Injectable Hyaluronic Acid Down-regulates Interferon Signaling and Increases the Disc Height in Injured Rat Tail Annulus Fibrosus

Zepur Kazezian1,2, Zhen Li3, Daisuke Sakai4, Mauro Alini3,4, Sibylle Grad2, Abhay Pandit1,4
1Centre for Research in Medical Devices (CUREM), National University of Ireland, Galway, Ireland; 2AO Research Institute Davos, Davos, Switzerland; 3Tokai University School of Medicine, Isehara, Japan; 4Collaborative Research Partner Annulus Fibrous Repair Program, AO Foundation, Davos, Switzerland.

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Introduction:
Intervertebral disc (IVD) degeneration, which is the main cause of low back pain, is characterized by degradation of extracellular matrix molecules resulting from an imbalance of anabolic and catabolic or inflammatory markers. Therefore, anti-catabolic or anti-inflammatory therapeutic approaches have been applied to prevent the degenerative process. We have been assessing the anti-inflammatory effects of high molecular weight hyaluronan (HA) hydrogels specifically in relation to annulus fibrosus (AF) repair. However, there is still a limited understanding about the signaling pathways through which HA can change the pathological conditions. We have previously identified specific downstream interferon IFNαβ signaling markers in degenerative AF such as Insulin-like growth factor-binding protein 3 (IGFBP3) and Interferon-induced protein with tetratricopeptide repeat 3 (IFIT3).

In this work, it was hypothesized that HA plays an anti-inflammatory role in suppressing the molecular markers IGFBP3 and IFIT3 and that it down-regulates pro-apoptotic proteins in the injured AF. Quantitative RT-PCR and immunohistochemistry were used to identify the effect of HA on the markers downstream of the IFNαβ signaling pathway in organ culture AF injury models without or with supplementation of exogenous IFNαβ. Moreover, the effect of HA was tested in an in vivo rat tail AF injury model.

Methods:
Hyaluronan hydrogel fabrication. 0.75% high molecular weight HA (850KD - 1.12MD) was prepared by crosslinking with 15%/4-ARM-PEG-NH2, 15% N-hydroxysuccinimide and 0.01% N-3Dimethylaminopropyl ethylicarboximid; Organ culture study: Caudal discs with cartilaginous endplates were dissected from 11 calves (4-5 months) and cultured for 3 days assigned to different groups: (1) Intact, (2) Defect (full thickness AF defect induced by 3mm puncher), (3) Defect treated with 40 μL HA, (4) Defect treated with 100U IFNαβ (added in the culture medium) and (5) Defect, treated, with IFNαβ and HA. After culture, 150 mg AF tissue was collected from the anterior defect side and processed for total RNA extraction by a modified TRI-spin method. Real time PCR. Gene expression analysis was performed using qRT-PCR, with particular focus on IFN signaling related genes (n=7/group). Expression differences between the groups were analyzed and statistically evaluated by one-way ANOVA, with p<0.05 being considered significant. Immunohistochemistry. After culture, bovine IVDs (n=4/group) were fixed in formalin, embedded in cryo-compound and sectioned 10μm thick. Sections were immunostained with anti-caspase 3 (p17) antibody and staining was quantified by image J. In vivo study: Sprague Dawley rats (n=5) were used to create AF defect by excising a window of 1mm by 1mm and 5μL HA was added in the treatment group. Disc height analysis. All rats were scanned pre- and post-surgery at 7 and 28 days and the disc heights were measured by the Impacts software for Xray analysis.

Results:
Organ culture study: qRT-PCR analysis shows that HA significantly *(p<0.001) down-regulates the gene expression of the IFNαβ receptors IFNAR1 and IFNAR2 in group D+IFN+HA compared to D+IFN (Fig. 1A, B). Analysis also shows that HA significantly *(p<0.01) down-regulates the expression of IGFBP3 and IFIT3 in D+IFN+HA compared to D+IFN (Fig. 1C, D). Protein expression analysis of fragmented caspase 3 (P17), which is a hallmark of apoptosis, shows significant up-regulation *(p<0.05) in the D and D+IFN groups compared to the intact and also significant down-regulation *(p<0.05) upon treatment of the D+IFN group with HA. In vivo study: Injection of HA in the rat tail AF injury model shows significant increase *(p<0.05) in the disc height in HA treated compared to the injured discs at day 7 and 28 (Fig. 2).

Discussion:
The aim of this study was to identify the anti-inflammatory, anti-apoptotic and regenerative properties of high molecular weight HA on the injured bovine and rat AF. Our PCR data demonstrate up-regulation of mRNA levels of IFN signaling molecules in Defect and Defect plus IFNαβ treated groups, while there is significant down-regulation upon injection of HA in IFNαβ treated groups. IGFBP3 is an important regulator of insulin-like growth factor (IGF) bioavailability that interferes with IGF-1 function and has been associated with the pathogenesis of osteoarthritis [1]. In addition, IGFBP3 has been associated with apoptotic signaling [2]. This is consistent with the fragmented caspase 3 expression, which was up-regulated in the defect and D+IFN and significantly down-regulated in D+IFN group treated with HA. Further studies on rat tail discs in vivo confirmed that HA was able to regenerate the injured disc AF leading to a significant increase in the disc height. Taking into account the results of both ex vivo and in vivo experiments, HA could be a non-invasive therapy candidate for the AF repair.

Significance:
Investigations into the effect of HA on the injured discs show that HA can suppress inflammatory IFN and apoptotic signaling and restore disc height. HA might be considered as a new non-invasive therapeutic approach for AF repair.

References:

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![Figure 1](image1.png)

**Figure 1.** Gene expression of IFNαβ receptors (A) IFNAR1 and (B) IFNAR2 and the downstream target genes (C) IGFBP3 and (D) IFIT3 in intact, injured (D), injured and HA treated (D+HA), injured and IFNαβ treated (D+IFN), and injured IFNαβ and HA treated (D+IFN+HA) groups. Mean fold changes ± SD. n = 7, *p<0.001 vs D+IFN, **p<0.01 vs Intact, D, D+HA, ***p<0.01 vs D+IFN.

![Figure 2](image2.png)

**Figure 2.** Disc height changes in the rat tail disc AF injury model: Bar graph represent the % disc height changes between sham, injured and HA treated groups over 7 and 28 days. Mean ± SD. n = 3, **p<0.05 vs injury.