A novel in vitro organ culture system for intervertebral discs with endplates

A NOVEL *IN VITRO* ORGAN CULTURE SYSTEM FOR INTERVERTEBRAL DISCS WITH ENDPLATES

+*Gantenbein, B; *Lee, C R; ** van Donkelaar, C C; *Alini, M; *, **Ito, K
+*AO Research Institute, Davos, Switzerland
benjamin.gantenbein@aofoundation.org

INTRODUCTION:

The limited nutrient supply and the harsh mechanical environment of the intervertebral disc (IVD) are two factors that are generally believed to contribute to the high prevalence of degenerative disc disease [1, 2]. Investigating these aetiologies and their interaction *in vivo* is challenging, making *in vitro* culturing systems an appealing alternative. However, traditional cell culturing methods are also limited because removing the cells from their native 3D extracellular matrix can alter cell behaviour and makes it nearly impossible to replicate the *in situ* mechanical and nutritional conditions. Thus, *in vitro* culturing of entire IVDs in order to maintain their biochemical and physical integrity would be an invaluable investigative model [2, 3]. Recently, C. Lee et al successfully cultured bovine IVDs under static compressive loads to avoid swelling [3]. However, cell viability was only maintained when discs were cultured without endplates, the removal of which substantially alters the biomechanics of the disc. In this study, a new preparation and loading technique aimed to preserve biomechanics and nutrient transport conditions was evaluated for *in vitro* culturing of intact ovine caudal IVD explants with adjacent endplates.

METHODS:

These animal experiments were approved by the veterinary authority of Canton Grison, Switzerland. Twelve Swiss alpine sheep (*Ovies aries*) were systemically anti-coagulated before sacrificed and the caudal vasculature evacuated post-mortem [4]. These sheep were from other ongoing studies at the AO Research Institute. The four most proximal caudal discs and adjacent endplates were then excised using a precision band saw to cut through the bony endplates (mean thickness 2.6 ± 0.7mm). Discs were placed in a standard incubator (37° C, 5% CO_2) under diurnal loading (0.2 MPa for 8 h and 0.8 MPa for 16 h) with media (high-glucose DMEM and 10% FCS, 1% penicillin/streptomycin) perfusing all surfaces. Two discs from each tail were analyzed on day 0 (fresh) and 2 discs after culturing for 7 days.

Cell viability was assessed using the LIVE/DEAD® staining kit (Molecular Probes). Samples of disc tissue (~ 75 mm^3 of annulus fibrosus or nucleus pulposus) were incubated under "free-swelling" conditions in serum-free DMEM for 3 hours [3]. Stained samples were visualized on an inverted CLSM (Zeiss). Total cell number and cell viability was counted from > 3 randomly chosen stacks of tissue with a custom image analysis macro (Zeiss). Each stack was 100µm deep, starting at about 50µm below the tissue surface.

Real time RT-PCR was performed according to standard protocols in order to measure expression of 3 anabolic (aggrecan, col I and col II) and 2 catabolic genes (ADAMTS-4, MMP-13) [5]. New ovine primers were designed on the intron-exon boundaries for col I, ADAMTS-4 and MMP-13 and are available on request.

Glycosaminoglycan (GAG) biosynthesis rate was determined using the ^{35}S-sulfate incorporation method [3]. Separate pieces of nucleus and annulus were incubated under "free-swelling" conditions for six hours in DMEM supplemented with 2.5 µCi/ml ^{35}S-sulfate. After radiolabeling, samples were lyophilized, weighed dry, and digested in proteinase K and dialyzed. The radioactivity of the dialyzed samples were measured by scintillation counting and normalized to sample dry weight.

Statistical analyses: Initially, a general linear model (GLM) was fit to the data in order to test for effect of sheep. In the absence of effect for sheep (cell viability and GAG synthesis rates) the data were unblocked. Non-parametric tests were used to assess differences in cell viability and GAG synthesis rates between day 0 and day 7. Gene expression measured as duplicate C_t values of the two discs were averaged within sheep. All means were normalized as ΔC_t values, relative to C_t values of 18S ribosomal RNA (house keeping gene). $\Delta\Delta C_t$ values were expressed relative to day 0 and then transformed using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was assessed by the non-parametric sign test comparing the values to a hypothetical mean of 1.

RESULTS:

There was a small but non-significant decrease in mean cell viability in both annulus and nucleus tissues over 7 days of culturing (90% at day 0, 84% at day 7, Figure 1). Gene expression in both tissue types revealed a non-significant down-regulation of anabolic genes and a significant "switching on" of catabolic genes at the end of the culturing period (Figure 2). GAG synthesis rates did not change significantly during culturing (Figure 3).

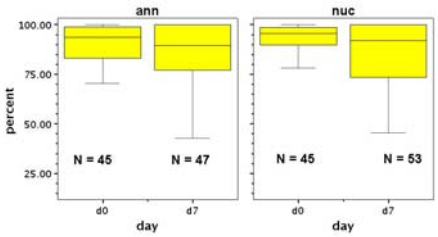

Figure 1: Percentage of viable cells at day 0 and after 7 days of tissue culture in the annulus (ann) and nucleus (nuc).

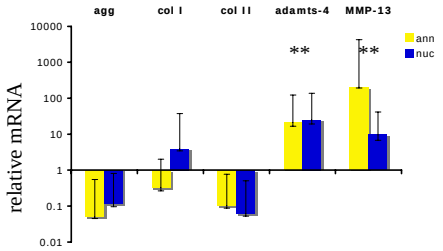

Figure 2: Gene expression of anabolic and catabolic genes at day 7 vs. day 0 in annulus (ann) and nucleus (nuc) (** $p < 0.01$).

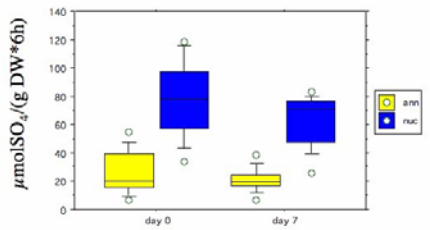

Figure 3: Glycosaminoglycan (GAG) synthesis rate measured as µmol incorporation of ^{35}S-sulfate standardized to dry weight in annulus (ann) and nucleus (nuc).

DISCUSSION:

To our knowledge, this is the first report showing *in vitro* maintenance of cell viability and GAG synthesis rates in large discs explanted with intact endplates. After only one week in culture, there were no obvious signs of matrix degradation to support the up-regulation of catabolic gene expression. Longer-term culture and analysis of enzyme activity and degradation products are necessary to better understand the consequences of the up-regulation of catabolic genes. It should not be surprising, however, to see this change in gene expression consistent with matrix remodelling considering that the discs were not exposed to physiological frequencies [6] of loading during the *in vitro* culture. Future studies will seek to more closely approximate physiological loading over an extended culture period (up to 21 days).

ACKNOWLEDGEMENTS:

We would like to thank P. Heil, T. Grünhagen, C. Sprecher and S. Grad for technical assistance. This work was supported by the AO Foundation.

REFERENCES:

[1] Urban J. et al. (2004) Spine 29: 2700-2709. [2] Ogata K. and Whiteside L.A. (1980) Spine 6: 211-217. [3] Lee C.R. et al (in press) Spine. [4] T. Grünhagen et al. (2004) EORS. [5] S. Grad et al. (2003) J Biomed Mater Res 66: 571-579. [6] MacLean et al. (2004) J Orthop Res 22: 1193-1200.

AFFILIATED INSTITUTIONS FOR CO-AUTHORS:

** Department of Biomedical Engineering, Eindhoven University of Technology, The Netherlands