Notochordal cell-based treatment strategies and their potential in intervertebral disc regeneration

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Supplementary Material

Supplementary table 1. Effects of Notochordal Cell-Conditioned Medium (NCCM) determined *in vitro* and *in vivo* (1999-2021)

Study	NCCM source (animal)	NCCM generation (medium, time)	NCCM generation (tissue/cells, culture system)	Cells/tissues where the effect of NCCM is determined on	Effect of NCCM
Aguiar, 1999 (1)	* Canine (NCD, *n*=?, age=?); cNCCM	* Hybridoma medium * 24 hours * NX	Alginate beads (10,000 cells/bead). Rest unknown	* Bovine adult NPCs (*n*=?, age=?) * Bovine fetal NPCs (*n*=?, age=?) * Both: alginate beads (10,000 cells/bead).	* 18 hours: cNCCM induced GAG synthesis
Boyd, 2004 (2)	* Porcine (*n*=?, 4-5 months); pNCCM	* OPTIMEM * 3 days * NX	Alginate beads (2*10^6 NCs/mL alginate), 30 beads in 2 mL medium /well (equals 1* 10^6 cells/well)	* Porcine NCs and AF cells (*n*=?, age 4-5 months) in alginate beads (2*10^6 cells/mL alginate), 30 beads/well	* 48 hours: pNCCM decreased *COL1*, *COL2* and *ACAN* expression in NP cells. NCCM increased *COL2*, *ACAN* expression in AF cells
Erwin and Inman, 2006 (3)	* Canine (NCD, *n*=?, 9-18 months); cNCCM	* DMEM * 4 days * NX	Alginate beads (unknown number of NCs/mL alginate); 20, 30, or 40 beads in 2 mL medium/well	* Bovine NPCs (*n*=3, age=?) in alginate beads (1*10^6 NPCs/mL alginate), 10 beads in 2 mL medium/well	* 3 days: cNCCM dose-dependently upregulated GAG production and increased cell proliferation (not dose-dependent)
Erwin, 2006 (4)	* Canine (NCD, *n*=5, 8-12 months); cNCCM	* DMEM * 4 days * NX	* Alginate beads (1*10^6 NCs/mL alginate) with 120 beads and 2.5 mL medium/well	Bovine NPCs (*n*=?, age=?) 100 alginate beads (rest unknown)	* 24 hours: cNCCM induced *ACAN*, *VCAN*, *Hyaluronic synthase-2* expression
Korecki, 2010 (5)	* Porcine (*n*=5, 2 years); pNCCM	* hgDMEM * 4 days * HX	* Alginate beads (2*10^6 NCs/mL alginate); 1*10^6 NCs per 5 mL NCCM	* Human BMSC (*n*=3) pellets of 250,00 cells	* 7 days: GAG/DNA content of pNCCM-treated pellets higher than control and TGFβ-treated pellets
Purmessur, 2011 (6)	* Porcine (*n*=8, 5-8 months); pNCCM	* lgDMEM * 4 days * HX	* NCA (2*10^6 NCs/mL alginate), 10 beads in 2 mL medium /well * NCT (NC-rich NP tissue) à 3 discs (0.9-1.3gram)/30 mL medium	* Human BMSC (*n*=3) pellets of 250,00 cells	*21 days: NCA - downregulated *COL1* and *COL3*, low *COLX*. GAG content not different from control and $TGF\beta_3$-treated pellets. NCT - GAG content induced and upregulated *COL2* and *SOX9* expression
Abbott, 2012 (7)	* Porcine (*n*=?, 2-8 months); pNCCM	* lgDMEM * 4 days * HX	* NCA (2*10^6 NCs/mL alginate), 10 beads in 2 mL medium /well * NCT (NC-rich NP tissue) à 3 discs (= 1.0 gram) per 30 mL medium * Both: 20,000 cells/mL NCCM	* Human NPCs (*n*=3, 2*10^6 NPCs/mL alginate), 10 beads/well, volume NCCM unknown	* 7 days: GAG/DNA content only upregulated by NCA. Matrix gene expression not upregulated in NCA and NCT conditions. *MMP1*, *MMP3* increased by NTC.
Erwin, 2011 (8)	* Canine (NCD, *n*=5-6, 8-12 months); cNCCM	*ADMEM/F-12 * 3 days * HX	* Alginate beads (1.5 *10^6 NCs/mL alginate), 80 beads in 6 mL medium /well	* Bovine NPCs (*n*= 5-6, 3 years) in monolayers of 0.5*10^6 cells/well	* 48 hours: IL-1β and FasL-mediated apoptosis rescued by cNCCM. Caspase 9 activity decreased by 2%cNCCM and

	* Bovine (*n*= 5-6, 3 years); BCCM		* 2% FBS-supplemented NCCM/ BCCM: 0.75 *10^6 NCs/mL, 80 beads in 3 mL medium/well		2%BCCM. Caspase 3/7 activity only reduced by 2%cNCCM. cNCCM increased *ACAN*, *COL2*, *ADAMTS4*, *TIMP1*, and decreased *IL6* and *MMP3* expression.
Gantenbein, 2014 (9)	* Porcine (*n*=4, 4-5 months); pNCCM	* hgDMEM * 7 days * HX and NX	* Alginate beads (4*10^6 NCs/mL alginate); 30 beads in 4 mL medium	* Bovine NPCs (*n*=4, 1 year) in alginate beads, alone or co-culture with porcine NCs. * Bovine AF cells (*n*=4, 1 year) in alginate beads, alone or co-culture with porcine NCs.	* 14 days: NCs activated NPCs in co-culture and by pNCCM addition (gene expression ratio of *ACAN*/*COL2*↑). AF cells unresponsive to pNCCM.
Potier, 2014 (10)	* Porcine (*n*=4, <10 weeks); pNCCM	* hgDMEM * 4 days * HX	* 1.0 gram NC-rich NP tissue / 30 mL medium	* Bovine NPCs (*n*=16; 4 pooled per repeat, 22-26 months) in alginate beads (3*10^6 NPCs/mL alginate) * Additive effect of bovine BMSC co-culture was also determined.	* 28 days: pNCCM increased NPC proliferation and GAG production to levels similar as TGFβ. NPC:MSC co-culture led to GAG synthesis similar to NPCs alone, which was slightly improved by pNCCM.
Bach, 2015 (11)	* Human (*n*=10, 20 weeks of gestation-3 months); hNCCM * Canine (NCD, *n*=4, 18-23 months); cNCCM * Porcine (*n*=4, 3 months); pNCCM	* hgDMEM * 4 days * HX (all species) and NX (human)	1.0 gram NC-rich NP tissue / 30 mL medium	* Human NPC (n=3, pooled, 47-63 years) micro-aggregates of 35,000 cells	* 28 days: All species NCCM increased the DNA and GAG content. pNCCM and cNCCM were more potent than hNCCM in inducing GAG deposition. Only hNCCM induced collagen type II production.
De Vries, 2015 (12)	* Canine (NCD, *n*=5, 1-1.5 years); cNCCM	* hgDMEM * 4 days * HX	* 1.0 gram NC-rich NP tissue / 30 mL medium	* CD canine NPCs and BMSCs (2-2.5 years) in alginate beads (3*10^6 NPCs/mL alginate)	* 28 days: cNCCM increased proliferation, GAG production, and expression of genes associated with a healthy NP-like phenotype in NPCs. cNCCM also increased GAG production in BMSCs. When NPCs were co-cultured with BMSCs (in cNCCM), no higher GAG content was observed vs. NPCs alone.
De Vries, 2015 (13)	* Porcine (*n*=10, <3months); pNCCM	* hgDMEM * 4 days * HX	* 1.0 gram NC-rich NP tissue / 30 mL medium	* Bovine NP tissue explants (n=5, 2 years, caudal discs) * Additive effect of 10^6 bovine BMSCs was also determined.	* 28 days: pNCCM increased GAG content BMSC addition did not increase the GAG or DNA content.
Purmessur, 2015 (14)	* Porcine (*n*=6, 6-8 weeks); pNCCM	* hgDMEM * 4 days * HX	* 4 NPs per 30 mL medium * Filtrate resuspended in an equal volume of either fresh control SH-SY5Y basal media (EMEM: F12K) or rat DRG Basal media (Neuronal Medium + growth serum).	* Human neuroblastoma SH-SY5Y cells and primary rat dorsal root ganglia monolayer culture * 10% and 100% NCCM tested * Mechanistic studies included to determine if CS is responsible for NCCM-mediated effects	* 48 hours: pNCCM inhibited neurite outgrowth from SH-SY5Y cells without dose or cytotoxic effects. Neurite growth from SH-SY5Y and DRG cells was not inhibited when cells were treated with pNCCM with digested CS.
Cornejo, 2015 (15)	* Porcine (*n*=8, 6-8 weeks); pNCCM	* hgDMEM * 4 days * HX	* Amount of tissue per mL hgDMEM unknown	* HUVEC monolayer culture, *n*=4 * 10% and 100% NCCM tested	* 16 hours: endothelial cell invasion inhibited by 10% and 100% pNCCM, 10 and 100 mg CS and 10 and 100 ng noggin

			* Filtrate resuspended in Medium 200PRF (Med200)	* Noggin and CS also tested (notochord is a source of anti-angiogenic factors, *e.g.* noggin, CS)	* 24 hours: 10% pNCCM, 10 and 100 mg CS and 10 ng noggin inhibited tubular formation. pNCCM decreased *VEGF-A, MMP-7* and *IL-6* mRNA, CS and noggin did not affect gene expression.
Müller, 2016 (16)	* Canine (NCD, *n*=10, 12-18 months); cNCCM	* ADMEM/F-12 with 2% FBS * Medium collected every 24 hrs, 5 days * HX	* Alginate beads ($1*10^6$ NCs/mL alginate). Rest unknown	Human chondrocyte (non-osteoarthritic; *n*=9 ,18–68 years and osteoarthritis; *n*=6, 60–82 years) in pellets of 2.5×10^5 cells	* 14 days: Healthy chondrocyte pellets recovered GAG content to baseline levels with cNCCM. cNCCM-treated OA pellets increased GAG content and levels of hyaluronic acid link protein, fibromodulin and SOX9. cNCCM reduced IL6, IL-8, MMP-3, MMP-13, and COX-2 expression/secretion.
Bach and de Vries, 2016 (17)	* Porcine (*n*=5, 3 months); pNCCM * Canine (NCD, *n*=8, 16-38 months); cNCCM	* hgDMEM * 4 days * HX	* 1.0 gram NC-rich NP tissue / 30 mL medium * Whole NCCM was also separated into a soluble (NCCM-S; peptides and proteins) and pelletable (NCCM-P; protein aggregates and extracellular vesicles) fraction	* Porcine NCCM was applied to bovine NPCs (*n*=4 repeats, pooled 2-2.5 years) in alginate beads ($3*10^6$ NPCs/mL alginate) * NCD canine NCCM was applied to CD canine NPCs (CD, *n*=4, 3-10 years) in albumin- and hyaluronic acid containing hydrogels	* 28 days: pNCCM-S exerted a more pronounced anabolic effect than pNCCM-P on bovine NPCs. cNCCM-S exerted a more pronounced anabolic effect than cNCCM-P on CD canine NPCs. pNCCM-P exerted a negligible effect on bovine NPCs. cNCCM-P enhanced GAG and collagen type II deposition by canine NPCs.
Bach, 2017 (18)	* Porcine (*n*=7, 1.5 months); pNCCM	* hgDMEM * 4 days * HX	* 1.0 gram NC-rich NP tissue / 30 mL medium (whole NCCM) * Extracellular vesicles (EVs) and proteins were separately isolated from whole porcine NCCM	* Canine NPC (*n*=4, 2-10 years of age, Beagles, pooled) and human NPC (*n*=4, 50-63 years of age, pooled) micro-aggregates of 35,000 cells	* 7 days (canine NPCs): pNCCM-derived EVs induced GAG deposition to a comparable level as pNCCM-derived proteins and whole pNCCM. * 21 days (human NPCs): pNCCM-derived EVs increased the DNA and GAG content to a lesser extent than whole pNCCM.
Mehrkens, 2017 (19)	* NCD canines (*n*=10, 12-18 months); cNCCM	* ADMEM/F-12 + 2% FBS * Medium collected every 24 hrs; 3-7days * HX	* 2-3 NPs per cell strainer within culture wells in 6 mL medium	* Human NPC (*n*=15, 23-80 years) monolayer culture	* 24 hours: cNCCM inhibited cytotoxic stress-induced caspase-9 and -3/7 and maintained mitochondrial membrane potential.
Matta, 2017 (20)	* NCD canines (*n*=10, age=8-14 months); cNCCM	* CD Hybridoma media * 1-2 days * HX	* 3 NPs were placed within tissue culture inserts with 40μm-filters in 6 well plates * cNCCM was centrifuged at 8000 rpm for 30 minutes and filtered through 0.2μm syringe tip filters	* *in vivo*: 12-week old female Wistar rats (*n*= 6 animals/group, 4 discs per animal), disc injury (caudal discs) with 26 G needle * 4 weeks post-injury: intradiscal injection (~8μL) of concentrated cNCCM (2.2μg/μL) or control medium with 32G needle * Evaluation after 6 weeks of treatment	* 6 weeks post treatment: NC-rich NPs with moderate Safranin-O staining in cNCCM injected rat-tail injured discs, * 6 weeks post treatment: increased aggrecan, collagen 2, brachyury, Oct4 and Nanog protein levels after cNCCM treatment

De Vries, 2018 (21)	* Porcine (*n*=5, 3 months); pNCCM	* hgDMEM * 4 days * HX	* 1.0 gram NC-rich NP tissue / 30 mL medium * HUVEC culture: filtrate resuspended in Medium * SH-SY5Y cell culture: filtrate resuspended in an equal volume of SH-SY5Y basal media (EMEM: F12K)	* HUVEC monolayer culture (pool of 10 donors, *n*=5 biological replicates) * Human neuroblastoma SH-SY5Y monolayer culture (poly-D-lysine coated well plate versus polystyrene culture surface; *n*=5 biological replicates)	* 24 hours (HUVEC): pNCCM induced vessel formation, more matured and organized than basal culture medium. Addition of CS alone decreased vessel growth. * 24 hours (SH-SY5Y, poly-D-lysine coated surface): pNCCM did not affect the percentage of neurite expressing cells or average neurite length * 24 hours (SH-SY5Y, polystyrene culture surface): pNCCM increased the percentage of neurite expressing cells

AF: annulus fibrosus, BMSC: bone marrow-derived mesenchymal stromal cell, CD: chondrodystrophic, CS: chondroitin sulfate, EV: extracellular vesicle, FBS: fetal bovine serum, GAG: glycosaminoglycan, HUVEC: human umbilical vein endothelial cell, HX: hypoxia (1-5% O_2), IL-6: interleukin-6, NC: notochordal cell, NCD: non-chondrodystrophic, NP: nucleus pulposus, NPC: nucleus pulposus cell, NX: normoxia (20% O_2), SOX9: SRY-box 9, VEGF: vascular endothelial growth factor

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