4-aminopyridine promotes bone-forming osteoblasts and accelerates tibial fracture healing

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Introduction: Delayed bone healing is an ongoing clinical challenge. Over the years, extensive research has been conducted to uncover cellular and molecular mechanisms of fracture healing. 4-aminopyridine (4-AP) is an US FDA-approved drug shown to improve walking in patients with chronic neurological disorders. Our recent study revealed a repurposing effect of 4-AP against peripheral nerve and muscle injury-induced bone loss. Given the suggestion that 4-AP may support a new approach to augment bone mineral content, we asked how 4-AP may affect fracture healing directly and hypothesized that 4-AP plays a direct role in improving fracture healing by promoting the differentiation of bone marrow mesenchymal stem cells into bone-forming osteoblasts.

Methods: All animal experiments conformed to Institutional Animal Care and Use Committee (IACUC) approved protocols at The Pennsylvania State University College of Medicine, Hershey, PA. Ten-week-old male C57BL/6J mice weighing 20-25g were anesthetized using intraperitoneal ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia. A 5 mm longitudinal incision was made along the anterior border of the tibia, which was then fractured with a scalpel blade and fixed with intramedullary pinning. The skin was closed with surgical staples, and post-operative slow-release buprenorphine (0.05 mg/kg) was given subcutaneously to all animals as an analgesic. X-ray images were acquired post-operatively and mice were randomly allocated into either 4-AP (1.6 mg/kg, IP, mass-adjusted dose based on approved systemic human 4-AP dosing for multiple sclerosis) or saline (0.1ml/mouse, IP) treatment groups daily for 21 days. Mice were euthanized on post-fracture day 21, and each tibia was harvested for micro-CT and histomorphology analysis. The effect of 4-AP on human bone marrow mesenchymal stem cells (hBMSCs) and osteoblast cells (hOBs) was tested *in-vitro* using cell viability, scratch testing, collagen deposition, and matrix mineralization assays in addition to bone-forming gene expression and protein level quantification. Data analyses were performed using GraphPad Prism 10. All results were presented as mean and standard error of the mean (SEM), and a P-value of 0.05 was deemed statistically significant.

Results: 4-AP significantly improved *in-vivo* fracture healing by enhancing BMD, BV/TV, trabecular number, and trabecular thickness following tibial fracture by day 21. Histomorphometric analysis of 4-AP treated tibial fracture has shown increased expression of BMP2, which supported matured total and cartilaginous callus with an increased bony callus and matrix components. Our *in-vitro* data confirmed an osteogenic effect of 4-AP via upregulating gene expression of RUNX2, OSX, BSP, OCN, and OPN in both hBMSCs and hOBs. 4-AP also enhanced BMP2 expression, which is responsible for activating these osteogenic mediators. 4-AP significantly enhanced osteoblast scratch assay closure, collagen deposition, and matrix mineralization.

Discussion: To the best of our knowledge, we are the first to demonstrate the accelerating effect of 4-AP on bone fracture healing. 4-AP stimulates the expression of BMP-2, which may act as a transcription factor to control osteogenic genes and bone-forming osteoblast cells. 4-AP is a potent osteogenic factor and may offer a new treatment approach for patients with impaired bone fracture healing. Ongoing and future studies will investigate the clinical potential of 4-AP in various bone fractures or disorders. 4-AP's role in fracture healing, though previously unappreciated, may be immediately translatable to humans given its current use in neurodegenerative disease.

Clinical Relevance: Our research has demonstrated that 4-AP has a unique therapeutic effect on bone fracture healing. Currently, there are no immediately translatable agents available to accelerate bone healing. We plan to proceed with Investigational New Drug approval from the United States Food and Drug Administration to conduct clinical trials in bone healing in the setting of simple and complex bone trauma.

Images:

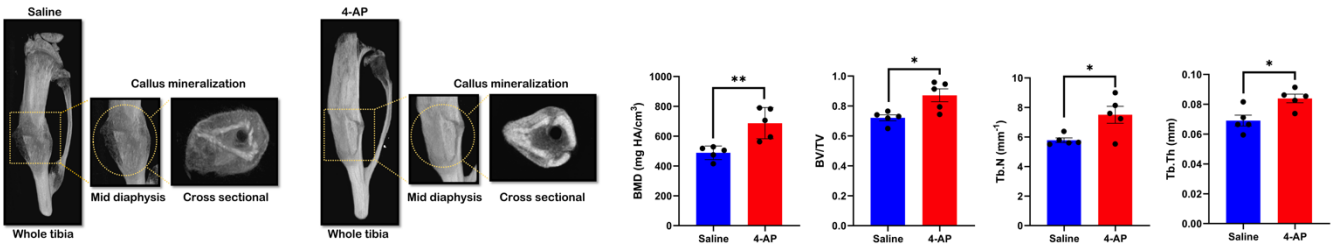

Figure: 4-AP enhances bone fracture healing in mice. Micro-CT images of the tibia and their quantification following fracture. Data are presented as mean± SEM (n=5). Values are significant at *p < 0.05.