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Development of an *in vitro* model to test the efficacy of novel therapies for IVD degeneration

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Abstract

Low back pain (LBP) is a major cause of disability worldwide that has been linked to intervertebral disc (IVD) degeneration. An improved understanding of the pathogenesis of disc degeneration is now developing, which is leading to the development of a number of possible future therapies targeted at the underlying pathology and regeneration strategies. Although results thus far are promising, the investigation of such therapies in an environment that mimics the mechanical environment of the human disc *in vivo* is problematic. The development of an *in vitro* model system that can maintain metabolically active IVD tissue within a loading environment pertaining to that of the human spine is crucial for testing the efficacy of future cell-based and tissue-engineering therapies for IVD degeneration. Here, using our novel loading rig, capable of mimicking the loading environment experienced within the human spine, we have cultured nucleus pulposus tissue explants, applied a daily hydrostatic loading regime for up to 2 weeks and investigated proteoglycan retention, metabolic activity and cellular phenotype. IVD tissue cultured under a loading environment pertaining to the *in vivo* loading environment maintained metabolic cell activity, proteoglycan content and cellular phenotype. Indeed, all parameters were improved in IVD tissue cultured with load compared to unloaded controls. Such a model is invaluable for investigations assessing the feasibility and efficacy of future therapeutic approaches to inhibiting degeneration or stimulating regeneration of the IVD, where the *in vivo* loading environment may be crucial to their success or failure.

Keywords

intervertebral disc; loading; culture system; therapeutic testing; tissue culture; low back pain

1. Introduction

Low back pain (LBP) is a major cause of disability, affecting approximately 11 million people within the UK for at least 1 week in each month (Borenstein, 2001). Current therapies are aimed at relieving the symptoms and importantly do not target the underlying cause of LBP, which in a significant proportion of cases has been attributed to intervertebral

disc (IVD) degeneration (Luoma *et al.*, 2000), which is associated with decreased matrix synthesis and increased matrix degradation resulting from altered cell function and reduced cell number (Le Maitre *et al.*, 2007b). An improved understanding of the pathogenesis of disc degeneration is now developing, which is leading to the development of a number of possible future therapies which are targeted at the underlying pathology and altered cell biology. These include therapies to inhibit the processes of disc degeneration (Wallach *et al.*, 2003; Le Maitre *et al.*, 2006, 2007a) and regeneration strategies involving cell-based therapies, growth factor treatments and tissue-engineering approaches (Richardson *et al.*, 2007).

Although results from these studies are promising, the investigation of such therapies in an environment that mimics the mechanical environment of the human disc *in vivo* is problematic. The upright stance of humans and the axial loading of the vertebral column bring with them biomechanical demands upon the lumbar spine, which are unparalleled in the animal kingdom. Although some levels of applied load appear necessary to maintain matrix homeostasis and overall disc health (Holm and Nachemson, 1983), excess load may be involved in the acceleration of disc degeneration (Iatridis *et al.*, 1999). Thus, the loading environment in which prospective therapies are tested could determine their potential success or failure, with the elimination of some therapies which do not work in an unloaded environment but may be well suited to the loaded environment prevailing *in vivo*. Conversely, those therapies that appear promising in unloaded environments may fail when placed under the loading environment seen *in vivo*. However, as man is the only animal that has an upright bipedal posture, the 'load environment' of human discs is markedly different from those experienced in experimental animals, making their use inappropriate for investigating the effect of load on cell/tissue function or the testing of potential therapies at levels which equate to the human disc loading environment.

As such, there is an urgent need for an *in vitro* system capable of culturing IVD tissue samples in a loading environment pertaining to the human spine *in vivo*. The culture of IVD tissue has been hampered by a number of aspects, particularly the high swelling potential of the nucleus pulposus (NP) (Urban and McMullin, 1985), which results in tissue expanding uncontrollably in tissue culture (Urban and McMullin, 1985). Recently the number of studies investigating IVD tissue in *in vitro* systems has increased, with several investigating the culture of whole organ or explant systems of rabbit (Chiba *et al.*, 1998; Haschtmann *et al.*, 2006a, 2006b), murine (Ariga *et al.*, 2003), rat (Risbud *et al.*, 2003, 2006), bovine (Lee *et al.*, 2006) and ovine (Gantenbein *et al.*, 2006) IVD tissues. Within these culture systems, disc swelling has been restricted in a number of ways, including constriction of the tissue, either by retention of end-plates and adjacent soft tissues (Risbud *et al.*, 2003; Ariga *et al.*, 2003; Gantenbein *et al.*, 2006; Haschtmann *et al.*, 2006b), embedding into gel systems such as alginate (Chiba *et al.*, 1998), application of hyperosmotic solutions (Urban and McMullin, 1985) and/or via the application of external load (Lee *et al.*, 2006; Gantenbein *et al.*, 2006; Haschtmann *et al.*, 2006b; Korecki *et al.*, 2007). Unfortunately, though, many of these still report poor cell viability, loss of proteoglycan from the tissue and altered cellular phenotype and behaviour that often mimics the degenerative phenotype (Haschtmann *et al.*, 2006a).

We previously described a semi-constrained culture system in which human IVD tissue explants could be cultured for up to 3 weeks (the maximum time investigated) without tissue swelling, and in which tissue integrity, cellular phenotype and viability were maintained for the time course investigated (Le Maitre *et al.*, 2004). However, at that time we did not address the problem of whether the system would still function under the load conditions pertaining to that of the human spine. A number of studies have investigated the application of load to explant or organ tissue cultures but these have either been single static loading regimes, or simple diurnal loading patterns which do not represent the loading patterns seen

in vivo, i.e. 0.2 MPa or 0.25 MPa static loading (Lee *et al.*, 2006; Korecki *et al.*, 2007) or diurnal loading of 12 h 0.1 MPa and 12 h 0.3 MPa (Korecki *et al.*, 2007) or 8 h 0.2 MPa and 16 h 0.8 MPa (Gantenbein *et al.*, 2006). Here, using our novel loading rig (Le Maitre *et al.*, 2008), which is capable of mimicking the loading environment experienced within the human spine, we have cultured bovine tissue explants in our Perspex ring culture system (Le Maitre *et al.*, 2004) and applied a daily loading regime designed using pressure measurements taken during daily activities in the human spine (Wilke *et al.*, 1999). The aim of this study was to develop an *in vitro* culture system capable of maintaining IVD tissue without inducing changes in cellular phenotype or altering tissue integrity, in order that such a system could be used for future *in vitro* testing of novel cell-based or biomaterial therapies under loading conditions experienced in the human spine.

2. Materials and methods

2.1. Tissue samples

Bovine caudal IVDs (age 9–18 months) were excised and NP tissue isolated macroscopically, with the interface zone discarded to ensure no contamination with annulus fibrosus (AF) tissue. Cores of NP tissue (ca. 0.5 cm diameter × 0.6 cm height) were formed and placed into the Perspex ring culture system described previously (Le Maitre *et al.*, 2004).

2.2. Loading apparatus

Our novel loading system, as described previously (Le Maitre *et al.*, 2008) (Figure 1) consists of two parts: a commercial hydraulic biomechanical testing system [bench-mounted HC5 material-testing loading frame (Zwick Testing Machines Ltd)] and an in-house designed stainless steel pressure vessel. Following the insertion of tissue explants, contained in sealed media filled bags, into the pressure vessel, the vessel is filled with water and sealed. Compressive force is then applied via the piston (which is controlled by the hydraulic biomechanical testing system) to the water-filled chamber, which results in the generation of controlled hydrostatic pressure to the tissue samples contained within the pressure vessel. Internal pressure is monitored using a pressure transducer connected to a Hydrotechnik 2042 handheld pressure monitor, and Hydrowin monitoring software enabling live real-time recording of the pressure experienced within the vessel and graphical display readings accurate to ± 1%.

2.3. Design of daily loading regime

Using data derived from studies of intradiscal pressure (Wilke *et al.*, 1999), we designed a daily loading regime consisting of a combination of static and dynamic loading regimes to mimic daily activities. These regimes included two regimes to mimic the load experienced during lying down, i.e. representing the increase in pressure observed during lying down (0.1–0.24 MPa) (Wilke *et al.*, 1999), and a selection of daily activities including walking, sitting/standing and jogging, ordered to mimic a standard daily routine (Table 1). This regime was repeated 24 hourly to generate constant loading.

2.4. Application of loading regime

Perspex ring tissue cultures were transferred to sterile Whirl-Pak® bags (Nasco) and 50 ml DMEM/F12 with HEPES medium (Gibco; to maintain pH in the absence of CO₂) supplemented with 10% v/v heat-inactivated fetal calf serum (FCS; Gibco), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 250 ng/ml amphotericin, 2 mM glutamine (Sigma) and 50 µg/ml ascorbic acid (Sigma) (complete cell culture medium), was added, after which any air was removed and the bags sealed. Unloaded controls were

maintained in six-well tissue culture plates in complete cell culture medium in a 37 °C incubator. Bags of Perspex ring tissue cultures for loading were hooked onto the carousel and placed within the pressure vessel (Le Maitre *et al.*, 2008). The pressure vessel was filled with water at 37 °C and sealed. The piston was attached and all air expelled from the system (Figure 1). Hydrostatic loading was then applied for up to 2 weeks, using a repetition of the daily loading regime (Table 1). The medium was changed on samples every 48 h during the 0.1 MPa static loading, to prevent the introduction of an interval in the loading regime. Samples were removed from the loading environment for a maximum of 10 min for each medium change (NB: atmospheric pressure = 0.1 MPa). Tissue samples were removed from loaded and unloaded cultures after 48 h, 1 week and 2 weeks.

2.5. Tissue processing

Following removal from culture, the tissue samples were weighed and at each time point three tissue samples were fixed in 4% paraformaldehyde/PBS overnight, prior to processing to paraffin wax, and three tissue samples were subjected to papain digest to assess GAG content.

2.6. Assessment of glycosaminoglycans

Following culture, triplicate tissue samples were used for quantification of GAG content, using the DMMB assay, and DNA was assayed using the PicoGreen assay. Tissue samples were digested overnight at 60 °C in 1 ml 20 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA, 2 mM dithiothreitol and 100 U papain (Sigma). DMMB assay was performed using 25 µl shark chondroitin sulphate (Sigma) standards (62.5, 31.25, 15.625, 7.81, 3.9 and 0 µg/ml) or 5 µl papain-digested tissue sample. Each sample was applied in duplicate in separate wells of the 96-well plate and 200 µl DMMB colour reagent, as described previously (Farndale *et al.*, 1986), was added to each well. Following mixing, absorbance at A_{525} was read immediately, using a Titertex Multiscan® MC (Labsystems). The concentration of GAGs present within tissue samples was then calculated, using standards. DNA within digested tissue samples was assayed along with calf thymus DNA standards, using the PicoGreen DNA quantification kit (Invitrogen) according to the manufacturer's instructions.

2.6.1. Statistical analysis—Total GAG per tissue explant was calculated and normalized to DNA content of respective tissue explants. Data were determined as non-parametric, using the Shapiro–Wilke test and Kruskal–Wallis tests, with Mann–Whitney U post hoc tests used to assess the effect of culture on level of GAGs/DNA in tissues.

2.7. Histological examination

Paraffin-embedded samples were subjected to Alcian blue staining, using standard protocols.

2.8. Immunohistochemistry

Paraffin-embedded samples were subjected to immunohistochemistry (IHC) for aggrecan and type I and II collagens, as described previously (Le Maitre *et al.*, 2005). Briefly, 4 µm paraffin sections were dewaxed and rehydrated and endogenous peroxidase blocked using hydrogen peroxide. After washing in distilled water, sections were then treated with a hyaluronidase enzyme antigen retrieval system [0.1% w/v hyaluronidase in TBS (Sigma), 30 min at 37 °C] for aggrecan and type II collagen, or hyaluronidase and trypsin enzyme retrieval system [0.01% hyaluronidase (Sigma), 0.02% trypsin (Sigma) w/v in TBS] for type I collagen. Following washing, non-specific binding sites were blocked at room temperature for 45 min with 20% w/v rabbit serum (Sigma) for aggrecan, type I collagen and type II

collagen. Sections were incubated overnight at 4 °C with mouse monoclonal primary antibodies against human aggrecan (1 : 25 dilution; AbCam, Cambridge, UK), collagen type I (1 : 250 dilution; ICN, Basingstoke, UK) and collagen type II (1 : 100 dilution; MP Biomedicals). Negative controls in which mouse IgGs (Dako) replaced the primary antibody (at an equal protein concentration) were used. After washing, sections were incubated in biotinylated rabbit anti-mouse antiserum (1 : 400 dilution, Dako) for 30 min at room temperature. Disclosure of secondary antibody binding was by the streptavidin–biotin complex (Dako) technique with 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma). Sections were counterstained with Mayer's haematoxylin (Raymond A. Lamb), dehydrated and mounted in XAM (BDH).

2.9. *In situ* hybridization for polyA mRNA

In situ hybridization for polyA mRNA was performed as an assessment of metabolic cell activity, as described previously (Marles *et al.*, 1991; Walsh *et al.*, 1993).

2.10. Image and statistical analysis

All slides were visualized using a Leica RMD research microscope and images captured using a digital camera and the Bioquant Nova image analysis system. Total cell counts were performed on uncultured control tissue explants and no significant difference was seen in total cell number between explants (data not shown). Thus, for analysis of immunohistochemistry and polyA mRNA, 200 cells per explant were counted and cell staining recorded as positive or negative and the percentage immunopositive cells calculated. Data were determined as non-parametric, using the Shapiro–Wilke test, and Kruskal–Wallis tests with Mann–Whitney U *post hoc* tests used to assess the effect of culture on polyA mRNA positive and immunopositive cells during the 2 weeks of culture.

3. Results

3.1. Tissue integrity and proteoglycan content

Tissue integrity was maintained, with no tissue swelling observed throughout the time course investigated in both unloaded and loaded samples. However, within unloaded samples a significant decrease in the proteoglycan content of tissue explants was observed with increasing time in culture ($p < 0.05$ 48 h vs. 2 weeks; Figure 2A). In contrast, an increase was seen in GAG/DNA content in loaded samples between 2 and 7 days, although this was not statistically significant (Figure 2A), with overall proteoglycan content within loaded explants being maintained throughout the time course with no significant decrease in GAG/DNA content observed at any time point compared with 48 h in culture ($p > 0.05$). No significant differences in proteoglycan content were observed between 2 or 7 days, but significantly less proteoglycan content normalized to DNA was seen in unloaded vs. loaded controls following 2 weeks in culture ($p < 0.05$; Figure 2A). Improved proteoglycan retention for the duration of the culture period within loaded disc explants compared with unloaded tissue was confirmed with Alcian blue histological staining (Figure 2B–D).

3.2. Metabolic cell activity

Cell metabolic activity was assessed using *in situ* hybridization for polyA mRNA. Within unloaded tissue samples, a decrease in the number of positively stained cells was observed during the time course investigated, 36% polyA mRNA positive cells in unloaded tissue cultured for 14 days compared to 54% in uncultured tissue (Figures 3, 4). However, samples subjected to the daily loading regime maintained cellular activity for the 2 weeks investigated (66% polyA mRNA-positive cells following culture under load for 14 days compared to 54% in uncultured tissue; Figures 3, 4).

3.3. Cellular phenotype

Type II collagen and aggrecan immunopositive cells were observed in the uncultured tissue (Figure 5). The number of collagen type II-immunopositive cells was not altered in unloaded tissue after 2 weeks, but in loaded samples a significant increase in the percentage of collagen type II-immunopositive cells was observed ($p < 0.05$; Figures 4, 5). Following culture for 2 weeks, both unloaded and loaded systems demonstrated a significant increase in the percentage of cells displaying aggrecan immunopositivity ($p < 0.05$; Figures 4, 5). Type I collagen immunopositivity was not observed in any of the tissue samples examined (Figure 5). No immunopositive staining was observed in IgG controls (Figure 5).

4. Discussion

The development of an *in vitro* model system which can maintain metabolically active IVD tissue within a loading environment pertaining to that of the human spine is crucial for testing the efficacy of future cell-based and tissue-engineering therapies for IVD degeneration, as well as investigating the effects of human loads on IVD cell phenotype/function. This study, for the first time, has successfully maintained IVD tissue *in vitro* whilst subjecting the living tissue to a load pertaining to that experienced in the human spine. Metabolic cell activity, tissue integrity and cellular phenotype in IVD tissue culture were unaltered for 2 weeks in this loading system. Such a model incorporating this loading regime is invaluable for investigations assessing the feasibility and efficacy of future therapeutic approaches for inhibiting degeneration or repairing the IVD, where the loading environment may be crucial to their success or failure.

We have demonstrated that when these tissue explants were cultured within a loading environment mimicking the complex loads experienced during a daily routine, tissue integrity, proteoglycan retention, cellular phenotype and metabolic activity were maintained to a greater extent than in tissue cultured under standard culture conditions without load. Previous studies have demonstrated poor maintenance of biosynthetic activity and cell viability within unloaded tissue culture systems. Risbud *et al.* (2003) demonstrated the maintenance of cell viability for 1 week, which was followed by a subsequent loss in cell viability, decreased matrix gene expression and biosynthetic activity after 3 weeks in tissue cultured within a high osmotic environment but without load (Risbud *et al.*, 2003). Such findings are consistent with our data, in that tissue samples cultured without load showed decreased metabolic activity, reduced matrix synthesis and GAGs retention compared to tissue explants maintained under a loaded environment. Our study, together with other data investigating the effect of physiologically normal loading regimes on IVD cells both *in vitro* and *in vivo*, demonstrates that load is important in maintaining a normal phenotype. Indeed, within the loaded environment investigated, tissue explants displayed increased matrix protein synthesis, as evidenced by immunohistochemistry over the 2 week culture period. Such results complement several studies that have shown the important role load plays in matrix homeostasis, where the application of 'normal' loads has been shown to increase matrix synthesis (Ishihara *et al.*, 1996; Handa *et al.*, 1997; Hutton *et al.*, 2001; Kasra *et al.*, 2003, 2006; Gokorsch *et al.*, 2004; Maclean *et al.*, 2005; Le Maitre *et al.*, 2008).

A number of studies to date have investigated organ culture systems for the *in vitro* culture of IVD tissue but these have often been hampered by poor cell viability, thought to be caused by poor nutritional diffusion through the end plates or due to the size of the tissue (Lee *et al.*, 2006; Korecki *et al.*, 2007). Some organ culture systems using smaller animal models have been developed and have shown improved cell viability (Risbud *et al.*, 2003, 2006; Haschtmann *et al.*, 2006a, 2006b). Here, we aimed to develop a system that could be transferred to use for human disc tissue samples, and thus favoured the development of an

explant culture system. Human samples are generally received as fragments of tissue following surgery, and thus culture of whole IVD organ cultures is not feasible.

Within the current study, we demonstrate the maintenance of metabolic cell activity (as an indication of cell viability) within tissue explants, with no decrease observed over 2 weeks of culture in a loaded environment. One of the key challenges of a successful tissue culture system appears to be the maintenance of cell viability, and studies to date have only succeeded in maintaining viability for periods up to 1 week (Risbud *et al.*, 2006; Gantenbein *et al.*, 2006; Korecki *et al.*, 2007). Within our study, samples cultured without load also showed a decrease in metabolic cell activity during culture; however, those cultured in our apparatus and under a loaded environment demonstrated improved metabolic cell activity. Thus, for the first time we have succeeded in maintaining metabolic cell activity in tissue cultured for up to 2 weeks, which could have been due to improved diffusion through the tissue induced by fluid flow as a result of the application of dynamic loading regimes (Ferguson *et al.*, 2004; Gantenbein *et al.*, 2006; Haschtmann *et al.*, 2006a), or alternatively due to survival signals provided to the cells by the applied loading regime (Holm *et al.*, 1981).

The culture system described here involved the culture of tissue samples in sealed bags for up to 2 weeks, with the medium changed only every 48 h, compared to tissue samples cultured under normal tissue culture conditions (i.e. 5% CO₂, 20% O₂). Within this 'bag' system, gas exchange may differ between the unloaded controls and the loaded samples, due to the sealed culture environment. Notably, however, these factors were not detrimental, but rather appeared to improve cell metabolic activity and matrix protein expression compared to unloaded cultures in standard conditions. Indeed, any resulting hypoxia, due to limited gas exchange, may have improved cell viability, as hypoxic conditions can provide survival signals to NP cells and rescue them from apoptosis (Risbud *et al.*, 2005).

In addition to problems with cell viability, a further limitation of tissue culture systems to date has been the loss of proteoglycans from the tissue and alterations in normal cell phenotype within tissue cultures (Risbud *et al.*, 2003; Haschtmann *et al.*, 2006a, 2006b). Our culture system demonstrated improved proteoglycan retention and cellular phenotype in loaded vs. unloaded samples, with no loss of either proteoglycan content or cell phenotype within loaded tissue samples. Such results suggest that the culture system employed in our loading apparatus, together with the loading regime, is a feasible system for the maintenance of the *in vivo* disc cell phenotype and tissue integrity.

To date, no studies have attempted to culture IVD tissue under loading regimes which mimic the human spine. Simple static and diurnal loading have been applied in the attempt to improve cell viability, tissue integrity and cellular phenotype of tissue culture systems to varying degrees of success (Ferguson *et al.*, 2004; Lee *et al.*, 2006; Gantenbein *et al.*, 2006; Haschtmann *et al.*, 2006a, 2006b; Korecki *et al.*, 2007). However, such static loading regimes have been reported to induce detrimental effects on IVD cells, in both *in vivo* and *in vitro* tests, inducing cell death and abnormal gene expression often associated with matrix destruction (Wang *et al.*, 2007). Similar findings have also been reported for IVD tissue subjected to static load, with a cellular phenotype more akin to that of the degenerate IVD being produced (Lee *et al.*, 2006; Gantenbein *et al.*, 2006; Korecki *et al.*, 2007). Our current study used intradiscal pressure measurements, taken by Wilke *et al.* (1999) during a series of activities, to derive a daily loading regime which could mimic the loads experienced during a conceivable daily regime. Obviously the regime designed is a simplified pattern, but it provides an important tool for testing future therapies and investigating complex loading patterns on the IVD. The designed loading rig is capable of applying any regime the user chooses, and thus is a vital advance for both feasibility and efficacy testing, but it will also

allow investigation of the effects of loading parameters on cells, tissues and tissue-engineered constructs. Although the explant system described here has the advantage that it could be used for human tissue samples from both normal and degenerate patients, its major limitation is that, here, we have only investigated NP tissue. However, the results illustrate an important proof-of-principle concept, in that IVD tissue can be maintained in the system for extended periods under load. As such, our system is ideally suited for testing future therapies in a loading environment pertaining to the human spine and could be expanded to further investigate the maintenance of whole-disc organ cultures.

Of course, *in vivo*, IVDs are exposed to low levels of glucose, pH and oxygen (Urban *et al.*, 2004; Bibby *et al.*, 2005), which we did not directly address within this study. These are important parameters which have been shown previously to affect cellular metabolism and gene expression (Wang *et al.*, 2001; Horner *et al.*, 2002), and which we aim to investigate directly in future studies using our culture system. Indeed, our culture system allows the humoral environment to be easily manipulated, enabling the complex system seen *in vivo* within the IVD to be mimicked.

5. Conclusion

This study has described a novel culture system in which NP explant tissue can be cultured in a defined humoral environment for extended periods under load regimes identical to that experienced within the human spine. The further development of this system, including the use of whole-disc explants, will become a vital tool in the efficacy and feasibility testing of future cellular, molecular and tissue-engineering approaches to disc degeneration.

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References

- Ariga K, Yonenobu K, Nakase T, et al. Mechanical stress-induced apoptosis of endplate chondrocytes in organ-cultured mouse intervertebral discs: an *ex vivo* study. *Spine*. 2003; 28:1528–1533. [PubMed: 12865839]
- Bibby SR, Jones DA, Ripley RM, et al. Metabolism of the intervertebral disc: effects of low levels of oxygen, glucose, and pH on rates of energy metabolism of bovine nucleus pulposus cells. *Spine*. 2005; 30:487–496. [PubMed: 15738779]
- Borenstein DG. Epidemiology, etiology, diagnostic evaluation, and treatment of low back pain. *Curr Opin Rheumatol*. 2001; 13:128–134. [PubMed: 11224737]
- Chiba K, Andersson GB, Masuda K, et al. A new culture system to study the metabolism of the intervertebral disc *in vitro*. *Spine*. 1998; 23:1821–1827. [PubMed: 9762737]
- Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta*. 1986; 883:173–177. [PubMed: 3091074]
- Ferguson SJ, Ito K, Nolte LP. Fluid flow and convective transport of solutes within the intervertebral disc. *J Biomech*. 2004; 37:213–221. [PubMed: 14706324]
- Gantenbein B, Grunhagen T, Lee CR, et al. An *in vitro* organ culturing system for intervertebral disc explants with vertebral endplates: a feasibility study with ovine caudal discs. *Spine*. 2006; 31:2665–2673. [PubMed: 17077734]
- Gokorsch S, Nehring D, Grottke C, et al. Hydrodynamic stimulation and long-term cultivation of nucleus pulposus cells: a new bioreactor system to induce extracellular matrix synthesis by nucleus

- pulposus cells dependent on intermittent hydrostatic pressure. *Int J Artif Organs*. 2004; 27:962–970. [PubMed: 15636054]
- Handa T, Ishihara H, Ohshima H, et al. Effects of hydrostatic pressure on matrix synthesis and matrix metalloproteinase production in the human lumbar intervertebral disc. *Spine*. 1997; 22:1085–1091. [PubMed: 9160466]
- Haschtmann D, Stoyanov JV, Ettinger L, et al. Establishment of a novel intervertebral disc/endplate culture model: analysis of an *ex vivo in vitro* whole-organ rabbit culture system. *Spine*. 2006a; 31:2918–2925. [PubMed: 17139222]
- Haschtmann D, Stoyanov JV, Ferguson SJ. Influence of diurnal hyperosmotic loading on the metabolism and matrix gene expression of a whole-organ intervertebral disc model. *J Orthop Res*. 2006b; 24:1957–1966. [PubMed: 16917902]
- Holm S, Maroudas A, Urban JP, et al. Nutrition of the intervertebral disc: solute transport and metabolism. *Connect Tissue Res*. 1981; 8:101–119. [PubMed: 6453689]
- Holm S, Nachemson A. Variations in the nutrition of the canine intervertebral disc induced by motion. *Spine*. 1983; 8:866–874. [PubMed: 6670021]
- Horner HA, Roberts S, Bielby RC, et al. Cells from different regions of the intervertebral disc: effect of culture system on matrix expression and cell phenotype. *Spine*. 2002; 27:1018–1028. [PubMed: 12004167]
- Hutton WC, Elmer WA, Bryce LM, et al. Do the intervertebral disc cells respond to different levels of hydrostatic pressure? *Clin Biomech (Bristol)*. 2001; 16:728–734.
- Iatridis JC, Mente PL, Stokes IA, et al. Compression-induced changes in intervertebral disc properties in a rat tail model. *Spine*. 1999; 24:996–1002. [PubMed: 10332792]
- Ishihara H, McNally DS, Urban JP, et al. Effects of hydrostatic pressure on matrix synthesis in different regions of the intervertebral disc. *J Appl Physiol*. 1996; 80:839–846. [PubMed: 8964745]
- Kasra M, Goel V, Martin J, et al. Effect of dynamic hydrostatic pressure on rabbit intervertebral disc cells. *J Orthop Res*. 2003; 21:597–603. [PubMed: 12798057]
- Kasra M, Merryman WD, Loveless KN, et al. Frequency response of pig intervertebral disc cells subjected to dynamic hydrostatic pressure. *J Orthop Res*. 2006; 24:1967–1973. [PubMed: 16900539]
- Korecki CL, Maclean JJ, Iatridis JC. Characterization of an *in vitro* intervertebral disc organ culture system. *Eur Spine J*. 2007; 16:1029–1037. [PubMed: 17629763]
- Le Maitre CL, Hoyland JA, Freemont AJ. Studies of human intervertebral disc cell function in a constrained *in vitro* tissue culture system. *Spine*. 2004; 29:1187–1196. [PubMed: 15167656]
- Le Maitre CL, Frain J, Fotheringham AP, et al. Human cells derived from degenerate intervertebral discs respond differently to those derived from non-degenerate intervertebral discs following application of dynamic hydrostatic pressure. *Biorheology*. 2008; 45:563–575. [PubMed: 19065005]
- Le Maitre CL, Freemont AJ, Hoyland JA. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis Res Ther*. 2005; 7:R732–745. [PubMed: 15987475]
- Le Maitre CL, Freemont AJ, Hoyland JA. A preliminary *in vitro* study into the use of *IL-1Ra* gene therapy for the inhibition of intervertebral disc degeneration. *Int J Exp Pathol*. 2006; 87:17–28. [PubMed: 16436110]
- Le Maitre CL, Hoyland JA, Freemont AJ. Interleukin-1 receptor antagonist delivered directly and by gene therapy inhibits matrix degradation in the intact degenerate human intervertebral disc: an *in situ* zymographic and gene therapy study. *Arthritis Res Ther*. 2007a; 9:R83. [PubMed: 17760968]
- Le Maitre CL, Pockert A, Buttle DJ, et al. Matrix synthesis and degradation in human intervertebral disc degeneration. *Biochem Soc Trans*. 2007b; 35:652–655. [PubMed: 17635113]
- Lee CR, Iatridis JC, Poveda L, et al. *In vitro* organ culture of the bovine intervertebral disc: effects of vertebral endplate and potential for mechanobiology studies. *Spine*. 2006; 31:515–522. [PubMed: 16508544]
- Luoma K, Riihimäki H, Luukkainen R, et al. Low back pain in relation to lumbar disc degeneration. *Spine*. 2000; 25:487–492. [PubMed: 10707396]

- Maclean JJ, Lee CR, Alini M, et al. The effects of short-term load duration on anabolic and catabolic gene expression in the rat tail intervertebral disc. *J Orthop Res*. 2005; 23:1120–1127. [PubMed: 16140193]
- Marles PJ, Hoyland JA, Parkinson R, et al. Demonstration of variation in chondrocyte activity in different zones of articular cartilage: an assessment of the value of *in situ* hybridization. *Int J Exp Pathol*. 1991; 72:171–182. [PubMed: 2015200]
- Richardson SM, Mobasheri A, Freemont AJ, et al. Intervertebral disc biology, degeneration and novel tissue engineering and regenerative medicine therapies. *Histol Histopathol*. 2007; 22:1033–1041. [PubMed: 17523081]
- Risbud MV, Di Martino A, Guttapalli A, et al. Toward an optimum system for intervertebral disc organ culture: TGF β 3 enhances nucleus pulposus and annulus fibrosus survival and function through modulation of TGF β R expression and ERK signaling. *Spine*. 2006; 31:884–890. [PubMed: 16622376]
- Risbud MV, Fertala J, Vresilovic EJ, et al. Nucleus pulposus cells upregulate PI3K/Akt and MEK/ERK signaling pathways under hypoxic conditions and resist apoptosis induced by serum withdrawal. *Spine*. 2005; 30:882–889. [PubMed: 15834331]
- Risbud MV, Izzo MW, Adams CS, et al. An organ culture system for the study of the nucleus pulposus: description of the system and evaluation of the cells. *Spine*. 2003; 28:2652–2658. [PubMed: 14673364]
- Urban JP, McMullin JF. Swelling pressure of the intervertebral disc: influence of proteoglycan and collagen contents. *Biorheology*. 1985; 22:145–157. [PubMed: 3986322]
- Urban JP, Smith S, Fairbank JC. Nutrition of the intervertebral disc. *Spine*. 2004; 29:2700–2709. [PubMed: 15564919]
- Wallach CJ, Sobajima S, Watanabe Y, et al. Gene transfer of the catabolic inhibitor TIMP-1 increases measured proteoglycans in cells from degenerated human intervertebral discs. *Spine*. 2003; 28:2331–2337. [PubMed: 14560079]
- Walsh L, Freemont AJ, Hoyland JA. The effect of tissue decalcification on mRNA retention within bone for *in situ* hybridization studies. *Int J Exp Pathol*. 1993; 74:237–241. [PubMed: 8392858]
- Wang JY, Baer AE, Kraus VB, et al. Intervertebral disc cells exhibit differences in gene expression in alginate and monolayer culture. *Spine*. 2001; 26:1747–1752. [PubMed: 11493844]
- Wilke HJ, Neef P, Caimi M, et al. New *in vivo* measurements of pressures in the intervertebral disc in daily life. *Spine*. 1999; 24:755–762. [PubMed: 10222525]

Figure 1.

Photomicrograph of the loading apparatus used in the application of hydrostatic pressure. Tissue explants were cultured in Perspex rings (top right), which for unloaded controls were placed in six-well tissue culture plates and cultured under standard conditions. For application of load, explants in perspex rings were inserted into bags containing medium, the air expelled and the bags sealed. The sealed bags were hung on a carousel (bottom right) to enable free floating in the water-filled pressure vessel (left)

Figure 2.

(A) Glycosaminoglycan (GAGs) content of tissue explants during culture with or without load. Error = bars standard error of the mean (SEM). * $p < 0.05$; $n = 3$. Photomicrographs of: (B) Alcian blue-stained tissue explants prior to culture; (C) following 2 weeks culture without load; or (D) following 2 weeks in culture with load, together with magnified images. Scale bars = 380 μm

Figure 3.

Photomicrographs representative of: (A) *in situ* hybridization for polyA mRNA within tissue explants prior to culture; and following 2 weeks culture without (B) or with (C) load. Magnified images are also displayed (Ai–Ci). (D–F) Negative control sections for polyA mRNA. Scale bars = 380 μ m

Figure 4.

Percentages of cells displaying positive for polyA mRNA and immunopositivity for type II collagen and aggrecan in uncultured tissue samples and those cultured without and with load for 2 weeks. Error bars = SEM; * $p < 0.05$; $n = 3$

Figure 5.

Photomicrographs representative of immunohistochemical staining for type II collagen, aggrecan, type I collagen and IgG control sections in tissue explants following 2 weeks of load or in unloaded controls. Scale bars = 380 μm

Table 1

Daily loading regime, designed using intradiscal pressure measurements

Step	Load (MPa)	Frequency (Hz)	Duration (h)
Lying 1	0.1	Static	4
Lying 2	0.24	Static	4
Walking	0.53–0.65	0.5	2
Sitting/standing	0.5	Static	4
Jogging	0.35–0.95	1.0	2
Sitting/standing	0.5	Static	4
Walking	0.53–0.65	0.5	2
Sitting/standing	0.5	Static	2

Pressure measurements from Wilke *et al.*, 1999.