A Model of Repetitive, Mild Traumatic Brain Injury and a Novel Pharmacological Intervention to Block Repetitive Injury Synergy

Gwen B. Effgen[1], Elise Gill[2], Barclay Morrison III[3]

Abstract Studies suggest that repetitive injury synergy (RIS) can occur during a period of heightened vulnerability following concussion. For athletes, who are at greater risk for multiple concussions, understanding the mechanical injury mechanisms of repetitive mild traumatic brain injury (mTBI) is required for preventing the potentially devastating outcomes of multiple concussions. Although a single sub-threshold mechanical stimulus will not cause cell death, it triggers a sub-injurious biological response. We postulate that activated biochemical cascades from multiple injuries in succession are additive, becoming supra-threshold for initiation of neurodegeneration and hypothesize that drugs, which target these cascades, can intervene to prevent their super-position. Organotypic hippocampal slice cultures subjected to 2 mechanical injuries (11.78 ± 0.44% equibiaxial strain, 20 s^{-1} strain rate) 24 hours apart exhibited a significant and super-additive increase in cell death in all regions of interest of the hippocampus 4 days following injury ($n > 15$, $p < 0.05$). A combination therapy of memantine and 17β-estradiol was used to combat excitotoxicity and oxidative stress. Post-injury treatment with the drug combination significantly reduced cell death ($n > 25$, $p < 0.05$). Our results suggest that therapeutic delivery following mTBI and subsequent mTBI exposure within 24 hours can prevent potential RIS in our model.

Keywords concussion, hippocampus, *in vitro*, mild traumatic brain injury, repetitive injury synergy

I. INTRODUCTION

Of the 1.5 million people that sustain a traumatic brain injury (TBI) in the US each year, as many as 75% of these cases are considered to be mild traumatic brain injuries (mTBI, [1]). A TBI event can be defined on a scale of mild, moderate, or severe depending on the clinical assessment; the Center for Disease Control and Prevention clinically defines mTBI as a TBI resulting in transient confusion, dysfunction of memory, impaired consciousness, or loss of consciousness for less than 30 minutes [2]. The economic costs of mTBI have been estimated to be nearly $17 billion annually in the United States, but mTBI carries substantial human costs such as long-term or permanent impairment [2]. Most mTBI symptoms spontaneously recover within a week, but in some instances patients are symptomatic up to 1 year following the injury. Experimental studies suggest that after an initial mTBI, risk for a second mTBI increases during a period of heightened vulnerability induced by the initial concussion [3]-[5]. Evaluation of college football players experiencing concussion over 3 playing seasons concluded that the likelihood for sustaining an mTBI increased with history of previous concussion in addition to decreased rate of recovery of neurological function. Of those players experiencing multiple concussions within one season, 95% of these players sustained repetitive injury within 10 days of the first concussion [6]. For these reasons, development of an mTBI model for identification of critical rest periods and drug delivery time points following concussion is essential to break the synergy of multiple concussions.

Experimentally, mTBI results in a period of heightened vulnerability to subsequent mechanical injury capable of producing a synergistic response with an increased rate of neurodegeneration in the immature pig and adult rodent [4],[7]-[10]. Metabolic, ionic, blood flow, and oxidative glucose metabolism changes following mTBI have been identified as potential mechanisms contributing to this period of increased vulnerability following a single mTBI [11]. While *in vivo* studies suggest that repetitive injury synergy (RIS) can occur within a given period of heightened vulnerability following injury, validating and controlling the tissue-level biomechanics is a significant challenge *in vivo*. Finite element models (FEM) have been employed in efforts to correlate macroscopic, external, or kinematic

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injury parameters to cell and tissue-level injury parameters; however, this approach is limited by our knowledge of the heterogeneous mechanical properties of the brain and regional-specific tolerance criteria [12]-[15]. An *in vitro* model for mTBI, which allows for the direct control and independent validation of the tissue-level injury biomechanics, will address these limitations by contextualizing resultant pathology to specific injury parameters, i.e. tissue strain and strain rate.

The effects of repetitive loading differ for living versus engineering (dead) materials. For example, fatigue of engineering materials is well understood and involves progressive, microscopic structural damage that propagates until the material fails. In contrast, the response of living tissue to loading is a dynamic process, initiating transient biochemical cascades involving active responses from the tissue such as processes of repair, protection, or degeneration that can have both short-term and long-term implications for function. For example, while drastic fluctuations in intracellular ion concentration and activation of proteases like caspase can induce neuronal death, mild injury results in less drastic changes such as slightly increased intracellular zinc concentration and slight activation of caspase-3, which remain below the threshold to induce apoptosis or necrosis and can even influence neuroprotection [16],[17]. Therefore, describing the brain's response to multiple mild mechanical injuries requires not only an understanding of the resultant degeneration manifested by cell death or functional changes but also evaluation of the response pathways initiated by each loading cycle [4],[6],[9],[18]-[20].

We postulate that activated biochemical cascades from multiple loading cycles in succession are additive, becoming supra-threshold for initiation of neurodegeneration, thereby resulting in RIS. Characterization of repetitive, mild, mechanical injury in this context can inform the timing of targeted therapeutic delivery to potentially attenuate or prevent RIS.

In the current study, we employed a well-characterized stretch-injury model previously used to define the regional, mechanical tolerance criteria of organotypic slice cultures of the hippocampus and cortex of the rat [21]-[26]. The use of organotypic slice cultures maintains *in vivo*-like neuronal and glial connections that have been shown to be important in modeling the biological cascades responsible for delayed degeneration after the primary injury, making the model more representative of the *in vivo* response [26]. *In vitro* models, with their ability to contextualize the resultant pathology to precise injury biomechanics, will complement *in vivo* models, which benefit from evaluation of the long-term tissue response to injury and neurobehavioral deficits. Together results from *in vivo* and *in vitro* models will form a more complete understanding of factors contributing to RIS, such as number of injuries, severity, and time between injuries.

II. METHODS

Organotypic Hippocampal Slice Culture

All animal procedures were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC). According to previously published culture methods, P8-10 Sprague-Dawley rat pups were decapitated, and their brains were removed [21, [23-25]. Hippocampii were excised and sectioned into 400 μm thick slices using a McIlwain tissue chopper (Harvard Apparatus, Holliston, MA). Organotypic hippocampal slice cultures (OHSC) were separated aseptically in ice-cold Gey's salt solution supplemented with 25 mM D-glucose (Sigma, St. Louis, MO) using blunt, plastic spatulas (Fisher, Pittsburgh, PA). Slices were plated onto silicone membranes coated with poly-D-lysine and laminin (Specialty Manufacturing, Saginaw, MI and Life Technologies, Grand Island, NY) and initially fed with Neurobasal medium supplemented with 1 mM GlutaMAX, 1X B27 supplement, 10 mM HEPES, and 25 mM D-glucose (Life Technologies). Culture medium was changed to conditioned full serum medium (50% Minimum Essential Medium, 25% Hank's Balanced Salt Solution, 25% heat inactivated horse serum, 2 μM GlutaMAX, 25 mM D-glucose, 10 mM HEPES, Sigma and Invitrogen) 3-5 days following plating, which was then changed every 2-3 days. OHSC were cultured at 37°C and 5% CO_2 and maintained on a rocker to aid gas exchange and diffusion.

After 11-16 days in culture, the baseline health of OHSC immediately prior to injury was assessed by quantifying pre-injury cell death with the fluorescent stain propidium iodide (PI, Invitrogen). OHSC with PI fluorescence greater than 5% in any region of interest (DG, CA3, CA1) at the pre-injury time point were not included in the study (see *Quantification of Cell Death*).

Stretch-injury

The stretch-injury device and loading mechanism have been described previously and characterized in detail 22],[23],[25]. In brief, culture medium was aspirated, and each culture well was placed on the injury device. An equibiaxial stretch-injury was achieved by displacing the well and silicone tissue-substrate over a hollow, cylindrical indenter (Fig.1). By controlling the speed of the displacement and the maximum displacement, strain rate and strain were controlled [22],[23],[25]. Strain histories followed a trapezoidal function, stretching at a constant strain rate to a maximum strain and returning to rest at a constant relaxation rate. Both substrate and tissue strains were verified via image analysis of high-speed video (1000 frames/s, Redlake Motion Pro,Tallahassee, FL and Elicar VHQ Super Macro MC 90mm f2.5, Tokyo, Japan) recorded during injury with custom MatLab code (Natick, MA). Cultures received either a mild stretch-injury or a sham-injury on days 0 and 1 (Fig.2). OHSC receiving the single, mild injury received the stretch-injury on day 0. OHSC receiving the double, mild injury were stretch-injured 24 hours apart on days 0 and 1. All samples not scheduled to receive a stretch-injury at a given time point were exposed to the sham-injury. For sham-injury, samples were clamped on the injury device; however the device was not fired. While on the injury device, cultures were kept at 37°C. Immediately following stretch-injury and sham-injury, cultures were returned to fresh full serum media and incubated.

Fig. 1. Immediately prior to injury wells were clamped on the injury device and the membrane was lowered to the surface of the indenter. During injury, an equibiaxial strain was imparted to the OHSC by displacing the well and silicone tissue-substrate over a hollow, cylindrical indenter.

Fig. 2. OHSC were evaluated for baseline health and PI fluorescence immediately prior to the first injury on day 0. 24 hours later OHSC received either a second mild stretch-injury or a sham-injury. Three days later (day 4) resultant cell death was evaluated with PI fluorescence before excitotoxic injury. Samples were imaged for excitotoxic-induced cell death 24 hours later (day 5) for normalization of stretch-induced cell death.

Drug Delivery

OHSC receiving drug therapy 1.5 nM 17β-estradiol (β-estradiol 3,17-disulfate dipotassium salt, E2, Sigma) and 1.5 μM memantine (memantine hydrochloride, Sigma) received the double mild injury 24 hours apart. 1 hour subsequent to both injury time points, full serum medium was replaced with full serum supplemented with the combination drug therapy (E2+memantine) or vehicle (phosphate buffered saline solution, PBS, Fig.3). At 4 days following the first injury time point, injury-induced cell death was evaluated, OHSC were subsequently glutamate-injured, and glutamate-induced cell death was evaluated 24 hours later (see *Excitotoxic Injury* and *Quantification of Cell Death*).

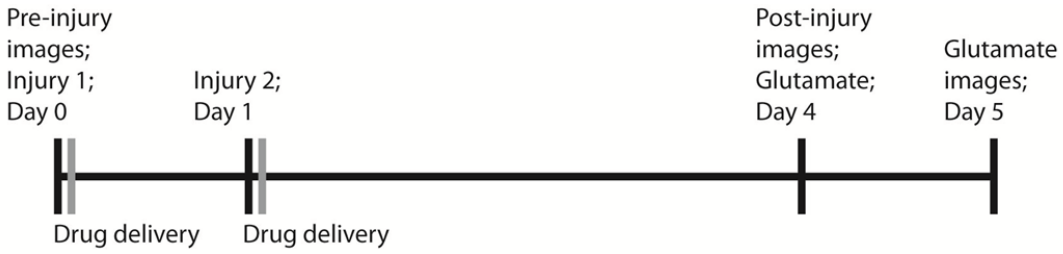

Fig. 3. OHSC were evaluated for baseline health and PI fluorescence immediately prior to the first injury on day 0. 24 hours later all OHSC received a second mild stretch-injury on day 1. Immediately following injury, cultures were returned to fresh culture medium. One hour following both injury time points culture medium was aspirated and replaced with media supplemented with the combination therapy or vehicle. Three days later (day 4) resultant cell death was evaluated with PI fluorescence before excitotoxic injury. Samples were imaged for excitotoxic-induced cell death 24 hours later (day 5) for normalization of stretch-induced cell death.

Excitotoxic Injury

To induce maximal cell death for normalization purposes, at the end of experimentation and 4 days after the first injury, culture medium was removed and OHSC were incubated with full serum medium containing 10 mM glutamate (Sigma) for 3 hours. Following excitotoxic injury, cultures were returned to fresh full serum media. At 24 hours following glutamate treatment, slices were analyzed for cell death (see *Quantification of Cell Death*).

Quantification of Cell Death

PI fluorescence was used to quantify cell death immediately prior to the first injury (to eliminate unhealthy cultures), 4 days following the first injury time point, and 1 day following excitotoxic injury. OHSC were incubated in 1.5 μM PI in serum-free medium for 30 minutes immediately prior to imaging. Images were acquired using an Olympus IX81 microscope with 568/24 excitation and 610/40 emission filters. Cell death was determined for specific anatomical regions of interest (DG, CA1, CA3), as previously described, using MetaMorph (Molecular Devices, Downingtown, PA, [27], [25]). In brief, the same threshold for fluorescence was used to analyze all images at each time point for an experiment. Tissue damage at a given time point was quantified as the percentage area of a specific region exhibiting fluorescence above the threshold. OHSC with greater than 5% cell death in any region of interest prior to injury were excluded from the study. Changes in percent cell death induced by stretch-injury were normalized to maximum cell death resulting from excitotoxic injury.

Statistical Analysis

A univariate general linear model was used to analyze the complete data set for each anatomical region with cell death as the dependent variable and experimental group as the fixed factor with a Tukey-Kramer *post hoc* test for sham, single, and double injury groups (SPSS v. 19, IBM, Armonk, NY, significance $p < 0.05$). A univariate general linear model was used to verify equal tissue-level strain for each group with a Tukey-Kramer *post hoc* test comparing x- and y-strain at both time points (significance $p < 0.05$).

III. RESULTS

OHSC were injured with a well-characterized stretch-injury model of inertial brain injury delivered 24 hours apart on days 0 and 1 (Fig.1 & 2, [22],[23]). This injury model benefits from a high degree of control and validation of the injury parameters imparted to the tissue [22],[23],[25]. High-speed video taken during each stretch-injury was used to calculate maximum strain imparted to the tissue samples (Fig.4). Injuries delivered to OHSC in both the single injury group and double injury groups (including samples receiving no drug, vehicle, and drug combination groups) were equibiaxial with a high degree of reproducibility for both groups at both injury delivery time points (Fig.4C). The average injury strain in the X-Y plane delivered to all OHSC was 11.78 ± 0.44% with a 20 s^{-1} strain rate.

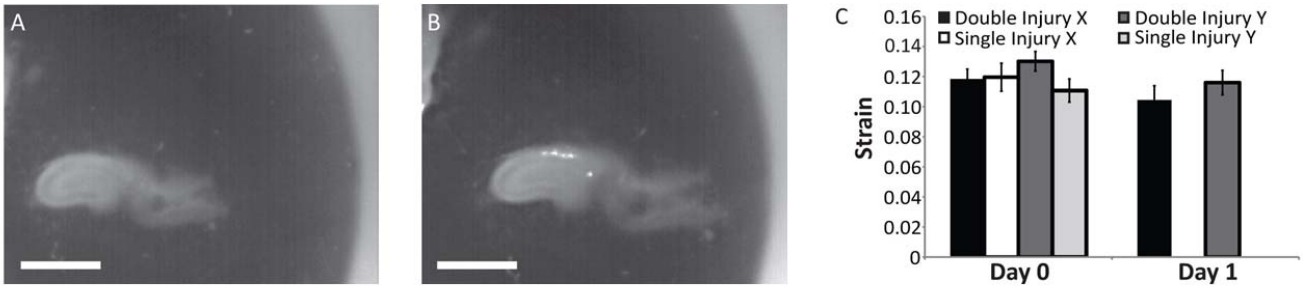

Fig. 4. A) Pre-injury images were used to calculate tissue baseline dimensions prior to injury (scale bar=2mm). B) Tissue dimensions at maximum stretch were used to calculate strain (scale bar=2mm). C) As characterized previously, induced strains were equibiaxial without shear strain in the X-Y plane and were not significantly different for each injured group at any injury time point (n > 47, SEM). High-speed video taken during each injury was used for evaluation of stretch-injury parameters (strain, strain rate).

OHSC stimulated with a single mild injury exhibited minimal cell death over the 4 day time period following injury, quantified by a change in percent PI fluorescence (< 5%, Fig.5). This change in percent cell death was not significant as compared to OHSC receiving the sham-injury; therefore, a single ~12% strain injury was sub-threshold for inducing cell death and defined as being mild for this injury model. OHSC receiving 2 mild injuries 24 hours apart demonstrated both a significant and super-additive increase in cell death in all regions of interest of the hippocampus as compared to the sham-injured controls and samples receiving the single mild injury (Fig.5A). Stretch-injured OHSC appeared darker 4 days following the first injury time point, indicative of ultrastructural changes and tissue damage (Fig.5B, [28]). Fluorescence from PI staining was minimal for OHSC receiving the single injury and was elevated for the double injury group. Sham-injured controls demonstrated minimal PI fluorescence over the 4 day period following exposure to the sham-injury. Glutamate injury of all OHSC resulted in increased PI fluorescence 24 hours following excitotoxic injury and was used for normalizing changes in percent cell death over the 4 day period following injury (Fig.5C).

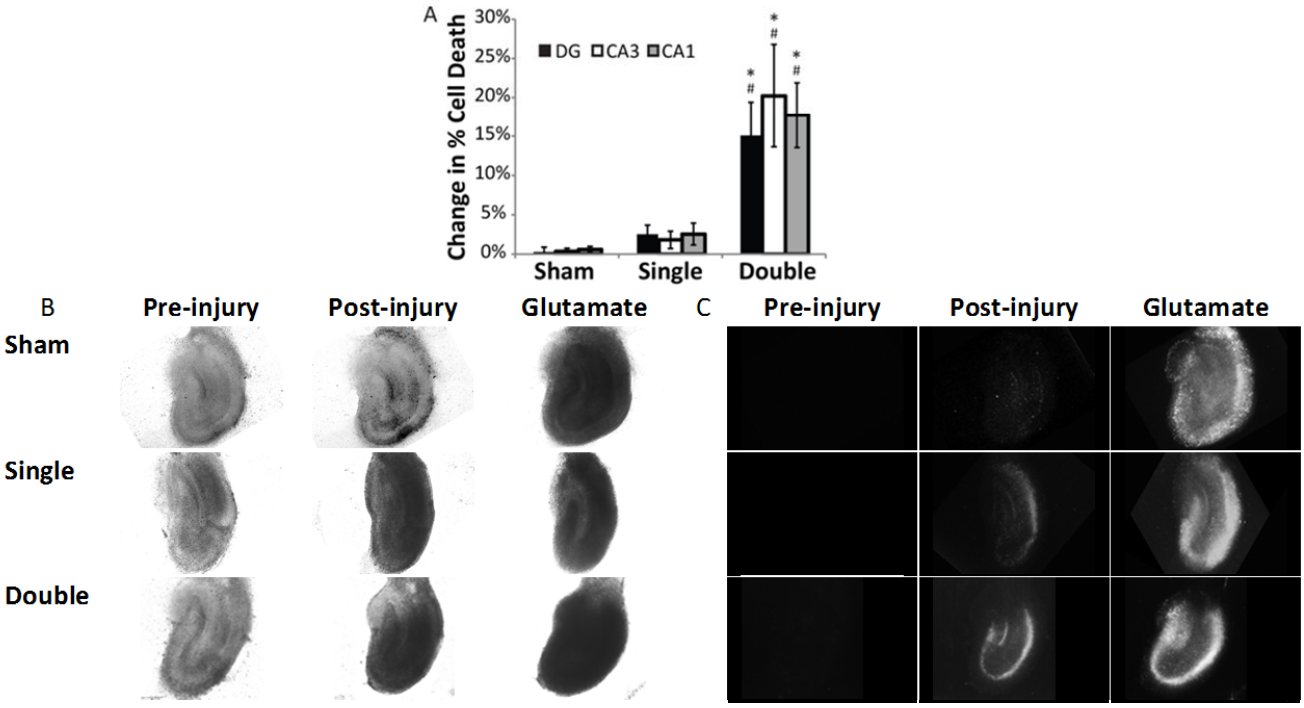

Fig. 5. A) Cell death increased minimally over the 4 day period following a single mild stretch-injury. Cell death increased significantly in all regions of interest of the hippocampus over the 4 day period following repetitive mild injury (n = 19) as compared to both the sham-injured group (n = 18, SEM, # $p < 0.05$) and the single injury group (n = 15, * $p < 0.05$). B) OHSC receiving the sham-injury maintained normal morphology 4 days following the injury delivery time point as indicated by bright-field images. OHSC receiving stretch-injury appeared darker and shrunken indicative of ultrastructural changes 4 days following the delivery of the first mild injury. C) PI fluorescence was minimal in OHSC 4 days following the sham-injury and the single injury. Cell death increased as indicated by PI fluorescence in all regions of interest of the hippocampus 4 days following repetitive injury. Excitotoxic injury and maximal PI fluorescence in the pyramidal cell layer indicated tissue integrity was maintained over the duration of the experiment.

To test the efficacy of the combination drug therapy in mitigating RIS, additional OHSC were subjected to the double injury and received both 1.5 μM memantine and 1.5 nM E2 (or vehicle) 1 hour following both injuries. OHSC receiving the combination therapy demonstrated a significant reduction in cell death over the 4 day period following injury (Fig.6A). As demonstrated by bright-field imaging, OHSC that received either the vehicle or the drug combination appeared darker 4 days following injury, which was exacerbated by excitotoxic injury (Fig.6B). PI fluorescence increased over the 4 days following the first injury time point. However, the extent of tissue damage as demonstrated by an increase in cell death was diminished for the sample group receiving the combination therapy (Fig.6C).

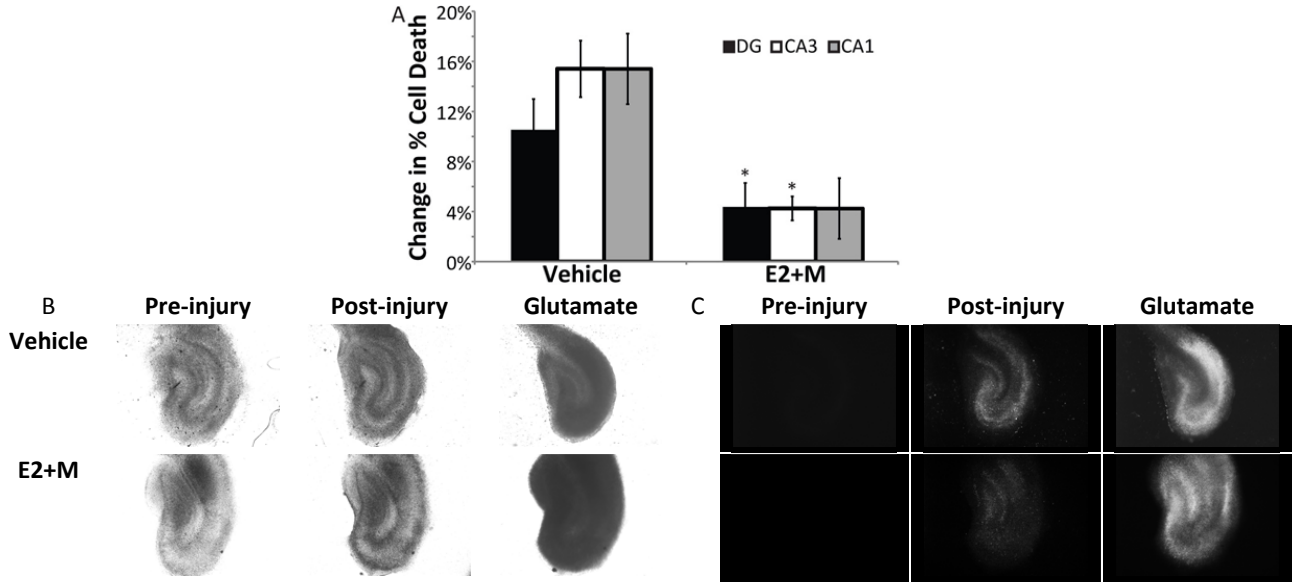

Fig. 6. A) Treatment with the combination therapy 1.5 μM memantine and 1.5 nM E2 1 hour following both injury time points reduced cell death in all regions of interest of OHSC receiving the double injury (n = 25, SEM, * $p< 0.05$) as compared to cultures receiving the vehicle (n = 40). B) Bright-field imaging revealed healthy morphology in OHSC prior to injury and over the 4 day time period following injury. Tissue cultures in both groups appeared darker following injury. C) PI fluorescence indicative of cell death was reduced in all regions of interest of OHSC receiving the combination therapy.

IV. DISCUSSION

Previous work on repetitive, mechanically-induced injury *in vitro* utilized a deformable silicone membrane and a Flexcell® injury device characterized by Ellis *et al.*, defining a mild injury as a 5.5 mm maximum deformation of the membrane resulting in a 31% membrane strain [5],[29]. Subsequent studies used this injury model to deliver multiple mild mechanical injuries either 1 or 24 hours apart to co-cultures of neurons and glia derived from the hippocampus and cortex. This work suggested that both the severity of the injury and the time interval between injuries were key parameters of the degenerative response [5],[18]. Cortical cells exposed to a mild, mechanical injury with the same model were more vulnerable to a subsequent ischemic injury [30]. Additionally, hippocampal-, cerebellar-, and cortical-derived neurons were also more vulnerable to an excitotoxic insult following a sub-lethal stretch-injury induced by the Flexcell® injury device [31]-[33]. These results suggest that an initial mild mechanically-induced injury sets in motion biochemical cascades which are sub-threshold for the induction of large scale degeneration. However, these activated cascades are capable of producing a subsequent period of heightened vulnerability to further insults.

Gilbert *et al.* characterized the strain profile for the stretchable cell culture membranes used with the Flexcell® injury device noting that radial and circumferential strain were not equal and varied with respect to radial position [34]. One limitation of these previous studies is that the applied mechanical stimulus (strain) was spatially inhomogeneous within a single culture well, making correlation between observed pathology and input biomechanics difficult. Paracrine signaling between cells in the same culture exposed to a nonhomogeneous injury further complicated correlation of observed physiological deficits to specific injury parameters. In addition, these studies did not verify strain delivered to the samples but assumed that cells were perfectly adhered to the substrate and experienced the substrate strain exactly.

The model of multiple mild mechanical injuries used in our study benefits from both precise control and validation of the mechanical stimulus at the tissue level (Fig.4). For the current study, we chose the injury severity and inter-injury interval based on reported literature. We chose the biomechanical parameters of the mechanical stimulus for the current study based on previous characterization of this injury model and mTBI studies measuring *in situ* deformation during injury. The mild injury parameters used here exceed tissue strain measured *in situ* with magnetic resonance imaging of volunteers exposed to sub-concussive mechanical stimuli [35]. Furthermore, characterization of the stretch-injury model used here demonstrates that a 10% strain injury is mild enough to result in a minimal (5%) cell death response consistent with the current study [21]-[26]. Previous *in vivo* and *in vitro* studies have identified a period of heightened vulnerability to subsequent injury following an initial mild brain injury that lasts at least 24 hours [4],[9],[32],[33]. In this study, RIS was demonstrated within the 24 hour time period following an initial mild injury (Fig.5A). This was consistent both with experimental and clinical data suggesting that subsequent concussions within a period of heightened vulnerability after an initial concussion can result in a super-additive degeneration of brain [3],[4],[6],[11],[18]. In this study, cell death was localized to the principal cell layers following repetitive injury similar to results from investigating OHSC exposure to a single stretch-injury of varying strain. These results indicate that the pyramidal cell layer is particularly vulnerable to mechanical injury (Fig.5C [25]).

Combination therapies have the capacity to mitigate injury cascades via multiple mechanisms. Following brain injury, changes in ion concentration and neurotransmitter release begin a series of biochemical cascades that have the potential to result in pathological calcium influx, impaired oxidative metabolism, initiation of apoptosis, and a multitude of ultrastructural changes [11]. Excitotoxicity in response to indiscriminant release of glutamate and oxidative stress resulting in free radical formation are two interrelated cell death pathways, which play a prominent role in TBI. Memantine and E2 were selected as the therapeutics in this study in an effort to target multiple injury cascades simultaneously. Memantine is an uncompetitive NMDA antagonist with the potential to reduce the effect of excitotoxicity following injury. Memantine reduced cell death when administered *in vivo* following ischemic injury without the negative side effects common to other NMDA antagonists [36-38]. An uncompetitive open-channel block may be an ideal therapeutic NMDA-antagonist because it is voltage and use-dependent, reduces the opening frequency and mean channel open time, and has increasing ability as an antagonist with increasing activity of the agonist [39]. It has been suggested that this distinction contributes to memantine's efficacy following injury and excitotoxic glutamate exposure without negatively influencing physiological levels of glutamate necessary for excitatory synaptic transmission. E2 has proven to be neuroprotective both *in vitro* and *in vivo* against oxidative

stress and ischemia [35],[40]-[42]. Activation of estrogen receptors within the nucleus resulted in increased expression of anti-apoptotic proteins, increased antioxidant activity, and reduced inflammatory response [35],[43]. It is important to note that these studies reported the neuroprotective effect of E2 administered to the animal or tissue culture prior to injury, which is important in determining the efficacy of a drug initially but is an unrealistic treatment for clinical application.

Studies investigating the neuroprotective effect of memantine and E2 when delivered after injury have been inconclusive; however, memantine and E2 pre-treatment have been effective at low concentrations both *in vivo* and *in vitro.* The dosages used here were chosen because they were among the lowest concentrations shown to be effective. Neuroprotection of OHSC has been demonstrated out to 72 hours following exposure to 30 minutes of oxygen-glucose deprivation (OGD) when cultures were pre-treated for 1 week prior to injury with 1 nM E2 [35]. For rat retinal ganglion cells, memantine at 1 μM effectively blocked a 200 μM NMDA-evoked response [39]. Memantine at 1 μM improved rat retinal ganglion cell survival and at 6 μM significantly increased survival when administered at the time of excitotoxic injury [44]. In this same study, co-culture of rat neurons and glia were protected from excitotoxic injury by 12 μM memantine administered at the time of injury [44]. In the adult rat, memantine and E2 have proven to be neuroprotective for ischemia models [36],[42]. A brain concentration of 1-10 μM memantine, which was chosen because it is similar to that found in patients chronically treated with memantine for Parkinson's Disease and is well-tolerated, reduced ischemic cell death and improved Morris Water Maze performance in the rat when administered 2 hours after injury [36]. Pre-treatment for one week with E2 to achieve serum concentrations of 10 pg/ml (3.7 pM), equivalent to endogenous, circulating levels in the adult female rat, reduced total brain infarct volume by 60% [42]. Future studies are necessary to determine the relative efficacy of the combination therapy as compared to monotherapy treatment and the ideal therapeutic window for E2 and memantine administered in combination in combating RIS.

While our model of *in vitro* repetitive stretch-injury is promising for evaluating mechanisms of RIS and therapeutic screening, the current study had some limitations. Cell death was evaluated 4 days following the first injury, a time point that was chosen based on previous reports indicating that cell death reached a maximum at this time point following a single stretch-injury [25]. Future studies will determine the temporal development of RIS with different inter-injury time intervals, different numbers of injuries, or different injury severities. A time-course evaluation will be important in defining the differences amongst injury paradigms. Drugs were delivered 1 hour following injury to simulate professional athletes receiving medical attention at a sports facility. For the average person, medical attention could be delayed beyond this time point for a variety of reasons including identification of concussion symptoms, travel time, or triage in an emergency room. The lack of a blood-brain barrier (BBB) in *in vitro* models simplifies therapeutic studies; but ultimately, the influence of the BBB on therapeutic access will need to be evaluated in future studies.

Unlike non-viable materials, brain has the potential for repair, protection, and degeneration. We postulate that the biochemical cascades that are initiated by mechanical stimulation of the tissue have the potential to result in all of these unique responses depending on their level of activation. Mild injury results in minimal, physiologic-level activation of caspase-3 and increases in intracellular zinc concentration that are sub-threshold to induce apoptosis and necrosis; however these same secondary messengers, with increased activity, induce cell death [16],[17]. Therefore, it is essential to determine the time-dependent vulnerability to a subsequent injury to prevent superposition of the sub-threshold biochemical activity that could lead to RIS. Our model benefited from precise control and validation of relevant parameters for concussion, which will be beneficial in contextualizing the observed pathobiology *in vitro* to the literature and other mechanical *in vitro* and *in vivo* models of repetitive mTBI. Additionally, advantages of models include ease of access to the sample and serially sampling to evaluate the pathology's development over time. Future studies will utilize the model described here to evaluate the time-course for the vulnerability that results from an initial mTBI to determine important rest periods following concussion and critical timing of drug-delivery to break RIS.

V. CONCLUSIONS

Our *in vitro* model for repetitive mTBI not only demonstrated RIS for a subsequent injury delivery within 24 hours of the first injury but also has proven to be an effective drug screening platform. Treatment of OHSC with the drug combination delivered 1 hour after both injuries, which can also be interpreted as prophylactic drug administration

23 hours prior to the second injury, significantly decreased resultant cell death by 50% 4 days following the first injury. These data suggest that the biochemical cascades activated following each mTBI can be attenuated within 1 hour following injury with memantine and E2, effectively breaking the super-position that results in RIS. Taken together, these results suggest that the necessary rest period between incidences of mTBI is at least 24 hours in our model and that therapeutics targeting oxidative stress and excitotoxicity may be effective in mitigating the devastating consequences of RIS.

VI. ACKNOWLEDGEMENT

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