Identification of the Compositional Traits and Permeabilities of the Cartilage Endplate that are Required for Nutrient Transport and Disc Cell Survival

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Introduction: Low back pain is the leading cause of disability and is closely linked to disc degeneration. Intradiscal biologic therapy is a promising strategy for managing disc degeneration. However, the poor nutrient environment of the disc is now recognized as an obstacle for biologic therapies that increase disc cellularity or matrix synthesis rates [1, 2]. One reason for the poor nutrient environment could be low cartilage endplate (CEP) permeability, since nutrients and metabolites entering and exiting the nucleus pulposus must diffuse across the CEP. **The goals of the study were to: 1) identify critical levels of CEP permeability needed to support cell densities associated with healthy discs; and 2) discover compositional characteristics of the CEP matrix that diminish its permeability and thereby hinder nutrient transport and disc cell function.** To do this, we first used diffusion chambers to test how human CEPs with a wide range of permeability impact nutrient transport and disc cell function. Then we probed the molecular components of the CEP with Fourier Transform Infrared Spectroscopy (FTIR) imaging to determine differences in the amount and spatial distribution of molecular markers that distinguish permeable CEPs from impermeable CEPs.

Methods: *Tissues*: Twelve intact human CEPs bordering the nucleus pulposus were harvested from six cadaveric lumbar spines (age range: 38–66 years old; mean age: 56 ± 10 years). *Permeability*: To determine the permeability of the CEP samples, we measured the diffusivity of a small fluorescent tracer (sodium fluorescein, 376 Da; 0.1 mg/ml in PBS) in the CEPs using fluorescence recovery after photobleaching. *Nutrient Transport*: Diffusion chambers were used to identify critical levels of CEP permeability needed for disc cell survival. The chambers consisted of two glass plates held apart by spacers; bovine nucleus pulposus cells cultured in the center of the chambers were separated from their nutrient source (low-glucose DMEM with 6% FCS) at the open sides of the chamber by the CEP samples. After incubating the chambers (48 hr; 21%/5% O_2/CO_2), cells were stained with a Live/Dead assay, and the viable distance was measured with a fluorescence microscope. We performed this procedure using two cell densities: 4 million cells/ml, which is the average cell density of an adult disc [3], and 8 million cells/ml. *CEP Composition*: The same CEPs were cryosectioned (thickness 7 µm) perpendicular to the surface of the CEP. Sections were mounted onto BaF_2 windows and air-dried. FTIR images were acquired with a spectral resolution of 4 cm^{-1} and pixel size of 6.25µm (Perkin Elmer Spotlight 400) and averaged for three adjacent CEP sections. We measured the depth-wise distribution of the following parameters: collagen content (peak area of 1595–1710 cm^{-1}), mineral/matrix ratio (ratio of phosphate's 895–1215 cm^{-1} P-O stretch peak area to collagen's Amide I peak area), and collagen maturity (absorbance ratio at 1660 cm^{-1} and 1690 cm^{-1} peaks [4]). *Statistics*: Results (Mean ± SD) were compared using t-tests.

Results: Diffusion chambers revealed that physiologic fluctuations in CEP permeability had a significant effect on disc cell viability, and that low CEP permeability hinders transport regardless of cell density (**Fig. A**). Solute diffusivity varied nearly 4-fold between the CEPs studied, and chambers with CEPs that had low diffusivity had a significantly shorter viable distance ($p < 0.01$). As expected, increasing cell density in the chambers shortened the viable distance; however, this effect depended on CEP permeability. Specifically, for CEPs with diffusivities <60 $\mu m^2/s$, there was no change in viable distance, which suggests that these CEPs may not allow for sufficient nutrient transport to satisfy cell nutrient demands. FTIR imaging showed significant differences in composition between CEPs with low vs. high diffusivity (**Fig. B-D**). Compared to CEPs with high diffusivity (>80 $\mu m^2/s$), those with low diffusivity (<40 $\mu m^2/s$) had higher collagen content ($p < 0.001$), greater mineral/matrix ratios ($p \leq 0.01$), and lower collagen maturity ($p < 0.001$) at all depths (**Table**).

Discussion: Our study set out to: 1) identify critical levels of CEP permeability needed for disc cell survival; and 2) discover compositional characteristics of the CEP that diminish its permeability and hinder nutrient transport. **Results demonstrated that nutrient diffusion across the CEP is insufficient to meet the metabolic demands of disc cells when solute diffusivity in the CEP is less than 60 $\mu m^2/s$, and that CEPs with low solute diffusivity have higher collagen content, higher mineral/matrix ratios, and lower collagen maturity.** Previous work using diffusion chambers showed that increasing disc cell density shortens the viable distance because doing so raises nutrient demands [5]. Here we found this effect depends on CEP permeability. Namely, when solute diffusivity in the CEP was <60 $\mu m^2/s$, viable distance was insensitive to cell density. This suggests that the limiting factor in these cases is low nutrient transport across the CEP and *not* the high nutrient demands of the disc cells. One implication of these findings is that patients with low CEP permeability may be poor candidates for biologic therapies intended to regenerate the nucleus pulposus. Instead, enhancing CEP permeability may be required. To determine compositional traits that distinguish the CEPs with low vs. high permeability and which could thus serve as possible targets for treatments to enhance CEP permeability, we performed FTIR imaging of the CEPs. Our finding that low solute diffusivity was associated with a greater amount of collagen, more mineral, and lower collagen maturity suggests that these characteristics may physically block nutrient diffusion. For example, collagen fibers can resist tissue swelling and hinder solute uptake [6]; mineral is highly impermeable; and lack of enzymatic crosslinks, which are increased in mature tissue, could destabilize the collagen network. Finally, unlike the zonal variations observed in articular cartilage [7], endplate cartilage composition was constant through the depth of the CEP, which implies that treatment strategies to enhance CEP permeability may need to consider the entire CEP.

Significance: The poor nutrient environment of the disc may hinder the success of intradiscal biologic therapies. Our findings are significant because they: 1) show that low CEP permeability may be a limiting factor for intradiscal therapy; and 2) identify compositional deficits in the CEP that prevent adequate nutrient diffusion and which thus represent targets for novel treatments to enhance CEP permeability.

References: 1. Huang+, *Nat Rev Rheum* 2014; 2. Zhu+, *J Orthop Res* 2015; 3. Maroudas+, *J Anat* 1975; 4. Paschalis+, *J Bone Miner Res* 2001; 5. Horner & Urban, *Spine* 2001; 6. Roberts+, *Spine* 1996; 7. Khanarian+, *J Bone Miner Res* 2014.

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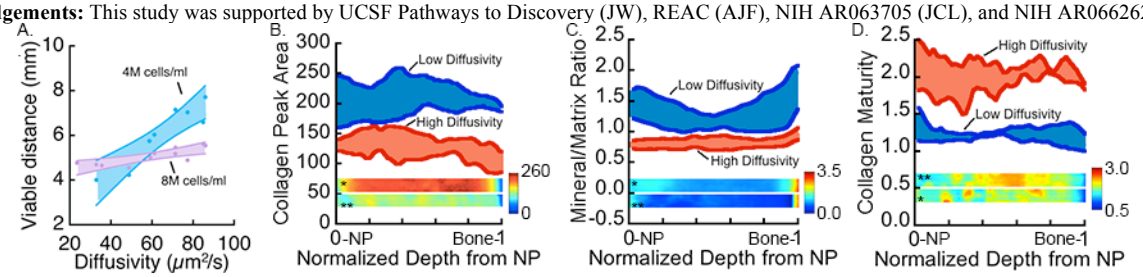

Figure. A. Viable distance in the diffusion chambers depended on cell density (nutrient demand) and CEP permeability (nutrient transport). Overlap in the relationships for CEPs with low diffusivity (<60 $\mu m^2/s$) suggests that nutrient transport is insufficient to meet cell demands. **B-D.** Representative FTIR data and images for CEPs with high and low solute diffusivity. Compared to CEPs with high solute diffusivity, those with low solute diffusivity had greater collagen content, greater mineral/matrix ratios, and lower collagen maturity across the CEP. Envelopes indicate ± 1SD for $n = 3$ adjacent sections per CEP.

Table. Compared to CEPs with high solute diffusivity, those with low solute diffusivity had significantly greater collagen content, greater mineral/matrix ratios, and lower collagen maturity. Compositional measurements did not vary significantly with depth. $n = 3$ CEPs per group.

	0.25 Normalized Depth from NP			0.50 Normalized Depth from NP			0.75 Normalized Depth from NP		
	Low Diffusivity	High Diffusivity	P-Value	Low Diffusivity	High Diffusivity	P-Value	Low Diffusivity	High Diffusivity	P-Value
Collagen Peak Area	210 ± 20	140 ± 30	< 0.001	210 ± 30	150 ± 20	< 0.0001	210 ± 40	150 ± 30	< 0.001
Mineral/Matrix Ratio	1.0 ± 0.3	0.6 ± 0.2	< 0.01	0.9 ± 0.2	0.6 ± 0.2	< 0.01	1.0 ± 0.4	0.6 ± 0.3	0.01
Collagen Maturity	1.4 ± 0.1	2.0 ± 0.4	< 0.001	1.3 ± 0.1	1.9 ± 0.3	< 0.001	1.4 ± 0.2	1.9 ± 0.3	< 0.001