Digital Pathology of the Femoral Head: Using a Novel RGB-Trichrome Stain for Tissue Quantification using HALO®

Ashton A. Amann[1], Ferenc Toth[1], Casey P. Johnson[1], Alexandra R. Armstrong[1]
[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota, United States
Amann029@umn.edu

DISCLOSURES: Ashton A. Amann (N), Ferenc Toth (N), Casey P. Johnson (N), Alexandra R. Armstrong (N)

INTRODUCTION: Identification and quantification of hard tissues is essential for understanding tissue remodeling and disease progression, particularly in models of musculoskeletal disorders. Although several histological techniques are available for evaluating bone, there is no reliable method to accurately quantify mineralized versus unmineralized bone in decalcified and formalin-fixed, paraffin-embedded tissue. Current options for determining mineralized vs. unmineralized bone require hard tissue processing, which is technically challenging and time-consuming. A modified alcian-blue/picrosirius red staining protocol, also referred to as RGB-Trichrome (RGBT), shows promise in addressing this need; however, its applicability in non-human tissues has not yet been evaluated [1]. The objective of this study was to utilize HALO® Image Analysis Software to: 1) compare the efficacy of a novel RGBT stain vs. a standard alcian blue-picrosirius red (ABPR) stain in quantifying mature bone, osteoid, cartilage, and marrow; and 2) determine how RGBT compares to Gomori's Trichrome (GT) staining in the quantification of fibrosis within the porcine femoral head.

METHODS: This study analyzed 13 femoral heads (7 female, 6 male) from piglet models of Legg-Calvé-Perthes disease (LCPD) that induce osteonecrosis of the hip. Piglets in this model utilized either 1) a surgically-placed ligature (n=7) or 2) fluoroscopic injection of embolic beads into arteries supplying the femoral head (n=6) to induce ischemia within the epiphysis [2,3]. Sample selection was based on histological appearance, with samples ranging from no lesions to extensive necrosis (bone, marrow) and fibrosis of the epiphysis. All procedures utilizing these piglets were overseen and approved by our local IACUC. Femoral heads were harvested either 1- or 2-weeks post-operatively and bisected at the level of the greater trochanter. Slides for histological assessment were obtained for each femoral head following three distinct staining techniques: RGBT, ABPR, and GT. The RGBT staining followed the same protocol as the ABPR staining with the addition of 0.04%v Fast Green. Slides were then digitally scanned at 40X magnification and uploaded to HALO® Image Analysis Software [4]. HALO®'s Tissue Classifier algorithm was used to calculate total area and percent composition of marrow, mature bone, cartilage, osteoid, and fibrosis within each annotated epiphysis. Osteoid was readily apparent within the setting of rapid bone growth/turnover within a subset of the examined femoral heads, consistent with previous experience in qualitative assessments of this model. To account for slide-to-slide stain variability, a pathologist with musculoskeletal expertise and extensive experience in the LCPD model confirmed 10 example annotations of each tissue type. Mean values (area, percent area) from HALO® were used to compare RGBT to ABPR as well as RGBT to GT for their ability to identify each of the tissues of interest using paired T-tests and treatment of ABPR and GT as the established gold standard.

RESULTS: RGBT demonstrated strong alignment with ABPR for total bone (RGBT: 24.87±11.35 mm², ABPR: 24.76±11.41 mm²) and marrow (RGBT: 87.08±26.66 mm², ABPR: 85.85±23.34 mm²), with no significant differences in total bone area ($p=0.925$) or percent bone area ($p=0.289$). Additionally, the staining techniques were strongly correlated for total bone ($r=0.93$ for area, $r=0.79$ for percent area) [Figure 2]. However, RGBT found cartilage (RGBT: 76.26±9.66 mm², ABPR: 65.47±10.48 mm²) and fibrosis (RGBT: 4.731±5.220 mm², GT: 8.703±5.660 mm²) to be statistically different from ABPR or GT, respectively, in terms of the total area ($p=0.016$, $p=0.030$) and percent area ($p=0.0056$, $p=0.0086$) [Figure 3]. Uniquely, RGBT was able to provide distinct measurements for the mean area of osteoid and of mature bone (5.865±2.987 mm² and 20.732±10.739 mm², respectively), distinguishing dark red osteoid in regions of healing from the peach-pale red mature, calcified bone [Figure 1].

DISCUSSION: RGBT was able to quantify the total bone area in a highly accurate manner as compared to the widely used ABPR, a staining technique known for its ability to delineate tissue types, particularly in assessments of the intervertebral discs and vertebrae [5]. RGBT also consistently differentiated osteoid, allowing HALO® quantification. With further validation of its specificity in distinguishing new from mature bone, RGBT may allow tissue analysis in the complex context of bone healing and repair. The significant difference in cartilage area between RGBT and ABPR is likely the result of tissue processing effects, given the frequency of cartilage folds observed in the examined sections, introducing variability. Surprisingly, RGBT detected less fibrosis than GT, suggesting the value of GT as a complementary technique in cases where fibrosis quantification is important. Limitations for this study include a lack of additional validation of osteoid vs. mature bone, such as using traditional hard tissue processing, and mild to moderate variation in staining intensity across slides despite staining being batched. Overall, RGBT shows promise as a technique to differentiate and quantify tissue types including osteoid and mature bone, improving on standard methods such as ABPR.

CLINICAL RELEVANCE: RGBT is a novel, time-efficient, non-destructive staining technique that may effectively differentiate mineralized (mature) from unmineralized (new) bone. This method may have clinical utility in elucidating the pathogenesis of bone disorders, such as LCPD.

REFERENCES: [1] Gaytan, Francisco et al. Scientific reports vol. 10,1 16659. 7 (Oct. 2020); [2] Kim, H K et al. JBJS vol. 83,5 (2001); [3] Novotny, Susan A et al. PloS one vol. 20,5.14 (May 2025); [4] HALO Image Analysis Platform version 4.0.5107 and HALO AI version 4.0.5107 (Indica Labs, Inc.); [5] Lee, Naomi N et al. *JOR Spine* vol. 4,2 e1162. 14 (June 2021).

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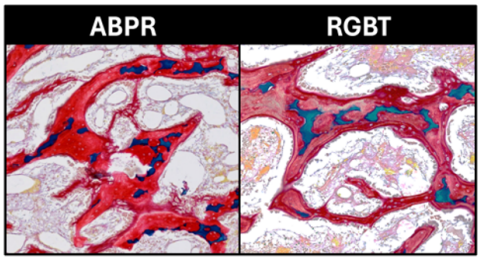

Figure 1: Comparison between ABPR and RGBT, highlighting the more dynamic color range in boney tissues (red) and cartilage (blue). Peach-colored regions on RGBT are consistent with mineralized bone while osteoid (unmineralized) shows up as a darker red.

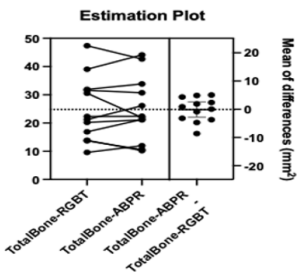

Figure 2: Estimation plot for total bone between RGBT and ABPR. Paired areas of total bone within femoral heads (left) and mean of differences within the 95% CI (right) show consistent total bone when osteoid is included with mature bone from RGBT stains.

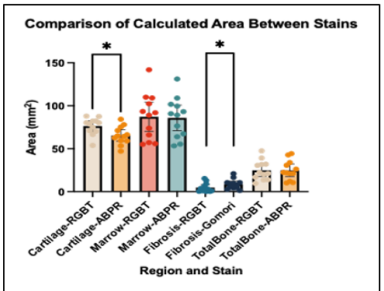

Figure 3: Calculated tissue area differences between RGBT, ABPR and GT. Cartilage and fibrosis were found to be significantly different between stains ($p < 0.05$) while marrow and total bone were similar.