Early Effects of Minimally Invasive Necrotic Bone Washing on a Piglet Model of Legg-Calve Perthes Disease in Late Avascular Necrosis Stage

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INTRODUCTION: Legg-Calve-Perthes disease (LCPD) is a childhood hip disorder caused by a loss of blood supply to the femoral head which produces extensive cell death and avascular necrosis (AVN). Chronic inflammatory repair response characterized by increased bone resorption, decreased bone formation, and progressive femoral head fragmentation and deformity occur over time[1]. A recent study showed that saline wash of the necrotic microenvironment 1 week after the induction of AVN in a piglet model of LCPD significantly improved bone healing by clearing some of the necrotic debris and inflammatory factors from the femoral head. The study found increased bone volume and bone formation and decreased pro-inflammatory macrophages in the repair tissue[1]. The timing of this intervention (1 week after AVN) represents the early avascular necrosis stage of LCPD (Stage 1a). Many LCPD patients, however, have delayed presentation and are diagnosis in the late avascular necrosis (Stage 1b) or early fragmentation stage (Stage 2a) of LCPD. The effects of necrotic bone washing at a later timepoint (Stage 1b or 2a) have not been studied. The purpose of this study was to determine the short-term effects of delayed necrotic bone washing at 3 weeks following the induction of AVN in the piglet model of LCPD, which is considered a clinically relevant time point.

METHODS: Right femoral head AVN was induced in 18 piglets by tying a ligature tightly around the femoral neck and cutting the ligamentum teres[2]. The animals were randomly divided into the experimental (Wash+NWB; n=8) and control (NWB alone; n=10) groups. All animals received above knee amputation 3 weeks after the AVN surgery to simulate the non-weight-bearing (NWB) recommendation used in clinical practice. The experimental group had the necrotic bone-washing procedure using a minimally invasive 3-intraosseous needle technique, as previously described[1] with a total saline flush volume of 480 mL. Initial 10 ml of the wash solution was collected and analyzed via bright field, live-dead cell assay, and H&E staining. All piglets were sacrificed six weeks after the AVN induction (i.e. 3 weeks after the wash treatment), and the femoral heads were assessed using radiography, micro-CT, and histology. The animals were given an intramuscular injection of xylenol orange (90 mg/kg) at 5 days and calcein (20 mg/kg) at 1 day prior to sacrifice to label new bone formation. Femoral head deformity was measured radiographically by determining the epiphyseal quotient, a ratio of the maximum femoral head height to the maximum femoral head diameter[2]. Micro-CT scans were analyzed with CTAn software to determine trabecular thickness, number, and separation in addition to bone volume/total volume. H&E stained sections were used to quantify % revascularized area using Image J. Tartrate-Resistance Acid Phosphate (TRAP) and McNeal Tetrachrome stained sections were used to quantify osteoblast (N.Ob/BS) and osteoclast numbers per bone surface (N.Oc/BS), respectively. Double labeling analysis was performed on BIOQUANT OSTEOIMAGER. All parameters of experimental and control groups were compared using an unpaired t-test and a p-value <0.05 was considered significant.

RESULTS: Analysis of the bone wash solution collected at the time of wash procedure revealed a wide variation in the presence of red blood cells, cell debris, and live-dead cell ratio in the animals of the experimental (Wash+NWB) group (Figure 2, 3). Abundance of red blood cells and increased live to dead cell ratios were found in some femoral heads indicating that the intraosseous needles were washing revascularized/repair areas of the femoral heads in the experimental group. Quantitation of the femoral head deformity using epiphyseal quotient revealed a presence of mild deformity in the experimental and control groups with no significant difference between the two groups (p=0.76). Micro-CT analysis also displayed no significant difference in trabecular thickness (p=0.68), separation (p=0.20), number (p=0.57), and bone volume/total volume (p=0.47) between the two groups. Histologic assessment revealed variable % revascularization of the femoral head in the experimental (mean 73 ± 39%, range 20 to 100%) and the control (mean 65 ± 32%, range 5 to 100%) group with no significant difference between the two groups (p=0.25). Interestingly, there was a significant increase in the mean N.Ob/BS (1/mm) in the experiment group compared to the control group (10.79 ± 7.11 vs. 4.62 ± 4.84, p=0.044). The mean N.Oc/BS (1/mm) was also significantly increased in the experimental group compared to the control group (2.48 ± 1.20 vs. 1.19 ± 1.13, p=0.032). Double labeling analysis showed no significant difference in the mineral apposition rate (p=0.72) and the bone formation rate (p=0.85) between the two groups.

DISCUSSION: Our study revealed statistically significant increase in the number of osteoblasts and osteoclasts per bone surface in the experimental group compared to the control group, but no significant differences in the femoral head deformity, micro-CT parameters, revascularization, mineral apposition rate, and bone formation rate. We postulate that the mixed results are due to following reasons. First, it is possible that bone wash at 3 weeks post-AVN in the piglet model may be too late to consistently obtain positive results due to variable degree of deformity and revascularization already present in the late AVN stage. It is also possible that bone washing procedure at a delayed time point may wash away live repair cells, as noted in our bone wash solution analysis from some femoral heads, which may had negative impact on the repair process. It is also possible that the 3-week time point from bone wash was too short to adequately determine the treatment effects compared to the 7-week time point used in our previous studies[1]. Given this, a longer follow up duration is indicated to determine if trabecular and histologic parameters will follow increased osteoblast and osteoclast numbers.

SIGNIFICANCE/CLINICAL RELEVANCE: Delayed necrotic bone washing at 3 weeks following the induction of AVN in the piglet model produced mixed results. While necrotic bone washing at 1 week following the induction of AVN (early AVN stage) improved necrotic bone healing[2], the efficacy of bone wash treatment in the late AVN stage appears to be reduced. Given that some femoral heads may already have some revascularization and initiation of the repair process, preoperative assessment of revascularization of the femoral head using a perfusion MRI is warranted followed by targeted washing of the necrotic region rather than the entire femoral head.

REFERENCES: [1] Kim HKW, et. al. Minimally Invasive Necrotic Bone Washing Improves Bone Healing After Femoral Head Ischemic Osteonecrosis: An Experimental Investigation in Immature Pigs. JBJS. 2021 Jul 7. doi: 10.2106/JBJS.20.00578. [2] Kim HK, Su PH. Development of flattening and apparent fragmentation following ischemic necrosis of the capital femoral epiphysis in a piglet model. JBJS. 2002 Aug;84(8):1329-34.12

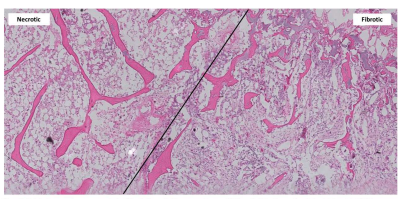

Figure 1. Histologic comparison or revascularized fibrotic tissue (right) compared to necrotic tissue (left) in a femoral head of a piglet model of LCPD.

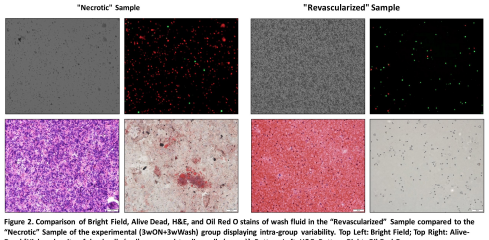

Figure 2. Comparison of Bright Field, Alive Dead, H&E, and Oil Red O stains of wash fluid in the "Revascularized" Sample compared to the "Necrotic" Sample of the experimental (3wON+3wWash) group displaying intra-group variability. Top Left: Bright Field; Top Right: Alive-Dead (Higher density of dead cells (red) compared to alive cells (green)); Bottom Left: H&E; Bottom Right: Oil Red O

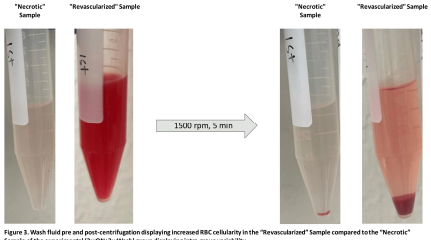

Figure 3. Wash fluid pre and post-centrifugation displaying increased RBC cellularity in the "Revascularized" Sample compared to the "Necrotic" Sample of the experimental (3wON+3wWash) group displaying intra-group variability.