Annulus fibrosus regenerative healing window in mice closes between postnatal days 14 and 28 and is modulated by collagen crosslink density

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INTRODUCTION: Annulus Fibrosus (AF) defects of intervertebral discs (IVDs) cause painful disability, and strategies are needed to repair AF defects and prevent progressive degeneration[1]. Previously, postnatal day 5 (p5) mouse IVDs were capable of functional AF regeneration while adults (4-6 months old) exhibited limited healing responses[2]. This study first applied a neonatal mouse *in-vivo* puncture model to narrow the age window of functional regenerative AF healing since narrowing this regenerative healing window can inform AF repair strategies and identify key factors in AF regeneration. This study secondly determined effects of crosslink density on AF stiffness and cell proliferation rate to determine their roles in AF regeneration since reduced crosslinking rescued cardiac regenerative healing with restored structure in neonatal mice[3].

METHODS: All experiments had IACUC approval. The regenerative window study had AF needle puncture injuries on caudal IVDs of p1, p5, p14 and p28 C57BL/6 ScxGFP reporter mice (needle gauge varied to produce AF defects ~80% IVD height & 50% IVD depth) with assessments 56 days post-injury (dpi). The crosslink density study administered β-aminopropionitrile (BAPN) to pregnant mice from embryonic day 5.5 until pups were p14. BAPN-treated mice were euthanized at p14 to measure AF crosslink density (multiphoton imaging), cell proliferation (Ki67 & DAPI immunohistochemistry), and tissue modulus (atomic force microscopy). AF healing was assessed on BAPN-treated vs control mice after p14 needle puncture injuries (when BAPN treatment was stopped) at 56 dpi. Regeneration was assessed on injured compared to uninjured adjacent control IVDs using disc height index (DHI), histology, and biomechanical testing. X-rays (Faxitron) were used to measure DHI. IVDs were stained with Picrosirius Red and Alcian Blue (PRAB) and imaged under brightfield and polarized light to assess morphology, % repair tissue in injury site, and semi-quantitative collagen disorganization grade (0-4 based on birefringence and organization of injury site and adjacent AF layers). DAPI determined total number of cells within injury sites. Injury sites were outlined in green, new tissue in white, and fibrous caps in purple. Yellow arrows point towards changes in AF birefringence. Separate cohorts were used for biomechanical testing of coccygeal 4/5 and 6/7 motion segments using axial and torsional test systems (TA Instruments). Axial testing involved 20 cycles of tension/compression to ±0.5N in displacement control at 0.1 mm/s; torsional testing involved 20 cycles of ±20º rotation at 0.5 Hz using established protocols[2]. Age matched (3 month) mouse coccygeal motion segments were also used for biomechanical testing with *ex-vivo* AF puncture to determine acute effects of injury without *in-vivo* healing. Students t-tests determined effects of injury and BAPN versus controls with n≥5 per group.

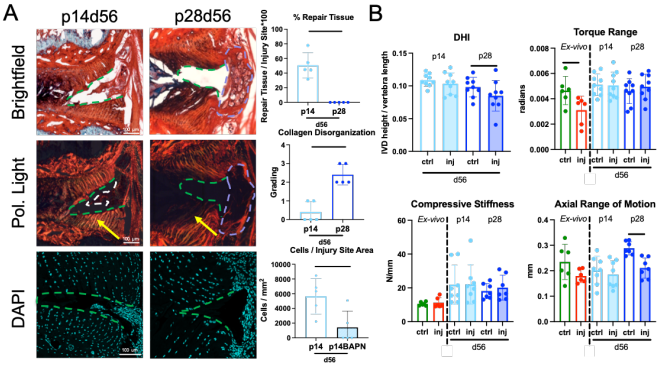

Fig 1: p14 mice healed with highly cellular matrix in injury sites and restored DHI and biomechanical function. (A) Imaging of p14 & p28 IVDs at 56dpi used to calculate % repair tissue in injury sites, collagen disorganization score, and cells/injury site. Scale bar = 100 µm (B) DHI and select motion segment biomechanical properties.

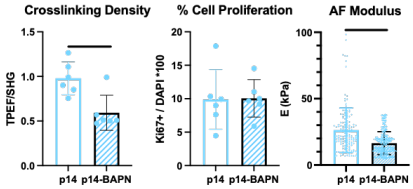

Fig 2: BAPN treatment limited AF crosslink accumulation, and reduced AF tissue modulus without affecting cell proliferation. Quantification of multiphoton crosslinking density, Ki67 % cell proliferation, and AFM AF modulus.

RESULTS: IVD puncture injuries produced large AF defects with nucleus pulposus herniation that were comparable across all groups at 3dpi (not shown). The p14 injuries exhibited regenerative healing at 56dpi (p14d56) with highly cellular matrix deposited in injury sites and maintained organization of adjacent AF layers (Fig 1A), as did p1d56 & p5d56, not shown. The p14d56 IVDs had restored DHI and functional biomechanical properties comparable to uninjured control levels and different from *ex-vivo* injuries (Fig 1B). The p28 IVDs had inferior healing at 56dpi with no cells or matrix within injury sites, disorganized adjacent AF layers, and loss of DHI (Fig 1A). The p28d56 IVDs also had a fibrous cap of disorganized (no birefringence on polarized light) collagen on the outer IVD edge, and decreased axial range of motion consistent with stiffening from fibrotic healing (Fig 1B). BAPN significantly decreased p14 crosslinking and AF tissue modulus compared to untreated p14 controls confirming its efficacy in reducing crosslink formation, yet did not affect AF cell proliferation (Fig 2). Injured IVDs with BAPN treatment had inferior healing at 56dpi with less matrix and cells in injury sites and substantial collagen disruption in adjacent AF layers (Fig 3A). The p14-BAPN IVDs had DHI loss and increased compressive stiffness consistent with lamellar buckling and tissue compaction (Fig 3B).

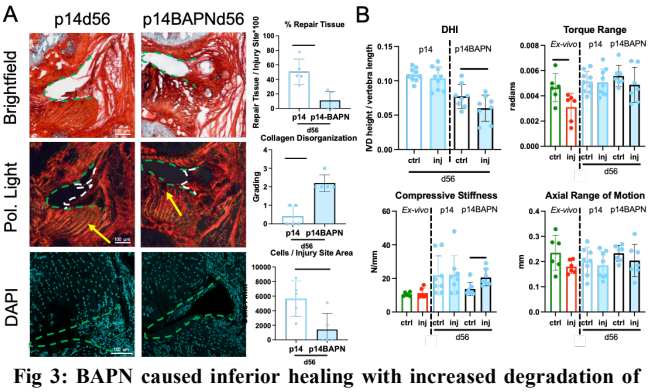

Fig 3: BAPN caused inferior healing with increased degradation of AF layers adjacent to injury site, loss of DHI, and impaired biomechanical function. (A) Imaging of p14 & p14-BAPN IVDs at 56dpi used to calculate % repair tissue in injury sites, collagen disorganization score, and cells/injury site. Scale bar = 100 µm (B) DHI and select motion segment biomechanical properties.

DISCUSSION: This study demonstrated the AF regenerative healing window, after a large AF defect, includes mice to age p14 and is closed by p28. Functional AF regeneration involved highly cellular matrix deposited in the injury sites, retained adjacent AF layers, and restored DHI and biomechanical function, but the lamellar structure did not regenerate within the injury site in mice of any postnatal age. AF healing in p28 mice was non-regenerative since it lacked matrix and cells in injury sites, had degraded adjacent layers, and had decreased DHI. The p28d56 IVDs healed with fibrous caps on the IVD periphery leading to decreased axial range of motion yet restoration of torque range suggesting a more robust fibrotic healing than previously reported for adult AF healing[2,4]. BAPN-treatment prevented crosslink accumulation, similarly to chick tendon[5], but this resulted in inferior AF healing with substantial degradation of adjacent layers, loss of DHI, and increased compressive stiffness suggesting matrix degradation and IVD collapse under compressive loading. Inferior AF healing with BAPN treatment contrasts the restored cardiac regeneration in neonatal hearts with BAPN[3]. BAPN did not affect AF proliferation levels suggesting high rates of cell proliferation are not sufficient for functional AF regeneration. The impaired AF healing in this BAPN study may result from greater susceptibility to matrix degradation from inflammation during healing in the less crosslinked tissue.

SIGNIFICANCE/CLINICAL RELEVANCE: This study showed AF regenerative healing requires cellular repair tissue in the injury site and retention of collagen structure next to AF defects and is modulated by crosslink density, which may be helpful in designing AF repair strategies for disabling back pain.

REFERENCES: [1] Iatridis+ *Spine J*, 2012 [2] Torre+ *FASEB J*, 2018 [3] Notari+ *Sci Adv*, 2018 [4] Martin+ *JOR*, 2013 [5] Marturano+ *Acta Biom*, 2014

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