The Effects of Folic Acid on C2C12 Cell Maturation

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INTRODUCTION

Supplements are widely consumed by athletes around the world. According to Sobal and Marqaurt, around half of the athletes in a sample of 10,274 people actively took supplements [1]. There are all kinds of supplements including minerals, vitamins, proteins, creatine, and other nutritional aids [2]. Common protein supplements like Muscle Milk® claim that their protein helps “contribute to recovery from exercise, gains in lean muscle mass, and healthy weight management” [3]. Unfortunately, there is a lack of literature and research to factually back that these supplements effect skeletal muscle on a cellular level.

Skeletal muscle is found throughout the human body and is connected to bones by tendons which aids in body movement. Initially, skeletal muscle cells originate as single nucleated cells called myoblasts. The myoblasts fuse together to form myotubes during development. These tubes become multinucleated fibers that can stretch throughout the body and form mature muscle. Moreover, skeletal muscle drives all motor functions throughout the body and is extremely important for humans, especially athletes [4]. Unfortunately, injuries and diseases can cause large areas of tissue to be lost or permanently damaged. For instance, systemic burns, cancer, gunshot wounds, and stabbings can all cause serious damage to skeletal muscle in the body. The damage of the tissue is treated often with transplantations and amputations. Therefore, researchers have focused on tissue engineering as a viable treatment option [5]. Researchers have successfully cultured cells outside of the body and aim to create replacement tissues and organs. This project focused on creating mature skeletal muscle fibers faster to improve maturation of cells for skeletal muscle replacement or regeneration.

For this project, the effect of folic acid on skeletal muscle cell growth and maturation was tested. Folic acid, a a synthetic form of Folate (vitamin B9), is a supplement found in leafy greens, peas, oranges, corn, and even meat [5]. Folate plays an important role in the synthesis of DNA and cell division. Folic acid is widely used because the body absorbs the synthetic form easier than folate itself [6]. The supplement is well known for its use by pregnant women as it has been found that it lowers the potential of neural tube defects [5]. Despite all the research that shows the positive benefits of folic acid on lowering potential neural tube defects, no literature was found explaining how folic acid works on skeletal muscle cells. Due to a lack of research, folic acid was chosen and tested for its effects on the growth of C2C12 skeletal muscle cells. The initial hypothesis was that the folic acid would promote the growth and maturation of C2C12 muscle cells faster compared to a control of no folic acid added.

MATERIALS AND METHODS

Folic Acid Supplement Media

Using the CDC recommended 400 mg of folic acid for pregnant woman as well as the knowledge of an average of 5 liters of blood in the human body, calculations were done to find that 0.008% g/mL (w/v) of folic acid should be added to media [7]. The specific media was Dulbecco’s Modified Eagle’s Medium (DMEM) with 1% (v/v) penicillin/streptomycin (P/S) and 10% (v/v) fetal bovine serum (FBS). To make the solution, folic acid (Schiff Nutrition International, Salt Lake City, UT) was crushed into a powder using a mortar and pestle, weighed out, and added to the media. Initially, the folic acid did not go into solution and was placed into a water bath for 45 minutes at 37 °C. Again, the folic acid did not go into solution. More calculations were done to lower the concentration of folic acid from the CDC recommended value. Using 0.0047% g/mL (w/v) of folic acid, the steps were repeated, and the solution placed into the water bath. The folic acid successfully went into solution and was filtered through a Millipore Stericup® 0.22 µm filter to ensure that it was sterile and ready for use in further experimentation.

Cell Study

C2C12 skeletal muscle cells at passage 9 were cultured in a tissue culture flask along with DMEM containing 10% FBS and 1% P/S and placed inside the incubator under standard conditions (37 °C, 5% CO_2). The cells grew for 8 days. On Day 0, 1,000 cells at passage 10 were seeded into 16 different wells on two different 12-well plates. One well plate was used for a 7-day trial while the other well plate was used for a 10-day trial. Cells were fed with DMEM supplemented with 10% FBS and 1% P/S until day 2. Then, the first four wells on both plates were fed with the positive control DMEM media containing 2% horse serum and 1% P/S, while the second row was fed with DMEM containing 1% P/S, 10% FBS, and 0.0047% folic acid solution. The seeded wells were fed three times per week with the respective media and incubated under standard conditions.

MTS Assay

An MTS assay was run at a ratio of 1:5 MTS assay solution:media (Promega, Madison, WI). At days 7

and 10, the old media was removed from the wells and MTS assay solution to fresh media was added in a 1:5 ratio. The plates were placed into the incubator under standard conditions for 2.5 hours. The well plates were read using a Bioteck Epoch Spectrophotometer at 490 nm to measure absorbance in each well.

Fixing, Permeabilizing, and Staining Cells

After completing the MTS assays, the cells were washed twice with phosphate-buffered saline (PBS) with a pH of 7.2-7.4. The samples were then fixed using 3.7% paraformaldehyde solution in PBS and the wells were washed twice with PBS. Next, they were permeabilized using 0.1% Triton-X in PBS solution and were again washed twice PBS. The PBS solution was removed and 1% bovine serum albumin (BSA) in PBS was added to each well. Phalloidin staining solution was used to stain the cytoskeletal actin and DAPI to stain the nuclei. The phalloidin and DAPI stains were obtained from Life Technologies Corporation in Eugene, Oregon.

Fluorescent Microscopy

With the Olympus IX70 microscope equipped with an Olympus SC35 camera and Photometrics CoolSnap$_{cf}$, four views of each well were taken. To keep consistency, the four views were taken in roughly the same areas on each well. In Figure 1, the green circle represents the microscope view and the blue dots show roughly where each image was taken in a single well. At each field of view, two pictures were taken, one for the cytoskeletal actin and one for nuclei, at 100x magnification in the same position to ensure that the images would merge properly. Using the program Image J, all the images were edited for brightness and contrast [8]. The DAPI and phalloidin fluorescent images for each field of view were merged together. Using the same program, analysis was done on each merged image to determine myotube length and number which was recorded. A myotube is defined as a minimum of three cells that have fused together. The data was taken using the program Image J and compiled into different graphs on Microsoft Excel.

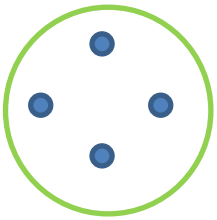

Figure 1: *Example of the four fields of view taken per well.*

RESULTS

Through a cell study, the effects of folic acid on C2C12 muscle cell maturation was investigated. Using an MTS Assay, data was compiled into figure 2 which shows absorbance vs. the two timepoints indicating cellular activity levels. The control group's mitochondrial activity stayed roughly the same with an absorbance of 0.156 ± 0.035 on day 7 and 0.155 ± 0.012 on day 10. The absorbance of the folic acid group was greater than the control on both days. It also increased from the day 7 mean absorbance of 0.353 ± 0.012 to 0.478 ± 0.052 on day 10.

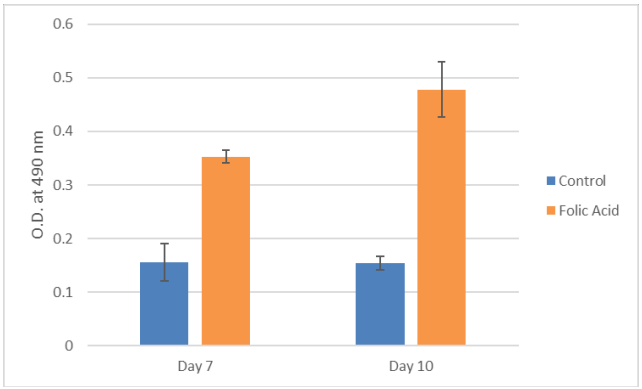

Figure 2: *Average absorbance values for control and folic acid (n=4).*

Figure 3 shows representative fluorescent images from both the control and folic acid groups at each timepoint. Myotube number and length were quantified for each group using the fluorescent images. Figure 4 shows a graph of total myotube number vs. timepoints for both the control and folic acid. The average myotube length vs. timepoints for both the control and folic acid is shown in Figure 5. For the day 7 control group, 72 total myotubes were counted throughout the images. The average length of the myotubes in this group was 131.26 $\pm$ 49.59 µm. The shortest myotube found was 48.51 µm and the longest was 283.80 µm. The day 10 control group had 41 myotubes counted with a mean length of 154.77 $\pm$ 68.74 µm and ranged from 79.68-406.18 µm. Control day 7 had more myotubes counted than day 10, but had a smaller average myotube length. Due to the standard deviations between the two days, there was large variability between the data.

For the folic acid day 7 group, 140 myotubes were counted with an average length of 95.55 $\pm$ 29.91 µm. The shortest myotube was recorded at 53.31 µm and the longest was 225.73 µm. In the folic acid day 10 group, 144 myotubes were counted with an average length of 119.79 $\pm$ 48.24 µm with a range from 47.73-303.38 µm. From day 7 to day 10, the number of myotubes stayed roughly the same; however, the average length of the myotubes

experienced a small increase from day 7 to 10. Again, the standard deviation shows a high variability in the data. Both timepoints of folic acid had a higher number of myotubes than the two timepoints of the control. On the other hand, both control timepoints had longer myotubes on average.

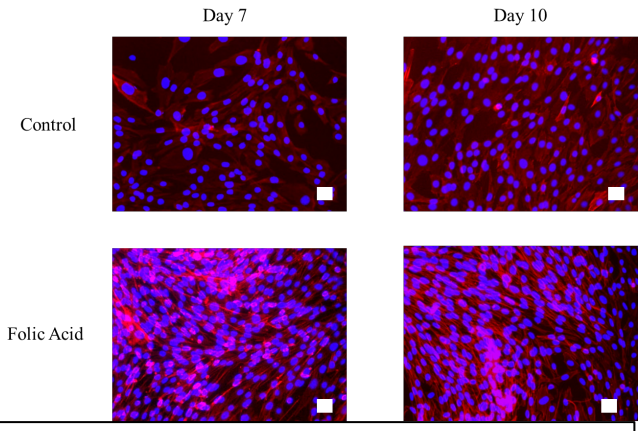

Figure 3: Representative images of Control and Folic Acid groups. Scale bar represents 75µm.

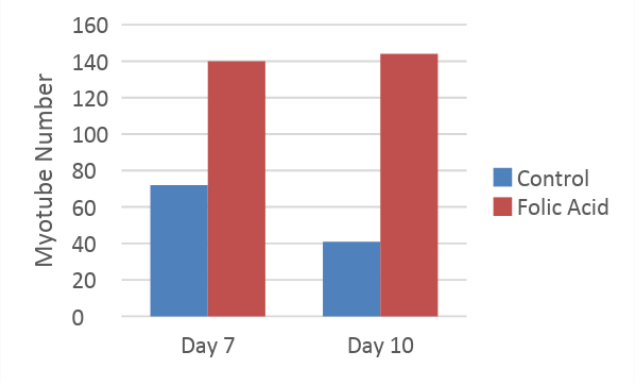

Figure 4: The total number of myotubes counted for control and folic acid supplemented cells.

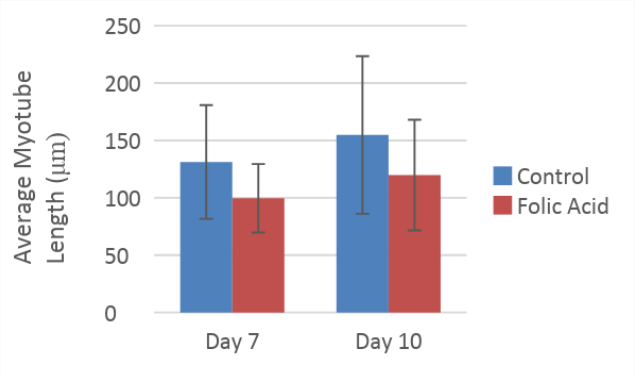

Figure 5: *Average myotube length for control and folic acid supplemented media.*

DISCUSSION

The folic acid data showed an increase in cellular activity and myotube formation over the 14-day period whereas the control only increased in average myotube length. The MTS assay data shows that mitochondrial activity stayed relatively the same between the control group on day 7 and day 10 (Figure 2). More importantly, the folic acid group showed an increase in cellular activity. Taken together, the data is indicative of a sustained and healthy cell environment.

The myotube number in the control group decreased from day 7 to day 10 (Figure 4). Likely, the smaller myotubes fused together, thus increasing the length. On the other hand, this could be a product of user error as the number of myotubes were interpreted by the researcher. However, the average myotube length of the control group increased from day 7 to day 10, which is to be expected because the DMEM with horse serum media used to feed the control group is known to promote cell maturation (Figure 5). The decrease in number and increase in length of myotubes is most likely a product of myotube fusion.

While the folic acid myotube number showed little to no increase from day 7 to day 10 (Figure 4), the number of myotubes stayed relatively the same between the two timepoints. Moreover, figure 5 shows an increase in myotube length from day 7 to day 10. Therefore, the myotubes that had formed already in the well continued to fuse with surrounding myoblasts and displayed an increase in cellular maturation. It must be noted that the myotube number may have stayed constant due to a high confluency in the wells. Moreover, analysis was done over multiple days which could have led to small discrepancies in what was considered a myotube.

CONCLUSION

The hypothesis that folic acid would promote more cell maturation than the control media was partially accepted. The data of the mean myotube lengths between the two groups overlap greatly when considering the high standard deviation and variability of the data. On the other hand, the folic acid timepoints both showed a much greater number of myotubes compared to the control group indicating that folic acid had a maturing effect on the cells. Moreover, there was an increase in cellular activity in the folic acid group. Due to the initial data and drastic myotube number difference between the two groups, folic acid should be considered for further research. Possibilities include investigating various folic acid concentrations including the CDC recommended value of 0.008% g/mL (w/v) using different techniques of solubilizing. The use of research grade folic acid is a possibility as there are concerns with the purity of the store-bought folic acid. Another possibility is increasing the time between the two timepoints to allow for more growth and maturation to further determine folic acids effects on skeletal muscle maturation.

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