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Investigating the mechanism of prolotherapy: the fibroblast response to dextrose *in vitro*

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Background & Hypothesis

The avascular nature of ligaments and cartilage can lead to limited healing from injury further resulting in joint laxity or osteoarthritis (OA). In 1958, Dr. George Hackett demonstrated ligaments could be strengthened by injection with a proliferative agent (1). Many proliferative agents have been used since then; however, 12.5% and 25% dextrose prolotherapy is particularly popular due to its relatively low cost and reduced side effect profile (2). Prolotherapy is thought to induce an initial inflammatory response surrounding the injection site, stimulating cellular proliferation and tissue repair, ultimately resulting in improved strength and stability. Increased cartilage thickness along with decreased pain and improved stability following prolotherapy treatment has been observed in patients with knee OA and other joint disorders (3-5).

Despite the observed clinical benefits, cell-based studies investigating the mechanism of prolotherapy are limited. Clinical trials investigating dextrose prolotherapy have varied greatly in the injection procedure, resulting in varied outcomes (2;6–9). Therefore, the 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of OA of the Hand, Hip, and Knee conditionally recommends against prolotherapy of the hip and knee, pending more research (10). We hypothesize that hypertonic solutions of 10-25% dextrose will incite an initially damaging, inflammatory response followed by the production of growth factors and enhanced fibroblast proliferation.

Materials & Methods

The human embryonic lung fibroblast cell line, MRC-5, was purchased from American Type Culture Collection and maintained in Eagles Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, and 1% penicillin/streptomycin at 37°C, 5% CO_2.

XTT assays were used to assess metabolic activity as an indicator of cell viability under several treatment conditions using 96-well plates. Cells were plated overnight prior to treatments at $2x10^4$ cells in 100uL media per well for each time point. Media control was compared to treatments ranging from 5-25% dextrose. Time points included 0.25, 0.5, 1, 2, 4, 6, 8, 10 and 12 hrs. Samples were run in duplicate from at least three independent experiments; significance of differences was determined by one-way ANOVA.

To assess the impact of dextrose-treated fibroblasts on metabolic activity of fibroblasts not previously exposed to dextrose, fibroblasts were exposed to varying concentrations of dextrose (5-25%) as indicated above. After reaching the appropriate timepoint (0.25, 0.5, 1, or 2 hrs), the supernatant fluid containing the dextrose was removed and replaced with fresh complete EMEM; the dextrose-treated fibroblasts were then incubated at 37°C, 5% CO_2 for 8 hrs at which point supernatant fluid was collected. Nascent fibroblasts plated overnight prior to treatments at $2x10^4$ cells were exposed to the 8-hr supernatants for 48 hrs. XTT assays were used as described above to determine how secreted factors from dextrose-treated fibroblasts affect cell viability of nascent fibroblasts. Samples were run in triplicate from five independent experiments; a mixed effect model was analyzed using ANOVA to compare control vs treatments.

Results

Direct Effect of Dextrose Prolotherapy on Fibroblast Viability Within the First Four Hours of Treatment

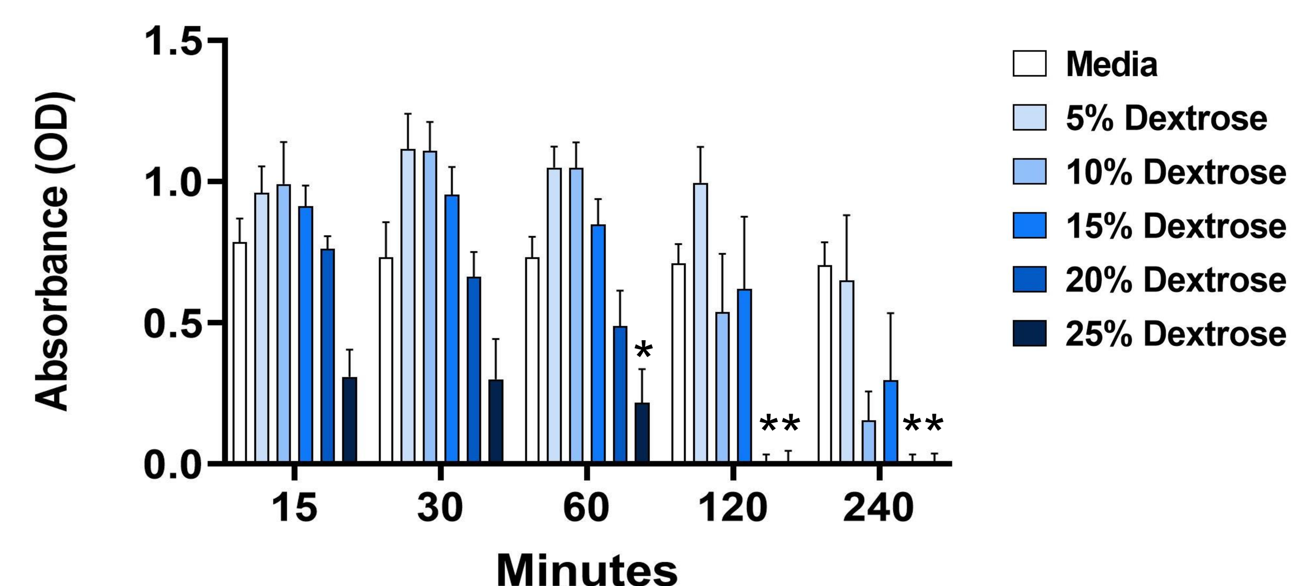

Figure 1. XTT viability assay of fibroblasts treated with 5, 10, 15, 20, and 25% dextrose compared to media control for 15, 30, 60, 120, and 240 minutes of exposure, run in duplicate, n = 5, SEM, *p<0.05

Direct Effect of Dextrose Prolotherapy on Fibroblast Viability Exposed for Longer Treatment Periods

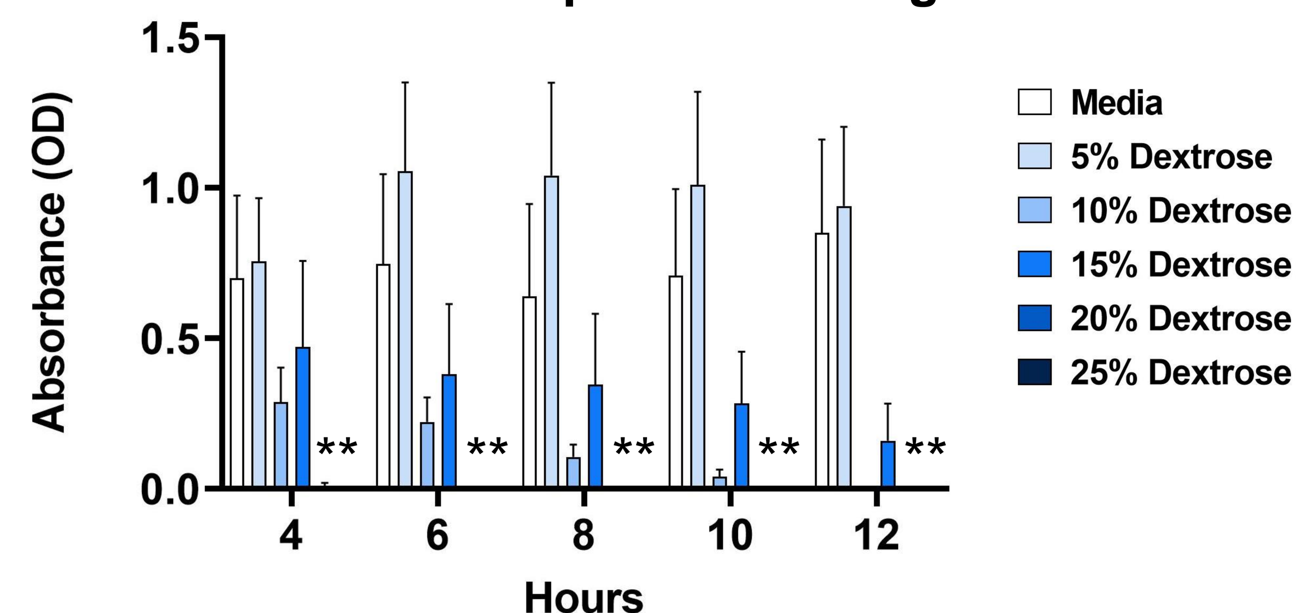

Figure 2. XTT viability assay of fibroblasts treated with 5, 10, 15, 20, and 25% dextrose compared to media control for 4, 6, 8, 10, and 12 hours of exposure, run in duplicate, n = 3, SEM, *p<0.05

Indirect Effect of Dextrose Prolotherapy on Fibroblast Viability Within the First Two Hours of Treatment

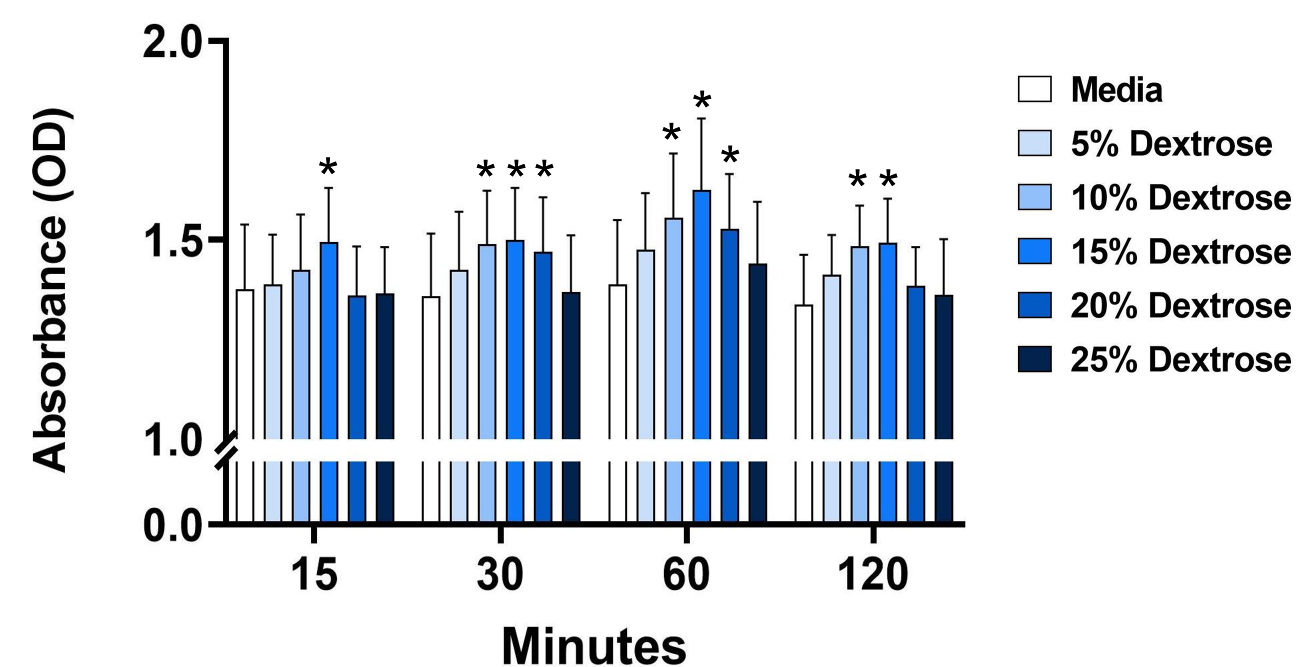

Figure 3. XTT viability assay of nascent fibroblasts treated with supernatant fluid from fibroblasts treated with 5, 10, 15, 20, and 25% dextrose compared to media control for 15, 30, 60, 120 minutes of exposure, run in triplicate, n = 5, SEM,*p<0.05

Discussion & Conclusions

<u>**Direct Effects of Dextrose on Fibroblasts:**</u>

- 20% dextrose for 120 minutes or longer decreases cell viability (p<0.05)
- 25% dextrose for 60 minutes or longer decreases cell viability (p<0.05)

<u>**Indirect Effects of Dextrose on Fibroblasts**</u>: (nascent fibroblasts exposed to the supernatant fluid from fibroblasts directly treated with dextrose)

- 10% dextrose for 30-120 minutes increases cell viability (p<0.05)
- 15% dextrose for 15-120 minutes increases cell viability (p<0.05)
- 20% dextrose for 30-60 minutes increases cell viability (p<0.05)

<u>**Overall Conclusions**</u>:

- Although higher concentrations of dextrose (20% or higher) may decrease cell viability of fibroblasts directly treated with dextrose, lower concentrations (<20%) do not demonstrate this effect.
- Supernatant fluid from fibroblasts directly treated with dextrose at 10-20% can stimulate a proliferative response by other fibroblasts.

The results of our study support our hypothesis that dextrose prolotherapy may incite an initially inflammatory and damaging response that then stimulates the production of growth factors and healing in the joint. We were also able to identify dextrose concentrations and timepoints of interest for multiplex and ELISA analysis which can be used to inform future experiments on other cell lines and primary cells. Continued exploration is still needed to further identify optimal therapeutic dextrose concentrations and what factors are secreted in response. Ultimately, a mechanistic understanding of dextrose prolotherapy may lead to improved clinical outcomes and evidence-based recommendations for use as an effective, nonsurgical option for patients with OA.

Acknowledgments

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