

Objective Tests of Gastocnemius Muscle Response for Duchene's Muscular Dystrophy

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This research involving non-human vertebrates was conducted under the supervision of an experienced researcher at a research institution and followed state and federal regulatory guidance applicable to the human and ethical conduct of such research.

Introduction

Adult stem cells maintain and repair tissue. Recent therapeutic approaches aim to use stem cells to regenerate and rebuild muscle cells. These are considered to be the best hope for curing degenerative diseases, such as Duchene's Muscular Dystrophy (DMD) (1). The goal of our project was to develop a methodology of non-invasive stimulated force assessment as a means to assess the potential of stem-cell therapy. To do this, we adapted the approach by Hong et al. to muscular dystrophic *Mus musculus* mice.

The research by Hong involved testing bicep muscle response to electrical stimulation in humans. Hong developed an objective procedure that eliminated many problems that occur during manual testing, the most commonly employed clinical method of assessing muscle strength (1). The problem with manual testing is that it relies on subjective assessment by a clinician, and as a result, it is difficult to detect small changes in the force produced by a muscle. As a consequence, any therapy that is applied to a muscle cannot be precisely measured (2). The Hong procedure included a stabilizing device that holds either the arm or leg in defined position. The device operates with a transducer that measures force produced by a given muscle group. This is coupled with electrodes for nerve stimulation; an amplifier signal; and a computer with data-acquisition software for recording, analyzing, and displaying all signals simultaneously (torque, EMG, applied stimulus) (2). The basic structure of the apparatus used in the Hong experiment was used to model the testing apparatus for our project. We used a protocol by Frommer that was a modification of the Hong procedure (3).

Our first objective was to determine the relative effects that various changes in anesthesia, breathing rate, or temperature may have on gastrocnemius muscle response and to find the electrode position that produced the greatest force with minimal voltage during stimulation. The purpose for testing these variables was to identify their effects on muscle

response and try to determine ways to eliminate variability produced by the protocol. The second objective of our project was to obtain baseline data from muscular dystrophic mice. To date, there have been few tests on muscular dystrophic mice, yet reproducible, non-invasive testing protocols are needed to determine long-term benefits of therapies used to treat muscular dystrophy (4).

An important aspect of studying muscle force is eliminating passive force, which is reserve energy that builds up as muscles recover. To eliminate passive force, the muscle has to be stretched completely out and stimulated for a short period of time at every position on its full range of movement. This removes excess energy that has been built up so that further stimulation of the muscle will measure only the actual strength of the muscle, not an overly high force that has been artificially bolstered by passive force. (3)

In order to develop a reliable-outcome protocol for testing the therapeutic benefits on the gastrocnemius muscle, it was important to eliminate all possible variables. For the first objective, we hypothesized that the following variables would significantly affect muscle response: breathing rate, time under anesthesia, ambient temperature, and electrode position. Our null hypotheses were:

- The lower the breathing rate, the more variable the muscle response, due to varying levels of CO₂ and O₂.
- The longer the mouse was under anesthesia, the more variable the muscle response, due to the effects of anesthesia.
- The higher the ambient temperature, the higher the muscle temperature, causing a greater the resultant muscle response.
- There exists an optimal one-cm² cell on the gastrocnemius muscle that produces the greatest force at a low voltage.

For the second objective, which was to obtain quantifiable testing data for muscular dystrophic mice, our null hypothesis was that the muscle response produced by the gastrocnemius muscle would be less than that of control mice.

Methods and Materials

Materials: The force assessment system consisted of a support frame, a swinging platform, a knee brace, and an ankle pin. The support frame measured 30.48 cm high with a 26.67 x 22.86 cm base, the swinging platform measured 20.32 x 7.62 cm, the knee brace positioned on top of the swinging platform measured 1.91 x 1.91 x 1.59 cm, and the ankle pin had a radius of 0.16 cm and a height of 1.59 cm. An electrode probe was used to apply voltage to the gastrocnemius muscle, up to 150 volts. The applied voltage did not harm the test mice, because voltage applied at very low charge is not harmful. The sensation that a mouse felt is similar to when a student touches a Van Der Graph Generator, which produces up to 100,000 volts.

Calibration of the Force Transducer: Using the program LabPro on a Compaq computer, the course gain knob was set to the scale of 1,000. The amplifier for the force transducer was first balanced to indicate 0.00 volts, and then the voltage output was calibrated by applying 20.0 g and 40.0 g weights to the transducer.

Mouse Preparation: The investigational protocol employed here received prior approval by the Animal Care and Use Committee of the University of Minnesota. Four *Mus musculus* Black-6 mice were obtained from the University of Minnesota Mouse Center. At the start of each study session, the mouse was placed in an anesthetic chamber into which 2% isoflurane was pumped at an airflow rate of 400 cm³/minute for approximately 45 seconds or until the mouse was unconscious. The mouse was then transferred to the swinging platform. To keep the mouse anesthetized, its head was placed into a small anesthetic hood, into which isoflurane was

continuously pumped at concentrations ranging between 1.0% and 1.5%. While under anesthetic, all hair was removed from the hind leg from the ankle up to the knee. Next, an electrode gel was applied. The test-leg ankle was taped to a small support post mounted on the support frame, and then the toes were adhered to the force-stimulator using super-glue. The foot was maneuvered to be parallel to the platform so that the angle of the ankle and toes formed a 90-degree angle.

Length-Tension Relationship: The passive force was removed and length-tension relationship of the gastrocnemius muscle was optimized in order to elicit the highest force-output by single-pulse (twitch) stimulation. To do this, the hind-leg gastrocnemius muscle was stimulated at ten different positions by moving the platform from 90 to 0 degrees at 10-degree decrements; the inter-pulse intervals used were five milliseconds. Then, the muscle was stimulated in 2-degree angles either side of the peak until the angle that produced the maximal force was found. After optimizing the force by modifying the ankle position, the resultant twitch force was further maximized by modifying the voltage in steps, up to 150 volts.

General Muscle Stimulation: Once the optimal muscle lengths and stimulation parameters were identified, three trials of single-pulse, double-pulse, triple-pulse, quadruple-pulse, and one tetanus¹ stimulation were applied to the hind-leg gastrocnemius muscle. The electrodes were removed from the leg, acetone was used to detach the foot from the force-stimulator, and the mouse was removed from the anesthetic hood. The mouse was then weighed and cleaned and allowed to rest with food and water for 24 hours before being re-tested.

Effects of Anesthetic: A control mouse was anesthetized as detailed above and allowed to rest for 30 minutes prior to obtaining force data as described above. This process was repeated

¹ A tetanus stimulation continually stimulates the muscle at 100 Hz for 0.25 sec until the muscle fails. This measures the maximum force the muscle can produce.

without the 30 minutes of rest before stimulation. The results were compared to see if time under anesthesia had an effect on the muscle contraction.

Effects of Breathing Rate: A control mouse was anesthetized, keeping its breathing rate at approximately 60-80 beats per minute (bpm) by regulating the flow of isoflurane through the anesthetic hood. This process was repeated two more times at approximately 100 bpm and approximately 120 bpm. The results were compared to see if the breathing rate of the mouse had an effect on muscle response.

Effects of Ambient Temperature: After preparing a control mouse, a heat lamp was placed above the mouse to manipulate the environmental temperature around the mouse. Subsequently the stimulation protocol was repeated at ambient temperatures at 25.0, 30.0, and 35.0 °C.

Electrode Position Optimization: Before applying electrode gel, a small 4 x 3 cm grid with 12 one-cm² cells was drawn on the hind leg of the mouse, using a black Sharpie pen. Each cell of the grid was stimulated at 120 volts.

Muscular Dystrophic Mouse Testing: A muscular dystrophic mouse was anesthetized and the preparation protocols were run. The results were compared to muscle stimulation data of control mice to determine the effects of muscular dystrophy on gastrocnemius muscle response.

Statistical Analysis: A F-test, a two-sample for variances data analysis tool, was run to calculate p-values for all data. A 0.05 significance level was set.

Results

Figure 1 shows deviation from the mean of the average force produced by the gastrocnemius muscle with initial anesthesia exposure and with an added 30 minutes of anesthesia exposure: no waiting time (blue) and 30-minute wait period (red). There was no statistical difference in elicited muscle forces between starting experimentation immediately after anesthetization and waiting 30 minutes after anesthetization, as shown by a p-value of 0.786.

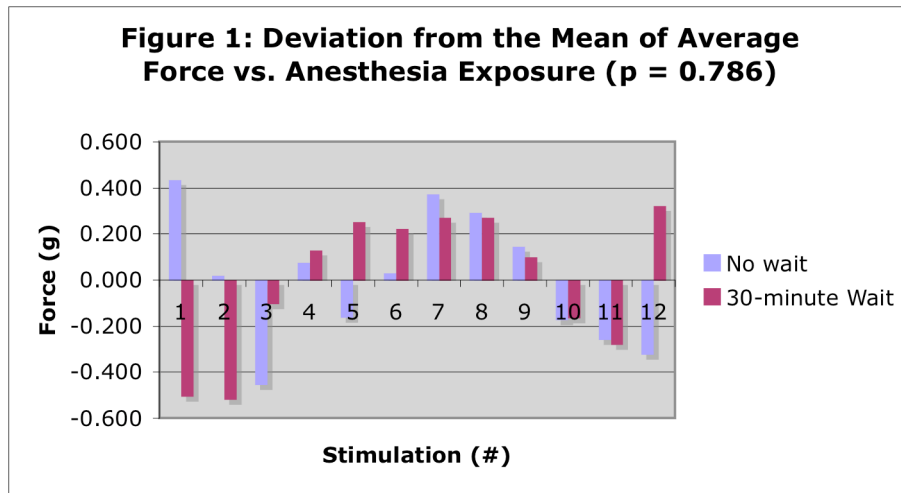


Figure 2 shows deviation from the mean of the muscle response produced by the gastrocnemius muscle at different breathing rates: 60-80 bpm (yellow), 100 bpm (blue), and 120 bpm (red). There was no statistical relationship between deviation from the mean of the muscle response and breathing rate, shown by the p-value of 0.460.

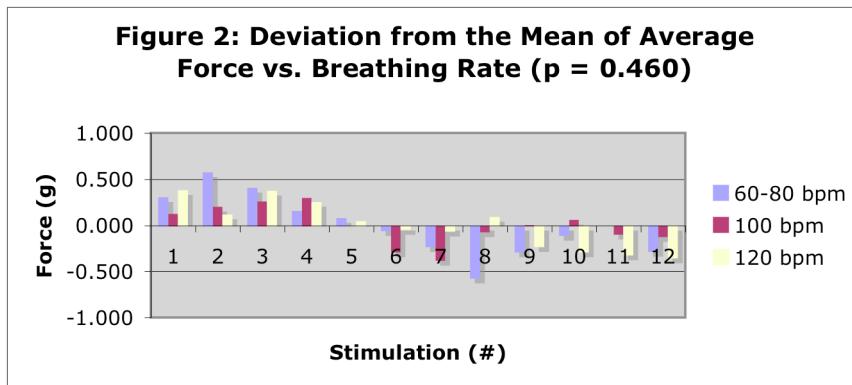


Figure 3 shows deviation from the mean of the muscle response produced by the gastrocnemius muscle at different ambient temperatures: 35.0 °C (yellow), 30.0 °C (red), 25.0 °C (blue). There was a significant statistical relationship between the deviation from the mean of the average muscle response and ambient temperature, as shown by a p-value of 2.53×10^{-5} .

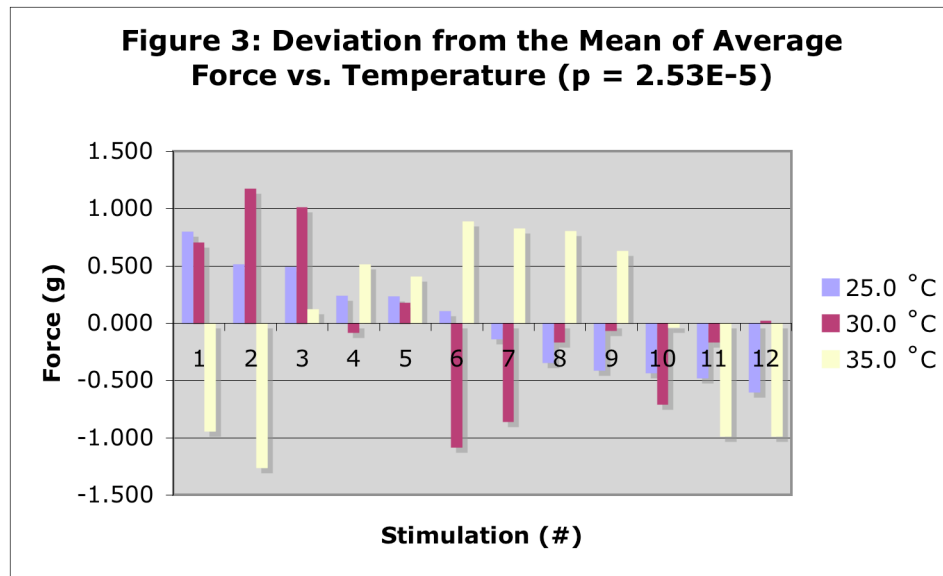


Figure 4 shows average muscle response produced by single stimulations at each of the 12 one-cm² cells, which had been drawn on the gastrocnemius muscle to test electrode position. There was no statistical relationship between electrode position and force, as shown by a p-value of 0.690.

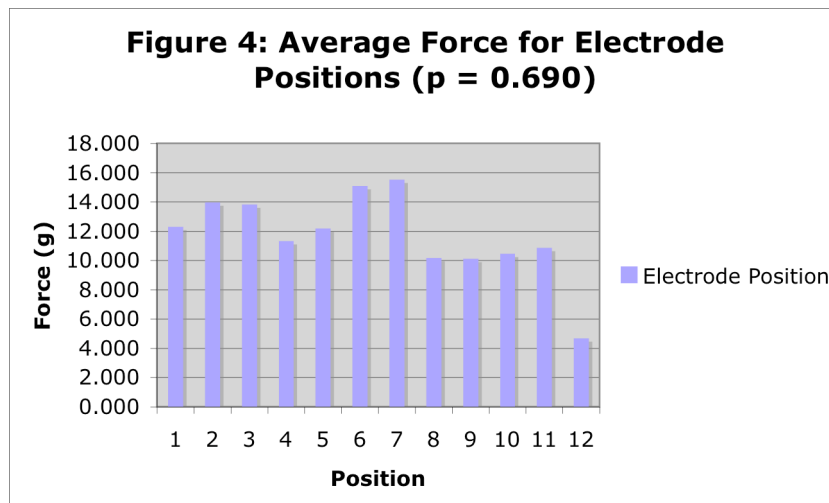
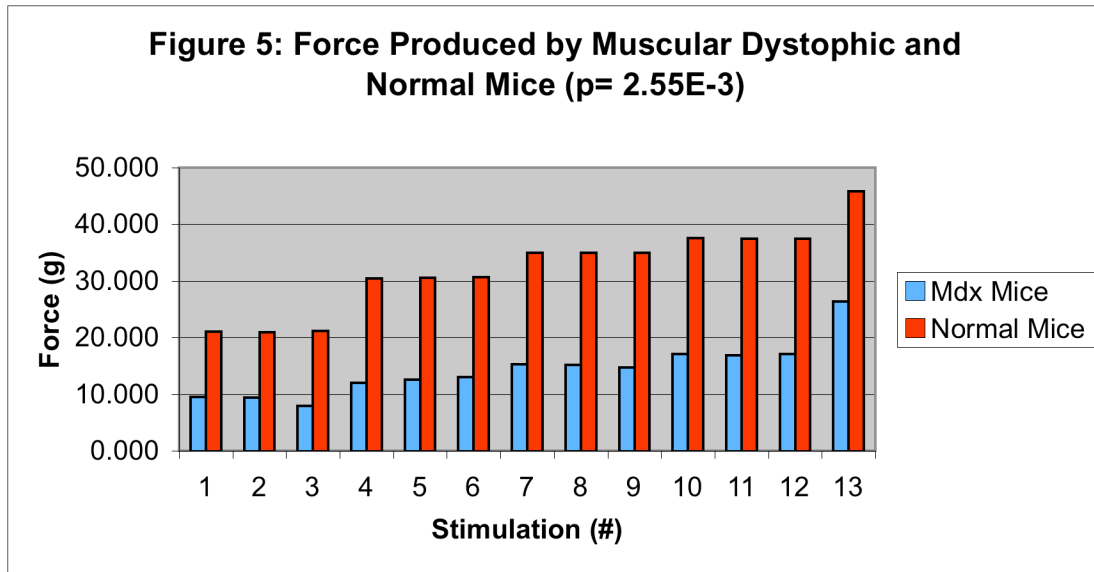


Figure 5 shows the average muscle response during stimulation of muscular dystrophic mice (blue) compared to control mice (red). There were significantly lower forces recorded from the muscular dystrophic mice compared to that of the control mice, as shown by a p-value of 2.55×10^{-3} .



Conclusion

Ambient temperature was the only variable that statistically affected the variability of the stimulated force of the gastrocnemius muscle response of the control mice ($p = 2.53 \times 10^{-5}$). Muscular dystrophic mice were found to be significantly weaker relative to control mice ($p = 2.55 \times 10^{-3}$). Therefore, we are confident that this assessment approach will be a viable means to study the therapeutic benefits of stem cell therapy for Duchene’s Muscular Dystrophy. It is important to note that these same stimulation methodologies can be employed in humans with a high degree of reproducibility (2).

The other hypotheses were not accepted. There was no effect on the variability of muscle response due to the length of time under anesthesia ($p = 0.786$). Lowering the breathing rate did

not affect the variability of response ($p = 0.460$). There was no single 1-cm² cell that maximized muscle response ($p = 0.690$).

We believe that there are two areas that would be important to continue testing in the future. While we ran up to 33 trials for the variability protocols, we ran only two trials on muscular dystrophic mice, so additional tests on a population of muscular dystrophic mice would be important to verify our results. Since our data showed high variability of muscle response at temperatures close to the body temperature of mice, and other studies have shown that human muscles work best if the ambient temperature is closest to the body temperature, future studies should determine if the muscle response is highest when the ambient temperature and body temperatures are similar (3). It might also be beneficial to identify the relationship between intramuscular and ambient temperatures. Our pilot data established that future tests on *Mus musculus* can be reliably performed at 25.0 °C and that the other variables tested will not affect reproducibility of data.

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Works Cited

1. Reyes, M. Lund, T., Lenvik, T., Aguiar, D., Koodie, L. Verfaillie, C.M., 2001, Purification and *ex vivo* expansion of postnatal human marrow mesodermal progenitor cells. *Blood*, **9**, 2615-2625.
2. Hong, J.B. and Iaizzo, P.A., 2002, Force assessment of the stimulated arm flexors: quantification of contractile properties. *Journal of Medical Engineering & Technology*, **26**, 28-35.
3. Frommer, Sarah. Personal Interview. 10 July 2005.
4. Brass, T.J., Loushin, M.K.H., Day, J.W., and Iaizzo, P.A. (1996) An Improved method for muscle force assessment in neuromuscular disease. *Journal of Medical Engineering and Technology*, **20** (2), 67-74.