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Institution: Rutgers, the State University of New Jersey
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1. Original aims of the project.

The original aims of the grant were as follows:

Specific Aim 1: To evaluate the potential of genipin to protect neurons in organotypic hippocampal cultures exposedto distinct injury-related stresses
Aim 1A: To evaluate the potential for genipin to protect from oxidative stresses
We will expose organotypic hippocampal cultures to superoxide and nitric oxide and assess the ability of genipin to ameliorate the cytotoxic effects.
Aim 1B: To evaluate the potential for genipin to protect neurons from amyloid peptide cytotoxicity
We will expose organotypic hippocampal cultures to beta amyloid peptides and assess the ability of genipin to ameliorate the cytotoxic effects.
Aim 1C: To evaluate the neuritogenic potential of genipin
We will quantify the number and length of neurite branches emanating from hippocampal neurons in organotypic cultures exposed to varying concentrations of genipin.
Specific Aim 2: To evaluate the therapeutic potential of genipin and identify a therapeutic window for its administration following controlled injury in organotypic hippocampal cultures.
We will expose organotypic hippocampal cultures to controlled biaxial stretch. We will quantify cellular loss and functional deficits after trauma and evaluate the neuroprotective and neuritogenic effects of genipin following injury.
Specific Aim 3: To test the therapeutic potential of genipin in vivo following cortical contusion
We will evaluate the neuroprotective and neuritogenic effects of genipin following induction of a cortical contusion using standard histological, immunohistochemical, and observational techniques.

2. Project successes.

The main objective of the research was to evaluate the potential of genipin as a therapeutic agent following TBI. This was motivated by a collection of different studies in the literature showing broad and varied effects of genipin in multiple systems as well as its use in traditional Eastern medicine. The research was a collaboration between Dr Shreiber and Dr. Barclay Morrison III from Columbia University Department of Biomedical Engineering.

Our primary focus was on genipin's potential use as a neuroprotective agent, possibly through anti-oxidant properties. We also aimed to use an organotypic hippocampus model to evaluate the effects because of the balance of physiological relevance and high throughput the model offers. A good portion of time was taken training new personnel in Dr. Morrison's lab, where the models was established, and personnel in Dr. Shreiber's lab, where the model was new.

To that end, we demonstrated:

A. A toxicity curve for genipin (Fig 1), showing that doses of 50uM and lower were safe. This was confirmed in both laboratories

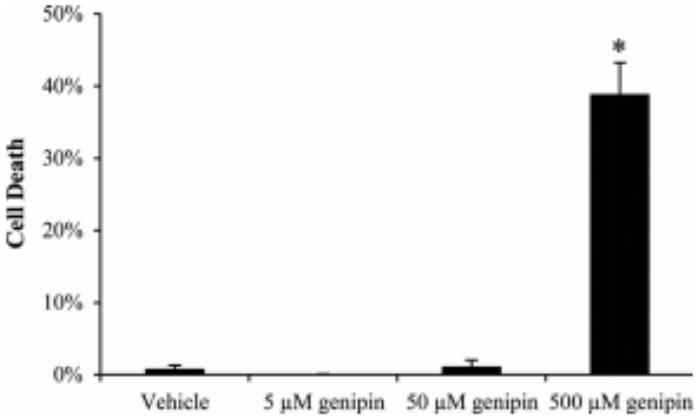

Fig 1: Toxicity curve for genipin in organotypic hippocampal slices

B. Genipin protects against glutamate excitotoxicity in a dose-reseponse manner. Slices were exposed to 10mM glutamate for 3hrs followed by treatment with genipin. The protection conferred by genipin was comparable to other known neuroprtoective compounds (Fig 2). Delaying addition of genipin by 5 hours (ie a therapeutic window) still offered neuroprotection, though not as much as MK-801 (Fig 3).

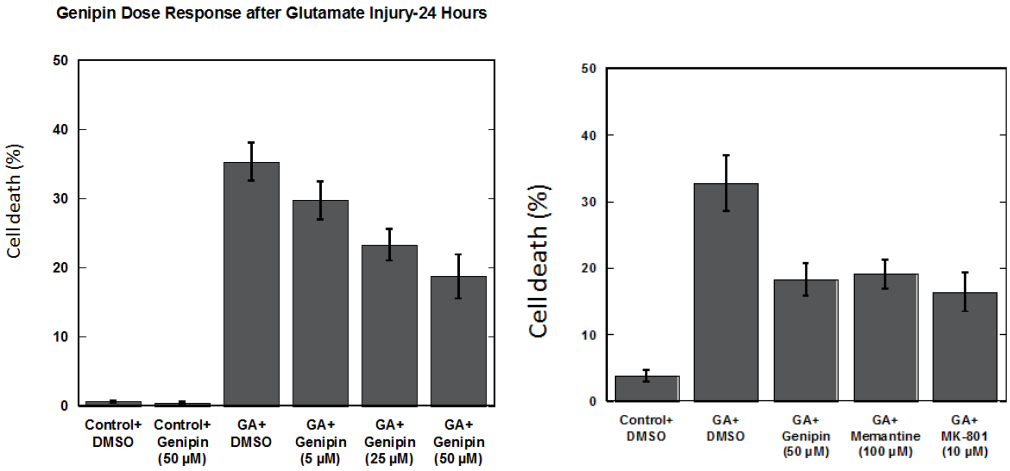

Fig 2: Left: Dose response neuroprotective effects of genipin following exposure to glutamate. Right: Genipin offered protection equivalent to known neuroprotective compounds MK-801 and memantine.

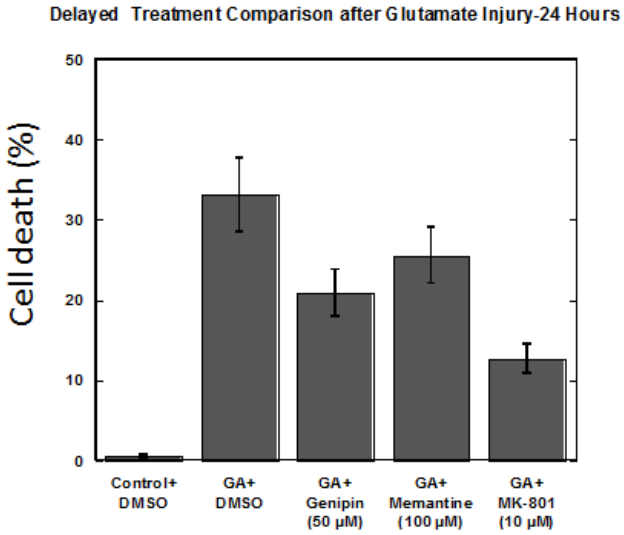

Fig 3: Delayed treatment with various neuroprotective compounds. Genipin offered significant neuroprotection even after a 5-hr delay after glutamate injury.

C. Genipin protects against individual receptor agonists. As an excitotoxic compound, glutamate is non-specific. No investigate the specifity of genipin protection, slices were treated with three known excitoxic compounds: AMPA, NMDA, and Kainate, which injure different cell layers of the hippocampus. Genipin protected against each of the compounds. (Fig 4)

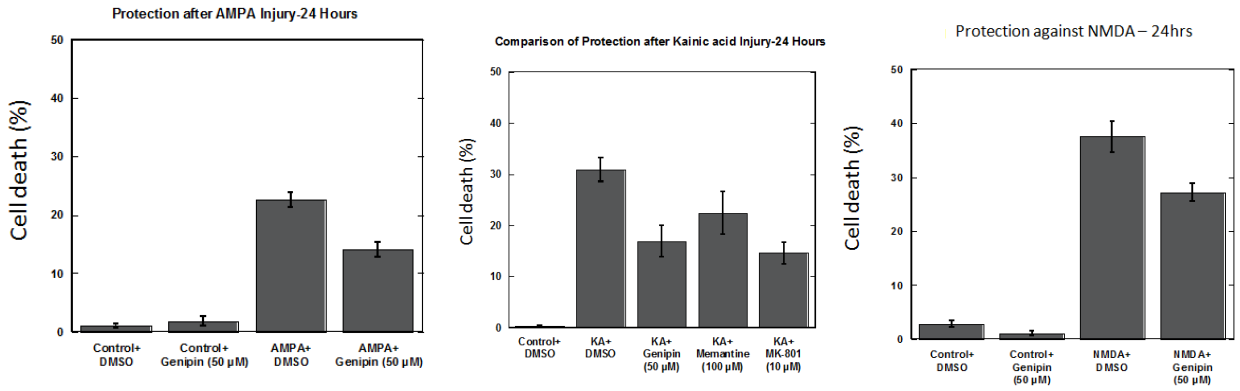

Fig 4: Genipin neuroroprotection against three different excitotoxic molecules: AMPA (left), Kainate (mid), and NMDA (right)

D. Genipin acts as a robust free radical scavenger. Slices were treated with the peroxide radical-generating molecule tBHP and treated with genipin or a known scavenger (ascorbic acid) (Fig 5). Treatment with genipin offered protection after as much as a 24 delay after injury with tBHP. A similar experiment was performed with rotenone, which generates superoxide free radicals by interrupting the mitochondrial electron chain

transport. For rotenone-induce injury, genipin offered protection with as much as a 6-hr delay in treatment (Fig 6). Finally, genipin protected against NO radical generation by S-Nitroso-N-acetylpenicillamine (SNAP), which is a direct NO donor. Genipin reduced nitrite formation, which coincided with significant neuroprotection (Fig 7).

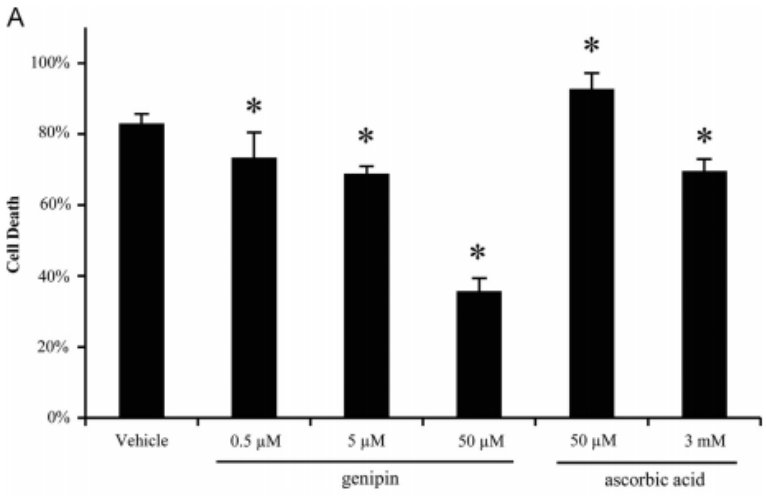

Fig 5: Genipin protects cells from tBHP radical-mediated death in a dose-response manner.

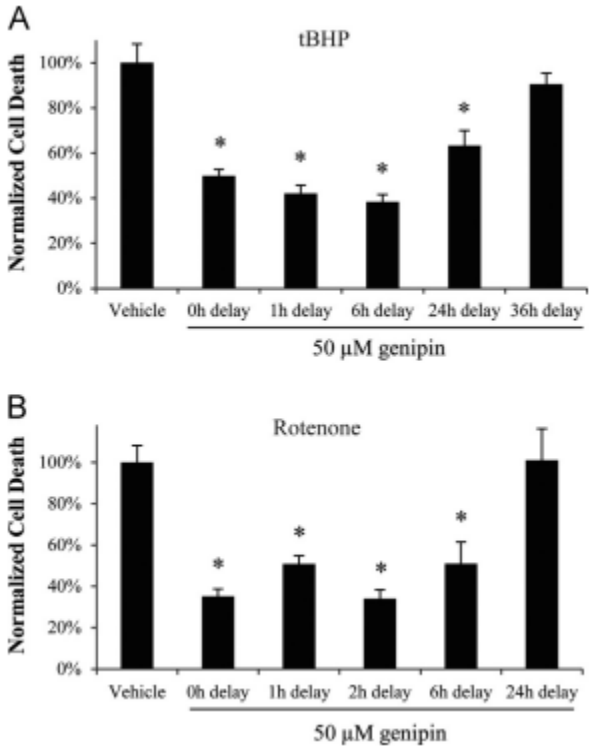

Fig 6: Delayed treatment with genipin protects against tBHP-generated peroxide radicals (A) and rotenone-induced superoxide radicals (B).

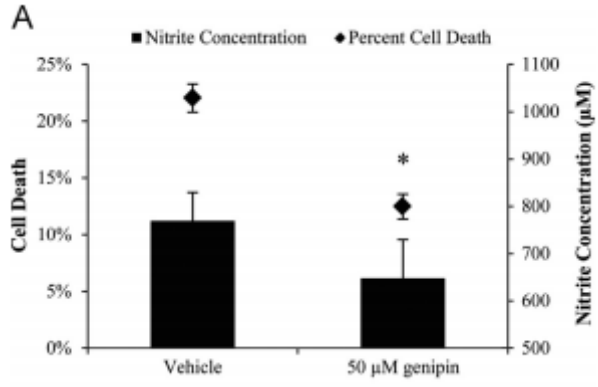

Fig 7: Cell Death and nitrite concentrations following SNAP injury and treatment with genipin. Genipin reduced the nitrite concentration and significantly protected against injury.

Summary: We successfully demonstrated neuroprotective effects of genipin in organotypic hippocampal models of secondary TBI. The protection was offered in a useful therapeutic window and protected against a broad array of insults. One mechanism for protection was free radical scavenging.

3. Project challenges

A. We are investigating genipin as a potential neuroprotective agent, but it also has been used to crosslink proteins, which induces fluorimetric changes in the tissue with a red/far red emission. Genipin-induced red fluorescence frequently confounded the data and often necessitated careful re-design of protocols where fluorescence was measured For example, the organotypic model established in Dr. Morrison's lab uses propidium iodide as n indicator of cell death. However, treatment with genipin induced fluorescence that overlapped with propidium iodide. Eventually, we switched to Sytox Green, which is another cell impermeant DNA-binding dye whose emission spectrum does not overlap with that from genipin-crosslinked proteins.

B. The organotypic model is difficult to master and even then can be difficult to maintain consistency. For example, despite training extensively with Dr. Morrison and replicating his laboratory's methods as closely as possible, cultures at Rutgers were not reliably viable, and the problem was ultimately traced to excessive evaporation. The humidity in the incubator was increased via an ultrasonic mister coupled to a timer for automatic misting. Evaporation was minimized, which improved the viability of the cultures. As another example, a change in medium formulation at Invitrogen caused confounding results in control conditions. The concentration of I-cycsteine was increased in Neurobasal medium, which caused cell death. Interestingly, genipin appears to protect against the toxicity induced by I-cycsteine. The toxicity of the neurobasal media caused problems interpreting data and prolonged the training period, since we thought that technique may have contributed to the cell death. However, as discussed

above, a retrospective consideration of that data strongly suggests that genipin was protective against stretch-induced trauma in combination with Neurobasal-induced excitoxicity. Eventually, we identified to source of the problems and switched to a different medium formulation.

C. The time required to build mosaic images of organotypic cultures was excessive at Rutgers. The slow acquisition was affecting data at the 4 hour time point, where substantial increases in cell death were observed in control conditions. and new macros were developed to improve the speed of image acquisition. Increasing the speed of acquisition – thereby limiting the time the cultures are out of the incubator –restored viability in control cultures to expected levels.

D. A significant investment in time is required to ensure objectivity data analysis. To dermine the extend (percentage) of cell death, images of the slices are taken in bright field and under fluorescence before injury and then at the desired time after injury. The regions of the hippocampus are traced by hand from the bright field "before" images and then superposed on the fluorescence "before" and "after" images. The images are then converted to binary images based on a threshold determined from the "before" epifluorescence images. Each of these steps involves a measure of subjectivity that only becomes consistent and "objective" after hundreds of hours of analysis. This and the challenge described in (C & D) demonstrate the steep learning curve associated with this model of injury.

4. Implications for future research and/or clinical treatment.

Genipin is a multi-potent compound that acts in a variety of tissue and cell systems with equally varied and unique outcomes. We have demonstrated it can be used as a neuroprotective agent, but it has also been used to dye leather, as a fingerprint reagent, as a crosslinker, as a therapy to improve glucose transport for type II diabetes, to name just a few applications. Discussions with Biotech professionals through our Office of Technology Commercialization and reviews from proposal submissions indicate that the promiscuity of genipin is a major impediment for its clinical use. A better approach, and one we hope to pursue, is to work with a medicinal chemist to identify if active motifs of genipin that confer specific properties can be identified, and then small molecule mimic that retain this motif generated.

5. Plans to continue this research, including applications submitted to other sources for ongoing support.

We are currently wrapping up a second manuscript for this research. We have submitted (unsuccessfully) to the NJ Commission on Spinal Cord Research and an R21 to the NIH. We have no immediate plans to continue to submit on the same subject, but are actively seeking the help of a medicinal chemist (see above).

In addition, resources from this award have trained our personnel in the organotypic model, and one student originally working and trained on this project used the organotypic model to evaluate the beneficial effects of encapsulated mesenchymal stem cells on TBI. This work actively continues, and this NJCBIR award was acknowledged in the relevant publication (see below).

6. List and include a copy of all publications emerging from this research, including those in preparation.

Publications:

Hughes, R.H., V.A. Silva, I. Ahmed, D.I. Shreiber, and B. Morrison, Neuroprotection by genipin against reactive oxygen and reactive nitrogen species-mediated injury in organotypic hippocampal slice cultures. Brain Research, 2014. 1543: p. 308-314. DOI: 10.1016/j.brainres.2013.11.020.

Stucky EC, Schloss RS, Yarmush ML, Shreiber DI. Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response. Cytotherapy. 2015 Jul 22. pii: S1465-3249(15)00910-X. doi: 10.1016/j.jcyt.2015.05.002.

Ahmed I, Stucky E1, Hughes RH, Morrison III BM, and Shreiber DI. Genipin protects against excitotoxic insults in organotypic hippocampal slice culture. In preparation for submission to Brain Research.

Oral Presentations:

Ahmed I, Stucky E1, Hughes R, Morrison III BM, and Shreiber DI (Speaker). Genipin Provides Neuroprotection Following Glutamate Exposure in Organotypic Hippocampal Slice Cultures, Annual Biomedical Engineering Symposium, Atlanta, GA, October 2012.

Poster Presentation:

Hughes RH (Speaker), Silva VA, Ahmed I, Shreiber DI, and Morrison III B. Neuroprotection by Genipin against Free-Radical Mediated Injury in Organotypic Hippocampal Slice Cultures (Poster). Neurotrauma Symposium, Phoenix, AZ, July 2012.

Shreiber DI (Speaker), Ahmed I, Hughes RH, Stucky E1, and Morrison III B. Genipin Protects against Glutamate-Mediated Excitotoxicity in Organotypic Hippocampal Slice Cultures (Poster). Neurotrauma Symposium, Phoenix,AZ July 2012.