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14. ABSTRACT Iron accumulation and deposition have been reported in patients with amyotrophic lateral sclerosis (ALS). Previous work in mutant SOD1 mice mouse models of ALS have indicated that iron chelation with a chemical agent extends lifespan. Therefore, we propose the use of apo-ferritin, the iron-storage protein ferritin that is iron poor, as a natural ionophore to sequester excess iron and redistribute it. The overall hypothesis is that infusion of apo-ferritin protein into the brain will provide neuroprotection by limiting the availability of excess iron to catalyze free-radical production. The most significant finding from the first year of funding is that infusion of artificial cerebrospinal fluid containing nutrients, including H-ferritin, increases lifespan and delays onset of disease in SOD1G93A mice. Furthermore, the number of motor neurons in the lumbar spinal cord at endpoint is greater in mice that received infusion versus those that did not. The potential clinical significance of this work is that increasing turn-over of cerebrospinal fluid should be further explored as a therapeutic option in ALS.					
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INTRODUCTION

Iron misregulation and oxidative stress have been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) (1) and are consistent features of the disease in humans (2, 3) and in animal models (4). Therefore, strategies to limit oxidative stress by iron sequestration are potentially effective methods to delay or attenuate ALS symptomology. In this project, our conceptual framework is that infusion of the iron storage protein, ferritin, into the murine brain is a means to provide neuroprotection by limiting the availability of excess iron to catalyze free-radical production. Overexpression of ferritin has been reported to be effective in *in vivo* (5) and *in vitro* (6) systems. The **purpose** of this work is to address the **hypothesis** that apo-ferritin (ferritin that is iron poor) is a viable therapy to attenuate symptom progression and prolong survival. The **scope** of the research will involve the use of two animal models. The first model is the superoxide dismutase 1 (SOD1) transgenic mouse (7), a model that is widely utilized in ALS research. The other model examines the contribution of SOD1 and an allelic variant of the *HFE* gene; this gene variant causes intracellular iron accumulation, and the variant is present in at least 1/3 of ALS patients. We are investigating the effect of intracranial infusion of apo-ferritin with ALS mouse models to study the effects of removal and redistribution of iron from the site of tissue damage. Our primary outcomes are examination of symptom onset and survival in the animals; secondary outcomes are biochemical and histological assessment of our intervention.

BODY

Project progress on each of the tasks is outlined per the approved Statement of Work. Task 1. Intracerebroventricular infusion of apo-ferritin into SOD1^{G93A} mice. Total anticipated duration of 12 months.

1a. Obtain regulatory review and approval. Purchase mice and supplies. Perform surgery at 70 days of age to implant cannula and osmotic pump into mice.

Progress: The Institutional Animal Care and Use Committee of the Pennsylvania State University approved the animal experiments outlined in the grant application on March 22, 2012. USAMRMC Animal Care and Use Review Office (ACURO) approval was granted on June 15, 2011.

When funds were released, efforts to generate apo-H-ferritin at a concentration of 2.0 mg/ml began. The laboratory routinely uses bacterial culture to generate ferritin protein, and the particular bacterial construct that is used in our laboratory contains a poly-histidine tag on the terminal end of the protein to aid in affinity purification using a nickel column. Although there were no technical problems in expressing or purifying the his-tagged H-ferritin, we experienced great difficulty in concentrating the ferritin to 2.0 mg/ml; the dose we wished to deliver. Instead of concentrating the protein in a small volume, the protein oligomerized and formed an insoluble precipitate. After weeks of troubleshooting using various concentrating devices (spin-column concentrator devices and stirred cell), we came to the conclusion that due to the size of the H-ferritin protein and the propensity of the monomers to oligomerize, steric hindrance from the histidine tag was preventing concentration of the protein to the desired concentration. We were left with two options: remove the histidine tag from the expressed protein after purification or use a new plasmid in which H-ferritin is not tagged with histidine and use a different purification method.

Due to the amount of ferritin protein that is required to perform the experiments, the more cost-effective option is to use the H-ferritin plasmid that does not contain a histidine tag. We have found that the untagged H-ferritin protein that we produce is able to be concentrated greater than 20 mg/ml (ten-fold greater than what our experiments require) and that this protein remains soluble at this concentration. Although troubleshooting ferritin production did delay our proposed timeframe of completion, it was necessary that we be able to generate highly concentrated ferritin for our experiments.

In January of 2012, the first group of SOD1^{G93A} mice were purchased from Jackson Laboratories. Animals were obtained in small batches rather than the entire approved amount so that we could generate proof-of-concept data. To our knowledge, this is the first time that H-ferritin has been infused into the brain of the SOD1^{G93A} mice and there are no pre-existing toxicity data.

Upon arrival, mice underwent training sessions on the rotarod apparatus prior to cannula and Alzet mini-osmotic pump surgeries at 70 days, which is historically a minimum of 30 days prior to disease onset. There was no decrease in locomotor ability due to the presence of the osmotic pump and neither the presence of H-ferritin or artificial cerebrospinal fluid (CSF) in the pump had any adverse effect on the animals.

We initially had a high mortality rate due to complications from the anesthesia regimen that we had been using; animals required deep sedation for surgery and were unable to recover from the anesthesia. After consultation with the veterinary staff, it was suggested that we alter our anesthesia formulation to include acepromazine, a compound that allowed us to decrease the amount of xylazine and ketamine by approximately 10% of what we had been using. The reformulation of the anesthesia allowed for appropriate anesthetization of the animals for surgery and a much more rapid recovery, which dramatically increased survival rates from surgery. We are amending our IACUC protocol to include this change in anesthesia. Once the challenges with the surgical procedures and ferritin production were overcome, we moved forward quickly and have evaluated a total of 25 animals in the first year of funding. We will complete the number of animals proposed for study in Task 1 by December 2012.

1b. Perform behavioral analysis of mice during disease phase. Mice that reach the pre-defined endpoint will be sacrificed during this time.

Progress: The behavioral measure of motor coordination was assessed via use of the rotarod apparatus. Latency to first fall is assessed using rotarod data collected from each individual mouse. When a mouse is not able to stay on the rotating rod for greater than one Standard Error of the Mean of its ability to stay on the rod during the pre-symptomatic phase, disease onset has occurred (8).

The median disease onset was not statistically significantly different (median values 114 days versus 112.5 days, respectively) in the group of animals treated with 2.0 mg/ml H-ferritin as compared to the No Surgery control group (Figure 1). It was noted that infusion with artificial CSF, which was the control group for the ferritin infusion group, had a tendency to postpone onset as compared to the No Surgery group (median value 119.5 days vs. 112.5 days). The combined data suggest that our infusion paradigm is not toxic to the animals and the concept of infusing a trophic or “cleansing” solution may provide a modest delay in disease onset. We will to continue to evaluate this

concept going forward and have provided some novel insights into this concept later in this report.

In terms of survival, endpoint is defined by the inability of the mouse to right itself within 30 seconds of being placed on its side or the inability to maintain basic grooming and feeding behavior (9, 10). The data in Figure 2 demonstrate that infusion with CSF or 2.0 mg/ml H-ferritin extends survival by approximately 5% as compared with control.

The result of infusion with 2.0 mg/ml H-ferritin was not sufficiently robust; therefore, we doubled the concentration of H-ferritin in another group of mice, as proposed as an alternative strategy in our grant application. The initial concentration of H-ferritin that was used for infusion was selected based on *in vitro* work (11), and 2.0 mg/ml of ferritin was the lowest dose reported to be effective. Therefore, a second set of SOD1^{G93A} mice were purchased in April of 2012 and were treated with H-ferritin at a concentration of 4.0 mg/ml. The data in Figure 3 indicate that treatment with CSF or 4.0 mg/ml H-ferritin did not significantly delay median disease onset as compared to the No Surgery control group. Similarly, there was no extension of lifespan due to infusion of H-ferritin 4.0 mg/ml (Figure 4).

One of the challenges in interpreting our results is the low number of animals in our groups; the aforementioned problems with anesthesia caused us to lose nine animals and another animal developed an unrelated health condition and was euthanized prior to behavioral endpoint. We will continue to add animals to these groups in the coming year. We have also evaluated the data by combining what was obtained from infusion of H-ferritin of both 2.0 mg/ml and 4.0 mg/ml concentrations. Subsequently, the data from the No Surgery and CSF infusion groups were pooled from the two sets of studies. The compilation of the data resulted in larger groups of eight or nine animals per condition and is shown in Figure 5. In this figure, median survival for the No Surgery Group is 112.5 days, 121 days for the CSF group, and 114 days for the H-ferritin group. As before, lifespan was increased by infusion with H-ferritin or CSF as compared to control by 4 or 6 days, respectively (Figure 6).

An interesting finding throughout our studies was that infusion of a trophic solution into the CSF is associated with delay in disease onset and prolonged life. As shown in Figure 5, infusion with artificial CSF increased median survival by 7.5% as compared to the No Surgery group, and the data in Figure 6 demonstrates that lifespan is increased by 5% as compared to the No Surgery group. Comparison of animals that received no surgery to animals that received CSF infusions with or without H-ferritin shows a significant extension of lifespan and a clear trend of increased survival (Figures 7 and 8).

Given that the only approved drug for ALS increases life-span by just 10%, our infusion studies may have clinical relevance. The concept that a general addition of fluid to the ventricles as a potential therapy to treat neurodegenerative disease has previously been purported (12), but not for ALS. There have been studies demonstrating that CSF from ALS patients causes motor neuron loss *in vitro* (13, 14) and that CSF obtained from ALS patients contains factors that promote cell death. These data suggest a buildup of toxic factors in the CSF in ALS. Moreover, protein content increases in CSF with age (15), most likely due to a decrease in CSF production (16). Therefore, a provocative outcome of our findings is to suggest that increasing CSF flow in the central nervous

system could be a therapy for ALS. This could be accomplished by increasing CSF turnover clinically by infusing trophic solutions through intrathecal pumps into the lumbar region similar to the current use of morphine pumps used clinically for pain.

The turn-over rate of CSF has been one of the challenges associated with using murine models of the disease, as the rates differ drastically between humans and mice. In humans, total CSF volume is 125-150 ml and turns over 3-4 times a day. In mice, the total CSF volume is approximately 35 μ l and the turnover rate is 18 μ l per hour. In two hours, CSF is completely overturned in the mouse, which is approximately three times faster than in humans. Although our paradigm utilizes a constant infusion of ferritin at a rate of 0.25 μ l per hour, it is possible that this rate is insufficient to maintain a high level of ferritin in the CSF given the high turnover rate. We are currently exploring ways to increase the amount of ferritin in the CSF, either by increasing the concentration of the ferritin in the pump or by using a different Alzet mini-osmotic pump with a higher flow rate. However, going forward we will have the improvement of the animals as a result of infusion therapy as a primary focus. We will be seeking funding in the future to evaluate combination therapy of Riluzole and infusion.

In order to understand why our H-ferritin infusion data were not as effective as treatment with a chemical iron chelator (4), a number of factors need to be taken into account, such as mouse strain, method of treatment, and gene copy number. Jeong et al. (2009) treated SOD1^{G37R} mice with a chemical chelator through intraperitoneal injection; our experiments utilize SOD1^{G93A} mice that receive direct infusion of H-ferritin into the lateral ventricle. The course of disease progression varies greatly among mutant SOD1 mouse strains, with onset in SOD1^{G37R} mice occurring at 4-6 months of age with slow progression over the course of 4-6 weeks (17); Jeong et al. reported survival in vehicle-treated SOD1^{G37R} mice ranging from 45-55 weeks. Based on our experience and elsewhere, onset in SOD1^{G93A} mice occurs at 3-4 months of age and progression lasts approximately 3 weeks (7). Gene copy number can vary from animal to animal within mutant SOD1 mouse strains, particularly within the SOD1^{G93A} mouse line (18). Consequently, gene copy number will need to be analyzed in our mice and, when paired with treatment and behavioral data, is expected to give insight to evaluate the efficacy of our infusion paradigm.

1c. Biochemical analysis of mouse tissue. Timeframe: months 9-12.

Progress The challenges associated with producing ferritin at the necessary concentration caused a delay in our original timeframe; therefore, the biochemical analyses are ongoing.

Immunoblotting was performed on lumbar spinal cord homogenates to examine changes in mitochondrial ferritin and L-ferritin. We examined tissue from animals at endpoint that did not receive surgery, animals that received infusion of CSF, and animals infused with H-ferritin 2.0 mg/ml. Because of the number of animals that died due to complications from anesthesia, we included these animals to generate a baseline group at 70 days of age. Mitochondrial ferritin levels were unchanged due to treatment or age (Figure 9).

L-ferritin levels were significantly lower in the 70-day-old mice as compared to the CSF-treated group, but not as compared to the H-ferritin-infused group (Figure 10). In this group of animals used for biochemical analyses, it may be worthwhile to note that

the average age of a CSF-infused mouse was 142 days, while the average age of the No Surgery group was 127 days, and 119 days for the H-ferritin 2.0 mg/ml group. At this point we are unable to determine if L-ferritin levels increase with age, but we will continue to add animals for this evaluation.

1d. Histological analysis of mouse tissue. Timeframe: months 9-12.

Progress Similar to our biochemical analyses of mouse tissue, the histological analyses are ongoing due to the delay in surgical start date from our initial problem in concentrating ferritin.

We have performed immunostaining using SMI-32 (Neurofilament H Non-Phosphorylated) antibody, which detects motor neurons in the spinal cord and have counted the number of neurons that survive at endpoint. The data presented in Figure 11 demonstrate that the number of surviving neurons at endpoint is elevated in CSF and H-ferritin 2.0 mg/ml groups as compared to the no surgery group. In Figure 12, the data suggest that infusion increases the number of surviving neurons as compared to no surgery, $p = 0.08$.

We are continuing to add animals to these groups to more accurately assess the histological impact of infusion.

Task 2. Intracerebroventricular infusion apo-ferritin into $SOD1^{G93A}/HFE^{wt/H67D}$ mice. Total anticipated duration is 12 months with portions of work concurrent with Tasks 1 and 3. Chronologically, work will occur during months 6-17.

2a. Purchase $SOD1^{G93A}$ mice and crossbreed with existing homozygous $HFE^{H67D/H67D}$ mice to generate experimental animals.

Progress The mice that were purchased in April 2012 for Task 1 were used as breeders to the homozygous $HFE^{H67D/H67D}$ mice that exist in our laboratory. We elected to use male and female offspring as experimental animals to determine if one gender responds better to H-ferritin infusion than the other.

2b. Allow animals to age to 70 days, at which time surgery to implant cannula and osmotic pump will be performed. It will take approximately two months for mice to reach this age and another 1 month to perform surgeries.

Progress We have performed surgeries on 12 animals using H-ferritin at a concentration of 2.0 mg/ml, as described for Task 1. If 2.0 mg/ml H-ferritin is ineffective, we plan to increase the concentration of H-ferritin to 4.0 mg/ml in another set of $SOD1^{G93A}/HFE^{wt/H67D}$ double transgenic mice.

2c. Perform behavioral analysis of mice during disease phase. Mice that reach the pre-defined endpoint will be sacrificed during this time. Timeframe: months 10-13.

Progress Behavioral data are currently being collected on 18 animals and disease onset data are not yet available.

Task 3. Intracerebroventricular infusion liposome-encapsulated apo-ferritin into $SOD1^{G93A}$ mice. Total anticipated duration is 12 months with portions of work concurrent with Task 2. Chronologically, work will occur during months 13-24.

Progress We are currently producing additional amounts of ferritin for the work described in Task 3.

Task 4. Communication of scientific findings via presentation and/or manuscript. We anticipate 2-3 presentations and/or manuscripts as a result of this research. Preparation will occur concurrent with the other tasks, most likely at the end of each task, e.g. months 12, 17 and 24.

Progress The data we have collected thus far are incomplete and publication at this point would be premature.

KEY RESEARCH ACCOMPLISHMENTS

- Intracerebroventricular infusion of artificial CSF or H-ferritin delays onset of ALS symptomology in SOD1^{G93A} mice.
- Intracerebroventricular infusion of artificial CSF or H-ferritin increases lifespan in SOD1^{G93A} mice.
- L-ferritin levels are increased in the CSF-infused SOD1^{G93A} mice at endpoint as compared to 70-day-old mice. This may reflect microglial activation.
- Motor neuron survival is increased in animals receiving a CSF infusion compared to untreated controls.

REPORTABLE OUTCOMES

None within the first year of funding.

CONCLUSION

To date, our results suggest that intracerebroventricular infusion of artificial CSF with or without H-ferritin into SOD1^{G93A} mice may be of therapeutic potential in ALS. Infusion of artificial CSF delays onset of symptomology and increases lifespan. A parsimonious explanation for our results is that increased flow of CSF (and subsequent dilution/removal of cytotoxic factors) out of the central nervous system is responsible for this benefit in our model. However, the artificial CSF contains essential nutrients such as glucose and salts. This idea could be directly tested by infusing phosphate buffered saline and performing the same behavioral analyses. This additional set of analyses cannot be performed in this application because of budget limitations, but we will pursue additional funds for this important evaluation. The potential outcome is important for the clinical implementation of our findings.

In terms of impact to humans struggling with ALS, increased flow of CSF could be used at the earliest stage of onset. The surgical implantation of a pump that would deliver a trophic solution into the lumbar spinal space would be similar in approach to that currently used with morphine pumps to treat chronic pain or baclofen for treatment of spastic muscular disease. Although still in its infancy, our study has presented an exciting unexplored, but easily clinically implemented, method to improve outcome in ALS.

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APPENDICES

None

SUPPORTING DATA

Figure 1

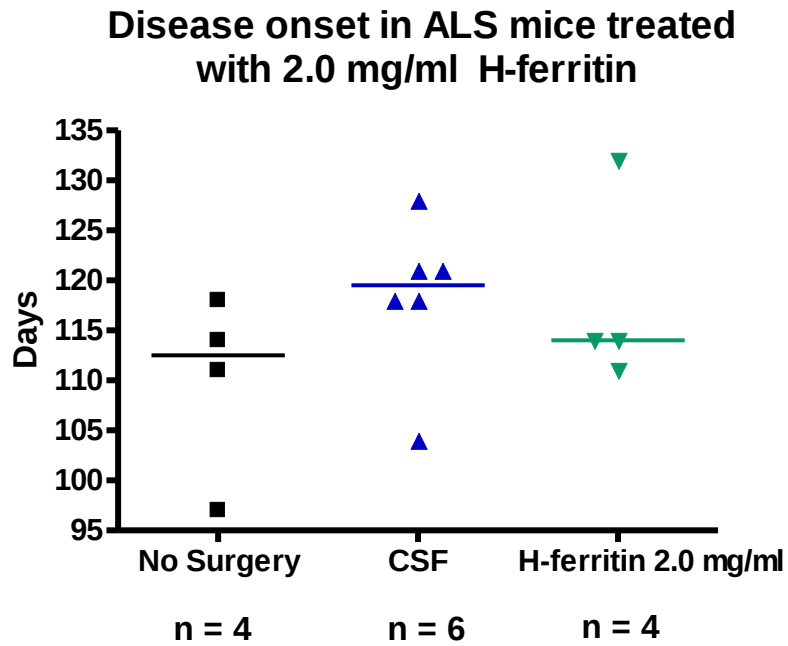


Figure 1. Disease onset as assessed by rotarod performance in SOD1^{G93A} mice. Onset was earliest in the No Surgery group as well as first individual animal to fail rotarod test (data not shown). Median values are displayed by horizontal lines. Infusion with H-ferritin or CSF is not toxic to the mice and modestly increases latency to disease onset.

Figure 2

Survival in mice treated with 2.0 mg/ml H-ferritin

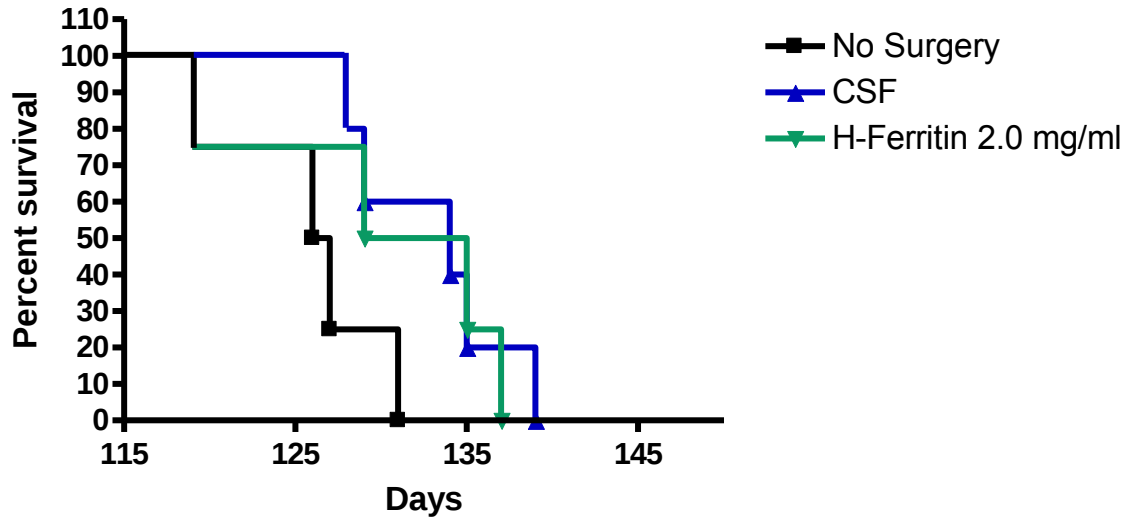


Figure 2. Survival is extended in $SOD1^{G93A}$ mice that received infusion of 2.0 mg/ml H-ferritin. Fifty percent survival rates are 126 days, 131 days, and 129 days in the No Surgery, CSF, and H-Ferritin 2.0 mg/ml groups, respectively. Treatment with H-ferritin increased survival by 5% as compared to the No Surgery control group. CSF infusion increased survival by 6% as compared to the No Surgery group.

Figure 3

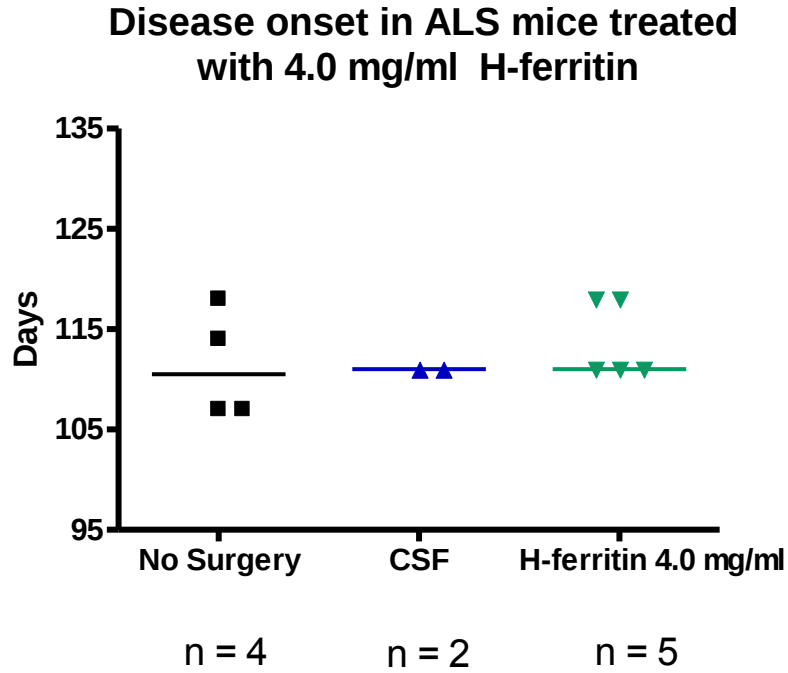


Figure 3. Disease onset in SOD1^{G93A} mice treated with H-ferritin at a concentration of 4.0 mg/ml. Neither treatment with CSF nor 4.0 mg/ml H-ferritin had an appreciable difference in delaying onset; median value for the No Surgery group is 110.5 days and 111 days in the CSF and H-ferritin 4.0 mg/ml groups. Median values are depicted by the horizontal lines.

Figure 4

Survival in mice treated with H-ferritin 4.0 mg/ml

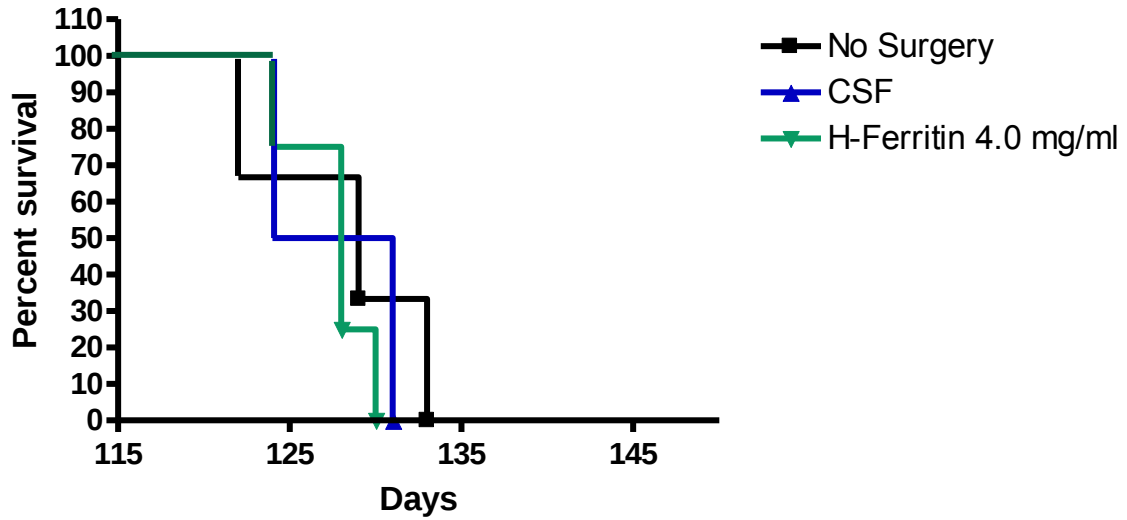


Figure 4. Survival data in $SOD1^{G93A}$ mice treated with 4.0 mg/ml H-ferritin. Survival was not extended by treatment with 4.0 mg/ml H-ferritin. The 50% survival values were 125.5 days in the No Surgery group, 124 days in the CSF group, and 126 days in the H-ferritin 4.0 mg/ml group.

Figure 5

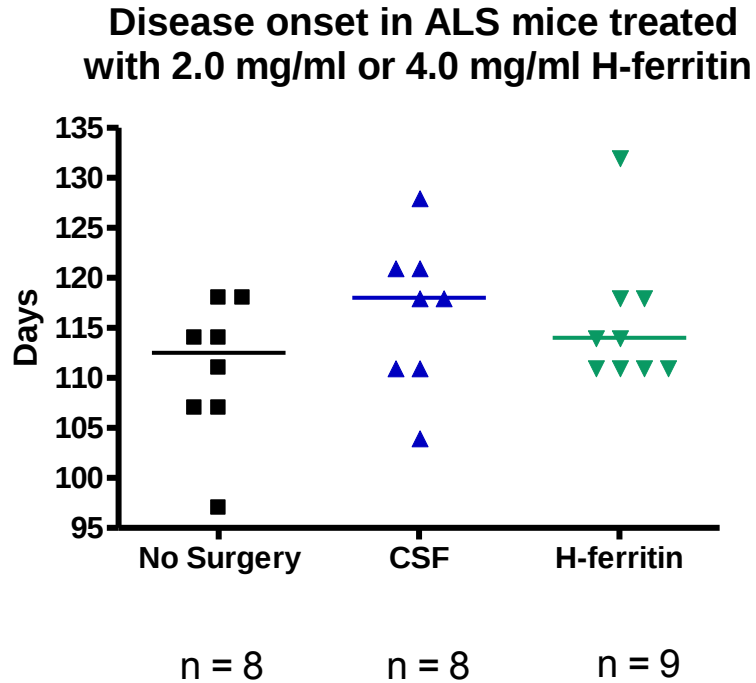


Figure 5. Disease onset in SOD1^{G93A} mice treated with H-ferritin. Data from mice treated with 2.0 mg/ml H-ferritin and 4.0 mg/ml were combined to create the H-ferritin group depicted in this graph. The horizontal lines represent median values. Median onset in the No Surgery group was 112.5 days, 121 days in the CSF group, and 114 days in the H-ferritin group. The data suggest that H-ferritin modestly postpones disease onset as compared to the No Surgery group.

Figure 6

Survival of Mice Infused with 2.0 mg/ml or 4.0 mg/ml H-ferritin

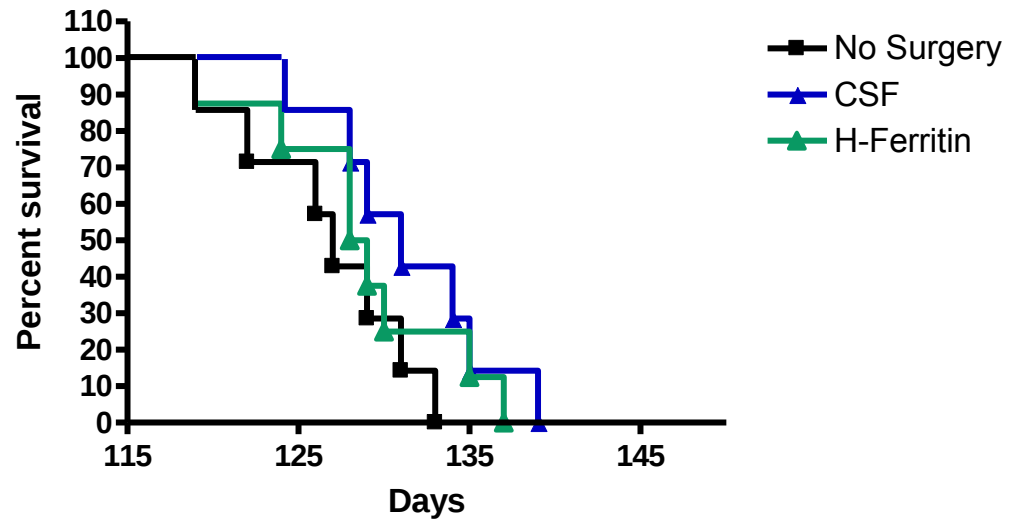


Figure 6. Infusion with H-ferritin increases lifespan as compared to SOD1^{G93A} mice that did not receive an infusion. Values for 50% survival in the No Surgery, CSF, and H-ferritin groups were 126.5 days, 130 days, and 128 days, respectively. The data suggest that infusion with CSF or H-ferritin prolongs survival in SOD^{G93A} mice.

Figure 7

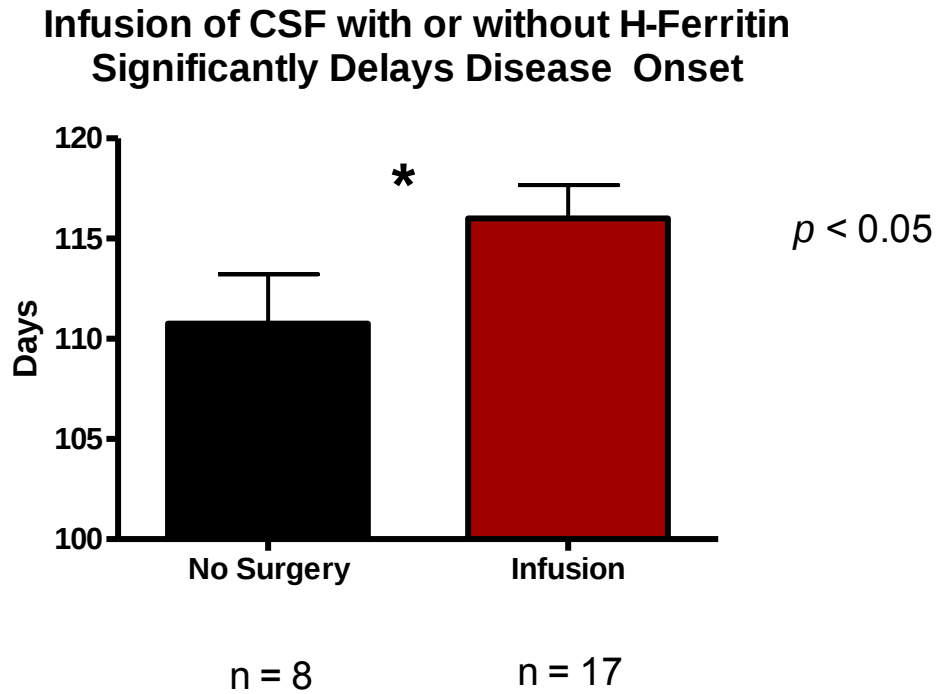


Figure 7. Infusion of CSF or H-ferritin delays disease onset in SOD1^{G93A} mice. Data collected from animals infused with CSF, H-ferritin 2.0 mg/ml, and H-ferritin 4.0 mg/ml were combined to generate an overall Infusion group. Average disease onset in the control (No Surgery) group was 110.8 days while the Infusion group's average onset was 116 days. This was a significant difference, as assessed by t-test ($p < 0.05$).

Figure 8

Survival Rate is Increased when Animals Receive Infusion with CSF or H-ferritin

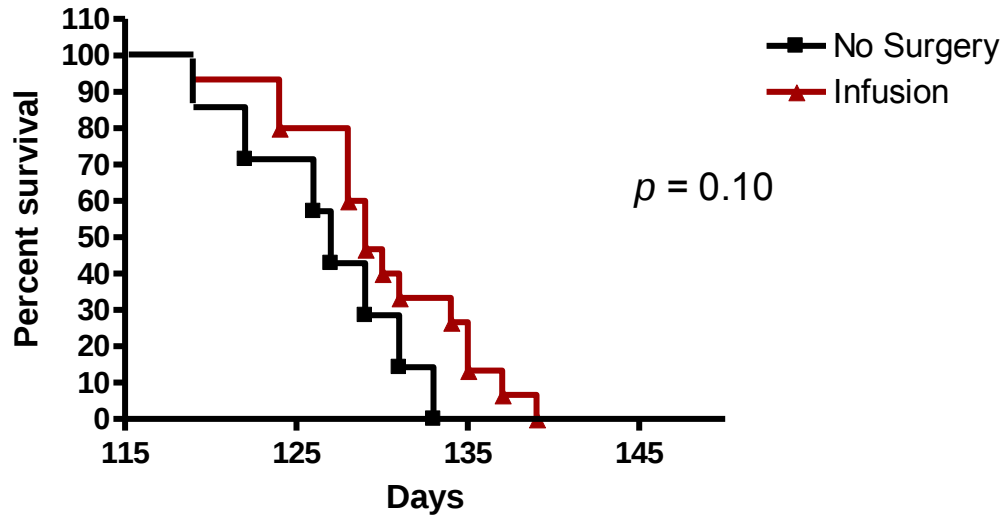


Figure 8. Infusion of CSF or H-ferritin increases survival rate in SOD1^{G93A} mice. Data collected from animals infused with CSF, H-ferritin 2.0 mg/ml, and H-ferritin 4.0 mg/ml was combined to generate the Infusion group. Times for 50% survival were increased in the Infusion group as compared to No Surgery (128.5 days to 126.5 days, respectively). Survival rate in the Infusion group ranged from 119-139 days as compared to 119-133 days in the No Surgery group. Extension of survival is trending towards significance, $p = 0.10$.

Figure 9

Mitochondrial Ferritin in Lumbar Spinal Cord Homogenates

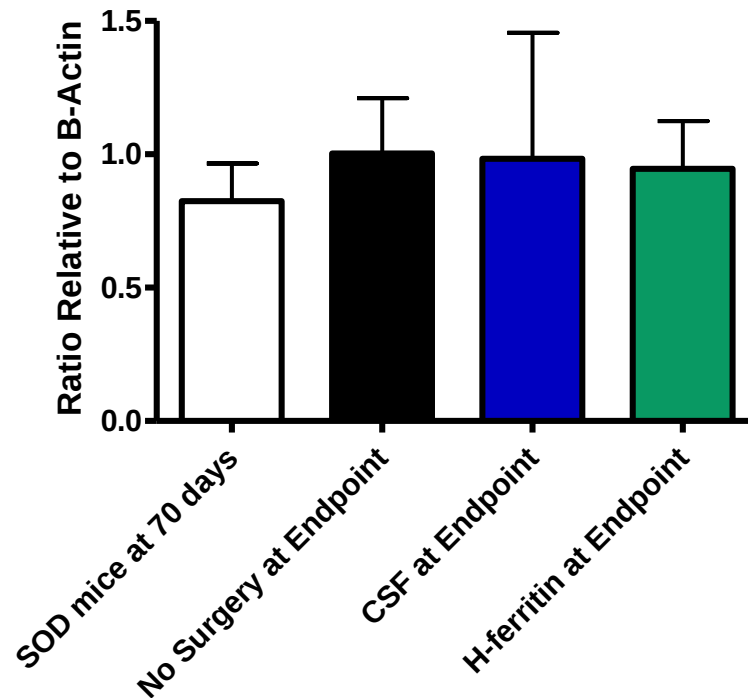


Figure 9. Mitochondrial ferritin levels in the lumbar spinal cord in SOD1^{G93A} mice are unchanged by treatment. Mitochondrial ferritin values were normalized to β -actin for each animal before each group mean was calculated. One-way ANOVA was used to compare groups and there was no change in mitochondrial ferritin levels. The mice sacrificed at 70 days were at least 30 days younger than the other three groups, but there was no significant increase in mitochondrial ferritin with age. Data are represented as mean $\pm$ S.E.M. $N = 2-4$ in each group.

Figure 10

L-ferritin Levels in Lumbar Spinal Cord Homogenates

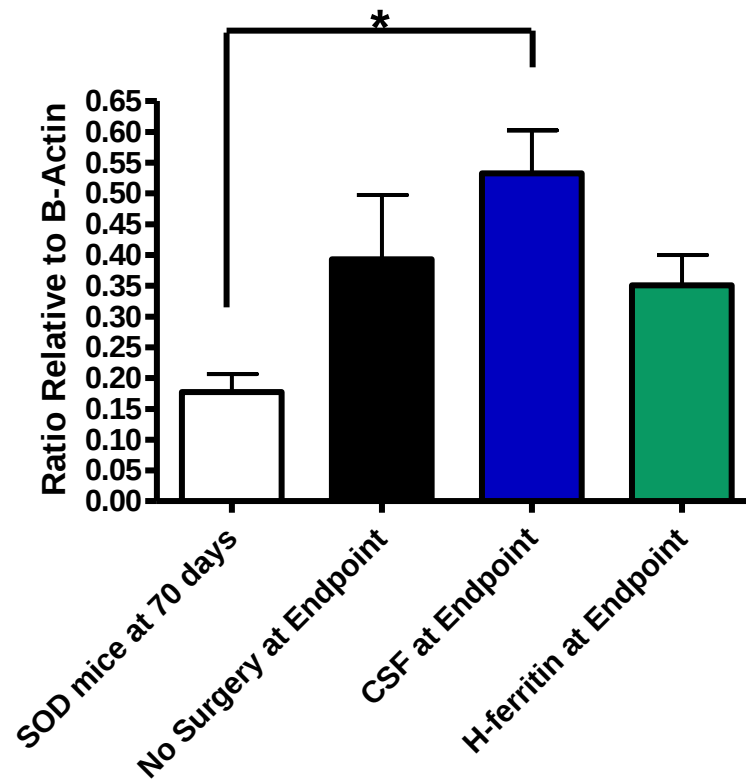


Figure 10. L-ferritin levels in the lumbar spinal cord in SOD1^{G93A} mice. The amount of L-ferritin in the lumbar spinal cord is significantly lower in 70-day-old mice than the CSF-treated mice at endpoint, as assessed by One-way ANOVA with Tukey post-test, $p < 0.05$. L-ferritin values were normalized to β -actin for each animal before each group mean was calculated. Data are represented as mean $\pm$ S.E.M. $N = 3-4$ in each group.

Figure 11

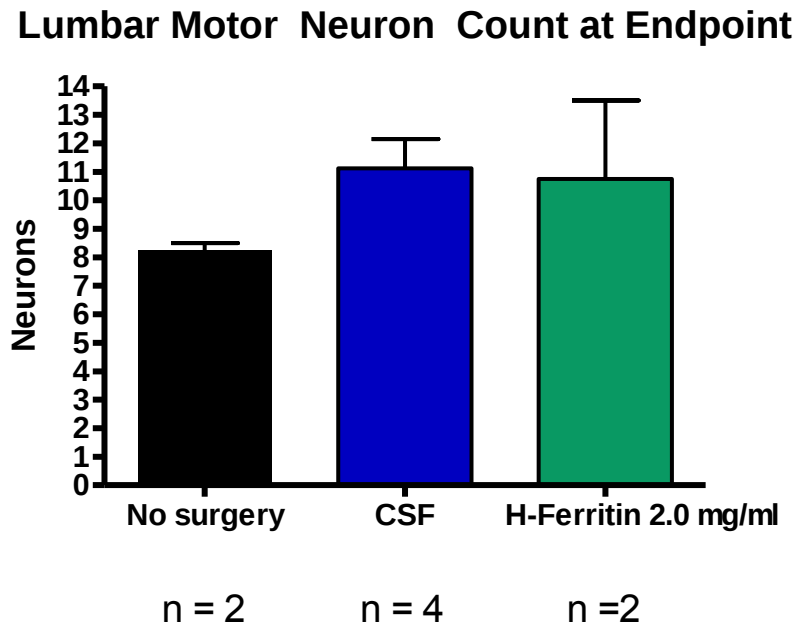


Figure 11. SMI-32 cell count in lumbar spinal cord sections in SOD1^{G93A} mice treated with 2.0 mg/ml H-ferritin. SMI-32-positive motor neurons on the left side of the ventral lumbar spinal cord with an area greater than 100 μm^2 were counted. At least two sections per animal were counted and the average value per animal was combined with averages from other subjects in the same treatment group. Although animals that received CSF or H-ferritin 2.0 mg/ml had 27% and 23% (respectively) more healthy neurons at endpoint, the effect is not statistically significant ($p=0.42$).

Figure 12

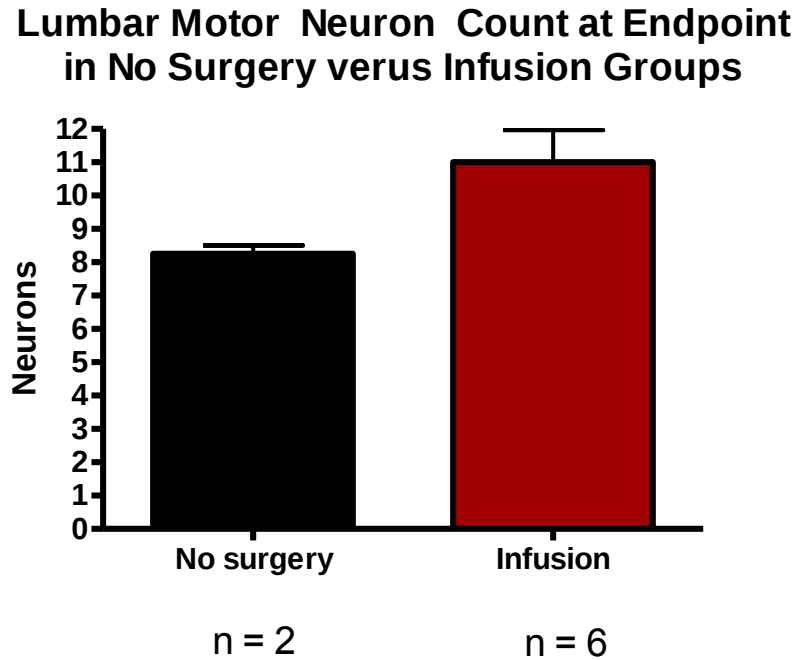


Figure 12. SMI-32 cell count in lumbar spinal cord sections in SOD1^{G93A} mice that received infusion of artificial CSF or no infusion. SMI-32-positive motor neurons on the left side of the ventral lumbar spinal cord with an area greater than 100 μm^2 were counted. At least two sections per animal were counted and the average value per animal was combined with averages from other subjects in the same treatment group. Infusion increased the number of healthy motor neurons at endpoint by 33%. T-test analysis indicates a trend towards significance, $p = 0.08$. More animals will be added in the second year of funding.