Modified Method For Assessing Cell Viability In Biopsied Skeletal Muscle Tissue Using Viability Dyes And Confocal Microscopy Imaging In Progressive Muscle Hypoxia Research

Jun Wei LIM[1,2], Derek Ball[2], Alan J Johnstone[1,2], Andrew Schmidt[3]
[1]Aberdeen Royal Infirmary, Aberdeen, UK, [2]University of Aberdeen, Aberdeen, UK, [3]Hennepin County Medical Center, Minnesota, USA
junwei.lim@nhs.scot

Disclosures: None

INTRODUCTION: Acute compartment syndrome (ACS) is a progressive form of muscle ischemia that is a surgical emergency and can have detrimental outcomes for patients if not treated optimally. The current problem is that there is no clear diagnostic threshold for ACS or guidance as to when fasciotomies should be performed. A new diagnostic method is necessary to provide real-time information about the extent of muscle ischemia in ACS. Given that lactic acid is produced by cells through anaerobic respiration, it may be possible to measure H^+ ion concentration and to use this as a measure of ischemia within muscle. Although we are familiar with the key biochemical metabolites involved in ischemia; and the use of viability dyes in cell culture studies to distinguish between living or dead cells is well recognized; research has not been undertaken to correlate the biochemical and histological findings of ischemia in skeletal muscle biopsies. Our primary aim was to investigate the potential for viability dyes to be used on live skeletal muscle biopsies (explants). Our secondary aim was to correlate the intramuscular pH readings with muscle biopsy viability in progressive muscle hypoxia research.

METHODS: Nine euthanized Wistar rats were used. A pH catheter was inserted into one exposed gluteus medius muscles to record real-time pH levels and muscle biopsies were taken from the contralateral gluteus medius at the start of experiment and subsequently at every 0.1 of pH unit drop. Prior to muscle biopsy, the surface of the gluteus medius was painted with a layer of 50µmol/l Brilliant blue FCF solution to facilitate biopsy orientation. A 4mm punch biopsy tool was used to take biopsies. Each muscle biopsy was placed in a base mold filled with 4% ultra-low melting point agarose. The agarose embedded tissue block was sectioned to generate 400 micron thick tissue slices with a vibratome with lowest speed (0 speed) and highest amplitude (10 amplitude). The tissue slices were then placed in the staining solution with 1mg/ml Hoechst 33342, 2mmol/L Ethidium homodimer-1 and 4mmol/L Calcein am. The confocal microscopy images were obtained using Zeiss LSM880 confocal microscope and ZEN acquisition software (Black edition, Zeiss) in 10x magnification and the smart setup settings in the program. The images were obtained in 16-bit format. The images were processed with Z-Stack and maximum intensity projection methods. To create a positive control (dead cells), 1ml of 0.05% Triton X-100 was added. To enable analysis of the image values, the images were then transferred to Image J where the image's contrast was adjusted but not saved to ensure that the pixel values remained unaffected (Figure 1 and 2). Individual cells were outlined with the selection tool in Image J. The mean gray value, representing the average gray value within the area of selection, was obtained. With the positive control, the threshold value for live cells in the green channel (Calcein AM) can be identified.

RESULTS SECTION: Viability dyes, used primarily for cell cultures, can be used with skeletal muscle explants. A total of 84 slides was examined. Among them, a total of 3996 cells was outlined. The combination dyes of calcein AM and Hoechst 33342 is a reliable method for measuring cells viability in skeletal muscle explants. The live cells displayed green fluorescence and easily distinguishable from dead cells where no green fluorescence was visible in the confocal images despite enhancing the green channel contrast (Figure 3). Majority of skeletal muscle cells were viable throughout the experiments, even at pH 6.0 and after 60 minutes of ischemia.

DISCUSSION: Our study also showed that despite a significant reduction in tissue pH concentration over time, that almost 100% of muscle cells were still viable at pH 6.0, suggesting that skeletal muscle cells are robust to hypoxic insult in the absence of reperfusion. A three-colour assay was used for simultaneous fluorescence staining of viable cells (Calcein AM), dead cells (EthD-I) and total cells (Hoechst 33342), but it was not possible to identify the dead cells with EthD-I. Skeletal muscle composed of individual multinucleated myofibres with nuclei positioned at the periphery and can contain hundreds of nuclei distributed across the cell surface. As each cell has different number of nuclei, it was not meaningful to calculate the percentage of EthD-I stained nuclei by the total nuclei count. Therefore, another way of quantifying the dead cells was used. The individual cells were outlined by following the nuclei stained with Hoechst 33342, and the mean gray value for calcein AM was obtained for both the positive and negative controls. From there, the threshold for live cells was determined.

SIGNIFICANCE/CLINICAL RELEVANCE: (1-2 sentences): Viability dyes can be used on skeletal muscle biopsies. Further research investigating the likely associations between direct measured pH using a pH catheter, the concentrations of key cellular metabolic markers, and muscle tissue histology using vitality dyes in response to ischemia, rather than hypoxia, is warranted.

ACKNOWLEDGEMENTS: The authors gratefully acknowledge the Microscopy and Histology Core Facility, University of Aberdeen, for their support & assistance in this work.

IMAGES AND TABLES:
Figure 1: The composite confocal image before contrast enhancement in Image J.
Figure 2: The composite confocal image after contrast enhancement in Image J.
Figure 3: The composite confocal image of positive control (dead cells).

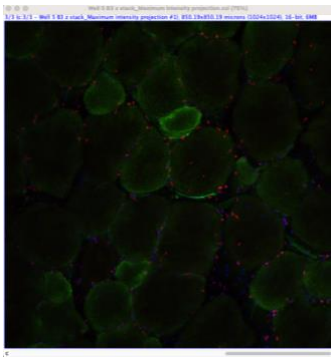

Figure 1

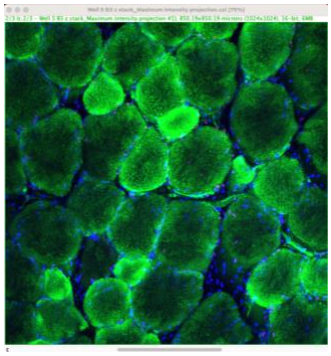

Figure 2

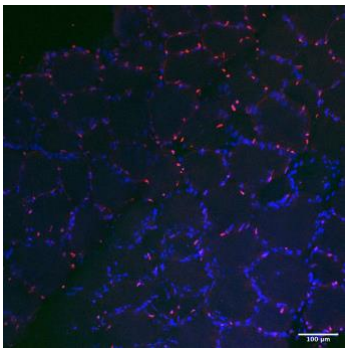

Figure 3