Investigation of Blood-mediated ACL Fibroblast $[Ca^{2+}]_i$ Shear Response and GPX4 Expression

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INTRODUCTION. The anterior cruciate ligament (ACL) is the most commonly injured ligament in the knee, with about 400,000 surgeries occurring each year.[1] Once the ACL ruptures, it does not spontaneously repair due to synovial fluid surrounding the joint space and mechanical stress exhibited during load-bearing.[2] Even after surgical intervention, the ACL still exhibits a poor healing capacity with increased incidence of post-traumatic osteoarthritis (PTOA).[3,4] ACL injury is typically accompanied by joint bleeding, but the individual contributions of whole blood components to joint damage are not fully understood.[5,6] Articular bleeding, known as hemarthrosis, has been shown to induce joint inflammation further contributing to the deterioration of other joint tissues, including synovium and cartilage.[6,7] Furthermore, maintaining iron homeostasis in the bloodstream plays a pivotal role in joint health.[8] Excess iron deposition can result in oxidative stress damage which is closely associated with conditions such as hemophilic arthropathy and osteoarthritis.[9,10] Earlier studies indicate that immediate blood exposure following injury elevates the production of pro-inflammatory cytokines and extracellular matrix (ECM) remodeling factors in joint tissues.[5] While blood itself may not persist in the joint, remnants of erythrocytes including hemoglobin and deposited iron (hemosiderosis) may have longer lasting or permanent effects.[5] This has been highlighted in the context of an iron-dependent, nonapoptotic cell death pathway known as ferroptosis.[11] To further characterize the effects of iron deposition following injury, the current study investigates the influence of red blood cell (RBC) lysates on human ACL fibroblast (ACLF) mechanosensitivity as well as glutathione peroxidase 4 (GPX4) expression, an enzyme that is a key regulator of ferroptosis.

METHODS. Erythrocytes from O-positive whole human blood (New York Blood Center) was separated using the Ficoll-Paque technique. Isolated RBCs were lysed to extract intracellular blood components. ACLF Culture: Healthy human ACL grafts were obtained from the Musculoskeletal Transplant Foundation (Edison, NJ) and cells were isolated via collagenase digestion. ACLF were expanded until confluency in DMEM supplemented with 10% fetal bovine serum (FBS) and 5 ng/ml FGF-2 followed by lysed blood treatment for 4 days. Previous dose response experiments have shown that 20% v/v is the minimum blood concentration to induce significant cell toxicity, but physiological concentrations following injury can be even greater. Therefore, lysed blood was added to culture media at 20% v/v with parallel osmotic control lysates. Photoacoustic Imaging: To quantify hemoglobin release in culture, photoacoustic (PA) absorbance spectra was measured across various blood components including intact RBCs and lysed groups. PA signal averages were obtained for 20% v/v blood treated samples to assess the effects of the lysed treatment.[12] Calcium Imaging: ACLFs were plated in silicone isolators on collagen-coated glass slides at a cell density of 4 x 10^4 cells/cm^2. Cells were pre-conditioned in 20% v/v lysed and osmolarity-controlled lysed blood groups, with parallel untreated controls. To track changes in intracellular calcium ($[Ca^{2+}]_i$), ACLF were stained using 5 μM Fura Red. Fluid flow-induced shear stress was applied in a parallel plate flow chamber at 0.1 Pa (**Fig. 2A**). Unidirectional flow experiments were conducted using Hank's Buffered Salt Solution (HBSS) supplemented with 0.1% FBS (n=100 cells/slide, 3 slides/treatment). Fluorescence intensity versus time measurements were taken for individual cells, where increasing $[Ca^{2+}]_i$ resulted in decreased fluorescence. Cells were considered responders if $[Ca^{2+}]_i$ increased 20% above baseline equilibrium measurements. Percentage of responding ACLFs was determined using a custom Matlab (MathWorks) code.[13,14] GPX4 expression: Following lysed blood exposure, ACLFs were stained with anti-GPX4 antibody (Abcam) with Phalloidin (Abcam) with and DAPI (Life Technologies) for cytoskeletal and nuclear visualization respectively. RNA was isolated from the same blood treated ACLF groups and GPX4 expression was assessed via qPCR. Target genes were normalized to GAPDH and control sample values using the $2^{\Delta\Delta CT}$ method. Statistical analysis: Differences across lysed blood groups and controls were analyzed via an ANOVA with Tukey post-hoc test for multiple comparisons using GraphPad Prism 9. Significance was determined at $p < 0.05$ for all output measures.

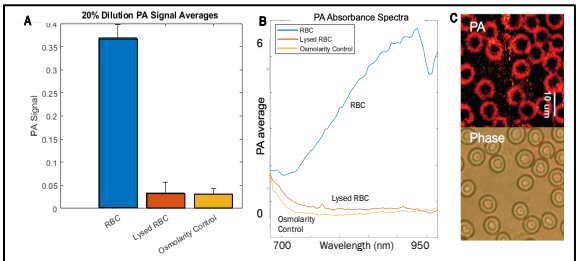

Figure 1: Photoacoustic imaging (**A**) signal intensity and (**B**) absorbance spectra for intact RBCs and lysates. (**C**) Representative PA image of blood smear showing RBC with corresponding bright field image (100X).

RESULTS. Photoacoustic average signal intensity and absorbance spectra were significantly decreased in blood lysate groups compared to intact RBCs (**Fig. 1A-B**). ACLFs $[Ca^{2+}]_i$ response to shear stress was significantly lower in 20% v/v lysed samples compared to non-blood treated controls with 31.7 ± 11.7% and 84.0 ± 11.1% responders, respectively (**Fig. 2B**). Lysed blood and osmolarity-controlled groups responded similarly, suggesting no osmolarity effects. ICC staining showed decreased expression for GPX4 following lysed blood exposure compared to non-blood treated controls (**Fig. 3A-B**). qPCR analysis yielded similar results with reduced GPX4 expression in ACLFs treated with lysed blood at day 2 (**Fig. 3C**; $p<0.0001$).

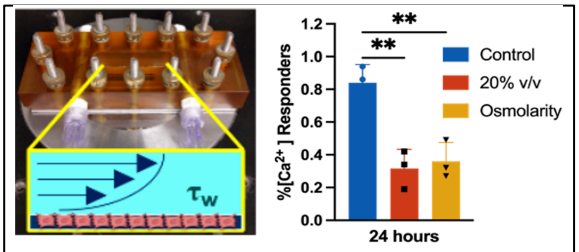

Figure 2: (**A**) Calcium imaging experimental setup with parallel plate flow chamber. (**B**) ACLF $[Ca^{2+}]_i$ response to shear stress following lysed blood exposure for 24h with osmolarity-treated controls. **p<0.01

DISCUSSION. The decreased photoacoustic signal intensity for the lysed blood samples may be indicative of greater hemoglobin release compared to intact RBCs. $[Ca^{2+}]_i$ imaging yielded similar results with decreased ACLF responders with lysed blood treatment suggesting diminished cellular activity. This reduced mechanosensitivity to shear stress may further contribute to downstream effects implicated in the ferroptosis pathway. Downregulation of GPX4, known for converting peroxidized lipids into lipid alcohols, suggest lysed blood exposure may activate ferroptotic cell death.

SIGNIFICANCE/CLINICAL RELEVANCE. These studies showed that decreased ACLF mechanotransduction and a maker of ferroptosis were linked to lysed blood exposure. Future work will aim to optimize clinical intervention and/or therapeutic targets to mitigate or reverse the negative effects of intra-articular bleeding following joint injury.

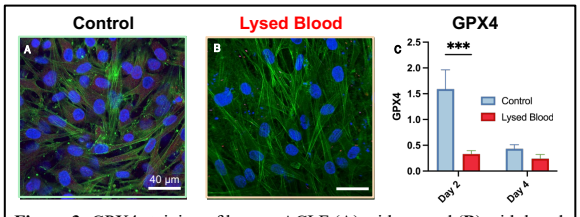

Figure 3: GPX4 staining of human ACLF (**A**) without and (**B**) with lysed blood exposure. Anti-GPX4 (red) with ACLF cytoskeleton (green) and nuclear (blue) counterstaining. (**C**) GPX4 gene expression of ACLF following lysed blood treatment at 2 and 4 days. ***p<0.0001

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