INTRODUCTION: High resolution, micro-computed tomography (µCT) scans of fracture calluses ex vivo are a routine experimental outcome in small animal models. The objective of many fracture repair studies is to determine whether a manipulation hastens or delays healing, and µCT can quantify such differences through measurements of callus volume (TV), fraction of callus that is mineralized (BV/TV), tissue mineral density of callus (TMD), and structural metrics (e.g., polar moment of inertia). While there are published guidelines on the application of µCT to assess the micro-structure of rodent bones [1], thresholding to segment mineralized tissue from air and soft tissue, especially for a callus, has no set rule other than to check the fidelity of the segmented image to the original image. Therefore, analyzing the uncertainty in selecting a threshold value, we tested the hypothesis that the time course of callus repair in mice, as assessed by µCT, depends on the thresholding procedure.

METHODS: All methods adhered to an IACUC-approved protocol. Following a retrograde insertion of a 25 gauge needle through the intercondylar notch, a calibrated, impact load was applied to the mid-shaft of the femur with the right leg of the anesthetized mouse resting on two supports. These closed, transverse fractures were generated in female C57/BL6 mice at 6 wk. of age. The mice were then euthanized between 2 weeks and 6 weeks of healing (n = 12 per time point). Upon removing the needle, a segment of each callus (4.92 mm in axial length) was imaged at an isotropic voxel size of 12 µm using the Scanco µCT40 at an integration time of 250 ms, peak tube voltage of 70 kVp, current of 0.114 mA, and an acquisition of 500 projections per 180°. We then used an automated procedure based on dual thresholding [2] to place contours around the callus. Standard Scanco evaluation scripts provided BV/TV, TMD, and polar moment of inertia (J) of each callus for 3 different thresholds – 244, 381, and 517 mg HA/cm³ – using the same noise filtering parameters (σ = 0.8 and support of 1). By flickering between the original image and the segmented images of several calluses, the intermediate value was manually chosen as being sufficient to resolve the mineralized tissue of the callus. The lower and upper values were 36% lower and higher, respectively, than the intermediate value. Lastly, the µCT-derived measurements were also obtained using the adaptive iterative thresholding (AIT) algorithm that utilizes the image’s histogram of attenuation values to converge on a threshold value being nearly equal to the grand mean of the background distribution and the distinct bone distribution [3]. A one-way ANOVA or non-parametric equivalent (Kruskal-Wallis) was used to determine whether there were differences in the µCT properties across the time course of healing for each thresholding procedure followed by a post-hoc, multiple pair-wise comparisons (Holm-Sidak method or Dunn’s method, respectively). Results are provided as mean ± standard deviation.

RESULTS: Even though increasing the thresholding value increased tissue mineral density and decreased polar moment of inertia per time point of healing, the changes in TMD and J over the time of repair were similar among the 4 threshold procedures (Fig. 1). That is, changing the threshold value did not alter which differences in TMD and J among time periods of healing were statistically significant. Changing the thresholding procedure however did affect the number of observed differences in the fraction of mineralized callus among the weeks of healing (Fig. 1). In the case of the intermediate value, there was no difference in BV/TV over time. This observation is not entirely unexpected since the callus size becomes narrower while calcified cartilage remodels away to a lamellar cortex from 2 weeks to 6 weeks of repair. With a decrease in the threshold value, BV/TV appears to decrease over time; whereas, an increase in the thresholding value (AIT derived) caused an increase in BV/TV from 2 to 3 weeks of healing with no further change in BV/TV over the later time points (Fig. 1).

DISCUSSION: With respect to the mineralization of callus tissue (TMD) and the structural resistance of the callus to torsion (J), the choice of the threshold value that segments bone from background is not overly critical. This is not to say the choice is not important, but these data suggest that thresholds within 30% of the optimal segmentation value do not significantly alter sensitivity. However, a low value can produce more variance in µCT properties than a high thresholding value (Fig. 1). In this study, the low value was just at the upper bound of the noise distribution on the image histograms. Since the original cortices were not excluded from the analysis of the callus, AIT converged on values approaching the mean TMD of cortical bone, and as such, finer callus structures, especially at the early time points, were absent (Fig. 2). Therefore, this thresholding approach may not be suitable for the callus unless the contribution of the original cortices to the TMD histogram can be excluded, a non-trivial task. Ultimately, a combination of multiple µCT properties contribute to callus strength [4], and as such, microstructural features of the callus need to be adequately resolved.

SIGNIFICANCE: Even though uncertainty exists in the choice of a threshold value for segmenting callus tissue from air, a less than non-optimal value is likely not going to affect the ability to observed differences in certain useful µCT properties over the time of healing.

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