Biomechanical Properties of Live Rat Brain Following Traumatic Brain Injury

by

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A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

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Abstract

Traumatic brain injury (TBI) has a 20% mortality rate and a 10-15% rate of resultant permanent disability. The consequences of TBI range from brief loss of consciousness, to prolonged coma or death. Mild TBI is amongst the common causes of admission to trauma centers all over the world. Future technologies such as magnetic resonance elastography and robotic surgery demand information about the physical properties of brain tissue. Walsh and Schettini described the mechanical behavior of brain tissue under normal status as nonlinear viscoelastic behavior and defined the associated biomechanical changes and responses in a quantitative measurement of the material changes. Yet, there is still a lack of data concerning time-dependent deformation and mechanical property changes associated with TBI.

My goal in this project was to describe these mechanical responses and to create a system for measuring and evaluating the mechanical response of brain tissue in vivo. This was to be achieved by inducing cortical contusions with a calibrated weight-drop method in seventy-four young adult male Sprague-Dawley rats. Instrumented indentation was performed on control brains and 1 hour to 3 weeks after contusion with intact dura using a 4-mm-diameter flat punch indenter to a maximum depth of 1.2 mm at loading. Loading rates did not exceed 0.34 N/min and 1.2 mm/min. In order to obtain force displacement data, we studied the elastic response of the traumatized brain tissue and the deformation process (creep) during the loading and unloading of indenter. After euthanasia, the brain was removed and evaluated histologically with different methods to reveal acute and chronic changes related to the contusion.

The results revealed that the biomechanical properties of the brain tissue were changed after cortical contusion. Brain tissue elasticity decreased in the edematous brain at one day following the contusion and increased at 3 weeks, in association with reactive astroglial changes. This experimental technique, combined with mathematical modeling, might eventually lead to a better understanding of the physical changes in brain following TBI.

Keywords: Brain. Traumatic brain injury. Weight drop. Viscoelasticity. Evans Blue. Brain Edema. Indentation. Magnetic Resonance Imaging. Gliosis. Rat. Viscous. Elastic. Creep.

Acknowledgements

I would like to thank my supervisor, Dr. Marc Del Bigio, for accepting me into his laboratory, for his constant support, and for providing me the opportunity to learn and explore the world of research. Dr. Del Bigio has been extremely patient, understanding, and very helpful throughout my tenure and his door was always open.

I also would like to thank all of the members of the Del Bigio lab for their support and assistance, especially Dr. Alex Shulyakov, who has continuously directed and assisted me in every aspect of my laboratory work and provided me with a large literature library to review. That is in addition to his great help in designing my experiment model and helping me during my indentation studies. Many thanks to Dr. Richard Buist for his advice and for doing the magnetic resonance imaging, and also to Terry Enno and Beatrice Buzahora for their technical assistance.

Many thanks to my friend Dr. Mahani for his continuous encouragement and endless support and advice. I would also like to thank Dr. Abdenor H. Timesguida, Program Director of the Libyan Cultural Section at the Libyan Embassy in Ottawa, for his continuous encouragement and support for this project and to the Libyan educational programm. Finally, I would like to extend a big thank you to the members of my committee, Dr. Maria Vrontakis and Dr. Brian Schmidt, for their time, patience, valuable advice and continued support.

I wish also to express my gratitude to my dear father and mother in Libya for their undying love and patience. They have thaught me to cultivate an inquisitive mind at a very early age and instilled in me the love of science, the importance of learning, and the

obligation to give back and contribute to the well-being of one's community and society. Without their inspiration and continuous support, I would never have envisioned pursing a medical career.

Naturally, this project would not have been undertaken, let alone achieved, without the unwavering love and support of my wife, Dr. Laila Ismail. She has been with me throughout, reading and discussing the different drafts, offering her ideas and insights, and providing constant encouragement despite her own hectic medical schedule and professional obligations. I will always be indebted to her and to our two children, Fatima and Mohammed, who fill our daily lives with unlimited joy and happiness.

Abbreviations

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TBI:	Traumatic Brain Injury
ANOVA:	Analysis of variance
CSF:	Cerebrospinal fluid
ICP:	Intracranial pressure
IOP:	Intraocular pressure.
SEM:	Standard error of the mean.
EBSD:	Evans Blue Spatial Distribution (% ratio to the brain slice area)
CL:	Core Lesion
L:	Lesion
E:	Young's modulus
SAH:	Subarachnoid hemorrhage
IVH:	Interventricular hemorrhage
V:	Velocity
L:	Length
Pa:	Pascal unit to measure the stress
PHe:	Petechial hemorrhage
SAH:	Subarachnoid hemorrhage
IVH:	Intraventricular hemorrhage
SDH:	Subdural hematoma
ICH:	Intracerebral hemorrhage
ID:	Indenter diameter
H:	Height
W:	Weight

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Chapter 1: INTRODUCTION AND LITERATURE REVIEW

1. 1. Brain Complexity

The brain is a semisolid tissue organ enclosed and protected by the skull. In adult humans it weighs about 1400g. It uses roughly 20% of the entire oxygen volume consumed by the body and receives approximately 15% of total cardiac output (Rockswold et al. 1994).[2] The brain has one of the most complicated geometries found in nature and there are corresponding complexities in the mechanical behavior of its components. The brain is composed of white and grey mater and blood vessels with cerebrospinal fluid (CSF) circulating inside and outside the brain. The brain is surrounded by skull bones and it is separated from those bones by meninges (Goldsmith et al. 2001).[3]

A volumetric change in any one of these components results in adjustments of the others, a process called compliance (Marmarou et al. 1978).[4] Traumatic brain injury (TBI) is one of the most frequent initiators of these changes because it can cause swelling and blood flow alterations (Ikeda et al. 1994).[5] TBI causes irreversible damage by at least three mechanisms: mechanical disruption of neurons or their projections, biomechanical or metabolic changes that are initiated by trauma, and reactive inflammatory or gliotic changes.

1.2.Traumatic brain injury

TBI is defined as "a blow or jolt to the head, which can disrupt the function of the brain.".[6] Such damage has the potential to negatively impact the life of the individual, their families, and society (Bruns et al. 2003, Olesenet al. 2003).[7] The consequences of TBI depend on the severity of the insult and anatomical region affected; results range from concussion syndrome, characterized by brief loss of conscious, to prolonged coma

or death. In mild cases, symptoms resolve within weeks to months, while the more severe cases involve longer periods of disability and have a 10-15% rate of permanent disability (Luerssen et al. 1988). [8]

The leading causes of TBI are falls, motor vehicle crashes, being struck by or against objects, and assaults (Rutland et al. 2005). [9] Blasts are a leading cause of TBI among active duty military personnel in war zones. Sports and recreation activities are also a major cause of TBI (Scott et al. 2005).[10] In North America, TBI is a leading cause of injury and death. In the United States, there are 1.4 million people diagnosed with a head injury each year; 50,000 of those victims die, while 80,000 suffer disabling injuries, and 235,000 are hospitalized (Rutland et al. 2005).[11] Approximately 5.3 million Americans, or 2% of the population, currently need long term or lifelong assistance to perform regular daily activities as a result of TBI. The costs associated with head injury totaled 60 billion dollars in 2000 (Thurman et al. 1999).[12] In Canada, the annual incidence of severe TBI is 11.4 per 100,000 and approximately half of deaths from all causes of trauma are due to head injury (Zygun et al. 2005).[13] The cost of hospital care, physician care and medical therapy totaled 151.7 million in 2000-2001.[14]

Classification of TBI. Although brain damage resulting from head injury is usually classified as either focal or diffuse, there are basically two main stages in the development of damage after head injury: primary and secondary (Scott et al. 1980).[15]

Primary Brain Injury. Primary brain injury usually occurs at the moment of injury, which is induced by any external mechanical force applied to the head directly or relative to acceleration-deceleration (Gennarelli et al. 1994, McLean et al 1997).[16] Primary brain

injury usually takes the forms of contusions, hematomas or diffuse brain injury (McIntosh et al. 1996).[17]

Secondary Brain Injury. TBI provokes a variety of disturbances and a series of destructive intracellular and extracellular pathologic processes, which include intracranial hematoma, disruption of the blood brain barrier, impairment in cerebral blood flow, brain edema and raised intracranial pressure (Conti et al. 1988, Fadenet et al. 1992, Katayama et al. 1990, Nilsson et al. 1993, Zipfel et al. 2000).[18] Systemic disturbances include hypotension, hypoxia, infection, and hyper and hypocapnia (Heegaard et al. 2007).[19] Identifying the degree and type of secondary brain insults is important in determining the final neurologic outcome of the patient who has suffered injury (Noppens and Brambrink 2004).[20] However, the mechanisms underlying secondary cellular death after TBI remain poorly understood.

1.3. Head Injury Models and Trauma Devices

Due to the complexity and heterogeneity of TBI subjects, a number of scientists have pointed out that there is no pharmacological treatment that has proven to limit the progression of secondary injury or to have a beneficiary effect on the outcome of TBI patients (Cernak 2005). [21] A comprehensive understanding of the pathophysiology, consequences and mechanisms behind primary and secondary injury after TBI is essential to the development of diagnostic tools, monitoring techniques and specific interventions that will improve the outcomes of traumatic brain injury and facilitate the development of therapeutic strategies for treatment TBI patients (Elf et al. 2002). [22] Such a comprehensive understandingof primary and secondary injury, in turn, requires experimental models of brain injury that can be characterized physiologically and biomechanically in order to

create better analytic modeling of tissue deformation. Lighthall and Anderson have explained the parameters needed for such models:

> "The purpose of experimental models of traumatic brain injury (TBI) is to replicate certain pathological components or phases of clinical trauma in experimental animals aiming to address pathology and/or treatment and the design and choice of a specific model should achieve the goal of the research by meeting the following criteria: the mechanical force used to induce injury is controlled, reproducible, and quantifiable, the induced injury is repeatable, quantifiable, the injury outcome, measured by morphological, histological, or mechanics parameters, is related to the used force , and the intensity of this force should predict the outcome severity" (Lighthall and Anderson 1994). [23]

All of the principle types of brain damage that occur in humans as a result of blunt injury have been reproduced in the laboratory using a range of techniques, species and methodologies designed to mimic clinically pertinent injury responses (Gennarelli et al. 1994).[24] Most of these studies focus on severe head injuries, while there have been just a few models developed to study mild traumatic brain injury. There are numerous TBI models designed to study the nature of human brain injury (Vander 2007, Cernak 2005).[25] For example, researchers have developed a pressure wave injury model (Ommaya et al.1970 , Feeney et al., 1981),[26] a cortical contusion model and a brain compression model (Lighthall et al. 1989). [27] Other studies have examined dynamic increases in intracranial pressure, fluid percussion injury (McIntosh et al. 1996, Dixon et al. 1988).[28] Models have been created of closed-head transitional acceleration (Marmarou et al. 1994),[29] and a model to measure high velocity injuries (Cernak et al. 2005).[30]

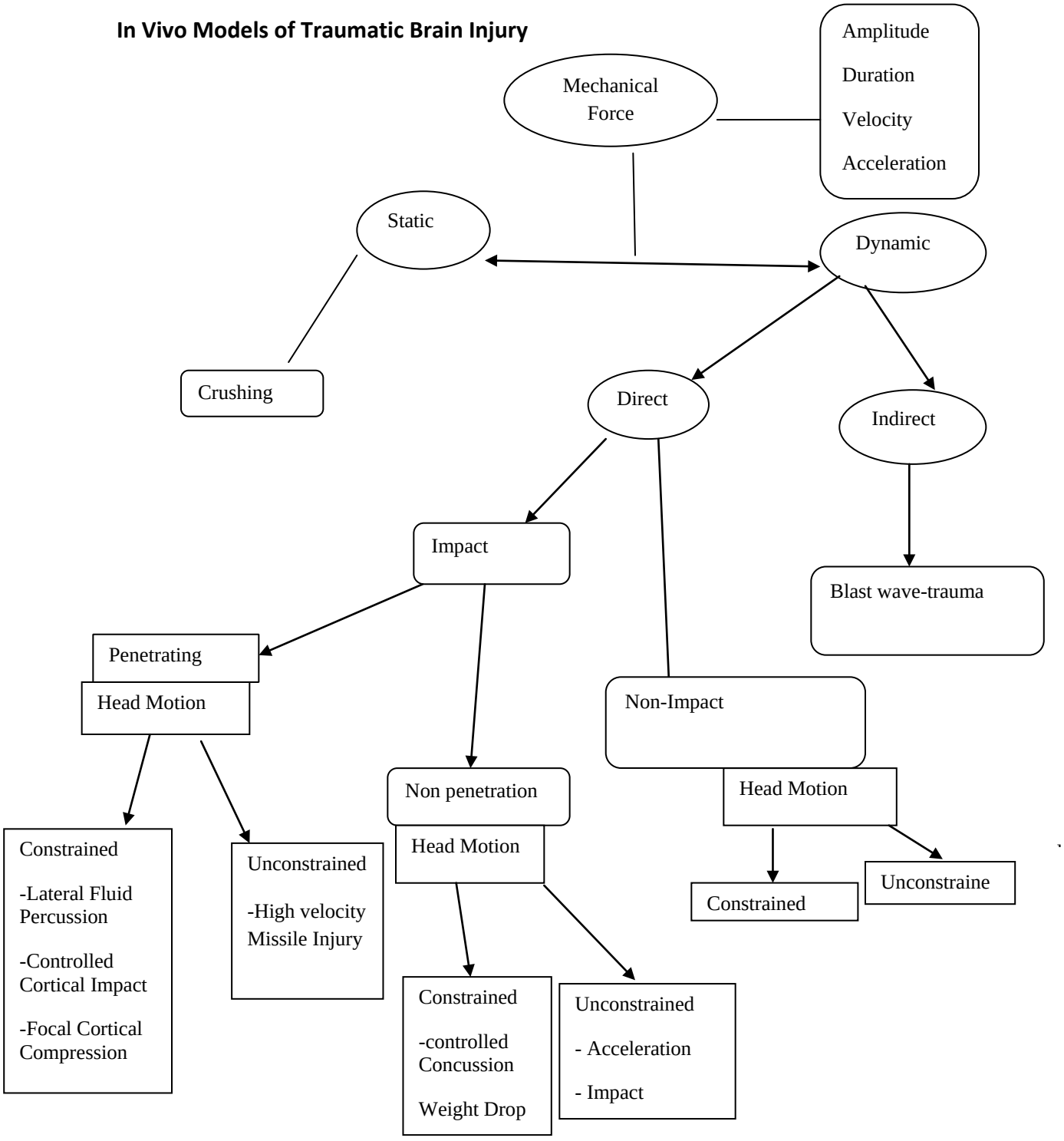

Figure 1: Animal Models of Head Trauma (adaptedfrom Cernak et al.).[31]

1.4. Animal Species

Laboratory animal models provide a wide range of neurophysiologic, molecular, and neurochemical information. Despite the many and varied opinions as to the preferred species to be used for modeling human TBI (Cenci et al. 2002)[32], many investigators have accepted rodent models as the most suitable for research. The relatively small size and cost of rodents permits repetitive measurements of morphological, biochemical, cellular, and behavioral parameters that require relatively large numbers of animals. However, differences between rodent and human systemic physiological and behavioral responses to neurotrauma have raised concerns about the use of rodents. Despite those doubts, rodents are still the most commonly used animals for modeling human TBI (Povlishock et al. 1995). [33]

1.5. Brain Contusion

Brain contusions occur most commonly in the frontal and temporal lobes and on inferior surfaces of the frontal and temporal lobes, where the brain tissue is in contact with bony protuberance. Contusions can extend through the cortex into sub-cortical white matter. Most of the time, they do not require neurosurgical intervention, unless there are serious injuries. [34]

There are certain categories of contusions, like fracture contusions, coup contusions, countrecoup contusions, and herniation contusion that are specifically associated with shifts of the brain within the head. Neuropath logical classification, in the early stages small contusions are merely collections of minute perivascular hemorrhages in the cortex, often only a few millimeters in diameter without any edema. In large contusions that begin as soft and granular but with time develop shrunken and often cystic

scars and areas of necrosismay also associated withsubarachnoid hemorrhageor with subdural or interventricular bleeding and swelling.(Hardam, J. 1979)[35]

In the early stage,microscopic examination of contusions usually shows hemorrhage, edematous tissue, and pyknotic (shrunken)neurons, necrosis cavitation in underlying white matter and perineural vascularization(Anderson and Opeskin 1998; ; Hausman and Betz 2000; Hausman et al. 1999, Loberg and Torvik 1989, Oehmichen et al. 1980, Feeney et al. 1981),[36]. In the following days to weeks the blood and necrotic material are removed by macrophages and replaced with hemosiderin deposition, foci of reactive astroglia and small clusters of microglia throughout the white matter. In order to assess contusion, a contusion index has been developed that allows the depth and extent of contusion in various parts of the brain to be assessed quantitatively (Adams et al. 1991). [37]

1.6. Swelling of the Brain Adjacent to Contusion

Brain edema is an abnormal accumulation of fluid within the brain parenchyma that results in volumetric swelling of the tissue (Dempsy et al. 2000, Gutierrez et al. 2001, Kuroiwa et al. 1997, Klatzo et al. 1967, Lighthall et al. 1989). [38] Brain edema is a common cause of brain swelling after TBI. If uncontrolled, it exhausts the volume reserves and leads to raised intracranial pressure and brain herniation (shifting of brain structures out of their normal anatomical location). Brain edema is usually due to a physical disruption of the tissue, damage to the blood brain barrier and loss of normal physiological regulation of arterioles (Miller et al. 2009). [39] It is associated with 50% of deaths that result from severe head injury.

Brain edema is classified into two types: vasogenic, and cytotoxic. Vasogenic brain edema occurs when an augmented vascular permeability results in increased edema fluid in the extracellular spaces. In cytotoxic brain edema, the primary characteristic is the swelling of the cellular constituents of brain parenchyma (Klatzo et al. 1967).[40] The cerebrovascular impermeability to serum proteins remains mostly intact in cytotoxic edema, while the augmented entry of water into brain parenchyma is mostly associated with osmotic gradients, which result from tissue damage and interference with cellular osmo-regulation. In addition, the regulation of water permeability at the cerebral capillary level might be influenced by the central noradrenergic system (Grubb Jr. et al. 1978). [41] Marmarou[42] noted that traumatic brain injury triggers a cascade of events, including mechanical deformation, neurotransmitter release (Katayama et al. 1990), [43] mitochondrial dysfunction, (Anonymous 2000, Dao et al. 2001, Okonkwo et al. 1999, Pettus et al. 1994, Xiong et al. 1997),[44] and membrane depolarization (Katayama et al. 1990). [45] Those events can lead to alterations in ionic gradients (Nilsson et al. 1993). [46] Excitatory amino acids released via mechanical deformation and membrane depolarization can activate ligandgated ion channels, which allow ions to move down their electrochemical gradients. These ionic disturbances result in cellular swelling and cytotoxic edema.

On the other hand, vasogenic edema, which usually resuls from the breach of the blood brain barrier (BBB), can be associated with traumatic lesions, tumors, and bleeding. The brain tissue undergoes secondary injury when edema is formed because raised intracranial pressures interfere with blood flow and tissue metabolism. This process may result in fatal complications, such as cerebral herniation, secondary hemorrhage and

infarction. The disruption of BBB can be assessed and quantified using T1-weighted magnetic resonance imaging following intravenous administration of contrast (Marmarou et al. 2003). [47] Vasogenic edema and extravasation of serum proteins can be assessed by trypan blue, or Evans blue injection into the blood (Klatzo et al. 1981 and 1987).[50] These bind to serum albumin and can be detected macroscopically in brain regions where the blood brain barrier is deficient.

1.7. Physical Properties Background

In order to understand the relationships between the brain and the mechanical forces that act upon the brain, some basic definitions must be explained. The following definitions are derived from authoritative references: (Klatzo et al 1981, Fung, Y. 1993, Sivaloganathan, S et al 2006, Goldsmith, W. 2001) [48] [52]

Biomechanics. The study of the mechanics of living organisms with a focus on forces and physical responses in static and dynamic biological systems. The role of biomechanics studies is to reveal the relationship between mechanical insult and its clinical consequences. There is also a focus on downstream pathological responses at the cell and tissue level that ultimately causes a pathophysiological injury response.

Creep. The permanent material deformation that occurs to reduce stresses. This deformation arises as a result of exposure to a stress that is greater than the strength of the material. The rate of this damage is the function of material properties, and depends on the magnitude of the stress used and its duration.

Deformation. The shape change produced in the tissue by the force. A rigid tissue would undergo very small deformations while non-rigid tissue can frequently endure noticeably large deformations.

Elasticity. The ability of a material to return to its normal shape after the stress ceases. The strain in an elastic material is instantaneously reversed with a return to the original state when the stress is removed. The yield point is the point along the stress-strain curve beyond which the material no longer exhibits elasticity. Elastic behavior can be expressed as the amount of stretching compared to the original length.

Elastic Modulus. The mathematical description of a material's tendency to be deformed when exposed to a force. It is calculated by the ratio of stress to strain, in which the stress representing the force causing the deformation is divided by the area exposed to the force. The strain will be represented by the ratio of the change caused by the stress to the original state of the object. The stress is measured in Pascals and strain is a unitless ratio. The units of Modulus E will be Pascals. The elastic modulus of the substance is always represented by the slope of the stress-strain curve (Modulus E = Stress/Strain). There are three types of elastic modulus, Young's modulus (E), which used to describe the tensile elasticity, Shear modulus (G),which is used to describe the shear tendency in the materials, and bulk modulus (K),which is used to describe volumetric elasticity.

Force. The action of one body on a second body. Force may increase the second body speed, or change its direction and/or its shape. A Newton (N) is a unit of force which, when acting along, causes a 1 kg mass to accelerate at a rate of 1 m/s^2.

Hysteresis. The process in which the body is subjected to acyclic loading. The stress strain relationship in the loading process is usually somewhat different from that in the unloading process.

Poisson's ratio. When the material stretched in one direction tend to get thinner in the other two directions, poisson's ratio measure this tendency, it is the ratio between transverse strain and extension strain.

Stiffness. The resistance of the elastic material (solid body) to deformation. This differs from the elastic modulus, which represents the properties of constituent material.

Strain. The action that is produced by stress on the body. It can be expressed as a change in size and/or shape. It can be estimated by calculating the change in the length of a line or by the change in the angle between two lines. The quantity strain can be measured by the following equation:

Equation (1): Strain= Deformation /Original = $\Delta L/L0$

Where L0 is the original length of the material, and ΔL is the change in length that results after the stress is applied.

Stress. The force strength that causes the deformation. Normal stress occurs when the first body contacts the second body perpendicularly to the second body's surface, whereas shear stress describes when that contact is tangential to the surface of the second body. The unit of measure is the Pascal (Pa); 1 Pa=1n/ m^2. The amount of mechanical stress created by a force is dependent on the size of the area over which the force is applied. Therefore, a given force acting on a small surface produces greater stress than the same force acting over larger surface.

Stress-strain relationship. Illustrated by a stress-strain curve, which is a plotted graph representing the measured stress in relation to the strain. The part of the curve that represents an increase in stress is called the loading curve, and the part that represents decreasing stress is called the unloading curve. The relationships between stress and strain are referred to as constitutive relationships and the resulting equations are used to define the mechanical response.

Equation (2): Stress= Force (F)/Area (A) where F is the force applied and A is the cross-sectional area of the material. Notice that the standard units of stress are [N/m^2] Newton per square meter; this is known as a Pascal, Pa.1 Pascal = 1 Pa = 1 [N / m^2]

Viscosity. The measure of a fluid's resistance to flow. A fluid with large viscosity resists motion because its molecular make-up provides it with a lot of internal friction. A fluid with low viscosity flows easily because its molecular make-up results in very little friction when it is in motion.

Viscoelastic. Materials that have both viscous and elastic characteristics when exposed to deformation. Viscoelasticity is the result of the diffusion of atoms or molecules inside of an amorphous material. Anelastic material is a special case of a viscoelastic material that recovers totally after removal of a transient load. Viscoelastic materials display a correlation between stress and strain that depends on time or frequency, and they have the following properties: hysteresis (the sum of all domains), stress relaxation (constant strain causes decreasing stress), and creep (stress causes increasing strain). When both creep response and load are separable, the creep response to loading in viscoelastic materials can be classified as a small deformation or in terms of linear viscoelasticity. When the response and load are not separable, the creep response is referred to as large deformation (non-linear) viscoelasticity.

1.8. Basics of Indentation Testing

Indentation (Bilton, L. et al. 1992)[53] is a well-known method used to illustrate the mechanical properties of materials and many soft tissues, such as the brain (Miller et al. 2000),[54] muscle, lung parenchyma, and the plantar fat pad (Gefen et al. 2001).[55] An indenter is pressed against the tissue and shear moduli are calculated from the applied load and extent of tissue deflection. In order to maximize the accuracy of the indentation method for soft tissue characterization, recommended that the indenter's tip radius R should be no more than 25% of the thickness of the tested tissue sample. As noted by Oliver and Phar:

> "Elastic modulus measurement by instrumented indentation techniques method was introduced in 1992, and has been widely used in characterization of mechanical behavior of materials at small scales, by this method mechanical properties can be determined by measuring the indenter Penetration depth, (h), under the applied force, (F), during loading and unloading cycles." (Oliver, W., and Pharr, G. 1992)[56]

For modeling purposes, the deformation during loading is assumed that elastic and plastic in nature as the permanent hardness. During unloading, it is assumed that only the elastic displacements are recovered. Elastic Modulus were defined as contact stiffness, or the slope of the upper portion (Tangent)of the unloading curve during the initial stages of unloading, were calculated directly from the force-displacement curve by measuring the maximum force, maximum displacement (h), and final depth of the penetration after the indenter unloaded: Equation (4) $S=dp/dh$

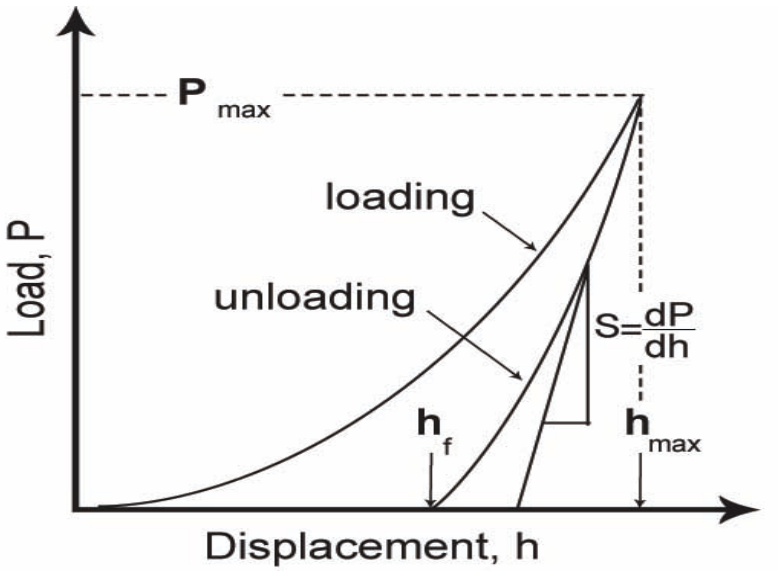

Figure 2: P-h curves. Schematic illustration of indentation load-displacement data showing important measured parameters: p = maximum load, h= maximum displacement, s = slope (stiffness) of unloading curve. *(Adapted from Pharr et al. 2004).*

The analysis used to determine the hardness, H, and elastic modulus E, is essentially an extension of the method proposed by Doerner and Nix.(Doerner, M., and Nix, W. 1986)[57] That method accounts for the fact that unloading curves are distinctly curved in a manner that cannot be accounted for using the flat punch approximation. In the flat punch approximation used by Doerner and Nix, the contact area remains constant as the indenter is withdrawn, and the resulting unloading curve is linear. In contrast, experiments showed that unloading curves are distinctly curved and usually well approximated by the power law relation.

Equation (5) $P = \alpha(h-hf)^m$, where α and m are power law fitting constants.

1.9 Neuronal Tissue Mechanical Properties

Due to the complexity of the brain and its meningial covering, information on living neural tissue mechanical properties is scant (Goldsmith et al. 2001, Ommaya et al. 1968), [58]

although many papers have been published concerning ex vivo properties.(Cheng S.et al 2008)[59] These properties depend on the structured elements of the brain tissue and its fluid contents. Using the method of Galford and Mcelhaney, [60] Ommaya was only able to determine the mechanical properties of brain tissue in vitro, on samples of dead brain tissue (Ommayaet al. 1970).[61] Fallenstein and a group of others scientists tried to investigate the mechanical response of the brain tissue after an impact to the head in postmortem human brain tissue (Fallenstein et al. 1969).[62] Walsh and Schettini measured a living dog brain's elastic response to impact by using a pressure displacement transducer, which permits simultaneous measurements of pressure and displacement (Walsh and Schettini, 1976). [63] After many experiments, these properties were characterized as inhomogeneous and found to exhibit nonlinear viscoelastic response behavior (Walsh and Schettini, 1976).[64]

It is crucial to have information regarding the physical property of the brain in vivo if we are to know the physical changes of the diseased brain and gain a better understanding of the physical mechanisms involved in TBI (Wataru et al. 1961).[65] Yamamoto tried to create a tool to assess the soft tissue stiffness quantitatively and intraoperatively in order to measure the tissue swelling, and he found that he can use this tool to assess the brain tissue intraoperatively (Yamamoto et al. 2004).[66] Although there was a heated debate concerning the mechanical properties of dead brain tissue and whether or not they apply to living tissues (Ommaya et al. 1970), [67] and despite the fact that there is little information on the effect of cerebral blood flow in live tissue, or on the effect of blood perfusion on brain mechanical properties, Gefen's experiments indicated that blood perfusion in living brain tissue has no effect on mechanical properties (Gefen et al. 2004). [68]

There have been the many studies of the mechanical and structural properties of the brain in normal conditions or after injury that have been conducted in approximately the past four decades (Gefen et al. 2003). [69] Yet, biomechanical models of TBI based on the study of the physiology and biomechanics of the brain, which could potentially be used to simulate and predict outcomes of biomechanical changes in the living brain after injury, are still not fully developed. Thus, there is much work to be done in this area. Biomechanical models of TBI are an important tool to enhance our understanding of the mechanical events occurring within the traumatically affected brain during the injury event (Kleiven et al.2002, Levchakov et al. 2006, Takhounts et al. 2003, Zhang et al. 2001).[70] The importance of these models in predicting physiological outcomes of injury relies on the specific mechanical parameters criteria of each part of the brain reported from previous experiments (Lighthall et al. 1994).[71]

1.10. Mechanical Response to Traumatic Insult

Brain tissue is considered to be more sensitive to shear strain than it is to extensional strain. For that reason, the loading that involves rotation of the head has been thought to result in more severe injuries, although this conclusion has been questioned recently (King et al. 2001).[72]

An understanding of injury biomechanics is essential to the development and interpretation of experimental studies in vivo and in vitro. Such understand might even lead to the ability to predict responses to deformation in pathological situations including trauma and surgical manipulation, like retraction or during robotic surgery (Darvish et al. 2001).[73] A traumatic insult to the brain will lead to a mechanical response of the tissue that is dependent on the mode, severity and anatomical location of the impact as well as on the

mechanical properties of the tissue. These may affect the initial injury or delayed damage. Tissue damage depends on the threshold of the tissue, which is dependent on the type and duration of the load. Applied loads are described as force and stress and the ensuing responses are deformation and strains (Bilton et al. 1992, Davis et al. 2006, Michael et al. 2000, Jaroslav et al. 2006).[74]

The mechanical properties of the brain tissue vary from individual to individual, depending on elements such as age and previous injuries or diseases (Prange and Margulies, 2002).[75] In addition, cellular orientation and tissue composition varies among anatomical regions of the brain, creating heterogeneous mechanical properties that directly affect structural and functional tolerances as well as the load distribution on the tissue upon mechanical loading. Because of the properties of soft tissues like brain, both the rate and the duration of the insult will also influence the response. Loads that are applied quickly may incur more damage due to the material properties of nervous tissue. When loads are applied at high rates, the tissue cannot absorb or reduce the force fast enough and can fail both structurally and functionally. On the other hand, slowly applied loads give the tissue time to reduce the force and generally result in less damage. In cases where there are short durations of force, much of the effects of the force are reduced. As the duration of force increases, less reduction occurs and therefore less force is needed to produce tissue deformation.

These behaviors are defined by the mechanical property termed “viscoelasticity behaviors” (Davis et al. 2006, Lakes et al. 2004, Mchedlishvili et al. 1979a and 1979b, Walsh et al. 1977).[76] It has been shown that the changes in the mechanical properties of

the brain tissue after craniotomy, apart from other abnormalities, were responsible for the fluid accumulation of the brain tissue in the course of edema development (Mchedlishvili et al. 1979).[77]Aoyagi et al. presented only one quantitative data analysis that has confirmed the increase of brain tissue elasticity after cryogenic injury (Aoyagi et al. 1982)[79]. Kuroiwa et al. attempted to study the chronological changes in cat brain tissue elasticity after cryogenic injury and during the development and resolution of vasogenic brain edema. It was found that there were significant decreases in tissue elasticity in the 24 hours after the injury and an increase in elasticity after ten days (Kuroiwa et al.).[81]
It must be underscored that no one to date has studied mechanical properties in living brain tissue following experimental contusion, which is clinically more relevant than cryogenic injury.

1.11. Hypothesis and Goals

- I hypothesize that the mechanical properties of brain tissue will change as the brain composition changes following contusion injury.

- The specific goals of this study are to:

- 1. Create calibrated cortical contusions in live rats using a weight-drop device with a stereotaxic constrained head and intact meninges.
- 2. Study the time-dependent deformation mechanics, such as creep and viscoelasticity in control and injured live rat brains.
- 3. Assess the correspondence between mechanical properties and histologic findings (cortical damage, hemorrhage, edema, reactive astroglial and microglial changes).

CHAPTER 2: MATERIALS AND METHODS

2. 1. Animal Preparation

For all experimental studies, 74 Sprague-Dawley (SD) rats purchased from Central Animal Care of the University of Manitoba were used. All rats were males, two months old and weighed between 300 and 350 grams. All animal procedures were conducted in strict accordance with the Canadian Council of Animal Care. Use of laboratory animals was approved by the institutional animal care and use committee of the University of Manitoba. All rats were kept under standard conditions: they had free access to food pellets and water and were kept in a temperature-controlled vivarium on a 12:12-hour light-dark cycle. They were handled daily before brain injury induction and experimental design.

2. 2. Weight Drop Device

Rats were subjected to mild traumatic brain injury (MTBI) with a weight drop device as described by Feeney (Feeney et al. 1981; Anderson et al. 1982) [82] and modified in the Marmarou model (Marmarou et al. 1994).[83] This device consists of dropping a standardized weight through a hollow vented guide tube held perpendicular to the brain with intact meninges. The weight strikes a light weight impounder resting on the dura, ensuing in brain injury. Injury severity is assessed by the g-cm product of drop weight mass and drop height; that is, it is assessed in terms of potential energy. It is important to standardize the weight used because even when the g-cm product is held constant, the functional and anatomical severity of injury can change significantly when different combinations of drop-weight mass and drop height are used. Errors can also arise due to

differences in the impounder contact area or from multiple impacts, because of drop-weight bounce. The model was used in its original and modified forms and the surgical procedure used was identical with all animals.

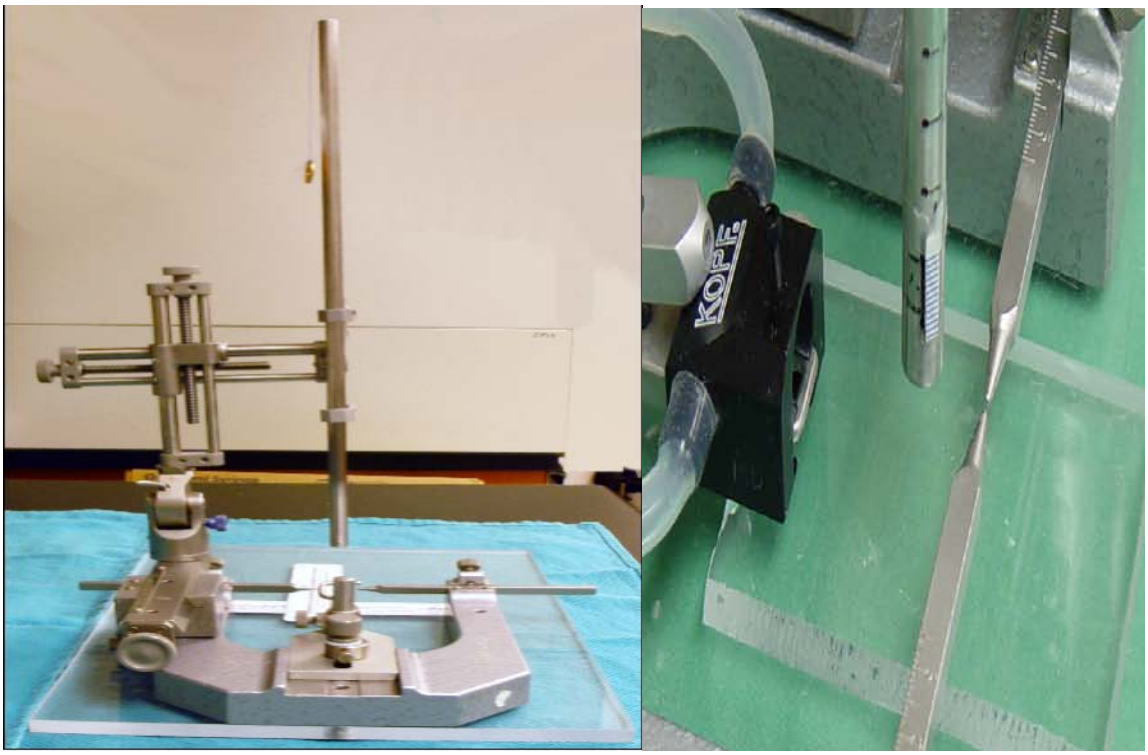

Figure 3: Weight drop device with stereotactic frame

2. 3. Surgical Procedure

Before surgery, rats were given Buprenorphine (0.03 mg/kg body weight) subcutaneously for pain relief. Rats were anaesthetized in induction chamber with 2% Isoflurane mixed with Oxygen, and then the scalp was shaved. The rats were placed in a prone position over a hard warming blanket, with all limbs fixed in place by tape and were anesthetized with isoflurane (3–5%): O2 (1 l/min); they were allowed to breathe spontaneously.

All procedures were performed under aseptic conditions, using Endure and 70% ethanol wash three times and final cleaning by Betadine, A 1.5 cm sagittal scalp incision

was made, with the removal of soft tissues, under the guide of an operating microscope. Using a motorized drill, a 5-6 mm craniotomy between the Bregma and Lambda on the right fronto-parietal side of the skull was created. Irrigation with saline during drilling prevented heating. Care was taken to leave the meninges intact. The heads of the rats were fixed in a stereotactic frame. The stainless steel tube, 45 cm in length, was adjusted to be at a 90° degree in relation to the cortical surface in order to allow the sterilized indenter (diameter 4 mm) to be positioned on the cortical surface of the right fronto-parietal cortex of the brain with intact meninges. We measured the penetration depth at this point .

To induce an identical injury profile, and after a pilot study to determine the force needed to produce repeatable injury, we exposed all rats to the same force by allowing 20 grams weight to fall from 20 cm height, on an indenter with a radius of respiratory 4 mm. To avoid depression, the anesthesia was decreased during injury induction. To prevent adhesion between the scalp and dura, we covered the craniotomy area with a layer of sterile bone wax then the scalp was stapled. For the control groups, only a craniotomy was done. During the procedure, animals were allowed to breathe spontaneously and the body temperature was kept at 37°C, as measured using rectal temperature, using a warming blanket (Hertog et al. 2007). [84]

As scheduled (see below), each group of rats was reanesthetized. Under the guidance of a surgical microscope, the incision was reopened and the scar tissue was carefully removed from the site of injury. Then, the femoral arteries were dissected and cannulated to inject Evans blue dye in order to assess integrity of the blood brain barrier. Finally, all animals were humanely killed under anesthesia and their brain tissues were

extracted from the control and experimental rats in order to begin brain cutting and histological studies.

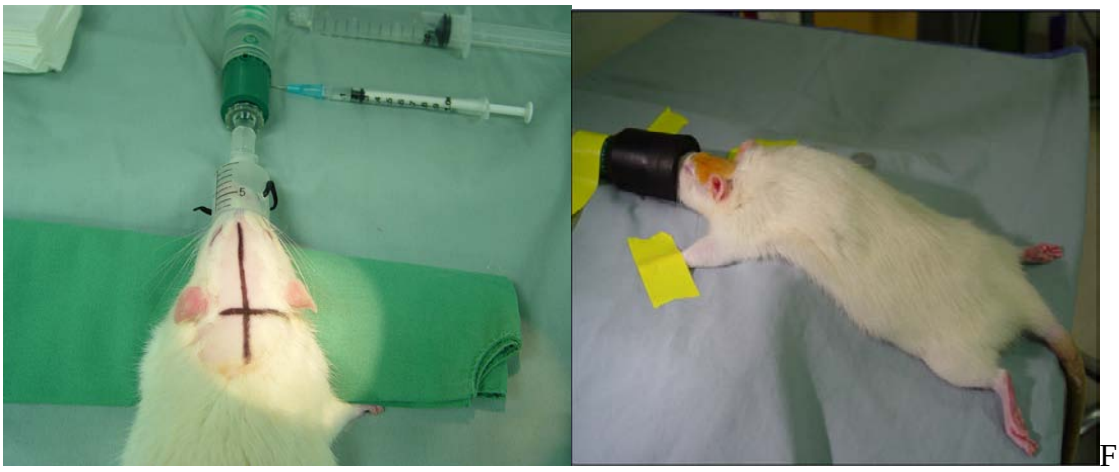 F

Figure 4: Skin incision landmarks, skin shaving and sterilization.

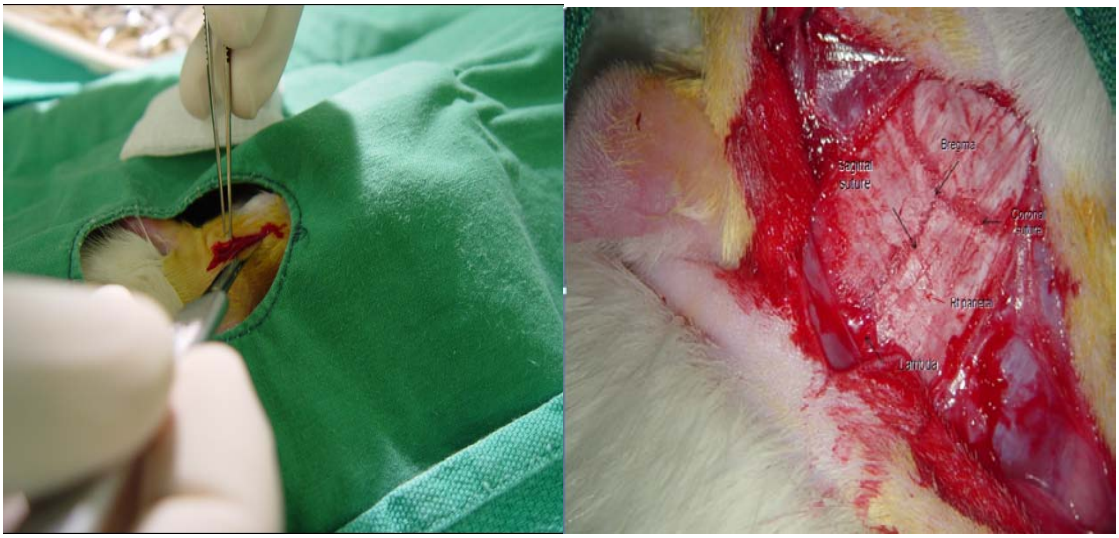

Figure 5: Incision of skin and craniotomy landmarks

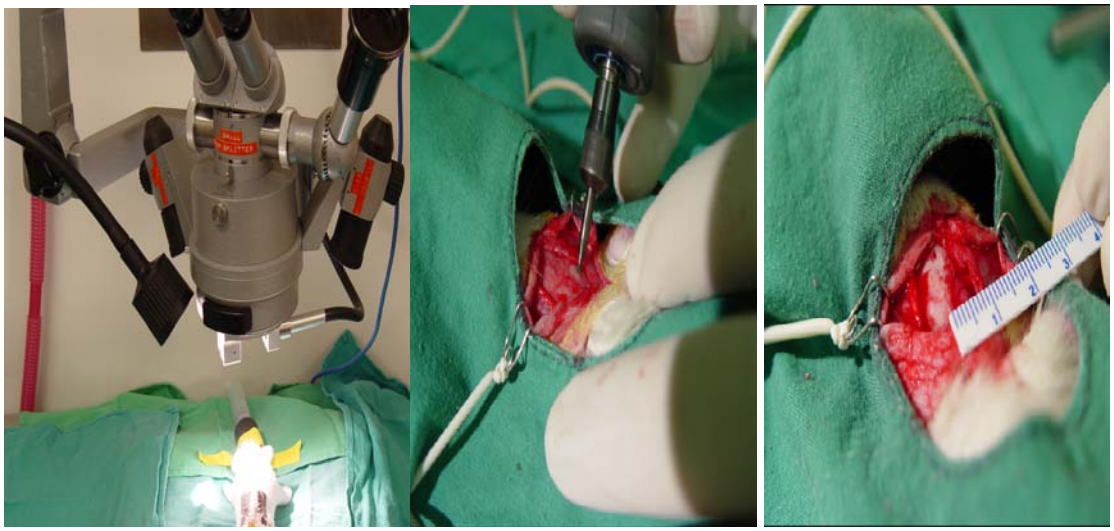

Figure 6: Positioning under the surgical microscope, drilling of the craniectomy, and measuring the craniotomy opening size (≈6mm)

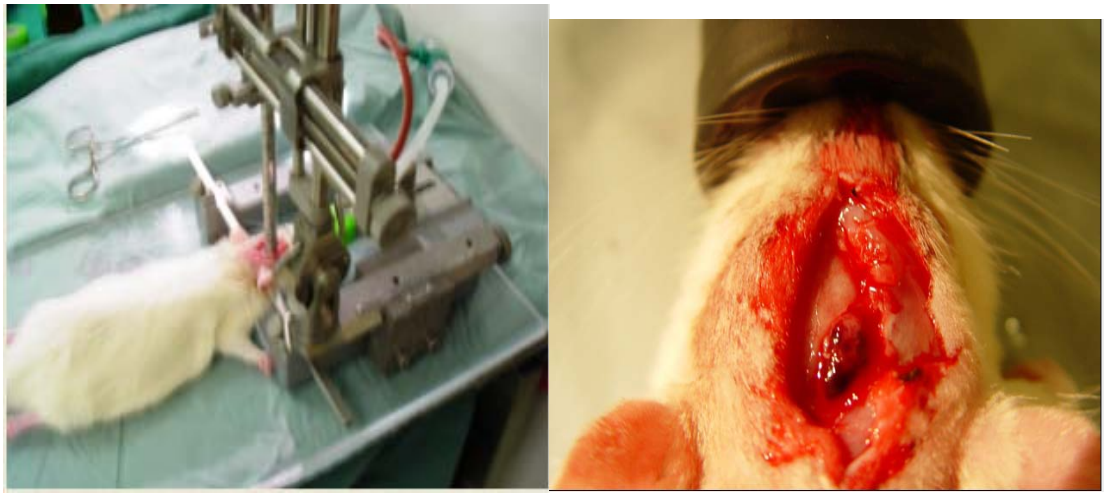

Figure 7: Weight dropping on dura and induced trauma

2. 4. Neurological Assessment

Immediately following the brain impact or craniotomy (sham group) and after suturing the skin, the animals were removed from the device and tested for pinch reflex response every 5 minutes, until the full recovery of reflex function was achieved. The time needed

for full recovery was up to one hour; the animals that failed to regain these functions died and were excluded from the study. Full recovery time for the sham group was approximately 20 minutes. To test pinch reflex, we applied a foot pinch using toothed forceps to detect withdrawal limbs. We conducted neurological assessments at 24 hours, 72 hours, and 7 days after injury.

2. 5. Magnetic Resonance Imaging

After craniotomy and contusion induction, and according to the indention test time schedule, all animals included in this study were re-anesthetized and magnetic resonance images (MRIs) were acquired using a 21 cm bore size 7 Tesla Bruker Magnet running Bruker Biospec/3 imaging software. Coronal T1 and T2 weighted images were acquired using a slice thickness of 1 mm (Albenis et al. 2000)[85] Animals were placed in supine position in Plexiglas holders with the body placed on a heating pad. Optic fiber temperature probes were then placed in the rectum and rectal temperature was maintained at 37˚C by adjusting the heating pad's temperature accordingly. In addition, respiratory motion detectors was fastened over the thorax to facilitate respiratory gating and avoid any haziness in the images by respiratory movements, while coronal images were collected throughout the brain regions of interest; multi-slice T2 weighted images were acquired to obtain four contiguous images; hyperintense lesions were noted on the side of trauma; and comparisons were performed between all experimental rat groups. The MRI of the sham groups (2h and 21 days) did not show any pathological lesions. In some cases, there was a small amount of hemorrhage at the site of craniotomy, which was detected as low signal regions on T2 images.

2. 6. Indentation Test

Instrumented brain indentation was performed with axisymmetric "flat punch" indenters of 2 mm radius. The standard sequence of indentations was as follows: 1. Simple non-preconditioned loading-unloading test; 2. Modified creep test; 3. Continuous multicycle loading-unloading test. All tests were made with recovery periods of approximately 15 minutes between tests. The optimum contact force setting, at which the indentation cycle is triggered, was previously determined to be 0.0040 - 0.0046 N (Shulyakov et al., 2009). [86] Force, depth, and timing parameters that allow acceptable loading-unloading indentation curves had been previously determined. The maximum force of 0.17 N was applied at a rate of 0.34 N/min and was reached after 30 seconds. These forces were chosen based on pilot data to achieve an indentation depth of ~1-1.2 mm. During the simple loading-unloading test, the indenter was immediately unloaded at the same rate to zero force. Force-deformation data points were recorded at the rate of 10 data points per second (10 Hz). The indentation test software (CSM Instruments, version 4.1) automatically computes the indentation modulus (EIT) from the slope of the tangent of the initial unloading curve using the power law fitting function (Doerner et al., 1986)[87] as reported by Oliver and Pharr (Oliver et al., 1992; Oliver et al., 2004)[88]. For both the automated calculations, a Poisson's ratio of 0.45 was used, for reasons previously discussed (Shulyakov et al., 2009). [89]

A true creep test with instantaneous loading followed by hold at constant force could not be attained for technical reasons and was therefore substituted with a modified test. The indenter was loaded over 30 seconds at the forces described above, and the maximal force was held for 60 seconds. Brain creep behavior (C) was measured as a

relative change between the initial and final penetration depth during the hold period and is reported as a percent. Another measure of viscous deformation was measured using an indentation series with the same maximum loading force applied over 10 seconds followed by unloading to 0 force at the same rate, repeated without pause over 5 cycles. We compared the maximum penetration depth on the first and fifth cycles and express the difference as a percent.

Under isoflurane anesthesia (breathing spontaneously) and with the head placed in a stereotactic frame, the rats were re-anesthetized and placed in a prone position over the warming blanket, with the head fixed in a stereotactic frame. After the incision, the site cleaned with Endure and 70 % ethanol three times, followed by the application of Betadine, using an operating microscope. A 1.5 cm sagittal scalp incision was used for reopening, with the removal of soft tissue scars, and the dura was kept intact, Indentation tests were conducted for all animals included in this study according to their trauma time as experimental groups (2 hrs, 6 hrs, 24hrs, 3 days, 7 days, and 21 days after injury), and two sham (craniotomy only) groups (2 h and 21 days of survival). Using a single indentation cycle and an assumed Poisson's ratio of 0.49 (Itoh et al. 2006), [90] the indentation modulus (E_{IT}), was automatically calculated by Oliver and Pharr [91] method with kPa as measuring unit. The creep and multi-cycle indentation were calculated using a computer program.

We chose to measure the elastic and viscous properties of the brain with the open platform micro-hardness tester (MHT, CSM Instruments SA; Peseux, Switzerland). MHT is an instrumented indentation system that allows measurements of mechanical properties by controlling the force or the depth at high resolution through load-unload in a

single or multi-cycle indentation in order to evaluate the plastic and elastic deformation in brain tissue specimen. The specific use of this device (figure 8) for in vivo brain testing was developed in the Del Bigio Laborartory (Del Bigio et al. 2008),[92] following the same principles used by Vanlandingham (2003) [93] and Randal (2003), [94] (Shulyakov, Del Bigio et al. 2009).[95] To probe the mechanical responses of different regions of the rat brain, we have used the following parameters: indenter radius (2 mm) and constant depth of penetration (δ= 1.4mm) probe. Shulyakov and Del Bigio designed a rat head-holder that includes a transparent acrylic frame with an anesthesia cone attached to gas delivery and exhaust hoses, and provides three-point skull fixation using an incisor bar and two ear bars. (*Figure 9*). The body platform, which is affixed directly to the indenter stage, is covered with a warming pad to maintain body temperature. The head holder was also designed on the same principles, utilizing a zygomatic clamp (Model 1240, David Kopf Instruments, Tujunga, CA).

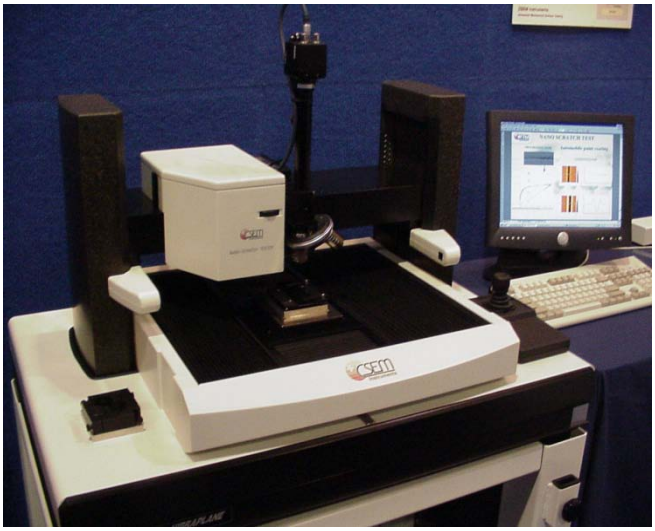

Figure 8: Open platform micro-hardness tester
(MHT, CSEM Instruments SA; Peseux, Switzerland)

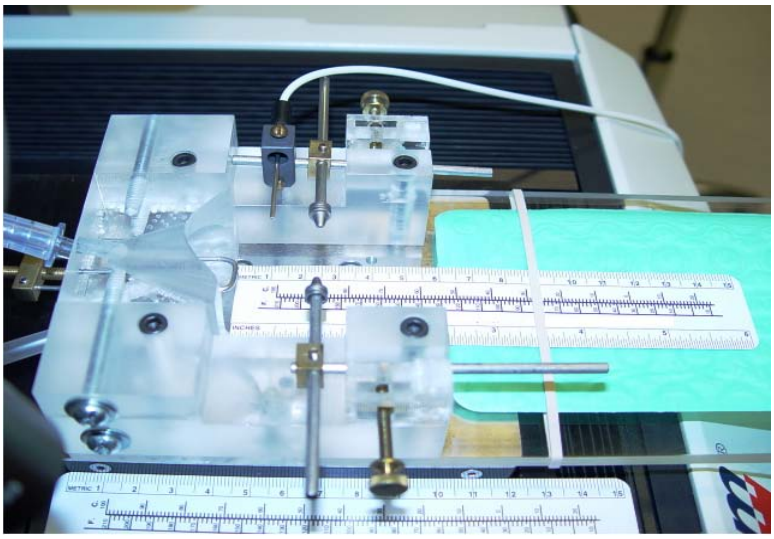

Figure 9: Side view of the rat head holder, includes ear bars, incisor bar, warm blanket, and nose cone.

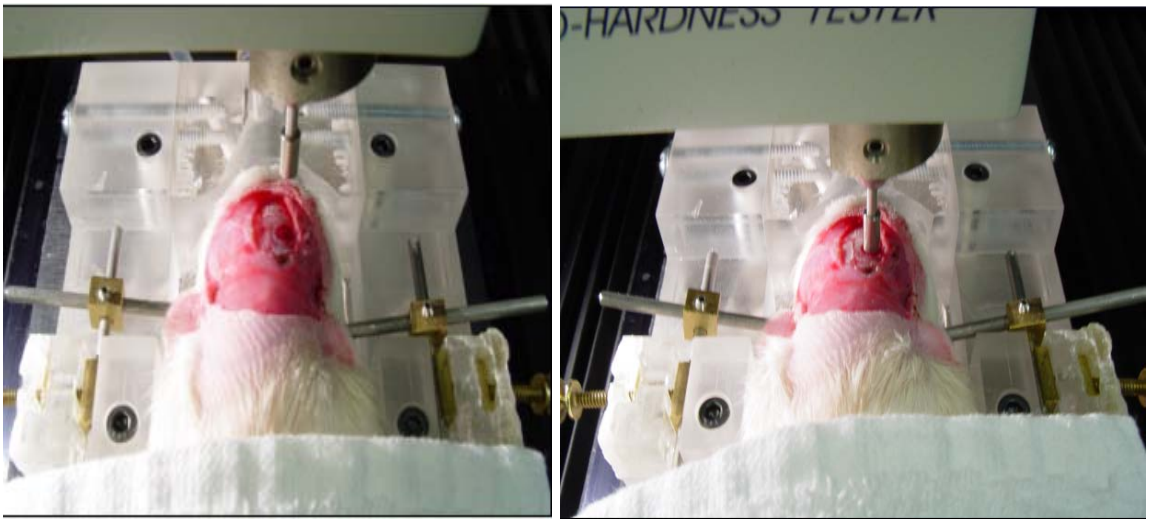

Figure 10: Fixation of Rat head for indentation

2. 7. Evans blue dye injection and trace to assess the Integrity of the Blood Brain Barrier

Before killing the rats, and under anesthesia and microscopic guiding, the left femoral vein was dissected and a catheter was placed to inject 2 % Evans blue dye (EBD) in saline (2 ml/kg). In the literature, it is known that Brain edema is apparent from 3 hours up to five days after injury. Edema peaks between 24-48 hours (Klatzo et al. 1981)[96], and then begins to decrease (Rapaport et al. 1976).[97]

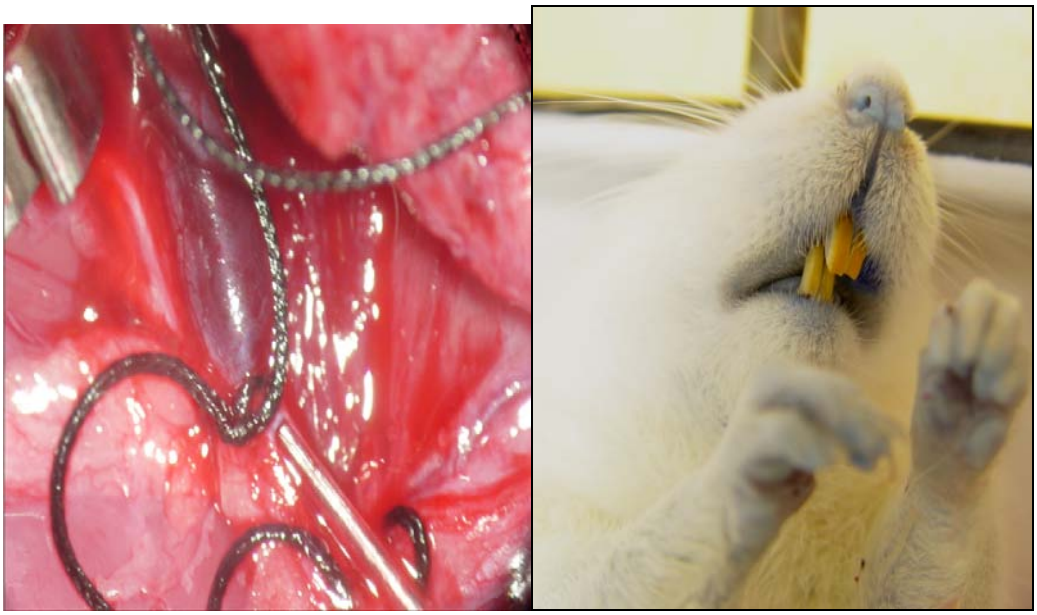

Figure 11: Insertion of left femoral vein catheter and distribution of Evans blue dye after injection (mucous membranes are bluish)

2. 8. Animal Euthanasia and Brain Cutting

At the set point, rats were humanely sacrificed. We exposed the animal to deep anesthesia, using 2% isoflurane with oxygen, then Animals were euthanized by CO2 narcosis then perfused through the heart. by opening chest rapidly and we introduced a perfusion catherer catheter into the left ventricle and incised the right atrium, (Fusion of 100-500 ml of 10% paraformaldehyde).

To remove the intravascular EB dye, the animals were perfused with saline through the left ventricle at 100 cm of water pressure until a colorless perfusion fluid was obtained from the right atrium. The brains were then removed and fixed in situ paraformaldehyde solution for 2 days before being removed from skull. The brain is put in the matrix and tissues including the contused brain were sliced in 1 mm coronal slices beginning at the periphery to avoid brain shift. The slices were photographed and returned back in formalin, and then were dehydrated and embedded in paraffin for optimal morphologic detail.

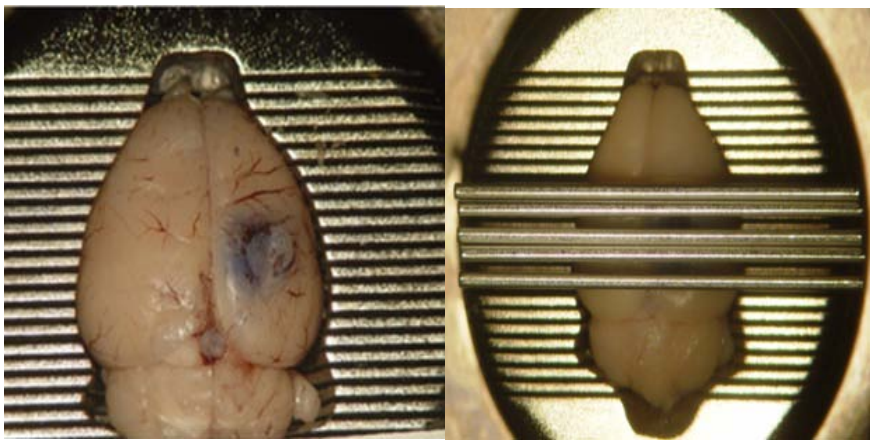

Figure 12: Brain cutting process

2. 9. Histopathological Evaluation

For histological processing, every 10th (µm) section was stained with hematoxylin and eosin (H&E). Reactive astrocytes were demonstrated using Immunohistochemistry with anti-glial fibrillary acidic protein (GFAP) and activated microglia were detected with Griffonia simplicifolia B4 isolectin (GSA I-B4). All slides were examined by light microscopy.

Histological observation of the samples was oriented predominantly to the estimation of the extent of lesions and the presence of edema and their characterization. Under microscopic examination, in order to assess contusion, a contusion index has been developed that allows the depth and extent of contusion in various parts of the brain to be assessed quantitatively by using this formula of contusion size (max width x max height / 2) assuming a roughly hemispherical shape. (Scott et al. 2005).[98]

2.10. Statistical Analysis

Statistical assessment utilized analysis of variance (ANOVA) with post hoc intergroup comparisons.

2. 11. Pilot Trials

Several preliminary experiments were done to produce repeatable, equal, and focal cortical contusion on twenty rats were randomly divided into nine groups, with two rats in each group. The weight drop model differed for each group, using different parameters with weights (10, 20, and 40 grams), heights (15, 20, 25 cm), and indenter diameters (2, 4 and 6 mm). The parameter were as following: Group I (40 g, 25 cm, 6 mm, n=2); Group II (40 g, 20 cm, 4 mm, n =2); Group III (4g, 15 cm, 2 mm, n=2); Group IV (20 g, 25 cm, 6 mm, n=2), Group V (20 g, 20 cm, 4 mm, n=2); Group VI (20 g, 15 cm, 2 mm, n=2); Group VII (10 g, 25 cm, 6 mm, n=2), Group VIII (10 g, 20 cm, 4 mm, n=2); Group IX (10 g, 15 cm, 2 mm, n=2) and two rats died from apnea. The following table shows the parameters used for each group to induce contusion as well as number of rats in each group.

Table 1: Summary of pilot study groups with the parameters used to induce the contusion

Groups	Parameters
I N=2	W=40g ID=6mm H=25cm
II N=2	W=40g ID=4mm H=20cm
III N=2	W=40g ID=2mm D=15cm
IV N=2	W=20 ID=6mm H=25cm
V N=2	W=20gm ID=4mm H=20cm
VI N=2	W20gm, ID=2mm H=15cm
VII N=2	W=10gm ID=6mm H=25cm
VIII N=2	W=10gm ID=4mm H=20cm
IX N=2	W=10gm ID=2mm H=15cm

W=weight, mm=millimeter, cm=centimeter, H=height, ID=indenter diameter.

2. 12. Measuring the Penetration Depth

To measure the PD, a ruler was placed at the lower end of the steel tube close to longitudinal opening, which was around 2cm in length. We lowered the steel tube until the indenter touch the meninges and the steel tube touch the skull. Then we measured (PD1) the penetration depth, and by releasing the pin to allow the impacter to fall on the right frontoparietal cortex and induce the injury, we measured (PD2) again and subtracted the first measured (PD1) from the last one (PD2) and from that we calculated the true penetration depth (PD=PD2-PD1).

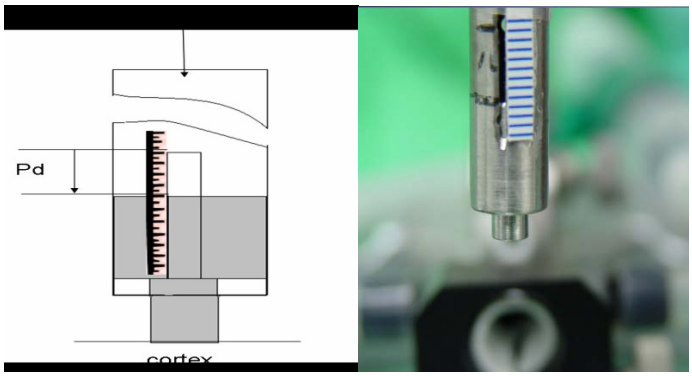

Figure 13: Measuring of penetration depth

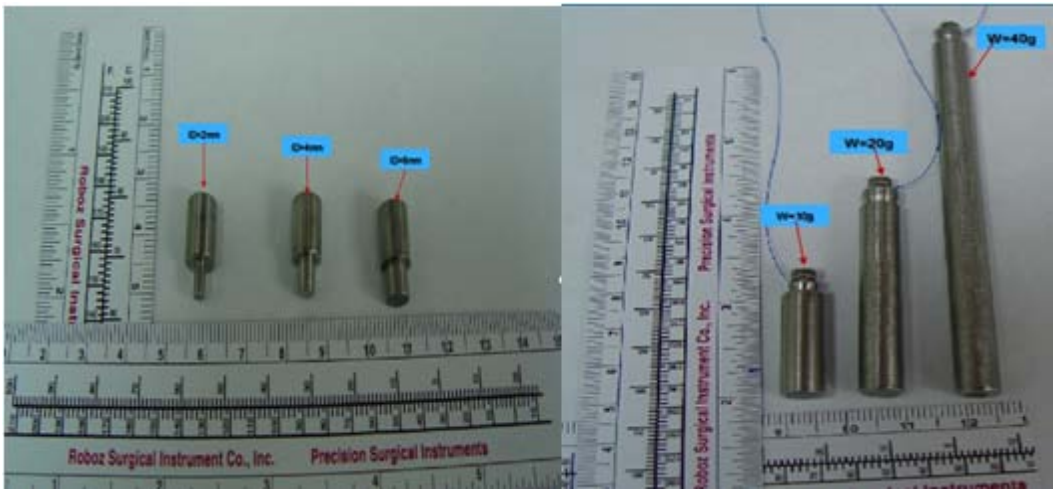

Figure 14: Different weights and different indenter diameters used in this study

2.13. Measurement of Impact Force

After measuring the penetration depth with each injury, and using the previous calculated kinetic energy KE ($KE=mv^2/2$) with different parameters, we calculated the forces needed in each case by using this formula: $F=KE/PD$

2. 14. Main Experiment Design

In our study fifty-four Sprague-Dawley young adult rats weighing 250-350g were randomly divided into 8 groups. There were 5 rats for each group. The six trauma groups were studied two hours, six hours, twenty four hours, three days, seven days, and twenty-one days after brain contusion. There were two control groups to study changes after only craniotomy after 2 hours and after twenty-one days. MRI testing was administered under anesthesia for all rats, and all rats were re-anesthetized for indentation testing followed by immediate euthanasia as scheduled for each group.

Chapter 3: RESULTS

3. 1. Standardization of the Contusion Method

Because the impacter has an upper surface, which is contacted by the dropped weight and the lower surface is in direct contact with the dura, we prevented the double impact, which would be induced by the dropped weight bouncing, by applying a Silicon sheet on the upper surface of the indenter.

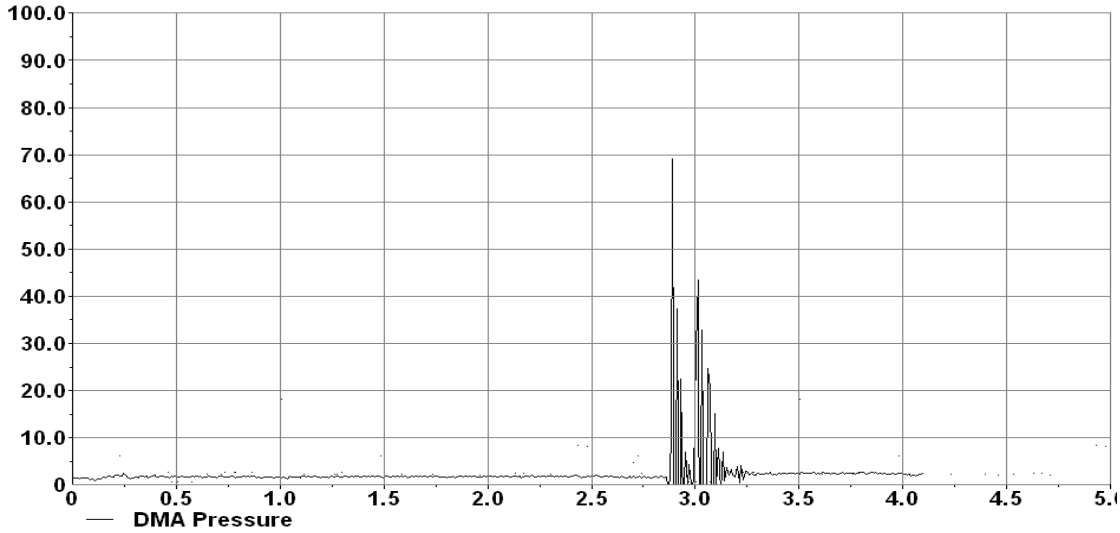

Figure 15: Diagram showing rebound phenomena of double impact. The diagram of pressure readings (in a fluid-filled chamber) clearly shows two peaks which indicate bouncing of the dropped weight.

The gross pathological findings in the pilot study, cortical contusion size varied according to the weight, impacter diameter and height used. All injured animals demonstrated unilateral contusion, as defined by gross evidence of hemorrhage in the cerebral cortex and the underlying white matter. Animals impacted by the 10g weight had only small, shallow contusions. The 40g weight caused severe, often bilateral contusion, some extending beyond the coronal suture. We noted that using an impacter with a 2mm radius made the induced trauma deeper than the trauma induced by using an impacter with a 6mm radius. Evans blue dye was obviously distributed in the lesioned cortex after brain injury, presumably representing sites of vasogenic brain edema.

Pilot Study Groups Histological Findings. To verify the severity of contusion, hematoxylin & eosin-stained coronal sections from rat brain were compared to all pilot

study groups 2 hours after contusion. Two rats were studied for each contusion condition. Serial sections through the area of contusion revealed a well demarcated area in the cerebral cortex with petechial hemorrhages. For trauma that was induced with a heavy weight (40g) or with a smaller impacter radius (2mm), there was subarachnoid hemorrhage (SAH), and cellular swelling with spongy appearance. Hemorrhages extended to the supraventricular areas, with pyknotic neurons and intraventricular hemorrhage (IVH). In some rats, due to severe trauma, we noted the presence of subdural hematoma with ruptured meninges. When the trauma was induced with lighter weights (10g, 20g) and larger impacter radius (4mm, 6mm) damage was restricted to the contusion area and displayed only localized petechial hemorrhage and subarachnoid hemorrhage with intact meninges. In the groups in which the trauma was induced by 10g weight or from a height of 25 cm was determined to be a hyperemic area rather than a contused area. The results are summarized in the following table 3:

Number of rats: 2	Parameters	PHe	SDH	SAH	IVH	Damaged Neuron	Hyper Cellularity	Vascular Congestion	Edema	ICH
I	**W=40g ID=6mm, H=25cm**	**++**	**++**	**+**	**+**	++	+	++	++	++
II	**W=40g ID=4mm, H=20cm**	**++**	**+**	**+**	**+**	+	+	++	++	++
III	**W=40g ID=2mm, D=15cm**	**++**	**++**	**++**	**+**	+	++	++	++	++
IV	**W=20, ID=6mm H=25cm**	**+**	**-**	**+**	**+, -**	+	++	+	++	**-**
V	**W=20gmI D=4mm H=20cm**	**+**	**-**	**+**	**-**	+	+	+	+	**-**
VI	**W20gm, ID=2mm H=15cm**	**+**	**+, -**	**+, -**	**-**	+	+	+	+	+
VII	**W=10gm ID=6mm H=25cm**	**+'-**	**-**	**-**	**-**	+,-	+	+	+	**-**
VIII	**W=10gm ID=4mm H=20cm**	**+**	**-**	**-**	**-**	+	+	+	+	**-**
IX	**W=10gm ID=2mm H=15cm**	+	**-**	+	**-**	+	+	+	+	**-**

Table 2: Summary of impact average force (F) and histological findings after using different weights, heights, and impacter diameters in each rat. SAH=Subarachnoid hemorrhage, ICH=Intracerebral hemorrhage, PHe=Petechial hemorrhage, SDH=Subdural hematoma, IVH=Intraventricluar hem

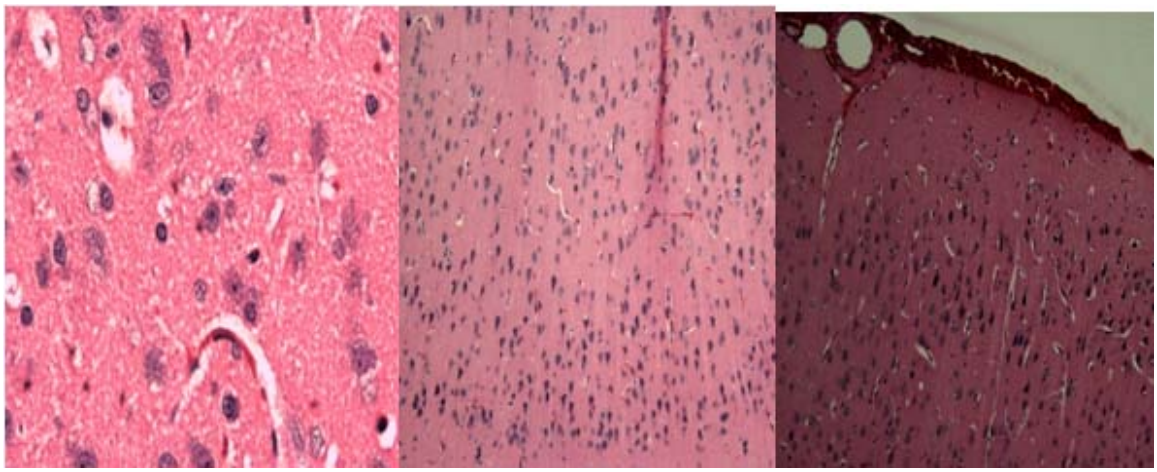

Figure 16: Cortical cortex showing incrustation of neurons after using 20gm weight drop (H & E stain x 100 &x 400).

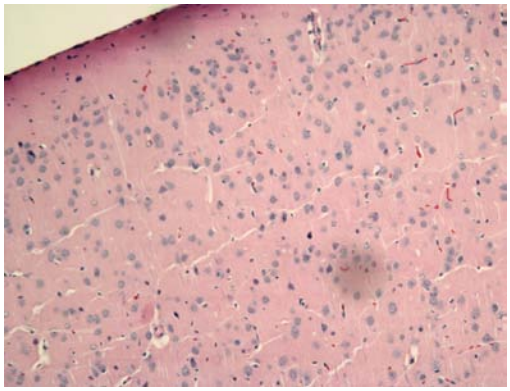

Figure 17: Cortical cortex showing incrustation of neurons after using 10gm weight drop (H & E stain x 100).

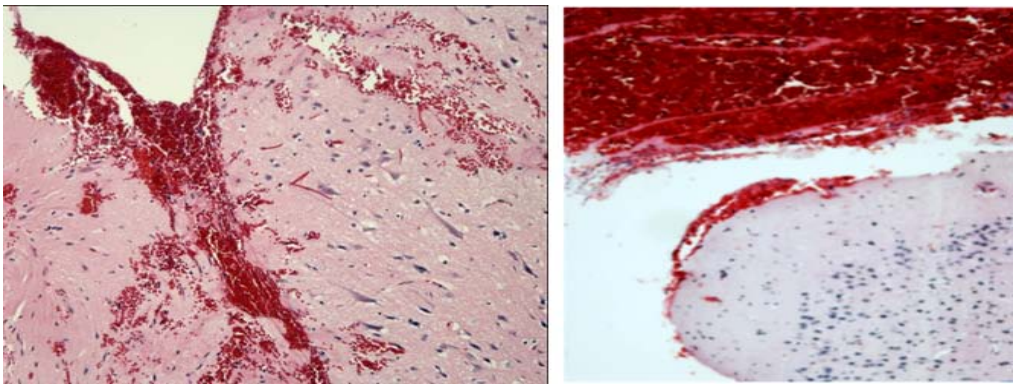

Figure18 : Cortical cortex showing icrustation of neurons, IVH , and ICH after using 40gm weight drop (H & E stain x 100).

3. 2. Determining the Trauma Device Parameters

The result of the pilot experiment showed that the reproducibility varied according to impacter radius, height and weight. We found that we were able to get reproducible cortical contusions using the 20g weight dropped from a 20cm height onto the 4mm impacter. Also we prevented the double impact, caused by bouncing of the dropped weight, by applying a Silicon sheet on the upper surface of the impacter.

3. 3. Experimental Groups

Almost all contused animals demonstrated temporary apnea episodes ranging from 5 seconds to 1 minute, and usually responded to resuscitation. Resuscitation was accomplished by decreasing the anesthetic gas to the lower limit and increasing the oxygen, along with a cardiac massage of the rat with fingers for approximately 5 minutes. After establishing a good heart rate, we continued our procedure. Ten of the 54 (17.9 %) rats died. Four rats died from included anesthesia overdose apnea (3 rats before craniotomy and 1 rat after craniotomy); we noted in this death group that the rat became blue, developed a very faint pulse and gradually died despite resuscitation. Four rats also died directly from induction of trauma; we noted in this death group, which occurred directly after weight drop, there was no blue discoloration and no pulse was palpable, suggesting a complete stoppage of the heart. Two others died during indentation testing, likely from anesthetic apnea also. There was no death in the control group after the craniotomy or indentation procedures. No rats died during the follow up period. Four rats shown to have dural injury were excluded from the study. The total excluded rats because of death or dura injury were fourteen. After the exclusion of 14 rats due to death or an injury to the meninges, 40 animals were used for the study analysis. Following impact, a

4-5 mm focal contusion was observed on the right front parietal cortex (the impact site) in all rats after weight drop. This was shown as a hyperemic area and bulging under the dura; increased eye bulging and redness was noted directly after trauma induction.

3. 4. Follow Up for Neurological Deficit, Animal Behavior and Wound Care

For all the animals that survived the trauma and recovered normally from anesthesia, their food and water intake was unaffected. Some animals appeared lethargic during the first day, yet there were no obvious focal motor deficits. Most of the rats gained weight. Five animals suffered from mild wound infections that were treated by removing the skin staples, cleaning the incision with antiseptic solution, and applying topical antibiotic gel twice daily for 7-10 days until the wound healed.

3. 5. Magnetic Resonance Imaging (MRI)

Magnetic resonance images were able to detect cortical contusions, edema, , and mass effect in all acute injured animals, and brain atrophy in those with chronic trauma.

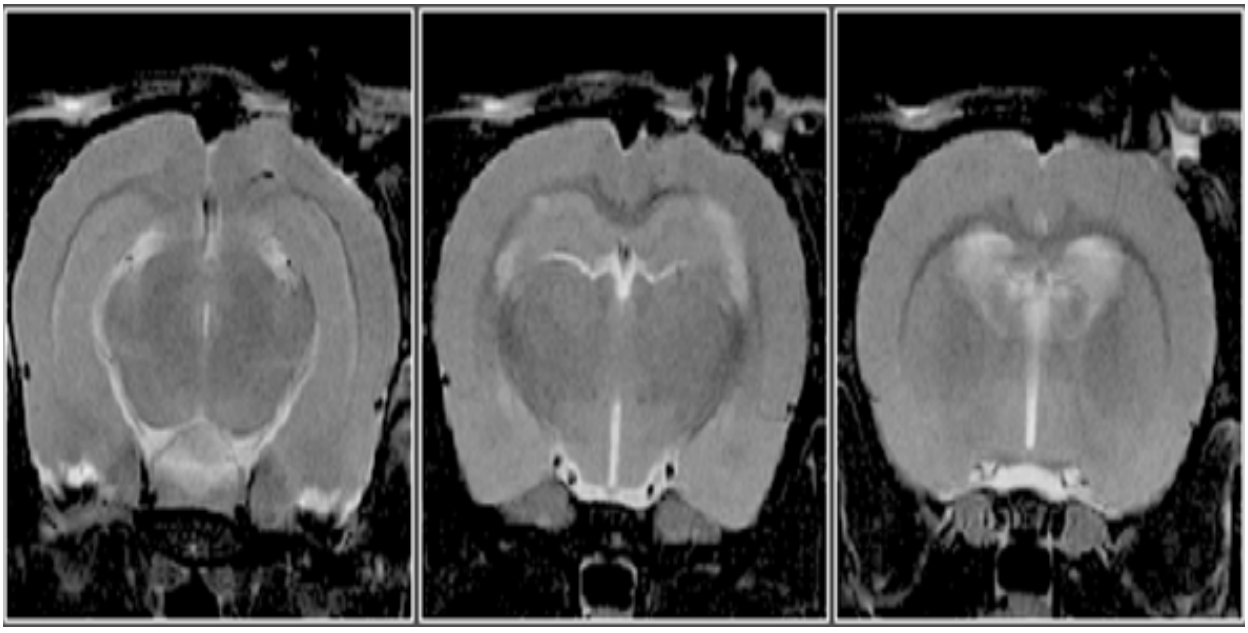

Figure 19: Serial coronal T2-weighted MRI obtained after 2 hours of unilateral cortical contusion, showing low or iso-isignal-ntensity.

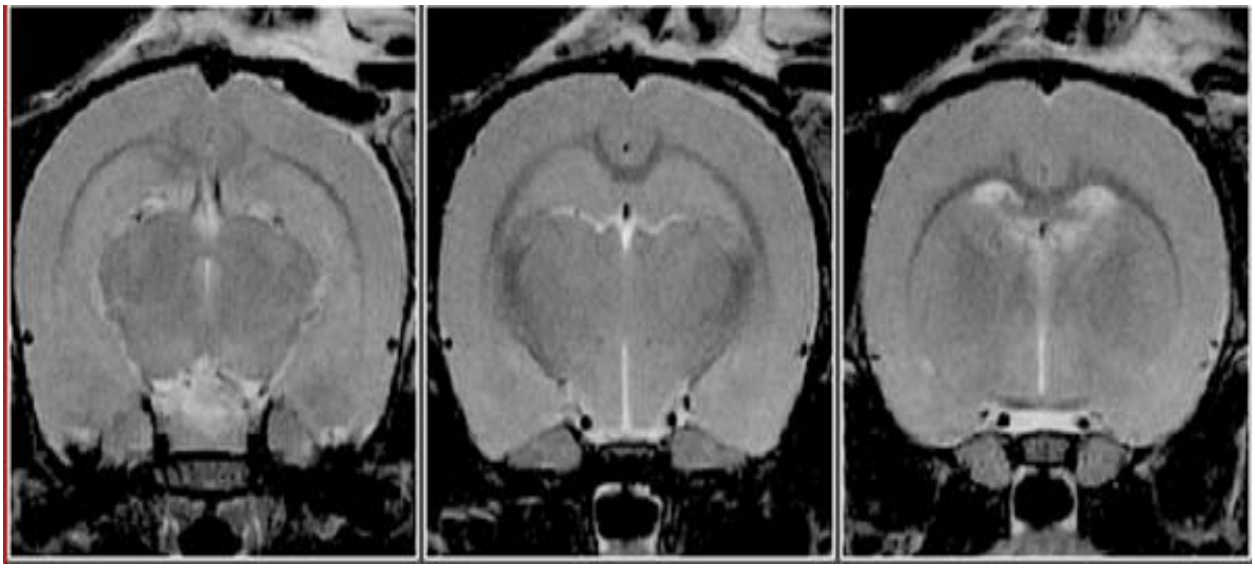

Figure 20: Serial coronal T2-weighted MRI, Showing changes in 2 hours control group showing no signal changes.

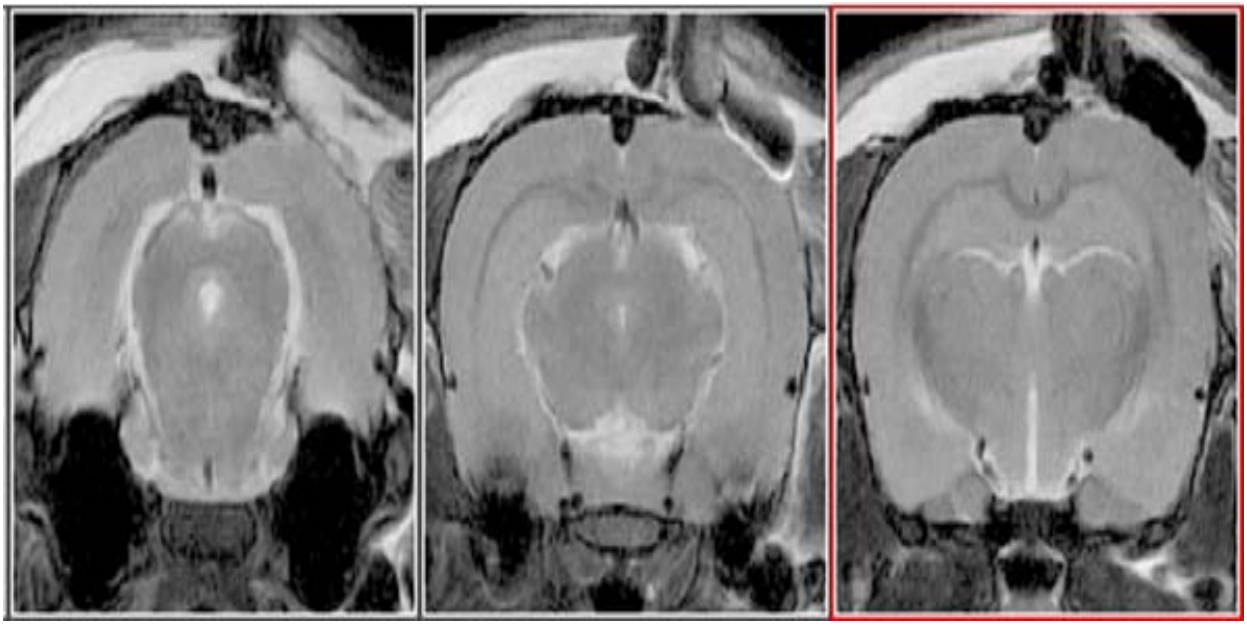

Figure 21: Serial coronal T2-weighted MRI, showing changes after 24 hours injury high-signal- intensity area is clearly seen in the cortex and compressed the underlying tissue under the impact site.

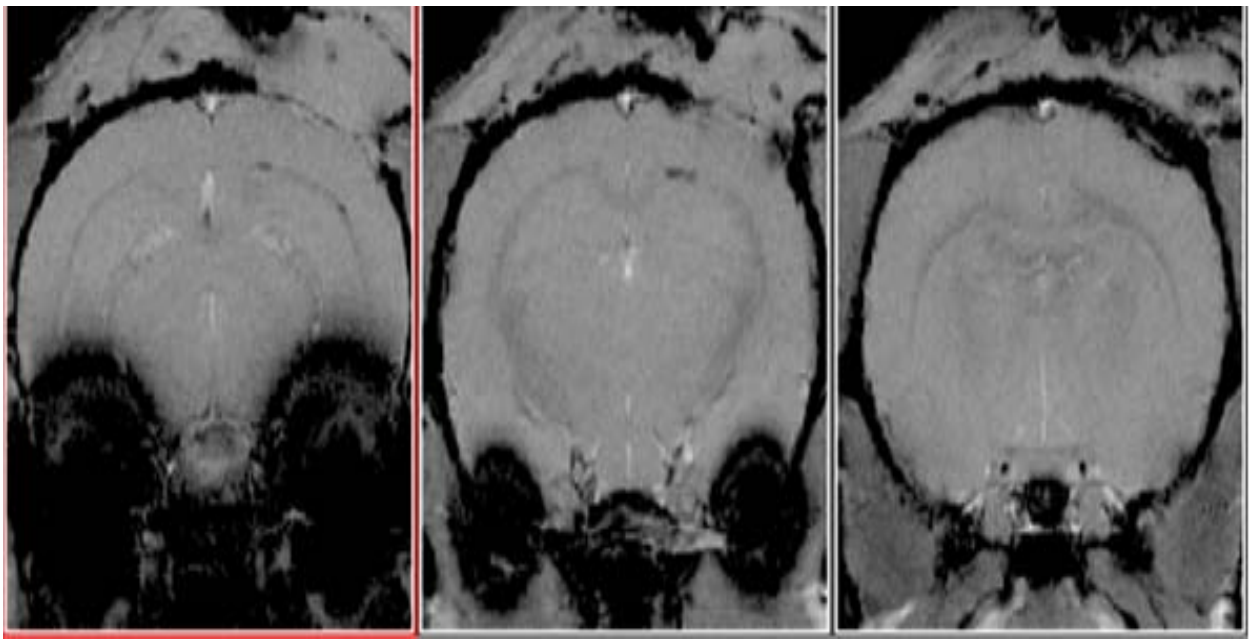

Figure 22: Serial coronal T2-weighted MRI obtained 3 days after cerebral contusion showing that the high –signal–intensity area is resolving but is still detectable.

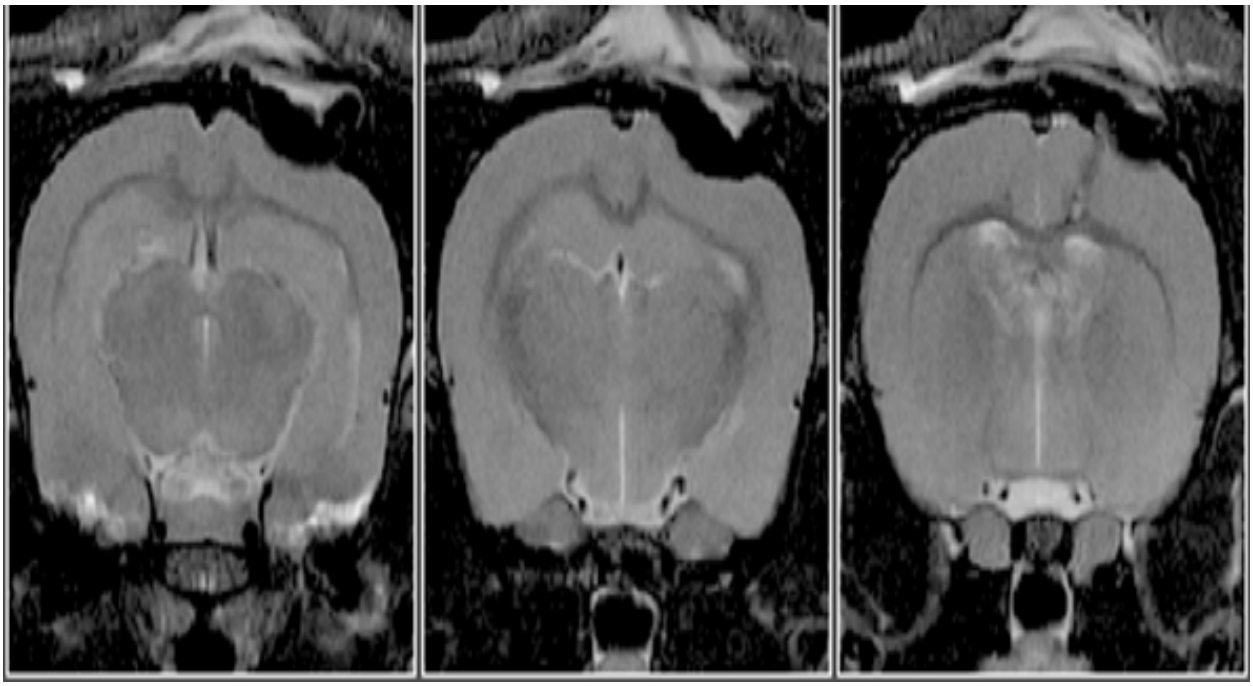

Figure 23: Serial coronal T2 weighted MRI, showing resolving of high-signal-intensity area and cyst formation after 21 days of unilateral cortical contusion.

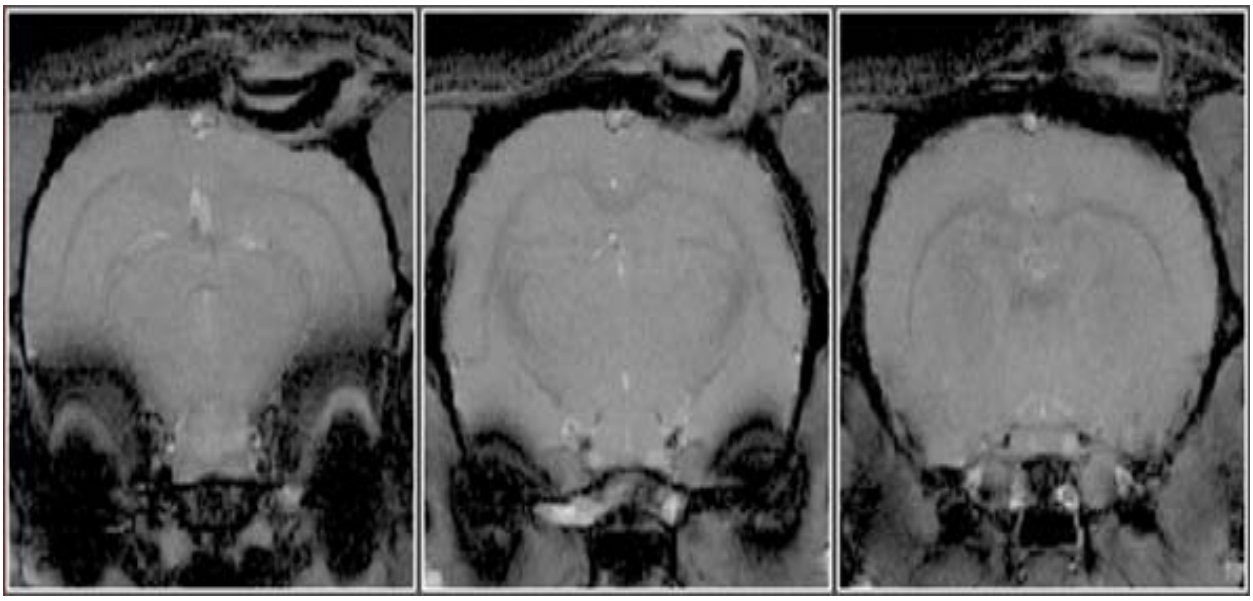

Figure 24: Serial coronal T2 weighted MRI of the Control 21 days rat (only craniotomy) showing no signal changes but scar of surgery.

3. 6. Gross Pathological Findings

After euthanizing the 40 rats according to the scheduled time for each group, we could identify gross evidence of injury in all acutely contused rats (2 hours group, 6 hours group, 24 hours group, 72 hours group). This was characterized by unilateral contusion located in the frontal area of the right cerebral hemisphere. In chronic injury rats, there was gross subarachnoid hemorrhage (SAH), for the 7 days and 21 days groups, in addition, some of them had some redness due to hyperemia which it could be from indentation process or brain extraction process after death, but there were no clear contusions on the brain surface. For the sham group (2hours group, 21 days group), in most of the cases we did not identify any trauma evidence but in some brains we identified some brain redness which could be related to the indentation process , or gross SAH; in some brains there were minor epidural hemorrhage.

3. 7. Tracing of Evans Blue Dye to Asses BBB Integrity

Using the previously described technique, the Evans blue stained area, which corresponds to the distribution of extravasated serum protein, was observed in the white matter under the lesion cortex. This area extended to the adjacent, deeper white matter, and showed brain edema in the area with Evans blue staining in experimental groups of 2h, 6h, 24h, 72h and sometimes in 7 days group. No Evans blue stained areas were noted in the 21 days group or in both control (2hrs and 21 days) groups.

Macroscopic examination in the area stained with Evans blue showed the peripheral spread of Evans blue in the cerebral cortex in all acute injured rats (2hrs, 6hrs, 24hrs, 3 days, and sometimes even with rates from the 7 days group). At 2 hours after head injury, visible dye had spread as far as 3 mm from the edge of isolated white matter contusions. At 6 hours after trauma, the dye had spread to adjacent subcortical white matter at level of contusion and it was about 6mm width. At 24 hours after trauma, there was up to 9 mm of caudal spread of the dye in the deep white compartment from the caudal edge of contusion. The spread then started to decreased. Throughout the dissection of the injured brain areas, the cortical areas stained with Evans blue dye suggested the formation of vasogenic edema at the area of contusion and revealed edematous white matter at 2h, 6h and reached the maximum at 24h. The spread started to decrease at 72 hours and there was no evidence of the edema in the 21 days group.

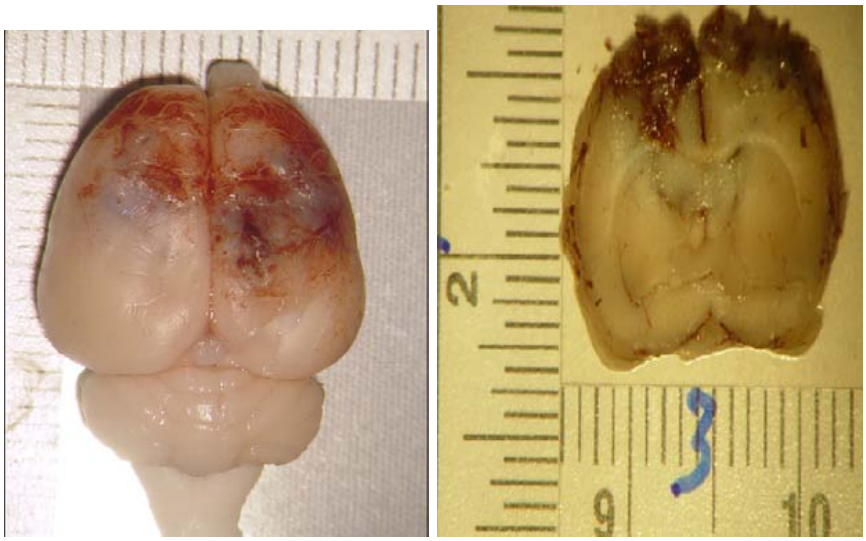

Figure 25: EBD spread in gross brain after acute injury 24hrs group

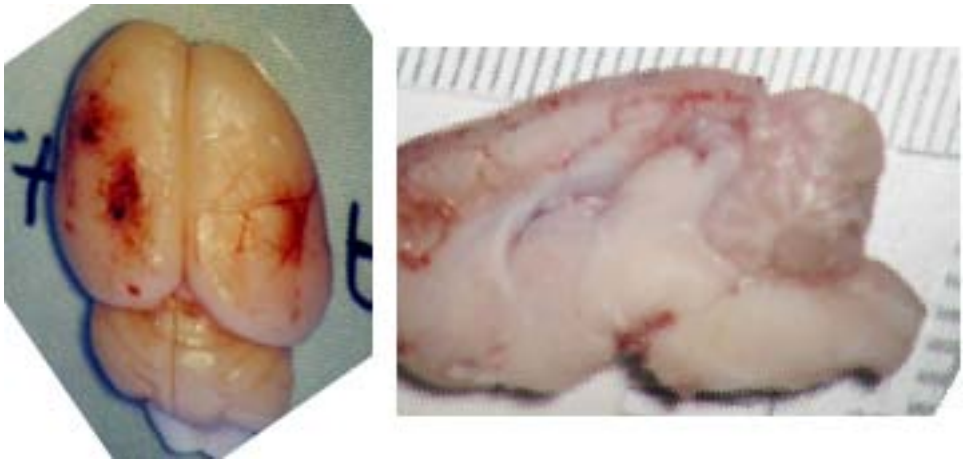

Figure 26: EBD distribution after three days

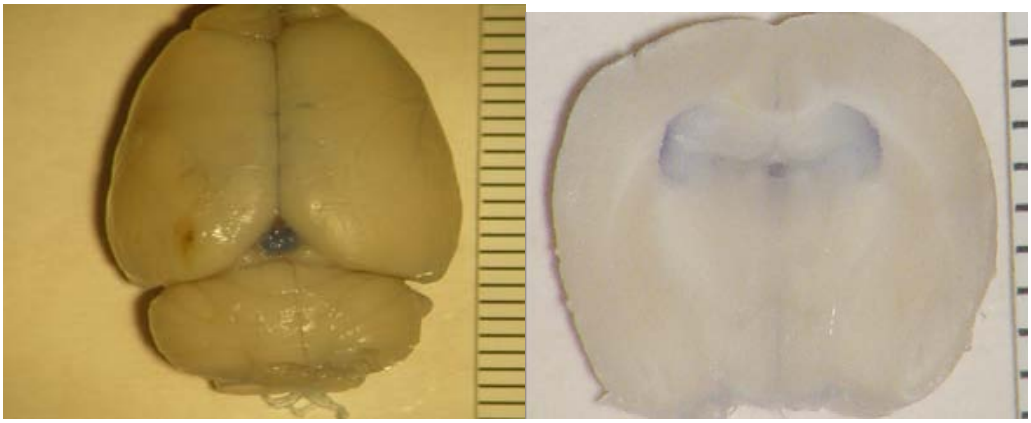

Figure 27: Absence of Evans blue dye in in 2hrs control group

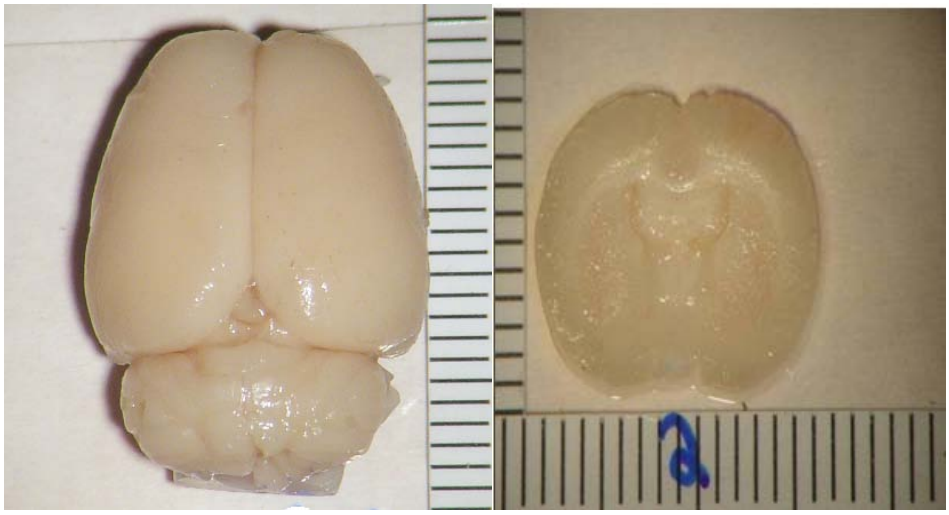

Figure 28: Absence of Evans blue dye in 21 days control group

3. 8. Histopathological Findings

We assessed the histological findings in rat brains at 2 hrs, 6 hrs, 24 hrs, 3 days, 7 days, and 21 days after the induction of TBI. To assess cellular changes after TBI at different time periods, hematoxylin and eosin-stained coronal sections were compared and samples were oriented to evaluate the extent of lesions. In addition, the presence of oedema, as well as their characterisation, and the reactive changes in injured brain regions was assessed. This was done semi-quantitatively, using immunostaining for detection of glial fibrillary acidic protein (GFAP) in reactive astrocytes and BS1 lectin in reactive microglia. This assessment was performed blindly and the histological slides were marked with numbers only.

Histological examination of sections at 2 hrs *(Figure 29).* showed subarachnoid hemorrhage, petechial hemorrhage, areas of condensed neurons and pallor areas (sponge) with sharply defined boundaries. After 6 hrs, brains demonstrated pyknosis and shrinkage to an irregular triangular shape with associated surface encrustations. The nucleus showed a loss of nucleolus and was surrounded by eosinophilic cytoplasm *(Figure 30*). After 24 hrs *(Figure 31)* cortical contusions were seen as well as the widespread activation of cells at the injury site along with subarachnoid hemorrhage and pyknotic neurons associated with a spongy appearance, vascular congestion, scattered macrophages and intraparenchymal hemorrhage at the border between gray and white matter. The edematous area, identified by rarefaction of tissue staining, became larger, and an obvious necrotic appearance surrounded by a zone of pyknotic shrunken neurons which developed with associated enlarged reactive astrocytes. At 3 days *(Figure 32,* lesion size became less

well demarcated microscopically and reduced in size, monocytes and macrophage response. This trend continued until 7 days after injury *(figure.33)*, along with a dense astrocytosis formation. The density of glial fibrillary acidic protein immunoreactive astrocytes was high in the white and gray matter of the cerebral cortex in the 21 days injury group *(Figure 34).*

In comparison, the histological examination of the sham group (2 hrs after craniotomy) showed no histological damage in the cortex, although mild subarachnoid hemorrhage was sometimes observed. In the delayed sham group (21 days), histological examination showed slightly thickened arachnoid layers but no evidence of cortical damage. GFAP immunostaining showed no reactive astrogliosis *(Figures 35, 36).*

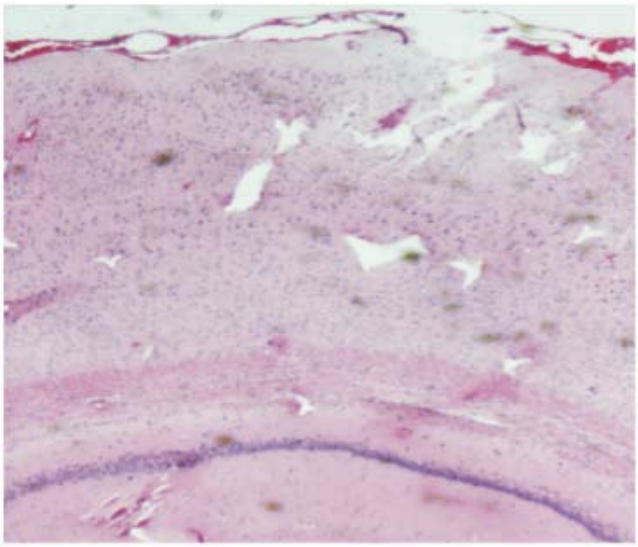

Figure 29: Cortical cortex showing swelling of glial cell, cytoplasm and SAH in case of 2 hours contusion group (H & E x 100).

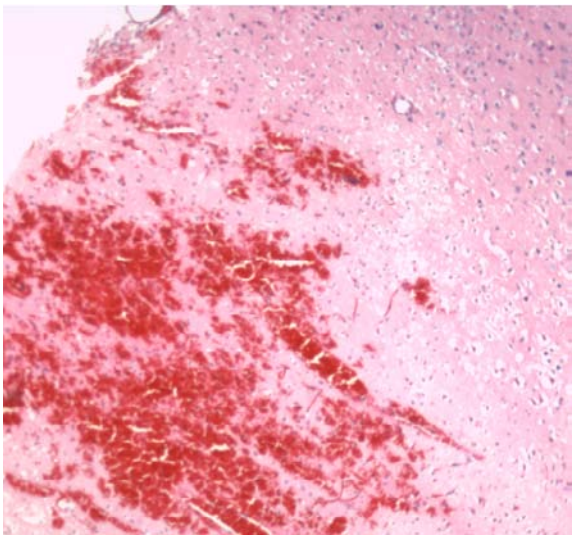

Figure 30: Light microscopic picture of cerebral cortex of rat survived after 6 hours of unilateral contusion of the cerebral cortex showing petechial hemorrhage (H & E x 100).

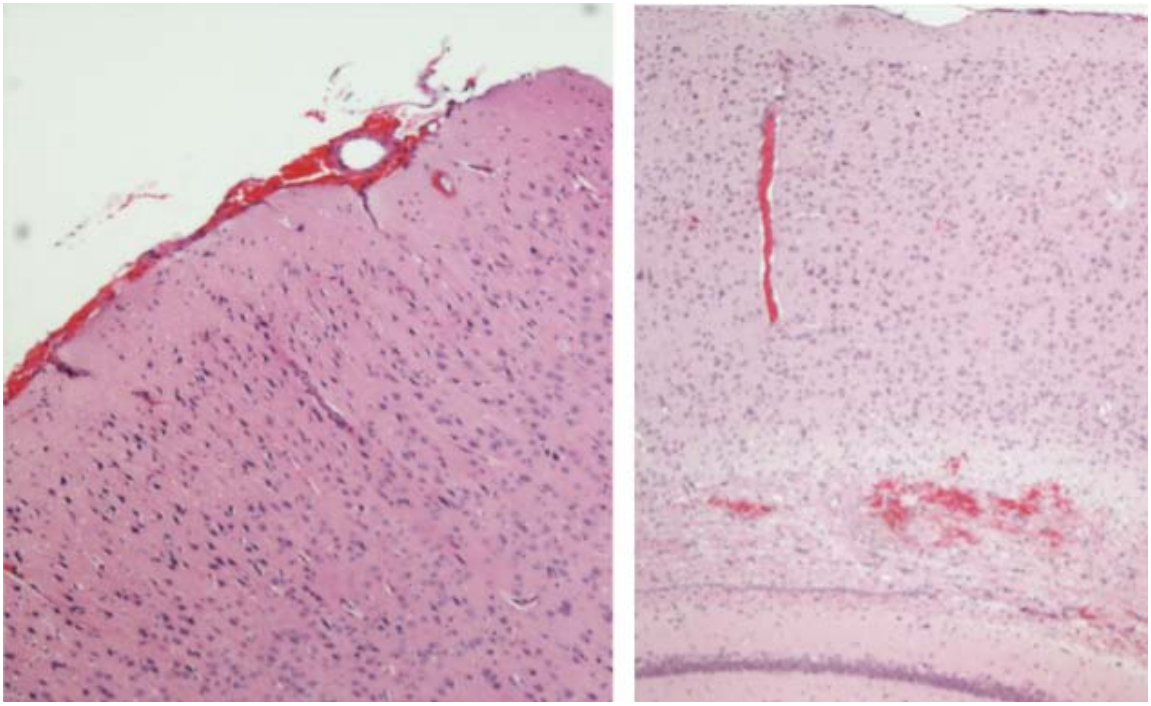

Figure 31: Cortical cortex of 24 hours trauma group showing SAH, petechial hemorrhages and neurona swelling (H & E x 100).

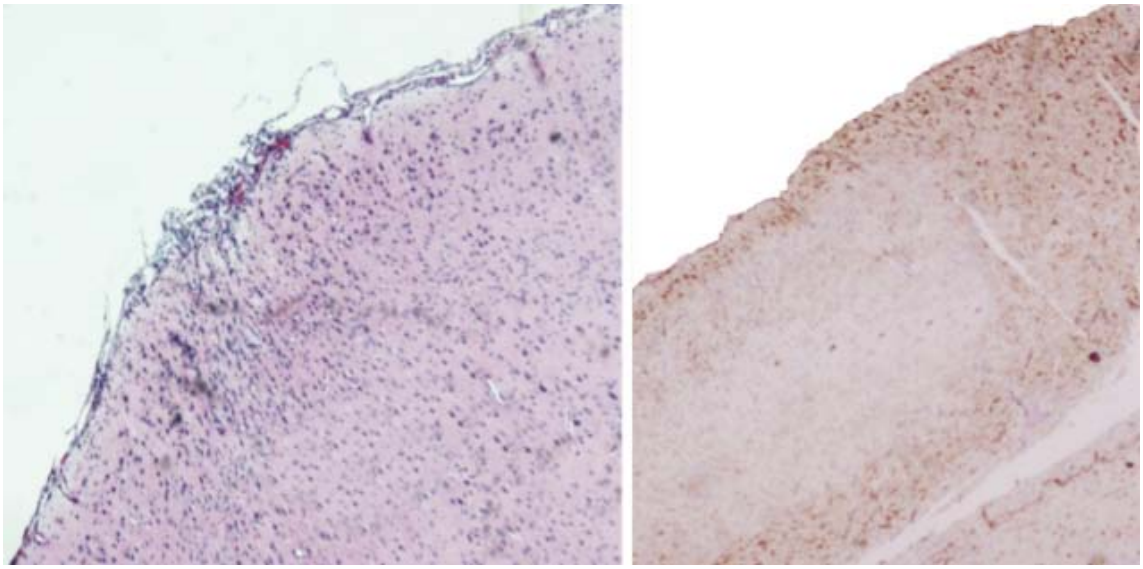

Figure 32: Cortical cortex of 3 days trauma group showing macrophages, hypercellularity in (H & E x 100) and GFAP slide showing glial staining.

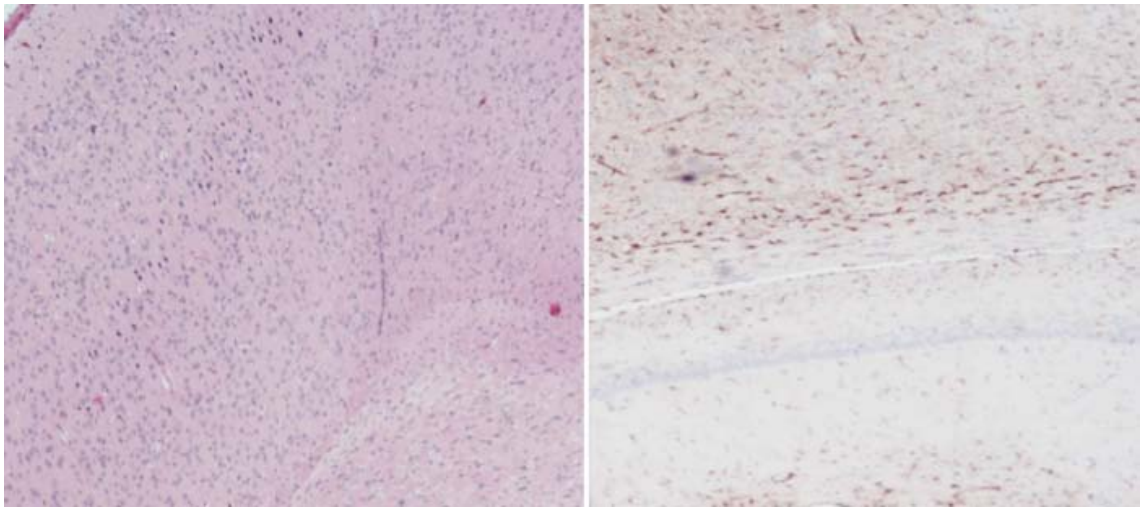

Figure 33: Light microscopic picture of the cerebral cortex of rat that survived 7 days after contusion of the cerebral cortex, H&E x 100 slides showing hypercellularity, and GFAP slide showing presence of reactive astrocytosis in the injured region of the brain.

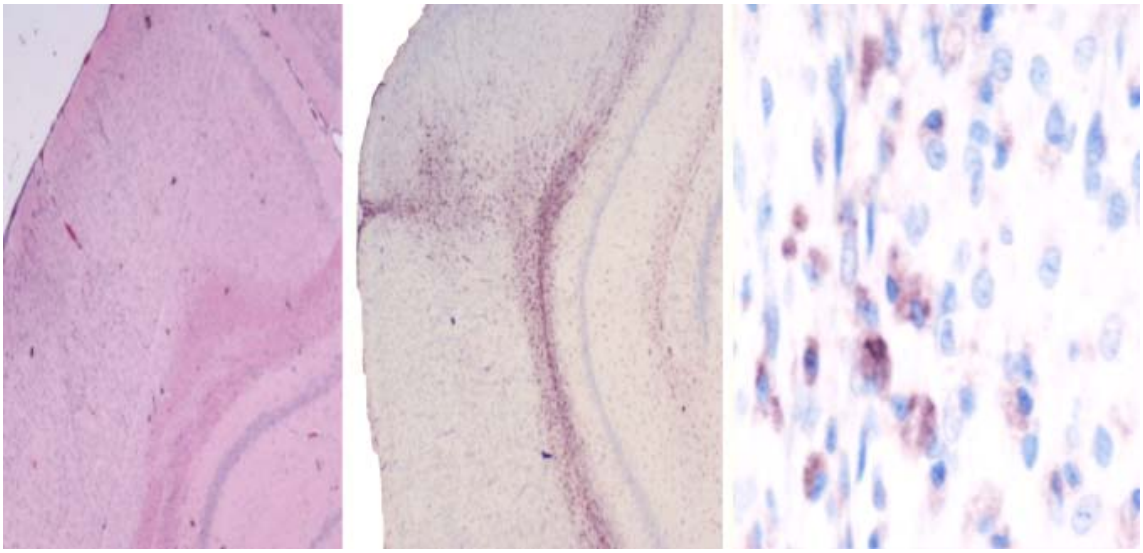

Figure 34: Light microscopic picture of cerebral cortex of rat after 21 days of unilateral contusion of the cerebral cortex H&E x 100 (left) showing , necrosis, few remnant pyknotic neurons and scattered macrophages, GFAP immunostaining (middle) shows an increase in immunoreactivity at the site of injury, and reactive astrocytes in the white and gray matter. BS1 lectin (Right) showing presence of reactive macrophage / microglia in the injured brain.

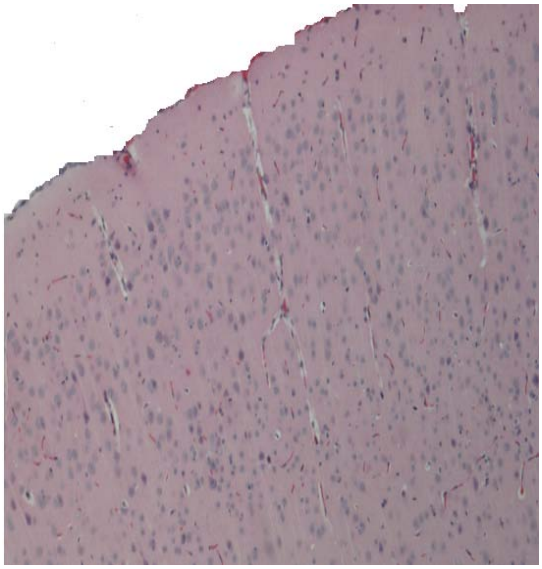

Figure 35: Photomicrograph (H&E x 100) showing normal cortical morphology in 2 hrs control.

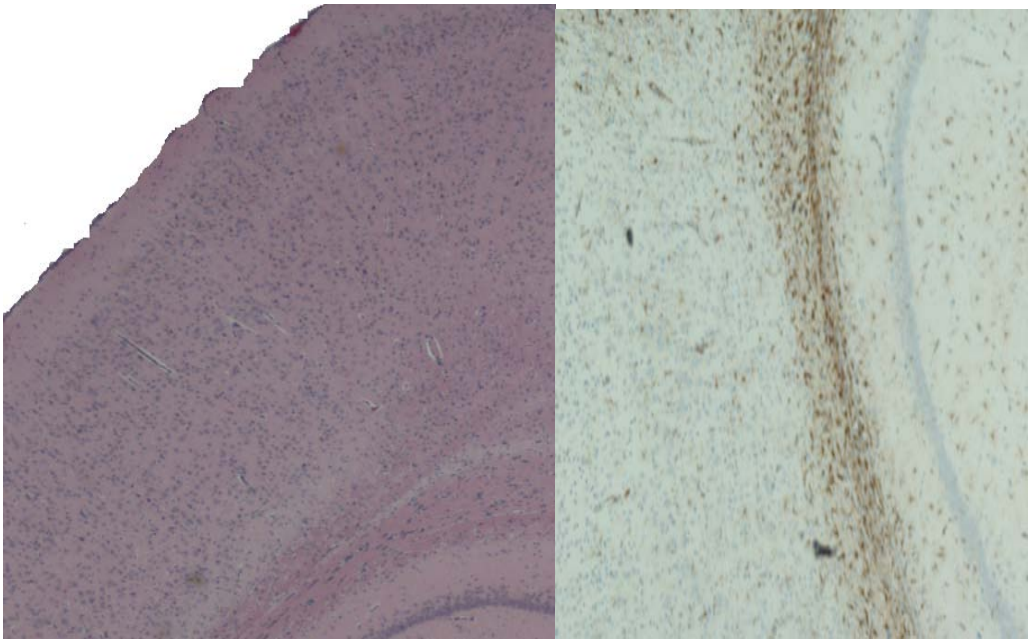

Figure 36: Photomicrograph (H&E x 100) showing normal cortical morphology and absence of GFAP immunoreactivity in 21 days control group.

3. 9. Indentation Test Analysis

Examples of indentation tests from rat brain in control and 24 hours following contusion are shown in *Figure 37*. Showing the force (vertical axis; mN in red) and the displacement (vertical axis; µm in green) plotted against time (horizontal axis; seconds). The force plots are very jagged because the forces of vascular pulsations are transmitted strongly through the brain and dura. Upon withdrawal of the indenter (return of the force to zero), the displacement does not return immediately to the starting point because the brain is viscoelastic rather than elastic. The upper pair shows the simple loading-unloading test without pause. The patterns of the plots differ between the control and contused group. Although both reach a peak force of ~90mN, the softer contused brain is indented more deeply (~1100µm vs. ~700µm). Also, the magnitude of vascular force pulsations is greater in the control state. The middle pair shows plots of the load-hold-

unload test. Here too, the magnitude of vascular force pulsations is greater in the control state. When the mean force is held constant, the displacement continues to change. This is indicative of creep, which tends to be greater in the softer contused brain. The lower pair show plots of the multicycle indentation. The force curve peaks at the same level each cycle then returns to zero while the displacement curve does not return to zero. Lastly. on each successive indentation the displacement is greater, particularly in the contused brains, this is indicative of creep or softening.

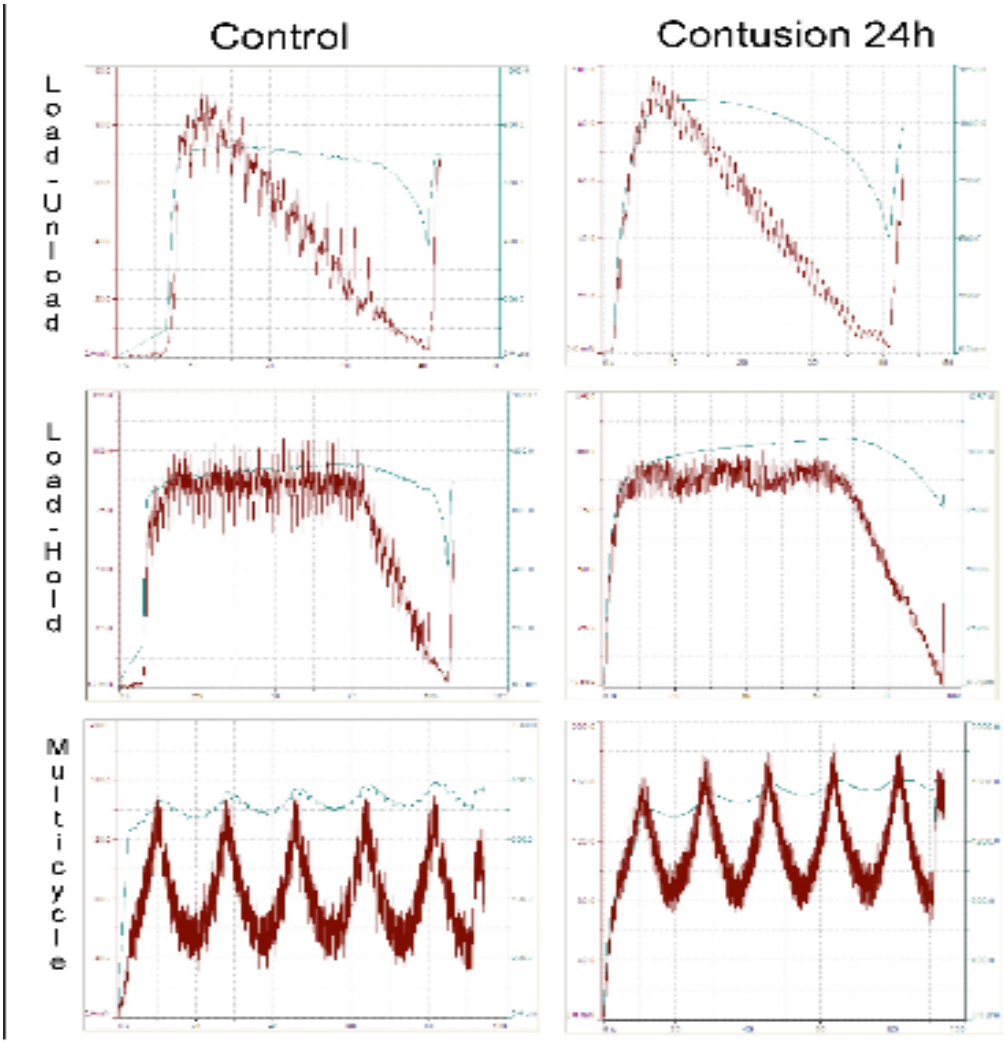

Figure 37: Control and 24 hours: Load-unload curve, creep curve, and multicycle curve.

As we noted there is decrease in E_{IT} after 24 hours of injury. According to Klatzo, after 24 hours, a peak of brain edema is reached during which ischemic insults are complicated with hypoperfusion, which may lead to hypoperfusion and secondary hypoxia. In addition, Marmarou (2007) [99] showed that TBI can trigger a cascade of events, including mechanical deformation, neurotransmitter and amino acid release, (Katayama 1990, Anonymous 2000, Pettus 1994) [100] and membrane depolarization, can lead to alterations in normal ionic gradients which result in cellular swelling and cytotoxic edema formation which reached their maximum limit at 24-48 hours, this give us a good explanation for decrease of EIT after 24 hrs.

3. 10. Statistics Analysis

Statistical analysis showed a decrease in tissue elasticity (E_{IT}) to 146.720 ± 36.052 at 24h after injury, and slight increases in the creep to 320.0333± 138.976 21 days after injury, but there was no significant difference in creep or multicycle between the two groups. EIT for control groups after 2 hours and 21 days was 230.900±91.478 and 265.875±167.462, respectively, showing no significant difference between the two groups. But when we combined control group EIT was 248.387±126.31 and showed a significant difference after 24 hours between the trauma group and both control groups as shown in the diagram, as shown in the following graphs:

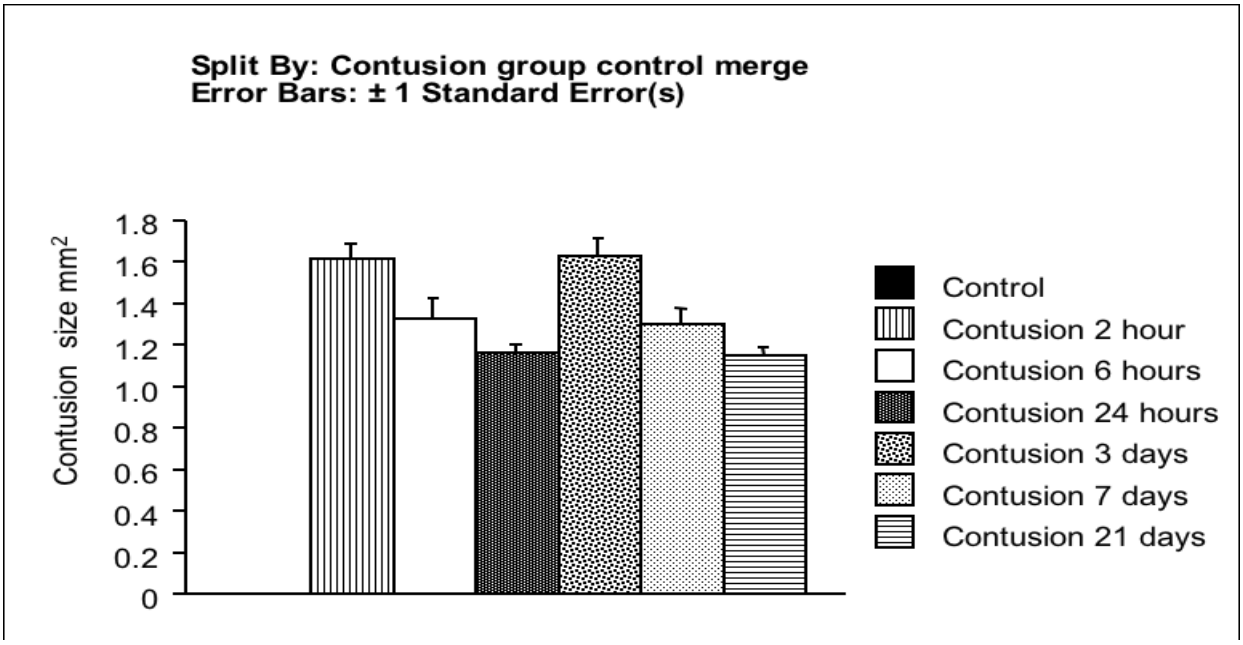

Figure 38: Graph showing no significant difference in size of the contusion between all trauma groups.

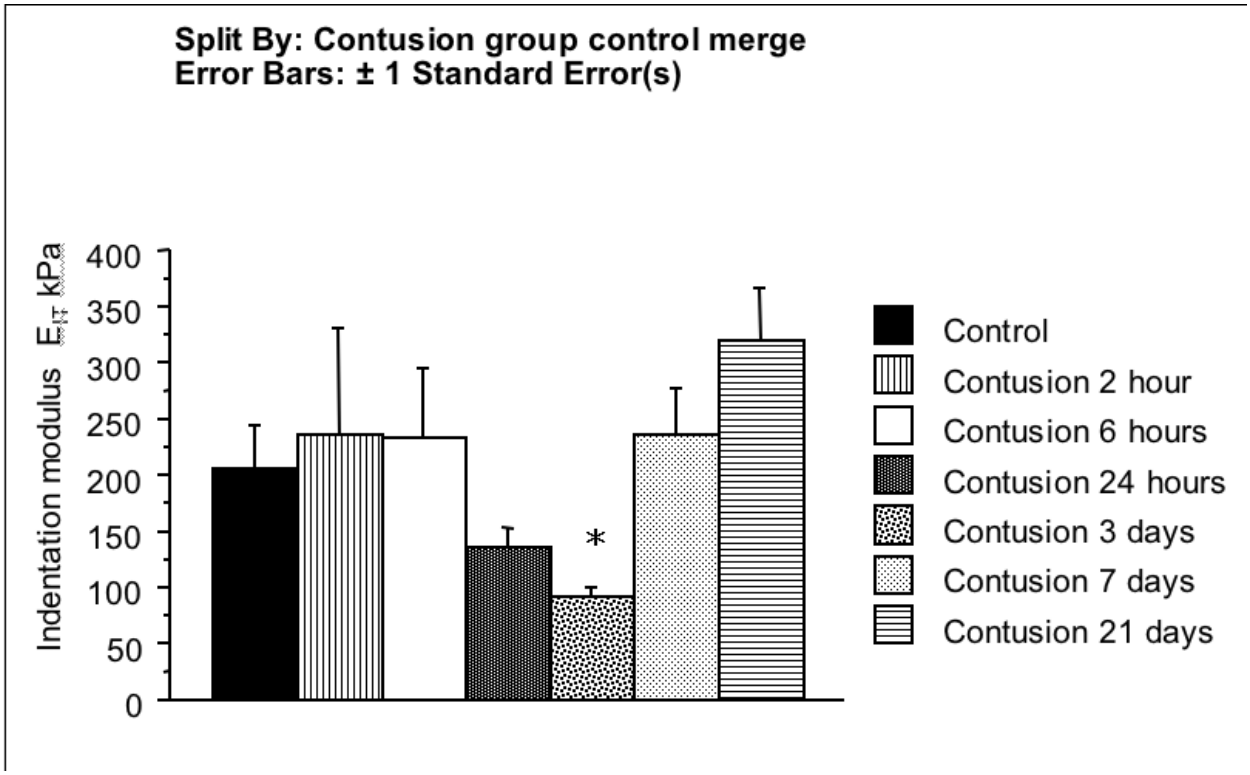

Figure 39: This graph shows decrease in tissue elasticity (E_{IT}) in the trauma group after 24 hrs and increase of the elasticity (E_{IT}) after 21 days of trauma.

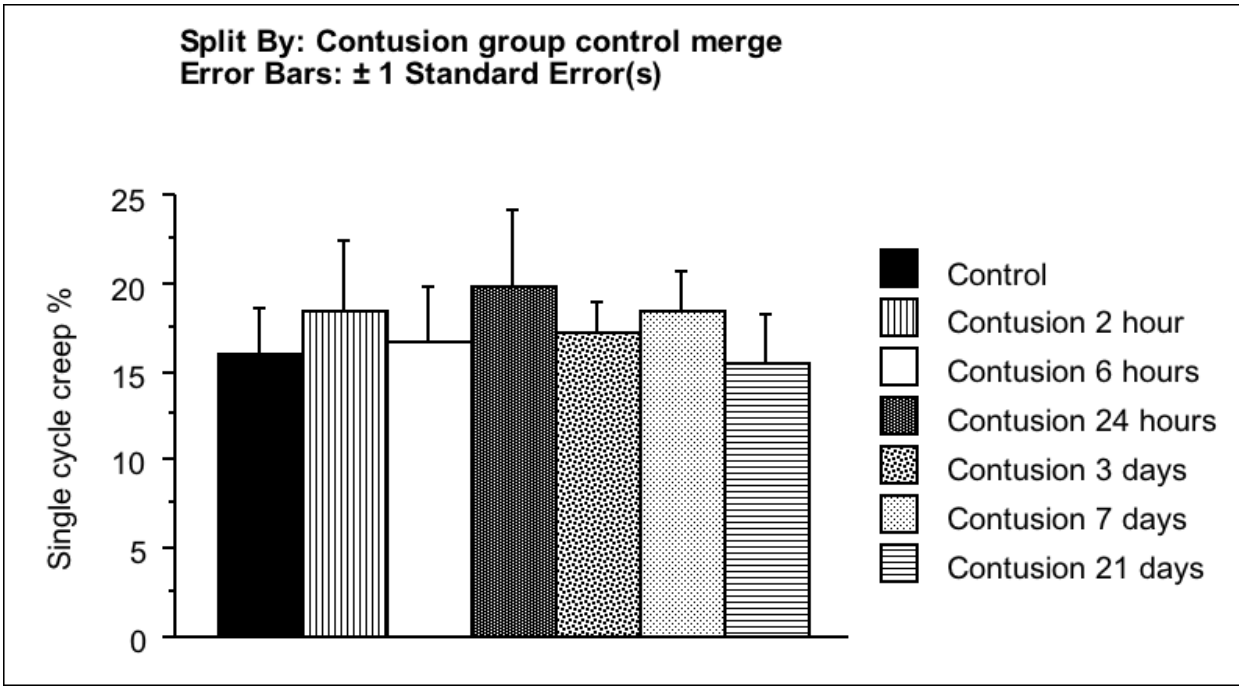

Figure 40: This graph shows no significant difference in single cycle creep between all trauma groups

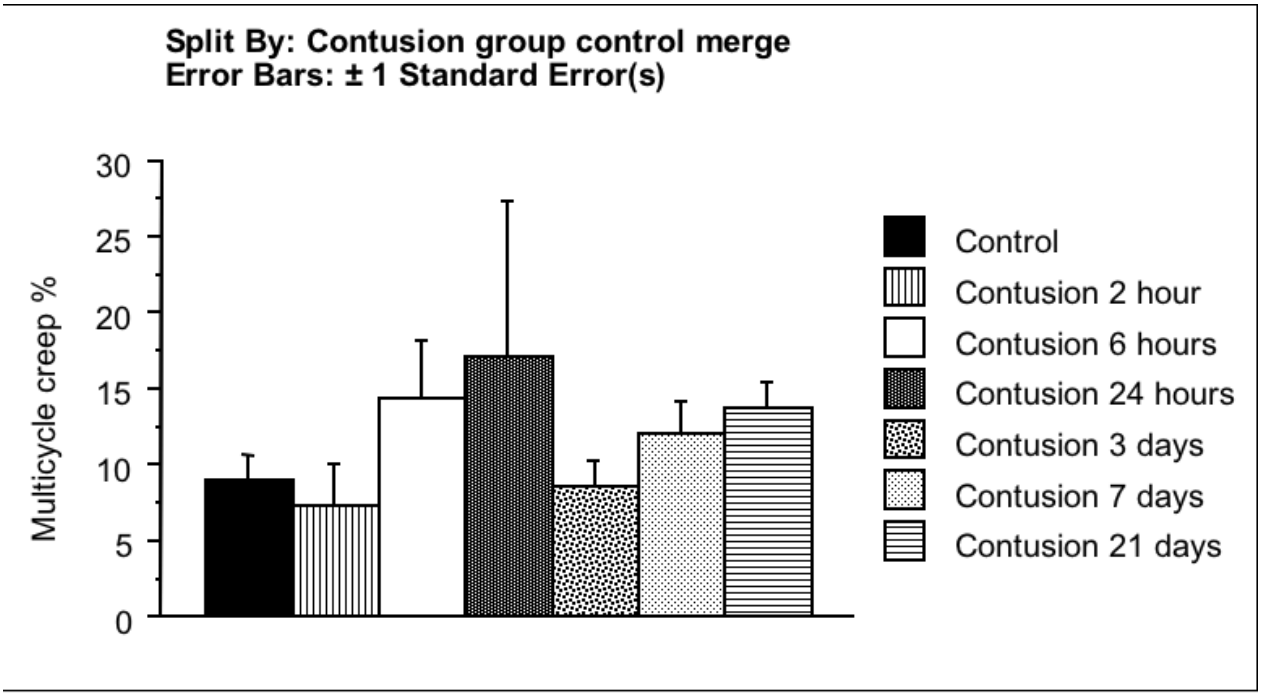

Figure 41: This graph shows a slight increase in the multicycle creep in trauma group after 24 hours.

We can summarize our histological and mechanical findings in the following table:

Group Finding	Control 2hr and 21 d	Contusion 2hr	Contusion 6hr	Contusion 24hr	Contusion 3d	Conusion 7d	Contusion 21d
Hemorrhage	nc	++	++	++	+	-/+	nc
Edema	nc	nc	+	++	++	-	nc
Necrosis or Cavity	nc	nc	nc	+	++	+	-/+
Astroglial activation	nc	nc	nc	nc	+	++	++
Microglial activation	nc	nc	nc	nc	+	++	+
Elasticity	nc	nc	nc	-	--	nc	+
Viscosity	nc	nc	+	++	nc	nc	nc

Table 3: Summary of histological and mechanical findings in all experimental groups
nc no change. Decrease: - ; inconsistent -/+; increase + ; pronounced increase ++.

Chapter 4: DISCUSSION

4.1. Discussion

Several scientists have developed experimental models of mechanical brain injury to evaluate different aspects of the biomechanical brain response after injury; each model was designed to meet the objectives and the goals of the scientist.

The first goal was to create model induce cortical contusion, cortical contusion generally defined as blunt head injury with brief period of loss of conscious , this blunt trauma associated with histopathological and mechanical changes (Elison, 1994).[116] Our experimental model reliably produced a traumatic focal lesion of the cerebral cortex of the live rat; there was no tearing of the cortex or the dura, and the histological examination revealed the restriction of the lesion to the cortex under the impact side by a specific force needed to induce cortical contusion.

The following three criteria distinguish our model from previous models of cortical contusion. Firstly, the weight drop felt on impactor touching the rat brain with contact meninges. Secondly, the use of the silicon sheet to cover the impactor tip to prevent bouncing phenomena and consequently prevent double impact. Lastly, the use of the MRI test to all experimental groups to confirm the induction of expected trauma in the cortical cortex.

The second objective of the study is to evaluate chronological changes in live traumatized brain tissue elasticity (measurement of regional tissue elasticity was done by indentation testing) in relation to the histological changes in traumatized brain tissue during formation and resolution of vasogenic brain edema and in presence of cavitations

and gliotic scar tissue over period of time (2 hours-21days). After 6 hours of injury, histological examination showed the lesion consisted of petechial hemorrhage, SAH, and dark neurons under contused area, by 24 hrs necrotic cavity appears at the site of the hemorrhage, this cavity expanded over the days up to 7 days when microphages and microglial started to appear. In the 21-day trauma group, the early hemorrhages were replaced by an expanding necrotic cavity, which shrank and became lined with macrophages. The two-hour control group showed evidence of SAH which could have resulted from the indentation process. In the 21-day group, examination showed slightly thickened arachnoid layers, but no evidence of cortical damage, and GFAP immunostaining showed no reactive astrogliosis either. The presence of microscopic hemorrhage indicates that blood vessels have been physically disrupted. Others have shown that cell bodies and axons are simultaneously undergoing degenerative changes, with substantial evidence of protein degradation by 24 hours (Clifton et al. 1991, Newcom, J. et al. 1997).[117] In humans, brain edema peaks from 1-3 days after ischemic damage (stroke) (Clasen et al. 1980, Rosenberg et al. 1999), [118] and ~2 days after traumatic injury (Bullock et al. 1990).[119] Experiments show that brain water content elevation begins at 1 hour following cryogenic injury (Schneider et al. 1994),[120] and peaks at 1 day in rats (Bareyre et al. 1997, Ikeda et al. 1994, Schneider et al.1994), [121] and 3-5 days in monkeys (Rieth et al. 1980). [122] Following experimental brain contusion in rats, intracranial pressure and edema peak at 1-3 days (Engelborghs et al. 1998, Jamali et al. 1998).[123] Evans blue dye (EBD) is a tracer that is injected into the blood stream where it binds to albumin. The EBD-albumin complex enters brain tissue at sites of blood-brain

barrier damage; it can persist in the brain up to 2 weeks (Fredericks et al. 1988).[124] We have confirmed in this contusion model the break of BBB integrity.

The third objective, viscoelastic properties of the living rat intracranial contents (e.g. brain elasticity and fluidity) were assessed by the indentation method at different times following brain contusion. Prior work (Shulyakov and Del Bigio unpublished)[125] has shown that the indentation modulus (E_{IT}) calculated automatically on the initial unloading curve and the Young's modulus of elasticity (E) calculated manually on the loading curve are highly correlated ($r>0.9$); therefore we reported only the indentation modulus, which was determined automatically by the testing device. The mean E_{IT} of ~200 kPa was shown to correspond to an E of ~50 kPa, which is in the range of values previously reported (~33 kPa) for dog brain tested through intact dura (Walsh et al. 1 1976)[126].

Previous studies have shown that intermediate filaments, such as glial fibrillary acidic protein (GFAP) in astrocytes, contribute to cell elasticity and stiffness (Miller et al. 2009, Lin et al. 2010). [127] However, our data demonstrated that tissue elasticity decreases in parallel with the evolving tissue damage and regional tissue elasticity decreased significantly during the initial 24h with maximium formation of edema (Engelborghs et al. 1998, Jamali et al.1998),[128] and continue decreasing up to 3 days. Subsequently, it increased in day 7 experimental groups, reaching a maximum at 21 days when astroglial scar tissue was shown to be present.

In this experiment, creep in the brain tissue increased in the 24-hour group and decreased in the 21-days group, which is not statistically significant compared to control the control groups. Although analysis of creep on the single cycle load-hold test showed

no significant difference between control and trauma groups, the multicycle test did show a tendency to increase at 24 hours after injury. The variability was broad, but if confirmed in an experiment with a larger sample size, this would indicate that the contused rat brain at the time of peak edema exhibits a higher degree of viscosity. Necrotic dog brains and brains made edematous by over hydration were previously shown to exhibit decreased elasticity and increased viscosity (Aoyagi et al. 1982).[129]

Although we have confirmed the previously reported data by Kuroiwa (1997) [130] which showed in injured cat brains by cryo trauma, decreased tissue elasticity with increased tissue fluidity at 24 hours, and increased tissue elasticity with decreased tissue fluidity at 10 days, and tissue fluidity was calculated by using SG values and Marmarou formula (1980). [131]

To date, there is no quantitative analysis has confirmed that after cortical contusion there is increase of brain tissue elasticity during formation of astrogliosis and decrease brain edema. Our data indeed showed that the elastic mechanical properties of the brain tissue after injury, estimated by indentation test changed significantly.

Precise understanding of material properties of the brain is required to create mathematical models of brain tissue that can be used for predicting and understanding responses to deformation in normal situations like response to blood pressure changes and in pathological situations like trauma other hand there is still need to study these mechanical properties in larger groups and in relation to age and gender to apply it for use in human being.

4.2. Clinical Application

Research of loading and unloading parameters and the resulting cell and tissue responses in the period of time is important to understand injury pathophysiological mechanisms and in developing experimental models to help understand these changes in human beings. This has lead to an interest in developing improved monitoring system to predict the limits of deformation associated with this injury monitoring such as magnetic resonance elastography (MRE), which could be used for brain monitoring after injury, to deal with associated ICP (Intracranial pressure) fluctations by imaging and waves correlation as in the case of spectroscopy to get more accurate and less invasive monitoring system or as a probe to identify intraoperative damaged brains and indentify the acute brain swelling or necrotic brain tissue (nonfunctioning (Fukuhara et al. 1996[132], Yamamoto 2004)[133] to determine how much resection needs to be done in cases of contused swollen brain tissue , or as probe in clinical conditions to reduce the strain on the brain tissue in situations such as surgical manipulation by determine tolerance limits before brain tissue injury during surgical procedures retraction or to identify this strain with structural disease like hydrocephalus , Furthermore to employ these data to create haptic learning systems for robotic surgery for more accurate surgical procedure and less intraoperative brain tissue traction .

4.3 Conclusions and Future Steps

We accept the hypothesis that mechanical properties of brain tissue change as the brain composition changes following contusion injury, this experiment is novel because no others have studied live brain mechanical properties following contusion, nor has anyone assessed the correspondence with histological changes. The nearest experiment is by Aoyagi and coworkers in which dogs with freeze injury of the brain were assessed. Physical analysis of tissue mechanical properties will lead to better understanding of how the cellular changes contribute to these properties, especially in large size experiment with different age groups.

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