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PRINCIPAL INVESTIGATOR: Gillian Muir DVM, Ph.D.

CONTRACTING ORGANIZATION: University of Saskatchewan
Saskatoon, SK S7N 5A2 Canada

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Gillian Muir

E-Mail: gillian.muir@usask.ca

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14. ABSTRACT

This research is part of a concurrent set of studies involving animals and human spinal cord-injured (SCI) subjects designed to test the effects of a novel therapy, termed acute intermittent hypoxia (AIH), on voluntary limb function following chronic SCI. The current research investigates the effect of AIH treatment in a rat model of cervical SCI. Within Year 1 of this 2 year study, we determined that AIH, in combination with daily motor training, elicits sustained improvement in skilled limb use during a ladder walking task in a rat model of SCI. Spinal-injured rats which underwent AIH treatment and daily motor training made fewer footslip errors on the ladder for up to 4 weeks after the end of treatment when compared to normoxia-treated, motor-trained control rats. In a separate experiment, spinal-injured rats treated with AIH without concomitant motor training did not show recovery on the ladder task. These results provide strong support for our proposed Year 2 experiments, which will directly test the effects of AIH treatment and motor training on recovery of function in SCI rats. These findings are important because they reveal that we can obtain consistent effects in an animal model for a promising SCI therapy. This therapy is also feasible, in that AIH has already been shown to augment motor function in persons with SCI.

15. SUBJECT TERMS
Spinal cord injury, acute intermittent hypoxia, recovery, motor, rat

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INTRODUCTION

The long term goal of this research is to improve functional recovery in persons with cervical spinal cord injury. The purpose of the current research is to assess the effectiveness of a novel therapy, termed acute intermittent hypoxia (AIH), to produce functional recovery in a rat model of cervical SCI. This therapy is based on the established finding that AIH (repeatedly breathing brief periods of low oxygen alternating with normal levels of oxygen) will strengthen synapses onto respiratory motoneurons by a mechanism known as long-term facilitation.[1] Briefly, exposure to hypoxia is known to trigger oxygen-sensitive chemoreceptors which activate brainstem neurons. This results in increased protein synthesis in respiratory centres in the spinal cord, which in turn augments neural activity and improves respiratory function.[1] In our preliminary data, we found that AIH also improves non-respiratory motor function in that forelimb function in spinal-injured rats and lower limb muscle activation in spinal-injured humans was improved with AIH exposure. Thus, over the 2 year scope of this research project, we proposed a concurrent set of studies involving animal and human experiments that provide a framework to quantify the effect of AIH on voluntary limb function following chronic spinal cord injury. The animal experiments quantifying functional recovery are the focus of the current report. Specifically, we proposed to (1) test the hypothesis that daily AIH elicits robust and prolonged improvement of voluntary limb function after chronic spinal injury (**Aim 1, Year 1**) and (2) to test the hypothesis that combining daily AIH with motor training enhances and prolongs improvements in voluntary limb function after chronic spinal injury (**Aim 2, Year 2**). In rats with chronic cervical injuries, we planned to assess multiple indicators of limb function, including horizontal ladder performance, grip strength, and maximal treadmill speed, both before and after exposure to AIH. In the second year of this project (not yet begun) we propose to combine daily AIH with daily motor training, with the rationale that together these therapies may provide a greater degree of recovery than either alone. We expect that the knowledge gained from this translational study will assist in the development of future clinical trials with an end goal of improving motor function in spinal-injured humans.

BODY

As per our Statement of Work, our research efforts during Year 1 have been focussed on **Specific Aim 1**, *to test the hypothesis that dAIH elicits robust and sustained improvement of voluntary limb function after chronic SCI*. Specifically, we have been working through **Task 1**, which was to quantify the effect of dAIH on limb function in rats with chronic cervical injuries. We have completed all of the subtasks (Subtasks 1a through 1e) identified for Task 1, which include the behavioural conditioning, surgery, limb function assessments, AIH treatment and post-treatment assessments in 60 rats. In order to maintain feasibility and quality of work, we carry out our experiments using sub-groups of 12 rats at a time. For the period under consideration, we have carried out 6 experiments (6 sub-groups of rats x 12 rats/gp = 72 rats); however, methodological complications (see below) were encountered early during these experiments and therefore not all of the data obtained could be used to address Aim 1. Fortunately, we have resolved these complications and we were able to obtain some promising results (Figures 1, 2).

Methodological Complications and Subsequent Improvements

1. **Problem: Post-surgical ischemic damage to distal appendages**
   In our first sub-group of rats, we encountered an early post-surgical complication in which skin was lost over the tail and from the toes. Working closely with the University Veterinarian and veterinary pathologists, animals and tissues were submitted for post-mortem and histopathological analysis. The pathologists concluded that the cause was loss of blood supply to the distal parts of the body (e.g. tail and toes). This ischemia was likely due to extremely low blood pressure during and immediately after surgery. We hypothesized that the low blood pressure was caused by the use of an injectable sedative, medetomidine, which was administered prior to anaesthesia induction and maintenance with inhalant anaesthesia (isoflurane).
   Solution:
   Under the direction of the University Veterinarian, we discontinued use of the injectable sedative in the subsequent experiments and instead solely relied on isoflurane for induction and maintenance of anaesthesia. In subsequent sub-groups, no post-surgical skin loss was observed.

2. **Problem: Lack of functional deficits following surgery**
   Following the improvements made to the anaesthetic regime described above, we began to notice that the majority of animals in subsequent experiments failed to show significant deficits on the ladder crossing task at 4 weeks after surgery (subgroups 2 – 5, Figure 3). In our spinal lesion model, which involves transection of the entire dorsal portion of the left lateral funiculus (e.g the dorsolateral funiculus) at spinal level C2, we normally expect post-surgery animals to make footslip errors with the left forelimb more than 40% of the time. Upon histological examination of the spinal cords of rats in subgroups 2 -5 , we determined that the spinal cord lesion did not encompass the entire dorsolateral funiculus. This suggested that, in our preliminary experiments, the low blood pressure caused by the injectable sedative used prior to aneasthesia (described above) had also exacerbated the spinal transection injury.
   Solution:
   Our surgical procedures have been adjusted in order to induce an appropriate-sized lesion, resulting in more distinct functional deficits post-surgery between (Figures 1, 2).

3. **Problem: Random assignment to treatment groups**
   Although the injuries produced in subgroups 2-5 were shown to be insufficient (as described in Problem 2), we carried these animals through the entire experiment as an opportunity to refine our methods and gain more information. By randomly assigning rats to AIH- or normoxia-treatment groups, we noticed in hindsight that this resulted in an unequal distribution of animals with respect to severity of injury (as assessed by ladder performance) between the two groups. This would cause any potential treatment effects to be questioned

since the treatment groups would not have equivalent functional capabilities prior to treatment.

Solution:

We have changed our protocol to ensure that animals have comparable and measureable deficits prior to being assigned to a treatment group. First, ladder performance in each animal within each subgroup is quantified before animals are assigned to groups. Next, we ensure that animals with equivalent deficits are distributed evenly between 2 groups in order to minimize the differences in functional deficit between treatment groups within each sub-group. We then randomly assign each treatment group to either AIH or normoxia treatment. With these steps, we have enhanced the repeatability of our findings and ensured more efficient use of animals within each experiment.

We have now overcome the methodological complications experienced with our first experimental groups, and have since obtained promising results in experiments using the ladder-walking task and grip strength measurements (Figures 2 and 3). We are currently working on experiments which assess performance on the treadmill task in SCI rats.

Effects of AIH on ladder performance in SCI rats

Data from our most recent experiments reveals that AIH treatment improves recovery on the ladder task in SCI rats, but *only when combined with motor training* (Figure 1). Rats with transection of the left dorsolateral funiculus were subject to either AIH or normoxia treatment at 4 wks post surgery. The data in Figure 1A shows results obtained from methods proposed in Aim 1 of this project, in that animals were treated daily with AIH but recording of ladder performance was only carried out on the last day of treatment, not on each day of treatment. With this protocol, there was no difference in ladder performance between IH and normoxia-treated control animals at any timepoint (Figure 1A). The data in Figure 1B shows results obtained when we repeated the methods used to obtain our preliminary data, namely that ladder performance was recorded for **each** day of the daily AIH treatment. In our laboratory, recording of ladder performance constitutes a motor training session as well, because rats repeatedly cross a horizontal ladder 10-15 times during digital videotaping for assessment of performance. SCI rats which receive daily AIH treatment and daily motor training made fewer errors on the ladder compared with animals receiving daily normoxia treatment and daily motor training (Figure 1B). The data in Figure 1B is important because it (1) demonstrates that we can replicate our preliminary findings, and (2) in combination with results in Figure 1A, strongly supports the experiments proposed in Aim 2 of this project. This latter Aim, which is to be completed during the second year of this project, is designed to directly test the relative effects of AIH treatment and motor training on recovery of function in SCI rats. Thus our latest findings highlight our next steps in this project, which are to proceed with experiments in support of Aim 2, as described in Task 5 in our original Statement of Work.

There is one other methodological difference between Fig 1A and 1B which is worth mentioning, although it does not affect our main conclusions. The data presented in Figure 1A was obtained with 5 days of AIH or normoxia treatment, whereas the data presented in Figure 1B was obtained with 7 days of treatment. Likewise, our preliminary data had been obtained using 7 days of AIH treatment in SCI rats and we had proposed the same in our original proposal. Early in the tenure of the current grant, our co-Principal Investigators suggested that we test the effect of 5 days of AIH treatment. This would provide useful information for the human experiments because 5 days of treatment would increase compliance amongst human subjects who were to be assessed in parallel with the animal experiments**.** We proceeded with this change in our methods. Nevertheless, we suggest that this change does not affect the differences in findings between Figure 1A and 1B, because in those animals receiving 7 days of AIH and motor training, functional improvements are apparent by Day 4 of treatment, consistent with our preliminary data. This suggests that 5 days of AIH treatment might still be sufficient to improve ladder performance if combined with daily training. We have applied for a separate grant to investigate whether fewer days of AIH exposure might be effective in improving functional recovery in SCI rats (see Reportable Outcomes).

Effects of AIH on grip strength in SCI rats

Our preliminary data and our initial experiments focussed on assessment of forepaw use in the ladder-walking task. We have also assessed forepaw grip strength to investigate whether AIH might promote recovery in more than one task, e.g. to determine whether AIH-induced recovery is robust. We assessed grip strength in SCI rats which received daily AIH treatment for 5 days and compared these to normoxia-treated control animals (Fig 2). AIH-treated animals showed improvements in grip strength compared to control animals starting at 2 weeks post-treatment. Improvements in grip strength were maintained until 8 weeks post treatment, the latest timepoint examined. Although RM-ANOVA did not reveal significant differences between AIH- and normoxia-treated animals, these findings are promising. We will repeat these experiments with more animals to increase the reliability of the findings.

KEY RESEARCH ACCOMPLISHMENTS

- Our experiments suggest that acute intermittent hypoxia elicits sustained improvement in skilled limb use during a ladder walking task when combined with daily motor training in a rat model of SCI.
- Our current data is insufficient to demonstrate that acute intermittent hypoxia elicits on sustained improvement in forepaw grip strength. This will be resolved with replicate experiments.
- We have made several key methodological refinements which will improve repeatability and ensure more efficient use of experimental animals.

REPORTABLE OUTCOMES

We have applied for a research grant from the Saskatchewan Health Research Foundation (SHRF Spinal Cord Injury Research) based on our findings from this project. Specifically, we have proposed to investigate the minimum number of days of AIH treatment sufficient to produce sustained functional recovery in a rat model of SCI.

CONCLUSION

In summary, our results demonstrate that AIH, in combination with daily motor training, elicits sustained improvement in skilled limb use during a ladder walking task in a rat model of SCI. Spinal-injured rats which underwent AIH treatment and daily motor training, in the form of repeated horizontal ladder crossings, made fewer footslip errors on the ladder for up to 4 weeks after the end of treatment when compared to normoxia-treated, motor-trained control rats. Spinal-injured rats treated with AIH without concomitant motor training did not make significantly fewer errors on the ladder task compared to normoxia-treated SCI control rats. These results are consistent with our preliminary findings and strongly support the experiments we have proposed in Aim 2, Year 2 of this project. This latter Aim is designed to directly test the effects of AIH treatment and motor training on recovery of function in SCI rats. Additionally, we have data which strongly suggests that AIH may improve forepaw grip strength in rats with cervical spinal injury - these findings will be confirmed with replicate experiments. Thus our latest results support our next steps in this project, which are to proceed with experiments in support of Aim 2, as described in Task 5 in our original Statement of Work. These findings are important because they reveal that we can obtain consistent effects in an animal model for a promising SCI therapy that is also feasible, in that AIH has already been shown to augment motor function in persons with SCI.[2] Thus, we have a translational framework with which to continue to investigate the effects of AIH on voluntary limb function following chronic spinal cord injury.

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SUPPORTING DATA

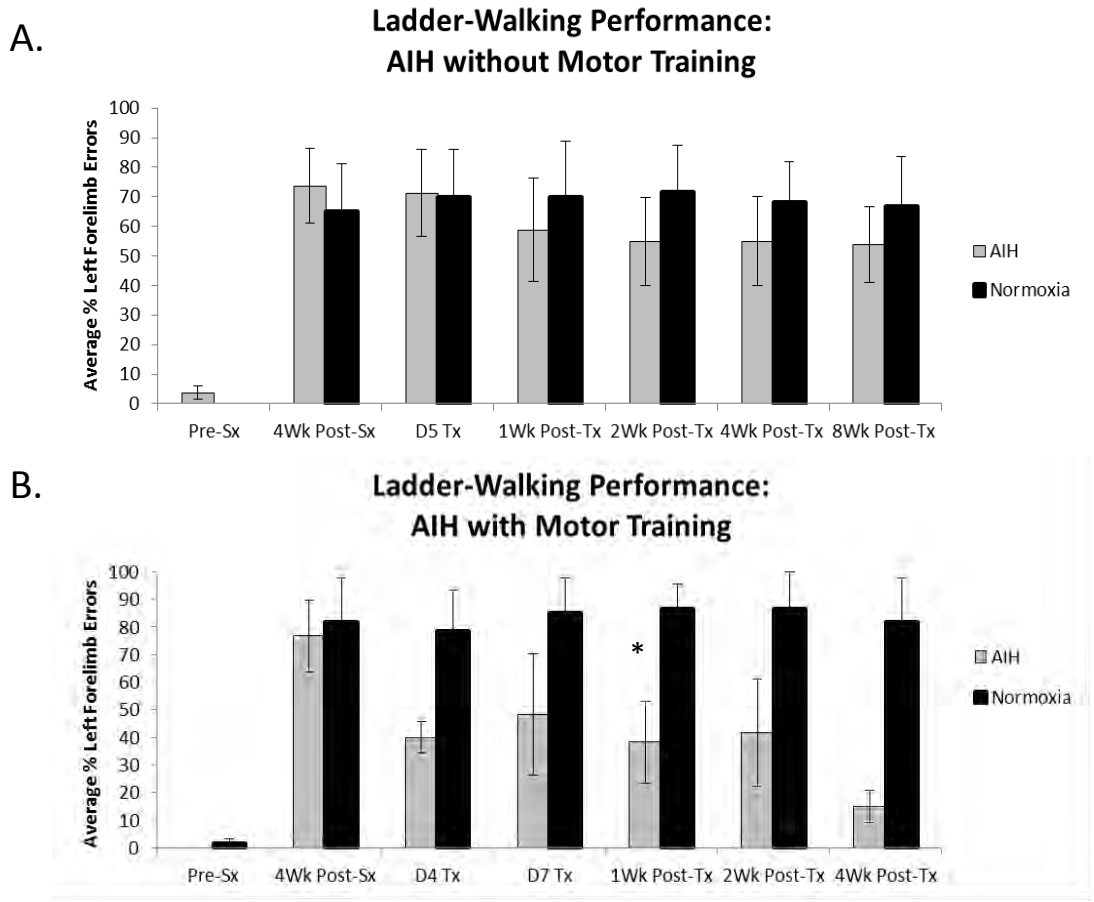

Figure 1. Ladder-walking performance in SCI rats exposed to AIH with or without motor training. A. Rats which received AIH but no motor training during the week of treatment showed no differences in left forelimb errors during ladder walking when compared to normoxia controls (RM-ANOVA, $p > 0.05$; n=4 AIH, n=3 normoxia). **B.** Rats which received motor training in the form of daily ladder crossing in combination with AIH treatment made fewer left forelimb errors when compared to animals which received the same training and normoxia treatment at 1, 2, and 4wk post-tx (RM-ANOVA, $*p < 0.05$; n=3/group). Treatments began 4wk after cervical spinal cord injury, and consisted of daily AIH exposure (5min 11% oxygen alternating with 5min room air, repeated 10 times), for 5 days (**A**) or 7 days (**B**) while normoxia-treated control animals were exposed to room air continuously for the same duration. Ladder-walking performance was assessed before surgery, 4wk after surgery (before treatment), on the last day of treatment, and at 1, 2, 4, and 8wk following treatment. In **B**, performance was also recorded for each of 7 days of treatment, but only day 4 is shown for clarity. Percent forelimb errors = # footslips/total number of steps. Sx = left dorsolateral spinal funiculus transection at C2, Tx = treatment (AIH or normoxia), D = day of treatment.

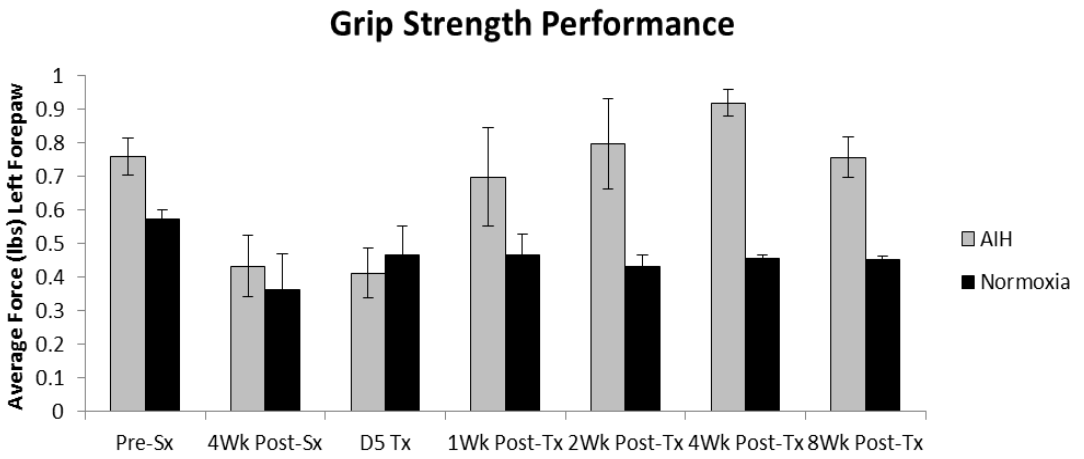

Figure 2. Grip strength performance in SCI rats exposed to AIH. Rats which received AIH but no motor training during the week of treatment showed no differences in grip strength when compared to normoxia controls (RM-ANOVA, $p > 0.05$; n=4 AIH, n=3 normoxia). Treatments began 4wk after cervical spinal cord injury, and consisted of daily AIH exposure (5min 11% oxygen alternating with 5min room air, repeated 10 times), for 5 days while normoxia-treated control animals were exposed to room air continuously for the same duration. Grip strength performance was assessed before surgery, 4wk after surgery (before treatment), on the last day of treatment (D5), and at 1, 2, 4, and 8wk following treatment. Sx = left dorsolateral spinal funiculus transection at C2, Tx = treatment (AIH or normoxia), D = day of treatment.

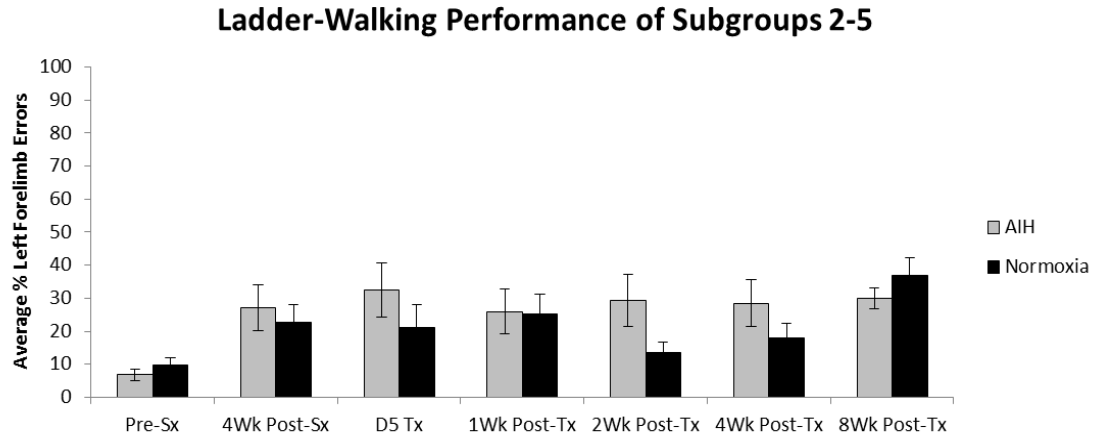

Figure 3. Ladder-walking performance in SCI rats within subgroups 2-5. Rats did not make significantly more errors after surgery compared to pre-surgery performance nor were differences found between AIH- and normoxia-treated animals at any time point (RM-ANOVA , $p > 0.05$; n=16/group for pre-sx, 4wk post-sx, D5 tx, and 1wk post-tx time points; n=15 for normoxia and n=16 for AIH for 2wk post-tx time point; n=13/group for 4wk post-tx time point; n=5 for normoxia and n=4 for AIH for 8wk post-tx time point). Treatments began 4wk after cervical spinal cord injury, and consisted of daily AIH exposure for 5 days while normoxia-treated control animals were exposed to room air continuously for the same duration. Ladder-walking performance was assessed before surgery, 4wk after surgery (before treatment), on the last day of treatment (D5), and at 1, 2, 4, and 8wk following treatment. Percent forelimb errors = # footslips/total number of steps. Sx = left dorsolateral spinal funiculus transection at C2, Tx = treatment (AIH or normoxia), D = day of treatment.