Elucidating the Role of Blood Constituents in ACL Injury

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INTRODUCTION. Anterior cruciate ligament (ACL) injury is typically accompanied by joint bleeding, but the individual contributions of whole blood components in the joint are not fully understood.[1,2] Hemarthrosis, or articular bleeding, has been shown to induce joint inflammation and has been implicated in the degeneration of various joint tissues, such as cartilage and synovium.[3] Additionally, iron homeostasis is vital to joint health as excess iron content can lead to oxidative stress damage which is closely associated with osteoarthritis and hemophilic arthropathy.[4] Previous work has shown that acute blood exposure increases pro-inflammatory cytokine production and cellular damage.[1] Initial research has also shown that TGFβ-1 is typically elevated in injury and plays an important role in fibrotic scarring during the wound healing process.[5] Here, we analyze the contributions of whole blood, intact red blood cells (RBCs), and lysed RBCs on the ACL in the context of biochemical content, oxidative stress, and various cell death pathways including ferroptosis, a specific type of cell death characterized by iron dependency and oxidative stress. We employed the use of a 3D collagen gel contraction assay as a measure of wound healing capacity. Using the contraction assay, this study will assess the impact of acute short-term blood exposure on the contractile phenotype of ACL fibroblasts (ACLF) as a measure of cell function and viability. We hypothesized that human ACLF seeded collagen constructs exposed to lysed RBCs and by extension, iron, will exhibit lower rates of contraction and increased forms of cell death and inflammation.

METHODS. RBC and ACL Fibroblast Culture: Whole human blood (O+, New York Blood Center) was separated via the Ficoll-Paque separation technique. Healthy human ACL was obtained from the Musculoskeletal Transplant Foundation (Edison, NJ). ACL fibroblasts were isolated via enzymatic digestion and cultured with various blood concentrations for 4 days. Previous dose response experiments have shown that 20% v/v is the minimum blood concentration to induce significant cell toxicity. Therefore, isolated intact RBC, lysed blood, and whole blood were added to CM at 20% v/v. Cellular Collagen Gel Assay: To study collagen contraction, human ACLF were cultured at 3520 cells/cm^2 until confluency in 10% DMEM supplemented with 5 ng/ml FGF-2. To cast collagen gel, 5X DMEM, rat tail type I collagen, 1N NaOH, and cell suspension were combined until homogeneously mixed. After solid gels formed at 37°C for 60 minutes in 24 well plates, 2 mL of experimental blood treatment group was added to each well. Wells were treated with CM (control), CM + TGFβ1 (1 ng/ml), and corresponding blood experimental groups (20% v/v). Images were collected at day 0, 1, 2, 3, 4 following gel preparation. Using FIJI, percent contraction was measured over time. Biochemistry: At day 4, collagen constructs were assessed for biochemical content (DNA and collagen). Collagen was normalized to DNA content. Gene expression: ECM genes: COL1, COL2, COL3 and proinflammatory gene: IL6 were measured in cell culture media compared to control using the $2^{-\Delta\Delta CT}$method. Target genes were normalized to GAPDH and CM values. Statistical analysis: Biochemical content and gene expression were analyzed with ANOVA on GraphPad Prism 9 with a Tukey post-hoc for multiple comparisons, and significance was determined at $p < 0.05$.

RESULTS. During the 3D collagen gel contraction assay, each condition was monitored over time to assess cell viability and the rate of contraction over time. Live/dead staining highlighted a larger number of dead cells in the lysed blood group demonstrating that this treatment group appears to induce more cell toxicity in comparison to other treatments **(Fig. 1K-O).** Although there were significant contraction differences between each treatment group by day 4, the intact RBC and whole blood treatment groups exhibited the lowest rates of contraction in comparison to the control group (**Fig. 1A-J, 1Q**; c = p<0.05: control vs. intact RBC; p=0.0005 for day 3 and p<0.0001 for day 4). Blood treatment significantly decreased DNA content in comparison to the control groups (**Fig. 2A**; p<0.0001 for day 4). Interestingly, the lysed group had the highest amount of DNA content in comparison to the whole and intact RBC treatment groups. Analysis of collagen content showed that there was significantly higher collagen content in the TGFβ-1 group in comparison to the control (**Fig. 2B**; p<0.0001 for day 4). There was no significant variability observed in COL1-3 activity between the treatment groups, but there were significant differences in IL6 activity between the TGF and whole blood treatment groups after 4 days in culture **(Fig. 3A-D**; p<0.05 for day 4).

DISCUSSION. The impact of blood on animal ACLF has previously been reported[6]. In the current study using human cells, we partially accept our hypothesis, with ACLF seeded collagen constructs exposed to lysed RBCs exhibiting more cell death and inflammation while not exhibiting lower rates of contraction compared to intact RBCs. Greater cell death in the lysed blood treatment group may indicate that blood lysing significantly induces ACLF cytotoxicity and potentially lowers their rate of cellular proliferation. After 4 days, the whole blood showed the least amount of contraction which could be due to a significant increase in fibrosis and DNA content, and may suggest that blood exposure may lead to induction of ferroptosis and inhibited contractility which can delay wound healing. Collagen production was significantly increased in the whole blood treatment group in comparison to all treatment groups. This potentially means that other blood constituents in whole blood aside from RBCs induce significant fibrosis within the wound healing response. The lysed blood treatment group did not exhibit as much collagen production which could reflect the lack of secretory factors by RBCs and other blood constituents. Although, the mechanisms behind the RBC secretory pathway are not currently well understood, previous research has shown that RBCs have a secretome and are capable of sending and receiving signals from other cell types that can further stimulate the release of pro-inflammatory markers and cytokines.[7] Our findings substantiate past work which highlighted that culturing lung fibroblasts + RBC conditioned media stimulated inflammatory signaling and corresponding mRNA expression.[7] Blood treatment did significantly induce inflammation through upregulation of IL6. Furthermore, upregulation of COL1, COL2, and COL3 suggest presence of RBCs and blood constituents increases general collagen production, indicative of fibrosis. Additionally, these experiments highlight that different blood constituents have varied effects on cell function and viability. In addition to ferroptosis, various other forms of cell death could be at play that are elicited by different blood components. Future studies will examine ferroptosis and other factors in whole blood to better understand their other impacts.

SIGNIFICANCE/CLINICAL RELEVANCE. This study attempts to further explore the early traumatic events in ACL injury and provide new knowledge that may be used to optimize clinical intervention of intra-articular bleeding in ACL treatment.

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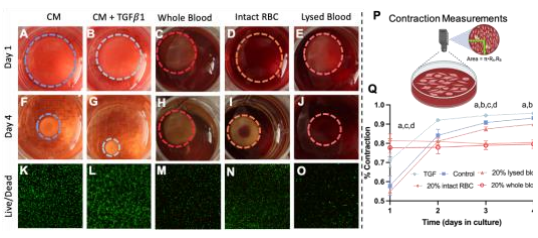

Figure 1: Human ACLF collagen gel-assay showing contraction from Day 1 **(A-E)** to Day 4 **(F-J)**. Cell viability **(K-O)** of collagen gels were assessed with calcein-AM (green, live) and ethidium homodimer-1 (red, dead) after 4 days in culture. Quantitative measurement of free gel diameter **(P-Q)** for control (CM), TGF (CM + TGFβ1), lysed red blood cells, intact red blood cells, and whole blood. p<0.05 for **labels:** a. TGF vs. control, b. control vs. lysed blood, c. control vs. intact RBC, d. control vs. whole blood.

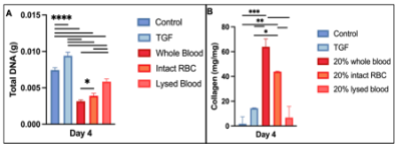

Figure 2: **(A)** Total DNA and **(B)** collagen content of human ACL fibroblast collagen gels cultured on 24-well plates with experimental treatment after 4 days in culture. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

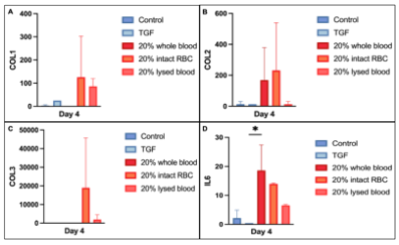

Figure 3: Gene expression of human ACL fibroblast 3D collagen gels after 4 days in co-culture with varying blood treatment groups. *p<0.05