UNDERGRADUATE SUMMER VACATION SCHOLARSHIP AWARDS – FINAL SUMMARY REPORT FORM 2016/17

NB: This report will be posted on the Society's website therefore authors should NOT include sensitive material or data that they do not want disclosed at this time.

Name of student:

Jamie Whitfield

Name of supervisor(s):

Professor Malcolm Logan

Project Title: (no more than 220 characters)

Exploring the role of irregular connective tissue in limb muscle development and repair.

Project aims: (no more than 700 words)

This study involved inducing injury to murine tissue, specifically, the tibialis anterior (TA) to visualise the destruction of the muscle connective tissue and its associated extracellular matrix. We injected the murine models with either two substances; cardiotoxin (CTX) or benzalkonium chloride (BAC) into the TA of the left hindlimb. Injecting the TA of the right hindlimb with a PBS solution, allowed us to identify a control experiment, ensuring the validity of the experiment. The forelimbs were unaffected in this study. CTX is a Protein Kinase C (PKC)- inhibitor, binding depolarises the skeletal muscle membrane, resulting in an increased contraction of the muscle cells. Affecting the potential difference of the cell membrane causes myofibre necrosis, although the surrounding connective tissue is unaffected. Due to its specificity, CTX is a useful tool in studying the regeneration and repair of muscle. However, its use is limited as it does not allow us to investigate specifically the role of muscle connective tissue (MCT).

BAC is a non-immunogenic, pancytotoxic surfactant, resulting in necrosis of myofibres and cells within the MCT. In this study, we injected the murine tissue with 0.5% BAC, which caused cell death throughout the muscle. Although BAC has been used to study the effect of cell ablation in many other tissues, this study is the first to look at its effect in the destruction of extracellular matrix (ECM) and muscle regeneration.

This study allows us to compare the repair of muscle following BAC and CTX injury to the murine TA. Our primary aim is to discover the function of MCT and how this contributes to muscle repair after injury. Our hypothesis is that damaging the MCT will disrupt the muscle repair process.

In this experiment, we examined the muscle tissue at different times from 3d to 28d when the muscle repair process is thought to be complete, however, there are varying opinions on how long the process lasts. We focused on muscle repair specifically at 3d, 5d, 7d, 10d, 14d and 28d following CTX and BAC-induced injury. The myofibre regeneration process was analysed by the expression of eMHC, the clearance of collagen deposited imeediately following injury (acute phase) and the diameter of the myofibres by 28d in both models. In addition to this, we expect to see eMHC expression more frequently in the later stages of BAC-injury models, along with a slower clearance of collagen and a slower re-establishment of myofibre diameter, reflecting the delayed repair process induced by damage to the connective tissue.

Project Outcomes and Experience Gained by the Student (no more than 700 words)

Tissue Preparation

I became competent in methods to prepare muscle tissue samples to retain as best possible their original anatomy. After careful dissection of TA muscles, the tissue was embedded in OCT compound and isopentane before being flash-frozen in liquid nitrogen. I also gained competence in using the cryostat machine to generate serial transverse cross-sections.

Immunohistochemistry

I learnt how to carry out immunohistochemical staining techniques. this started by washing the transverse sections with PBS and then blocking them with blocking solution for 30 mins. The primary antibodies were added to the blocking solution and then applied to the sections at 4ºC and incubated overnight. I then washed the sections three times for 10 minutes each in 0.05% Tween 20/PBS [Sigma]. The secondary antibodies were also prepared in blocking solution and applied to the sections for 1 hour at room temperature. It was important, after this step, to keep the sections protected from light as the secondary antibodies were light sensitive. After this, I washed the sections with 0.05% Tween 20/PBS and added DAPI for 5 minutes, before washing the sections again for 5 mins in PBS and adding coverslips. Coverslips were mounted using Dako Fluorescent Mounting Medium [Dako], with sections left to incubate at 4ºC until dry. All procedures were carried out at room temperature, unless specified.

Picrosirius Red Staining

The cryosections were air-dried at room temperature for 1 hour, before being treated as paraffin-embedded sections. I treated the sections twice with Xylene in the fume hood for 10 minutes each. After this, the sections were hydrated in descending concentrations of alcohol (100%, 70%, 30% and then purified water) for 10 seconds each. I then stained the sections with Picrosirius red solution for 1 hour before being quickly rinsed using acetic acid. Sections were then dehydrated in ascending concentrations of alcohol. The sections were cleared in two washings using Xylene for 10 minutes each. Finally, coverslips were then mounted using DPX Mountant. All procedures were performed at room temperature.

Imaging

For the immunostained sections, images were obtained on a Leica DM8i microscope using 10x objectives. The images were obtained using Leica LAS-X software. By using the tilescan option, I was able to photograph the entire section. The images of the eMHC, laminin and DAPI staining were tiled to view the entire section with all staining. For Picrosirius Red stained sections, the same process was used to photograph the section in its entirety.

All the images were adjusted for brightness and contrast.

Quantification

Quantification of the cells with eMHC expression, where counted manually using 3 unit areas located across the whole image of the section. The mean cross-sectional diameter of the centro-nucleated regenerating myofibres was obtained, using the same 3 areas, by measuring the Feret diameter of each fibre with the software, ImageJ. Feret's diameter of the non-injured peripherally nucleated myofibres, was measured identically to act as a control. The quantification process was completed across three sections for each condition per time point. This was repeated for three biological samples for 3d, 5d, 7d and 10d and 14d and 28d overall.

Quantification of the muscle connective tissue was obtained using the area stained with the PicroSirius Red using ImageJ. Images were converted to a binary image and threshold was adjusted for the red-stained laminin. The area that stained red was measured and presented as a percentage of the total section area. The quantification process was also completed across three sections for each condition per time point. This was repeated for three biological samples for 3d, 5d, 7d and 10d and 14d and 28d overall.

Statistical Analysis

Data from 3 mice per condition per time point were represented as a mean of the three biological replicates using Excel. This was used for eMHC expression, size of regenerating myofibres and amount of laminin. Tables were used to represent the significant differences between condition and time points.

Outcomes

Overall our data suggest that muscle repair following damage to the muscle tissue with CTX or BAC takes longer than 28 days. The muscle begins the regeneration period at 3 days post injury and finalises between 14 and 28 days post injury. The remodelling phase succeeds this and we have found this takes longer than 28 days post injury. Further studies are required to establish the true time-frame of the remodelling phase. Significantly, in this study, we also established that by injuring the cells in the MCT, by injecting BAC, lead to delayed muscle repair. In BAC injured muscle, the myofibres took longer to regenerate. This was highlighted as eMHC expression peaks around 10d compared to 5d in the CTX injured tissue. Although, following injury with BAC and CTX; the myofibres are full regenerated by 28 days post injury, these are much smaller and have more collagen deposition in the BAC injured tissue. These results are likely to occur due to significant muscle repairing events such as the delay in such as deregulation of anti-inflammatory macrophages and the damage to fibroblasts and other cell populations residing in the MCT.

Please state which Society Winter or Summer Meeting the student is intending to present his/her poster at:

Summer Meeting

Proposed Poster Submission Details (within 12 months of the completion of the project) for an AS Winter/ Summer Meeting – (no more than 300 words)

I propose to complete the poster by January 2018, I will then discuss to Professor Malcolm Logan that my poster is acceptable, covering enough information to gain interest without including sensitive material. I think it will be appropriate to present the poster to Malcolm and the group, answering any question they have before submitting my poster and presenting in the Summer Meeting 2018.

Brief Resume of your Project's outcomes: (no more than 200-250 words).

The title of your project and a brief 200-250 word description of the proposed/completed project. The description should include sufficient detail to be of general interest to a broad readership including scientists and non-specialists. Please also try to include 1-2 graphical images (minimum 75dpi). NB: Authors should NOT include sensitive material or data that they do not want disclosed at this time.

Exploring the role of Muscle connective tissue (MCT) in muscle repair.

In my Project, I studied the role of a population of cells that surround muscle fibres and muscle bundles called muscle connective tissue (MCT). The MCT is thought to be important for muscle formation and repair and disriuption of the MCT has been implicated in muscle disease. We have little understanding of exactly how MCT carries out is functions ort how they have been disrupted in disease. To demonstrate the importance of MCT on muscle repair and to begin to understand what it is doing during the repair process we analysed muscle tissue injured in different ways using either of two chemicals, CTX and BAC. CTX is a Protein Kinase C (PKC)- specific inhibitor that depolarises skeletal muscle membrane resulting in death of the cell, while other cells and tissues are unaffected. BAC is a non-immunogenic, pancytotoxic surfactant that will kill all cell types when injected in muscle tissue. By comparing the repair process in both conditions we are able to compare the presence or absence of MCT on the process of muscle repair. I studied the repair process by analysing the expression of a markers of newly formed muscle tissue, embryonic Myosin Heavy Chain (eMHC), the rate of diameter growth of the repairing myofibres and the remodelling of extracellular matrix molecules (collagen) laid down during the acute phase of injury that are removed as the muscle repairs itself.

I was able to show that rate of muscle repair and quality of muscle repair was reduced in the absence of MCT cells.

Other comments: (no more than 300 words)

I am unable to attend the Winter Meeting in Dundee. Although dates have not been posted for the Summer meeting, it is more likely I can attend this to present my poster.

Signature of student. ..Date.16-10-17

Signature of supervisor Date.16-10-17

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