The Potential Of Notochordal Cells To Stimulate Nucleus Pulposus Cells Via Soluble Factors Compared To Other Therapeutical Approaches.

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Introduction: Low back pain is a widespread disorder with tremendous socio-economic impact. While this condition is multifactorial, intervertebral disc degeneration is one of its main causes. Disc degeneration is characterized by the inability of the resident cells (nucleus pulposus cells, NPCs) to maintain its matrix rich tissue due to a change of their phenotype and their decreasing number [1]. Bone marrow stromal cells (BMSCs) have been proposed to complement the declining NPC population. Though able to stop the degenerative process, BMSCs could not restore the disc to a healthy state [2, 3] and complementary methods should be explored. Looking at development, when a large amount of matrix is produced, the disc contains a second cell type, the notochordal cells (NCs), potential regulator and/or progenitor of NPCs. As NCs are not present in adult humans, their potential for disc regeneration has been poorly explored. Few studies, however, showed that NCs can promote, via soluble factors present in conditioned medium (NCCM), matrix synthesis rate of NPCs [4, 5] and BMSCs [6] in short term studies. The goal of this study was therefore to compare, in a simplified in vitro model, the potential of different approaches to stimulate disc regeneration: (i) stimulation of resident cells with molecular factors or regulator cells; (ii) addition of exogenous cells and (iii) a combination of both biochemical stimulation and exogenous cells. The effects on matrix production and cell phenotype were evaluated after 28 days of culture and compared to standard TGFβ stimulation.

Methods: NPCs were extracted from caudal discs of cows (4 independent donors pooled per repeat) and NCs from spinal discs of young pigs (1 donor per repeat). BMSCs were isolated from bone marrow of calves and expanded up to P3 (2 independent donors pooled per repeat). All slaughterhouse materials were obtained in accordance with local regulations. NPCs and BMSCs alone were seeded at 3 millions cells/ml in alginate beads. For cell mixture groups, NPCs and BMSCs (NPC+BMSC) were mixed to a 1:1 ratio and seeded to a final concentration of 6 millions cells/ml. NPCs were also mixed with NCs to a 1:1 ratio (6 millions cells/ml). All groups were cultured for 4 weeks in serum-free high glucose DMEM (Plain) at 5 % O2. NPCs+BMSCs, BMSCs, and NPCs were also cultured in NCCM and chondrogenic medium (Ch; Plain medium supplemented with TGFβ3 and dexamethasone). NCCM was produced by culturing intact porcine NPs rich in NCs in serum-free high glucose DMEM for 4 days under 5% O2 (1 donor per repeat). At day 1 and 28, cell viability was evaluated by confocal imaging (calcein and propidium iodide staining). At day 0 and 28, extracellular matrix composition by biochemical assays (DMMB, Hoechst dye and Chlormarin-T assays for glycosaminoglycan (GAG), DNA and hydroxyproline (HYP) content, respectively) and gene expression profiles were determined by RT-qPCR (for collagen types I, II and X, aggrecan, and SOX9). 4 independent repeats were done for each group and condition. Statistical significance was evaluated with a non-parametric Kruskal-Wallis test followed by a post-hoc Dunn's procedure. Statistical significance was assumed for p < 0.05.

Results: Viable cells were observed in all groups at day 1 and 28. GAG and DNA contents increased, though not significantly, when NPCs where exposed to NCCM, in levels similar to Ch medium (Fig.1). Addition of BMSCs (NPC+BMSC(Plain)) also led to improved GAG production (Fig.1B). When this Mix was cultured under NCCM, GAG and DNA contents also increased, but to a lesser extent than under Ch medium (Fig.1). Direct co-culture of NPC with NCs (NPC+NC), on the other hand, did not promote proliferation nor GAG production (Fig.1). BMSCs alone displayed the same trend than the NPC+BMSC for both DNA and GAG contents, with more pronounced differences between media. Collagen production (HYP assay) was limited and only detected in groups containing BMSCs, with no effects of medium. Gene expression of aggrecan, type II collagen, and SOX9 followed the same pattern: their expression slightly decreased in all NPC groups (0.03-0.24 x Day 0). The NPC+BMSC groups, on the other hand, showed a mild up-regulation of these markers (2.24-13.97 x Day 0), whereas BMSCs displayed much higher stimulation (2.8-118,420 x Day 0). For all groups, medium conditions had no effect on expression levels of these markers. The highest up-regulation of type X collagen was observed for BMSCs (up to 9,000 x Day 0). This up-regulation, however, decreased under NCCM and even more under Ch medium. Type I collagen expression did not vary much except for NPCs in Ch medium and NPC+NC (up to 30 x Day 0). Histological analysis is ongoing, and the last repeat is currently analyzed.

Discussion: These results showed that molecular factors expressed by NCs and present in the NCCM are able to substantially stimulate both NPC’s proliferation and GAG production, even though gene expression profiles of the NPCs did not substantially change during culture. Addition of BMSCs to NPCs also had stimulatory effects. Though the BMSCs added to NPCs were undifferentiated, the GAG/DNA was similar to NPCs alone, suggesting that either BMSCs became functional NPCs or that they stimulated GAG production by NPCs. BMSC’s differentiation, however, is more likely as, cultured alone, BMSCs showed a high
up-regulation of several disc markers. Hence, the slight up-regulation of disc marker gene expression observed for NPC+BMSC may be explained by the presence and differentiation of the BMSCs. NCCM, however, did not have an additive regenerative effect on the combination of NPC+BMSC. As both NPCs and BMSCs alone responded well to NCCM, this suggests that the co-stimulation of BMSCs and NPCs in the NPC+BMSC groups make these cells less sensitive to any additional stimulation. When NCs were directly added to NPCs (NPC+NC), no positive effects were observed, indicating that the observed influence of NCCM may be linked to the soluble factors secreted by the freshly isolated NCs during the generation of the conditioned medium. The lack of a regenerative effect in NPC+NC group may therefore be explained by a loss of the NC-phenotype during the 4 weeks of culture, as shown before [7]. To conclude, this study showed that NPCs can be substantially stimulated, and to a similar extent, by NCCM and co-culture with BMSCs. The use of NCCM as a therapeutical approach, however, may be more attractive as it does not require the injection of a large number of cells (NPC:BMSC ratio 1:1 used). Moreover, its effect can be amplified using a higher concentration of NCCM, though the best approach will be to identify the molecular factor(s) involved in NCCM’s effects. **Significance:** This study provides new and important insight on the role of NCs in disc regeneration and constitutes an additional step toward a new biological therapy to treat one of the most common disorders in modern society.

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Fig.1: [A] DNA content (µg) per bead at day 0 (D0) and 28 (D28). [B] GAG content (µg) per bead at day 28. Values are means ± Standard deviation. N = 3; * p < 0.05 compared to NPC D0; † p < 0.05 compared to NPC D28 Plain; ‡ p < 0.05 compared to NPC+BMSC D28 Ch; § p < 0.05 compared to NPC+NC D28 Plain.