

# Hindlimb Unloading and Reambulation Differentially Affect Murine Fracture Healing via Regulation of Callus Vascularity and Osteoclastogenesis

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**INTRODUCTION:** Patients with bone and muscle loss (Osteosarcopenia) from prolonged disuse have higher risk of falls and subsequent fragility fractures.<sup>1</sup> Osteosarcopenia and impaired mobility prior and during fracture healing is associated with worse clinical outcomes.<sup>2,3</sup> Hindlimb unloading (HLU) via tail suspension has been shown to increase cellular apoptosis, osteoclastogenesis, and disrupt vascularity in uninjured long bones, key processes implicated in bone repair.<sup>4</sup> However, little to no data exists detailing how unloading and reambulation may affect these bone healing processes following disuse. Therefore, the goal of this study was to better inform post-fracture rehabilitation strategies by investigating how immediate physical reambulation affects fracture healing. We hypothesized that disuse by hindlimb unloading would decrease callus bone formation by decreasing angiogenesis and increasing cellular apoptosis and osteoclastogenesis, processes which would be attenuated with reambulation.

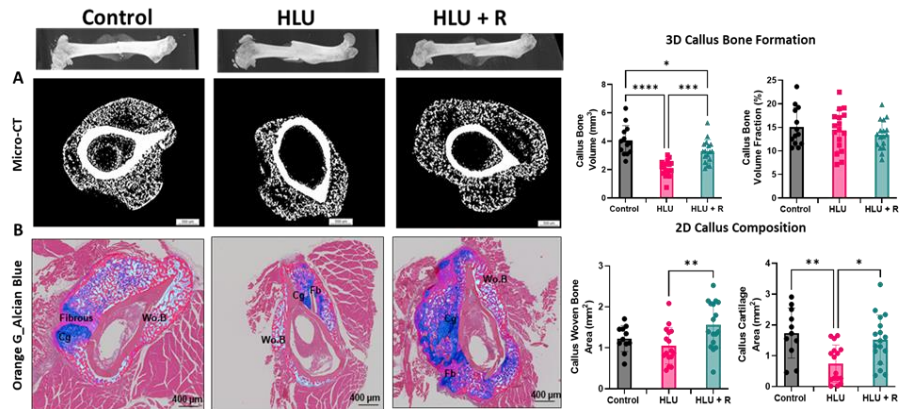
**METHODS:** All animal procedures were approved by the VCU IACUC. Skeletally mature, male and female C57BL/6J mice (18 weeks old) underwent HLU for 3 weeks. Then, mice had their right femur fractured by open surgical dissection (stabilized with 24-gauge pin). Next, mice were randomly assigned to continued HLU or allowed normal physical weight-bearing remobilization (HLU + R). Mice given normal cage activity throughout the experiment served as controls. All mice were sacrificed 4-days or 14-days following fracture (6-10 mice per treatment/sex). Primary outcomes were fractured femoral bone formation by micro-CT, and callus histology for cartilage formation (Alcian Blue), osteoclast density (TRAP<sup>+</sup> multinucleated cells), cellular apoptosis (cleaved-caspase-3<sup>+</sup> cells), and bone vasculature (endomucin<sup>+</sup> structures). Data were analyzed for the effects of treatment (HLU, HLU+R, control) by 1-WAY ANOVA with Tukey's multiple comparisons test (\*p<0.05).

**RESULTS:** There was no evidence of mineralized callus formation at day 4 among any experimental group. However, by day 14, HLU showed significant decreases in absolute callus volume and bone formation compared to controls (Fig 1). In contrast, HLU + R significantly increased callus bone volume compared to HLU mice, although not to control levels. Histology at D14 reinforced micro-CT results, as HLU resulted in significantly reduced callus cross-sectional area, woven bone area and cartilage area compared to reambulated mice (Fig 1). Cellularly, there was significantly decreased vessel volume and trends toward greater osteoclast density in woven bone from HLU versus control and HLU + R mice at day 14 post-fracture (Fig 2). At day 4 post fracture, we saw elevated cellular apoptosis in HLU mice but this wasn't significant (Fig 2).

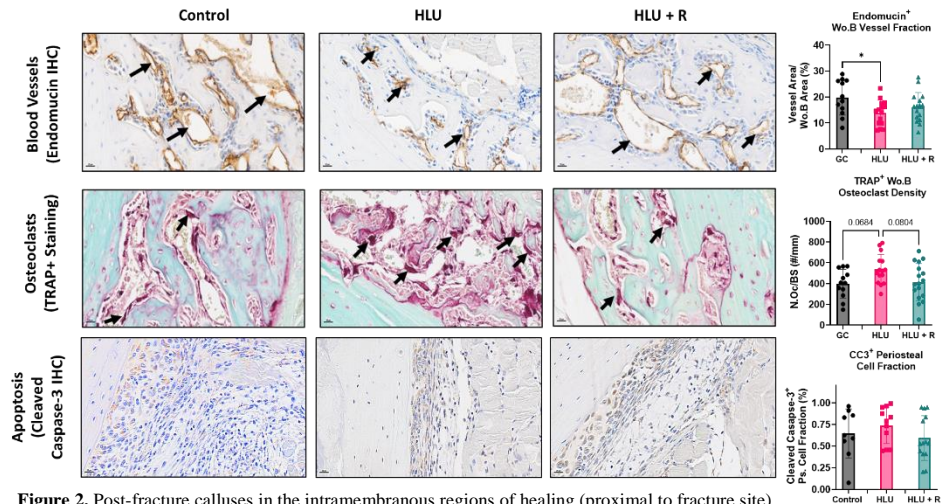
**DISCUSSION:** Within the fracture callus, bone formation and chondrogenesis were attenuated due to continued HLU, similar to other reports<sup>5-7</sup>, suggesting some level of diminished healing. In support of our hypothesis, physical reambulation immediately after bone injury was able to increase callus bone formation, although not to levels seen in control mice. Furthermore, our histological results suggest that this improved bone formation due to reambulation results at least partly from increased chondrogenesis, blood vessel volume and decreased callus resorption, which has been shown to be enhanced previously in bone injury with mechanical loading.<sup>8,9</sup> A limitation of our study is the lack of control over physical loading parameters and use of skeletally mature versus aged mice, which may more accurately represent the clinical osteosarcopenic cohort. Future work will seek to remedy this and also investigate controlled loading regimens to increase callus healing with disuse.

**SIGNIFICANCE:** Our results suggest that weight-bearing reambulation immediately following fracture may improve callus healing by stimulating blood vessel volume and decreasing callus resorption compared to minimal or delayed rehabilitation regimens.

**REFERENCES:** 1. Huo, Y.R., et al., 2015. 2. Kamel et al. 2003. 3. Ariza-Vega et al. 2014 4. Globus and Morey-Holton. 2016. 5. McKenzie, J.A., et al. 2017. 6. Hsia, A.W., et al. 2018. 7. McCabe, N.P., et al. 2013. 8. Palomares, K.T., et al., 2009. 9. Liu et al. 2019.



**Figure 1.** Day 14 post fracture calluses (excluding original cortical bone) were analyzed for woven bone and cartilage formation. Mean ± SD. \*\*\*\* p < 0.00005 \*\*\* p < 0.0005 \*\* p < 0.005 \* p < 0.05 significant effect by Tukey Post-Hoc following 1-WAY ANOVA.



**Figure 2.** Post-fracture calluses in the intramembranous regions of healing (proximal to fracture site) were analyzed for blood vessel formation (day 14), osteoclasts (day 14) and cell apoptosis (day 14). Mean ± SD. \* p < 0.05 significant effect by Tukey Post-Hoc following 1-WAY ANOVA.