Improving Efficiency and Ensuring Rigor in Non-Rodent Bone Growth Research

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INTRODUCTION: The growth plate (GP) or physis of bones is a complex and dynamic cartilaginous structure that allows bones to grow longitudinally. While mid- to large animal models may be more clinically relevant due to their size, activity, and ultimate physeal closure at maturity than their rodent counterparts, histomorphometric analysis of growth rates and structural changes within the physes of these larger animals can be tedious. Further, it is still being determined whether techniques described in rodents are translatable to larger animal specimens. In this project, we measured the reliability of pulsed fluorochrome labeling[1] (Growth Rate Determination) using manual and computer-assisted measurements. We then measured the reliability of the Column Index[2] (CI; Linearity of Proliferative Chondrocytes) similarly.

METHODS: 48 tibial physes from 12 skeletally immature (age 9-17 weeks) New Zealand White (NZW) rabbits were pulsed with fluorochrome-labeled Alizarin Red complexone (red) first followed by Oxytetracycline (green) 72 hours later; tibiae were harvested and processed into ~1 mm central slabs of the bone that were then analyzed using one of three methods: (1) Fully Manual Technique (FMT); (2) Manual Digital Measurement (MDM); and (3) Computer Assisted Image Processing (AIP) (in lab Matlab program) to measure distances between the bone labels across the bone (Figure 1). Pearson's correlation coefficient was calculated between measurement modalities. Pairwise Bland Altman Analyses[3] were performed to assess differences in the distances obtained from the different techniques. The Intraclass correlation coefficient (ICC, Two Way Random Effects, p=0.05) of the MDM were then determined by 5 independent readers reading a sample of 10 of the fluorochrome-labeled physes, and duplicate measures of a single reader, similarly the ICC of the AIP, was determined. The CI was then manually digitally measured across 10 decalcified and Hematoxylin and Eosin-stained rabbit tibial growth plates while one reader measured the CI of the 10 samples in duplicate to determine the ICC. To assess CI in the growth plate, we used Image J software to connect centroids of individual chondrocytes in columns, calculating the angles between them using a segmented line Image J software plug-in. We averaged these angles within each column and measured eight random columns in seven areas across the entire growth plate to determine overall alignment (Figure 2). Reliability for this study was defined as less than 0.50: poor reliability, between 0.5 and 0.75: moderate reliability, between 0.75 and 0.9: good reliability, greater than 0.9: excellent reliability[4].

RESULTS: The correlation between the FMT and MDM methods was $r(46)=.98$, $p < .001$. Bland Altman analysis revealed a bias of 29.42 µm and a standard deviation of 21.94 µm (Figure 3a). The correlation between MDM and AIP was $r(49)=.99$, $p < .001$, with a bias of -9.94 µm and a standard deviation 14.48 µm (Figure 3b). The correlation between FMT and AIP methods was $r(44 =.98$, $p < .001$, with a bias of 18.59 µm and a standard deviation of 20.86 µm (Figure 3c). ICC between independent readers using the MDM was 0.997, and intra-reader ICC 0.998. ICC between readers using the AIP was 0.992, and intra-reader was 0.998. The CI measurement ICC between readers was 0.48 and within readers 0.45 in these rabbit samples.

DISCUSSION: Growth rate measurement techniques demonstrated high correlation and excellent reliability (>0.99 intra- and inter-reader) regardless of the technique used in these samples. The AIP technique allows for more rapid data acquisition in these large samples without losing reliability. A higher agreement between MDM and AIP is expected as the same digital image was used for the analysis, whereas individual microscopic fields of view are used in FTM. Unlike the high ICC found in the growth rate measurements, the CI measurements demonstrated poor reliability (<0.5, inter- and intra-reader indicating that improvements in the sampling (more clusters per GP given the larger GP size) or an alternative measurement strategy may be needed to reliably assess overall chondrocyte alignment in these larger samples. Alternatively, this may indicate significant regional differences of chondrocyte alignment throughout the larger physis that must be better controlled for during data acquisition and processing. Methods to improve reliability are being explored.

SIGNIFICANCE: The data from this study demonstrates that increasing the size of the animal model may affect the reliability of measurements designed in rodent models and that such measurements should be validated in larger models. While measuring the distance between pulsed fluorochrome labels proved very reliable regardless of technique, improved sampling or measurement techniques may be necessary to better assess the more complex characteristics of the physis. Further computer-assisted image processing may prove critical in improving the reliability and efficiency of such measurements on larger tissue specimens.

REFERENCES:

1. Halanski MA, Yildirim T, Chaudhary R, Chin MS, Leiferman E. Periosteal Fiber Transection During Periosteal Procedures Is Crucial to Accelerate Growth in the Rabbit Model. *Clin Orthop Relat Res* 2016; **474**(4): 1028-37.
2. Killion CH, Mitchell EH, Duke CG, Serra R. Mechanical loading regulates organization of the actin cytoskeleton and column formation in postnatal growth plate. *Molecular Biology of the Cell* 2017; **28**(7):1862-70
3. Bland JM, Altman DG (1986). Statistical methods for assessing agreement between two methods of clinical measurement" *Lancet*. **327** (8476): 307–10.
4. Koo TK, Li MY. Erratum to "A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research" *J Chiropr Med.* 2017 Dec;**16**(4):346.

FIGURES:

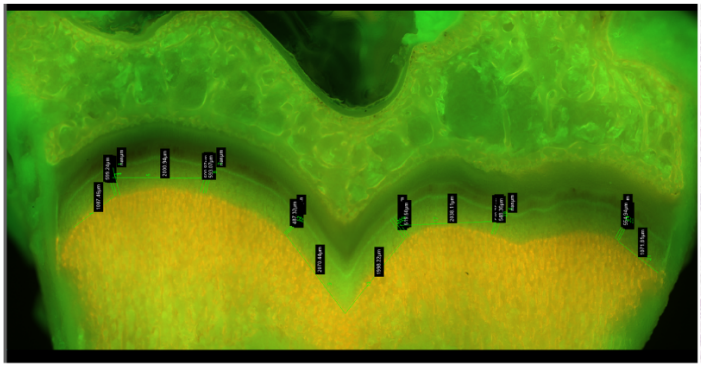

Figure 1: Growth rate measurement using pulsed fluorochrome labeling

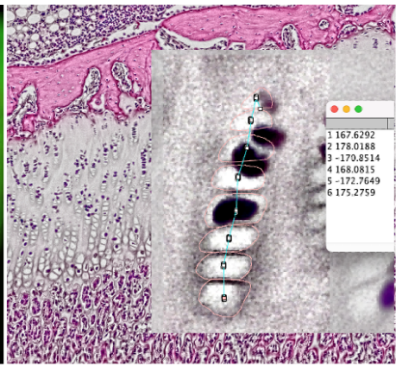

Figure 2: Chondrocyte Alignment Measurement

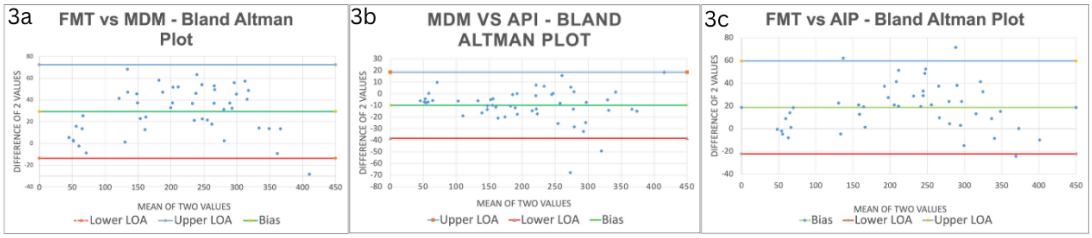

Figure 3: (3a) Shows Bland Altman plot of FMT vs. MDM. (3b) Shows Bland Altman plot of MDM vs. AIP. (3c) Shows Bland Altman plot of FMT vs AIP.