

Senolytic Drug Ruxolitinib and Deflazacort Synergistically Improved Bone Quality and Muscle Histopathology of Dystrophin^{-/-}/Utrophin^{-/-} Double Knockout Mice

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Introduction: Duchenne's Muscular Dystrophy (DMD) is a severe genetic muscle disease due to the mutation of the dystrophin gene [1]. DMD patients also are at high risk of bone fracture [2-4]. Dystrophin^{-/-}/Utrophin^{-/-} (DKO-Hom) is a mouse model that recapitulates the clinical manifestations of DMD better than dystrophin^{-/-} (Mdx) mice with severe muscle histopathology including muscle necrosis, fibrosis, fat infiltration, heterotopic bone formation (HO), kyphosis and shortened lifespan [1]. DKO-Hom mice exhibit bone osteopenia as early as 4 weeks secondary to muscle pathology as well as delayed fracture healing [5, 6]. Both DKO-Hom mice and Mdx mice display cellular senescence and senescence-associated secretory phenotypes in skeletal muscle. Ruxolitinib, has been found to improve bone quality in the spine trabecular bone in addition to muscle histopathology (Gao X et al ORS 2023 abstract). The aim of this study is to investigate if Ruxolitinib in combination with Deflazacort can further improve the bone quality and muscle pathology of DKO-Hom mice.

Methods: 1. Animal breeding. DKO-Hom mice were generated using dystrophin^{-/-}/Utrophin^{-/-} mice bred at the Animal Facility of Colorado State University. All experiments were approved by IACUC animal protocol of CSU (#1234). **2. Mice treatment.** DKO-Hom mice at 4 weeks-old were divided into 3 groups (n=8/group including both males and females) and treated with vehicle (5% DMSO in PBS), Ruxolitinib (60mg/kg/d), and Deflazacort (0.9mg/kg/d) by oral gavage daily. Mice were then sacrificed at 12 days after treatment. **3. MicroCT and histology analysis.** Lumbar spine, right tibia, femur bones and fixed, MicroCT scanning were performed using 15µm voxel size using Viva-CT 80. Histology included Herovici's staining for bone tissues and H&E staining for muscle tissues. Gastrocnemius muscle tissues were collected for immunofluorescence staining including β-galactosidase (GLB1a)/CD68, α-L-Fucosidase (FUCA1)/CD68 and FCA1/CD31 double staining to detect macrophage and endothelial cells senescence. Data were analyzed using Graphpad Prism 10.

Results: 1. Ruxolitinib and Deflazacort synergistically improved bone microarchitecture parameters. Treatment of DKO-Hom mice with Ruxolitinib alone significantly increased bone volume/total volume (BV/TV) and trabecular thickness (Tb.Th) of spine L5 trabecular bone. However, treatment of DKO-Hom mice with Deflazacort+Ruxolitinib significantly increased BV/TV, trabecular number (Tb.N), Tb.Th and decreased trabecular separation (Tb.Sp) of spine L5 trabecular bone when compared to control and significantly increased BV/TV, Tb.N, and decreased Tb.Sp compared to Ruxolitinib. (Fig.1A-E). For the proximal tibia, treatment with Deflazacort+Ruxolitinib significantly increased BV/TV, Tb.N, Tb.Th and decreased Tb.Sp compared to control and Ruxolitinib alone (Fig.1F-J). For the femur cortical bone, neither Ruxolitinib alone nor Deflazacort + Ruxolitinib significantly changed cortical thickness (data not shown). However, Deflazacort +Ruxolitinib significantly increased BV density of femur cortical bone compared to the control or Ruxolitinib group (Fig.1K,L). **2. Deflazacort +Ruxolitinib improved bone and muscle histology.** Herovici's staining showed Ruxolitinib alone increased collagen 1 positive trabecular bone thickness and Deflazacort+Ruxolitinib increased the collagen 1 positive trabecular bone number and thickness, compared to the control. (Fig.2A). Similar results were found for proximal tibia trabecular bone (Fig.2B). H&E staining showed less muscle damage and inflammation in the Ruxolitinib group, and further improvement was found for Deflazacort+Ruxolitinib group compared to control (Fig.2C). **3. Deflazacort+Ruxolitinib decreased senescent macrophages.** GLB1/CD68 double staining showed Deflazacort+Ruxolitinib decreased senescent macrophage (GLB1⁺/CD68⁺) number and senescent macrophage percentage (GLB1⁺CD68⁺/Total CD68⁺) compared to control or Ruxolitinib alone. Ruxolitinib+Deflazacort did not significantly decrease total macrophages (CD68⁺) and non-senescent macrophage (GLB1⁻CD68⁺) cell number compared to the Ruxolitinib group (Fig.3A-E). FUCA1/CD68 double staining showed that both Ruxolitinib alone and Deflazacort+Ruxolitinib significantly decreased FCA1⁺/CD68⁺ cell number compared to control. No significant differences were identified between any two groups of FUCA1⁺CD68⁺ cells or the FUCA1⁺CD68⁺/Total CD68⁺ cell percentage (Fig.3F-I). FUCA1/CD31 double staining indicated neither Ruxolitinib alone nor Ruxolitinib+Deflazacort decreased senescent endothelial cells (FUCA1⁺/CD31⁺). Furthermore, both groups significantly decreased FUCA1⁺CD31⁺ cells (non-endothelial senescent cells). Ruxolitinib significantly decreased FUCA1⁺CD31⁺ cells and total CD31⁺ cells while Deflazacort+Ruxolitinib further decreased these cells' populations (Fig.3J-N).

Discussion and conclusion: Our results indicated that Ruxolitinib+Deflazacort synergistically improved bone microarchitecture of spine bone and long bone of DKO mice. The combined treatment not only increased Tb.Th (existing bone) but also increased Tb.N (new bone). Further, the combined therapy can improve bone and muscle histopathology by targeting senescent microphages. The decrease of non-senescent endothelial cells by Ruxolitinib or Deflazacort+Ruxolitinib was likely due to their effects on improvement of muscle pathology. In conclusion, Ruxolitinib can enhance the beneficial effect of Deflazacort on muscle in DMD. **Significance/clinical relevance:** Targeting cell senescence with Ruxolitinib and in combination with Deflazacort can be a novel therapy to improve bone and muscle health of DMD patients. **Acknowledgements:** This project was funded by NIH R01AR065445 to Dr. Huard.

