Effects of Adenosine Treatment on Extracellular Matrix Biosynthesis and Intracellular ATP Production in Intervertebral Disc Cells

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Introduction: The intervertebral disc (IVD) is formed by the nucleus pulposus (NP), which is rich in proteoglycan (PG) and resists compression; and the annulus fibrosus (AF), which is rich in collagen and allows bending and torsion of the disc. Maintenance of a proper ECM biosynthesis in the IVD is suggested to be an important aspect preventing disc degeneration. Our recent study reported high accumulation of extracellular ATP in the NP of young healthy porcine IVD (~ 165 μM), while compressive loading reduced extracellular ATP content in the NP, suggesting that ATP hydrolysis may be promoted by mechanical loading [1]. ATP hydrolysis may increase extracellular accumulation of adenine derivatives such as adenosine in the IVD due to its avascular nature. Since adenosine has demonstrated its ability to maintain ECM homeostasis [2] and to regulate different cellular activities via P1 purinergic receptors [3], extracellular adenosine may potentially influence cellular activities in the IVD. Therefore, the objective of this study was to investigate the effects of extracellular adenosine on the ECM biosynthesis of porcine NP and AF cells.

Methods: NP and AF tissues were harvested from the spine of young pigs within 2 hours after sacrifice. Cells were isolated and cell-agarose samples of 1x10^6 cells were obtained by mixing IVD cells at a 1:1 ratio with 4% agarose gel. Agarose constructs were incubated at 37°C, 5% CO2 in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. The experimental groups of samples were cultured under different concentrations of adenosine (Sigma-Aldrich) for 21 days (Control, 20 μM and 100 μM adenosine treatment groups; NP: n = 9; AF: n = 9 for each group). Adenosine was administered three times a week in each medium change. At the end of the experiment, the dimethylmethylene blue dye binding assay was used to quantify PG content and the hydroxyproline assay was performed as an indirect method to measure collagen levels in each sample. The PG and collagen content of each sample were normalized by its DNA content and each treatment group was normalized by its respective Control group. A comparison of collagen accumulation between different treatment groups of the same cell type was performed by one-way ANOVA following by post hoc SNK test. To examine the effect of adenosine on intracellular ATP content, the agarose constructs were cultured with 100 μM adenosine for 2 hours (Control and 100 μM adenosine treatment groups; NP: n = 9; AF: n = 9 for each group). Intracellular ATP was measured using the Luciferin-luciferase method (Sigma-Aldrich). The results were normalized by DNA content in each sample. Comparisons of the intracellular ATP content between the Control and the treatment group of the same cell type were performed by student’s t-tests. To examine the effect of adenosine on gene expression, the agarose constructs were treated with 100 μM adenosine for 16 hours. mRNA levels of aggregan and collagen type II were measured using real-time PCR and t-tests
were performed to compare gene expression levels between the Control and treated groups. Significance was taken at p < 0.05 in all statistical analysis.

**Results:** For NP cells, the adenosine treatment groups exhibited significantly higher PG and collagen levels than the Control group, while the 100 μM adenosine group showed a significant higher increase in the contents of PG and collagen than the 20 μM adenosine group after 21 days of treatment (Figures 1A and 1C). For AF cells, the 100 μM adenosine treatment significantly increased PG content than the 20 μM adenosine and Control groups after 21 days of treatment. No significant difference in PG was found between the Control and the 20 μM adenosine groups (Figure 1B). Moreover, the adenosine treatment groups showed significantly higher collagen levels than the Control group while the 100 μM group showed a significant higher increase in collagen content than the 20 μM adenosine group after 21 days of treatment (Figure 1D). The intracellular ATP content significantly increased in NP and AF cells after 2 hours of 100 μM adenosine treatment compared to their Control groups (Figure 2). NP and AF cells expressed a significantly high relative gene expression of aggrecan and type II collagen after 16 hours of 100 μM adenosine treatment compared to control conditions (Figure 3).

![Figure 1. Proteoglycan and collagen contents accumulated by IVD cells treated with adenosine at different concentrations for 21 days. A. PG by NP cells. B. PG by AF cells. C. Collagen by NP cells. D. Collagen by AF cells. (n = 9; **p < 0.01 indicates statistically significant differences between groups)](image-url)
Discussion: ECM deposition and gene expression enhanced by adenosine treatment suggests that the adenosine treatment may promote ECM biosynthesis in IVD cells by upregulating the gene expression of aggrecan and type II collagen. Moreover, a considerable amount of ATP is needed for protein synthesis, especially PG biosynthesis that also uses ATP as a building block [4]. Since the avascular nature of the

Figure 2. Intracellular ATP content of IVD cells treated with 100 μM adenosine for 2 hours. (n = 9; **p < 0.01 indicates statistically significant differences between groups)

Figure 3. Aggrecan and type II collagen gene expressions of NP and AF cells treated with 100 μM adenosine for 16 hours. (n = 9 for NP, n = 9 for AF; *p < 0.05 and **p < 0.01 indicate statistically significant differences between groups)
IVD limits nutrient supply for energy (i.e. ATP) production, an increase in the intracellular ATP content of IVD cells by the adenosine treatment suggests that extracellular adenosine may promote ECM biosynthesis by increasing intracellular ATP production in the IVD. The findings indicated that adenosine promoted ECM biosynthesis by two different pathways, suggesting that adenosine may play a crucial role in maintaining the integrity of the IVD.

**Significance:** Insufficient maintenance of the ECM may promote disc degeneration, which is associated to low back pain and afflicts millions of people in the US. The results of the present study suggest that adenosine may play an important role in the maintenance of the ECM in the IVD.

*ORS 2015 Annual Meeting*

**Poster No:** 1599