

Differentiation of Mesenchymal Stem Cells (MSCs) to NP-Like Cells in Chitosan/Glycerol Phosphate : Implications for Tissue Engineering of The Intervertebral Disc (IVD)

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INTRODUCTION: IVD degeneration is a major cause of low back pain. Surgery has limited success and novel technologies, such as tissue engineering to regenerate the IVD are being developed. One approach is to use a hydrogel seeded with cells, to replace the area most affected functionally by degeneration, the gelatinous nucleus pulposus (NP). NP cells can not be used because removal from a healthy disc causes degeneration and NP cells removed from a degenerate disc behave abnormally. The cells of the NP are chondrocyte-like and an attractive cell source is MSCs, which have the capacity to differentiate down the chondrocytic lineage. However, there is not a method available to differentiate MSCs in monolayer. Instead 3D cultures, such as alginate beads are usually used, which have been demonstrated to support chondrocytic differentiation of MSCs. In this study, we have investigated whether the novel thermoreversible hydrogel chitosan-glycerol phosphate (C/Gp), can support MSC differentiation and compared it to the alginate culture system. C/Gp has the potential to be used for non-invasive injection into the IVD and possesses *in situ* gelation, characteristics that can not be achieved using alginate. These are important features for tissue engineering of the IVD when attempting to replace the NP of the degenerate IVD with a hydrogel seeded with differentiated MSCs. The hypothesis for this study is that MSCs can differentiate into chondrocyte-like NP cells within C/Gp layer systems.

METHODS: MSCs were directly isolated from bone marrow and expanded in monolayer, before transferring to a novel hydrogel layer culture system. Layers were seeded at 1, 5, 10 or 20 million cells/ml and cultured for 1 or 2 weeks. 1.2% (w/v) alginate layers were set by polymerisation with 102mM calcium chloride. C/Gp layers composed of 3% (w/v) chitosan with 0.5M Gp were set in 10 minutes at 42°C. Cell viability was assessed using live/dead staining. Relative gene expression was assessed by RNA extraction, reverse transcription and real-time

PCR for the chondrocytic marker genes type II collagen, SOX9, and aggrecan, the fibroblastic marker type I collagen and endogenous control 18S. Data was analysed using the $2^{-\Delta\Delta Ct}$ method.

RESULTS: The live/dead assay showed that MSCs were viable in alginate layers, although this was affected by time in culture and cell density. At 1, 5, or 10 million cells/ml viability reduced from about 85 to 75% after 3 weeks of culture. However, at 20 million cells/ml viability was reduced from 58 to 52 % after 3 weeks of culture. Real-time PCR demonstrated that alginate layers induced chondrocytic differentiation of MSCs compared to monolayer. The relative gene expression was affected both by time in culture and cell density. After 2 weeks of culture, the markers began to decrease to similar levels for all cell densities. The highest gene expression of chondrocytic markers was seen at 5 million cells/ml after 1 week of culture. Nuclear staining of MSCs within the C/Gp layers demonstrated that the cells were dispersed throughout the gel at all densities, although this was more uniform at lower densities and cell clumping increased with density. The live/dead assay for MSCs in optimised C/Gp layers at 5 million cells/ml demonstrated that MSC viability fell from 90% after 2 days of culture to 75% after 3 weeks. Real-time PCR demonstrated that MSCs in C/Gp layers strongly expressed chondrocytic markers. For 1, 5, or 10 million cells/ml this required 2 weeks of culture and cell density had little effect on gene expression levels.

DISCUSSION & CONCLUSIONS: We have shown for the first time that human MSCs can distribute within and be cultured with high viability in C/Gp layers, at a similar level to alginate. In addition, we have shown that this novel hydrogel causes differentiation of MSCs to NP-like cells *in vitro*, after 2 weeks of culture as demonstrated by a change in expression of phenotypic marker genes. The 'smart' properties of C/Gp, coupled with its ability to induce NP differentiation of MSCs, suggest great potential for C/Gp in tissue engineering of the IVD.