic epilepsy is 5-7% in closed head TBI and 11% in severe closed head TBI. However, the risk is 35-50% if the TBI is due to an open wound. Risk factors for post-traumatic seizures include focal injuries, wounds involving penetration of the dura mater, and severe rather than mild TBI (Christensen, 2001b). If an individual meets these criteria he or she is usually put on an antiepileptic drug. A very rare complication of TBI is hydrocephalus which is due to increased amounts of cerebrospinal fluid under increased pressure (Christensen, 2001b). This can occur when the brain shrinks in size after TBI due to the loss of many neurons. Cerebrospinal fluid takes up the excess space and only becomes a problem when the pressure increases. For individuals with hydrocephalus, a ventriculoperitoneal shunt can be inserted to drain the cerebrospinal fluid from the ventricles into the abdomen to relieve the pressure (Christensen, 2001b).

Motor impairment is another consequence of TBI, but it usually is not severe in most patients. Most severe TBI patients will move and react more slowly than prior to the injury but they will still retain function. Patients who have endured comas lasting a few weeks or more will usually have some type of obvious motor impairment, but a substantial amount of improvement can be made through physical therapy (Christensen, 2001b). High muscle tone is an issue for most severe TBI cases, but it can also be helped using physical therapy techniques such as stretching, splinting, and positioning of the limbs and body (Christensen, 2001b). Eating and drinking can also pose a problem after TBI for several reasons. First, the individual may have difficulty swallowing due to weakness and/or lack of coordination of the muscles involved in chewing and swallowing. This can lead to taking a very long time to eat and also having food go down the trachea instead of the esophagus. Feeding tubes can be used if the individual cannot eat at all or a special diet can be implemented until he or she regains sufficient muscle control to eat normal food (Christensen, 2001b).

In the weeks following TBI, hormone regulation may be affected since the brain regulates the structures which, in turn, regulate hormones (Christensen, 2001b). This may cause symptoms associated with thyroid problems, cessation of menstruation in women for 3-6 months, and/or too much or not enough urine production (Christensen, 2001b). The immune system may also be weakened after TBI and secondary infections such as pneumonia or oral thrush may manifest (Christensen, 2001b).

TBI can lead to an increased incidence of psychiatric issues which include depression and psychoses and also disinhibition, inattention, and impulsivity which can lead to secondary attention-deficit/hyperactivity disorder (Christensen, 2001b). Secondary AD/HD affects many

individuals, especially children, who have had a moderate to severe TBI. Stimulants such as Ritalin or Dexedrine can be given to improve attention (Christensen, 2001b).

"Memory problems have been identified as the most common and most persistent consequence of TBI" (Brady, 2001). Declarative memory—or memory for facts and experiences—is much more likely to be impaired than procedural memory, which is the memory for motor skills, conditioning, and that which is subconsciously retrieved (Brady, 2001). In declarative memory, old memories are often unaffected by TBI, but the retrieval of new and short-term memories and the learning of how to do new things is often hindered by TBI. (Brady, 2001)

Difficulty with the visual system is also a likely consequence of TBI because swelling, bleeding, or other sources of pressure put force on the cranial nerves—in particular the oculomotor (III), the trochlear (IV), and the abducens (VI). Problems that may result are ptosis (drooping of the eyelid), pupil dilation which causes difficultly in seeing things that are near, and strabismus which occurs when one eye is deviated upward, inward, and/or outward so that both eyes cannot focus on the same spot resulting in double vision (Brady, 2001). Usually these problems get better in the days following the injury due to decreased swelling and therefore decreased pressure on the cranial nerves. (Brady, 2001)

Visual perception problems may also arise from TBI. An afflicted individual may experience blind spots, but this is very uncommon in TBI as it is a consequence of localized injury to the visual primary zone (Brady, 2001). A more common problem is not being able to tell where one object ends and another begins, not being able to assimilate many features to recognize a whole, or not being able to recognize an object if it is at an unfamiliar angle or in silhouette (Brady, 2001). These problems worsen when the visual field has many stimuli such as

in a crowded area or a busy street. Visual-auditory integration problems may arise which lead to difficulty in naming something that is seen and in reading. Visual-motor integration is the ability to "see" a route or a picture and then carry it out (Brady, 2001). Complications in this area can manifest as problems in moving through a space or writing and drawing. Another uncommon problem that is associated with damage to the right side of the brain in the tertiary zone of the sensory unit is unilateral neglect (Brady, 2001). This phenomenon is when an individual neglects objects on the left side of the body and even the left side of the body itself. This can cause problems in reading, navigating the body through spaces, and even crossing the street due to neglecting to look left. (Brady, 2001)

One of the most common consequences of closed head TBI injury is executive function deficits. Executive function is a term for "the cluster of cognitive processes that are needed for organized, goal-directed behavior" (Brady, 2001). These functions are controlled by the frontal lobes which are responsible for sensory output. In closed head TBI the frontal lobes are almost always involved which affects them and also their pathways with almost all other regions of the brain due to the stretching and tearing of neuronal axons (Brady, 2001). The frontal lobe is essential in impulse control which allows a person to keep impulses from distracting them from working towards an end goal or saying or doing something inappropriate. It is also important in abstract thinking. Individuals with TBI often think in concrete terms and are unable to grasp abstract ideas such as innuendo, sarcasm, humor, symbolism, and other forms of indirect communication (Brady, 2001). These individuals also usually have difficulties with frustration control because they cannot grasp the concept of waiting for something if it does not come immediately. They may also have difficulties with flexibility, creativity, and initiation of new ideas especially in problem solving and daily living. Lastly, TBI sufferers may lack empathy,

insight, and responsibility because they cannot evaluate how their actions affect others and may therefore be seen as selfish and uncaring (Brady, 2001). Therefore, frontal lobe injury has a large impact on an afflicted individual and can make social interactions awkward and difficult.

Speech and language problems may also arise after TBI and vary with the severity of the injury and the way in which the brain moved within the skull. If the brain just hit one side of the skull, speech and language problems will most likely be less than if the brain rebounded off one side of the skull and hit another side and if bleeding and edema were involved (Schoenbrodt, 2001). These types of problems can be transitory, but many can last for years or be permanent. Speech issues can occur in any of the three phases of speech: respiration, speech sound production, and vocal (Schoenbrodt, 2001). Speech problems are less common than language problems because language is closely related to cognitive and executive functions which may also be damaged. Language difficulties can be in the ability to understand others' language, the ability to use language to express oneself, or in both of these areas (Schoenbrodt, 2001).

Lastly, behavioral problems may be seen in TBI victims, especially those who otherwise seem to be recovered, in particular if they suffered damage to the frontal lobe. There are two types of behavioral problem patterns: over stimulation and under stimulation. If an individual is over stimulated he or she will most likely display "distractibility, irritability, agitation, hyperactivity, impulsivity, disinhibition, social inappropriateness, and aggression" (Tucker, 2001). If an individual is not stimulated enough, he or she will usually display "apathy, lack of motivation and initiation, shyness or social withdrawal, depression, and physical complaints such as stomachaches, fatigue, or general malaise" (Tucker, 2001). A person with TBI can exhibit either one of these behavioral patterns can fluctuate between the two (Tucker, 2001).

There are several indicators that are used to assess the severity of TBI, and depending on the severity of the case, it may be conjectured which, if any, of the aforementioned problems may arise in the afflicted individual. The severity of the brain injury can be classified as mild, moderate, or severe and can be measured by three means: the Glasgow coma scale, the duration of the coma, and the length of the posttraumatic amnesia (Christensen, 2001a). This classification is important as it allows the patient and doctors to know what to expect and look for as well as what treatment should be given. Traumatic brain injury severity is also linked to age as children and adolescents usually fair much better than adults, but older adolescents do better than younger children (Christensen, 2001a). This can be attributed to the fact that TBI affects learning, and older adolescents have already learned many things that younger ones have not. It can also be due to the different physical and chemical properties of the young brain. Secondary injuries such as hypoxic ischemic injury and stroke will also increase the severity of the brain injury (Christensen, 2001a).

The damage that is incurred by the neurons and astrocytes as well as other glial cells may result in cell death which causes the brain to lose mass. This leaves excess space which is then occupied by enlarged ventricles that have an increased amount of cerebral spinal fluid (Bigler, 1997). Using brain imaging techniques the ventricles can be viewed over the ensuing months, and it can be determined in what areas of the brain the tissue loss has occurred (Bigler, 1997). This allows health specialists to surmise which symptoms a patient may display.

The brain injuries that cause the aforementioned problems in humans can be separated into immediate and delayed injuries. Immediate injuries include contusions (bruises) from an impact and diffuse axonal injury in which the axons of nerves are severed due to the shearing forces of the brain trauma (Christensen, 2001a). Delayed injuries include bleeding, herniation

syndrome, edema, and damage from the neurotoxic cascade (Christensen, 2001a). Depending on the severity of these injuries and the time in which they are treated, treatment may be successful with the exception of treatment for the neurotoxic cascade. Currently there are no effective treatments to alter the neurotoxic cascade.

Bleeding is caused by blood vessels being torn due to trauma and can occur within the brain itself, into the ventricles, into the spaces surrounding the brain, between the skull and the dura mater, under the dura mater, or under the arachnoid layer into the cerebrospinal fluid (Christensen, 2001a). When bleeding occurs pressure is put on the brain because there is not enough room in the closed skull for the extra blood. This may lead to reduced blood flow through capillaries which can lead to neuronal injury due to lack of oxygen and blood flow which is termed hypoxic ischemic injury (Christensen, 2001a).

Herniation syndrome occurs when a localized mass or lesion, usually blood or swollen brain tissue, pushes on the brain and forces it to deform to fit into another intracranial space. This can cause further localized brain injury and stroke (Christensen, 2001a). Edema is the swelling of the brain which can cause increased intracranial pressure resulting in the same problems as bleeding (Christensen, 2001a). In most severe TBI cases an intracranial pressure monitor is temporarily surgically implanted in the head so that increases in intracranial pressure can be detected and treated early (Christensen, 2001a).

Damage from the cellular neurotoxic cascade is a new focus in treating TBI but there is still much that is unknown so research is focused on ways to modify or prevent the secondary injuries due to this cascade pathway (Christensen, 2001a). At the cellular level, neurons have been the main focus of TBI research, and the supporting glial cells have been neglected. However, more attention has been directed toward the roles of glial cells, specifically astrocytes,

in the body's response to TBI. These glial cells constitute approximately 40% of the volume of brain tissue and outnumber neurons ten to one (Walz & Juurlink, 2002). In the uninjured brain, astrocytes have many diverse roles. First, these glial cells are an integral part of the blood-brain barrier. Astrocytes have cytoplasmic extensions that end in expanded processes that wrap around the endothelial cells of the capillaries found in the brain (Martini, 2004). These cells secrete chemicals which are necessary for maintaining the special permeability characteristics of the capillary endothelial cells (Martini, 2004). If the astrocytes are damaged and cease stimulating these endothelial cells, the blood-brain barrier does not work properly. When this occurs, hormones and other chemicals can exit the blood and enter the interstitial fluid which may damage neurons (Martini, 2004). These glial cells are also an important part of the physical structuring of the brain. Astrocytes create a three-dimensional framework for the CNS because they contain many microfilaments that extend across the cell body and its processes which provide structure (Martini, 2004). In the embryonic brain, astrocytes "appear to be involved in directing both the growth and interconnection of developing neurons" (Martini, 2004). They also are essential in repairing damaged neural tissue since neurons seldom regain normal function after injury. In this case, astrocytes move to the site of injury to make structural repairs that stabilize the tissue by isolating the damaged area, thereby preventing further injury. (Martini, 2004)

Another very important role of astrocytes is controlling the interstitial environment by adjusting its composition. Astrocytes have a variety of ways in which they accomplish this, including regulating the concentrations of sodium ions, potassium ions, and carbon dioxide, providing a system for transport of nutrients, ions, and dissolved gases between capillaries and neurons, controlling the volume of blood flow through capillaries, absorbing and recycling

certain neurotransmitters, and releasing chemicals that enhance or suppress communication across synaptic terminals (Martini, 2004). Astrocytes are able to accomplish these duties because they have a diverse collection of receptors which include those for neurotransmitters, glutamate, acetylcholine, ATP, and histamine as well as voltage-gated calcium ion channels (Floyd & Lyeth, 2007). They also provide metabolic support to neurons by supplying them with nutrients such as glucose and lactate. Astrocytes are able to do this as they allow glutamate to enter through their processes contacting capillaries, and then they can deliver glutamate to neurons for the pentose phosphate pathway (Walz & Juurlink, 2002). Astrocytes also store energy in the form of glycogen which can be broken down into lactate and supplied to neurons when there is a shortage of glutamate (Walz & Juurlink, 2002). Astrocytes are able to communicate and alter neuronal signaling too, and accomplish this through the release of glutamate and ATP (Floyd & Lyeth, 2007).

When an injury first occurs, neurons release large quantities of glutamate and other excitatory neurotransmitters due to the triggering of many action potentials by nonspecific depolarization (Madikians & Giza, 2006). This causes an efflux of potassium into the extracellular space and also the opening of neuronal ligand-gated calcium channels (Madikians & Giza, 2006). Calcium travels through these channels into the neuron which can cause "overstimulation of phospholipases, plasmalogenase, caplains, protein kinases, guanylate cyclase, nitric oxide synthetase, calcineurins, and endonucleases" which in turn cause overproduction of free radicals, disruption of cytoskeletal organization, and activation of apoptotic signaling (Madikians & Giza, 2006). This does not always result in cell death, but it does affect the metabolic machinery of mitochondria so that they are unable to produce energy when needed which can then lead to cell death (Madikians & Giza, 2006).

Cell death can trigger secondary neuronal injury including axonal swelling, loss of membrane integrity, and perpetuated free radical injury (Hatton, 2001). Cerebral ischemia can then trigger "chemical activation of mediators that perpetuate continued neuronal death despite correction of the initiating event" (Hatton, 2001). Ischemia, toxic insult, and physical insult can then all cause neuronal necrosis to occur. Necrosis is characterized by "cellular swelling and membrane disruption in conjunction with lysis of nuclear chromatin" (Hatton, 2001). This allows the contents of the cells, including ions and glutamate, to spill into the extracellular fluid and cause inflammation and excitotoxicity in other cells which allows the cycle of cell death to continue which can then lead to large areas of tissue death (Hatton, 2001). These signs of delayed axonal injury can begin within 2 to 3 hours post-injury and continue for at least 24 hours. (Hatton, 2001)

In response to injury and the reactions of neurons to injury, astrocytes swell, change their ion channel pattern, their ionic composition, and increase their production of growth and trophic factors within minutes (Walz & Juurlink, 2002; Liberto et al., 2004). "If local cerebral blood flow falls below 20% of normal, energy depletion occurs in surrounding cells, and ion homeostasis is subsequently lost" which results in anoxic depolarization (Walz & Juurlink, 2002). The swollen astrocytes exhibit "pale and watery cytoplasm and dissociation of filament bundles" which is not to be confused with the hypertrophy visible in the reactive astrocytes of the delayed response stage (Walz & Juurlink, 2002). Astrocytes will also swell within thirty minutes if exposed to glutamate, and they will swell within seconds of exposure to excess potassium (K^+) as they clear this molecule and other anions from the extracellular space (Walz & Juurlink, 2002).

In the normal extracellular space K^+ levels are 1 mM with the passage of one action potential and, during increased stimulation or during seizure, these levels may reach a ceiling value of 12 mM (Walz & Juurlink, 2002). However, during an injury such as one caused by hypoxia/ischemia, trauma, or hypoglycemia, K^+ levels may increase to 25 mM (Walz & Juurlink, 2002). When spreading depression waves occur, K^+ concentration levels can transiently reach 30-80 mM (Walz & Juurlink, 2002). Potassium ion increases that are higher than 5 mM lead to hyperexcitibility and affect the efficacy of synaptic transmission (Walz & Juurlink, 2002). Astrocytes help in removing excess K^+ from the extracellular space using two mechanisms. They can remove it via current loops through the astrocytic syncytium which allows one K^+ ion to enter as another leaves at a distant site from the active neurons, or they can remove K^+ by uptake and transfer into adjacent astrocytes (Walz & Juurlink, 2002).

The swollen astrocytes are most likely a large component in the increased intracranial pressure seen after TBI. This is due to "alterations in ion-transport mechanisms that leads to osmotically obligated water entry" (Floyd & Lyeth, 2007). The astrocytic response of swelling also negatively impacts neurons as swollen astrocytes take up extracellular space and restrict exchange with the blood vessels. The decreased extracellular space causes an increase in excitatory substances such as K^+ and glutamate which will also damage neurons (Walz & Juurlink, 2002). The astrocytes eventually decrease in size through a regulatory volume decrease in which amino acids are released. The astrocytes then depolarize as depolarized neurons release K^+ and glutamate which in turn causes the astrocytes to release intracellular calcium ions (Walz & Juurlink, 2002). Since injured neurons release an excess amount of glutamate, astrocytes remove it from the extracellular space and convert it into glutamine which can then diffuse out of the cell and is taken back up by the presynaptic neuron to be made back into glutamate (Floyd &

Lyeth, 2007). However, in TBI the excess glutamate in the extracellular space can result in excitotoxicity due to over activation of "ion-channel linked and G-protein linked glutamate receptors" (Floyd & Lyeth, 2007).

The delayed response phase begins within days of the trauma and is when reactive astrocytes appear. These reactive astrocytes are marked by "hypertrophy, increase in filaments and in processes, changes in the expression of enzymes, different extracellular matrix composition, and different secretion products" (Walz & Juurlink, 2002). If neuronal loss is also occurring, proliferation of astrocytes can be seen. Reactive astrocytes respond differently depending if the injury is diffuse or focal, and within the focal injuries there are two types of response astrocytes: local and remote (Walz & Juurlink, 2002). Both the local and remote response astrocytes express an increased amount of glial fibrillary acidic protein (GFAP) which is an 8-9 nm intermediate filament in the cytoskeletal protein family (Eng et al., 2000; Walz & Juurlink, 2002). This is permanent in the local area astrocytes but returns to normal levels in the remote area astrocytes. Hypertrophy is also similar as the astrocytes in the local area stay larger and the ones in the remote areas return to normal size (Walz & Juurlink, 2002). Vimentin is also expressed in the local astrocytes but is not expressed at all in the remote astrocytes (Walz & Juurlink, 2002). These factors support that local area response astrocytes serve different purposes than remote area astrocytes and are triggered by different mechanisms (Walz & Juurlink, 2002).

These reactive astrocytes have been found to have both positive and negative effects on neurons. The remote response astrocytes have a neuroprotective effect which may be due to "the secretion of neurotrophic factors, such as nerve growth factor, basic fibroblast growth factor, neurotrophin 3, and ciliary neurotrophic factor" (Walz & Juurlink, 2002). They also help to

protect neurons as they clear K^+ from the extracellular space even though they cannot do this as well as uninjured astrocytes. However, reactive astrocytes can also be detrimental as there is evidence that they inhibit axonal regeneration which may be a result of proteoglycan expression (Walz & Juurlink, 2002). The proteoglycans are only expressed by astrocytes that "demarcate the borders of cysts and the interface between activated macrophages-microglia within the necrotic core area and surrounding reactive astrocytes" which suggests that the local response astrocytes are responsible for the negative effect on neuron repair and axonal regeneration (Walz & Juurlink, 2002). This process serves to create what is known as a "glial scar" which marks the border of the damaged area and prevents its spread into nearby tissues (Floyd & Lyeth, 2007; Walz & Juurlink, 2002).

It is known that reactive astrocytes increase in number in the area around a wound following TBI through mitotic division (Takamiya et al., 1988). It is has also been shown that reactive astrocytes are characterized by rapid GFAP synthesis which is "directly involved in cell resilience and the maintenance of tissue integrity" (Eng et al., 2000). Glial fibrillary acidic protein is vital in astrocyte-neuronal interactions and in "modulating synaptic efficacy in the CNS" (Eng et al., 2000). It is also important in altering the motility and shape of astrocytes by providing astrocytic processes with structural stability which is particularly important following TBI (Eng et al., 2000). The rapid GFAP synthesis occurring in reactive astrocytes after injury can be visualized by immunostaining with GFAP antibody or shown by an increase in protein content using methods such as Western blotting (Eng et al., 2000). Hypertrophy and hyperplasia might account for the increase in GFAP staining following injury due to an associated increase in size and therefore the number of GFAP-positive cells (Kraig et al., 1991).

There are many different ways to simulate TBI in the lab, which include *in vivo* and *in vitro* models. The four most commonly used *in vivo* methods of injury include fluid percussion, impact acceleration, controlled cortical impact, and weight drop models (Kulbatski et al., 2008). *In vivo* models are useful in studying the effect of injury at the organismal level, but they do not allow for easy study of individual cell types such as neurons and glial cells (Ellis et al., 1995). However, using *in vitro* models these cells and their responses to injury can be observed with greater ease and accuracy. In *in vitro* models concerning the effects of injury on astrocytes, the cells plated can be purely astrocytes, co-cultures of astrocytes and neurons, or co-cultures of astrocytes and microglia, depending on the goals of the research. The cells used for these cultures are usually obtained from young uninjured animals, and are then injured *in vitro* using techniques such as scraping the cells, exposing the cells to chemicals such as NMDA or glutamate, or stretching the cells (Wu & Schwartz, 1998). All of these models have their pros and cons in respect to what is to be measured or observed, the type of injury that is to be examined, and the ease of use.

In order to injure the astrocytes in our experiment, we used a stretch-induced injury model developed by E.F. Ellis and collaborators. According to Ellis et al. (1995), the purpose of this model was to "produce simple, reliable, and consistent injury of large cell populations grown in culture" that "inflicts mechanical forces that are relevant to those which might occur *in vivo*." This model uses stretch to injure cells which is "a key component in the acceleration/deceleration type of closed human TBI" (Floyd & Lyeth, 2007).

In this model the cells of interest are plated on Flex Plates which have wells with deformable silastic bottoms. The cells are injured by deforming the silastic membranes that they are sitting on using pressurized air. The air pressure and the duration of the pulse of air delivered

to the wells is regulated by the Cell Injury Controller. Once the air pulse is delivered to the well, the air between the well and adapter is vented out which allows a rapid deformation and rebound of the silastic membrane (Ellis et al., 1995). Different levels of stretch can be achieved by varying the air pressure delivered to the well. The controller can be calibrated by seeing how far the deformed membrane pushes a sliding plastic pointer mounted on a ruler at a certain air pressure. Once this is determined the cells can be measured at known levels of stretch by dialing in the desired pressure on the Cell Injury Controller (Ellis et al., 1995).

This model has been used in many diverse experiments which include those examining mitochondrial membrane potential and cellular ATP in astrocytes and neurons (Ahmed et al., 2000), the effects of 17β-estradiol on intracellular calcium changes and neuronal survival after injury (Lapanantasin et al., 2006), the effects of injury on calcium and sodium in cortical astrocytes (Floyd et al., 2005), and activation of extracellular signal-regulated kinase in astrocytes (Neary at al., 2003). The efficacy of this *in vitro* model of TBI has been shown as the injured cells exhibit many of the following known signs of injury which include "intracellular lesions to the mitochondria, Golgi, and cytoskeletal elements in astrocytes and neurons, and increases in astrocyte and neuronal permeability," and rises in intracellular calcium and sodium levels in astrocytes (Ellis et al., 1995).

As it has been demonstrated, TBI has profound effects on individuals and society, but there still is a great deal to discover, especially on the cellular level. Due to the diverse effects that astrocytes have on the injured brain and the recent attention that they have been receiving, more research is needed to further understand their role in TBI. According to Floyd and Lyeth (2007), further research is needed to uncover the mechanisms mediating cell damage after TBI and also to define the time course of astrocyte damage after injury.

In the current study, we used the stretch-induced cell injury model to injure astrocytes at increasing levels of stretch and at increasing time intervals. Since GFAP is upregulated due to astrocyte injury, it was used as a marker to determine astrocyte response to different levels of stretch as well as to astrocyte response at increasingly longer times after injury. Glial fibrillary acidic protein was assessed using immunofluorescence and Western blotting. It was hypothesized that GFAP expression would increase with higher levels of stretch and be detectable at 6-24 hours after injury.

Methods

1. Astrocyte Cell Culture

Dissection

Astrocytes were derived from 1-3 day postnatal mice (Hilltop Lab Animals, Scottdale, PA) that were housed in the Lynchburg College animal facility with a 12 h light/12 h dark cycle and food and water provided *ad libitum*. The mice were sacrificed by rapid decapitation and their heads were rinsed twice in culture dishes containing 70% ethanol to remove debris. The heads were transferred to a culture dish of dissecting medium (Hanks Balanced Salt Solution without Ca^{++} or Mg^{++} (Fisher Scientific, Rockford, IL) supplemented with sucrose, dextrose, and Hepes) on ice in a horizontal flow hood under a dissection microscope.

Micro dissecting forceps were used to peel the skin away from the skull and the skull away from the brain. The brain was removed and placed in another dish of dissecting medium, placed ventral side up, and the meninges were peeled away. It was then turned over and the dorsal meninges were then removed. Next, the hemispheres were pulled open and the basal forebrain, hippocampus, and striatum were removed and the cortices were transferred to a third

dish with dissecting medium. The cortices were mechanically minced with forceps and added to a 15 ml centrifuge tube of thawed 0.025% trypsin in dissecting media using a transfer pipette. The tissue was incubated in a 37 °C water bath for 20-30 minutes to start the initial breakdown of tissue to a single cell suspension.

Initial Cell Plating

The trypsin tube containing the cortical hemispheres was centrifuged at 1000 rpm for 5 minutes, and the supernatant was aseptically decanted and replaced with a small amount of plating medium (Modified Earle's Media supplemented with glucose (final concentration 20mM), 10% fetal bovine serum, 10% calf serum, L-glutamine (2mM), penicillin (50 IU/ml)-streptomycin (50μg/ml) and epidermal growth factor (10 ng/ml)). The mixture was triturated with a medium bore pipette followed by a small bore pipette until homogeneous and then diluted with plating medium (~1.2 cortical hemispheres/10 ml). Ten milliliters of cell mixture was plated into T75 flasks and incubated at 37 °C with 5% CO_2/95% air.

Subculturing Astrocytes onto Flex Plates

After the astrocytes became near contact-inhibited (~1 week) they were subcultured onto 25 mm silastic membranes of six-well Flex Plates (Flexcell International, NcKeesport, PA) at a density of $4x10^5$ cells/ml. To subculture the cells, the flasks were rinsed twice with Hanks Balanced Salt Solution (HBSS), exposed to 0.25% trypsin in HBSS for one minute, and then incubated with fresh HBSS at 37 °C for five minutes to loosen the cells. The cells were knocked off the flask and then centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was removed and replaced with plating medium and the mixture was triturated until homogeneous. Five ml of plating medium with was added to the cells for every flask subcultured. The cells were counted with a hemocytometer and then diluted with plating

medium to $4x10^5$ cells/ml before plating onto Flex Plates. After growth had become confluent the cells were treated with 9 μM cytosine β-D-arabinofuranoside for 4-7 days to reduce the microglia population in the astrocyte enriched cultures. After this treatment, the cultures were fed with growth media (Modified Earle's Media supplemented with 10% calf serum, L-glutamine (2mM), and penicillin (50 IU/ml)-streptomycin (50μg/ml)).

2. Astrocyte Cell Injury

Cell Injury

The day before injury the growth media in the Flex Plates was replaced and the next day the cells were injured using a Model 94A Cell Injury Controller Device (Commonwealth Biotechnology, Richmond, VA) in a horizontal flow hood. For the injury response study, cells were injured at 3, 4, 5, 6, and 7 mm of stretch (Appendix A) and one well was uninjured as a control. After injury the cells were allowed to recover in the incubator at 37 °C with 5% CO_2/95% air for either 24 or 48 hours. For the time course study, all wells of a plate except for the control were either injured at 6 or 6.5 mm of stretch, and evaluated at 15 minutes, 2, 6, 24, or 48 hours after injury. After each injury time point, the cells were returned to the tissue culture incubator.

3. GFAP Expression Evaluation

Western Blotting

To isolate the proteins, the astrocytes were rinsed twice on ice with cold PBS prior to manually rocking with radioimmunoprecipitation (RIPA) buffer (Boston BioProducts, Worcester, MA) for 5 minutes. The wells were scraped, triturated, and the buffer was pipetted

into centrifuge tubes. The lysates were centrifuged (14,000 rpm, 30 minutes, 4°C) to remove cellular debris. The supernatants were collected, the protein concentrations were determined using the BioRad assay (BioRad, Hercules, CA) with a bovine serum albumin standard, and then the volume of sample containing 2.5 μg of protein was calculated from the standard curve (Appendix B). All remaining samples were aliquoted and frozen at -20°C until needed.

After determining the protein concentration of each lysate, 2.5 μg protein was separated on a 10-well 4-12% Bis-Tris mini gel (Invitrogen) with a MOPS running buffer (Invitrogen) under reducing conditions. The proteins were loaded at 2.5 μg so that the total volume of sample, dH_2O, loading buffer, and reducing agent loaded was 25 μl (Appendix C) with SeeBlue standard molecular weight markers (Invitrogen). After the gel was loaded it was run at a constant 200 V for 50 minutes. The proteins on the gel were transferred to nitrocellulose paper using 30 V for 1 hour in the XCell SureLock Mini-Cell. The blots were rinsed twice with PBS, dried on filter paper and stored at -20°C until needed.

To complete the Western blotting, the blots were brought to room temperature and rehydrated with PBS. They were blocked with blocking solution (0.05% Tween-20, 5% non-fat dry milk in PBS) for one hour at room temperature and then the primary antibody, rat α-GFAP (1:7500 in blocking solution; Invitrogen) was applied for ~20 hours while rocking at 4°C. The next day, the blots were rinsed three times with blocking solution for five minutes with rocking at room temperature, and then 3 more rinses were repeated using PBS. The secondary antibody, donkey α-rat horseradish peroxidase (1:10,000 in PBS; Invitrogen) was applied for one hour with rocking at room temperature. The blots were washed four times with PBS with rocking for five minutes and then were exposed for one minute to SuperSignal West Pico Chemiluminescent (Pierce, Rockford, IL) while manually rocking to ensure entire coverage. The blots were then

placed in a plastic development sheet in a cassette and exposed to film for 5-60 seconds in the dark room.

Immunofluorescence

To fix the cells they were rinsed twice with phosphate buffered saline (PBS), pH 7.4, exposed to 4% paraformaldehyde in PBS for 15 minutes, and then rinsed twice again with PBS. Cells were stored in PBS at 4°C until needed.

To prepare for the addition of the GFAP antibody, the cells were rinsed twice with PBS and then they were permeabilized with 2N HCl/0.1% Triton-X 100 for 30 minutes at 37°C. Two 5 minute washes with sodium borate while rocking were completed to neutralize the acid and then the cells were rinsed 3 times with PBS. Nonspecific binding of the primary antibody was blocked by rocking the cells with 4% horse serum for one hour at room temperature. The primary antibody, rabbit α-GFAP (1:500 in blocking solution; Dako, Carpinteria, CA), was put on the cells and rocked for 20 hours at 4°C. The following day, the cells were washed three times with PBS for 5 minutes while rocking. The fluorescent secondary antibody, Alexa Fluor 488nm goat anti-rabbit IgG (1:750 in PBS; Invitrogen, Camarillo, CA) was put on the cells for one hour in the dark at room temperature while rocking. Under dimmed lights, the cells were rinsed three times with PBS for five minutes while rocking.

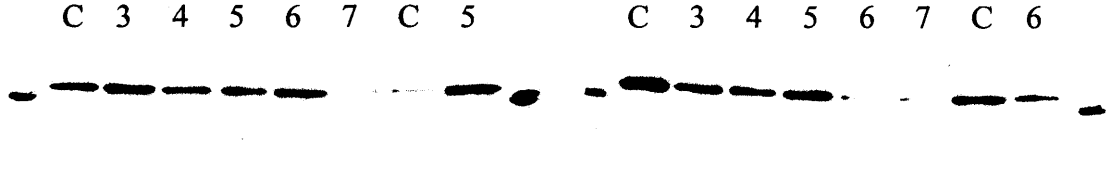
In order to view the cells under a fluorescent microscope (Olympus CK40), the bottom of the Flex Plate wells were popped out into PBS, the curved edges of the membrane were removed, and they were mounted on slides with Fluoromount-G (SouthernBiotech, Birmingham, AL). Fluorescent pictures were taken with an Olympus camera attached to the fluorescent microscope and captured with PictureFrame software.

Results

1. Astrocyte Response to Magnitude of Injury

Western Blotting

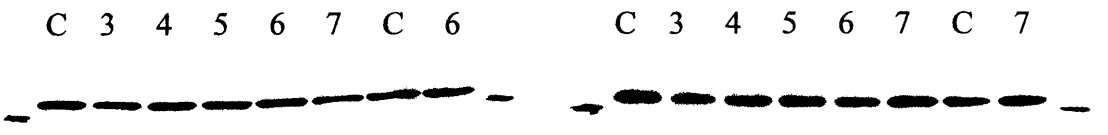
The Western blots for injury level at 24 and 48 hours showed bands right above the molecular weight marker of 51 kDa (blue line in Figs. 1 and 2). There was also only one band for each sample with no apparent shadows above or below the bands. In Fig. 1, the lanes containing the 7 mm sample and the second run of the control in blot (a) have very light bands, and this also occurred in the lanes containing the 6 mm and 7 mm samples in blot (b). In blot (a) at 24 hours it appears that the sample at 3 mm and the second 5 mm sample are the largest bands. Also, the first band from the 5 mm sample appears to be smaller than the second band from the same 5 mm sample. In blot (b) at 24 hours the first control band appears to be the largest band, but this band is larger than the second control band which was taken from the same sample.



(a) (b)

Fig. 1. Western blots of GFAP expression in astrocytes 24 hours after injury between 3 mm and 7 mm stretch. From left to right, the first 6 bands in each blot correspond to astrocytes injured at 0 (control), 3, 4, 5, 6, and 7 mm of stretch respectively. The 7^{th} band in both blots is a repeat of the control sample, and in blot (a) the 8^{th} band is a repeat of 6 mm and in blot (b) it is a repeat of 5 mm. Both blots were exposed for 30 seconds. Blot (a) is from 1/16/09 plate #1 and blot (b) is from 1/16/09 plate #2.

In both blots (a) and (b) at 48 hours, all of the bands within in each blot appear very similar to one another and it is not possible to detect the subtle differences with the unaided eye.



(a) (b)

Fig. 2. Western blots of GFAP expression in astrocytes 48 hours after injury between 3 mm and 7 mm stretch. From left to right, the first 6 bands in each blot correspond to astrocytes injured at 0 (control), 3, 4, 5, 6, and 7 mm of stretch respectively. The 7th band in both blots is a repeat of the control sample, and in blot (a) the 8th band is a repeat of 6 mm and in blot (b) it is a repeat of 7 mm. Both blots were exposed for 15 seconds. Blot (a) is from 1/16/09 plate #5 and blot (b) is from 1/16/09 plate #6.

Immunofluorescence

Since GFAP is an intermediate filament that helps to provide astrocytes their shape, the fluorescent green-stained GFAP can be used to determine cell morphology alteration after injury. As would be expected, GFAP is everywhere in the cell except for the nucleus which shows up as a circular black area in the middle of the cell. The uninjured control astrocytes (Fig. 3a.) have a circular morphology with several processes. At the mild injury levels of 3 mm and 4 mm of stretch (Fig. 3b and c), the astrocytes increase the lengths and numbers of their processes. At the moderate levels of 5 mm and 6 mm of stretch (Fig. 3d and e), the processes become even more prominent and take on a stringy appearance. These cells are also more difficult to bring into focus under the microscope because they are not as flat as the uninjured and mildly injured cells

potentially due to swelling. At the severe injury level of 7 mm of stretch (Fig. 3f), the astrocytes have the elongated, more numerous processes, but there are also areas where astrocytes are absent. This is most likely due to astrocytes having been detached from the bottom of the Flex Plate due to the magnitude of the stretch and then being washed away in the cell fixing process.

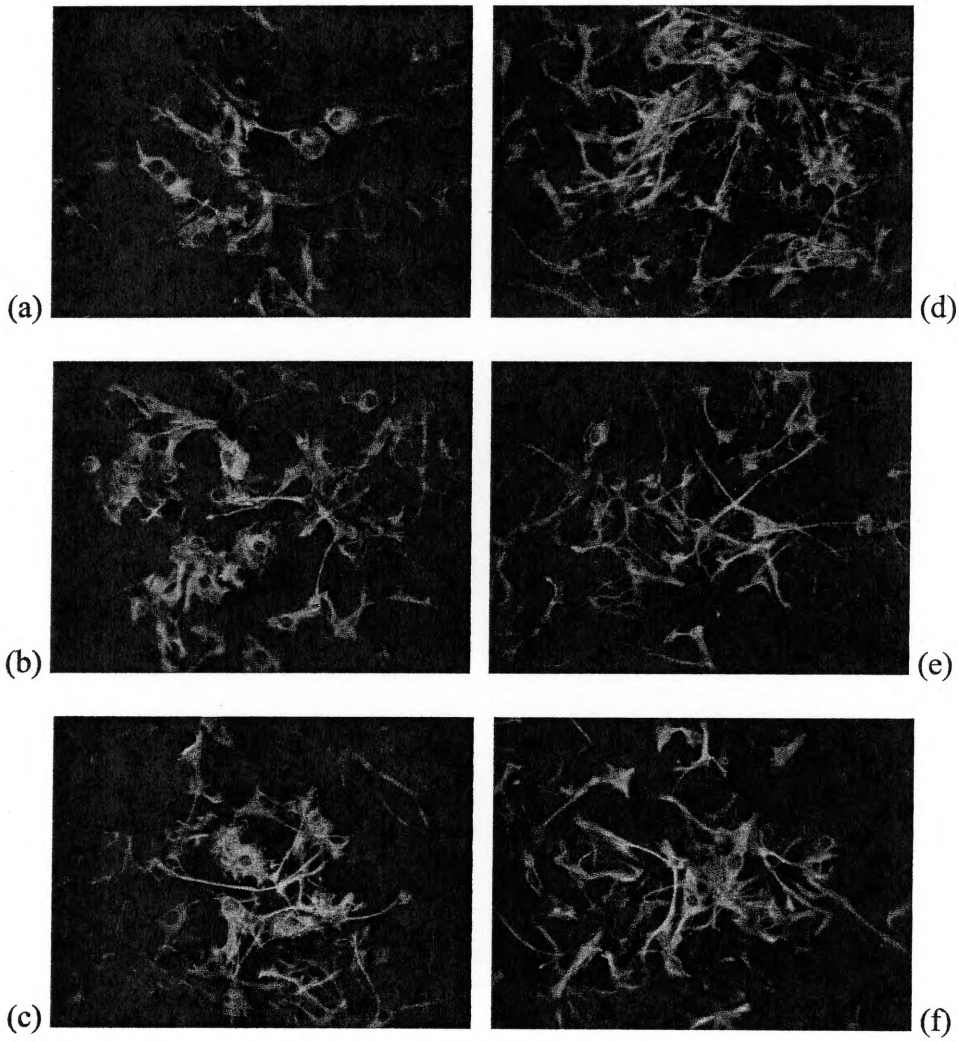


Fig. 3. Astrocyte response to injury levels at 24 hours. The astrocytes in picture (a) are uninjured controls. The cells in (b) and (c) were injured at a mild level of stretch (3 mm and 4 mm respectively), the cells in (d) and (e) were injured at moderate levels of stretch (5 mm and 6 mm respectively), and the cells in (f) were injured at a severe level of stretch (7 mm).

2. Astrocyte Response at Time Points after Injury

Western Blotting

The Western blots for GFAP expression at times after injury also showed bands right above the 51 kDa molecular weight marker which is indicative of GFAP. There was also only one band per sample. In Fig. 4 blot (b) bands are missing for the lanes containing the samples for the first run of the control, 15 minutes, and the second run of the control. In Fig. 4, blot (a) shows a noticeably larger band at the 6 hour mark indicating increased GFAP expression but the rest of the bands look very similar to each other. In blot (b) it appears that the band at 24 hours after injury is the largest.

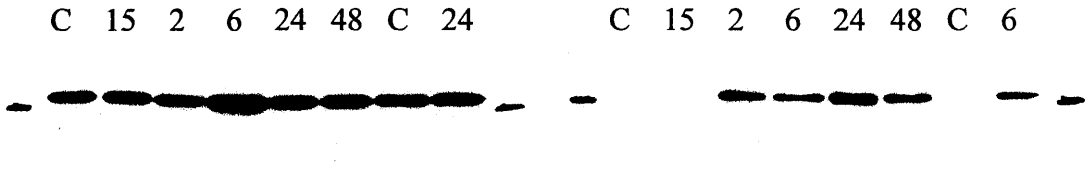


Fig. 4. Western blots of GFAP expression in astrocytes at times between 0.25 and 48 hours after injury of 6 mm. From left to right, the first 6 bands in each blot correspond to astrocytes at times of N/A (control), 0.25, 2, 6, 24, and 48 hours after injury respectively. The 7^{th} band in both blots is a repeat of the control sample, and in blot (a) the 8^{th} band is a repeat of 24 hours and in blot (b) it is a repeat of 6 hours. Both blots were exposed for 15 seconds. Blot (a) is from 1/17/09 plate #1 and blot (b) is from 1/17/09 plate #2.

Immunofluorescence

As in the control astrocytes from the injury response, the uninjured astrocytes of the time course also have a circular morphology with a limited number and length of processes. At 15

minutes post-injury (Fig. 5b) the astrocytes do not show any characteristics of reactive astrocytes. However, by 2 and 6 hours after injury (Fig. 5c and d) they show elongated and stringy processes, and also areas with thickened processes which is also characteristic of reactive astrocytes. At 24 hours post-injury (Fig. 5e) the astrocytes continue to have stringy and thick processes. By 48 hours the processes are not as prominent and cell division is apparent as two nuclei are seen in many of the cell bodies.

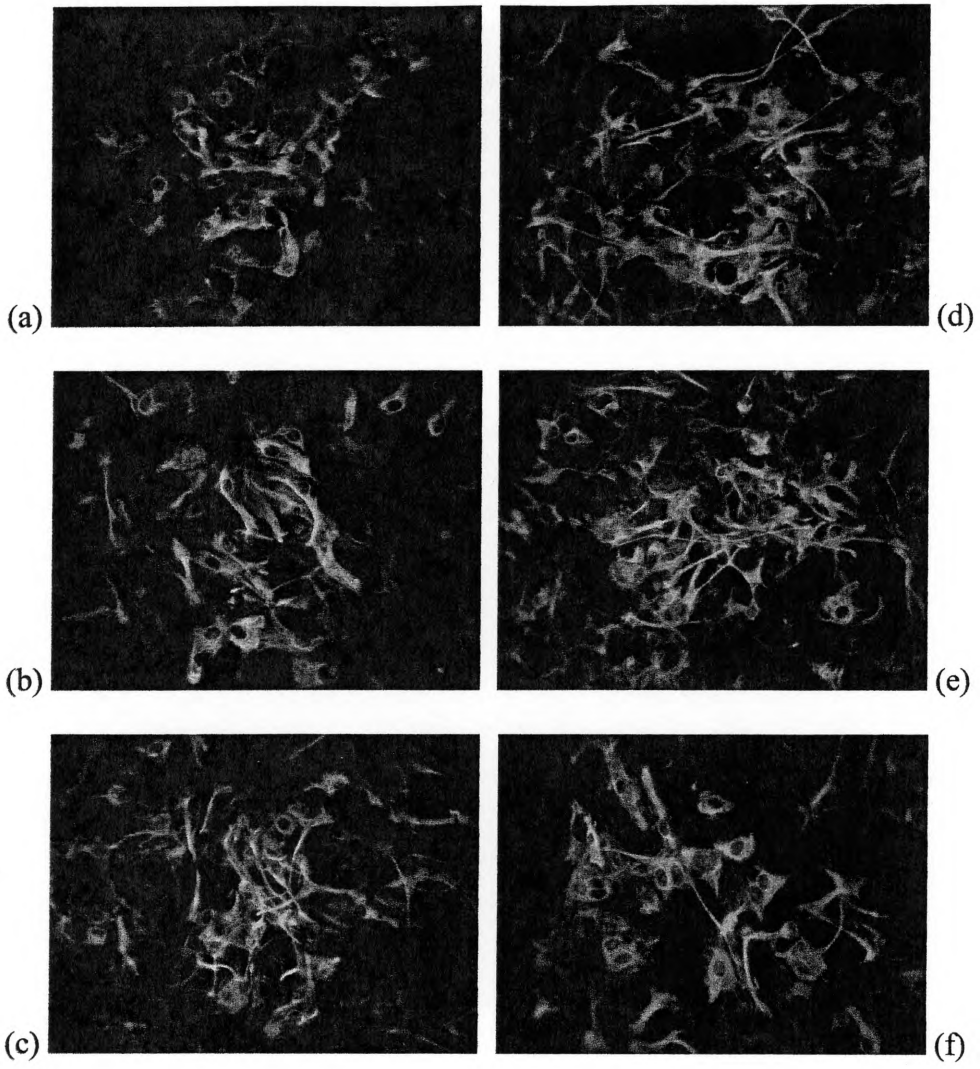


Fig. 5. Astrocyte injury response time course at 6 mm. The astrocytes in picture (a) are uninjured controls. The cells in (b) have recovered from injury for 15 minutes. The cells in (c) and (d) have recovered for 2 hours and 6 hours respectively, and the cells in (e) and (f) have recovered for 24 hours and 48 hours respectively.

Discussion

In Western blotting on SDS-PAGE gels, it is known that GFAP runs at approximately 55 kDa, and sometimes an additional band at 48 kDa can be seen which results from a breakdown product of the 55 kDa band ("Monoclonal Antibody"). From the appearance of the Western

blots, it can be concluded that the primary antibody used selectively stained for GFAP due to the bands appearing at approximately 55 kDa and the absence of any other bands on the blot, including break-down products. GFAP was present in all of the samples except time course blot (b) lanes containing the first control, 15 minutes after injury, and the second control. This is most likely due to a mistake that was made during protein isolation when RIPA buffer was put on the cells in the control and 15 minute wells too early and then discarded. There was also very low GFAP expression in the injury level response at 24 hours on blot (a) in the lanes containing the samples for 7 mm and the second control, and on blot (b) in the lanes containing the samples for 6 mm and 7 mm. This cannot be due to decreased GFAP levels in the samples loaded because in blot (a) the lane containing the second run of the control is the same control that was loaded in the first lane, which is much darker. There are several explanations for these light bands such as incomplete loading of the protein sample, but the cause is unknown. The sizes of the bands within each blot are very similar to each other, particularly in figure 2, which makes it difficult to determine differences in GFAP expression between the treatments.

For the samples that did show GFAP expression, it was difficult to analyze the amount of GFAP in each control and injured sample as the bands were all relatively similar in size to each other. This is probably due to the fact that GFAP is a relatively highly expressed protein in uninjured astrocytes, therefore making it more difficult to detect subtle changes in its expression. In the future, it would be beneficial to analyze the pixel density of the bands using computer software, and also to increase the number of replications.

From the immunofluorescence results it can be concluded that GFAP expression showed that astrocytes became more reactive with increasing magnitudes of stretch. This is supported by the increased number of astrocytic processes, the increased "stringyness" of the processes, the

thick bundles of GFAP, and the swelling of the astrocytes which was found as they were difficult to focus on as they were not in a single plane. The injury response data in the current study correlates with the findings of Ellis et al. (1995). Using the same stretch model of injury, Ellis and colleagues found that astrocyte injury was proportional to the degree of stretch as evidenced by cell morphology seen using electron microscopy, lactate dehydrogenase (LDH) enzyme release, and propidium iodide dye exclusion (1995). They found that at a moderate level of stretch (5.7 mm), the cells appeared swollen, exhibited structural abnormalities in their mitochondria, and had isolated vacuolization in the Golgi apparatus and rough endoplasmic reticulum. At increasing levels of stretch, the cells retracted and became stellate which is an indication of cytoskeletal disruption. At 7.5 and 8.6 mm of stretch, the cells were retracted with intercellular spaces in addition to the other abnormalities but at a more dramatic level of expression. (Ellis et al., 1995)

For the time course of GFAP expression after injury at 6 mm of stretch, our immunofluorescence data shows that change in astrocyte morphology begins around 2 hours post-injury and continues through 48 hours post-injury. The astrocytes display increased number of processes between 2 and 24 hours but have a reduced number of processes at 48 hours. Also, at 24 hours post-injury astrocyte cell division is seen and this is even more pronounced at 48 hours after injury. In the study by Ellis and colleagues (1995), it was found that astrocyte recovery after injury was dependent on the level at which they were injured. They discovered that cells injured at low levels of stretch began to recover as soon as 2 hours after injury and were able to completely exclude propidium iodide at 24 hours which shows that their cell membranes had regained function. At the more severe levels of stretch (7.5 and 8.6 mm), the cells did not begin to recover until 6 hours post-injury, and at 8.6 mm of stretch, the astrocytes

had not even reached 50% recovery by 24 hours after injury. However, these severely injured cells were able to repair themselves to look like the control cells by 2-3 days post-injury. (Ellis et al., 1995)

In addition to animal studies, human studies have also been conducted in order to determine astrocytic response to traumatic brain injury. In humans with cerebral contusions, electron microscopy of brain tissue has shown evidence of astrocytic swelling as early as 3 hours post-injury, lasting up to 3 days after injury (Zhao et al., 2003). Human research has also focused on GFAP levels in cerebrospinal fluid (CSF) and serum following brain injury. Glial fibrillary acidic protein is a good marker of traumatic brain injury as it was found that "GFAP is not released after multiple traumas without brain injury" having occurred (Nylén et al., 2006). Other proteins expressed following brain injury such as S-100 β have been used as markers of TBI, but this protein is also elevated in peripheral trauma and therefore is a less specific indicator. Studies first showed that acute CNS injury significantly increased CSF-GFAP levels, and astrogliosis increased CSF-GFAP levels but by a lesser degree. However, obtaining CSF from brain-injured patients is potentially dangerous and usually contraindicated. Therefore, several studies were conducted where serum was collected instead of CSF, and it was found that increased levels of GFAP were present in the serum after injury, which is probably due to a damaged blood-brain barrier that allows GFAP to enter the systemic circulation. (Nylén et al., 2006)

In healthy, uninjured individuals, the average serum-GFAP level is only 0.061 μl/L. In a study conducted by Nylén and colleagues (2006), serum-GFAP levels were measured in individuals with severe TBI, and it was found that those with unfavorable outcomes (death, vegetative state, or severe disability) exhibited median GFAP serum levels of 2.72 μg/L which

was significantly higher than those with favorable outcomes (moderate disability or good recovery), who had median GFAP serum levels of 0.85 μg/L. This study found that the highest GFAP serum levels usually occurred by day 1 and in no case later than day 4, and then they gradually declined. After one year, serum-GFAP levels were normalized in all cases except for 3 patients who had slightly elevated levels. This research is clinically beneficial as "there is a possibility to predict death or unfavorable outcome in patients with extremely elevated serum-GFAP levels." (Nylén et al., 2006)

New, clinically significant findings will only continue if further research is conducted in both the therapeutic and cellular areas of TBI. Future work in the present study includes quantifying the Western blots, testing the time course of injury response at other levels of stretch, and lengthening the duration of time course observations. The astrocytes in our model were kept in growth media after injury which does not factor in the effects of secondary insult such as hypoxia or ischemia which frequently occur after injury and have significant effects on neural tissue. In the future it would be interesting to observe the effects of hypoxia on GFAP expression in astrocytes by altering the post-injury environment. Ellis and colleagues (1995) have also suggested that the rate of cell stretch is an important determinant in the degree of cell injury and another possible research area of interest.

Much remains unknown about the consequences of traumatic brain injury at the cellular level and how these events influence patient outcome. This is especially true of the response of astrocytes and their interaction with neurons post-injury since this has not become a research focus until recent years. The stretch-injury model is a good way to learn more about these responses as it mimics the major biomechanical aspects of traumatic brain injury, and allows for easy observation of *in vitro* cell response. This model allows researchers to observe the injury

response of one cell type such as astrocytes, or the interaction between multiple cell types such as astrocyte-neuronal co-cultures. The stretch-induced injury model also allows researchers to manipulate many variables such as the duration and magnitude of stretch injury, and post-injury conditions such as oxygen deprivation. Through research directed at elucidating the normal roles of astrocytes and their roles in response to injury, knowledge can be amassed that can lead to drug development targeting specific mechanisms. Since there are not any highly successful drug therapies to treat traumatic brain injury, both cellular and therapeutic research must be continued in order to better understand and hopefully successfully treat traumatic brain injury.

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Appendices

A. Cell Injury Controller Calibration

B. Protein Concentration Calculations

C. Proteins for Western Blots

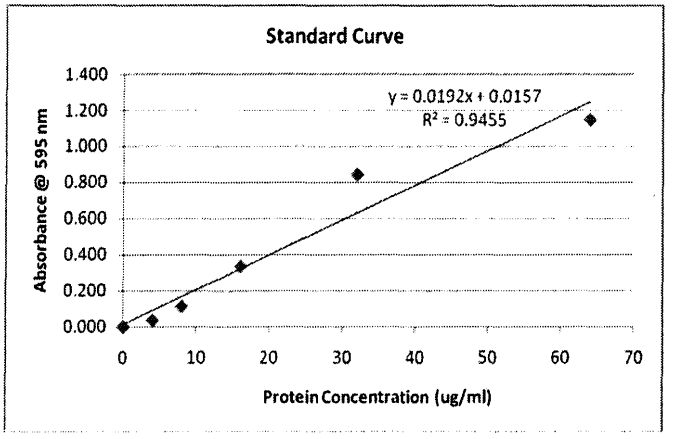
Appendix A

Cell Injury Controller Calibration Performed on 02/07/09

Force (PSI)	Deformation (mm)
10	3
12	3.5
16	4
20	4.5
24	5
28	5.5
32	6
36	6.5
40	7
44	7.5

Appendix B

Protein Concentrations for 1/16/09 Plates #1, 2, 5, & 6 and 1/17/09 Plates #1 & 2



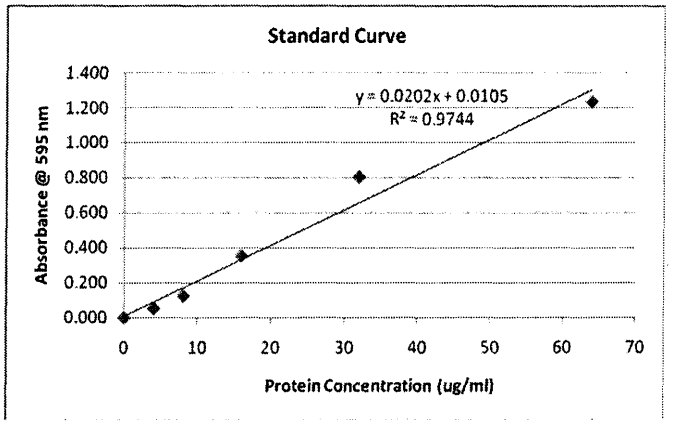
	Standards (ug/ml)	Absorbance (595 nm)
H2O	0	0.000
#5	4	0.037
#4	8	0.115
#3	16	0.337
#2	32	0.844
#1	64	1.148

Sample Identity	Absorbance (595 nm)	Sample (ug/ml)	Sample (ug/ul)	for 1ug:	for 2ug:	for 2.5ug:	for 5ug:	for 10 ug:
1/16/09 Plates								
Plate #1: Control @ 24 hr	0.236	11.474	2.29	0.44	0.87	1.09	2.18	4.36
Plate #1: 3mm @ 24 hr	0.121	5.484	1.10	0.91	1.82	2.28	4.56	9.12
Plate #1: 4mm @ 24 hr	0.175	8.297	1.66	0.60	1.21	1.51	3.01	6.03
Plate #1: 5mm @ 24 hr	0.168	7.932	1.59	0.63	1.26	1.58	3.15	6.30
Plate #1: 6mm @ 24 hr	0.068	2.724	0.54	1.84	3.67	4.59	9.18	18.36
Plate #1: 7mm @ 24 hr	0.160	7.516	1.50	0.67	1.33	1.66	3.33	6.65
Plate #2: Control @ 24 hr	0.100	4.391	0.88	1.14	2.28	2.85	5.69	11.39
Plate #2: 3mm @ 24 hr	0.153	7.151	1.43	0.70	1.40	1.75	3.50	6.99
Plate #2: 4mm @ 24 hr	0.168	7.932	1.59	0.63	1.26	1.58	3.15	6.30
Plate #2: 5mm @ 24 hr	0.137	6.318	1.26	0.79	1.58	1.98	3.96	7.91
Plate #2: 6mm @ 24 hr	0.185	8.818	1.76	0.57	1.13	1.42	2.84	5.67
Plate #2: 7mm @ 24 hr	0.177	8.401	1.68	0.60	1.19	1.49	2.98	5.95
Plate #5: Control @ 48 hr	0.163	7.672	1.53	0.65	1.30	1.63	3.26	6.52
Plate #5: 3mm @ 48 hr	0.210	10.120	2.02	0.49	0.99	1.24	2.47	4.94
Plate #5: 4mm @ 48 hr	0.190	9.078	1.82	0.55	1.10	1.38	2.75	5.51
Plate #5: 5mm @ 48 hr	0.188	8.974	1.79	0.56	1.11	1.39	2.79	5.57
Plate #5: 6mm @ 48 hr	0.204	9.807	1.96	0.51	1.02	1.27	2.55	5.10
Plate #5: 7mm @ 48 hr	0.254	12.411	2.48	0.40	0.81	1.01	2.01	4.03
Plate #6: Control @ 48 hr	0.214	10.328	2.07	0.48	0.97	1.21	2.42	4.84
Plate #6: 3mm @ 48 hr	0.261	12.776	2.56	0.39	0.78	0.98	1.96	3.91
Plate #6: 4mm @ 48 hr	0.210	10.120	2.02	0.49	0.99	1.24	2.47	4.94
Plate #6: 5mm @ 48 hr	0.216	10.432	2.09	0.48	0.96	1.20	2.40	4.79
Plate #6: 6mm @ 48 hr	0.229	11.109	2.22	0.45	0.90	1.13	2.25	4.50
Plate #6: 7mm @ 48 hr	0.229	11.109	2.22	0.45	0.90	1.13	2.25	4.50
1/17/09 Plates								
Plate #1: Control @ 6mm	0.214	10.328	2.07	0.48	0.97	1.21	2.42	4.84
Plate #1: 0.25 hr @ 6mm	0.219	10.589	2.12	0.47	0.94	1.18	2.36	4.72
Plate #1: 2 hr @ 6mm	0.239	11.630	2.33	0.43	0.86	1.07	2.15	4.30
Plate #1: 6 hr @ 6mm	0.040	1.266	0.25	3.95	7.90	9.88	19.75	39.51

Plate #1: 24 hr @ 6mm	0.166	7.828	1.57	0.64	1.28	1.60	3.19	6.39
Plate #1: 48 hr @ 6mm	0.228	11.057	2.21	0.45	0.90	1.13	2.26	4.52
Plate #2: Control @ 6mm	0.148	6.891	1.38	0.73	1.45	1.81	3.63	7.26
Plate #2: 0.25 hr @ 6mm	0.153	7.151	1.43	0.70	1.40	1.75	3.50	6.99
Plate #2: 2 hr @ 6mm	0.220	10.641	2.13	0.47	0.94	1.17	2.35	4.70
Plate #2: 6 hr @ 6mm	0.214	10.328	2.07	0.48	0.97	1.21	2.42	4.84
Plate #2: 24 hr @ 6mm	0.212	10.224	2.04	0.49	0.98	1.22	2.45	4.89
Plate #2: 48 hr @ 6mm	0.202	9.703	1.94	0.52	1.03	1.29	2.58	5.15

Protein Concentrations for 2/18/09 Plates #1 & 2 and 2/24/09 Plates #1 & 3

	Standards (ug/ml)	Absorbance (595 nm)
H2O	0	0.000
#1	4	0.052
#2	8	0.125
#3	16	0.354
#4	32	0.805
#5	64	1.234



Sample Identity	Absorbance (595 nm)	Sample (ug/ml)	Sample (ug/ul)	for 1ug:	for 2ug:	for 2.5ug:	for 5ug:	for 10 ug:
2/18/09 Plates								
Plate #1: Control @ 24 hr	0.093	4.084	0.82	1.22	2.45	3.06	6.12	12.24
Plate #1: 3mm @ 24 hr	0.136	6.213	1.24	0.80	1.61	2.01	4.02	8.05
Plate #1: 4mm @ 24 hr	0.218	10.272	2.05	0.49	0.97	1.22	2.43	4.87
Plate #1: 5mm @ 24 hr	0.215	10.124	2.02	0.49	0.99	1.23	2.47	4.94
Plate #1: 6mm @ 24 hr	0.161	7.450	1.49	0.67	1.34	1.68	3.36	6.71
Plate #1: 7mm @ 24 hr	0.153	7.054	1.41	0.71	1.42	1.77	3.54	7.09
Plate #2: Control @ 24 hr	0.194	9.084	1.82	0.55	1.10	1.38	2.75	5.50
Plate #2: 3mm @ 24 hr	0.196	9.183	1.84	0.54	1.09	1.36	2.72	5.44
Plate #2: 4mm @ 24 hr	0.194	9.084	1.82	0.55	1.10	1.38	2.75	5.50
Plate #2: 5mm @ 24 hr	0.198	9.282	1.86	0.54	1.08	1.35	2.69	5.39
Plate #2: 6mm @ 24 hr	0.207	9.728	1.95	0.51	1.03	1.28	2.57	5.14
Plate #2: 7mm @ 24 hr	0.219	10.322	2.06	0.48	0.97	1.21	2.42	4.84
2/24/09 Plates								
Plate #1: Control @ 6mm	0.167	7.748	1.55	0.65	1.29	1.61	3.23	6.45
Plate #1: 15 min. @ 6mm	0.212	9.975	2.00	0.50	1.00	1.25	2.51	5.01
Plate #1: 2 hr @ 6mm	0.198	9.282	1.86	0.54	1.08	1.35	2.69	5.39
Plate #1: 6 hr @ 6mm	0.233	11.015	2.20	0.45	0.91	1.13	2.27	4.54
Plate #1: 24 hr @ 6mm	0.224	10.569	2.11	0.47	0.95	1.18	2.37	4.73
Plate #1: 48 hr @ 6mm	0.217	10.223	2.04	0.49	0.98	1.22	2.45	4.89
Plate #3: Control @ 6.5mm	0.223	10.520	2.10	0.48	0.95	1.19	2.38	4.75
Plate #3: 15 min @ 6.5mm	0.211	9.926	1.99	0.50	1.01	1.26	2.52	5.04
Plate #3: 2 hr @ 6.5mm	0.213	10.025	2.00	0.50	1.00	1.25	2.49	4.99
Plate #3: 6 hr @ 6.5mm	0.214	10.074	2.01	0.50	0.99	1.24	2.48	4.96
Plate #3: 24 hr @ 6.5mm	0.192	8.985	1.80	0.56	1.11	1.39	2.78	5.56
Plate #3: 48 hr @ 6.5mm	0.188	8.787	1.76	0.57	1.14	1.42	2.85	5.69

Appendix C

Calculations for Western Blot Samples

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	1/16/09 Plate #1: Control @ 24 hr	1.09	15.11	6.30	2.50
3	1/16/09 Plate #1: 3mm @ 24 hr	2.28	13.92	6.30	2.50
4	1/16/09 Plate #1: 4mm @ 24 hr	1.51	14.69	6.30	2.50
5	1/16/09 Plate #1: 5mm @ 24 hr	1.58	14.62	6.30	2.50
6	1/16/09 Plate #1: 6mm @ 24 hr	4.59	11.61	6.30	2.50
7	1/16/09 Plate #1: 7mm @ 24 hr	1.66	14.54	6.30	2.50
8	1/16/09 Plate #1: Control @ 24 hr	1.09	15.11	6.30	2.50
9	1/16/09 Plate #1: 6mm @ 24 hr	4.59	11.61	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	1/16/09 Plate #2: Control @ 24 hr	2.85	13.35	6.30	2.50
3	1/16/09 Plate #2: 3mm @ 24 hr	1.75	14.45	6.30	2.50
4	1/16/09 Plate #2: 4mm @ 24 hr	1.58	14.62	6.30	2.50
5	1/16/09 Plate #2: 5mm @ 24 hr	1.98	14.22	6.30	2.50
6	1/16/09 Plate #2: 6mm @ 24 hr	1.42	14.78	6.30	2.50
7	1/16/09 Plate #2: 7mm @ 24 hr	1.49	14.71	6.30	2.50
8	1/16/09 Plate #2: Control @ 24 hr	2.85	13.35	6.30	2.50
9	1/16/09 Plate #2: 5mm @ 24 hr	1.98	14.22	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	1/16/09 Plate #5: Control @ 48 hr	1.63	14.57	6.30	2.50
3	1/16/09 Plate #5: 3mm @ 48 hr	1.24	14.96	6.30	2.50
4	1/16/09 Plate #5: 4mm @ 48 hr	1.38	14.82	6.30	2.50
5	1/16/09 Plate #5: 5mm @ 48 hr	1.39	14.81	6.30	2.50
6	1/16/09 Plate #5: 6mm @ 48 hr	1.27	14.93	6.30	2.50
7	1/16/09 Plate #5: 7mm @ 48 hr	1.01	15.19	6.30	2.50
8	1/16/09 Plate #5: Control @ 48 hr	1.63	14.57	6.30	2.50
9	1/16/09 Plate #5: 6mm @ 48 hr	1.27	14.93	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	1/16/09 Plate #6: Control @ 48 hr	1.21	14.99	6.30	2.50
3	1/16/09 Plate #6: 3mm @ 48 hr	0.98	15.22	6.30	2.50

4	1/16/09 Plate #6: 4mm @ 48 hr	1.24	14.96	6.30	2.50
5	1/16/09 Plate #6: 5mm @ 48 hr	1.20	15.00	6.30	2.50
6	1/16/09 Plate #6: 6mm @ 48 hr	1.13	15.07	6.30	2.50
7	1/16/09 Plate #6: 7mm @ 48 hr	1.13	15.07	6.30	2.50
8	1/16/09 Plate #6: Control @ 48 hr	1.21	14.99	6.30	2.50
9	1/16/09 Plate #6: 7mm @ 48 hr	1.13	15.07	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	1/17/09 Plate #1: Control @ 6mm	1.21	14.99	6.30	2.50
3	1/17/09 Plate #1: 0.25 hr @ 6mm	1.18	15.02	6.30	2.50
4	1/17/09 Plate #1: 2 hr @ 6mm	1.07	15.13	6.30	2.50
5	1/17/09 Plate #1: 6 hr @ 6mm	9.88	6.32	6.30	2.50
6	1/17/09 Plate #1: 24 hr @ 6mm	1.60	14.60	6.30	2.50
7	1/17/09 Plate #1: 48 hr @ 6mm	1.13	15.07	6.30	2.50
8	1/17/09 Plate #1: Control @ 6mm	1.21	14.99	6.30	2.50
9	1/17/09 Plate #1: 24 hr @ 6mm	1.60	14.60	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	1/17/09 Plate #2: Control @ 6mm	1.81	14.39	6.30	2.50
3	1/17/09 Plate #2: 0.25 hr @ 6mm	1.75	14.45	6.30	2.50
4	1/17/09 Plate #2: 2 hr @ 6mm	1.17	15.03	6.30	2.50
5	1/17/09 Plate #2: 6 hr @ 6mm	1.21	14.99	6.30	2.50
6	1/17/09 Plate #2: 24 hr @ 6mm	1.22	14.98	6.30	2.50
7	1/17/09 Plate #2: 48 hr @ 6mm	1.29	14.91	6.30	2.50
8	1/17/09 Plate #2: Control @ 6mm	1.81	14.39	6.30	2.50
9	1/17/09 Plate #2: 6 hr @ 6mm	1.21	14.99	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	2/18/09 Plate #1: Control @ 24 hr	3.06	13.14	6.30	2.50
3	2/18/09 Plate #1: 3mm @ 24 hr	2.01	14.19	6.30	2.50
4	2/18/09 Plate #1: 4mm @ 24 hr	1.22	14.98	6.30	2.50
5	2/18/09 Plate #1: 5mm @ 24 hr	1.23	14.97	6.30	2.50
6	2/18/09 Plate #1: 6mm @ 24 hr	1.68	14.52	6.30	2.50
7	2/18/09 Plate #1: 7mm @ 24 hr	1.77	14.43	6.30	2.50
8	2/18/09 Plate #1: Control @ 24 hr	3.06	13.14	6.30	2.50
9	2/18/09 Plate #1: 5mm @ 24 hr	1.23	14.97	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	2/18/09 Plate #2: Control @ 24 hr	1.38	14.82	6.30	2.50
3	2/18/09 Plate #2: 3mm @ 24 hr	1.36	14.84	6.30	2.50
4	2/18/09 Plate #2: 4mm @ 24 hr	1.38	14.82	6.30	2.50
5	2/18/09 Plate #2: 5mm @ 24 hr	1.35	14.85	6.30	2.50
6	2/18/09 Plate #2: 6mm @ 24 hr	1.28	14.92	6.30	2.50
7	2/18/09 Plate #2: 7mm @ 24 hr	1.21	14.99	6.30	2.50
8	2/18/09 Plate #2: Control @ 24 hr	1.38	14.82	6.30	2.50
9	2/18/09 Plate #2: 6mm @ 24 hr	1.28	14.92	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	2/24/09 Plate #1: Control @ 6mm	1.61	14.59	6.30	2.50
3	2/24/09 Plate #1: 15 min. @ 6mm	1.25	14.95	6.30	2.50
4	2/24/09 Plate #1: 2 hr @ 6mm	1.35	14.85	6.30	2.50
5	2/24/09 Plate #1: 6 hr @ 6mm	1.13	15.07	6.30	2.50
6	2/24/09 Plate #1: 24 hr @ 6mm	1.18	15.02	6.30	2.50
7	2/24/09 Plate #1: 48 hr @ 6mm	1.22	14.98	6.30	2.50
8	2/24/09 Plate #1: Control @ 6mm	1.61	14.59	6.30	2.50
9	2/24/09 Plate #1: 6 hr @ 6mm	1.13	15.07	6.30	2.50
10	STD	0.00	0.00	0.00	0.00

Well	Sample ID	Sample (ul)	dH2O (ul)	Loading Buffer (ul)	Reducing Agent (ul)
1	STD	0.00	0.00	0.00	0.00
2	2/24/09 Plate #3: Control @ 6.5mm	1.19	15.01	6.30	2.50
3	2/24/09 Plate #3: 15 min @ 6.5mm	1.26	14.94	6.30	2.50
4	2/24/09 Plate #3: 2 hr @ 6.5mm	1.25	14.95	6.30	2.50
5	2/24/09 Plate #3: 6 hr @ 6.5mm	1.24	14.96	6.30	2.50
6	2/24/09 Plate #3: 24 hr @ 6.5mm	1.39	14.81	6.30	2.50
7	2/24/09 Plate #3: 48 hr @ 6.5mm	1.42	14.78	6.30	2.50
8	2/24/09 Plate #3: Control @ 6.5mm	1.19	15.01	6.30	2.50
9	2/24/09 Plate #3: 2 hr @ 6.5mm	1.25	14.95	6.30	2.50
10	STD	0.00	0.00	0.00	0.00