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## Organ Culture Bioreactors – Platforms to Study Human Intervertebral Disc Degeneration and Regenerative Therapy

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### Abstract

In recent decades the application of bioreactors has revolutionized the concept of culturing tissues and organs that require mechanical loading. In intervertebral disc (IVD) research, collaborative efforts of biomedical engineering, biology and mechatronics have led to the innovation of new loading devices that can maintain viable IVD organ explants from large animals and human cadavers in precisely defined nutritional and mechanical environments over extended culture periods. Particularly in spine and IVD research, these organ culture models offer appealing alternatives, as large bipedal animal models with naturally occurring IVD degeneration and a genetic background similar to the human condition do not exist. Latest research has demonstrated important concepts including the potential of homing of mesenchymal stem cells to nutritionally or mechanically stressed IVDs, and the regenerative potential of “smart” biomaterials for nucleus pulposus or annulus fibrosus repair. In this review, we summarize the current knowledge about cell therapy, injection of cytokines and short peptides to rescue the degenerating IVD. We further stress that most bioreactor systems simplify the real *in vivo* conditions providing a useful proof of concept. Limitations are that certain aspects of the immune host response and pain assessments cannot be addressed with *ex vivo* systems. Coccygeal animal disc models are commonly used because of their availability and similarity to human IVDs. Although *in vitro* loading environments are not identical to the human *in vivo* situation, 3D *ex vivo* organ culture models of large animal coccygeal and human lumbar IVDs should be seen as valid alternatives for screening and feasibility testing to augment existing small animal, large animal, and human clinical trial experiments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

## Keywords

Bioreactor; cell viability; mechanical loading; intervertebral disc; mesenchymal stem cell; torsional loading; engineering; nutrition

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## 1. INTRODUCTION

Low back pain is often related to intervertebral disc (IVD) degeneration. While painful IVD degeneration is not a lethal disease, it has been recognized as a major social burden with a high socioeconomic impact with many people often not being able to return to work for several weeks [1,2].

Two main hypotheses for the onset of painful IVD degeneration have been raised: The first hypothesis comes from Michael Adams and his colleagues, who investigated if IVDs would fail and degenerate based on pure mechanical overloading [3,4]. This hypothesis implies that the mechanical damage, i.e. micro-fissures in the tissue, occurs first and is followed by cells failing to maintain the homeostasis, and matrix breakdown. On the other hand, there is the limited nutrition hypothesis raised by Jill Urban and others [5,6], who state that at first there is a shortage of nutrition induced for the disc cells by blockage of diffusion through the cartilaginous endplate (CEP) route, which then leads to cell loss over the years and subsequently, the tissue homeostasis cannot be maintained, which then leads to mechanical failure of the IVD. The initiation and progression of IVD degeneration is a complex interaction of mechanical, biological, and physiological factors that are modulated by inflammatory responses, the genetic background and general health of the individual, which seems to be the most appropriate explanation for the development of IVD degeneration [7].

In order to test such hypotheses in IVD research many studies have been undertaken, including *in vitro* diffusion studies [8,9] and *in vivo* studies [10,11]. These studies demonstrated the importance of solute transport distances in defining cell density and metabolic rates. As a result, studies addressing nutritional compromise in the IVD require the use of IVDs from large animal models. Many issues were raised concerning *in vivo* animal studies regarding their limited application for the human situation [12]. For example, several biological differences exist among animal models, and quadrupeds may have distinct biomechanical properties from the upright human spine [12], however the relative contributions of gravitational forces and muscular contraction to spinal loading has not been thoroughly quantified in quadrupeds. Also in terms of the anatomy there are important differences, which make a direct comparison questionable. For instance, the IVDs of the porcine spine are thickest in the cerebral region [13]. For the porcine model there seems to be biomechanical similarity of thoracic and human lumbar IVDs [13,14]. Recent studies also proposed the minipig as an animal model for studying IVD regeneration approaches such as injection of mesenchymal stem cells (MSCs) [15]. There are a number of mechanical and biological parameters to be considered, including cell density, which is considerably higher in many animal models than in human IVDs [16,17]. From a biomechanical perspective, there is some evidence that rodent and lagomorph (i.e. rabbit) IVDs have mechanical parameters that scale similarly to human IVDs although some important differences exist

[17-19]. Some animals keep the notochordal cells throughout their life-time and other animals such as the human lose these cells when they are mature. In this respect, the presence or absence of notochordal cells has been discussed and their presence might produce outcomes that are more favourable in promoting regeneration and repair [20]. Their function has not yet been fully clarified but their regenerative potential has been described based on co-culture studies where these cells up-regulate glycosaminoglycan production [21-24] and even seem to release protective proteins that down-regulate apoptotic pathways [25]. In terms of range of motion (ROM) and creep, the coccygeal IVDs are very similar to lumbar discs [19,26-28].

3D organ culture models for the IVD are very appealing [12], as the effect of specific treatment strategies can be investigated in a controlled environment while *in vivo* animal studies add complexity and thus, might result in higher variance. Further, due to the absence of true upright spine animal models, *ex vivo* culture of human cadaveric IVDs in bioreactors represents a valid alternative to animal models. Monkeys, kangaroos and giraffes are not true upright walking animals, and there are also important functional and anatomical differences to the human. Thus, besides the ethical concerns one has to conclude that there is no good animal model for the human spine. In the following sections we will briefly discuss the basic concepts, which are needed to culture IVDs successfully.

## 2. OVERVIEW OF BIOREACTOR SYSTEMS

In recent years, several research groups have realized a variety of organ culture systems, which have been optimized over the years based on user experience. Here, we provide an almost complete list of these systems and summarize in short the major findings and limitations in terms of cell survival and function (Table 1). A number of bioreactor systems exist [29-35], all devoted to maintain the IVD explant, and designed specifically to fulfil the need of the different IVD sizes. Especially, the distance in the sagittal orientation from cartilaginous endplate to endplate determines the number of cells to be maintained and also the nutritional limits due to passive diffusion. In the last decade we have seen the emerging of several actively diffused bioreactors with tubing systems [7,31,36-39] as well as the development of static culture systems which do not require additional media tubing if the nutritional pathways can be maintained and the media to tissue volume ratio is high enough [35,36,40] (Table 1). Considering uni-axial compression, it becomes obvious that disc surface area is relevant and thus determines the force range of the bioreactor force cell and the dimensions of the culture chambers. Disc height and creep determines the required range of displacement [33,41]. Figure 1 depicts a general design of a bioreactor for IVD culture and summarizes current research foci for the establishment of *in vitro* degeneration and regeneration models. It has also been shown repeatedly that some sort of “physiological” loading of the IVD is essential to maintain the disc matrix content and the continuous solute exchange. Free-swelling culture, on the other hand, mimics the condition of spines and IVDs under low or microgravity [42,43] and could possibly affect cell viability by changes in osmotic pressure [44]. Recently, a wedge-loaded biomechanical system has been tested using bovine IVDs [45]. With this system it was found that asymmetric loading involving excessive bending can induce delamination of the annulus fibrosus, up-regulate metalloproteinases (MMPs) and pro-inflammatory cytokines and this lead to increased cell

death. The simulated hyperflexion in this system is known to cause IVD injury [46]. Jim *et al.* [33] and Haglund *et al.* (2011) [34] have well demonstrated that the load distribution across the IVD is not homogenous in the bovine coccygeal discs as compared to a human lumbar disc. This discrepancy likely arises from the anatomy of the cartilaginous endplate (CEP) of the bovine tail, which is concave in structure whereas the human lumbar disc has a flat CEP [34]. Considering the anatomy of the bovine disc, the team of Haglund *et al.* (2011) developed a novel bioreactor with exchangeable part for fitting the anatomy of both human and bovine discs [34] (Figure 2). However, in order to enable torsional loading, a flat bone surface was needed to prevent slippage and to obtain a smooth hysteresis curve of the IVDs during the loading cycles; thus, no such adjustments considering the concave shape of the CEPs could be realized [40]. An overview of bioreactor systems published within the last decade by research groups around the globe is depicted in Figure 3. Obviously, all bioreactors have in common that they use biocompatible materials, which are supposedly noncytotoxic and easy to clean after culture. These materials are either polycarbonate (Figure 3, A, B) or combinations of glass and polyoxymethylene (POM) (Figure 3, D, E) PEEK or even of ULTEM® (Figure 3, C). Most bioreactors use a fluidic muscle system for force application, while hydraulic valves were used to apply the high forces in the range of 4kN to harbour cadaveric human IVDs [29]. For smaller disc specimens (e.g. rabbit), a cost-effective alternative is a multi-station loading system, which is integrated into a standard materials testing machine [47], providing the advantage of high throughput for simultaneous stimulation of multiple samples. In terms of loading, most bioreactors focus on uniaxial compressive loading except one that can also investigate torsion as a 2<sup>nd</sup> degree of freedom (DoF) by using rough-surface titanium plates to enable torsion [41]. More complex loadings by increasing the DoF up to six have been recently undertaken with formalin fixed samples [48] and studies are under way to use also unfixed live tissue (Costi, personal communication). Recently, it was also reported that bioreactor studies could be used to validate numerical modelling data [49,50]. Castro *et al.* [50] predicted the disc height variation of chondroitinase ABC injected IVDs under a given diurnal disc loading by their osmo-poro-hyper-viscoelastic and fiber-reinforced finite element model; data validation was performed by comparing the data obtained from ex-vivo loading of the goat discs in a bioreactor. The studies by Paul *et al.* (2012; 2013) [7,51] demonstrated that the creep of the IVD is happening in the first 4h of “high” loading. They also provide a finite element model of the enzymatic digestion process, which better predicts the enzymatic break-down of the ECM.

So, there is a general trend from uni-axial compressive loading to more complex loading using multiple DoF loading. In addition, there is a trend to keep more structures around the motion segments such as ligaments and the facet joints, which also contribute significantly to a more realistic load distribution [52]. Nevertheless, these complex motion segments also require more nutrition, as there are much more cells involved, and could be limited by a reduced cell viability of the tissue overall especially the IVD.

### 3. MINIMAL REQUIREMENTS TO GET STARTED

One of the most frequent questions is what is required to maintain IVD explants *in vitro*? The tissue obtained from the slaughterhouse should be transported to the laboratory as

quickly as possible as the tissue will start to decay. However, the cells in the inner part will stay unaffected during this processing time [40]. One of the first steps before starting experiments is to certify that the biological material has a high cell viability at so-called “day 0” of the experiment. This is especially advisable in case of obtaining material from an abattoir one is not very familiar with. Secondly, during the preparation and dissection process the end plate needs to be maintained permeable or made permeable again for nutrients diffusion from and into the organ explant. Here, different approaches have been undertaken. In an ovine study it was shown that cell viability could be improved by flushing of the lower extremities by systemic injection of heparin before euthanasia and immediate post-mortem flushing with ringer solution containing heparin [36]. Bovine coccygeal IVDs harvested from a local abattoir within 3-24 h post-mortem have advanced to a more available and reproducible model [53]. This turned out to be a very reliable animal source of healthy IVDs with the possibility to select for specific sizes of segments for various purposes [40,54]. A significant challenge for all IVD research, regardless of species, is to maintain the original tissue structure during the preparation of the tissue. Obviously, removing tissue, which is always under a certain hydrostatic pressure, causes swelling [55]. When muscular loading is removed and IVDs are isolated, the tissue exhibits a propensity to swell in standard culture media as a result of development of large osmotic gradients [56]. Notably, if bovine coccygeal disc is isolated without the superior and inferior end plates, its weight increases drastically compared to discs with bone-covered end plates. This rapid increase in wet weight of IVD without end plates is apparent within the first 1.5 hours (% increase of initial wet weight for discs after 10, 20, and 90 minutes was 10%, 16%, and 22% for IVDs without any end plates = no endplate, NEP), while the change was 2%, 4%, and 5% for discs with bone-covered end plates. After 15 hours, the weight of IVDs without end plates increased around 22% compared to 10% when cultured with bone-covered end plates (= BEP). A similar finding was observed by Gawri *et al.* (2011) [57] who compared swelling of human IVDs with cartilage end plates (= CEP) versus IVDs without end plates; after 66 hours in culture, the observed increase in wet weight was 21% in the cartilage end plates group, compared with 44% for the IVDs without end plates [54,57]. When the same experiment was done using bovine discs a 20% increase was found in CEP-prepared disc whereas NEP discs increased by 60-100% of the initial weight [33].

Another appealing approach is to use punched-out tissue of a pre-defined shape and surface area instead of an intact disc. This can be achieved for instance with biopsy punches. For the culture, it is important to maintain the glycosaminoglycans (GAGs) inside the tissue over time [58]. Such a system was established by Potier *et al.* (2014) [59]. This group proposed to maintain the GAGs content of the tissue by containing the tissue in dialysis membranes with a molecular cut-off of 15 kD or by raising the osmolality by addition of polyethyleneglycol (PEG) to the medium [58]. Their data also showed a better outcome in terms of cell viability and GAG/DNA ratio if the tissue was contained with the dialysis membrane (Table 1). A similar approach was reported by Haschtmann *et al.* (2006) [60] to achieve “diurnal” swelling and shrinking by increasing osmolality by addition of sucrose: They created a diurnal environment and cultured the IVDs daily for 8 h in iso-osmotic media followed by 16 h in hyperosmotic media. An improved cell viability was achieved by culturing the IVDs

daily for 8 h in iso-osmotic media, followed by 16 h in hyperosmotic media (test group). In this way a convective transport could be enabled.

#### 4. ORGAN CULTURE, BIOMATERIALS AND STEM CELL THERAPY

Adult mesenchymal stem cells (MSCs) have been shown to be a promising cell source for regenerative therapies for several decades [61,62]. MSCs have the potential to differentiate along various lineages, including the skeletal lineages [63] and bone marrow derived MSCs are the most studied MSCs. While differentiation into cartilaginous cell types is well established, recent *in vitro* and *in vivo* studies indicated the potential of MSCs to differentiate also towards a phenotype similar to nucleus pulposus cells [63-72].

The regenerative potential of MSCs was further indicated by their migration potential towards injured and compromised (stressed) tissues such as bone defects [73]. Recent data also indicated that MSCs can actively “home” towards stressed IVDs by migrating through the CEP [74]. In this bovine IVD organ culture model fluorescently labelled primary human bone marrow MSCs were applied onto “stressed” IVDs and their migration potential towards the disc center was determined by confocal laser scanning microscopy. IVD cell stress was initiated through nutritional deprivation and/or unphysiological biomechanical loading [30,37] and annular puncture of the IVD with a needle. MSC migration potential was further confirmed in migration assays; MSCs migrated towards the conditioned medium of “stressed” IVDs, demonstrating the chemo-attractive effect of the medium [74]. One potential initiator of the observed cell migration is the chemokine RANTES/CCL5. This chemokine is expressed by NP cells and AF cells and is thought to play a central role in the biology of attracting stromal cells [75].

Yet, undifferentiated MSCs may have difficulties to survive in an IVD environment, which is typically depleted of oxygen, is more acidic, and has a higher osmolarity compared to other tissues [76,77]. Chan *et al.* [77] demonstrated in a papain-induced bovine caudal IVD degeneration organ culture model that MSCs could not thrive even in the healthy IVD matrix and many of the MSCs actually died as demonstrated by LIVE/DEAD stain and confocal microscopy [77] after a 10-day culture. Later, the same group [78] demonstrated MSC proliferation in IVD organ cultures under relatively mild loading conditions when the MSCs were suspended in a thermo-reversible hyaluronan-based hydrogel, i.e. [poly(N-isopropylacrylamide), HA-pNIPAM] prior injection into papain digested cavities. However, the HA-pNIPAM hydrogel was found to lose a large portion of the initial volume after 7 days of mild static compressive loading at 0.2 MPa [78]. Though the compromised stability under mechanical load is a limitation of the HA-pNIPAM hydrogel, other investigations demonstrated its potential to support MSC differentiation towards a disc-like phenotype. Indeed, human MSCs encapsulated within the HA-pNIPAM hydrogel survived and differentiated within an IVD cavity without the need for pre-culture and/or growth factor supplementation [79]. MSC survival was further investigated in an *in vivo* rat-tail model: Crevensten *et al.* (2004) [64] injected MSCs suspended in a 15% hyaluronan gel and observed that while the cell number of surviving MSCs significantly decreased after 7 and 14 days, after 28 days the MSC population returned to the initial amount of injected cells and viability was 100%. Further, a trend of increased IVD height compared to blank gel



suggested an increase in matrix synthesis. From these findings the authors concluded that MSCs could survive and proliferate in IVDs [64].

Other recently published data by the Canadian disc researcher team by Mwale *et al.* (2014) [80] could show successful MSC survival without any pre-conditioning with growth factors if injected into bovine coccygeal organ culture model along with the short peptide link-N. They injected only about  $10^5$  cells into the bovine disc and found a high cell viability after 21 days of organ culture. However, a crucial factor seems to be number of MSCs that are supplied and that the IVDs are kept under high nutrition. On the effect of the number of injected MSCs there has been only one study systematically undertaken so far in an *in vivo* canine model [81]. Serigano *et al.* (2010) [81] injected  $10^5$ ,  $10^6$ , or  $10^7$  per canine lumbar disc and found only after injecting at least  $10^6$  cells the expected regenerative effects by partial restoring disc height on MRI. Live/dead stain also confirmed higher cell viability if  $> 10^6$  cells were applied.

During recent years the application of various hydrogels for NP and AF repair has been widely followed-up [80,82-85]. One approach is the injection of fibrin-genipin adhesive hydrogel to seal large AF defects [86,87]. In a bovine organ culture model, bovine-derived fibrin cross-linked with genipin successfully closed an AF wound that was induced by a stab with a scalpel blade [86,87]. The hydrogel with gelation times of less than 15 min successfully sealed large AF defects, promoted functional restoration with improved motion segment biomechanics, and served as a biocompatible adhesive biomaterial that had greatly enhanced *in vivo* longevity compared to fibrin alone. The authors concluded that fibrin-genipin would offer promise for AF repair strategies and would warrant further material development and evaluation for clinical application [86]. Thus, hydrogels might find their way into the operation rooms as commercially available products are already FDA-approved such as Tisseel© from Baxter, inc. Another approach is the combination of genipin reinforced hydrogels with adherence molecules or anti-inflammatory drugs such as anti-TNF $\alpha$ . In this respect, drug delivery systems consisting of hydrogel carriers have been investigated such as a hyaluronan-gel releasing stromal cell derived factor-1 (SDF-1) to enhance homing of MSCs [88]. Many other 3D hydrogels were investigated in particular for NP regeneration. These are summarized in [89,90].

Recently, viral and non-viral gene delivery has been investigated for their feasibility to be applied directly to the IVD [89,91]. DNA approaches have traditionally been undertaken using viral vectors onto monolayer cells [89] or in some cases using pellet or alginate bead cultures [91,92]. Wallach *et al.* (2006) [92] investigated adenovirus Ad-TGF- $\beta_1$  and Ad-BMP-2 as therapeutic factors for the IVD, as a potential treatment strategy for degenerative disc disease. However, non-viral transfection methods might be key for future translational medicine as their acceptance is much higher and toxic issues to cells are much less than with viral methods. Such an approach has been recently investigated in a pilot study using a papain-cavity bovine organ culture model where transiently GDF-5-transfected MSCs embedded in a polyethylene glycol hydrogel (PEG) hydrogel were injected [91]. This study showed potential for such a gene therapy approach by achievement of increased GAG after injection of the transfected cells into the bovine organ culture model [91].

Bioreactors could serve as a “priming tool” if they achieved FDA approval and/or CE label. *In vitro* and *in vivo* studies have demonstrated that MSCs can be directed towards the discogenic pathway by application of growth factors such as GDF5 and GDF6 or hypoxic conditions [71,72,93]. It has been multiple times demonstrated that MSCs injected into the microenvironment of the disc start to differentiate into disc-like or more precisely into NPC-like cells either after preconditioning of the MSCs with suitable growth factors or directly driven by the transplantation into the microenvironment [78]. Other studies confirm direct discogenic differentiation by injection of undifferentiated MSCs after a prolonged culture time of 14 days into AFC-like precursor cells [94]. Concerning the “priming” of naïve MSCs towards a discogenic phenotype, there has been to the best of our knowledge no clinical trial so far involving an *ex vivo* step using bioreactors. Of course, the idea would be appealing to inject patient's autologous MSCs, which were expanded *in vitro* on plastics and then “primed” into IVD precursors by simulating the IVD-specific microenvironment. This could be accomplished by culturing MSCs under hypoxic conditions and application of specific mechanical or hydrostatic loading. It is hypothesized that MSCs “primed” by a mechanical bioreactor mimicking relevant loading conditions may show improved survival and activity after injection into the disc. The risk remains that the “primed” MSCs may be injected into an inflammatory microenvironment; this could have a negative or a beneficial influence on the MSCs, though the role of the inflammatory milieu is not very clear yet [95].

The results from these presented studies confirm that organ culture is a useful tool to investigate regenerative approaches such as cell therapy, gene therapy or biomaterial injection. Furthermore, these devices could hold the potential to “prime” MSCs or other stem cells for re-injection into the patient.

## 5. ANIMAL MODELS FOR ORGAN CULTURE

Much concern has been raised whether tail IVDs, the IVDs from the elongation of the spine should serve as a model system. From a mechanical point of view it was demonstrated that tail IVDs of different species could be used as model systems as their creep behaviour and compressive stiffness was comparable to the lumbar IVD in principle [26,96]. Beckstein *et al.* (2008) [27] compared nine animal species and concluded after correction for geometry that the disc tissue material properties are largely conserved across animal species, which were calf, pig, baboon, sheep, rabbit, rat, and mouse lumbar discs, in addition to the cow and rat tail IVD [27]. The coccygeal IVDs have a similar range of motion as lumbar IVDs in flexion and extension, bending and torsion [27]. Torsion angle of failure of coccygeal discs for rat and cattle was found to be very high at  $\sim 30^\circ$  [97,98] in contrast to the human lumbar disc where the failure range is smaller  $\sim 10^\circ$  [52]. Biochemical composition, cell density and the cell population lacking notochordal cells of the bovine tail IVD are highly similar to human lumbar IVD [99,100].

## 6. ENZYMATIC DISC DEGENERATION MODELS

In recent years, several approaches were undertaken to develop disc degeneration models involving proteinases that digest mainly GAGs or collagenases. Among various enzymes, chondroitinase ABC, trypsin and papain (serine protease) have been used to induce IVD



degeneration. Chondroitinase ABC has been widely used to remove painful tissue in disc herniation but was also intensively investigated to induce disc degeneration *in vitro* and *in vivo* [101-107] (Table 2). Papain has been repeatedly shown to induce cavity formation if injected in various doses [77,108]. Recently, it has been demonstrated that loss of disc integrity and stiffness induced by injection of trypsin or by non-physiological loading can be partially recovered by applying a physiological loading [109]. Recently, Furtwängler *et al.* (2013) [110] injected more physiologically relevant enzymes such as aggrecanase (ADAMTS4) collagenase (MMP3) and high temperature requirement serine protease A1 (HTRA1) into the IVD [110,111]. It has been repeatedly demonstrated that ADAMTSes and MMPs are up-regulated in IVD degeneration. However, *in vitro* injection of higher doses of these enzymes did not induce major matrix break down as compared to papain and trypsin. Thus, neither injection of activated MMPs nor HTRA1 did cause visible matrix depletions as it was found with papain [110] with diurnal loading with 0.4 MPa and free-swelling recovery for 16 hrs (Table 2). It was noticed that different enzymatic treatment of IVDs caused similar T2\* or T1ρ MRI sequences as different stages of IVD degeneration [106,112-114]. Several studies demonstrated a correlation between severity of disc degeneration and MRI signal. Along this line Purmessur *et al.* (2013) [115] investigated a role of TNFα, which is known to alert cells as a pro-inflammatory cytokine initiator of degenerative disease. They found that a 7-day incubation period with elevated levels of recombinant TNFα (200 ng/mL) induced a non-recoverable catabolic shift under static loading conditions [115], although it was noted that physiological loading may have reduced these effects. Gawri *et al.* (2014) [109] could also demonstrate that there is a connection between matrix homeostasis and the applied compressive loading. When they digested bovine coccygeal IVD with trypsin and did not apply any loading the matrix break-down was very high with a decrease of the proteoglycan content. However, with the application of physiological loading the matrix-breakdown could be recovered, which confirmed that some physiological loading is beneficial for intervertebral disc culture and homeostasis. It is likely that this dynamic physiological loading directly stimulates anabolic metabolism of the cells while also enhanced convective transport of large metabolites into the disc.

## 7. Mechanical Disc Degeneration Models

Beside enzymatic break-down there is also the possibility to induce mechanical damage to the IVD. This is often performed by creating an annular defect. Bovine coccygeal disc organ cultures showed localized cell death and altered disc mechanics when punctured with large and small needles [116]. A 22-Gauge needle puncture showed increased signs of degeneration when combined with high intensity, or 'degenerative' loading conditions. This was demonstrated by increased cell death and elevated MMP gene expression in punctured IVD and was also associated with enhanced MSC migration into the tissue [74]. Organ cultures have also been utilized to investigate new AF repair strategies. Injection of platelet-rich plasma stimulated ECM synthesis in a bovine AF defect model [94]. A bovine annulotomy model was used to investigate a combined cell and biomaterial approach for AF rupture repair. Implantation of MSC-seeded scaffolds with a sutured membrane could preserve the disc height and prevent NP protrusion under bioreactor guided mechanical load

[94]. Modified fibrin was also able to seal the AF defects and restore disc height following substantial compressive loading in a one week organ culture experiment [86].

An alternative access route, the transpedicular approach, has been proposed rather than injection of enzymes or cells through the outer and inner annulus fibrosus into the center of the IVD [117]. This approach involved the penetration of the vertebral body and the cartilaginous endplate. This approach has been tested *in vitro* in bovine coccygeal organ culture nucleotomy models. In particular, this endplate approach has been valuable and successful for testing the survival and differentiation of NP cells or MSCs encapsulated in various injectable hydrogels and implanted into partially nucleotomized discs. Examples include a thermo-reversible HA-pNIPAM hydrogel [79,118] and a biomimetic fibrinhyaluronan hydrogel for NP regeneration [119]. The advantage of the endplate approach is that the AF remains intact, minimizing the risk for leakage of injected cells or hydrogels under mechanical load, provided that the endplate is properly sealed, although the clinical translation of endplate routes for delivery of therapies is substantially more complicated.

## 8. ORGAN CULTURE & ENDPLATE FRACTURE MODEL

In the case of trauma, endplate damage has been proposed as a major cause for subsequent IVD degeneration. Dudli *et al.* (2011; 2014a; 2014b) [35,47,120] cultured rabbit IVD explants from the thoracolumbar region and analyzed the biological consequences of high-energy burst fractures. In these studies, impact energy was delivered by a dropped weight. A threshold impact energy value of approx. 0.75J was found to produce bone fractures in 50% of specimens. *In vitro* culture of rabbit IVDs following impact demonstrated that a fracture is needed in the BEP was required to induce degeneration and inflammation; an equi-energetic impact but without burst fracture yielded a significantly milder degenerative response [120]. Subsequent studies with this model have shown that nuclear depressurisation alone is not the mechanism for post-traum degeneration, pointing towards an immune-regulated response [35], and that degeneration persists also in the presence of post-traumatic dynamic loading [47]. Recently, injury-loaded IVDs by Alkhatib *et al.* (2014) [121] were able to induce acute mechanical trauma and to cause the catabolic cascade of disc degeneration. Thus, there is evidence that the CEP is of key importance to maintain a healthy disc and more investigations should be undertaken to better understand the relevance of disc injury/trauma for accelerated disc degeneration and the development of pain.

## 9. ORGAN CULTURE INVOLVING HUMAN IVDS

Recently, human lumbar IVD organ culture was established by Gawri *et al.* (2011) [57] and by Walter *et al.* (2014) [29]. Both groups managed to keep cells throughout the discs viable and metabolically active for more than 4 weeks. The usage of human cadaveric IVDs has to be considered of high clinical relevance and directly translational to human disease. Important advantages compared to other model systems are that the discs come with naturally occurring degeneration of varying grade, and they have a cell and matrix composition directly comparable to live human patients. As in other *ex vivo* model systems the effect of loading, inflammatory environment and nutritional status can be manipulated,

allowing the effect of these factors to be factored into the regenerative potential of various treatment modalities. As explained earlier the upright position of the spine is unique for the human and cannot easily be reproduced in any animal model [12]. Of course, the culture of cadaveric human IVD requires the collaboration and the permissive law of transplantation since IVD should be obtained within 24h postmortem for successful organ culture. Two isolation methods have been used for the culture of human IVDs Walter *et al.* (2014) [29] applied a preparation method leaving a thin layer of BEP attached. A jet lavage system was used to clean the trabecular bone to enable nutrient diffusion. This method has been proven to be successful also for bovine coccygeal IVD [29,40]. Gawri *et al.* (2011) [57] removed the calcified parts of the endplates leaving a disc with intact CEP. In this case no further manipulation is necessary to enable sufficient nutrient supply. Discs isolated using this method have been shown to survive for a up to 4 months in culture enabling long term studies to be conducted. More recently, such an *in vitro* cadaveric human IVD culture has been used to investigate the effect of peptide therapeutic treatment. The injection of short peptide sequences could be much more straightforward approach to be clinically feasible than the more risky methods involving autologous differentiated or progenitor cells. Here, Link N, a peptide sequence of Link protein, has been demonstrated to be a possible target for direct drug delivery [57,80]. Recent work by Mwale *et al.* (2014) [80] demonstrated that a single injection of link-N could increase proteoglycan production in the NP region of degenerated IVDs in organ culture. The same effect was observed when isolated cells were exposed to the peptide [122].

## 10. CONCLUSION

We conclude that organ culture of IVDs is highly appealing for the study of the interplay of mechanical forces and biological responses. IVD organ culture bioreactors are mainly force-controlled devices that are able to mimic the natural forces, so far mainly in uni-axial compression. There are small- and large animal, as well as cadaveric human IVD models. Investigations requiring simulation in a large, nutritionally compromised IVD can be modelled using organ cultures of IVDs that are large in size. The characteristic dimension of interest is the sagittal disc height from CEP-disc-CEP, which determines the number of transport rate of nutrients and metabolites and ultimately has a profound effect on the density of live cells in a IVDs [9,99]. Bovine coccygeal IVDs are appealing to meet this constraint because of their large IVD height. Physiological loading on IVDs from all regions, including coccygeal IVDs, are dominated by muscle contraction forces, and result in similar resting pressure as in the IVDs. However, peak forces are lower and ranges of motion are greater in caudal IVDs than lumbar IVDs so that caution should be exercised used when designing experiments and interpreting results. With this limitation, however, cell therapy using allogenic or autologous mesenchymal stem cells, injection of peptides such as link N, and injection of biomaterial hydrogels can be tested *in vitro* in an environment with many similarities to human IVDs. Enzymatic matrix break-down, addition of key inflammatory cytokines or mechanical damage have been investigated as IVD degeneration models to better simulate the diseased human IVD state. These 3D organ culture models bridge basic science and translational medicine and allow *in vitro* experiments with substantial control while maintaining cells in a native tissue

microenvironment that approaches the in vivo state more closely than other monolayer and 3D culture environments can achieve.

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## LIST OF ABBREVIATIONS

<b>AF</b>	Annulus fibrosus
<b>BEP</b>	Bony endplate (bone form vertebral body)
<b>CEP</b>	Cartilaginous Endplate
<b>DoF</b>	Degree of freedom
<b>EP</b>	Endplate
<b>IVD</b>	intervertebral disc
<b>Link N</b>	short peptide sequence, which bridges the hyaluronic acid with the polysaccharide chains in the aggrecan molecule, naturally occurring peptide that can stimulate proteoglycan synthesis
<b>MSC</b>	mesenchymal stem cells
<b>MRI</b>	Magnetic resonance imaging
<b>NEP</b>	No endplates, i.e. detachment of cartilaginous endplates and free-swelling condition
<b>NP</b>	Nucleus pulposus
<b>pNIPAM</b>	polyamide poly-N-isopropylacrylamide
<b>POM</b>	Polyoxymethylene
<b>ULTEM®</b>	polyetherimide, which is an amorphous thermoplastic polyetherimide (PEI) material which combines exceptional mechanical, thermal, and electrical properties

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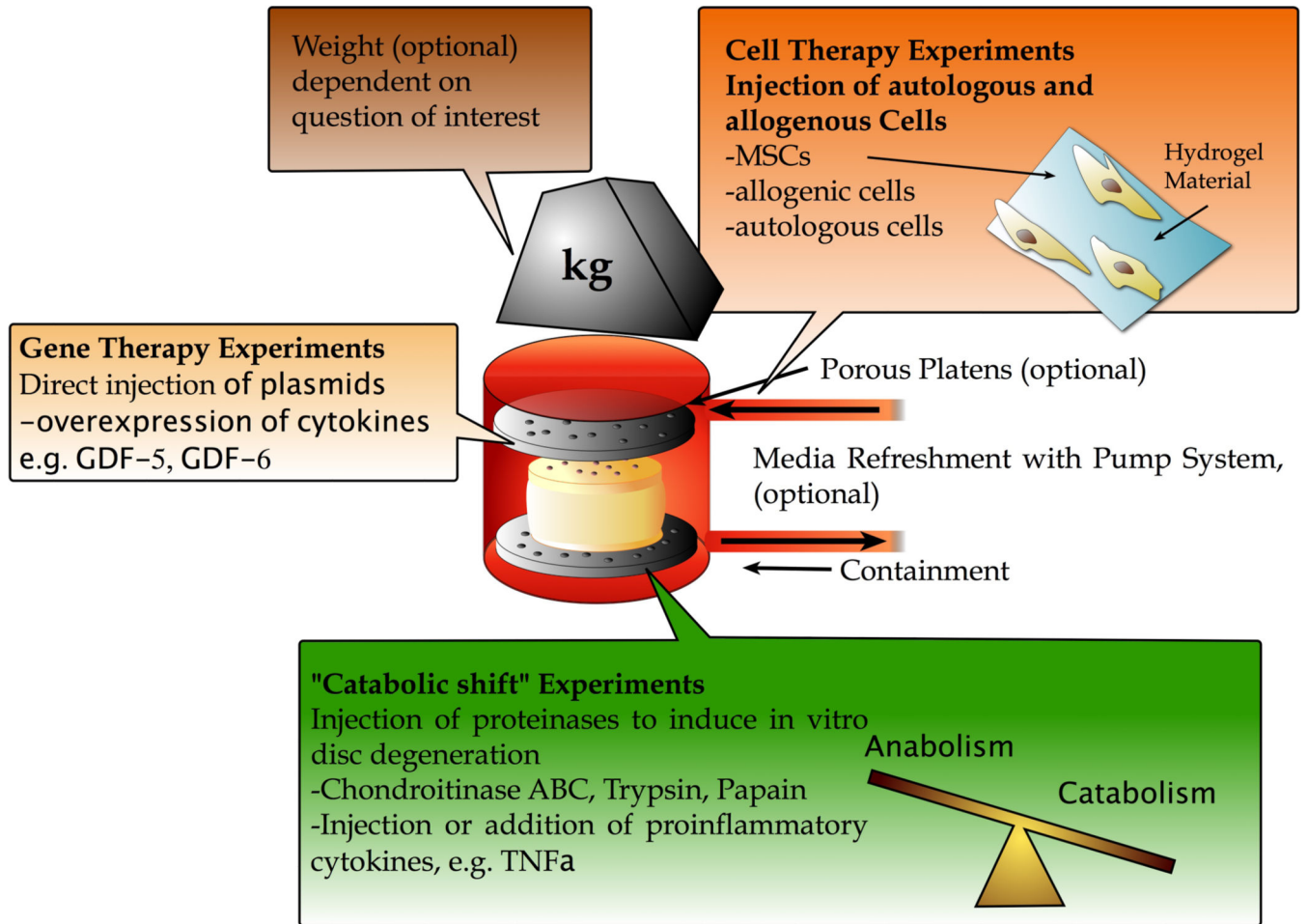
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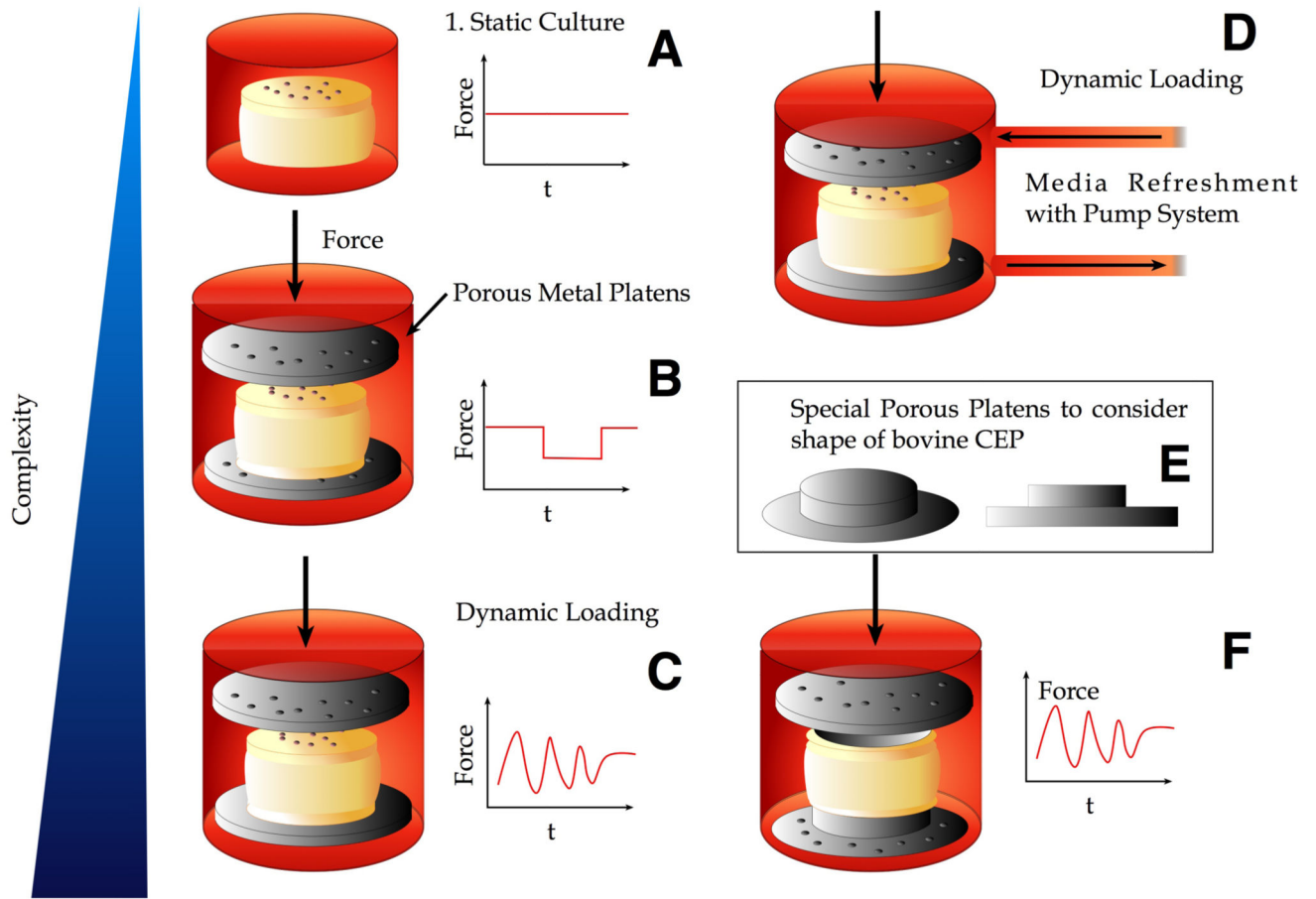


**Disc organ culture: current trend for studying disc degeneration and regeneration**

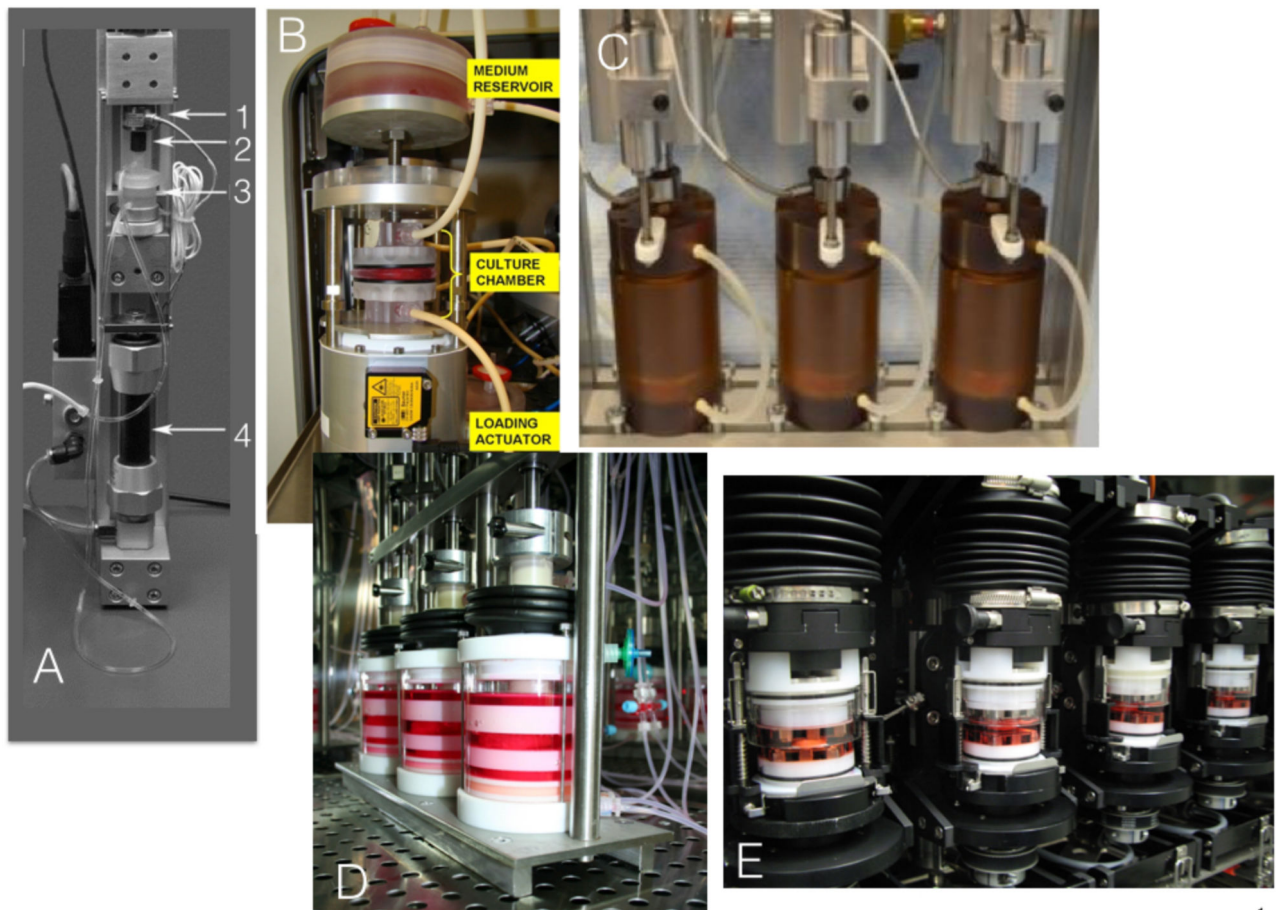


**Figure 1.** General set-up of a loading chamber and overview of current main questions addressed for degeneration and regenerative approaches. The material of the containment is ideally glass or cytocompatible biomaterial that can be cleaned and sterilized easily. A media refreshment pump and media tubing can help to maintain cell viability longer. Loading of the disc can be achieved by application of uni-axial compression or complex multiple degree of freedom loading.





**Figure 2. The evolution of intervertebral disc organ culture systems. From relatively simple designs with “free-swelling static” to dynamically loaded systems including media refreshment**  
**A.** free-swelling organ culture in a simple plastic beaker. **B.** Organ culture using porous platens and uni-axial compression. Possibility to apply diurnal loading by using defined weights on top of the culture system. **C.** Cyclic compression system allowing for frequency control . **D.** Cyclic compression and media refreshment system using a peristaltic pump. **E.** Inlet: Adjusted platens to adapt the concave shape of the CEP in the case of the coccygeal bovine IVD. **F.** Culture chamber fitting the special porous platens.



**Figure 3. Five examples of Bioreactors recently published by different research teams**

A. Polycarbonate chamber with gas-permeable silicon membrane [36,37]. Key feature: media refreshment system with tubing; culture of IVD with BEP, a steel ball in the axis to ensure parallel and even force transition. 1. Force cell, 2. Coupling with steel ball, 3. Polycarbonate culture chamber, 4. Pneumatic actuator “fluidic muscle”. B. Polycarbonate culture system to allow IVD culture with BEP; key feature: perfusion of culture media through large medium containers [31]. C. Key feature: high force hydraulically actuated bioreactor, Instrumented with load cells and inductive way sensors to allow measurements of IVD height and mechanics throughout testing and culture, chambers are made from Ultem® [29]. D. Glass and Polyoxymethylen (POM) press fit design with uniaxial compression. key feature: specialized adjustments to allow bovine and human IVD culture [33]. E. Glass and POM bioreactor [32]; key feature: presence of serrated titanium plates to allow torsion and compression, special release mechanism to hook and un-hook the bioreactor stations.

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Table 1

## Overview of Intervertebral Disc Organ Culture Systems.

Loading	Preparation of discs	Model	Media Refreshment	Culture System	Findings	Reference
<b>Unloaded systems (free-swelling)</b>						
Unloaded, free swelling	Embedding of disc in alginate gel	Rabbit	Static culture	Culture well (standard lab plastics)	↑ proteoglycan synthesis and maintained a higher content of extracellular matrix components	[55]
No loading, free swelling	With CEP,	Bovine coccygeal, Human cadaveric IVD	Static culture	Sterile plastic beaker culture	Injection of LINK N and or MSCs	[122]
<b>Mechanically loaded systems</b>						
Static loading 0, 0.2, 0.4, 0.8, and 1 MPa	With CEP	Mouse lumbar	Static culture	Culture bottles (glass)	Apoptosis was absent in discs without load, but was particularly ↑ noticeable in loaded discs (load weight, 1.0 MPa).	[123]
Static loading with ~0.4MPa	NEP	Bovine coccygeal	Pump	PEEK reactor, media refreshment with pumping	↑ Cell survival without CEP good, keeping CEP and BEP very ↓ cell viability	[54]
Papain injection free-swelling and then static loading with ~0.4MPa, injection of pNIPAM material and with MSCs	with BEP	Bovine coccygeal	Static culture	Glass/POM chamber	MSCs differentiated toward IVD cells, cell viability of MSCs stayed high, hydrogel volume was drastically reduced	[78]
Diurnal loading, 0.2MPa for 16h and 0.8MPa for 8h	with BEP	Ovine	Pump	Polycarbonate	day 7 loading, diurnal superior over static, ↑ cell viability, ↑ diffusion	[36]
Cyclic compression unloaded (UL) or loaded (L) (0.1MPa for 16h/day and 2 intervals for 2h/day at 0.1Hz with 0.3MPa ± 0.1MPa	with CEP	Bovine, Ovine, Mouse	Pump or static	Custom-made glass/POM reactor, pressfit design	The discs were cultured for 14 days ↑ cell survival with CEP than with BEP	[33,34,109]
Cyclic compression, static loading, simulated physiological loading, (SPL = -0.1MPa for 16h/day, high 3× 30min/day blocks of dynamic loading from 0.1-0.6MPa, compared to high dynamic and high static loading.	with BEP (including CEP)	Bovine coccygeal, Ovine	Pump or static	Custom-made polycarbonate reactor	The ↑ cell viability with CEP than with bony endplate ↑ cell viability if	[45,98,124]
Cyclic compression, static loading, simulated physiological loading, (SPL = -0.1MPa for 16h/day high, 3×	with BEP (including CEP)	Caprine	Pump	Custom-made polycarbonate reactor	The ↑ cell viability with CEP than with bony endplate	[7,31,49-51]

Loading	Preparation of discs	Model	Media Refreshment	Culture System	Findings	Reference
30min/day blocks of dynamic loading from 0.1-0.6MPa, compared to high dynamic and high static loading.					↑ cell viability if	
Cyclic compression physiological loading (0.2MPa for 16h/day, 0.6MPa±0.2MPa for 3×2h/day with 2h resting/day, test of low and high frequency, i.e. 0.2 vs 5 Hz	With BEP	Ovine, Bovine coccygeal	Pump or static	Custom-made polycarbonate reactor	Cell survival depends on loading frequency and nutrition, synergistic effects of nutrition and frequency causes ↓ cell viability	[37,38,125]
Complex cyclic torsio -compression loading, i.e. 0.6±0.1MPa for 8h and 16h free-swelling or low load 0.2MPa	With BEP	Bovine	Static	Custom-made glass/POM reactor	↓ cell survival with extended complex loading in NP but not in AF (≠twisting, combined compression and torsion	[32,98]
Cyclic compression 0.1Hz 0.4-0.6 MPa for 8h, recovery at 0.2 Mpa for 16h	With BEP	Human cadaveric IVD	Pump	Custom-made chambers, made of Ultem	System is capable of monitoring IVD height and mechanics during experiment	[29]
Cyclic compression, 1 Hz, 1 MPa, 2500 cycles per day	With hemi-vertebrae	Rabbit	Static	Loading inserts in Falcon tubes, 6-station system mounted in Instron dynamic testing frame	Persistent degenerative changes after spinal trauma with dynamic loading	[47]
<b>Osmotically adjusted systems</b>						
Osmotically adjusted with salt to iso-osmotic media (335 mosmol/kg) or were diurnally exposed for 8 hours to hyper-osmotic conditions (485 mosmol/kg)	with BEP	Rabbit	static	well-plate design free-swelling or with osmotic pressure	↑ cell viability for osmotically loaded IVDs compared to iso-tonic IVDs	[60,126,127]
Osmotically adjusted using PEG and dialysis membrane ((430 mOsm/kg); standard medium 8.2% w/v PEG; Hypertonic to native in situ NP (570 mOsm/kg	NP explants	Bovine	static	Culture well , standard lab plastics	Cultured for up to 42 days, ↑ cell viability, ↑ GAG/DNA ratio of hypertonic medium.	[58,128]
Osmolality adjusted by increasing NaCl to 410 mOsm/kg	IVD explants	Rat	static	Culture well, standard lab plastics	Biochemical and molecular assays reveal viable and functional nucleus pulposus cells; TGF-beta1, TGF-beta3 elevated the expression of critical matrix genes, ↑ GAG/DNA ratio	[129,130]

**Table 2**

Summary of enzymatic and mechanical disc degeneration (= “catabolic shift”) experiments.

Stress factor	Organ Culture	Animal Model	Outcome	Reference
<b>Enzymatic Degeneration Models</b>				
Injection of Catabolic Enzymes: Papain	BEP	Bovine coccygeal	↑ Injection of MSC, cell survival was improved in papain digested IVDs, MSC differentiated toward IVD phenotype	[77,78,91,108]
Injection of Catabolic Enzymes: Trypsin	BEP	Bovine coccygeal	↑ cell viability for osmotically loaded IVDs compared to isotonic IVDs	[33,80,108,109]
Injection of HTRA1, MMP3 and ADAMTS4	BEP	Bovine coccygeal	No major cavity formed, no major changes in disc matrix observed in histology	[110]
Injection of chondroitinase ABC	BEP	Coat, rabbit, rat coccygeal	Altered mechanical loading, secondary to biochemical changes in the NP	[106,107,131,132]
Injection of proinflammatory Cytokines, TNF $\alpha$	BEP	Bovine coccygeal	Addition of TNF $\alpha$ resulted in a catabolic shift, ↑ MMPs, ↑ ADAMTses	[115]
<b>Mechanical Injury Models</b>				
Disc endplate fracture model, customized droptower to induce burst fractures in BEP	BEP, preserving 1/3 of a vertebral body	Rabbit	induced IVD degeneration much higher in burst fractures, ↑apoptosis, ↑MMPs, ↑interleukins in burst fractures, ↑LDH activity released to media	[35,47,120]
Single-ramp compression consistently cracks cartilaginous CEPs of healthy human IVDs, 5 % (non-injured) or 30 % (injured) strain	CEP	Bovine coccygeal	Mechanical injury resulted in about 40-50 % cell death viability in the NP, ↑Apoptosis, ↑inflammatory cytokines, ↑MMPS, ↑ADAMTses	[121]
Needle-stab or stab incision induced IVD degeneration	BEP	Bovine coccygeal	Needle stab models express ↑nitrite oxide (factor for stress), ↑LDH, stressed IVD releases factors that attract homing of MSCs	[74,87,116,133-135]