In-Vitro Approaches for Studying Blast-Induced Traumatic Brain Injury

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Abstract

Traumatic brain injury caused by explosive or blast events is currently divided into four phases: primary, secondary, tertiary, and quaternary blast injury. These phases of blast-induced traumatic brain injury (bTBI) are biomechanically distinct, and can be modeled in both *in-vivo* and *in-vitro* systems. The purpose of this review is to consider the mechanical phases of bTBI, how these phases are reproduced with *in-vitro* models, and to review findings from these models to assess how each phase of bTBI can be examined in more detail. Highlighted are some important gaps in the literature that may be addressed in the future to better identify the exact contributing mechanisms for bTBI. These *in-vitro* models, viewed in combination with *in-vivo* models and clinical studies, can be used to assess both the mechanisms and possible treatments for this type of trauma.

Key words: biomechanics; blast; brain injury; *in-vitro* models

Introduction

SOME OF THE FIRST DESCRIPTIONS OF BLAST EFFECTS on humans originated in World War I, where the term "shell shock" was first coined (Southborough, 1922). At the time, it was not known whether the basis of the associated symptoms was due to a psychological or biological condition. A series of reports by military medical officer Charles Myers (Myers, 1940; Myers, 1915), and the influence of neurologist Gordon Holmes (Macleod, 2004) led to the first descriptions of such symptoms to include a temporary altered mental state or confusion immediately following the blast. In more severe forms, unconsciousness would occur. Reports on this form of blast injury appeared periodically over the years, but not until very recently was the true extent of injury better elucidated (Pary et al., 1988; Macleod, 2004; Jones et al., 2007). The current Iraq conflict and the prominent role of improvised explosive devices (IED) dramatically increased the fraction of traumatic brain injuries (TBI). This perhaps led to the well publicized view that blast-induced traumatic brain injury (bTBI) is the signature brain injury for combat troops in today's military.

The Centers for Disease Control and Prevention (CDC) defines blast injury in four phases. However, the bulk of bTBI occurs in the first three phases: the primary injury phase is comprised of the response of brain tissue to the blast wave (an intense overpressurization impulse component of the blast). The secondary injury phase results from shrapnel penetration into the head. The tertiary injury phase results from head contact/acceleration forces as the body is moved by the "blast wind" (a forced super-heated air flow). The quaternary injury phase incorporates any injury not covered in the other three phases such as some of the extracranial injuries or "polytrauma" including hemorrhagic shock and chemical or thermal burn injuries (Lew, 2005; Scott et al., 2006) that can occur. This quaternary phase of bTBI can significantly alter the timing and consequences of the primary damage occurring in the first three phases, and therefore can be a major contributor to overall brain pathology. This may be particularly true in mild bTBI, where there are either minor or no complicating factors.

The purpose of this review is to consider the mechanical phases of bTBI, how these phases are reproduced with *in-vitro* models, and to review findings from these models to assess how each phase of bTBI can be examined in more detail. Also described, where relevant, is how the potential secondary mechanisms have been studied in these models of TBI. These *in-vitro* models, viewed in combination with *in-vivo* models and clinical studies, can be used to assess both the mechanisms and possible treatments for this type of trauma.

Defining Blast Injury

No one model can mimic the clinical and mechanical complexity resulting from a bTBI. For injuries in the civilian population, the mechanical factors are often grouped into

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either the *focal/contact* or *acceleration/inertial* forces that produce the different mixture of clinical injuries ranging from concussion to skull fracture, contusions, and diffuse axonal injury (DAI). However, bTBI are grouped by injuries resulting from the different physical aspects of the blast phenomenon; in short, primary bTBI is from the shockwave, secondary bTBI is from shrapnel, tertiary bTBI is from the blast wind, and quaternary covers the remaining mitigating factors. These groupings apply not only to studying brain injuries, but also to other body regions susceptible to injury from blast. This distinct method of grouping blast injuries may seem inconsistent with the classifications often used for civilian TBI resulting from falls/assaults or motor vehicle accidents; however, a fair amount of knowledge gained from civilian TBI studies can be used to study the military problem.

Primary blast injury

The primary blast injury phase is limited to injuries caused by the rapidly expanding blast wave (Scott et al., 2006). Past work identified typical pressure wave profiles generated during blast events (Wharton et al., 2000), and how this pressure wave propagates through air and biological tissues (Clemedson, 1956; Clemedson et al., 1956; Clemedson and Pettersson, 1956). Shock-waves from a blast are typically characterized as a rapid rise and fall of high pressures (Depalma et al., 2005). The shockwave passes on the order of milliseconds, although the exact profile and range is dependent on the size, type, and shape of the explosive. In air, the shockwave oscillates between over- and underpressure segments, but these waves dampen out quickly. In water, the shockwave maintains a normal pressure profile over a longer range than in air. The presence of structures and fluid interfaces complicate this pressure profile.

Most structural damage occurs when the shockwave travels from water to air. For this reason, a large number of studies focused on the fluid-air interfaces in the body, as they are highly vulnerable to the passing pressure waves. Studies in these areas have generated injury thresholds for the pulmonary system and the gut/bowels (Yang et al., 1996). As this pressure wave travels through the organs, there are stress concentrations that arise at tissue-air interfaces, reflections of stress waves at tissue interfaces, and possible constructive stress wave interference within internal points of tissue that can further complicate the pattern of injury (Depalma et al., 2005). Models to study the primary injury phase should account for both the blast pressure transmitted through the organ of interest and shock-wave reflection/transmission behavior at tissue interfaces.

Secondary blast injury

The secondary blast injury phase covers both the penetrating and nonpenetrating injuries that occur when high-velocity projectiles/fragments impact the head (Scott et al., 2006). Certainly, these injuries share some common ground with the ballistic wounds seen in civilians, but are considerably more complex because of the non-uniform size and number of the impacting fragments. This results in a myriad of injuries compared to traditional penetrating lesions. At the mechanistic level, these injuries are best modeled as a tissue laceration, with the obvious complicating mechanisms related

to blood in the extracellular space, and the potential for secondary brain injuries such as hypoxia.

Tertiary blast injury

The tertiary blast injury includes the effects when the primary blast wind causes the person to collide with fixed or mobile objects. These types of injuries share the most in common with contact/acceleration injuries in civilians, where both the contact and acceleration forces can contribute to the different intracranial injuries that appear. Helmets worn by troops reduce the likelihood of injuries from direct contact for most of the head. However, current helmets are not designed to specifically reduce rotational acceleration following a helmeted impact (Richter et al., 2001; Pellman et al., 2003). There still is a high potential for inertially-based injuries that include subdural hematoma and DAI. As a result, the predominant mechanism for tertiary blast injuries in helmeted troops is the intracranial deformations caused by the head striking an object, or the head being struck by an object with sufficient mass to cause a significant inertial load. In non-helmeted victims, tertiary blast injuries can also include skull fracture or contusions from the focal contact forces when the unprotected head strikes an object or surface. Models to study this phase are best used to examine how tissue and cellular deformation change neuronal and glial functions in the CNS over time.

Quaternary blast injury

Quaternary blast injury includes conditions or the exacerbation of pre-existing conditions that develop from exposure to blast not covered in the other three phases. One such example would be the development of asthma and other breathing problems due to inhaling smoke and dust resulting from the blast. Any resulting burns would also be covered in this phase of injury. Other conditions could also include angina, hypertension, and crush injuries. Unconventional explosive devices such as nuclear weapons and dirty bombs will also emit harmful energy such as radiation that can further complicate sustained injuries. Traditionally, injury from toxic by-products of the explosion would be considered part of quaternary blast injury. However, it is now being considered a fifth emerging category. Injuries sustained in this phase can include the toxic residue of the explosives themselves (Steevens et al., 2002), their metabolites (Lachance et al., 2004), and the generation of toxic gases such as carbon monoxide (Deitchman et al., 1998; Bakke et al., 2001). This fifth emerging category of bTBI will not be discussed in this review because it needs more precise characterization (e.g., how these contaminants eventually surface in the brain parenchyma) before any systematic examination in an *in-vitro* setting.

Superimposed secondary insults

Briefly mentioned above, there are numerous complications that occur in blast injury not limited to the head that occur in each of the four phases. In the primary injury phase, the high pressure waves can cause damage and rupturing of air-filled cavities in the body such as the lungs, middle ear, and the gastrointestinal tract. In the second injury phase, the flying debris can cause penetration injuries to any part of the body, such as the eye, and may even cause loss of limbs. In the tertiary injury phase, the force at which the person is

thrown can cause fracture and traumatic amputation type injuries. In the quaternary phase, complications such as burns or the development of asthma would make recovery or rehabilitation from bTBI more difficult. Much of these superimposed insults such as loss of limb or internal hemorrhage from damaged organs can cause secondary insults such as hypovolemic shock, hypoxia, and hypoxic-ischemia complications. While some of the secondary insults cannot be modeled *in vitro*, hypoxia and ischemia are secondary insults commonly modeled with an oxygen-glucose deprivation chamber or through chemical treatments. Where appropriate, the *in-vitro* studies that combined secondary insults with the mechanical effects are highlighted.

Blast-to-bench translation

Each of the first three categories of blast injury is biomechanically distinct, allowing each to be studied relatively independently. Primary blast injury, in a reduced form, consists of pressure wave transmission through tissue. However, the portion of this wave most injurious to the brain is unknown. Secondary blast damage is best reduced to a direct tearing or transection-type lesion. The mechanism of tertiary blast damage is modeled simply by stretching *in-vitro* preparations directly, but below the point of cellular structural failure. The nature of *in-vitro* models provides a tool to explore each mechanism, and can be made progressively more complex when adding in elements of secondary brain injury (such as excitotoxicity), or potentially damaging agents to the extracellular space.

The next sections are a review of current findings from the literature that fall within one of these three biomechanically distinct categories of blast injury. Most of the current literature for *in-vitro* models falls within tertiary blast injury, where the intracranial strains and stresses are of primary importance. For this reason, this area of the literature is reviewed first, followed by studies that provide some mechanistic insight into secondary blast injury. A summary of *in-vitro* work involving the mechanism of primary blast injury is next addressed, as this area has been the least studied. A brief discussion of possible factors that may induce quaternary bTBI is last, since this phase has the least biomechanical distinction in terms of injury mechanism.

Tertiary blast injury

Modeling considerations in tertiary bTBI.   The acceleration/deceleration of the head that occurs in tertiary bTBI causes a shearing of the intracranial tissue. The membrane between the hemispheres separating the cerebrum from the cerebellum amplify these shearing strains in some brain regions, while limiting the strains in others (Meaney et al., 1995; Margulies et al., 1990). A number of increasingly sophisticated finite element (FE) models of the brain are excellent computational tools for relating head movement and brain structure to predicted areas of damage (Viano et al., 2005; Zhang et al., 2001; Kimpara et al., 2006). These models can estimate damage in regions for injuries with simple geometries. With more knowledge of the physical properties and measurements of the brain (Chu et al., 1994; Zhang et al., 2002), the exact levels of strain can be modeled. Injured and uninjured regions in a cell can also be distinguished. Although a number of critical issues remain, these computational models provide a "virtual

window" into the brain for predicting tissue deformation that can occur during the tertiary bTBI. Investigators have used these estimates of intracranial motions and deformations to develop precise models to understand how cellular functions change in response to mechanical deformation resulting from sudden acceleration or deceleration. These models, termed *cell-deformation* or *cell-stretching models*, distinguish areas of damage from regions where no injury is expected.

Reducing this complex pattern of deformation within the brain to an *in-vitro* model presents its own challenges. First, one must decide how to mimic the predominant strain within the brain (i.e., shearing of the tissue) in a two-dimensional system. Second, one must also balance the convenience of an experimental design against the similarity to the *in-vivo* environment. Converting an *in-vivo* (three-dimensional) shearing strain into an *in-vitro* (two-dimensional) equivalent strain field that can be applied to a cell monolayer is fairly straightforward. Namely, the monolayer can be extended in one direction, allowed to contract in a perpendicular direction, and remain unchanged in a third direction (Fig. 1A). This is the primary purpose of the uniaxial stretch system used in the past to mechanically injure dissociated cells and organotypic tissues (Lusardi et al., 2004a; Pfister et al., 2003). An alternative system used more widely is the biaxial system (Fig. 1B), which produces a stretch in two perpendicular directions across the culture surface. The *in-vivo* equivalent of the equibiaxial stretch system is relatively easy to construct, as it consists of a uniform expansion along a plane and a corresponding contraction out of plane. For non-equibiaxial stretch, the approximate *in-vivo* strain is broken into two components: a uniform expansion and an equivalent shear. The choice of which system to use is left to the investigator and likely depends upon past experience, simplicity, and cost. For tertiary bTBI, the uniaxial model offers an advantage because of its simple correlation to pure shear. However, the biaxial system has been more commonly used by past investigators, and therefore offers more past results for direct comparison.

A summary of the major findings from the work in this category appears in Table 1. In the following paragraphs, these studies are described more completely.

In-vitro *models of tertiary bTBI*

In-vitro astrocyte deformation models.   One of the earliest cell stretching systems studied was of primary astrocytes on both the single-cell and cell-monolayer level. For the single cell, injury was provoked by the mechanical poking of an astrocyte with the blunt end of a glass pipette tip (Charles et al., 1991). While this process can initiate a series of intercellular calcium waves that radiate from the stimulated cell, the exact level of mechanical stimulus used to create these waves was not well quantified. However, the use of astrocytic monolayers allowed for a correlation between levels of mechanical deformation and cellular injury. The cells in the monolayers showed cytoskeletal alterations, organelle disruption and derangement, and apparent changes in intracellular calcium ion influx (Ellis et al., 1995). These largely ultrastructural changes increased in severity as the level of mechanical deformation increased. At one point, significant cell death could be produced within 24 h of the initial mechanical insult. Unlike most *in-vitro* models used at the time, cell death did not propagate from an initial and immediate site of
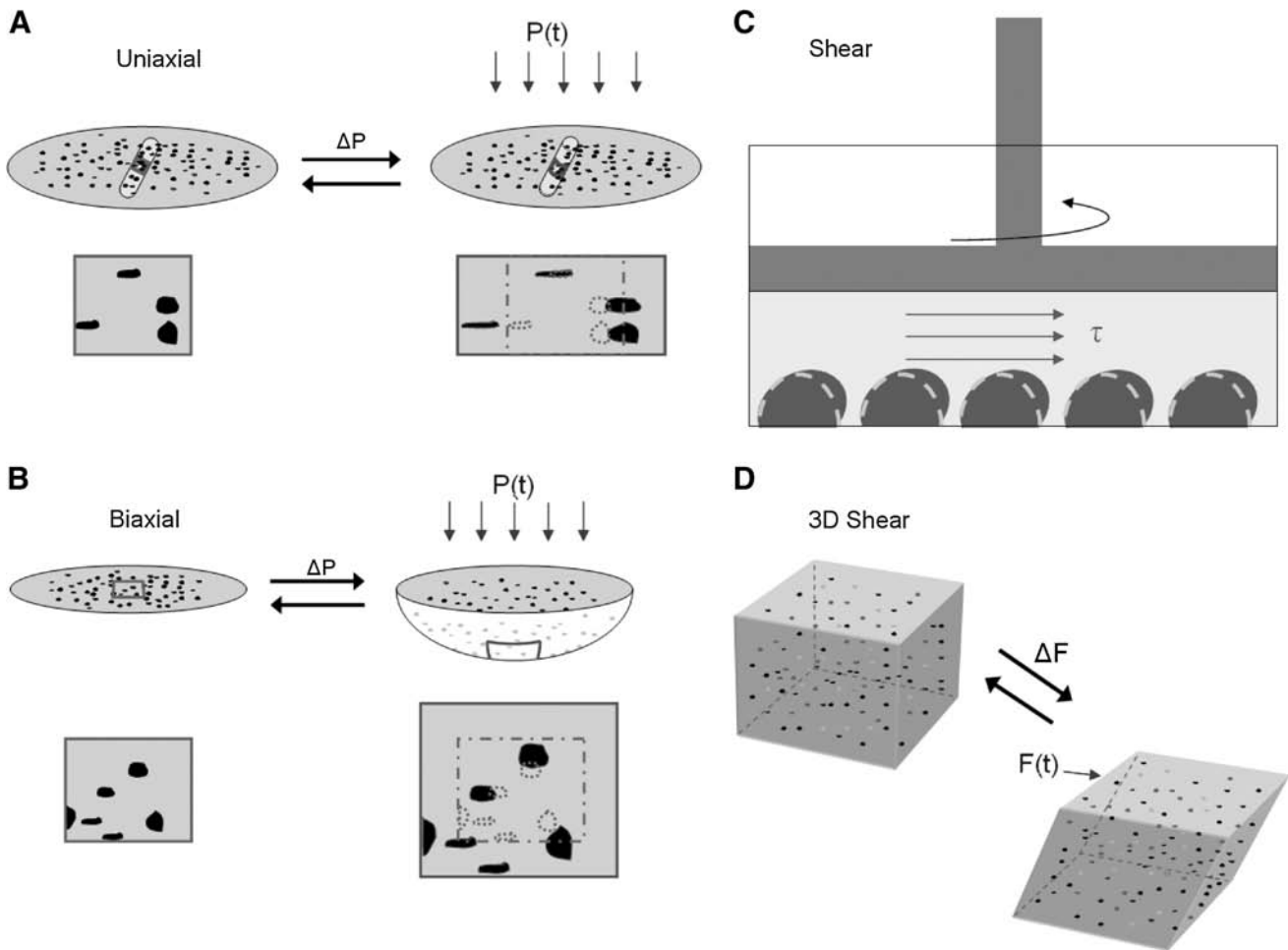
FIG. 1. Models of tertiary bTBI. (**A** and **B**) For two-dimensional monolayer cultures, the cells are typically plated on a transparent flexible membrane and the membrane is either deformed using an air pressure pule (P, ΔP) to strain the cells in both directions (biaxial), or primarily only in one direction (uniaxial). Shown are illustrations of both techniques, with highlights of how the cells in a region of the membrane (the dark objects) are deformed from their initial shape (dashed lines), and then returned rapidly to their initial shape. (**C**) A less commonly used technique on monolayer cultures is using hydrodynamic shear (λ), which is applied to the surface of the cell monolayer, to induce a deformation in the cells. (**D**) A new three-dimensional technique to study the response of cells embedded within a three-dimensional structure is also shown. The cells (the dark spots) are within a three-dimensional hydrogel that is deformed in shear by applying a force (F(t), ΔF) to the top of the gel.

mechanical failure (i.e., the cells did not lyse and cause secondary death in neighboring cells). Rather, death was driven by the mechanically initiated intracellular events.

In a series of follow-up studies, this same group identified a potentially novel role for calcium in astrocytic injury (Rzigalinski et al., 1997), as well as the mechanisms responsible for calcium elevation (Rzigalinksi et al., 1998; Rzigalinksi et al., 1997; Chen et al., 2004). Lamb and associates (1997) studied the role of membrane repair over time in injured astrocytes. Ahmed and colleagues (2000) studied mitochondria and ATP regulation. Other researchers examined how the complex cascades regulating free radical generation and IP_3 signaling contributed to the stretch injury response in astrocytes over time (Hoffman et al., 2000; Floyd et al., 2001). Recent work shows that mechanical stress leads to the release of proteins that can further exacerbate the initial injury (Ellis et al., 2007).

These works assembled a more complete picture on how disruption of potential physical linkages among intracellular structures can lead to transient calcium cascades, an important and unique step in the mechanoactivation cascade in astrocytes (Floyd et al., 2004). Other efforts have shown that mechanical injury leads to changes in volume-activated ion channels within astrocytes (Di et al., 2000), alterations in intracellular sodium (Floyd et al., 2005), production of endothelin-1 to influence vascular tone (Ostrow et al., 2000), and a potential crossover influence on the N-methyl-D-aspartate (NMDA) receptor (NMDAR) in strain-injured neurons (Lea et al., 2002). In addition, several studies now link the initial mechanical insult to the activation of purinergic receptors and the subsequent activation of intracellular signaling cascades (Neary et al., 2003; Neary et al., 2005; Neary and Kang, 2006).

In-vitro neuron deformation models. Although several studies discuss astrocyte response to a controlled mechanical stretch, the neuron is by far the most studied cell type in this area. A number of different devices are available for studying

Table 1. Summary of Injury Mechanisms from Tertiary bTBI

Reference	In-vitro injury	RM	IM	IC	O	Major finding
Charles et al., 1991	Astrocyte deformation		X	X		Ca^{2+} signaling after stretch
Floyd et al., 2004	Astrocyte deformation		X	X		Uncoupling of IP_3 signaling from Ca^{2+}
Ellis et al., 1995; Rzigalinski et al., 1997; Rzigalinski et al., 1998; Chen et al., 2004	Astrocyte deformation		X		X	Ca^{2+} influx; cytoskeletal alteration; organelle disruption
Lamb et al., 1997	Astrocyte deformation			X	X	Generation of hydroxyl and oxyradicals
Ahmed et al., 2000; Ahmed et al., 2002	Astrocyte deformation			X	X	Decreased ATP and mitochondria membrane potential; increase in intracellular free $[Ca^{2+}]$
Hoffman et al., 2000; Floyd et al., 2001	Astrocyte deformation			X	X	Free radical generation; IP_3
Ellis et al., 2007	Astrocyte deformation				X	Mechanical threshold for release of S100B, neuroprotective protein
Di et al., 2000	Astrocyte deformation		X			Volume-activated ion channel changes
Floyd et al., 2005	Astrocyte deformation		X			Intracellular $[Na^+]$ increases after injury
Ostrow et al., 2000	Astrocyte deformation	X	X	X		Cytoplasmic Ca^{2+}, IP_3 increases, leading to increased endothelin-1 production
Lea et al., 2002	Astrocyte deformation	X				mGluR5 regulates NMDAR in mild, but not in severe stretch
Neary et al., 2003; Neary et al., 2005; Neary and Kang, 2006	Astrocyte deformation	X		X		Purinergic receptor activation, signaling cascades
Zhang et al., 1996	Neuronal stretch	X				Stretch-induced relief of Mg^+ blockade of NMDAR
Geddes et al., 2003a; Serberst et al., 2005; Serberst et al., 2006	Neuronal stretch				X	Increases in membrane permeability after injury
Goforth et al., 1999, 2004	Neuronal stretch	X		X		Loss of AMPAR desensitization and potentiation of AMPA-evoked currents after injury
Kao et al., 2004	Neuronal stretch	X		X		Potentiation of GABA currents
Tavalin et al., 1997	Neuronal stretch	X	X			NMDAR delayed depolarization; mediated by electrogenic Na^+ pump
Geddes et al., 2003b; Lusardi et al., 2004b	Neuronal stretch			X		Changes in cytosolic Ca^{2+} from NMDAR activation and mechanical pores
Chen et al., 2004	Neuronal stretch	X	X			mGluR anatogonists prevent Ca^{2+} depletion of stores
Arundine et al., 2003	Neuronal stretch	X	X			Stretch induces signaling cascade through NMDAR
Lea et al., 2003	Neuronal stretch	X				Glia affects mGluR-1-mediated NMDAR modulation in neurons
Weber et al., 2001; Weber et al., 1999	Neuronal stretch			X	X	Intercellular Ca^{2+} store mediated signaling changes
Pike et al., 2000	Neuronal stretch				X	Calpain/caspase activation follows injury
Slemmer and Weber, 2005	Repeated neuronal stretch				X	Cumulative damage with repeated injuries
Movsesyan and Faden, 2006	*In vivo* controlled cortical impact	X				mGluR2 activation is neuroprotective
Wolf et al., 2001; Iwata et al., 2004	Axonal stretch			X		NaCh dysregulation leads to influx of Ca^{2+}
Cargill and Thibault, 1996	Neuronal stretch and hypoxia			X		Acute Ca^{2+} influx depends on rate and severity of mechanical injury
Glass et al., 2002	Neuronal stretch and hypoxia					Combined stretch and hypoxia resulted in increased cell death over single stretch or hypoxia
Engel et al., 2005	Neuronal stretch and hypoxia					Increased cell death with combined treatment; neuroprotective agents not effective

RM, receptor mediated; IM, ion mediated; IC, intracellular signaling; O, other.

neuronal response to biaxial stretch (Ellis et al., 1995; Morrison et al., 1998; Geddes and Cargill, 2001) (Fig. 1B), uniaxial stretch (Lusardi et al., 2004a; Pfister et al., 2003) (Fig. 1A), and general deformations in neurons or axons by using a fluid shear pulse (LaPlaca and Thibault, 1997; Edwards et al., 2001; Nakayama et al., 2001) (Fig. 1C). Early studies showed that calcium signaling initiated from the site of primary mechanical injury was predominantly mediated by electrophysiological changes in the NMDAR (Zhang et al., 1996). An alternative mechanism of injury suggests that nonspecific increases in plasma membrane permeability contribute to neuronal fate (Geddes et al., 2003a; Serbest et al., 2005; Serbest et al., 2006). Activating the NMDAR is key for the desensitization of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) after stretch (Goforth et al., 1999, 2004), potentiation of γ-aminobutyric acid$_A$ currents (Kao et al., 2004), and changes in the mitochondria membrane potential (Ahmed et al., 2000, 2002). All this may explain the potential vulnerability of hippocampal neurons (Geddes et al., 2003b) to mechanical trauma. However, the NMDAR is critical only for the induction of a stretch-induced delayed depolarization that can often accompany stretch injury (Tavalin et al., 1997); the maintenance of this modestly increasing depolarization occurring hours following injury is mainly mediated by the electrogenic sodium pump in the membrane. Acute and long-term changes in cytosolic calcium are not only related to NMDAR activation (Zhang et al., 1996; Geddes et al., 2003b; Lusardi et al., 2004b), but are also influenced by other glutamate receptors (Chen et al., 2004). It is important to note that changes in the NMDAR after stretch are not universally observed across different laboratories (Arundine et al., 2003). Changes in neuronal NMDARs are also sensitive to the presence of glia in the culture (Lea et al., 2003; Lea et al., 2002).

The progression of damage to injured neurons *in vitro* continues to reveal important new insights into how the traumatically injured brain may respond to mechanical injury over the ensuing days. This is important when considering short- and long-term treatment options. Post-injury changes in intracellular calcium signaling can lead to altered neuronal function under agonist stimulation (Weber et al., 2001, 2002; Weber et al., 1999), the consequences of which are yet to be fully understood. Activations of cysteine proteases, calpain, and caspase-3 also occur hours following injury, suggesting that the injury includes both apoptotic and necrotic forms of cell death (Pike et al., 2000). Furthermore, recent evidence showed that different mechanoactivation cascades occur when neurons are subjected to different mechanical strain fields (uniaxial or biaxial) (Geddes-Klein et al., 2006), indicating that moving *in-vitro* findings into *in-vivo* models, with their associated complex strain fields, will be important to consider when interpreting findings from the *in-vitro* work. Mild TBI *in vitro* appears to increase cellular susceptibility to secondary excitotoxic insults. For a single mild injury, the interplay between receptors and molecules in the synapse also extends to receptor cross-talk. This is mediated in part by the coupling between the NMDAR and molecules within the post-synaptic density (Arundine et al., 2003; Arundine et al., 2004).

Undoubtedly, issues involving long-term changes in neuronal function will become more complex as the field moves towards using systems designed for three-dimensional strain/stress fields along with improved tissue culture methods. This new paradigm can increase understanding of how the orientation of neurons within the tissue can affect the injury response (Cullen and Laplaca, 2006; Laplaca et al., 2005) (Fig. 1D). New information on neuronal susceptibility will likely show new interactions among receptor subtypes that might influence therapy development.

Another factor that can affect therapeutic development is the frequency of injury. Repeated mild injuries have an effect on neuronal viability and the release of neuronal proteins that is distinct from the effects of a single mild TBI. Repeated mild TBI are modeled as multiple mechanical simulations and may have particular relevance to bTBI, where there is the possibility for repeated blast exposures. The length of time between repeated insults to mechanical stress can change the timing of protein release and nuclear dye uptake (Slemmer and Weber, 2005). Information on treatment strategies gleaned from these *in-vitro* models of repeated injury will certainly make these strategies more sophisticated.

In-vitro axon deformation models.   DAI is one of the most important common pathologies of civilian TBI and may also be a common pathology in bTBI. Accordingly, Smith and associates (1999) developed an *in-vitro* model of dynamic axon deformation to mimic the mechanical loading conditions of *in-vivo* DAI. This model induces uniaxial stretch only to axons spanning two populations of neurons. In an initial study, it was found that rapid axonal stretch injury induced a loss of elasticity, demonstrating direct mechanical damage to the axonal cytoskeleton. Subsequent studies demonstrated that traumatic axonal injury induces a deleterious feed-forward cycle linked to calcium influx into the axoplasm and causes dysregulation of the voltage-gated sodium channel (NaCh) (Wolf et al., 2001). Although the sodium channel is not a direct pathway for calcium entry following axon stretch injury, its sensitivity to traumatic mechanical stimulation is the upstream mechanism. Specifically, pathologic sodium entry through a dysregulated NaCh induces activation of voltage-gated calcium channels and reversal of sodium/calcium exchangers. The resultant increase in intra-axonal calcium levels triggers proteolysis of the inactivation loop of the NaCh. As such, sodium influx through the NaCh is unmitigated, resulting in increasing levels of calcium. This in turn fuels additional pathologic changes that may result in axon degeneration or may impair the ability of the axon to maintain normal function (Iwata et al., 2004), similar to other channelopathies reported in the literature. *In-vivo* evidence also shows that sodium channel proteolysis is a component of axonal damage after TBI, but concludes further that myelinated axons respond differently to stretch injury than unmyelinated axons in the same brain region (Reeves et al., 2005). Future *in-vitro* studies may begin exploring some of the potential mechanisms that regulate NaCh proteolysis, as well as the differences between myelinated and unmyelinated axons, using more sophisticated culturing techniques to myelinate axons *in vitro*. Parallel *in-vivo* DAI studies using TBI models such as a weight drop impaction to the head (Kallakuri et al., 2003) can further determine the effects of laceration in a more complicated system.

In vitro cell combination, brain slice, and organotypic deformation models.   In recent years, the continued refinement of knowledge for mechanotransduction in both astrocytes and neurons has been complemented with emerging

information on microglia, past work on endothelial cells, and a continuing series of investigations on cross-talk among cell types (McKinney et al., 1996; Ellis et al., 2007). The organotypic brain slice culture—literally a living slice of brain cultured over time—maintains the approximate architecture and proportion of cell types in the brain. These models are a reasonable extension of studies using either pure neuronal or astrocytic populations, or even studies using mixed neuronal glial cultures that are not designed to mimic the *in-situ* architecture of the brain. Data from these organotypic slice culture models have already established that neuronal death in the cortex has a mechanical threshold distinct from that seen in hippocampal slices (Cater et al., 2006; Morrison et al., 2003). Moreover, the genes expressed by injured organotypic tissue have some level of correspondence with activated genes reported for *in-vivo* TBI (Morrison et al., 2000a, 2000b), therefore this preparation can also be used to study gene expression changes over time. These models now also extend to isolated segments of white matter to study DAI (Shi and Pryor, 2002; McBride et al., 2006; Shi and Whitebone, 2006). Ultimately, treatment strategies must consider injury severity (Cater et al., 2007) and protect other cell type populations such as astrocytes and oligodendrocytes that also die during injury (Deridder et al., 2006).

Modeling secondary insults with tertiary bTBI *in vitro*

While not all types of complications can be modeled *in vitro*, hypoxic-ischemic conditions can be mimicked in slices and cell cultures with the use of an oxygen-glucose deprivation chamber or through chemical means; the application of sodium azide or potassium cyanide with iodoacetic acid both deplete cellular ATP. Several labs have performed combined mechanical stretch and hypoxia treatments to determine how the combined injuries affect neuronal survivability. Cargill and Thibault (1996) showed that at high rates of mechanical stretch, chemical hypoxia did not affect acute response. At low rates of deformation, calcium influx was independent of stretch but dependent on hypoxic-ischemic conditions. Glass and colleagues (2002) and Engel and associates (2005) each found that the combination of moderate stretch and hypoxic treatments resulted in increased cell death over a stretch or hypoxia alone. Furthermore, agents that have shown neuroprotection for either hypoxia or mechanical injury are not effective in treating combined insults (Engel et al., 2005). This suggests that the combined insult needs careful consideration when developing effective treatments (Glass et al., 2002), especially for those suffering from polytrauma.

Secondary blast injury

Increasing effort is being made to study secondary bTBI since there is a higher mortality rate associated with penetrating brain injuries. According to the CDC, firearm use is the leading cause of TBI-associated death. Since penetrating injuries from complex skull fracture are not uncommon in motor vehicle crashes, this also adds to the sequelae of more diffuse types of injuries. Although the penetrating injury involves both vascular and neural tissue in the brain parenchyma, little is known about how the two factors interact *in vitro* to create a picture similar to what is observed clinically. At the *in-vitro* level, penetrating injuries are best reproduced using a direct laceration or scratching of cell monolayers. Although relatively simple technology is needed, this type of model has received less attention in the past decade compared to the cell-stretch models previously described. Similar to the summary of tertiary bTBI models, the

TABLE 2. SUMMARY OF INJURY MECHANISMS FROM SECONDARY bTBI

Reference	In-vitro *injury*	RM	IM	IC	O	Summary
Regan and Choi, 1994	Stylus	X	X			Injury mediated by excessive NMDAR activation and dihydropyridine-sensitive mechanisms
Regan and Panter, 1995	Stylus	X			X	Injury was reduced with MK801 and free radical scavengers
Mukhin et al., 1997a, 1998	Stylus	X				Glutamate receptor antagonists were neuroprotective
Mukhin et al., 1997b; Mukhin et al., 1996	Stylus	X				Activation of mGluR1 exacerbates injury
Faden et al., 1997	Punch laceration (stylus)	X				Group III mGluR agonists are neuroprotective
Wang et al., 2005	Injured tissue from humans/rodents				X	Performed proteomics on injured tissue to determine biomarkers of injury
Lucas et al., 1985; Emery et al., 1987; Kirkpatrick et al., 1985	Laser transection				X	Damage to cytoskeleton, neurofilaments microtubules, organelles (especially mitochondria)
Lucas et al., 1991	Laser transection			X		At low $[Ca^{2+}]$, Na^+ mediates injury
Rosenberg et al., 1996a	Laser transection				X	MPSS helps stabilized damaged membranes
Rosenberg et al., 1996b; Rosenberg et al., 2001	Laser transection			X		Na^+ and Cl^- ions may mediate injury
Balentine et al., 1988	Weight drop				X	*In vitro* injury is similar to *in vivo* spinal cord trauma
Epstein, 1971	Spinner bottle				X	Determined viability and oxidative metabolism of rat cortices after stir-bar-induced trauma

RM, receptor mediated; IM, ion mediated; IC, intracellular signaling; O, other.
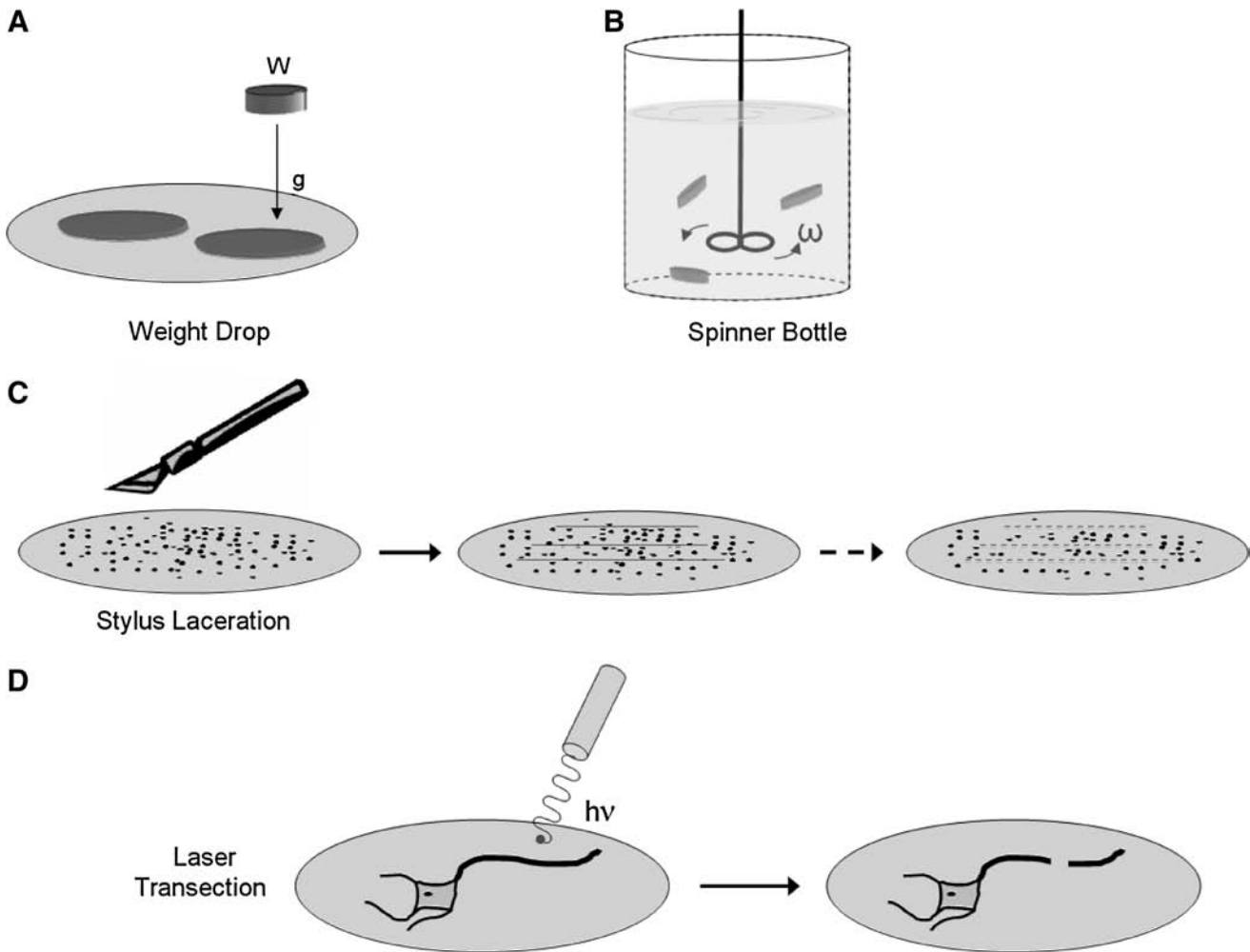
FIG. 2. Models of secondary bTBI. (**A** and **B**) The most simple models used on tissue slices and aggregates involve using either a dropped weight (W) or rotating spinner (ω) bottle to induce the primary injury. (**C**) More recent techniques use a stylus to induce a primary injury, which can be designed to injure several regions of the culture simultaneously. (**D**) A less commonly used approach is to use a laser (energy hv) to precisely injure local regions of the cell to study the regenerative or repair response.

past *in-vitro* work relevant to secondary bTBI is summarized in tabular form in Table 2, and in the following paragraphs.

Some of the earliest *in-vitro* models used to study TBI fall under this category. Slices of the mouse spinal cord were subject to a direct weight drop to approximate the mechanical forces seen in the cortex of a depressed skull fracture (Balentine et al., 1988) (Fig. 2A). Similarly, small chunks of the cortex were placed in a spinner bottle to detect potential mechanism(s) of damage that were mediated by the forces during transection (Epstein 1971) (Fig. 2B). Even though these studies were among the first to use laceration on more complex tissue, there were no further reports comparing *in-vitro* laceration models on dissociated cultures to these tissue structures.

Several years later, Tecoma and colleagues (1989) used a stylus to scratch a region of cells containing a mixture of neurons and glia, tearing the cell layer to study cell death in the surrounding regions of the micro-laceration (Fig. 2C). Interestingly, mostly neurons died while glial cells were spared. Cell death could be controlled by administering two different NMDAR antagonists, supporting concurrent *in-vivo*

controlled cortical impact studies used to develop NMDA-based treatments for TBI (Raghavendra Rao et al., 2001; Dempsey et al., 2000). Subsequent scratch studies showed that dihydropyridines could augment the protection offered by NMDAR antagonists (Regan and Choi, 1994); 21-aminosteroids showed a similar effect (Regan and Panter, 1995). In a later series of studies, Mukhin and associates (1997a, 1998) used this laceration model as a method to simultaneously test different pharmacological approaches, with the goal of achieving a model for high-throughput screening. Notable improvements in this field included a precise dose-response for the laceration technique in producing cell death, and an expansion in the number of therapeutic targets studied. Beginning with NMDAR antagonists, work from this new model soon showed that different glutamate receptors (mGluR1) work both alone and in combination with traditional NMDAR antagonists (Mukhin et al., 1997b, 1996). Intriguingly, new approaches using mGluR3 receptor agonists would also prove neuroprotective in this model (Faden et al., 1997), suggesting a more complex interplay between neuronal glutamate receptors during recovery from a laceration injury.

This *in-vitro* model also has the potential for testing injury biomarkers (Wang et al., 2005). A possible *in-vivo* model of the *in-vitro* laceration model could be a simple cortical ablation.

The cell laceration model can be further refined to study the response of a single neuron. Laser microsurgery, as a technique, has evolved to where an investigator can harness the power of a laser to cut out regions of an individual cell (Gross et al., 1983) (Fig. 2D), or even transiently permeabilize cell regions to deliver genetic material for subsequent study (Barrett et al., 2006). The location of transection in a neuron is crucial. Nuclear damage occurs readily if the transection point is close to the soma, and decays to nearly zero if the lesion is far from the cell body ($>300\,\mu$m) (Lucas et al., 1985). Injury from this very controlled and focal disruption of the neuronal structure consists of cytoskeletal damage to the neurofilaments and microtubules, as well as damage to the organelles, most notably the mitochondria (Lucas et al., 1985; Emery et al., 1987; Rosenberg et al., 1996a; Kirkpatrick et al., 1985). Extracellular calcium does little to control the damage observed in the soma from this type of laceration, but significant changes were observed when different extracellular calcium conditions were used to study the changes in dendritic morphology and structure (Lucas et al., 1990). In one of the earliest reports that expanded on the calcium hypothesis, it was suggested that other ions (potassium and sodium) could play a key role in dendritic integrity after dendritic transection (Rosenberg and Lucas, 1996b; Rosenberg et al., 2001). Laser ablation *in vivo* would be a comparable model (Nishimura et al., 2006a; Davalos et al., 2005).

Despite the relatively simple technology needed to mimic secondary bTBI *in vitro*, there is little published work that examines the role of secondary insults on either the exacerbation of initial injury cascades, or the promotion of new, more complex cascades of damage. Given the likelihood that the secondary injury associated with hemorrhagic shock, hypoxic-ischemic injury, or free radical generation are high in the moderate and severely brain-injured patients, this area remains ripe for new exploration in understanding not only the mechanisms of cell death that may be activated, but also the potential pro-survival signals that are possibly activated with these injuries.

Primary blast injury

What is currently known of primary bTBI. As noted in the introduction, the initial phase of blast injury consists of the pressure wave transmitting through the body, as well as the associated wind force that can cause direct damage to external tissues. Remarkably little is known about the response of CNS cultured cells from either form of mechanical input. The cellular response to shockwaves is often mentioned only in passing as a potential complication when interpreting data from the effects of ultrasound on tissue structure or function. The transmission of shockwaves through the scalp and skull is also considerably complex, so proper representation of shock input to tissue or cell culture preparations remains to be defined. One open question is how the shockwave affects tissue as it travels through. Nevertheless, the limited reports on such effects suggest that the shockwave is capable of primary tissue damage *in vivo* that resembles, but is not exactly similar to, the direct laceration damage that can appear with penetrating fragments and secondary brain injury. Certainly, the laser-induced shock-wave pulse can be controlled to create nanosurgical procedures capable of *in-vivo* transection of individual nerve fibers within the roundworm *Caenorhabditis elegans* (Yanik et al., 2004). Less clear is whether this shock-wave pulse can also produce regions of transient permeability changes in the plasma membrane without long-term transection damage. The shock-wave transmission at the interface between different structures (e.g., the white/gray matter interface and the solid/fluid interface at the blood-brain barrier) also presents an opportunity for additional damage.

Aside from the complex shock-wave patterns that can emerge in blast, what about the general influence of pressure on biological functions? Few studies have looked at pressure-mediated effects *in vitro* despite past concussive-based *in-vivo* insult studies that showed the ill effects of pressure on the nervous system (Denny-Brown and Russell, 1940; Gurdjian et al., 1962). Many also consider the peak intracranial pressure as a reasonable predictor of tissue injury (Stalhammar and Olsson, 1975). High negative transient pressures seen during a blast event can cause cavitation-based damage, but achieving similar pressure levels at the *in-vitro* level is difficult. Quasi-static hydrostatic pressures on cells causes cellular function and potential membrane-based changes in neurons and astrocytes (Murphy and Horrocks, 1993; Salvador-Silva et al., 2004), but the time-scales in these experiments are considerably longer than any blast event.

By far the most commonly used device to study the effect of pressure on the CNS is a barotrauma chamber, often built from existing or slightly modified fluid percussion devices used in TBI studies (Shepard et al., 1991) (Fig. 3A). Transient pressures from this type of chamber are sufficient to cause production of cytokines, specifically interleukin-6, and may contribute to cerebrovascular dysfunction (Hariri et al., 1994). This pressure chamber is readily amenable to test treatments for reversing pressure-mediated injury; successful treatments include a peptide to control Bcl-2 activation, and inhibitors for leukotrienes, nitric oxide, ADP-ribosylation, and the anticonvulsant felbamate (Wallis et al., 1995, 1996; Girard et al., 1996; Panizzon et al., 1996, 1998). Another type of transient pressure model is the rapid acceleration injury (RAI) model (Fig. 3B). This model can be used to study multiple impacts, creating a brief pressure gradient during the impact. Lucas and Wolf (1991) showed that neurons exposed to multiple acceleration/pressure forces caused soma swelling, nuclear prominence, and sometimes neuronal death, depending on the severity. Even though *in-vitro* models do not yet study changes in cell viability after transient pressurization, they strongly suggest that the biological effects of the pressure transient need to be explored more thoroughly in the future.

A current method that can be used to generate high-amplitude, transient pressure pulses is extracorporeal shock-wave lithotripsy (ESWL) (Fig. 3C). ESWL is currently used to treat kidney stones; the high-pressure shockwaves physically break up the stones. However, some also use this technology to study shock-wave damage on tissue. Two aspects of shock-wave pressure that have been shown to cause damage is the high transient pressure and the generation of cavitation bubbles in fluids subjected to high tensile stress. Howard and Sturtevant (1997) used ESWL *in vitro* on thin membranes immersed in tissue-mimicking fluids to study the mechanism of failure. The contributing factors that caused membrane
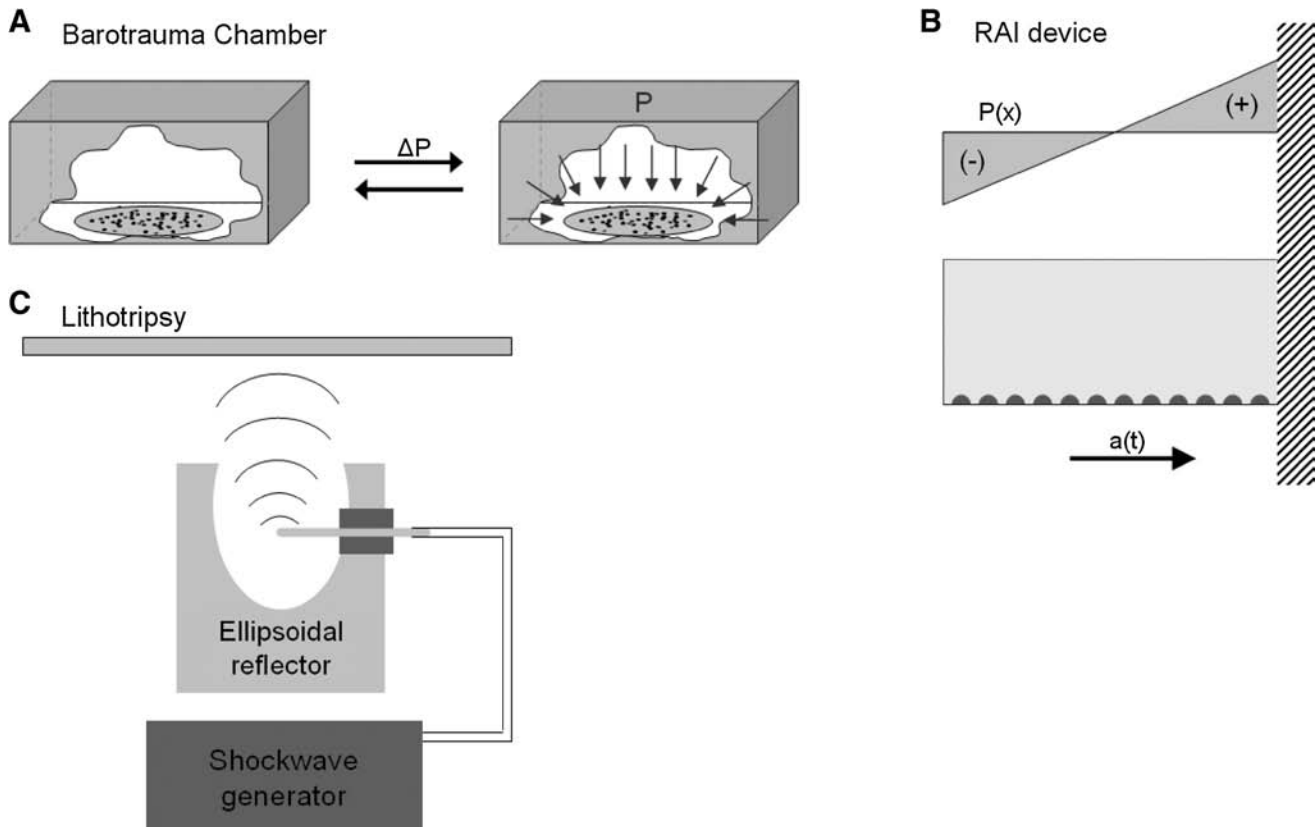
FIG. 3. Models of primary bTBI. (**A**) In the barotrauma chamber, even pressure (ΔP) is applied to all cells and slices to induce pressure-mediated injury. In a hydrostatic chamber, the pressure is quasistatic; in a fluid percussion chamber, the pressure is transient. (**B**) In the rapid acceleration injury (RAI) model, a flask containing the cells is swung on a pendulum from specified heights. A pressure transient (P(x)) is induced by the fluid upon impact of the pendulum/flask against a stationary object at the lowest point of the trajectory. The resulting injury is from a combination of acceleration (a(t)) and pressure forces. (**C**) In lithotripsy, shockwaves are generated and reflected off the ellipsoidal reflector.

failure were: shock compression causing the membrane to fail in shear, bubble collapse also caused membrane failure, and inhomogeneity of the tissue. These mechanisms of damage are not cavitation-based, as they can occur in fluids where cavitation does not occur. The threshold for the damage depends on the magnitude of the shock, the number of exposures, and physical properties of the medium. There is also a pressure-induced compression and lateral extension that can induce shear damage to structures that include the plasma membrane, organelles, and intracellular membranes (Howard and Sturtevant, 1997). The impedance properties of the medium focus and defocus the entrant shockwave, leading to a complex pattern of damage. This membrane-based damage or "nanoshearing" is currently unexplored in the blast literature.

A second phenomenon associated with shockwaves is the generation of cavitation/bubble formation, which seems to be dependent on fluid properties. The higher the presence of cavitation nuclei in the fluid, the greater the number of cavitation bubbles that will generate with a passing shockwave. Degassing the fluid reduces the amount of cavitation nuclei and subsequent bubble formation (Pa et al., 2005). Other labs have shown that the formation and collapse of cavitation bubbles from the transmission of a shockwave through tissue can lead to minute local injury. Laser pulses can produce

controlled cavitation *in vivo* to create damage limited to a single microvessel in the cortex (Nishimura et al., 2006b), or cut off individual nerve fibers in simple neural networks *in vivo* (Yanik et al., 2004). These *in-vitro* experiments can be analyzed along with *in-vivo* blast studies, such as using a bioshock tube to expose the whole body to blast, to further elucidate how cells respond to fast and extreme changes in pressure (Wang et al., 1998; Cernak et al., 2001a, 2001b) and to the presence of cavitation. Possible future endeavors to study primary bTBI could include FE simulations to model injury from a shockwave. FE tools used to model tertiary bTBI could possibly be coupled to Eulerian based hydrocodes designed to simulate shock situations. Current hydrocodes have been coupled to Lagrangian-based codes to model blast damage to structures (Cullis, 2001; Clutter and Stahl, 2004). Such programs could possibly be expanded upon with the aid of experimental data (Chavko et al., 2007; Clemedson, 1956; Clemedson et al., 1956; Clemedson and Pettersson, 1956) to determine shock-wave transmission through bone and tissue.

A final important area that is not yet explored is the role of secondary factors in the survival and function of cells following primary bTBI. It is not surprising that this particular area is unexplored, as the development and use of the *in-vitro* techniques to study this part of the force spectrum are not yet available.

TABLE 3. SUMMARY OF INJURY MECHANISMS FROM PRIMARY bTBI

Reference	In-vitro *injury*	RM	IM	IC	O	*Major findings*
Murphy and Horrocks, 1993	Hydrostatic pressures				X	Pressure induces cellular membrane damage
Salvador-Silva et al., 2004	Hydrostatic pressures				X	Pressure induces changes in human optic nerve head astrocyte migration
Hariri et al., 1994	Fluid percussion				X	Production of IL-6
Wallis et al., 1995; Wallis et al., 1996; Girard et al., 1996; Panizzon et al., 1996; Panizzon et al., 1998	Fluid percussion	X		X	X	Potential treatments: Bcl-2 activation; inhibition of leukotrienes, nitric oxide, ADP-ribosylation, NMDAR; felbamate; metalloporphyrins
Nishimura et al., 2006b	Laser pulse					Produced three forms of vascular insult: rupture, extravasation, blockage
Howard and Sturtevant 1997	Lithotripsy				X	Cellular membranes fail in fatigue by shear

RM, receptor mediated; IM, ion mediated; IC, intracellular signaling; O, other.

The results of the *in-vitro* models used to study primary bTBI have been summarized in Table 3.

Quaternary blast injury

Simply put, the quaternary injury phase comprises any conditions not covered in the previous three phases. For conventional explosives, types of quaternary injury would involve burns or the onset of asthma due to dust inhalation. However, there are other types of weapons that may cause further TBI. Radiological dispersal devices, often called a "dirty nuke" or "dirty bomb," are used to spread sub-lethal levels of radioactive materials over a large area. Nuclear weapons also emit electromagnetic pulses (EMP) in addition to spreading thermal radiation, radioactive material, and emitting an intense blinding light.

The radioactive, electromagnetic, and thermal energy components need consideration for their long-term consequences on the brain. Much of the work on radiation toxicity is *in vivo* and studies the radiation-induced injury to the CNS due to tumor treatments. *In-vitro* studies that have looked at x-ray exposure to neuronal cultures show that free radical scavengers can be neuroprotective (Noel and Tofilon, 1998). *In-vitro* studies have shown that ionizing radiation induces apoptosis in endothelial cells *in vitro* (Haimovitz-Friedman et al., 1994) and CNS endothelial cells *in vivo*, thereby damaging vasculature (Peña et al., 2000). Further radiation studies in culture show that astrocytes provide protection to neurons through the release of proteins (Noel and Tofilon, 1998). Information on electromagnetic radiation effects on the brain generally stem from studies on the effects of mobile phones on people (Munshi and Jalali, 2002; Christ and Kuster, 2005).

Mobile phones generate a relatively weak, but constant electromagnetic field, whereas the EMP generated from an explosion is considerably higher, but passes quickly. Mechanisms for injury based on weak electromagnetic fields are not often well described, and it would be difficult to scale these mechanisms to the much stronger level of an EMP generated by a nuclear explosion. One potential effect of the EMP to consider is how cortical activity can be modulated by a single, brief EMP. Early studies on transcranial magnetic stimulation (TMS) were met with some concern on how the technology would affect cortical function. Laser studies show that this potential side effect is not a problem with TMS. However, a much larger EMP has the potential to alter the existing neural network in some manner and warrants at least some consideration in the future. Thermal effects from the blast are not well studied either, although the transient nature of the thermal exposure would make significant transfer of energy into the brain unlikely. More severe circumstances would result in burns. The results of the *in-vitro* models used to study quaternary bTBI have been summarized in Table 4.

Conclusions and Future Perspectives

Just as there is no single *in-vitro* or *in-vivo* model that can be favored as the model to study civilian TBI, it is equally difficult to pick a particular model as the signature *in-vitro* model for bTBI. The blast phenomenon will change depending on the type of IED used, the presence of surrounding structures, atmospheric conditions (i.e., wind), and the direction of the blast with respect to the person. There is a high degree of variation in each blast situation. Certainly the advantage with

TABLE 4. SUMMARY OF INJURY MECHANISMS FROM QUATERNARY bTBI

Reference	In-vitro *injury*	RM	IM	IC	O	*Summary*
Noel and Tofilon, 1998	Radiation (x-ray)			X		X-ray induces neuronal apoptosis; astrocytes secrete neuroprotective proteins
Haimovitz-Friedman et al., 1994	Radiation (ionizing)			X		Radiation interacts with the cellular membrane and induces apoptotic signals
Munshi and Jalali, 2002	–					Review of cell phone hazards
Christ and Kuster, 2005	–					Factors that affect energy absorption

RM, receptor mediated; IM, ion mediated; IC, intracellular signaling; O, other.

the *in-vitro* models over *in-vivo* models is the ability to control the mechanical injury and reduce other potentially complicating factors. On the whole, current *in-vitro* TBI models capture the scope of the response at both the molecular and cellular scale, but further examination is needed. For injuries related to the blast environment, past *in-vitro* studies are strongly biased towards injuries sustained in the tertiary blast phase. Eventually, studies related to the mechanism of tertiary bTBI will likely include more functional measures of injury, test therapeutics over a longer treatment window, and begin evaluating how different brain regions may be vulnerable to different secondary brain injury mechanisms. Undoubtedly, this work will impact the treatment of injuries not only related to the blast environment, but also cross over into treating similar injuries from the traditional TBI environment (e.g., motor vehicle accidents, falls, and assaults). When considering *in-vitro* approaches for studying secondary blast phase related injuries, there remain large areas yet to be examined. For example, while early laceration studies *in vitro* provided some insight into the mechanisms and treatments for neuronal death, a considerable amount of work is needed to identify more effective treatments with a wider therapeutic window. Further studies will also determine whether increased neuroprotection corresponds to better cellular function. A significant issue that needs resolution is how the presence of factors in the extracellular space (e.g., blood, serum, and activated macrophages) can complicate recovery from transection injury. Moreover, the time of death for neuronal and glial cells following transection needs better definition, as the transection location has been shown to affect the window for cellular survival.

The most unexplored area are the mechanisms causing the primary blast injury phase. Even though *in-vivo* experiments show that significant intracranial pressures can transmit through the brain during a blast, there is very limited information on how these pressures can affect biological function, and how this shockwave is transmitted/reflected through the brain, as well as how this wave can cause damage at the cellular and molecular scale. Moreover, the influence of repeated exposures to a blast event, and their long-term effect on neuronal function, are not known.

Quaternary blast injury is likely the most difficult to study simply because it encompasses so much. Multiple factors contribute to injury in this phase. Because there is not a single physical force that can be directly tied to the injury mechanism, it lacks a distinct biomechanical form of study. Therefore *in-vitro* models from the three phases—primary, secondary, and tertiary—should be used to examine how the toxic products can contribute to the injuries observed in this phase.

In closing, it is worth noting that the relative wealth of new research directions in understanding bTBI with *in-vitro* approaches presents a very simple challenge: which ones should be studied first? It is natural to believe that as the biomechanical environment within the brain during a blast event is better characterized, the precise *in-vitro* model best suited to study primary bTBI will become clear. This may be one of the most important next questions, as relatively little is known about the cellular and molecular transduction events in primary bTBI. In addition, the effects of secondary insults on the primary, secondary, and tertiary phases of injury are also critically important and need clarification, as these can help better identify the potential therapies for the broader picture of bTBI. Similarly, it could also be interesting to combine two or more *in-vitro* models to study the multiple effects of blast. Regardless of the specific questions addressed by an investigator, there is opportunity for quick and meaningful progress in the field of bTBI that will have an immediate impact on both military and civilian suffering.

Author Disclosure Statement

No conflicting financial interests exist.

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