Effects Of Beta-aminopropionitrile And Exercise On Bone Mechanics, Composition, and Collagen Morphology In Mice

Max A. Hammond, B.E.¹, Joseph M. Wallace, Ph.D.².
¹Purdue University, West Lafayette, IN, USA, ²Indiana University-Purdue University at Indianapolis, Indianapolis, IN, USA.

Disclosures:

Introduction: The specifics of how the nanoscale morphology of type I collagen affects larger length scales in bone’s structural hierarchy is poorly understood. Osteolathyriusm alters collagen morphology through reduced lysyl oxidase (LOX)-dependent enzymatic crosslinking, which is modeled experimentally using β-aminopropionitrile (BAPN) treatment to irreversibly block the LOX active site. Exercise improves post-yield mechanical properties in murine bone, likely due to changes in collagen. The purpose of this study was to examine the effects of reduced enzymatic crosslinking, exercise, and exercise-mediated compensation of altered crosslinking on collagen morphology, tissue composition, and nano and microscale mechanical properties. We hypothesized that disease-induced alterations in collagen can be compensated for via exercise.

Methods: 8 week old female C57BL/6 mice were separated into 4 weight-matched groups (n=5 per group) undergoing normal cage activity (Sed) or running (Ex: 12 m/min, 30 min/day for 21 consecutive days) with daily 200 μl subcutaneous injections of either phosphate buffered saline (PBS) or BAPN (300 mg/kg in PBS; IUCAC #SC210R). After sacrifice by CO₂ inhalation at 11 weeks of age, femora and tibiae were excised and stored wrapped in saline-soaked gauze at -20°C until needed. Each femur was mounted onto a steel disk with the ends removed and the anterior surface was polished with a 3 μm diamond suspension. Left femurs were treated with 0.5M EDTA (pH 8.0) for 14 min followed by 5 min of sonication in H₂O for 3 cycles for atomic force microscopy (AFM). Four 3.5 μm x 3.5 μm images were acquired per bone and D-spacing was measured from 15-20 individual fibrils per image (~70 total per bone) using 2D Fast Fourier Transforms. Nanoindentation was performed with a diamond Berkovich indenter along the anterior polished surface of hydrated right femora at 300 μN/s to 3000 μN, held for 10 sec, and unloaded at 300 μN/s. Reduced modulus, Eᵣ, and hardness, H, were calculated from five locations along each bone. Raman spectroscopy was performed at five locations distal to the tibia-fibula junction (TFJ) on each left tibiae while hydrated using a 660 nm laser focused to a 10 μm spot. After baseline correction, band area ratios were calculated to reveal matrix mineralization (PO₄³⁻ v1/Amide I, PO₄³⁻ v1/ CH₂ wag, and PO₄³⁻/Amide III) and carbonate substitution (CO₃²⁻ v1/ PO₄³⁻ v1). Crystallinity was defined as 1/full width at half max (FWHM) of the PO₄³⁻ v1 peak. Reference Point Indentation (RPI) was performed at five locations along the left tibia distal to the TFJ while hydrated (10 N, 10 cycles, 2 Hz). A custom Matlab code was used to calculate 1st cycle indentation distance (ID 1), 1st cycle energy dissipation (ED 1), 1st cycle unloading slope (US 1), 1st cycle creep indentation distance (CID 1), indentation distance increase (IDl), total indentation distance (TID), total energy dissipation (ED Tot), average creep indentation distance (CID), average energy dissipation from cycles 3-10 (ED), and average unloading slope (US). A two-way ANOVA examined mean differences due to the effects of BAPN and exercise. D-spacing distribution differences due to BAPN (Sed BAPN vs Sed PBS), exercise (Ex PBS vs Sed PBS), and exercise-induced modulation of BAPN treatment (Ex BAPN vs Sed PBS) were analyzed with a Kolmogorov-Smirnov (KS) test. Statistical significance was defined as p<0.05.

Results: Data are presented as mean ± standard deviation. One animal was excluded from the Ex PBS group (n=4) due to an inability to run. Mean D-spacing was not significantly different for either BAPN treatment or exercise. However, Sed BAPN had a significantly different D-spacing distribution compared to Sed PBS (p<0.001; Fig. 1). The distribution of Ex PBS was not different from Sed PBS (p=0.429). The distribution of Ex BAPN was not significantly different from Sed PBS (p=0.726; Fig. 1). Crystallinity was significantly increased due to BAPN (p=0.025) but was not affected by exercise (p=0.979). No compositional changes were observed for either Ex or BAPN treatment for carbonate substitution or matrix mineralization. Nanoindentation did not reveal significant changes in Er or H. No RPI parameters were statistically different for either main effect.
Figure 1. Cumulative distribution plots by group. BAPN has a significant effect on D-spacing distribution in Sed animals, but the presence of Ex rescues this effect despite Ex not having a discernible effect on its own (p=0.429; not shown). Sed PBS n=326, Sed BAPN n=252, Ex PBS n=355, Ex BAPN n=332.

**Discussion:** As seen from Fig. 1, BAPN administration has an effect on the nanoscale morphology of collagen under normal conditions. While exercise does not independently alter the D-spacing distribution, it does modify the effects of BAPN alone (Fig. 1). A possible mechanism of exercise’s ability to restore the D-spacing distribution in BAPN mice to levels indistinguishable from Sed PBS animals is that exercise induces increased expression of enzymes involved in collagen synthesis (e.g. LOX), a necessary step if bone formation were to increase as an adaptive response to mechanical stimulation. In Ex PBS, increased LOX does not induce a morphology change because under normal conditions, enzymatic crosslinking levels are tightly regulated. However, in Ex BAPN where there is a deficiency in functional LOX, increasing LOX expression would result in more functional unbound LOX, possibly leading to normal crosslinking and thereby restoring the D-spacing distribution to control levels. In this study, bone near the periosteal surface was imaged, which due to the rapid growth in 8 week old mice is likely newly formed tissue. Other experiments suggest exercise can influence preexisting bone, which would allow for additional mechanisms to be possible in those areas.

Work by El Rouby et al suggests reduced mineralization with BAPN treatment which would explain the increased crystallinity here in BAPN treated animals due to the reduction of newly formed disorganized crystallites and a greater contribution of larger more perfect crystals undergoing secondary mineralization. The discrepancy between results here and a recent study showing no effect of BAPN on mineral could be the difference in dosing, method of BAPN administration (injection vs ingestion), or animal model used (mouse vs rat).

The lack of observable mechanical effects may be due to the low sample size or the ability of AFM to detect changes at small length scales near the insult of BAPN whereas other techniques measuring over larger scales are unable to detect this difference within the short 3 week timeframe. The changes in D-spacing distribution and crystallinity may indeed affect properties measured with micro and nanoindentation techniques and could possibly be observed with longer administration or a lag time between administration and sacrifice.

In conclusion, exercise in the presence of BAPN treatment restores the collagen D-spacing distribution to normal in murine bone, suggesting a beneficial effect on enzymatic crosslinking despite the disease state. The altered D-spacing distribution produced no observed mechanical effects although mineral crystallinity was altered with BAPN treatment. Future experiments will investigate if rescued collagen morphology translates into restored mechanical integrity at the collagen fibril level using AFM indentation and study potential effects in tissue formed before and after the treatment.

**Significance:** The contribution of this work will be to examine the effects of altered enzymatic crosslinking, exercise, and exercise in the presence of disease on the morphology, composition, and mechanics of bone. If bone mechanical integrity can be rescued as a result of this change, exercise could be a potent non-invasive treatment for osteolathyrism and other collagen-based diseases.

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**References:**