Dose-Dependent Effects of Beta-Aminopropionitrile on Osteoblast Gene Expression and Collagen Production

Silvia P. Canelon[1] and Joseph M. Wallace[1,2]

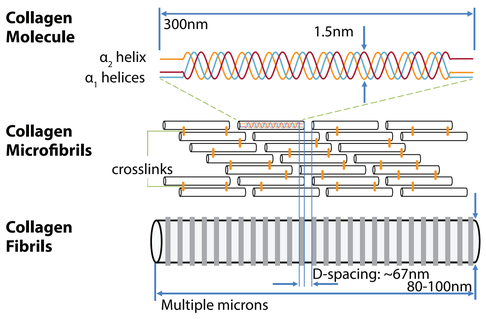
[1]Purdue University, Weldon School of Biomedical Engineering, West Lafayette, IN

[2]Indiana University-Purdue University at Indianapolis, Department of Biomedical Engineering, Indianapolis, IN

INTRODUCTION

Type I Collagen

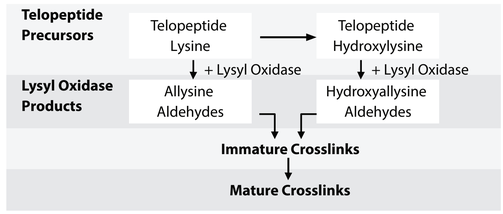
- Cells secrete three alpha helices which form a helical procollagen molecule
- Terminal telopeptide ends of molecules are cleaved by proteinases
- Inline self-assembly of molecules forms microfibrils
- Microfibrils arrange in a quarter-staggered array into fibrils with repeating gap and overap regions and are stabilized by crosslinks



- Periodicity of the gap and overlap region is referred to as the D-spacing and exists as a distribution of values
- Changes in the D-spacing distribution are reflective of disease, tissue type, and drug treatment

Collagen crosslinking

- Crosslinks stabilize collagen molecules within the fibrillar structure and the staggered array
- Enzymatic crosslink formation initiated in telopeptides by lysyl oxidase (LOX) enzyme reaction



Osteolathyrism

- Disease characterized by crosslink deficiency resulting in mechanical defects to bone and connective tissues
- Caused by high dietary consumption of osteolathyrogenic compounds such as beta-aminopropionitrile (BAPN)
- BAPN irreversibly binds to the active site of the LOX enzyme, preventing it from acting on telopeptide precursors

Study motivation

- Research dosage-dependent effects of BAPN on MC3T3-E1 osteoblast collagen-related gene expression, nanoscale collagen morphology, and collagen crosslinking

RESULTS

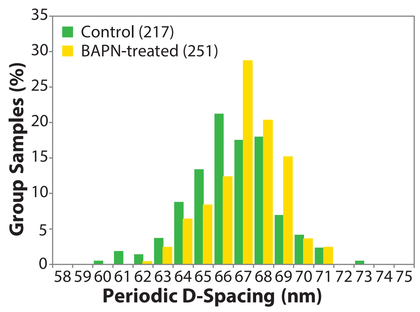
Quantitative reverse transcription polymerase chain reaction

Target Gene	Fold Change	0.125mM	0.25mM	0.5mM	1.0mM	2.0mM
Lysyl Oxidase	LOX	0.783	**0.580***	**0.540***	0.727	0.912
Bone Morph. Protein-1	BMP-1	1.044	**0.675***	0.071	**0.666***	0.945
Type I Collagen α_1	COL1A1	1.236	0.918	0.872	0.941	1.001
Type I Collagen α_2	COL1A2	1.215	0.878	0.930	1.123	1.396

*Indicates statistically significant changes ($p<0.05$)

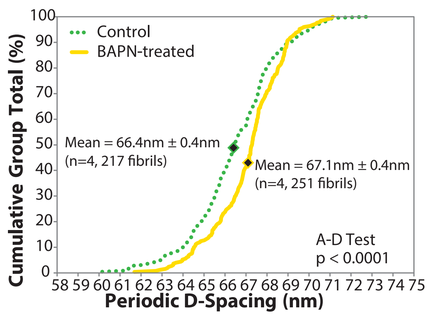
- Significant downregulation of LOX and BMP-1 at 0.25mM, 0.5mM, and 1.0mM
- No difference in genes coding for Type I collagen

Atomic force microscopy



Mean D-spacing
- Control: 66.4 nm ± 0.4 nm
- BAPN: 67.1 nm ± 0.4 nm

Non-parametric U-test revealed p=0.060

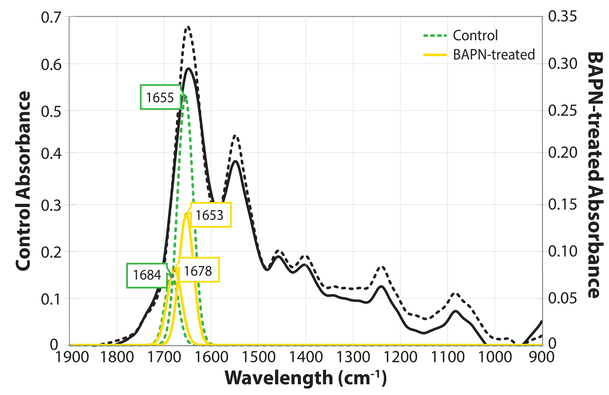


D-spacing distribution range
- Control: 60.2 nm - 72.9 nm
- BAPN: 61.7 nm - 71.1 nm

Anderson-Darling test revealed p<0.0001

Fourier Transform Infrared Spectroscopy

		Mean Peak Percent Area	Mean Area Ratio
Control	~1660 cm^{-1}	16.2868 ± 4.1089	3.9068 ± 1.6353
	~1690 cm^{-1}	4.7963 ± 2.2037	
BAPN	~1660 cm^{-1}	8.2149 ± 3.4959	1.9865 ± 0.6145
	~1690 cm^{-1}	4.4880 ± 2.3100	
p-value	~1660 cm^{-1}	**0.0048**	**0.0338**
	~1690 cm^{-1}	0.8177	



Significant decrease in collagen crosslink ratio driven by a reduction in the mature crosslink peak percent area

MATERIALS AND METHODS

Cell culture and collagen synthesis

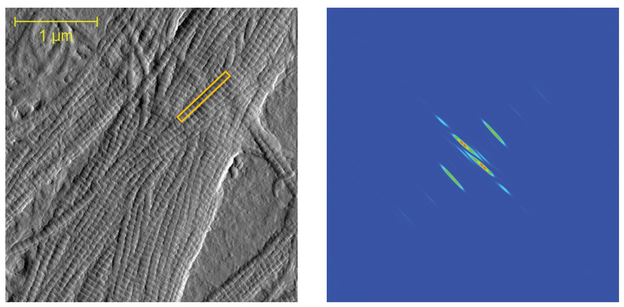
- Murine preosteoblasts (MC3T3-E1) cultured in proliferation medium and differentiated with 50 µg/mL ascorbic acid
- 500,000 cells were seeded into 60 mm dishes (density: 177 cells/mm^2)
- Experimental cultures were supplemented with 0.125, 0.25, 0.5, 1.0, or 2.0mM BAPN for qRT-PCR and 0.14mM for AFM and FTIR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

- Cells seeded into 10 dishes, 5 dishes per group (control or BAPN, n=5 each), and differentiated for 1 week
- SYBR Green primers and master mix used in determining mRNA expression
- Sample/gene combinations run in triplicate with β-actin as reference gene
- Expression fold change found using an efficiency-calibrated mathematical model of the REST® program

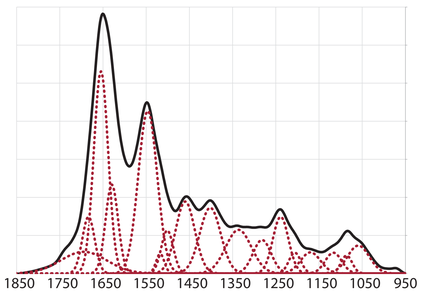
Atomic Force Microscopy (AFM)

- Cells seeded into 8 dishes, 4 dishes per group (control or 0.14mM BAPN, n=4 each), and differentiated for 2 weeks
- Media was removed and cells were treated with 10 mM EDTA to promote detachment from the extracellular matrix
- Matrix was rinsed with water and air-dried
- 5 locations per dish were imaged in air by atomic force microscopy
- 3.5 µm x 3.5 µm images from each location
- 2D Fast Fourier Transform (2D-FFT) performed on 10 collagen fibrils per location for D-spacing analysis
- Minimum of 50 fibrils per dish and 200 fibrils per group



Left: AFM error image of collagen with one fibril outlined for 2D-FFT.
Right: 2D-FFT corresponding to the outlined fibril.

Fourier Transform Infrared Spectroscopy (FTIR)



- Cells seeded into 6-well plates, 6 wells per group (control or 0.14mM BAPN, n=6 each), and differentiated for 2 weeks
- Media was removed and cells were rinsed with PBS and water then transferred to BaF_2 window
- Peaks under amide I region found with 2nd derivative spectroscopy
- Underlying peaks at ~1660cm^{-1} (mature crosslinks) and ~1690cm^{-1} (immature crosslinks) fit

DISCUSSION

- Significant effects of BAPN treatment on gene expression, as well as the morphology and enzymatic crosslinking in Type I collagen produced *in vitro*
- Fewer crosslinks are initialized and formed due to BAPN binding to LOX active site
- qRT-PCR confirmed a dose-dependent response of LOX and BMP-1 to BAPN treatment
- 0.25mM BAPN dosage
  - Irreversible binding of LOX by BAPN drives decrease in LOX expression, potentially driven by decreased BMP-1
- Lack of a coupled effect with higher dosages suggests BAPN binding alone may downregulate LOX and BMP-1, or that other contributing factors exist
- Low 0.14mM BAPN dosage
  - Decreased ratio of mature to immature crosslinks, driven by reduction in mature crosslink HP (hydroxylysylpyridinoline)
- Low 0.14mM BAPN caused an increase in the D-spacing distribution of collagen
  - Crosslinks may compress fibrils driving lower D-spacing in normal collagen
  - Fewer crosslinks in the BAPN group could account for the increase in D-spacing

CONCLUSION

BAPN is able to produce post-translational nanoscale structural changes to the collagen matrix in absence of a response in expression of genes relating to collagen synthesis or enzymatic crosslinking