HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Bone. 2015 December ; 81: 327–337. doi:10.1016/j.bone.2015.07.030.

Exercise Increases Pyridinoline Cross-linking and Counters the Mechanical Effects of Concurrent Lathyrogenic Treatment

Erin M. B. McNerny[1], **Joseph D. Gardinier**[2], and **David H. Kohn**[1,2,*]

[1]Department of Biomedical Engineering, College of Engineering and Medical School, University of Michigan, MI USA

[2]Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, MI USA

Abstract

The collagen cross-link profile of bone, associated with bone strength and fracture toughness, is tightly regulated (affecting cross-link quantity, type, lysine hydroxylation and maturity) and may contribute to the improvements in bone quality during exercise. We hypothesized that 1) exercise promotes mature cross-link formation, 2) increased mature cross-linking is accompanied by shifts in lysine hydroxylation, and 3) these changes in collagen cross-link profile have positive effects on mechanical properties. Growing male C57Bl6 mice were treated with 30 min/day of running exercise, 350 mg/kg/day β-aminopropionitrile (BAPN) injected subcutaneously to inhibit enzymatic collagen cross-linking, or both exercise and BAPN, from 5 to 8 weeks of age. Bone collagen cross-linking profile, mechanical properties, morphology, and mineralization were measured from the tibiae. Cross-link measures, including immature, pyridinoline, pyrrole and pentosidine cross-links, ratios reflecting cross-link maturity and hydroxylation, and mineralization were tested for their importance to mechanical properties across 8 week groups through correlation analyses and step-wise linear regressions.

BAPN treatment significantly reduced lysylpyridinoline, pyrrole, hydroxylysinorleucine, and total mature collagen cross-linking, resulting in decreased bone elastic modulus and increased yield strain despite a marginal increase in TMD. Exercise caused a shift toward pyridinoline cross-linking, with increased hydroxylysylpyridinoline and decreased pyrrole cross-linking resulting in total mature cross-linking and estimated tissue level mechanical properties matching sedentary control levels. Exercise superimposed on BAPN treatment increased total mature cross-linking from BAPN to control levels, but did so by increasing pyridinoline, not pyrrole, cross-links. Exercise also counteracted the BAPN effects on modulus and strain, without a change in TMD. Pyrrole cross-linking was the strongest correlate of modulus ($r=0.470$, $p<0.01$) and yield strain ($r=-0.467$, $p<0.01$). Cross-links with similar levels of telopeptide lysine hydroxylation to pyrrole (lysylpyridinoline and hydroxylysinorleucine) also correlated with modulus and strain to a lesser

*Corresponding Author 1011 N University Ave., Ann Arbor, MI, 48109 USA. Tel.: +1 734 764 2206, fax: +1 734 647 2110, dhkohn@umich.edu.

extent. In conclusion, exercise in growing mice promotes pyridinoline collagen cross-linking in bone, the resulting increase in total mature cross-linking is sufficient to counteract the mechanical effects of concurrent cross-link inhibition, and this responsiveness to loading is a potential means by which exercise might improve bone quality in diseased or otherwise compromised bone.

Keywords

Collagen cross-linking; mechanics; lathyrism; exercise; β-aminopropionitrile; bone quality

1. INTRODUCTION

The amounts and ratios of different types of cross-links in bone (e.g. immature vs. mature, enzymatic vs. non-enzymatic, hydroxylation level) are highly regulated to meet specific, but not fully understood, functional purposes.[1–8] Cross-linking is a tightly controlled process with tissue-specific patterns of hydroxylation, location and quantity.[9–14] Healthy human bone collagen has a particular pattern of cross-links distributed at the N and C termini,[9,15] but shifts in cross-link profile occur with aging and many musculoskeletal pathologies and can be detrimental to bone quality.[1,8,16–19]

The quantity of enzymatic cross-links formed is determined by lysyl oxidase (LOX) enzyme activity. Extracellular LOX transforms telopeptide lysine residues to reactive allysines, which are required precursors for cross-link formation. LOX also has homologs, lysyl oxidase like proteins 1-4 (LOXL1-4), which may have tissue specificity.[13] Initial cross-link formation in bone following LOX activity produces divalent, immature cross-links, quantified as their reduced forms dihydroxylysinorleucine (DHLNL) and hydroxylysinorleucine (HLNL). Some of these cross-links further react to form trivalent, mature cross-links, including hydroxylysylpyridinoline (HP), lysylpyridinoline (LP) and pyrroles.

Inhibition of LOX and LOXLs by the lathyrogen β-aminopropionitrile (BAPN) reduces cross-link formation,[21–24] negatively impacting bone and soft connective tissue mechanical integrity.[24–27] We recently developed a BAPN dose-controlled lathyrism mouse model and demonstrated that a reduction of only mature enzymatic cross-links in growing mice is sufficient to decrease bone strength and fracture toughness.[28] While mature, trivalent cross-links are greater contributors to bone strength than the less stable, divalent cross-links,[28,29] bone is unique among fibrillar collagenous tissues in the persistence of large quantities of immature cross-links.[14,30]

While LOX is required for enzymatic cross-links to form, the species of cross-links to form is determined by intracellular lysyl hydroxylase (LH) activity. Three isoforms of LH are known, LH1-3, with LH2 being a telopeptide-specific lysyl hydroxylase that controls the relative proportions of pyridinoline and pyrrole cross-links.[10,31–33] Pyrrole cross-links may be interfibrillar and directly provide greater mechanical advantage than pyridinolines.[29,34] It is also possible that the ratio of pyrrole to pyridinoline cross-links controls mineralization and mechanical properties of bone, as suggested by decreases in mineralization observed with increased LH2 expression and pyridinoline levels.[1,35]

As tightly as bone's cross-link profile is regulated, and with its association with bone quality, it is plausible that cross-linking has a role in bone's adaptive responses to external cues such as mechanical loading. The classic understanding of the effect of mechanical loading on bone is that it promotes an anabolic bone formation response.[36] However, exercise can also influence tissue quality, illustrated by improvements in mechanical properties and fatigue resistance in the absence of significant new bone formation.[37,38] One indication that exercise-induced changes in tissue quality are mediated by changes in cross-linking is that exercise protected against a fatigue-induced drop in the Raman ~1660/1690 band area "cross-link" ratio.[38] Further evidence that cross-link profile is altered by changes in loading is that unloaded subchondral bone is cross-link deficient compared to loaded bone[39] and that young rats increase lysyl pyridinoline levels in trabecular bone following 10 weeks of moderate running.[40]

In summary, changes in cross-linking as a result of mechanical loading may contribute to changes in bone quality. However, it remains to be elucidated how the complete cross-link profile, i.e. the full battery of immature (divalent), mature (trivalent pyridinoline and pyrrole) and glycation cross-links, changes in response to exercise and which specific cross-link changes contribute to coinciding improvements in mechanical properties. We hypothesized that exercise promotes mature cross-link formation and may concurrently modify cross-link chemistry through shifts in lysine hydroxylation, that these changes have positive effects on mechanical properties, and that as a result of this control, exercise can be used to improve the quality of diseased or otherwise compromised bone. To test this hypothesis and better understand the role of cross-links in bone's response to mechanical loading, we combined our murine model of running exercise[41] with our injected BAPN lathyrism model[28] and measured the effects of exercise, BAPN, and their combination on: 1) bone collagen cross-link profile, 2) bone cross-sectional geometry and mineralization, and 3) bone mechanical properties. We then explored the relationship of individual cross-link measures and mineralization with mechanical properties through step-wise linear regression.

2. METHODS

2.1 Animals

All animal procedures were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. Male C57Bl6 mice (Charles River) were weight matched at 5 weeks of age into five treatment groups: Baseline, PBS-Sedentary, PBS-Exercise, BAPN-Sedentary and BAPN-Exercise (n=20/group). Baseline mice were sacrificed on experiment day 0 at 5 weeks of age. The remaining groups received their assigned treatment for 3 weeks (experiment days 1-21). PBS and BAPN treated mice received daily subcutaneous injections of PBS or 350mg BAPN/kg (β-aminopropionitrile fumarate, Sigma Aldrich) in PBS, respectively. This dose of BAPN induces lathryism and leads to a significant reduction in bone strength and fracture toughness.[28] All groups had access to normal cage activity. Exercise groups additionally ran 30 min/day on a motorized treadmill (12m/min, 5 degree incline; Columbus Instruments, OH).[37,38] Non-Baseline mice received fluorochrome injections on experiment days 1 (alizarin complexone, 25mg/kg), 7 (calcein, 15mg/kg), 13 (xylenol orange, 90mg/kg) and 19 (tetracycline, 25mg/kg) to

mark the location, direction and amount of bone formation in order to quantify the percentage of cortical area formed during treatment. Mice were sacrificed by CO_2 inhalation on day 22 at 8 weeks of age. Both tibiae were immediately harvested, cleaned of soft tissue, and stored frozen in gauze soaked in calcium buffered PBS.

2.2 Collagen Cross-link Quantification

Right tibial diaphyses were isolated, flushed of marrow, and used for quantification of collagen cross-linking (n=12/group). Mature (HP, LP and pyrrole), reduced immature (DHLNL and HLNL) and glycation (pentosidine, PEN) cross-links were all quantified from the same bone and normalized to collagen content, as reported.[28] In brief, cortical bone samples were demineralized in EDTA and then reacted with sodium borohydride to reduce and stabilize the immature cross-links. Reduced samples were digested with trypsin, and an aliquot of trypsin digest was used for quantification of pyrrole cross-links by colorimetric assay in a 384 well plate. The remaining trypsin digest was acid hydrolyzed and used for colorimetric hydroxyproline assay, SPE column clean-up, and subsequent HPLC injection for quantification of HP, LP, DHLNL, HLNL and PEN. Pentosidine in these samples was at the detection limit and is not presented.

2.3 μCT

Left tibiae (20/group) were scanned by μCT over the entire length (μCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 12μm, 70kVp, 114μA, 0.5mm AL filter, and integration time 500ms. Scans were reoriented using Scanco IPL to match the alignment of each bone *in silico* with its eventual alignment during mechanical testing. A 19 slice standard region of interest was taken from the mid diaphysis at the point 23% of the distance from the tibia-fibula junction to the proximal end of the tibia. This site, located near the center of the mechanical testing span, was used for quantification of bone cross-sectional geometry and mineral density. Thus, the cortical site analyzed was reproducible across samples and appropriate for calculating tissue level properties using classic beam theory. Geometry, tissue mineral content (TMC) and tissue mineral density (TMD) were measured using a fixed threshold of 276 “per mille” (equal to a linear attenuation coefficient of 2.21 [1/cm]). Three-dimensional analysis of the trabecular bone structure was conducted on each sample using a 500 μm thick volume of interest 50 μm below the proximal growth plate. Using the manufacturer’s software and direct 3-dimensional method provided, the following trabecular parameters were calculated: bone volume fraction (BV/TV), tissue mineral density (TMD), trabecular number (Tb.N), spacing (Tb.Sp), thickness (Tb.Th), connectivity density (Conn.D), and structural model index (SMI).

2.4 Four-Point Bending

Following μCT–scanning, left tibiae (n=20/group) were tested in 4 point bending on an eXpert 450 Universal Testing Machine (Admet; Norwood, MA) as described.[42] Bones were hydrated with Ca-buffered PBS at all times, oriented with the medial surface in tension (bending about the anterior-posterior axis) and loaded at a displacement rate of 0.025mm/s (loading span: 3mm, support span: 9mm). Using beam theory and the standard site cross-sectional geometry measured by μCT, recorded load and displacement data were normalized

to estimate stress and strain. Both whole bone (load, displacement) and estimated tissue level (stress, strain) properties were quantified at the yield and ultimate points. Yield was defined from the stress-strain curve using the 0.2% strain offset method and converted to identify yield on the load-displacement curve. Whole bone stiffness and tissue elastic modulus were calculated by linear regression fitting of the linear-elastic pre-yield region. Pre-yield work and pre-yield toughness (resilience) were calculated as the area under the load-displacement and stress-strain curves, respectively, up to the previously defined yield point. Failure point data were not collectable due to large post-yield deformations that necessitated test termination prior to catastrophic fracture, as in our prior study using mice of the same age and strain.[28]

2.5 Embedding, Sectioning, and Imaging of Fluorochrome Labels

Following mechanical testing, left tibiae (n=6-8) from non-Baseline groups were dehydrated in a graded ethanol series, cleared with Clear-Rite 3 (Thermo Scientific), and infiltrated and embedded using Koldmount (SPI supplies). Sections ~150μm thick were cut from the mid-diaphysis near the failure site using a low-speed sectioning saw (Model 650; South Bay Technology, San Clemente, CA) with a diamond wafering blade (Mager Scientific). Sections were mounted on glass slides and hand ground and polished using wet silicon carbide paper to a final thickness of 75-100μm. Fluorochrome labels were imaged on a Nikon E800 fluorescence light microscope equipped with a FITC filter and Photometrics coolsnap monochrome camera. The goal of fluorochrome administration in this study was not to quantify bone apposition rate but rather to quantify the location and amount of tissue formed during treatment. Areas of new bone growth were discriminated from pre-existing tissue by the location and order of fluorochrome labeling. Alizarin red marked the earliest boundary between pre-existing tissue and bone formed during the experiment, while the remaining labels positively identified the locations and directions of new bone apposition throughout the experiment. The perimeters defining new and total bone areas were traced and the new, pre-existing, and total bone areas were quantified using ImageJ software.

2.6 Statistics

Statistical analyses were performed using SigmaStat and SPSS software. The effects of skeletal maturation during sham treatment on TMD, cross-link and mechanical measures were tested by comparing PBS-Sedentary to Baseline with Student t-tests or with non-parametric Mann-Whitney U tests. In the case of mouse and bone size parameters, where it was of interest to confirm that all 8 week old groups grew significantly from Baseline, one way ANOVA with Holm-Sidak post-hoc testing was used to compare each of the four treatment groups to Baseline. Main factor and interaction effects of BAPN and Exercise across the non-Baseline groups were tested by two-way ANOVA with Holm-Sidak post-hoc testing between all groups. Relationships between mineral, cross-link and tissue-level mechanical properties were tested using Pearson correlation and step-wise multiple linear regression. Correlations and linear regressions were performed for the 8 week old groups only, to avoid the confounding effect of tissue maturation. In all analyses, $p<0.05$ was considered significant, and $p<0.1$ was considered marginally significant.

3. RESULTS

3.1 Exercise and BAPN Reduce Mouse and Cortical Bone but not Cancellous Bone Growth

Mice in all groups sacrificed at 8 weeks of age significantly grew in bodyweight and tibia size compared to Baseline (Table 1). Exercise and BAPN both significantly reduced final mouse weight (Factors tested by 2-way ANOVA, $p<0.001$ and $p=0.010$ respectively), but only exercise had a statistically significant effect on tibia length, cortical area, cortical thickness, and bending moment of inertia about the medial-lateral axis (Table 1). The lack of significant effects of BAPN and Exercise on the bending moment about the anterior-posterior axis, as well as the averaged traced cortical morphometry (Figure 1), illustrate that the differences in bone size between the treatment groups are primarily attributable to differences in cortical expansion along the anterior-posterior axis. Periodically administered fluorochrome labels positively identified areas of bone formed between 5 and 8 weeks (Figure 2); there were no significant differences in the percentage of cortical tissue formed during BAPN and/or exercise treatment (Table 1).

Unlike overall mouse growth and cortical expansion, neither BAPN nor exercise significantly affected cancellous bone. Cancellous bone showed significant changes with growth from baseline in all treatment groups, with increased total volume, bone volume, bone volume fraction, trabecular number, trabecular thickness, and connective density (Table 1, total volume and bone volume not shown). Trabecular spacing and structural model index decreased with growth from baseline (Table 1). There was a trend of an increase in connective density with exercise ($p=0.10$, Table 1).

TMD was increased from Baseline in both cancellous and cortical bone (Figure 3). In cortical bone, there was a marginally significant interaction between BAPN and Exercise treatments (Figure 3). PBS-Exercise had significantly greater cortical TMD than PBS-Sedentary, and BAPN-Sedentary had marginally increased cortical TMD compared to PBS-Sedentary controls.

3.2 Cross-link Maturity Increased from Baseline in Sedentary-PBS Controls

Total mature cross-links were increased in the 8-week old PBS-Sedentary mice compared to 5-week old Baseline mice (Figure 4-G), reflecting significant and marginally significant increases in pyrrole (F) and pyridinolines (C), respectively. In the absence of significant changes in the immature cross-links (D, E, H), the ratio of mature to immature cross-links was significantly increased (Figure 5-A) from Baseline in PBS-Sedentary mice.

3.3 Exercise Increases Pyridinoline Cross-linking, Improving Total Mature Cross-linking in BAPN Treated Bones

The effects of BAPN and Exercise on enzymatic cross-link content differed and included significant interactions (Figure 4A-I). BAPN caused a reduction of cross-linking with significant factor effects for LP (B), HLNL (E), Pyrrole (F), and total mature cross-links (G). Exercise countered the negative effects of BAPN on pyridinoline content; BAPN-Exercise mice had significantly increased HP, LP and total pyridinolines compared to BAPN-Sedentary mice (A-C). Exercise did not alter total mature cross-links in PBS treated

mice but countered the negative effect of BAPN, with BAPN-Exercise mice having significantly more mature cross-links than BAPN-Sedentary mice (G). Exercise alone increased pyridinoline cross-links, with significant factor effects on HP (A) and total pyridinoline (C). Exercise and BAPN both significantly reduced pyrrole as separate treatments, but pyrrole content was not further decreased by BAPN in exercised mice (F).

3.4 BAPN and Exercise Differentially Modulate Cross-link Maturity and Hydroxylation

Causing a greater reduction of mature than immature cross-links (Figure 4), BAPN significantly reduced the ratio of mature to immature cross-links in sedentary mice (Figure 5A). In addition, BAPN increased helical lysine hydroxylation of cross-links, having significant factor effects on pyridinoline (Figure 5B) and immature cross-link hydroxylation (Figure 5D). In contrast, exercise did not alter pyridinoline or immature cross-link hydroxylation, but increased the ratio of pyridinoline to pyrrole cross-links (Figure 5C). A shift from pyrrole to pyridinoline formation reflects an increase in telopeptide lysine hydroxylation.[15]

3.5 Exercise Counteracts BAPN-Induced Reductions of Modulus and Increases of Yield Strain

Tibiae from PBS-Sedentary mice exhibited significantly increased structural stiffness, strength and pre-yield work over tibiae from Baseline mice (Figure 6A-D). BAPN significantly reduced bone stiffness (Figure 6A) and yield strength (Figure 6B). However, post hoc tests revealed significant differences in stiffness from BAPN treatment in sedentary mice, but not in exercised mice, and BAPN significantly increased yield deformation in sedentary mice but not in exercised mice (Figure 6E). Thus, exercise counteracted BAPN-induced decreases in stiffness and increases in yield deformation. In both PBS and BAPN-treated exercised mice, yield and ultimate deformation (Figure 6E-F) were not significantly different from PBS-Sedentary. Exercise had negative effects on whole bone ultimate strength (Figure 6C) and pre-yield work (Figure 6D).

Although the bones of exercised and BAPN treated mice were smaller than those of PBS-Sedentary controls (Table 1, Figure 1), whole bone mechanical properties (Figure 6) largely reflected differences in estimated tissue level properties (Figure 7). PBS-Sedentary bones had increased modulus and material strength (Figure 7A-C) over Baseline. Pre-yield toughness (Figure 7D) and yield strain (Figure 7E) were also increased in PBS-Sedentary bones compared to baseline. BAPN significantly reduced bone tissue modulus (Figure 7A) and yield stress (Figure 7B). However, exercise partially counteracted the BAPN reduction of modulus. Post hoc tests revealed significant differences in modulus from BAPN treatment in sedentary mice, but not exercised mice. BAPN significantly increased yield strain (Figure 7E) in sedentary mice, but not in exercised mice, further indicating a counteractive effect of exercise on the effects of BAPN treatment Exercise had a marginally significant effect on ultimate stress (Figure 7C). In both PBS and BAPN-treated exercised mice, yield and ultimate strain (Figure 7E-F) were not significantly different from PBS-Sedentary.

3.6 Pyrrole Cross-links Significantly Correlate with Modulus and Strain

Within treatment groups sacrificed at 8 weeks of age, cortical TMD had no significant correlation with any mechanical property and a marginally significant, but negative, correlation with modulus (Table 2). In contrast, pyrrole, total mature and HLNL cross-links were significant positive correlates of modulus. No cross-links were significant correlates of tissue strength (regressions not shown), but yield strain was significantly negatively related to pyrrole, LP, total mature, and HLNL cross-links. Reflecting its positive correlation with modulus and negative relationship with yield strain, pyrrole was a significant negative correlate of pre-yield toughness. Total immature and total enzymatic cross-links were marginally significant negative correlates of strain. Pyridinoline hydroxylation (HP/LP) was a significant negative correlate of modulus and a marginally significant positive correlate of yield strain. Overall, higher levels of the less hydroxylated cross-links (pyrrole, LP and HLNL) were associated with tissues having a higher rigidity and lower yield strain.

Stepwise linear regressions were also performed to explain tissue level mechanical properties. All cross-link measures, as well as TMD, were available for entry into the models. However, in all cases, only pyrrole was included in the stepwise model (or no significant model was found). Thus, the significant models are equivalent to the bivariate correlations (Table 2).

To explore the most significant relationships visually, correlations between modulus and cross-link metrics were plotted, regression lines were fit with their 95% confidence intervals, and data points were scaled in size according to each sample's TMD (Figure 8). Illustrating the non-significant findings of the correlation analysis and step-wise regressions for TMD, no patterns of TMD covariance are apparent (Figure 8A-I). The plots highlight the strength of the relationship between pyrrole cross-links and modulus (Figure 8G); pyrrole content explains 22% of the variance in bone rigidity. Total mature cross-links are also a significant regressor (Figure 8H), but given the poor explanatory value of total pyridinolines (Figure 8F), this significance is attributable primarily to the pyrrole fraction. The next strongest regressor is HLNL, which is a more predominant precursor of pyrrole formation than DHLNL.[1]

4. DISCUSSION

This work demonstrates that exercise can shift cross-link chemistry toward the pyridinoline pathway (Figure 4), prevent or rescue the loss of mature cross-links from concurrent cross-link inhibition (Figure 5), and counter the effects of cross-link inhibition on mechanical properties (Figure 7). Inhibiting lysyl oxidase with BAPN significantly reduced LP, pyrrole, and total mature cross-links (Figure 4B,F,G) and led to significant reductions in modulus and increases in yield strain (Figure 7), despite marginally increased cortical TMD (Figure 3). Exercise superimposed on BAPN treatment counteracted these mechanical changes, as modulus (Figure 7A) and yield strain (Figure 7E) were no longer significantly different between BAPN treated sedentary and exercise groups. This recovery effect of exercise was not explained by greater mineralization, given that exercise superimposed on BAPN treatment did not increase TMD compared to BAPN treatment alone (Figure 3). However, exercise did increase the number of HP, LP, total pyridinoline, and total mature cross-links

(Figure 4A-C,G), as well as relative cross-link maturity (Figure 5A) in BAPN treated bones. Thus, the ability of exercise to counter the mechanical effects of cross-link inhibition may be attributable to an increase in total mature cross-links.

This study provides new insight into the importance of collagen cross-link specificity, showing not only the importance of pyrrole cross-links to mechanical properties in young bone, but also an association between mechanics and the immature and mature cross-link types most like pyrrole in location and degree of lysine hydroxylation. Pyrrole cross-links, which involve less telopeptide lysine hydroxylation than pyridinolines, had the strongest association with bone modulus (+), yield strain (−), and pre-yield toughness (−) (Table 2, Figure 8). These findings are in accordance with pyrrole cross-links having stronger associations than pyridinolines with mechanical properties in osteoporotic avian bone.[43] However, our study extends this finding to young bone and additionally shows that the immature (HLNL) and pyridinoline (LP) cross-links with the greatest overlap in formation pathway with pyrroles also correlated with modulus and strain (Table 2, Figure 8). Notably, collagen cross-link chemistry, rather than simple quantity or maturity, was most important in explaining bone mechanical properties. The most abundant cross-link, DHLNL, was not significantly associated with any mechanical factor. Also, the less hydroxylated immature (HLNL) and pyridinoline (LP) cross-links were less abundant than their hydroxylated counterparts, yet were more significantly correlated with mechanical properties.

Interestingly, although pyrrole cross-linking was a stronger correlate of bone mechanical properties, exercise increased pyridinoline cross-linking and counteracted the mechanical effects of BAPN treatment. The shift from pyrrole to pyridinoline content from exercise in PBS treated mice did not cause mechanical detriment. Thus, although pyrrole content was the most sensitive predictor of mechanical properties, the maintenance of total mature cross-linking is also important to mechanical quality.

The complexity of bone as a composite and living material makes it difficult to define universally applicable rules for relationships between its composition and mechanical properties. Despite this, the “rule of thumb” in explaining the mechanical properties of bone is that the mineral controls pre-yield rigidity and strength, and the organic matrix provides bone its ductility and toughness. The results of this study challenge the blanket nature of this concept. Tissue mineral density did not significantly correlate with any mechanical property within 8-week old mice whereas collagen cross-links were significantly associated with tissue modulus, yield strain, and pre-yield toughness.

We used young, growing mice in this study to ensure that sufficient amounts of cross-link deficient bone would be formed during the course of BAPN treatment. One concern was that reduced growth from BAPN or exercise treatment could skew the fractions of cross-link-inhibited and normal tissue found in the final bone sample. However, the percent new cortical tissue area was not significantly different across groups, with the bones of all groups comprised of >25% new tissue (Table 1). Also, neither BAPN nor exercise impaired cancellous bone development from baseline, suggesting that osteoblast function was not directly inhibited. A second concern was the order of treatment each day and whether effective BAPN dose might be altered by exercise. The fact that immature (HLNL, DHLNL,

total immature) and total enzymatic crosslinks were equally reduced in BAPN Sedentary and BAPN Exercise groups is supportive of successful and similar BAPN dosing in these groups. Mice were injected with BAPN within 60 minutes after the completion of exercise each day. We chose to perform injection after exercise to minimize loss of BAPN by diuresis or increased agitation of the subcutaneous injection site while exercising.

The results of this study related pyrrole and its precursor crosslinks to increased tissue modulus, reduced strain and reduced pre-yield toughness; thus, increases in pyrrole explained an increase in rigidity with yield occurring at a fixed stress after less energy absorption. In contrast, in our previous study testing the dosage effects of BAPN in growing mice, pyridinoline crosslinks were correlated with strength and, as part of a maturity ratio, fracture instability toughness.[28] Thus, pyridinolines were associated with the stress needed to initiate yield or crack propagation. The comparisons between Baseline and PBS-Sedentary bones in this study highlight the rapidity with which cross-links and mechanical properties are established in this young tissue. Mice in both studies were from the same vendor and prescribed age, but had different mean bodyweights. One possibility is that the larger baseline mean body weight (17.9 ± 1.1g vs. 15.3 ± 1.0g) in this study caused BAPN treatment to be imposed during a later phase of tissue development and maturation so that bone strength and modulus may have been more developed, affecting these properties' sensitivity to subsequent cross-link inhibition. Despite differences in the specific mechanical changes induced by BAPN in these two studies, cross-links with less lysine hydroxylation were consistently identified as the cross-links of greatest importance to tissue quality.

In human bone, pyridinoline cross-links form approximately equally at the N and C-termini, but greater levels of LP and pyrrole cross-links occur at the N terminus.[15] This site-specific distribution of cross-link species and levels of hydroxylation may play roles in mineralization and could contribute differently to mechanical properties due to differences in molecular packing and cross-link connectivity at these sites.[1,15,44,45] An improved ability of pyrrole cross-links compared to pyridinolines to form interfibrillar linkages could explain the positive relationship between pyrrole crosslinks and modulus (Table 2, Figure 8G), since interfibrillar crosslinks would better resist fibril sliding, increasing tissue rigidity. Alternatively, alterations in fibril diameter or packing are means by which cross-linking profile could affect mechanical properties.[46] Increasing LH2 expression in MC3T3 cells increases pyridinoline, delays mineralization and reduces collagen fibril diameter despite otherwise normal markers of differentiation.[32,35] In contrast, BAPN treatment increases the median and broadens the distribution of collagen fibril diameter in chick osteoblast cell culture.[47] The possibility that BAPN is similarly increasing fibril diameter *in vivo* and exercise counteracts the mechanical effects of BAPN by controlling fibril diameter through increased pyridinoline cross-linking is deserving of further study.

This study established the effects of exercise and its combination with BAPN treatment on cross-link profile but did not assess the mechanisms controlling these changes. The increase in pyridinoline formation from exercise could be the result of altered lysyl hydroxylase expression or activity. Without direct knowledge of how BAPN is metabolized in mice and how exercise changes BAPN clearance or lysyl oxidase expression patterns, we must speculate as to whether this model represents exercise having a preventative or rescue effect

of BAPN crosslink inhibition. BAPN reduces cross-linking by covalently binding and inhibiting the LOX active site; exercise could potentially counter this effect by promoting LOX expression to levels greater than the BAPN dose is capable of inhibiting or by promoting the maturation of immature to mature cross-links in order to offset the losses from BAPN treatment.

In conclusion, this study demonstrates the dynamic nature of collagen cross-linking in bone, with nearly all aspects of bone's cross-link profile displaying sensitivity to both exercise and cross-link inhibition. Exercise influenced the types and quantities of cross-links formed, providing further evidence that mechanical loading influences bone tissue quality, in addition to quantity. Although this exercise program reduced weight gain and bone size in these young, rapidly growing, mice, exercise increased the number of mature cross-links in BAPN treated bone and counteracted the effects of BAPN on modulus and strain. Cross-link type, rather than quantity, defined the strength of association between cross-link species and mechanical properties. Namely, pyrroles and cross-link species with similar telopeptide lysine hydroxylation (HLNL, LP) had the strongest relationships with modulus. As hypothesized, exercise promoted mature cross-linking with concurrent changes in lysine hydroxylation, reflected as shifts in the ratio of pyrrole and pyridinoline formation, and successfully improved mechanical properties in a model of impaired bone quality. These results contribute to a growing body of evidence that alterations in collagen cross-linking chemistry, from factors such as exercise, aging, and disease, can affect bone quality. Continued efforts to understand the cellular mechanisms underlying this responsiveness and to better define the parameter space that defines the "desirable" cross-link profile for bone will provide potential strategies to control and optimize cross-linking for improved bone health and fracture resistance.

ACKNOWLEDGEMENTS

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under award numbers R01 AR056657 and F32-AR064668, and by the National Institute of Dental and Craniofacial Research of the National Institutes of Health under award number T32 DE007057. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

REFERENCES

[1]. Eyre DR, Weis MA. Bone collagen: new clues to its mineralization mechanism from recessive osteogenesis imperfecta. Calcif Tissue Int. 2013; 93:338–47. doi:10.1007/s00223-013-9723-9. [PubMed: 23508630]

[2]. Karim L, Tang S, Sroga G, Vashishth D. Differences in non-enzymatic glycation and collagen cross-links between human cortical and cancellous bone. Osteoporos Int. 2013

[3]. Buehler MJ. Nanomechanics of collagen fibrils under varying cross-link densities: atomistic and continuum studies. J Mech Behav Biomed Mater. 2008; 1:59–67. doi:10.1016/j.jmbbm.2007.04.001. [PubMed: 19627772]

[4]. Turecek C, Fratzl-Zelman N, Rumpler M, Buchinger B, Spitzer S, Zoehrer R, et al. Collagen cross-linking influences osteoblastic differentiation. Calcif Tissue Int. 2008; 82:392–400. doi:10.1007/s00223-008-9136-3. [PubMed: 18488133]

[5]. Willems NMBK, Mulder L, Bank RA, Grünheid T, den Toonder JMJ, Zentner A, et al. Determination of the relationship between collagen cross-links and the bone-tissue stiffness in

the porcine mandibular condyle. J Biomech. 2011; 44:1132–6. doi:10.1016/j.jbiomech.2011.01.023. [PubMed: 21333996]

[6]. Puig-Hervás MT, Temtamy S, Aglan M, Valencia M, Martínez-Glez V, Ballesta-Martínez MJ, et al. Mutations in PLOD2 cause autosomal-recessive connective tissue disorders within the Bruck syndrome--osteogenesis imperfecta phenotypic spectrum. Hum Mutat. 2012; 33:1444–9. doi:10.1002/humu.22133. [PubMed: 22689593]

[7]. Schwarze U, Cundy T, Pyott SM, Christiansen HE, Hegde MR, Bank RA, et al. Mutations in FKBP10, which result in Bruck syndrome and recessive forms of osteogenesis imperfecta, inhibit the hydroxylation of telopeptide lysines in bone collagen. Hum Mol Genet. 2013; 22:1–17. doi:10.1093/hmg/dds371. [PubMed: 22949511]

[8]. Saito M, Marumo K. Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. Osteoporos Int. 2010; 21:195–214. doi:10.1007/s00198-009-1066-z. [PubMed: 19760059]

[9]. Eyre DR, Weis MA, Wu J-J. Advances in collagen cross-link analysis. Methods. 2008; 45:65–74. doi:10.1016/j.ymeth.2008.01.002. [PubMed: 18442706]

[10]. Van der Slot AJ, Zuurmond A-M, Bardoel AFJ, Wijmenga C, Pruijs HEH, Sillence DO, et al. Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. J Biol Chem. 2003; 278:40967–72. doi:10.1074/jbc.M307380200. [PubMed: 12881513]

[11]. Uzawa K, Grzesik WJ, Nishiura T, Kuznetsov SA, Robey PG, Brenner DA, et al. Differential expression of human lysyl hydroxylase genes, lysine hydroxylation, and cross-linking of type I collagen during osteoblastic differentiation in vitro. J Bone Miner Res. 1999; 14:1272–80. doi:10.1359/jbmr.1999.14.8.1272. [PubMed: 10457259]

[12]. Sricholpech M, Perdivara I, Nagaoka H, Yokoyama M, Tomer KB, Yamauchi M. Lysyl hydroxylase 3 glucosylates galactosylhydroxylysine residues in type I collagen in osteoblast culture. J Biol Chem. 2011; 286:8846–56. doi:10.1074/jbc.M110.178509. [PubMed: 21220425]

[13]. Lucero H, a; Kagan, HM. Lysyl oxidase: an oxidative enzyme and effector of cell function. Cell Mol Life Sci. 2006; 63:2304–16. doi:10.1007/s00018-006-6149-9. [PubMed: 16909208]

[14]. Robins, SP.; Brady, JD. Collagen Cross-Linking and Metabolism. In: Bilezikian, JP.; Raisz, LG.; Martin, TJ., editors. Princ. Bone Biol. 3rd. Academic Press; San Diego: 2008. p. 319-34.doi:http://dx.doi.org/10.1016/B978-0-12-373884-4.00035-5

[15]. Hanson DA, Eyre DR. Molecular Site Specificity of Pyridinoline and Pyrrole Cross-links in Type I Collagen of Human Bone. J Biol Chem. 1996; 271:26508–16. [PubMed: 8900119]

[16]. Avery NC, Bailey a J. Enzymic and non-enzymic cross-linking mechanisms in relation to turnover of collagen: relevance to aging and exercise. Scand J Med Sci Sport. 2005; 15:231–40. doi:10.1111/j.1600-0838.2005.00464.x.

[17]. Saito M, Fujii K, Mori Y, Marumo K. Role of collagen enzymatic and glycation induced cross-links as a determinant of bone quality in spontaneously diabetic WBN/Kob rats. Osteoporos Int. 2006; 17:1514–23. doi:10.1007/s00198-006-0155-5. [PubMed: 16770520]

[18]. Remst DFG, Blaney Davidson EN, Vitters EL, Blom AB, Stoop R, Snabel JM, et al. Osteoarthritis-related fibrosis is associated with both elevated pyridinoline cross-link formation and lysyl hydroxylase 2b expression. Osteoarthritis Cartilage. 2013; 21:157–64. doi:10.1016/j.joca.2012.10.002. [PubMed: 23069856]

[19]. Bank RA, Tekoppele JM, Janus GJ, Wassen MH, Pruijs HE, Van der Sluijs HA, et al. Pyridinium cross-links in bone of patients with osteogenesis imperfecta: evidence of a normal intrafibrillar collagen packing. J Bone Miner Res. 2000; 15:1330–6. doi:10.1359/jbmr.2000.15.7.1330. [PubMed: 10893681]

[20]. Eyre D, Shao P, Weis MA, Steinmann B. The kyphoscoliotic type of Ehlers-Danlos syndrome (type VI): differential effects on the hydroxylation of lysine in collagens I and II revealed by analysis of cross-linked telopeptides from urine. Mol Genet Metab. 2002; 76:211–6. [PubMed: 12126935]

[21]. Trackman PC. Diverse biological functions of extracellular collagen processing enzymes. J Cell Biochem. 2005; 96:927–37. doi:10.1002/jcb.20605. [PubMed: 16167328]

[22]. Ito H, Akiyama H, Iguchi H, Iyama K, Miyamoto M, Ohsawa K, et al. Molecular cloning and biological activity of a novel lysyl oxidase-related gene expressed in cartilage. J Biol Chem. 2001; 276:24023–9. doi:10.1074/jbc.M100861200. [PubMed: 11292829]

[23]. Borel A, Eichenberger D, Farjanel J, Kessler E, Gleyzal C, Hulmes DJ, et al. Lysyl oxidase-like protein from bovine aorta. Isolation and maturation to an active form by bone morphogenetic protein-1. J Biol Chem. 2001; 276:48944–9. doi:10.1074/jbc.M109499200. [PubMed: 11684696]

[24]. Barrow MV, Simpson CF, Miller EJ. Lathyrism: a review. Q Rev Biol. 1974; 49:101–28. [PubMed: 4601279]

[25]. Di Cesare PE, Nimni ME, Yazdi M, Cheung DT. Effects of lathyritic drugs and lathyritic demineralized bone matrix on induced and sustained osteogenesis. J Orthop Res. 1994; 12:395–402. doi:10.1002/jor.1100120312. [PubMed: 8207593]

[26]. Oxlund H, Barckman M, Ørtoft G, Andreassen T, Ortoft G. Reduced concentrations of collagen cross-links are associated with reduced strength of bone. Bone. 1995; 17:365S–371S. [PubMed: 8579939]

[27]. Brüel A, Ortoft G, Oxlund H. Inhibition of cross-links in collagen is associated with reduced stiffness of the aorta in young rats. Atherosclerosis. 1998; 140:135–45. [PubMed: 9733224]

[28]. McNerny EMB, Gong B, Morris MD, Kohn DH. Bone Fracture Toughness and Strength Correlate with Collagen Cross-Link Maturity in a Dose-Controlled Lathyrism Mouse Model. J Bone Miner Res. 2014 doi:10.1002/jbmr.2356.

[29]. Knott L, Bailey AJ. Collagen cross-links in mineralizing tissues: a review of their chemistry, function, and clinical relevance. Bone. 1998; 22:181–7. [PubMed: 9514209]

[30]. Gineyts E, Borel O, Chapurlat R, Garnero P. Quantification of immature and mature collagen crosslinks by liquid chromatography-electrospray ionization mass spectrometry in connective tissues. J Chromatogr B Analyt Technol Biomed Life Sci. 2010; 878:1449–54. doi:10.1016/j.jchromb.2010.03.039.

[31]. Salo AM, Sipilä L, Sormunen R, Ruotsalainen H, Vainio S, Myllylä R. The lysyl hydroxylase isoforms are widely expressed during mouse embryogenesis, but obtain tissue- and cell-specific patterns in the adult. Matrix Biol. 2006; 25:475–83. doi:10.1016/j.matbio.2006.08.260. [PubMed: 16996725]

[32]. Pornprasertsuk S, Duarte WR, Mochida Y, Yamauchi M. Lysyl hydroxylase-2b directs collagen cross-linking pathways in MC3T3-E1 cells. J Bone Miner Res. 2004; 19:1349–55. doi:10.1359/JBMR.040323. [PubMed: 15231023]

[33]. Yamauchi M, Sricholpech M. Lysine post-translational modifications of collagen. Essays Biochem. 2012; 52:113–33. doi:10.1042/bse0520113. [PubMed: 22708567]

[34]. Knott L, Bailey AJ. Collagen biochemistry of avian bone: comparison of bone type and skeletal site. Br Poult Sci. 1999; 40:371–9. doi:10.1080/00071669987476. [PubMed: 10475635]

[35]. Pornprasertsuk S, Duarte WR, Mochida Y, Yamauchi M. Overexpression of lysyl hydroxylase-2b leads to defective collagen fibrillogenesis and matrix mineralization. J Bone Miner Res. 2005; 20:81–7. doi:10.1359/JBMR.041026. [PubMed: 15619673]

[36]. Turner CH, Robling AG. Exercise as an anabolic stimulus for bone. Curr Pharm Des. 2004; 10:2629–41. [PubMed: 15320750]

[37]. Wallace JM, Ron MS, Kohn DH. Short-term exercise in mice increases tibial post-yield mechanical properties while two weeks of latency following exercise increases tissue-level strength. Calcif Tissue Int. 2009; 84:297–304. doi:10.1007/s00223-009-9228-8. [PubMed: 19283427]

[38]. Kohn DH, Sahar ND, Wallace JM, Golcuk K, Morris MD. Exercise alters mineral and matrix composition in the absence of adding new bone. Cells Tissues Organs. 2009; 189:33–7. doi:10.1159/000151452. [PubMed: 18703871]

[39]. Brama PA, Bank RA, Tekoppele JM, Weeren P, Van Weeren PR. Training affects the collagen framework of subchondral bone in foals. Vet J. 2001; 162:24–32. doi:10.1053/tvjl.2001.0570. [PubMed: 11409926]

[40]. Salem GJ, Zernicke RF, Martinez DA, Vailas AC. Adaptations of immature trabecular bone to moderate exercise: geometrical, biochemical, and biomechanical correlates. Bone. 1993; 14:647–54. [PubMed: 8274308]

[41]. Wallace JM, Rajachar RM, Allen MR, Bloomfield SA, Robey PG, Young MF, et al. Exercise-induced changes in the cortical bone of growing mice are bone- and gender-specific. Bone. 2007; 40:1120–7. doi:10.1016/j.bone.2006.12.002. [PubMed: 17240210]

[42]. Wallace JM, Golcuk K, Morris MD, Kohn DH. Inbred strain-specific response to biglycan deficiency in the cortical bone of C57BL6/129 and C3H/He mice. J Bone Miner Res. 2009; 24:1002–12. doi:10.1359/JBMR.081259. [PubMed: 19113913]

[43]. Knott L, Whitehead CC, Fleming RH, Bailey AJ. Biochemical changes in the collagenous matrix of osteoporotic avian bone. Biochem J. 1995; 310:1045–51. Pt 3. [PubMed: 7575401]

[44]. Wassen MH, Lammens J, Tekoppele JM, Sakkers RJ, Liu Z, Verbout AJ, et al. Collagen structure regulates fibril mineralization in osteogenesis as revealed by cross-link patterns in calcifying callus. J Bone Miner Res. 2000; 15:1776–85. doi:10.1359/jbmr.2000.15.9.1776. [PubMed: 10976997]

[45]. Yamauchi M, Katz EP. The post-translational chemistry and molecular packing of mineralizing tendon collagens. Connect Tissue Res. 1993; 29:81–98. [PubMed: 8403898]

[46]. Christiansen DL, Huang EK, Silver FH. Assembly of type I collagen: fusion of fibril subunits and the influence of fibril diameter on mechanical properties. Matrix Biol. 2000; 19:409–20. [PubMed: 10980417]

[47]. Gerstenfeld LC, Riva A, Hodgens K, Eyre DR, Landis WJ. Post-translational control of collagen fibrillogenesis in mineralizing cultures of chick osteoblasts. J Bone Miner Res. 1993; 8:1031–43. doi:10.1002/jbmr.5650080903. [PubMed: 8237472]

HIGHLIGHTS

- Growing mice were treated daily for three weeks with an inhibitor of enzymatic collagen cross-linking (BAPN), running exercise, or both.
- Exercise alone shifted cross-link chemistry from pyrrole formation toward pyridinoline cross-linking, without changing total mature cross-links, TMD, or mechanical properties.
- BAPN alone reduced mature and immature collagen cross-linking, marginally elevated TMD, decreased bone modulus and increased bone yield strain.
- Exercise superimposed on BAPN treatment counteracted BAPN's effects, increasing pyridinoline cross-linking, recovering total mature cross-linking, and rescuing mechanical properties.
- Pyrrole cross-links were the strongest correlate of mechanical properties, while TMD was not a significant correlate of any mechanical property.

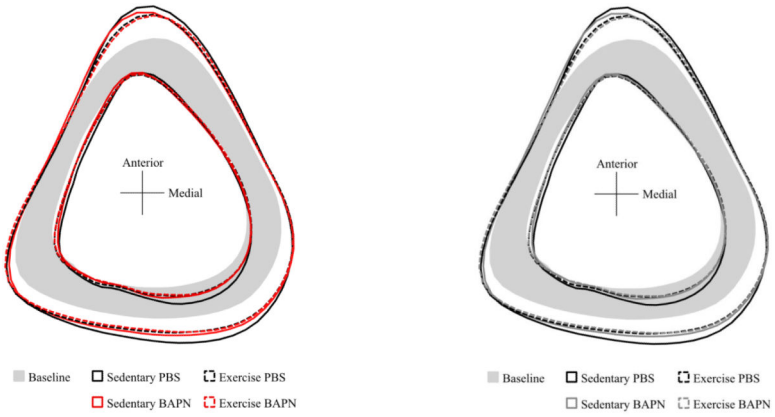

Figure 1. Traces of tibia cortical geometry

Cortical geometry was traced from μCT slices from all bones at the standard site and averaged within each group. All treatment groups (outlines) show significant growth from Baseline (shaded), but exercise (dashed lines) significantly reduced cortical area and thickness compared to Sedentary PBS mice (see quantitative data in Table 1). The greatest differences in bone size are due to differences in cortical expansion along the A-P axis. *Single column image, color online, grayscale print*

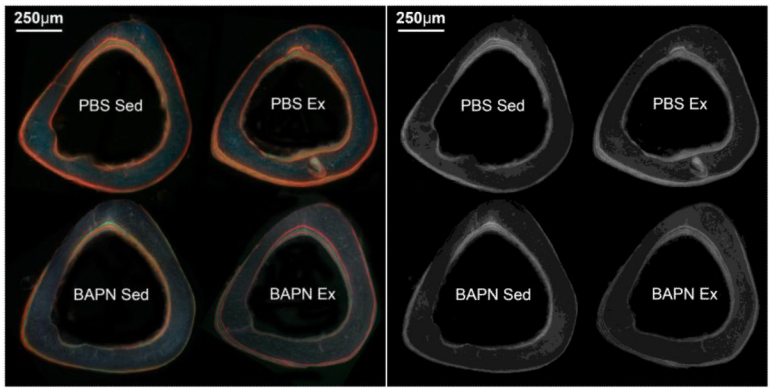

Figure 2. Fluorochrome labeling of new bone

Areas of cortical bone formed during the experiment were distinguished from pre-existing tissue by fluorochrome labeling, assessed at the tibia mid-diaphysis. Images were analyzed using ImageJ software by tracing and quantifying regions of new bone and total cortical area. No group differences in percent new tissue were found (Table 1). *Single column image.Color online, grayscale print*

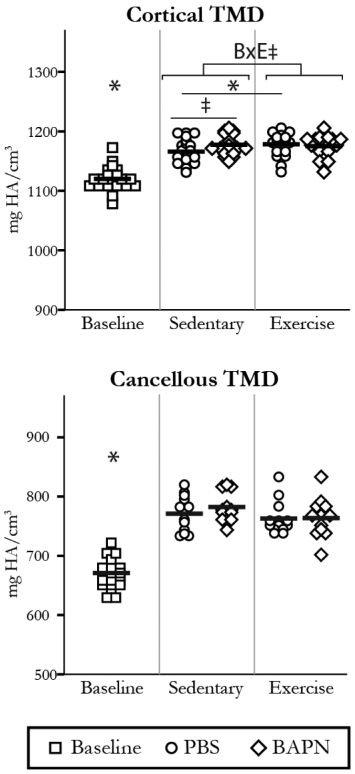

Figure 3. Tissue Mineral Density as a Function of BAPN and Exercise Treatments

Over Baseline indicates significant difference from PBS Sedentary (Student t-test) for both cortical (A) and cancellous (B) bone. Treatment affected cortical (A) but not cancellous (B) TMD. BxE indicates marginally significant factor interaction for BAPN and exercise measured by 2-Way ANOVA. Specific group differences tested by Holm-Sidak post hoc are additionally noted. ($p<0.05$, ‡$p<0.1$). *Single column image*

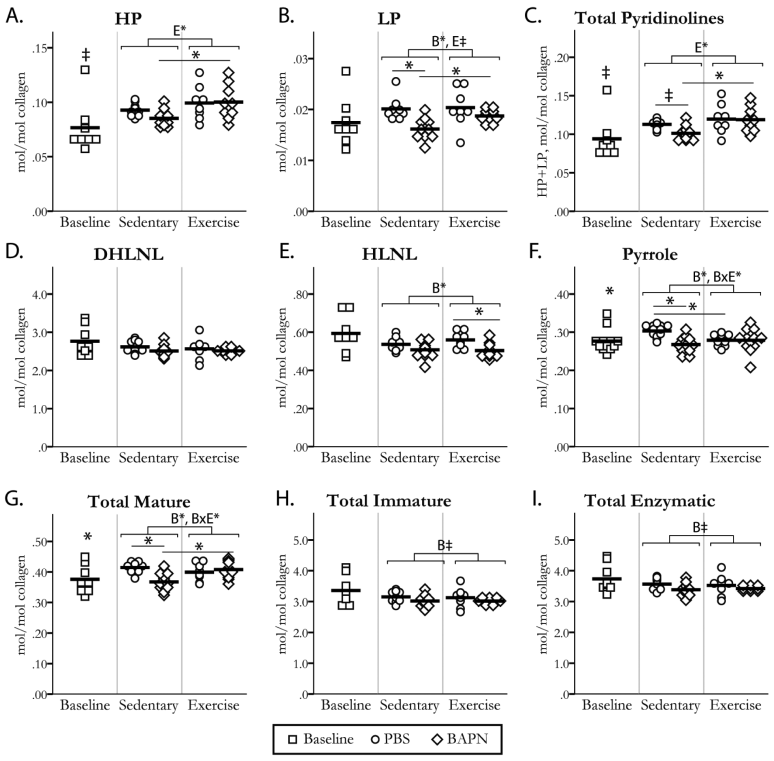

Figure 4. Collagen Enzymatic Cross-link Content as a Function of BAPN and Exercise Treatments

BAPN treatment reduced mature collagen cross-linking, with post-hoc group differences identifying significant reductions in LP (B), Pyrrole (F), and Total Mature (G) cross-links in BAPN compared to PBS treatment in sedentary mice.. Exercise increased pyridinoline cross-linking, particularly in BAPN treated mice, evidenced by significantly higher HP (A), LP (B) and Total Pyridinolines (C) in BAPN Exercised compared to BAPN Sedentary mice. * or ‡ above Baseline indicates significant ($p<0.05$) or partially significant ($p<0.1$) difference, respectively, from PBS Sedentary (Student t-test or Mann-Whitney). B, E and BxE indicate significant factor effects of BAPN, Exercise, or their interaction, respectively, by 2-Way ANOVA with specific group differences tested by Holm-Sidak post hoc additionally noted. (*$p<0.05$, ‡$p<0.1$). *Double column image*

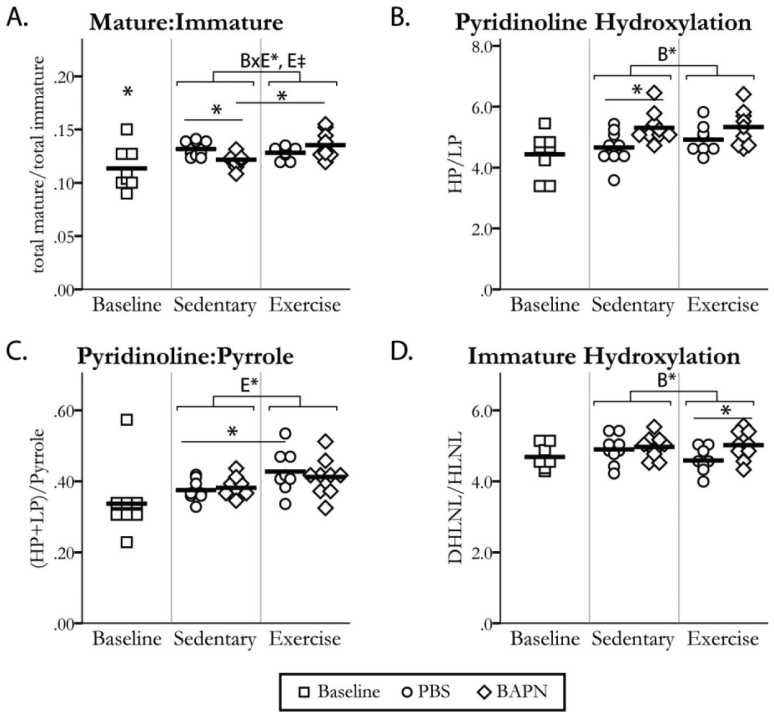

Figure 5. Cross-link Profile Ratios as a Function of BAPN and Exercise Treatments

BAPN treatment reduced relative cross-link maturity in sedentary mice (A) and was a significant factor in increasing pyridinoline (B) and immature (D) hydroxylation. Exercise countered BAPN's reduction of cross-link maturity (A) and increased the ratio of Pyridinoline to Pyrrole formation (C). * or ‡ above Baseline indicates significant ($p<0.05$) or partially significant ($p<0.1$) difference, respectively, from PBS Sedentary (Student t-test or Mann-Whitney). B, E and BxE indicate significant factor effects of BAPN, Exercise, or their interaction, respectively, by 2-Way ANOVA with specific group differences tested by Holm-Sidak post hoc additionally noted. (*$p<0.05$, ‡$p<0.1$). *1.5-column image*

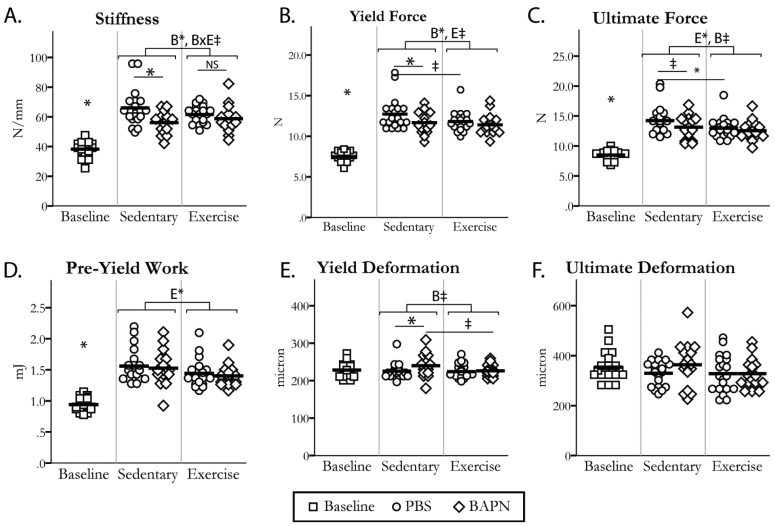

Figure 6. Whole Bone Mechanical Properties as a Function of BAPN and Exercise Treatments

BAPN reduced bone stiffness (A), yield force (B), and ultimate force (marginally significant, C), and increased yield deformation (E) in sedentary mice. Exercise reduced ultimate force (C) and pre-yield work (D) but negated the effects of BAPN on yield deformation (E). * or ‡ above Baseline indicates significant ($p<0.05$) or partially significant ($p<0.1$) difference, respectively, from PBS Sedentary (Student t-test or Mann-Whitney). B, E and BxE indicate significant factor effects of BAPN, Exercise, or their interaction, respectively, by 2-Way ANOVA with specific group differences tested by Holm-Sidak post hoc additionally noted. (*$p<0.05$, ‡$p<0.1$). *Double column image*

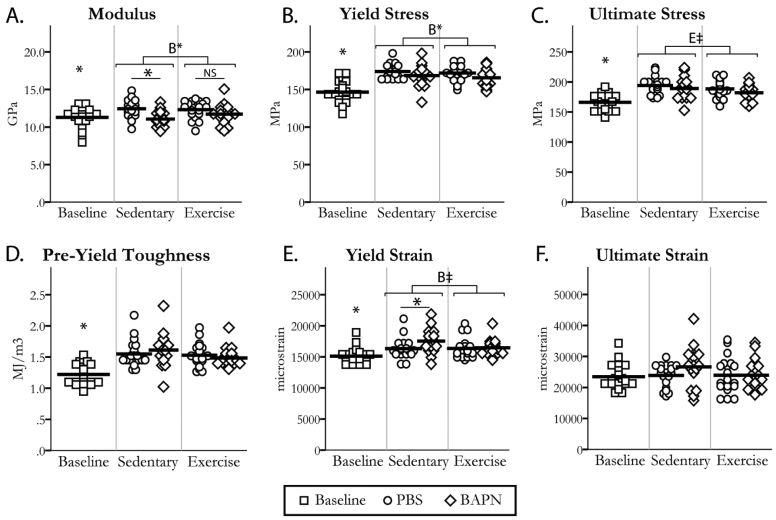

Figure 7. Tissue Level Mechanical Properties as a Function of BAPN and Exercise Treatments

BAPN reduced bone modulus (A) and yield stress (B) and increased yield strain (E) in sedentary mice. Exercise negated the effects of BAPN on yield strain (E). * or ‡ above Baseline indicates significant ($p<0.05$) or partially significant ($p<0.1$) difference, respectively, from PBS Sedentary (Student t-test or Mann-Whitney). B, E and BxE indicate significant factor effects of BAPN, Exercise, or their interaction, respectively, by 2-Way ANOVA with specific group differences tested by Holm-Sidak post hoc additionally noted. (*$p<0.05$, ‡$p<0.1$). *Double column image*

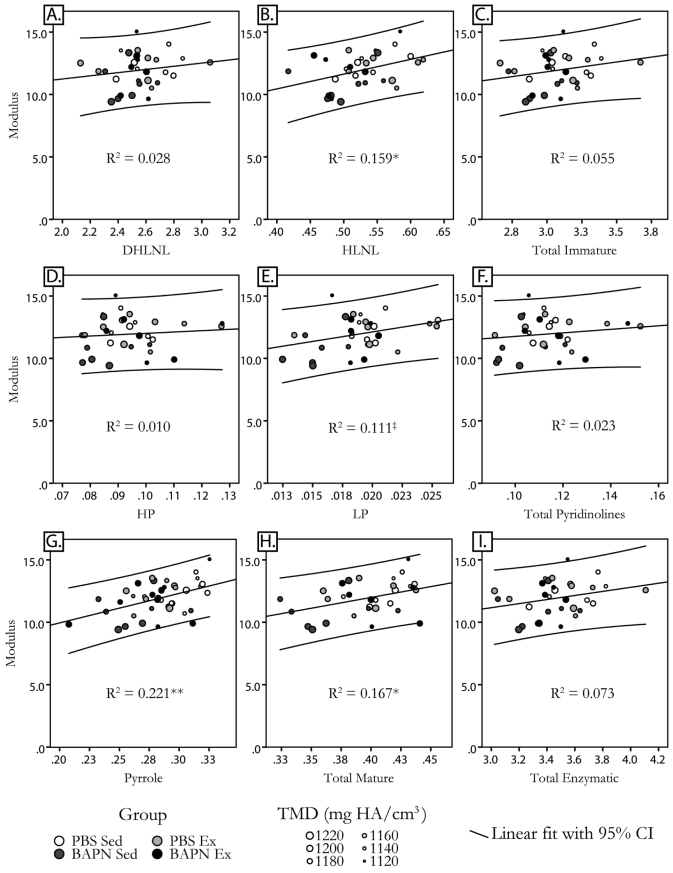

Figure 8. Linear regression tested associations between cross-linking measures and modulus

The greatest explainer of modulus is Pyrrole cross-links (G), accounting for 22% of modulus variability. The immature HLNL cross-link (B), a precursor of pyrrole cross-links, is also significantly associated with modulus. Of the pyridinolines, only LP (E), which also forms using HLNL as a precursor, is marginally associated with modulus. Data point size is proportional to each sample's cortical TMD (see legend for scale). TMD was not a significant correlate of any cross-link or modulus. Total Pyridinolines=HP+LP. Total Mature=HP+LP+Pyrrole. Total Immature=DHLNL+HLNL. Total Enzymatic=Total Mature +Total Immature. **p<0.01, *p<0.05, ‡p<0.1 *Double column image*

Table 1

Mouse Body Weight and Tibia Morphology as Functions of BAPN and Exercise Treatments

	Baseline	*PBS Sed*	*PBS Ex*	*BAPN Sed*	*BAPN Ex*	*Factor Effects*[a]	
						Exercise	**BAPN**
Bodyweight (g)	17.9[b] ± 1.1	22.8 ± 1.8	21.5 ± 1.8	21.8 ± 1.3	20.5 ± 1.5	**p<0.001**	**p=0.010**
Tibia Length (mm)	16.0[b] ± 0.15	17.2 ± 0.26	17.1 ± 0.24	17.2 ± 0.28	17.0 ± 0.28	**p=0.014**	p=0.580
Tibia Cortical Measures							
Ct.Area (mm^2)	0.385[b] ± 0.020	0.580 ± 0.071	0.535 ± 0.047	0.552 ± 0.053	0.535 ± 0.048	**p=0.016**	p=0.264
Ct.Th (mm)	0.129[b] ± 0.005	0.177 ± 0.014	0.16 ± 0.012	0.170 ± 0.010	0.165 ± 0.007	**p=0.003**	p=0.113
I_{ML} (mm^4)	0.048[b] ± 0.006	0.098 ± 0.023	0.082 ± 0.013	0.087 ± 0.016	0.081 ± 0.015	**p=0.008**	p=0.142
I_{AP} (mm^4)	0.040[b] ± 0.004	0.062 ± 0.011	0.059 ± 0.008	0.060 ± 0.009	0.059 ± 0.010	p=0.395	p=0.704
New Tissue (% of Ct.Area)	N/A	28.3 ± 3.8	27.0 ± 3.0	27.4 ± 5.7	26.3 ± 4.7	p=0.479	p=0.637
Tibia Cancellous Measures							
BV/TV	0.14[b] ± 0.01	0.21 ± 0.04	0.23 ± 0.04	0.22 ± 0.04	0.23 ± 0.04	p=0.324	p=0.931
Tb.N (1/mm)	4.54[b] ± 0.35	5.94 ± 0.37	6.13 ± 0.49	6.06 ± 0.22	6.17 ± 0.52	p=0.219	p=0.517
Tb.Th (mm)	0.050[b] ± 0.002	0.055 ± 0.004	0.056 ± 0.004	0.055 ± 0.006	0.054 ± 0.002	p=0.824	p=0.565
Tb.Sp (mm)	0.22[b] ± 0.02	0.155 ± 0.004	0.151 ± 0.004	0.151 ± 0.010	0.149 ± 0.018	p=0.466	p=0.494
Conn.D ($1/mm^3$)	95[b] ± 20	172 ± 35	193 ± 39	188 ± 33	205 ± 48	p=0.100	p=0.244
SMI	2.7[b] ± 0.17	2.2 ± 0.29	2.1 ± 0.30	2.2 ± 0.24	2.1 ± 0.30	p=0.141	p=0.952

[a] 2-Way ANOVA; Interaction term was not significant and is not shown.

[b] Sig. different from all other groups (ANOVA Holm-Sidak post-hoc or ANOVA on Ranks Dunn post-hoc, p<0.05)

Table 1

Pearson Correlations between Cross-links and Tissue Mechanical Properties of 8 Week Old Mice

Pearson Coefficient[a]	Modulus	Yield Strain	Pre-Yield Toughness
Cortical TMD	−0.200^		
Pyrroles	0.470**	−0.467**	−0.342*
Pyridinolines			
HP			
LP	0.334^	−0.350*	
Total Mature [b]	0.408*	−0.447*	−0.341^
DHLNL			
HLNL	0.399*	−0.392*	
Total Immature [c]		−0.315^	
Total Enzymatic [d]		−0.348 (p=0.051)	
Mature/Immature			
HP/LP	−0.365*	0.300^	

** p<0.01,

* p<0.05,

^ p<0.1;

[a] Only coefficients with p<0.1 are shown for clarity.

[b] Sum of Pyrroles and Pyridinolines.

[c] Sum of DHLNL and HLNL.

[d] Sum of Pyrroles, HP, LP, DHLNL and HLNL.