Mechanical Unloading Attenuates the Innate Immune Response to Impair Enthesis Regeneration in Adult Zebrafish

Ella J. Kim[1], Jason C. Marvin[2,3], Benjamin Peterson[2,3], Maddie Burke[2], Jenna L. Galloway[2,3]
[1]Harvard University, Cambridge, MA, [2]Massachusetts General Hospital, Boston, MA, [3]Harvard Medical School, Boston, MA
ellakim@college.harvard.edu

Disclosures: JCM serves on an ORS committee (9). All other authors declare no disclosures (N).

INTRODUCTION: Tendon-bone attachment ('enthesis') ruptures account for 30% of musculoskeletal consultations[1] but have limited therapeutic options, contributing to low treatment efficacy and high re-injury rates. Standard care combines surgical repair and rehabilitation; however, post-surgical scarring fails to restore the native tissue structure required for proper force transmission and injury variability complicates the development of targeted therapies[2]. Furthermore, successful enthesis regeneration relies on a tightly regulated immune response, with early immune activation facilitating recruitment of reparative immune and progenitor cells[3,4]. Emerging evidence suggests that immune activity is linked with loading dynamics, where mechanical stimuli promote immune cell recruitment to the injury site[5]. Despite this, no published studies have explored how mechanical unloading influences immune activity, limiting our ability to define the mechanical conditions required for optimal immune-mediated healing. To address this, our group leverages adult zebrafish, which, unlike mammals, fully regenerate both the midbody[6] and enthesis of their maxillary superficial tendons (MSTs) following acute injury. Our preliminary data show that localized injection of Botulinum Toxin B (BoNTB), a muscle paralysis neurotoxin, effectively induces unloading in zebrafish, providing a model to examine the effects of unloading on the post-injury immune landscape. We hypothesized that BoNTB-induced unloading impairs enthesis regeneration by attenuating the recruitment and infiltration of neutrophils and macrophages to the injury site.

METHODS: All animal procedures were approved by IACUC and conducted in adult male and female zebrafish aged 6-8 months. To establish a baseline regeneration timeline, we surgically transected the MST enthesis in Tübingen/wild-type (WT) zebrafish and zebrafish deficient in tenascin-C (*tnc-/-*), an immunocompromised mutant line, both expressing *scxa:mCherry* (tenocyte reporter). Both groups were sacrificed at 7 days post-injury (dpi), when tendon reattachment is expected, and imaged using whole-mount multiphoton microscopy (n=6/group). To assess the effects of unloading on initial reattachment and immune cell recruitment, we injected WT zebrafish expressing *scxa:mCherry* and either *mpx:EGFP* (neutrophils) or *mpeg1:EGFP* (macrophages) with 5 µL of BoNTB (unloading), PBS, or no injection (control) into the jaw adductor muscle, then immediately transected the MST enthesis. Zebrafish were sacrificed and imaged at 1, 3, and 7 dpi (n=4-7/group). To determine whether unloading-induced deficits could be pharmacologically rescued, we first treated zebrafish with BoNTB and then co-treated with either recombinant prostaglandin E2 (dmPGE2, 10 µM in 10 µL), a pro-inflammatory mediator, or PBS (control) via intraperitoneal injection at -1 dpi and every 2 days until 7 dpi. Zebrafish were sacrificed and imaged at 1, 3, and 7 dpi to assess initial reattachment and immune cell dynamics (n=3-5/group). An additional group received BoNTB with either dmPGE2 or PBS through 7 dpi, then recovered in system water until sacrifice at 56 dpi to evaluate long-term regenerative outcomes under unloading (n=2-3/group). MST reattachment was quantified by the linear gap distance between the transected MST and bone. Collagen organization was assessed via second harmonic generation (SHG) signaling. Immune cell infiltration was quantified by the number of *mpx:EGFP+/mpeg1:EGFP+* cells per total tendon area, visualized via Draq5 (nuclei) staining. Statistical analyses were conducted in GraphPad Prism with significance set at $*p \leq 0.05$. Injury gaps across no injection, PBS, and BoNTB groups were compared using a one-way ANOVA with Tukey's post hoc, while all other pairwise comparisons used unpaired Student's t-tests. Data shown as mean ± STD.

RESULTS: At 7 dpi, WT zebrafish fully reattached the transected MST, with tenocytes repopulating the injury site and organized collagen visible by SHG, while *tnc-/-* zebrafish showed persistent gaps and reduced tenocyte presence, confirming their non-regenerative phenotype (data not shown). BoNTB-treated zebrafish exhibited significantly increased gap distances at 7 dpi compared to controls, reflecting disrupted initial reattachment and subsequent poor regeneration (**Fig. 1A-D**). Consistent with our hypothesis, neutrophil (**Fig. 1E-G**) and macrophage (**Fig. 1H-J**) infiltration at their respective peaks (1 and 3 dpi) were significantly reduced in BoNTB-treated samples compared to controls. dmPGE2 treatment robustly rescued both macrophage infiltration at 3 dpi and enthesis reattachment at 7 dpi, despite unloading (**Fig. 2A-B**). Although both PBS- and dmPGE2-treated zebrafish achieved full or near-full enthesis reattachment by 56 dpi, PBS-treated tendons displayed disorganized, kinked collagen fibers, indicating compromised structural integrity, whereas dmPGE2-treated tendons exhibited well-aligned, continuous fibers (**Fig. 2C-D**). These initial findings suggest dmPGE2 may enhance tissue organization and mechanical function, warranting further validation in larger cohorts.

DISCUSSION: BoNTB-induced unloading disrupts early enthesis regeneration by attenuating neutrophil and macrophage recruitment, highlighting the interplay between mechanical stimulation and immune-mediated repair. While post-injury immobilization (i.e., casts, splints, or braces) protects the tendon and alleviates pain, it also dampens the innate immune response critical for healing. Our findings suggest that dmPGE2, not previously explored in musculoskeletal contexts, restores neutrophil and macrophage infiltration under unloading and promotes early enthesis reattachment, revealing a potential strategy to combine immobilization with precise, tailored pharmacological support. Additional work is needed to quantify the biomechanical strength of tendons following dmPGE2 treatment. Ongoing experiments target the more mechanically active pelvic fin tendon to determine conserved enthesis repair processes and implement custom live-imaging approaches for longitudinal tracking of immune cell dynamics.

SIGNIFICANCE: Mechanical unloading compromises enthesis healing through suppression of the innate immune response. Our results support the potential of pairing clinical immobilization with pharmacologic dmPGE2 to preserve the regenerative benefits of loading while protecting against reinjury.

REFERENCES: [1]Peniche Silva et al., 2024 (DOI: 10.26481/dis.20240417cs); [2]Yang et al., 2013 (PMID: 4041869); [3]Crosio et al., 2022 (PMID: 35178698); [4]Jiang et al., 2024 (PMID: 39568806); [5]Gracey et al., 2020 (PMID: 32080619); [6]Tsai et al., 2023 (PMID: 37726307)

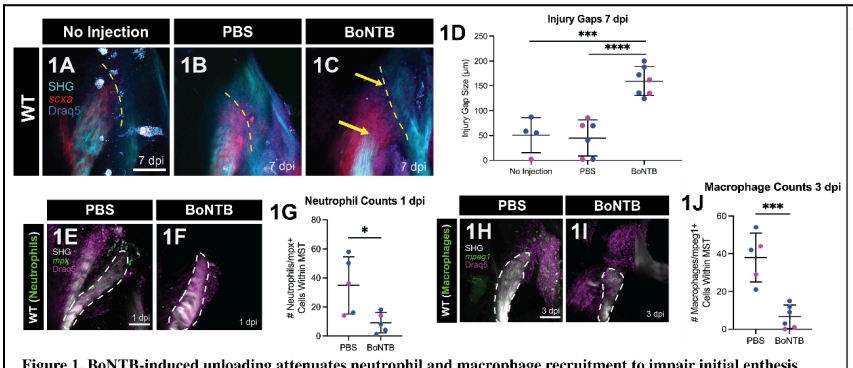

Figure 1. BoNTB-induced unloading attenuates neutrophil and macrophage recruitment to impair initial enthesis reattachment. **(A-C)** WT *scxa:mCherry* tendons at at 7 dpi. **(D)** Injury gaps at 7 dpi are significantly larger with BoNTB treatment (n=4-7/group). **(E-F)** WT *mpx:EGFP* tendons at 1 dpi. **(G)** Neutrophil/*mpx+* counts per tendon area are significantly reduced with BoNTB (n=5/group). **(H-I)** WT *mpeg1:EGFP* tendons at 3 dpi. **(J)** Macrophage/*mpeg1+* counts per tendon area are significantly reduced with BoNTB (n=5-6/group).
Yellow dotted lines and arrows: enthesis. White dotted lines: MST. Draq5: counterstain. Scale bars: 100 μm. Male and female animals. *p≤0.05, ***p≤0.001, ****p≤0.0001.

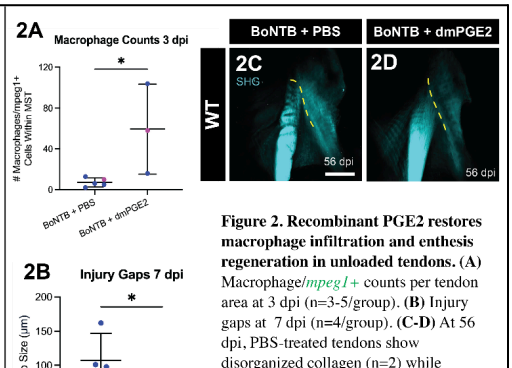

Figure 2. Recombinant PGE2 restores macrophage infiltration and enthesis regeneration in unloaded tendons. **(A)** Macrophage/*mpeg1+* counts per tendon area at 3 dpi (n=3-5/group). **(B)** Injury gaps at 7 dpi (n=4/group). **(C-D)** At 56 dpi, PBS-treated tendons show disorganized collagen (n=2) while dmPGE2-treated tendons show aligned, continuous fibers (n=3).
Yellow dotted lines: enthesis. Scale bars: 100 μm. Male and female animals. *p≤ 0.05, ***p≤0.001, ****p≤0.0001.