**INTRODUCTION**: A healthy nucleus pulposus (NP) is a highly hydrated tissue owing to the abundant chondroitin sulfate (CS) proteoglycan, which withstands compressive spinal loading and facilitates multidimensional spinal movement. In the progression of NP degeneration, inflammatory cytokines and proteinases e.g., interleukin (IL)-6 and matrix metalloproteases (MMPs) have a potential to break down the proteoglycan into fragmented CSs and other glycosaminoglycans (GAGs). It is known that fragmented CSs accelerate osteoarthritis via Toll-like receptor (TLR). However, the biological roles of fragmented CSs in NP cells are unclear. We thus hypothesized that the fragmented CSs produced by the cytokines and proteinases stimulate catabolic turnover via TLR. We tested this hypothesis using bovine NP (bNP) cells and human NP (hNP) cells.

**METHODS**: We harvested bNP tissues from five proximal intervertebral discs (IVDs) of bovine tails (livestock animals 1–2 years old, n=6) and hNP tissues (Pfirrmann grade 3 and grade 4, n=3 and 5) from the subjects who underwent surgery due to lumbar disc herniation or spinal stenosis (n=8; age 40±11.1 [27–63]; sex, 2 M and 6 F) under IRB approval (No. 2017P001306). The isolated NP cells were seeded onto 1.5% cell-culture-grade agarose-coated 12-well plates and then pre-incubated for 2 days. The culture medium was replaced with bovine CS (Sigma-Aldrich) contained medium (0, 4, 16, 64 mg/ml) and incubated for 24 h with/without TAK242 (TLR4 antagonist) 1 µM /10 µM or C29 (TLR 4 antagonist) 10 µM /100 µM. The specification of CS was validated by electrophoresis with coomassie blue and toluidine blue staining. The cells were harvested for cell viability (cytotoxicity test), gene expression assessment (Acn, Col2a1, Colla1, Csgalnact1, Mmp3, Mmp13, Il6, Timp2, Tr2, Tlr2, Tlr4) with RT-PCR, and immunocytochemistry (MMP13, TLR2, TLR4) with a laser confocal microscopy. One-way ANOVA with the Tukey-Kramer post hoc test was used with a significance of p<0.05.

**RESULTS**: Bovine CS had approximately 20000 Da of molecular weight with various amounts of sulfated GAG. Distinct cytotoxicity was not shown at any dose. In bNP cells, significantly upregulated catabolic Mmp3, Mmp13, and Il6 expression in a dose-dependent manner, whereas CS did not significantly change anabolic Acn, Col2, and Csgalnact1 expression. Tlr2 and Tlr4 were markedly upregulated with CS. In human NP cells, CS also significantly upregulated catabolic MMP3, MMP13, IL6, and TLR2 expression in a dose-dependent manner. In particular, Tlr2 expression was dramatically upregulated in 64 mg/ml CS to approximately 400 times in bNP cells and 80 times in hNP cells compared to that without CS (p=0.023 and 0.009) (Fig 1). These gene upregulations at CS 16 mg/ml were significantly diminished by TLR2 antagonist (C29 100µM) (Fig 2). Immunocytochemistry for MMP13 demonstrated dose-dependent increase in the number of granules around nucleus. Immunocytochemistry for TLR2 demonstrated markedly higher expression at the cellular membrane with CS 16 mg/ml and 64 mg/ml. These findings at CS 16 mg/ml were greatly diminished by the TLR2 antagonist. Immunocytochemistry for TLR4 showed mild dose-dependent increase in TLR4 expression compared to MMP13 and TLR2 (Fig 3).

**DISCUSSION**: The present study demonstrated that fragmented CSs stimulate catabolic turnover in bNP and hNP cells mainly via TLR2. Growing evidence implicates the involvement of TLR and their activation through not only infection but damage-associated molecular patterns (DAMPs). However, no studies demonstrated the effects of fragmented CS on metabolism in NP cells. Although supplementing 64 mg/ml CS into culture medium provides high osmolality physiologically relevant to that in the NP (450 mOsm/kg H2O), fragmented CSs were recognized as a degraded product from proteoglycan and activated DAMPs mainly via TLR2. Given that TLR2 expression in NP tissue is dependent on the degree of degeneration, fragmented CSs may cause degeneration in the IVD via TLR2, which can be a therapeutic target to prevent IVD degeneration.

**SIGNIFICANCE/CLINICAL RELEVANCE**: We demonstrated that fragmented CSs stimulate catabolic turnover via TLR2 in the NP cells. Inhibiting TLR2 cascade is a promising therapeutic strategy to prevent IVD degeneration.


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**Fig. 1**: Gene expression in bNP and hNP cells with CS

**Fig. 2**: Gene expression in bNP cells with CS with/without TLR antagonists

**Fig. 3**: Immunocytochemistry in bNP cells with CS with/without TLR antagonists