Wake Forest School of Medicine
Department of Neurobiology and Anatomy

Blazeman Foundation for ALS

Progress Report: Administration of Hsp70 maintains muscle innervation in the SOD1 mouse- a new therapeutic approach?

Principal Investigator: Carol Milligan, Ph.D.
Institution: Wake Forest School of Medicine

March 17, 2016

We wish to thank the Blazeman Foundation and all ALS Warriors for continued support of our work. While this progress report focuses on our recombinant Hsp70 protein as a potential therapeutic for ALS, below we have included pictures of the members of our lab and the projects on which they focus their efforts. We wanted to take this opportunity to let everyone who supports the Blazeman Foundation know that our lab is dedicated to furthering our understanding of ALS with the ultimate goal of contributing to an effective therapy. Additionally, you will see that many in our lab are students ranging from high school to graduate school. We are equally dedicated to training future scientists to continue working to understand motoneuron development, physiology, pathology that is the foundation for developing cures for ALS.

In the photo on left (from left to right): Jane Strupe, Carol Milligan, Rachel Gillespie, Phonepasong (Pop) Arounleut, Leea Richardson, Miles Lyon, Marlena Wosiski- Kuhn.
In phone on right, Liz Forbes and Sophia Pauca (seated).

The Miligan Lab members:
Jane - part time lab technician who assures the lab is maintained and compliant to function on a daily basis.
Rachel - MS student who is looking at potential role of inflammatory cytokines in neuromuscular junction (NMJ) denervation in the SOD1 ALS mouse model.
Pop- Senior Lab Tech who is looking at the role of muscle in regulating motoneuron susceptibility to NMJ denervation and dysfunction in ALS. Pop also maintains the SOD1 mouse colony.
Leea - MS student looking at role of distinct microglia activation in ALS pathology.
Miles - MS student working on the Hsp70 project.
Marlena - MD, PhD student working on IL-6 and IL-6 receptor expression in patients as biomarkers for ALS and disease progression.
Sophia - high school student learning how to process tissue.
Liz - Research Associate. Liz is working primarily on the Hsp Project, but she helps with the projects all students are working on.
Theresa Urquhart- college senior at WFU who has worked in the lab over the past year.

Project Summary:
Researchers at Wake Forest School of Medicine have found a new treatment that may delay the onset of symptoms and increase the lifespan for those who are afflicted with ALS, or Lou Gehrig's disease. The researchers have determined that injections of a particular type of protein called heat shock protein (Hsp) 70 may benefit ALS suffers. The study was conducted in the mutant SOD1 mouse model of ALS. When the mice were given treatments of Hsp70, their survival was increased. This study focused on protecting the motor neurons in the mice that had ALS. The injected protein was not detected in the nervous system of the mice. Rather, this treatment appeared to work where the neurons and muscles contact each other. When the neurons and muscles loose contact, muscle weakness occurs, the prominent symptom of ALS. In a second study, the group found that the contact between neurons and muscle was maintained much longer in treated mice as compared to untreated mice. While these studies have shown positive signs, researchers caution that many more studies are needed before they can begin to conduct clinical trials in people. The group has a smaller fragment of the protein that also shown positive effects and is currently being more intensely tested. The group is determining the best way to efficiently produce the proteins to assure they will function properly. This is the first step to develop the protocols for protein production and develop GMP large-scale production strategies that will facilitate IND application and clinical trials. With support from the Blazeman Foundation, Wake Forrest School of Medicine is the only medical center that is engaged in researching this potential ALS treatment.

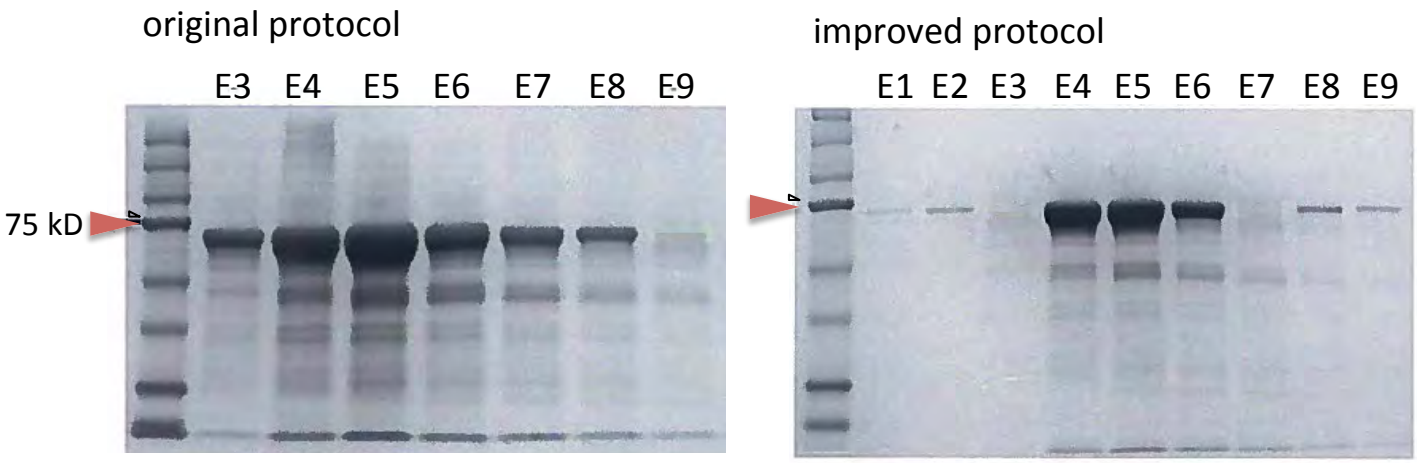
Progress:

Purification of Protein in Native Conditions

Summary*: We have established a SOP that results in increased yield, low endotoxin, native and functional protein.*

An established standard operating procedure (SOP) for the purification of recombinant human heat shock protein 70 (rhHsc70) was previously completed in this lab in both denaturing and native conditions; however, we aimed to refine these SOP's to allow for easier replication and increase in product. We chose to continue the shift toward greener chemistry and refine the SOP to purify an injectable rhHsc70 in native conditions. In creating this protocol, we aimed to achieve the following: (1) A consistent elution profile, (2) an endotoxin free product, & (3) an increased yield and purity from the previous procedure. In order to achieve all of these results we needed to start by creating a sterile environment for production and establish secondary protocols to maintain sterility and decontaminate products if necessary. We then re-tuned our buffer system to maintain a more specific activity during nickel-affinity column purification.

We have been able, to produce a consistent elution profile that allows us to selectively gather the purified product, even when changes were made to the experimental protocol to achieve reduced endotoxin levels and greater purity. We are still fine tuning the SOP to achieve a final product that is consistently endotoxin negative at a value of 0.06 EU/mL, the point of sterility at which a product can be directly injected into the central nervous system. We are closing in on this goal rapidly and aim to have the first batch of injectable protein ready to continue our dose-response studies within the month. While we are not conducting experiments where direct injection to the CNS is necessary, we want to produce a product that is clean enough for any application going forward in experimentation and treatment. With respect to yield, we have achieved a concentration greater than previously achieved using similar conditions in the lab, however we want to continue working on conditions that optimize the amount of protein we produce per batch. Once we achieve the desired endotoxin level, we will send a sample of our protein for mass spectroscopy analysis to determine the final purity.

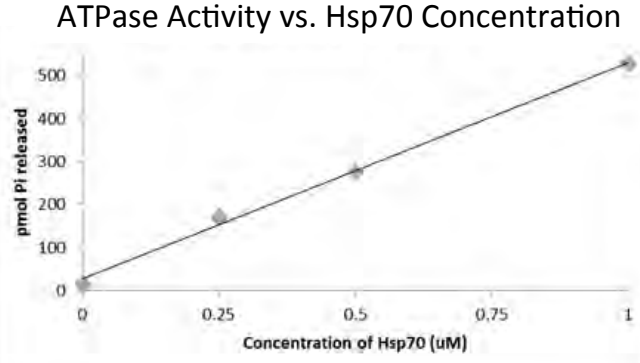


Shown are two PAGE gels with eluted fragments from Nickel affinity column purifications from the stadard SOP we originally developed (left). The gel on the right shows the same eluted fragments following modifications to the protocol have resulted in protein being eluted over a tighter range (large, dark band slightly below the 75 kD marker in fractions 4-6 as opposed to 3-8). These modifications have also resulted in fewer, smaller proteolytic fragments (bands below the 70 kD band). In subsequent steps of the protocol the lower fragments are easily removed. With these modifications, we have accomplished a more pure preparation with increased yield- a major goal of this project

Analysis of Protein Activity

Summary: *We can now routinely measure activity of the recombinant protein and treatments are now expressed as units activity.*

Small batch purification of any product varies from large scale production at a corporation, and some of that variability results, in this case, in fluctuations in protein activity, which is why we are working to establish a continually replicable SOP. We wanted to determine how much variation exists between the recombinant protein we produced in our lab previously and in commercially available protein from established companies. We spent the time to optimize assay conditions and have determined that the activity level of our protein is comparable to commercially available products. Activity assay results suggest that the commercially available Hsp700 is significantly less pure than our preparations. We are currently confirming this. Understanding the activity level of the purified protein in comparison to that of other companies was required to establish the correct dosing for treatment of our animal models.



We have established the ATPase activity assay and to produces consistent and reproducible results. Activity Assay results indicate a linear proportion of ATPase activity with increasing recombinant protein concentrations.

RhHsc70 Injections in Experimental Animals

Summary: *We are performing dose response experiments to determine optimal dose of recombinant protein to delay NMJ denervation.*

We have injected a small cohort of 8 animals with previously purified rhHsc70, dissected out the muscles of interest, and are in the process of examining the effects of the protein on the denervation of the

neuromuscular junction. The preliminary results that we have seen provide a baseline concentration, based on calculated protein activity level, that we will use to create a dose-response curve in treated SOD1 animals. Additional cohorts of animals are currently being treated at different doses.

Determining the Localization of rhHsc70 in vitro

Because Hsp70 proteins are highly conserved across species and as result, we do not have an antibody that specifically recognizes our recombinant protein as opposed to the mouse endogenous protein. Using a commercially available antibody to the His-Tag of our protein, we are currently processing muscle from treated SOD1 mice to determine the localization of protein at either the muscle or the presynaptic terminal of the NMJ. We will also determine if we can use the His-tag antibody to biochemically isolate protein from treated animals. This will be a critical first step for experiments to investigate how the recombinant protein is delaying NMJ denervation.

Substrate Binding Domain Purification

Following the optimization of the SOP for the production of full-length protein, we will begin adapting the purification for the substrate-binding domain (SBD) and the ATPase domain of the protein. We have previously generated primed stocks for the SBD and will begin the purification process once we complete th dose response studies currently underway.