VALIDATION OF AN IMPROVED INJURY DEVICE FOR IN VITRO STUDY OF NEURAL CELL DEFORMATION

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ABSTRACT

Traumatic Brain Injury is hypothesized to occur as a function of the strain and strain rate experienced by neural tissues during a traumatic event. *In vitro* studies of TBI at the cellular level have used a variety of methods to subject neural cell cultures to potentially injurious strains and strain rates. The Advanced Cell Deformation System (ACDS) has been developed which has the ability to independently control strain and strain rate and can strain cell cultures grown on a stretchable membrane from 0.1 to 0.60 at rates up to 25 s^{-1}. The ability to control strain and strain rate independently or to simulate quick repetitive loading was not available in previous devices. Here we present the experiments testing the ability of the ACDS to replicate the results of *in vitro* experiments of neural cell deformation conducted by earlier researchers. This is a first step toward future experiments which will use the more advanced capabilities of the ACDS.

Keywords: Mild Traumatic Brain Injury, Experiment, Neural Injury Model, Neural Tissue

INTRODUCTION

Traumatic Brain Injury (TBI) in humans is hypothesized to be a function of the strain and strain rate experienced by neural tissue. Previous work [1] has estimated that diffuse axonal injury, a condition associated with TBI, occurs in humans at strains between 0.05 and 0.1. Other studies [2, 3] have found that strains of 0.20 at strain rates of 10 [s^{-1}] produce moderate injury in rat brain slices, with higher strain rates resulting in irreparable damage and cell death. Thus, the current estimated threshold for cell level injury in brain tissue is approximately 0.20 strain at rates greater than 10 [s^{-1}].

Ellis [4] developed a model of strain-induced neural tissue injury using neural cells cultured on an elastic membrane. If strong adhesion between the cultured cells and membrane is assumed, then the strain experienced by the cells can be taken to be the observed strain of the membrane. This membrane, which forms the bottom of a culture well, is strained by applying a pulse of pressurized air or nitrogen to the well via a special fitting (Figure 1). As the elastic membrane expands, the cells on its surface are strained, causing injury.

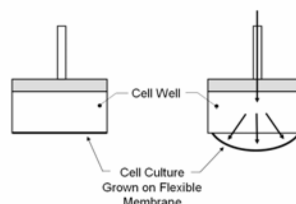

Figure 1: Operating principle of the Ellis injury model.

The system used in [4] to control the pressurized air pulse delivered to the culture well was called the Cell Injury Controller, and has several important limitations. Strain and strain rate could not be controlled independently of one another and no control over the shape of the pressure pulse (other than duration and pressure) was available. Additionally, peak membrane deflection (and thus strain) was

measured via "calipers" consisting of a metal slider on a ruler; this method is known to have limited accuracy [5], and provides no means of obtaining a time history of membrane deflection.

A device which addresses these issues has been developed in prior work [5]. Called the Advanced Cell Deformation System, it can generate pressure pulses of arbitrary shape, can control strain and strain rate independently and uses a digital high-speed camera (likely not even available at the time of [4]) for recording the entire time history of membrane deformation. Figure 2 shows a labeled photograph of the ACDS components.

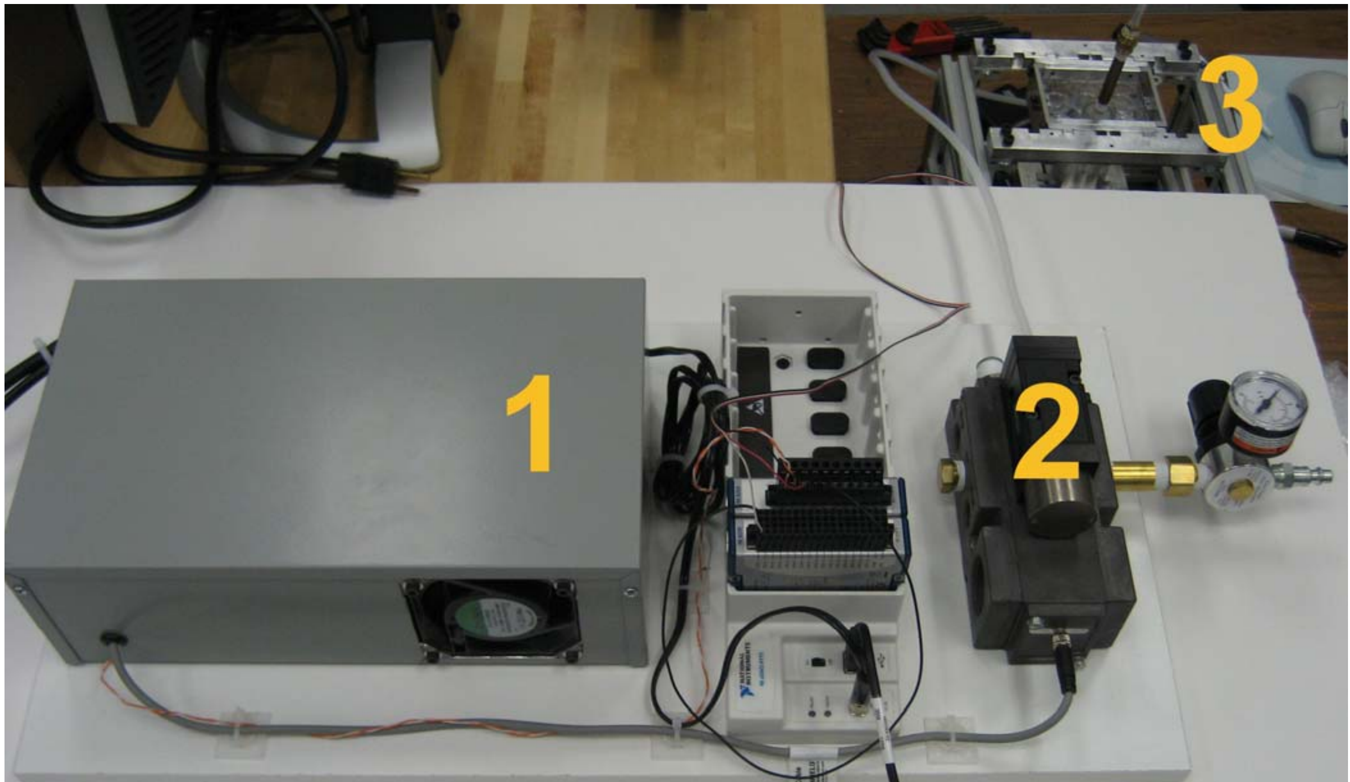

Figure 2: The components of the ACDS: (1) is the control circuitry, consisting of a valve controller and a modular data acquisition system, (2) is the pneumatic valve that regulates the flow of compressed gas/nitrogen into the cell well and (3) is the chassis that holds the culture well trays during testing (shown here with the gas fitting inserted into a well).

METHODS

Since it had yet to be tested in laboratory use, this study aimed to validate the ACDS by using it to replicate the cell injury observed with the Ellis injury model. In particular, it was decided to replicate the study of astrocyte injury using propidium iodide uptake in [4], due to its relative simplicity. First, astrocyte cultures were injured using the strain-based injury levels of mild, moderate and severe identified in [4]. Each of these injury levels corresponds to a different amount of peak membrane deflection (and thus strain) at very roughly the same deflection rate (and thus strain rate). Before any cultures were actually injured, an empty culture well tray was used to calibrate the ACDS to yield these deflections and rates as discussed below. Each culture tray contained 6 wells – 5 were injured, and the remaining well served as a control. For the Ellis model injury levels, media is left in the culture wells

during injury. In order to investigate whether motion of this media had any effect on astrocyte injury rates, a single tray was injured in addition to the others at the moderate level, but with only 100 [µL] of media present – just enough to cover the bottom of the well and keep the cultured cells from drying out and dying during the injury process.

Immediately after injury, all culture wells were stained with 10 [µL] of 1 [mg/mL] propidium iodide. This dye becomes fluorescent when it binds to a cell nucleus, but can only enter cells with damaged membranes, thereby providing a means to readily identify injured cells. At 15 minutes post injury, the number of injured cells was assessed via fluorescence microscopy. Injured cells were counted in 3 or 5 randomly selected fields in each well (more fields for wells with sparser counts to manage variability levels), and the counts for that well averaged and multiplied by the correct scaling factor. Each well average was then transformed to a percent of the control average, and the average percent of control for all the wells in each injury level reported (Table 1). Ellis reported injury rates in [4] as injured cells per [mg] cell protein - no protein assay was conducted in this study, so it cannot be said with scientific rigor how much protein was in each well. However, the value was almost certainly between 0.45 [mg] and 0.50 [mg]: the value of 0.475 [mg] was used to get an estimation of cells injured per [mg] cell protein for comparison to the results in [4] (Figure 3).

The injury levels of mild, moderate and severe identified in [4] correspond to strains associated with peak membrane deflections of 5.7 [mm], 6.3 [mm] and 7.5 [mm] respectively (note that the 7.5 [mm] deflection is called "moderately-severe" in [4]; this level was later named "severe" and is referred to thusly here). In the original study, the CIC was calibrated for membrane deflection via analysis of video taken on a standard video recorder (digital high-speed video was likely not available to Ellis) with 33 [ms] between frames. Because the pressure pulse duration used was only 50 [ms], it seems unlikely that the Nyquist criterion was adequately satisfied and thus these deflection level measurements likely contain a great deal of uncertainty. Based on these same videos, the time of peak deflection for these injury levels was placed between 33 [ms] and 66 [ms] after deflection began [4]. High-speed video (270 [µs] frame interval) was used by ACDS to record membrane deformation for each well, and the average deflection for each injury level in this study calculated (Table 2). In order to replicate the Ellis model as closely as possible, the ability of the ACDS to shape the pressure pulse was utilized to place the peak deformation within this time interval. A triangular pulse shape with a duration of 50 [ms] placed the peak deflection in all runs between 38 [ms] and 44 [ms], comfortably within the interval observed in [4].

RESULTS

Sterility of the device during the injury process was checked by injuring a culture tray and incubating for 24 hours. No signs of contamination were present, so the tests were executed as planned. Table 1 gives the results of the testing: the observed average control level was 1706 injured cells per control well, or 1.76 per observed field.

Table 1: Cell injury rates corresponding to each of the injury levels tested. The average injured cell count in the control wells was 1760 injured cells/well.

	Mild	**Moderate**	**Severe**	**Moderate – No Media**
Percent of Control Injured [%]	251.4	1693	9801	7127
Standard Error [%]	57.00	387.0	944.9	1087

Using the assumption of 0.475 [mg] cell protein per culture well, the number of cells injured per [mg] of protein was estimated. Figure 3 shows the results; while the exact values are just estimates, the observed dose-response between injury level and cell injury rate is identical to that seen in the percent of control results. The original results from the study in [4] are also shown in Figure 3. These values used were extracted from the original graphic in [4] via WinDig (a data digitizer) and re-plotted here to facilitate comparison. As with any data obtained by such a digitizer, these are probably very slightly different from the actual values used to make the original graphic.

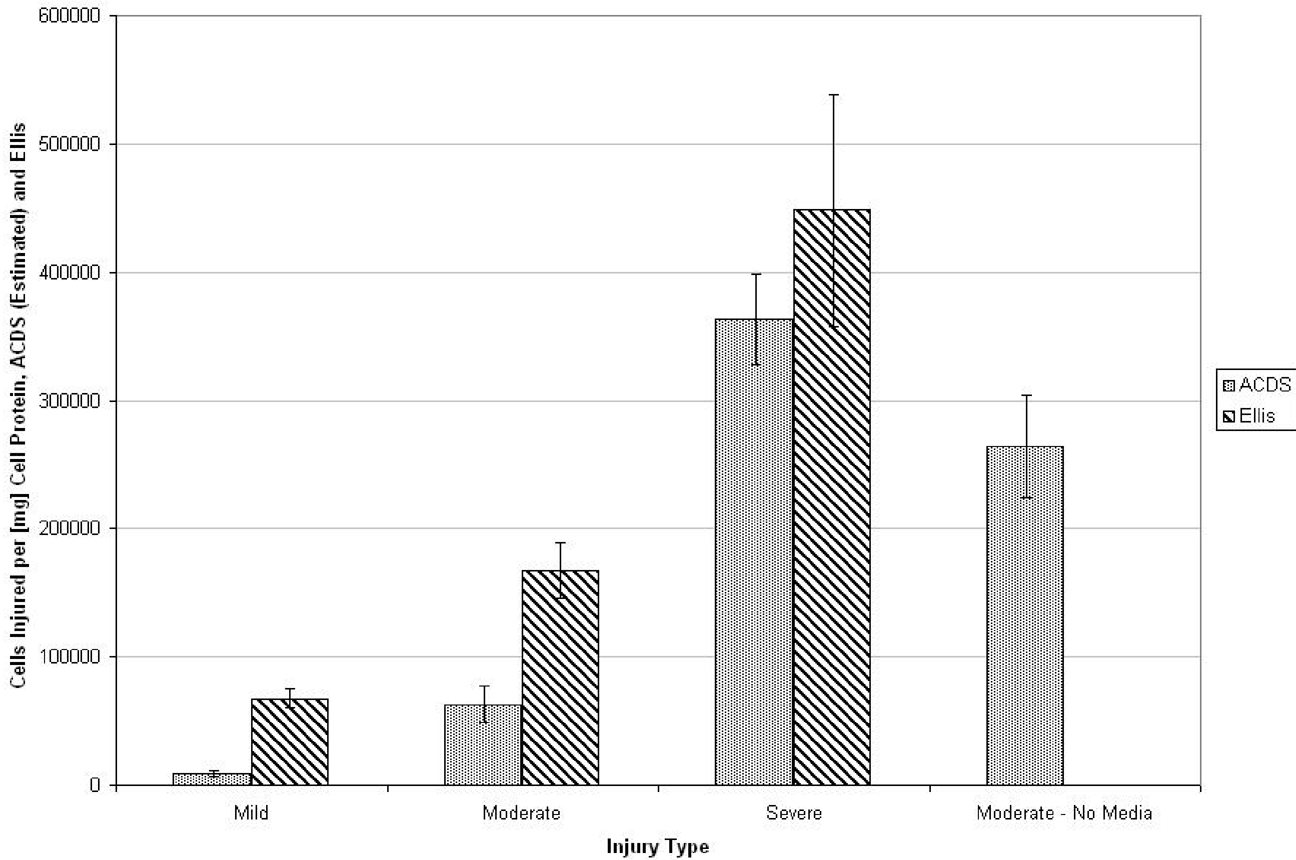

Figure 3: Estimated cells injured per [mg] protein. Values were calculated based on an assumed cell protein content of 0.475 [mg] per well, and are intended only for comparison to the Ellis results [4], which are provided for comparison. Note: Ellis values were extracted from the original graphic via WinDig, so are not rigorously precise. Error bars are +/- 1 Standard Error.

The ACDS can records high speed video for each well injury. As verification that the injuries generated were in fact those specified by the Ellis model, the peak deflections for each well were recorded. The average deflection level achieved at each injury level is presented in Table 2. Note that there is some uncertainty in displacement measurements taken in this way, as the resolution of the video used constrains the resolution of measurement. All deflection measurements taken in this study have uncertainties less than 0.15 [mm].

Table 2: Average measured membrane deflection for each injury level assessed in this study.

	Mild Injury	Moderate injury	Severe Injury	No Media
Average Membrane Deflection [mm]	5.37	6.45	7.58	6.32

DISCUSSION

Overall, the results seem to indicate that the ACDS can replicate the Ellis model. The results, when reported in the same format as the study in [4], exhibit the same strong dose-response between astrocyte injury and peak membrane deflection. Comparison of the ACDS and Ellis results in Figure 3 show that, for every injury level, the estimated ACDS cell injury rates are less than those observed by Ellis - this could be attributed to simple experimental variability. However, the possible uncertainty in the deflection levels specified by Ellis could also be a factor, especially given the strong dose-response observed in both that and this study.

Of significant interest is the difference between the moderate injury levels with and without media, shown in both Table 1 and Figure 3. It shows that, without the presence of media in the culture well, the astrocyte injury rate for a moderate injury level becomes more representative of that seen in a severe injury. This raises a number of interesting questions which this study is not suited to answer.

It has been hypothesized that the media may protect the cultured cells from direct exposure to the pulse of pressurized air expelled by the ACDS (or CIC), and that removing this buffer allows the pressure pulse to injure cells directly, raising injury rates. Examination of the high-speed video for the tests confirms that the air pulse never penetrates the layer of media covering the well bottom, so this seems plausible. However, testing this with the ACDS or the CIC would be essentially impossible, as both induce membrane deflection by pressurizing the inside of the culture well. An injury device which creates a deflection in some other way, such as a negative pressure applied to the outside of the well, would be required for such a test.

The high-speed video of the tests also revealed that there is tremendous disturbance of the media during the injury process. Figure 4 shows two frames from the video of one of the severe injuries: the frame on the left shows the resting state of the media (prior to injury), and the right frame is representative of the media immediately after the pulse has subsided. A droplet (circled) of media can be seen falling back into the culture well in the right frame.

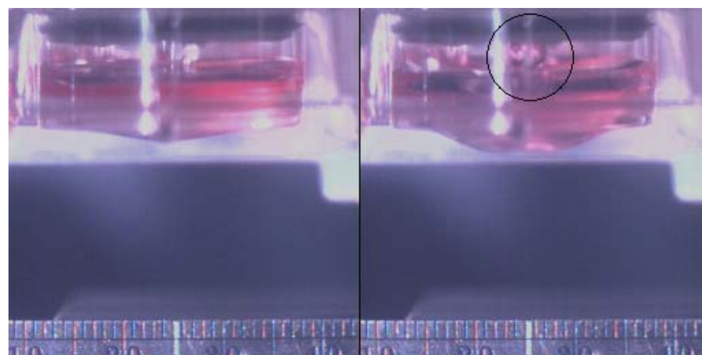

Figure 4: The culture media on the left is shown at rest before injury. The frame on the right shows the same well, and is representative of the perturbed state of the media during and immediately after injury. In particular, the large droplet of media (circled) falling back into the culture well is indicative of the degree to which the media is disrupted by the pressure pulse.

It is unknown whether the degree of shear seen in the bulk media is representative of that seen in intracellular fluid in actual neural tissue. While the fluid in the well can move freely, the fluid in actual tissue is much more restricted in the tiny spaces between cells. This also naturally raises the question of

whether neuron cultures would be affected by this injury model in the same way as astrocytes. Astrocytes lie flat against the culture membrane, with an entire side of the cell for attachment and very little protruding structure. Neurons on the other hand are only attached by a few points on their membranes and have much structure that protrudes far into the media, which would seem to make them somewhat less susceptible to membrane strain, but far more susceptible to shear in the media. Further studies using cultured neurons seem to be a logical extension of this observation.

CONCLUSIONS

Given the peak deflection and time of peak deflection corresponding to an Ellis model injury level, the ACDS device is capable of reasonably replicating the cell injury rates predicted by the model in astrocyte cultures. Additionally, the presence or absence of media in the culture wells during testing is observed to have a significant effect on injury rates. Determination of why this is so, and whether the observed motion of the culture media is representative of *in vivo* neural tissue injury, warrants further investigation.

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