Molecular Investigation of Collagen Damage in Annulus Fibrosus from Degenerative Disc Disease Patients using a Collagen Hybridizing Peptide

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INTRODUCTION: Degenerative disc disease (degen) is accompanied by both mechanical and biochemical changes in the intervertebral discs (IVD) [1]. The IVDs in these patients often exhibit severely degenerated annulus fibrosus (AF) tissue at the time of surgery, performed to alleviate significant pain and improve mobility. Extra-cellular matrix (ECM) degradation of the AF has been correlated with the degeneration of the IVD [2]. Collagen is a vital component of the ECM of the IVD [3]. Collagen Hybridizing Peptide (CHP) is an engineered protein that can quantify and monitor the collagen damage by binding to the degraded collagen directly [4]. CHP mimics the triple-helical molecular structure of natural collagen and binds to glycine-proline-hydroxyproline amino acid repeat sequences. We hypothesized that surgical AF tissue from degen patients will have decreased structural collagen integrity compared to the samples from non-degen ("normal") patients. The objective of this study was to measure the mean fluorescence intensity and the percentage of positively stained area with CHP from surgical and normal fresh AF tissue samples.

METHODS: Fresh human AF tissue from patients undergoing surgery for the degen condition and "normal" samples from organ donors obtained through the Southern Alberta Tissue Donation program were collected (Ethics ID: REB18-1308, University of Calgary). Fresh tissue with minimum dimensions of 2 mm X 2 mm X 2 mm was embedded immediately in Optimal Cutting Temperature (OCT) compound and stored at -80°C until cryo-sectioning. The tissue was sectioned at 6 μm thickness and Hematoxylin and Eosin (H&E) staining was performed for morphologic assessment. CHP solution (1 μM, 3Helix) conjugated with Cy3 was heated to 85°C for 5 minutes, quenched on ice for 1 minute, and applied immediately onto the sections. After incubation with CHP and washing, hard-set mounting media with DAPI was applied with coverslips. The slides were scanned using the Zeiss Axio Scan.Z1 Scanner (Zeiss Group). 3 regions of interest (ROI), with a size 900 μm squares, were selected as representative of the section. Mean fluorescence intensity was calculated from the Zen software (Zeiss Group) as the mean pixel intensity of the Cy3 channel on a 16-bit image. Percentage positive ROI area was calculated using a custom ImageJ algorithm to identify the area of the Cy3 channel as a percentage of total image area. Both parameters were calculated and averaged for each ROI. One-tailed Mann-Whitney U-test and Welch t-test based on Shapiro-Wilk normality and F-test were performed to assess the differences between the degen and normal samples. The Type I error was set to $\alpha = 0.05$ and outliers were removed based on Tukey's outlier criteria ($k = 1.5$).

RESULTS: Detailed patient demographic information is presented in Table 1. Surgical samples stained with H&E exhibited qualitative morphological structure changes with tissue organization (Figure 1). DAPI showed increased cellularity (white dots in the DAPI + CHP and DAPI channels) in the ROI for degen samples (Figure 1). Normal and degen samples had an average mean fluorescence intensity of 6301 ± 1653 and 10103 ± 3370, respectively ($p < 0.001$) (Figure 2). Normal and degen samples had an average percent positive ROI area of 18.8 ± 11.0 % and 46.5 ± 18.9 %, respectively ($p < 0.001$) (Figure 2).

DISCUSSION: Through qualitative differences observed through H&E and DAPI staining, we showed that there are structural changes at the tissue level, which may develop from more collagen damage observed using CHP at the molecular level. Quantification of collagen damage through increased mean fluorescence intensity and percentage positive ROI area showed increased concentration and spatial variation of collagen damage in degen samples compared to the non-degen samples. Previous studies have focused on CHP assessments on murine models, with only one study using clinical human samples from disc herniation patients [5]. This is the first study to use CHP on clinical degenerative disc disease samples compared with samples from young donors as controls. This study also had some limitations. Only three serial sections (one slide) per sample were stained with CHP instead of using multiple slides selected systematically to investigate the damage profile throughout the tissue. Next, CHP may have been bound to damaged collagen instead of just having the collagen binding regions exposed for interacting with CHP due to other molecules such as aggrecan and decorin being displaced from the collagen trimer [6]. However, this study has provided quantitative evidence that the structural integrity of collagen may be decreased in surgical patients with the degen condition compared to the non-degenerated/normal patients.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrated that CHP has the potential to detect structural degenerative AF changes in samples from surgical patients. With an expanded cohort of degen and spine deformity patients, the results will provide new insights into the structural changes of collagen at the molecular level and its role in the development and progression of these spine conditions.

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IMAGES AND TABLES:

	Normal	degen
Sample Size	12	14
Number of Patients	6	13
Age (years)	34 ± 10	45 ± 10
Sex (Male/Female)	(8/4)	(9/5)
Level (L4-L5/L5-S1)	(6/6)	(4/10)

Table 1: Basic demographics information of the included patients. The age is calculated based on the number of patients whereas sex distribution and level distribution are based on the number of samples.

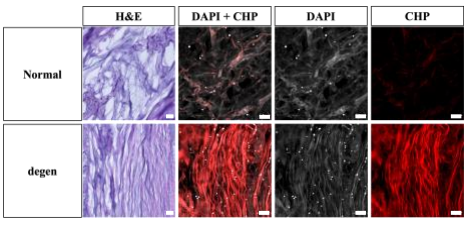

Figure 1: Comparison of the Normal and degen samples with H&E, DAPI + CHP, DAPI, and CHP channels in the ROI. CHP shows the fluorescence from the damaged collagen. Red shows the positive fluorescence and the intensity of red depicts the concentration of the damaged collagen in a particular pixel/location. Scale Bar = 100 μm.

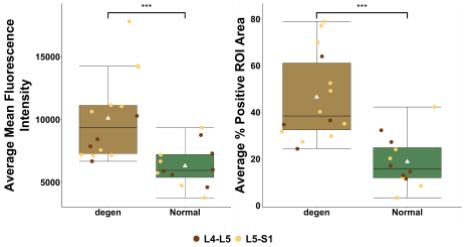

Figure 2: Comparison of the average mean fluorescence intensity (left) and average % positively stained ROI area (right) between degen and Normal samples. Males and females are combined. The horizontal line in the middle of the boxplot represents the median and the white triangle represents the mean of the group. *** represents $p \leq 0.001$.