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Age's Effect on Regenerative Capabilities of Myocytes Through Satellite Cell Analysis

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Abstract

The objective of this study was to investigate potential effects aging had on muscle fiber area and satellite cell count in myocytes. This research could help elucidate the detrimental effect age has on regenerative capabilities whether in terms of satellite cell function or satellite cell number. Satellite cells are primarily responsible for generating new muscle tissue after being activated through mechanotransduction of injury. This study utilized immunofluorescence to examine the presence of the PAX7 gene expression, a unique marker of satellite cells, within a 12 month and 18 month old population of mice. The PAX7 marker was co-stained with DAPI to identify nuclei and mark positive satellite cells. Fiber area was calculated with ImageJ image processing, and the satellite cells were counted by hand. The difference in fiber area of the two populations was negligible, but the satellite cell count was shown to be higher in younger populations.

Introduction

Volumetric muscle loss is a debilitating condition that is the result of the loss of musculoskeletal tissue within the body. Due to this loss of muscle tissue, the affected patients typically have severely reduced functionality in terms of both flexion and extension [3]. The individuals most commonly affected are those involved in traumatic injury such as war veterans, victims of motor vehicle accidents, or even patients undergoing major surgeries [7].

Under minor circumstances, the body does relatively well to repair injury, however, when substantial damage has been sustained to the musculoskeletal tissue, the body's normal healing mechanics are not sufficient to maintain functionality. To ameliorate this clinical issue, physicians and biomedical researchers are investigating tissue engineering as a possible solution [1]. To form new methods to synthesize new muscle, the natural muscle repair process must be fully understood. The key to understanding native muscle repair lies within satellite cells and their role within the body.

Satellite cells are a critical component in muscle regeneration, but in order to understand their importance, myogenesis must be examined in a broader context. Myogenesis is the formation of new muscle tissue, and there are two types of myogenesis, embryonic and adult. Embryonic muscle formation begins from the paraxial mesoderm with stem cells forming into somites which transition into dermamyotomes, and finally into myotomes [4]. The body then expresses multiple transcription factors

which stimulate the myotomes into myoblasts which ultimately derive the skeletal muscle. The muscle is generated by the fusion of mononucleated myoblasts into multinucleated myotubes [10].

In adults, without the pluripotentcy of stem cells, satellite cells step in to form new tissue. Satellite cells are quiescent myoblasts. They are located on the periphery of the basal lamina and the sarcolemma and become activated after experiencing mechanical stimuli such as high load or injury. After activation, they migrate to their associated muscle fiber to differentiate into myoblasts and begin the process of muscle generation anew [8].

From this analysis of myogenesis, it quickly becomes apparent that myosatellite cells must be able to regenerate themselves or they would deplete rather quickly. As mentioned earlier, the body can do this with minor injuries, but fails to do so with large traumas. **Figure 1** shows how satellite cells repair themselves in minor injury and **Figure 2** shows how fibrosis and extraneous extracellular matrix impedes muscle functionality.
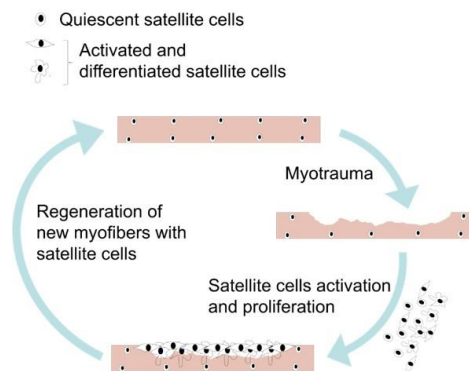
Figure 1: Depiction of healthy muscle regeneration process. The satellite cells are able to proliferate and construct new myofibers [9]
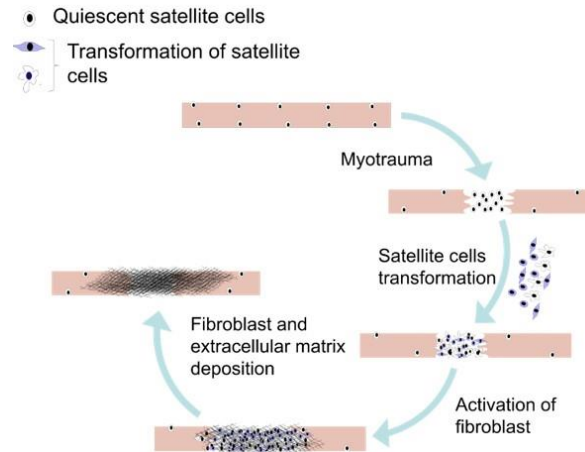
Figure 2: Depiction of traumatic injury repair. The fibrosis of the tissue does not allow for regeneration of satellite cells [9]

Taking a step back and looking from a clinical standpoint, patients who are younger typically recover better from injury than patients who are older [2]. In addition, it has been shown that aged populations typically have less muscle mass as well [6]. Assuming that the satellite cells are responsible for the majority of muscle regeneration, this peculiarity suggests that either satellite cell functionality or number is affected by age.

In order to closely examine satellite cells, immunofluorescence can be utilized. The PAX7 gene is uniquely expressed in satellite cells, and DAPI is a stain the allows for the staining of the nucleus [5]. Co-staining PAX7 with DAPI and identifying co-localizations allows for positive markers of satellite cells. The objective of this study was to determine if there was a significant difference in satellite cells and muscle fiber area in aged and young mice.

Methods

In order to investigate the effect age has on satellite cells, tibialis anterior (TA) muscle tissue and gastrocnemius tissue were harvested from mice aged at 12 months and at 18 months. A total of 10 samples were collected, 5 young samples and 5 old samples.

Tissue Preparation

This tissue was prepped using a novel cryopreservation technique to attempt to improve the quality of immunofluorescence images. The tissue was originally fixed by incubating in a 4% paraformaldehyde/10% formalin solution for 24 hours at 4 degrees Celsius. After the tissue was incubated, it was rinsed in 1x Phosphate Buffer Solution (PBS) for 30 minutes. The samples were then patted dry, with care taken not to press too hard on the samples. After this rinse, a 10% sucrose solution was made by mixing 3g of sucrose into 30 mL of PBS solution. This solution was thoroughly vortexed for 3 minutes to ensure solvation of the sucrose. The samples were allowed to incubate for 24 hours in this solution. After 24 hours, a 20% solution was made with 6g of sucrose into 30mL of PBS, and the samples were allowed to incubate in the 20% solution for an additional 24 hours. Finally, a 30% solution was made with 9g of sucrose into 30mL of PBS, and the samples were allowed to incubate for another 24 hours.

Cryopreservation

After the samples had been cryoprotected with a sucrose gradient, they were frozen in a minus 80 degree Celsius freezer. The samples were cut in half along the horizontal cross section to create top and bottom halves and also fit the samples into cryomolds. This maintained natural fiber alignment while freezing the tissue. The samples were then covered in Optimal Cutting Temperature (OCT) solution taking care to not allow air bubbles into the viscous solution. Any accidental air bubbles were removed via pressure by putting the samples into a vacuum chamber. The samples were then partially frozen by placing the bottom of the mold into a liquid nitrogen bath. Only the plastic mold was allowed to touch the liquid nitrogen, the tissue sample and OCT were kept out of contact with the liquid nitrogen. The OCT was allowed to partially freeze, but the last portion of solution was placed into a -80 degree Celsius freezer to complete freezing.

Sectioning

To examine the tissue under the fluorescence microscope, a cryostat was used to section the samples at 8 microns each. The samples were removed from the -80 degree Celsius freezer and placed into the cryostat and allowed to equilibrate at -20 degrees Celsius for 30 minutes. OCT was utilized to freeze and adhere the samples onto the sectioning plates that were then mounted on the slicer. The slices were slowly cut taking care to only place samples that had not been rolled on to the slides. VWR SUPERFROST

PLUS Microscope Slide Adhesion slides were used to obtain best binding to the slides from the frozen tissue. Ten slides with three sections were made for each sample.

Immunostaining

In order to visualize the co-localization of PAX7 and the nuclei, a PAX7 stain and DAPI stain were utilized. PAX7 utilized a primary antibody to adhere to the PAX7 protein and a secondary fluorophore to detect the protein in the samples. The protocol for staining involved rinsing the slides with PBS solution by soaking them for 5 minutes in PBS and then rinsing the slide with PBS solution. This process was repeated 3 times for each sample to ensure excess OCT was not present. Then slides were incubated in 0.1% Triton-x solution for 20 minutes. The triton solution served to permeabilize the cells so the antibodies could reach their target proteins. After triton, the samples were rinsed again in PBS for 5 minutes each repeated 3 times. The samples were then allowed to incubate in a block serum to prevent nonspecific binding of the proteins for 24 hours in the 4 degree Celsius refrigerator. After incubation, the block was discarded and then the primary antibody was placed onto the slides and allowed to incubate for a minimum of four hours. After incubation, the primary antibody was recollected and then the samples were rinsed 3 times with PBS. After this, the secondary antibody was added and allowed to incubate for 1 hour at room temperature. During this step, the samples were kept in a dark place to prevent photobleaching. After the secondary was added, the slides were rinsed again and the DAPI stain was applied to the slides for 7 minutes. After 7 minutes,

the samples were rinsed again using the PBS solution. After the stains were applied, the slides were considered photosensitive and shielded from all sources of light.

Imaging

Photographs of the slides were taken at both 358nm and 488nm excitation wavelengths to image DAPI and PAX7 respectively. The DAPI images were blue in color and the PAX7 images were green in color. ImageJ image processing was used to determine average fiber area of samples, and co-localization areas were counted by hand in samples. ImageJ processing involved taking the 20x image and opening it ImageJ, analyzing the image and setting the scale to 4.17 microns per pixel and applying it globally. This number was changed to 2.08 microns per pixel when examining 10x images. The brightness and contrast of the image were manipulated to create clear fibers from the background. The image was converted to an 8-bit image. The threshold was set and the slide bar was adjusted until the fibers were mostly filled with a red color. Image processing tools were utilized as needed for the pictures. The picture was then set by setting the measurement area and limiting the results to the chosen threshold. The particles were analyzed using the analyze particles function and the discrimination size was set to a range of 100-infinity pixels. The masks were then overlaid on top of each other with the holes not included and the edges of the image excluded. This highlighted the fibers of interest and allowed the data to be exported to an excel file that contained the area in microns. This data set was averaged for each

picture to get an average value for each image. This process was repeated 3 times for

each mouse and then the averages were compared for statistical significance.

Results:

The results of the stains were varied across the samples. Some stains worked
successfully as shown in **Figure 3**, while others failed to work correctly as shown in
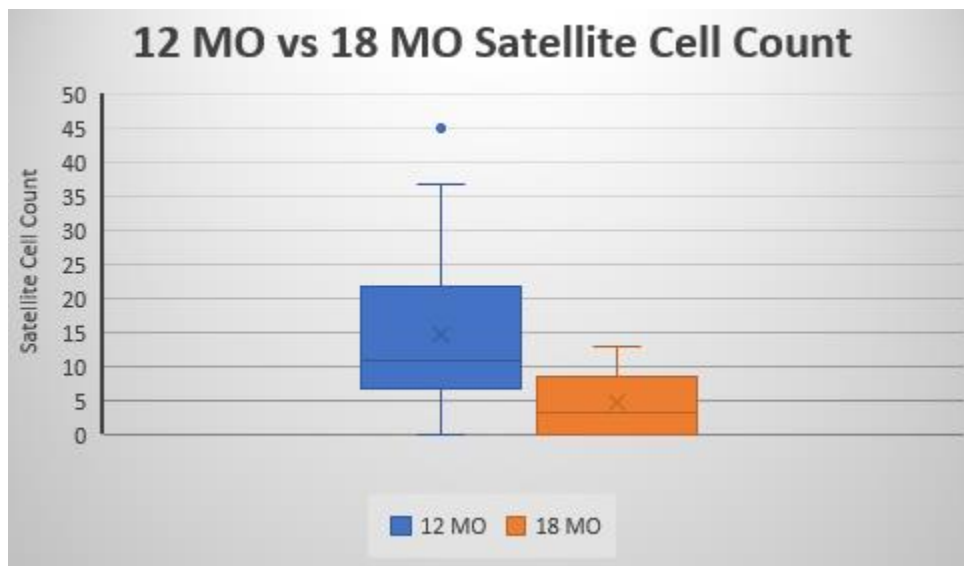**Figure 4.**
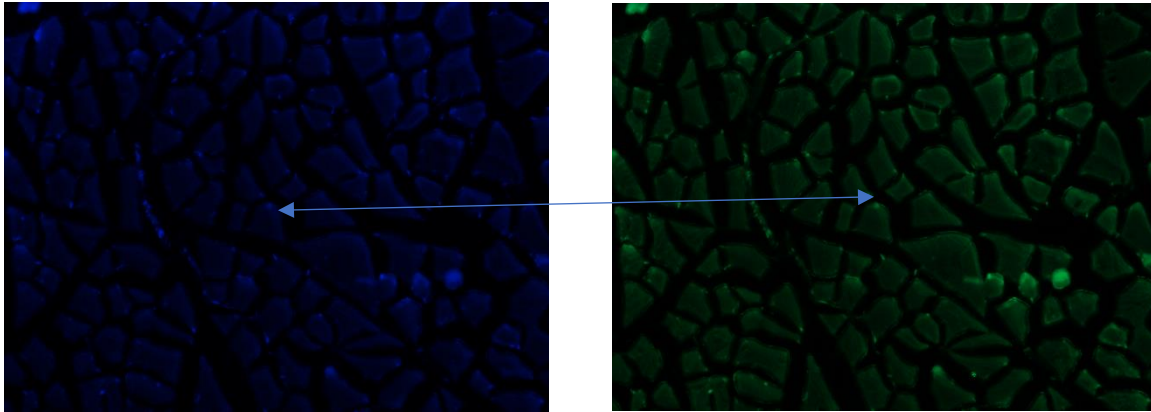
Figure 3: Depicts 12 MO 20x sample with easily identifiable PAX7 positive cells. The
arrow is indicating a PAX7 Positive cell.
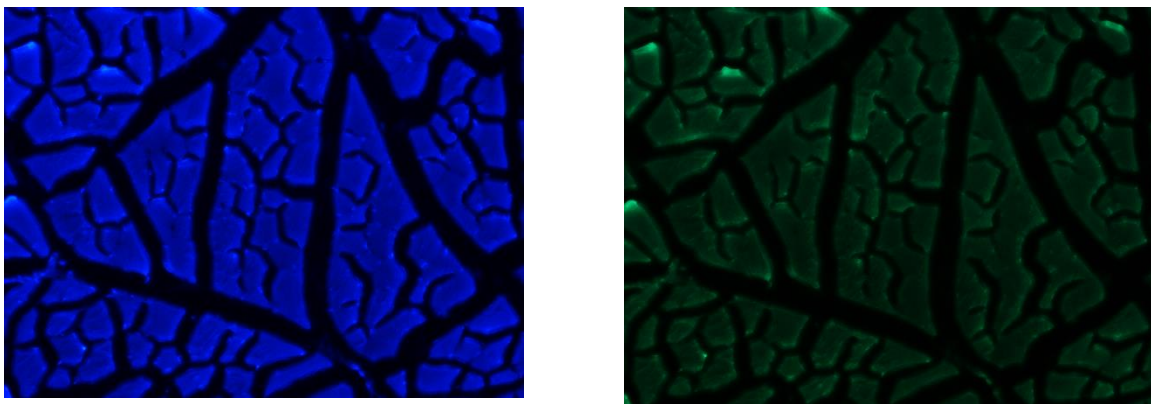
Figure 4: Depicts a 12 MO 20x sample with prevalent photobleaching. The nuclei
become very difficult to see due to background noise.
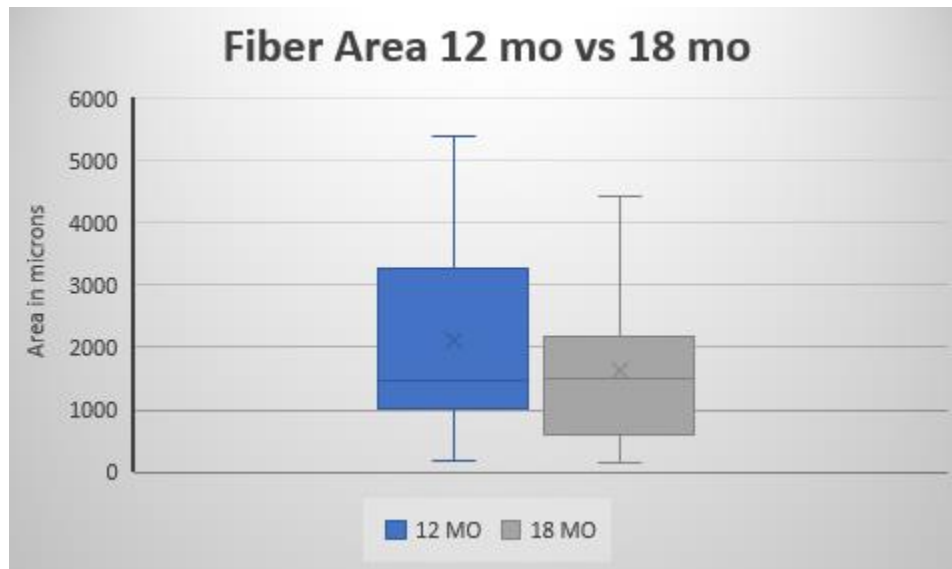
Figure 5: Depicts box and whisker plot of Fiber area. N=15. P = 0.265. Average for 12 MO = 2108.518 microns squared. Average for 18 MO = 1642.969 microns squared.

Figure 6: Depicts box and whisker plot of satellite cell count. N=16. P = 0.013. Average 12 MO =14.8125. Average 18MO = 4.875.

Conclusions

Figure 6 indicated that there was a statistical difference between the two samples with a P-value of 0.013 and supports the hypothesis that there is an increased number in satellite cells in a younger population of mice (12mo) in comparison to an older population (18mo). **Figure 5** supports that there was no statistical difference between the two populations in terms of muscle area fiber due to the P-value of 0.265.

The inconsistency of the staining results indicated a source of error in the results of the satellite cell experiment. Photobleaching washed out the signal of positive cells so many cells in the sample could have been miscounted therefore leading to an inaccurate reading. The slides initially had a clear readable signal, but under prolonged exposure to the microscope (~5 minutes) the background noise had become so prevalent, readable pictures could no longer be taken. One possible solution would be to use greater amounts of block to minimize the background noise in the sample and prevent nonspecific binding of the stains. The error could have also stemmed from the permeabilization stage that could have led to the failure of the block. Another key addition that could have been added to the protocol to improve the histological readability of samples would be mounting the slides in a cytoseal solution to preserve the stains better. If this experiment were to be repeated, it would help to have fresh reagents as the ones used in this experiment were dated and could have lost efficacy over time.

Overall, there are a lot of challenges in determining satellite cell count through the use of PAX7 staining. For future research, histological readability and reliability of stains are two huge factors that could be improved upon to obtain better results in regards to this study.

References

1. Bian W, Bursac N. Tissue engineering of functional skeletal muscle: challenges and recent advances. IEEE Eng Med Biol Mag 2008;27:109-13.

2. Gould L, Abadir P, Brem H, et al. Chronic Wound Repair and Healing in Older Adults: Current Status and Future Research. Journal of the American Geriatrics Society. 2015;63(3):427-438. doi:10.1111/jgs.13332.

3. Grogan, Brian F., and Joseph R. Hsu. Skeletal Trauma Research Consortium. "Volumetric muscle loss." Journal of the American Academy of Orthopaedic Surgeons 19. suppl 1 (2011): S35-S37

4. Hollway GE, Currie PD. Myotome meanderings. Cellular morphogenesis and the making of muscle. EMBO Rep. 2003;4:855–860.

5. Sambasivan R, Yao R, Kissenpfennig A, Van Wittenberghe L, Paldi A, Gayraud-Morel B, Guenou H, Malissen B, Tajbakhsh S, Galy A. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. Development. 2011 Sep 1;138(17):3647-56.

6. Seene, T. and Kaasik, P. (2015) Age-Associated Changes in Skeletal Muscle Regeneration: Effect of Exercise. Advances in Aging Research, 4, 230-241. doi: 10.4236/aar.2015.46025.

7. Wu, Xiaowu, et al. "A standardized rat model of volumetric muscle loss injury for the development of tissue engineering therapies." BioResearch open access1.6 (2012): 280-290

8. Yin H., Price F., Rudnicki M.A. Satellite cells and the muscle stem cell niche. Physiol. Rev. 2013;93:23–67. doi: 10.1152/physrev.00043.2011

9. Yoseph B, Soker S. Redefining the satellite cell as the motor of skeletal muscle regeneration. J-ournal of Science and Applications: Biomedicine. 2015; 03:76-82

10. Zammit, Peter S., Terence A. Partridge, and Zipora Yablonka-Reuveni. "The skeletal muscle satellite cell: the stem cell that came in from the cold." Journal of Histochemistry & Cytochemistry 54.11 (2006): 1177-1191.

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