In-vitro models of disc degeneration – A review of methods and clinical relevance

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Abstract

The intervertebral disc (IVD) provides flexibility, acts as a shock absorber, and transmits load. Degeneration of the IVD includes alterations in the biomechanics, extracellular matrix (ECM), and cellular activity. These changes are not always perceived, however, IVD degeneration can lead to severe health problems including long-term disability. To understand the pathogenesis of IVD degeneration and suitable testing methods for emerging treatments and therapies, this review documents in-vitro models of IVD degeneration including physical disruption, hyperphysiological loading, ECM degradation by enzyme digestion, or a combination of these methods. This paper reviews and critically analyses the models of degeneration published since the year 2000 in either in human or animal specimens. The results are categorised in terms of the IVD biomechanics, physical attributes, ECM composition, tissue damage and cellularity to evaluate the models with respect to natural human degeneration, and to provide recommendations for clinically relevant models for the various stages of degeneration. There is no one model that replicates the wide range of degenerative changes that occur as part of normal degeneration. However, cyclic overloading replicates many aspects of degeneration, with the advantage of a dose-response allowing the tuning of damage initiated. Models of severe degeneration are currently lacking, but there is potential that combining cyclic overloading and enzymatic digestion will provide model that closely resembles human IVD degeneration. This will provide an effective way to investigate the effects of severe degeneration, and the evaluation of treatments for the IVD, which would generally be indicated at this advanced stage of degeneration.

1. Introduction

The function of the human spine is to support and transmit loads, protect neural structures, and stabilise the human posture and motion (Oxland, 2016). The complex structure comprises vertebral bones connected via intervertebral discs (IVDs) and articular facet joints, with additional stability provided through ligaments and an extensive musculature (Adams and Dolan, 2005; Shapiro and Risbud, 2014). Degenerative disc disease (DDD) is a leading cause of low back pain (LBP) (Iatridis et al., 2013; Vergroesen et al., 2015), which is a worldwide burden for healthcare systems, not only for the number of patients with this condition but also the high costs associated with treatments and therapies (Belfiore et al., 2018; Whatley and Wen, 2012).

IVD degeneration is a multi-factorial process that involves alterations in biomechanics, the structure of the extracellular matrix (ECM), and genetic and cellular activity (Daly et al., 2016; Vergroesen et al., 2015) (Figure 1). IVD degeneration is often asymptomatic, whereas DDD can be defined as when IVD degeneration progresses to being symptomatic, for example, through pain resulting from nerve ingrowth into the IVD (Freemont et al., 1997). Although IVD degeneration is extremely common, and some level of degeneration occurs as part of the ageing process, the aetiology of DDD remains unclear (Kushchayev et al., 2018; Urban and Roberts, 2003; van Dieën et al., 1999), and this leads to a huge challenge in the development of treatment strategies to mitigate, repair or regenerate the damaged IVD.

Ageing leads to notochordal cells in the nucleus pulposus (NP) of the IVD being gradually replaced by chondrocyte-like cells, and this is accompanied by a change in the NP from vacuolated to fibrocartilaginous tissue (Kim et al., 2003). Following this, the CEPs gradually become thinner and more calcified. As the IVD is predominantly an avascular structure, the transport of nutrients occurs via diffusion and convective transport through the annulus fibrosus (AF) and the CEPs, which is affected by loading and recovery relating to daily activities and the diurnal cycle (De Geer, 2018; Ferguson et al., 2004; Gullbrand et al., 2015;

Urban et al., 2004; Zhu et al., 2012). Ageing and degeneration can negatively impact the nutrient supply, which may decrease cell activity and cause cell death (Adams and Roughley, 2006), causing further compromise in the IVD structure, ultimately leading to a degenerative cascade (Huang et al., 2014; Urban et al., 2004).

Degeneration of the IVD is also characterised by a loss of proteoglycans (PGs) and alterations in the collagen chains, which can reduce the water-binding capacity (Vergroesen et al., 2015), and lead to a reduction in the intradiscal pressure (IDP). In the early stages of degeneration the IDP decreases slightly, but more severe degeneration leads to drastic reductions (Stefanakis et al., 2014), which can increase the deformation of the AF, leading to tears, delamination and fissures (Adams and Roughley, 2006). Additionally, the loss in water content can result in a reduction in the IVD height (approximately 3% per year) (Adams and Dolan, 2012), which can compromise the load-carrying ability of the IVD, and load transfer through the spinal column, leading to degeneration in structures such as the facet joints.

As degeneration progresses, the stability and stiffness of the spinal segments are affected (Maquer et al., 2014). Measurements carried out in human degenerated IVDs (graded with Pfirrmann (Emanuel et al., 2015), Thompson (Amin et al., 2016), Otsu (Maquer et al., 2014) and Boos (Boos et al., 2002) methods) have shown that mild and moderate IVD degeneration leads to a decrease in stiffness, whereas severe degeneration in which bone-to-bone contact can occur, leads to an increase in stiffness (Emanuel et al., 2015).

A variety of methods have been used to promote and replicate the above aspects of IVD degeneration to provide a greater understanding of the mechanisms of degeneration, and for the pre-clinical evaluation of novel treatments and therapies, which may be designed to eliminate pain, and restore function and biomechanics. The use of in-vitro models can be advantageous compared to in-vivo models because of (1) reduced experimentation time, (2) greater cost-effectiveness, (3) greater control of experimental conditions, and (4) ethical considerations (An and Masuda, 2006; Daly et al., 2016).

In-vitro models of disc degeneration can be broadly defined under three experimental schemes: mechanical; biochemical; and hybrid (Figure 2). Mechanical models involve a direct disruption to the IVD (Elliott et al., 2008; Korecki et al., 2008a; Michalek and Iatridis, 2012) or the application of loading to induce damage (Adams et al., 2000; Berger-Roscher et al., 2017; Wade et al., 2014; Wilke et al., 2016). Biochemical models employ digestive enzymes or similar chemical agents to replicate the degradation of the ECM that occurs as part of degeneration (Chan et al., 2013; Roberts et al., 2008; Smith, 1964). Finally, hybrid models combine aspects of both mechanical and biochemical models (Gawri et al., 2014; Growney Kalaf et al., 2014).

The purpose of this review is to examine, discuss and evaluate the state-of-the-art in replicating human disc degeneration in-vitro. The review includes the methods used to compile and assess existing models of degeneration (Section 2), descriptions of the range of degenerative initiators and test protocols used in models of IVD degeneration (Section 3), discussion relating to the advantages and disadvantages, clinical relevance, and comparisons between models (Section 4), and recommendations of the most suitable models of disc degeneration, along with potential areas for future research (Section 5).

2. Methods

The reviewed papers were found in the PUBMED and Google Scholar databases between March 2020 and September 2021. The research keywords for general papers included the terms: *'disc degeneration, in-vitro, intervertebral disc, lumbar spine'.* After refining and classifying information, the following exact keywords were added to focus on mechanical models: *'(puncture OR stab), (overloading OR physiological loading OR cyclic loading OR static loading)'.* For the case of biochemical methods, these exact terms were used: *'digestive enzyme, (proteolytic OR trypsin OR papain OR collagenase)'.* 'Hybrid' methods were classified considering the degenerative initiator as the combination of mechanical and biochemical agents. To further expand the research, citations of the papers and related

articles were also tracked. Articles published after 2000 were considered in this review. Studies were included that refer to IVD herniation or injury if the methods and outcomes were similar or relevant to models of degeneration. This paper includes 51 models: 29 mechanical; 14 biochemical; and 8 hybrid. The level of degeneration achieved by the reported methods was evaluated by using a grading scheme (Table 1) based on the Thompson (Thompson et al., 1990) and Pfirrmann (Pfirrmann et al., 2001) scales, histological classification (Boos et al., 2002), literature relating to IVD degeneration (Adams and Roughley, 2006; Maquer et al., 2014) and data from in-vitro studies of IVD degeneration relating to microstructural and biomechanical characteristics (Amin et al., 2016; Emanuel et al., 2015; Stefanakis et al., 2014).

3. In-vitro models of disc degeneration

In-vitro models of disc degeneration may adopt mechanical, biochemical, or hybrid methods to replicate aspects of natural IVD degeneration (Figure 2), which can be used to provide a greater understanding of degenerative mechanisms, and assist in the development and evaluation of treatments and therapies for DDD.

3.1. Mechanical methods of IVD degeneration

In-vitro disc degeneration through mechanical initiation (Table 2) has been carried out using two main methods: (1) physical disruption; and (2) overloading (Figure 2). However, each may be altered to produce differing levels of damage, for example the needle gauge in disc puncture models, or the loading magnitude in dynamic overload models. Similarly, there are a variety of outcome measures that can be employed (Figure 2), including biomechanical measures, physical attributes, disc composition, assessment of physical damage, and parameters such as cell viability in bioreactor-based whole organ culture models.

3.1.1. Physical disruption

The basis of models using physical disruption is that by damaging components of the disc the degenerative process can be initiated. Puncturing the AF and NP with a needle (Hsieh et al., 2009; Korecki et al., 2008a; Lin et al., 2019; Liu et al., 2017; Michalek et al., 2010; Snow et al., 2018) or making a minor incision (partial annulotomy or nucleotomy) (Bostelmann et al., 2017; Chiang et al., 2011; Techens et al., 2020; Varma et al., 2018) has been shown to result in cell death, and the loss of PGs and glycosaminoglycans (GAGs) in the proximal region to the punctured zone after 6 days in a culture system (Korecki et al., 2008a). Similar findings have been reported in cultured murine IVDs 21 days after puncturing the AF with a 27G needle, with a significant reduction in PGs (11 to 5 ug/mg) and collagen (3 to 1.9 ug/mg) (Liu et al., 2017). Perforation of the endplate has also been used to initiate disc degeneration (Dudli et al., 2014), and this results in the depressurisation of the NP, and a reduction in GAG content after 28 days in culture. These results suggest that disruption of the IVD via a puncture through the AF/NP or CEP may lead to transport of PGs and collagen out of the IVD, reduce IVD hydration, alter nutritional pathways.

Mechanical tests conducted after puncturing the AF of rat, bovine, and ovine IVDs has shown a decrease in the IDP (Elliott et al., 2008), and reductions in compressive stiffness (25 %), dynamic modulus (20 %) (Korecki et al., 2008a), and neutral zone stiffness under dynamic axial compression-tension (60 %) (Elliott et al., 2008; Torre et al., 2019). Although similar outcomes are achieved in terms of IDP depressurisation and stiffness reduction when both the AF and NP are punctured, the magnitude of those changes is greater compared to puncturing of the AF alone (Bostelmann et al., 2017; Elliott et al., 2008; Torre et al., 2019). Additionally, the size of the implement used to puncture the disc is important; disc punctures with a relatively small needle (a needle diameter/disc height ratio of <0.3) do not significantly reduce disc height, compared to needles with a needle diameter/disc height ratio of 0.5-1.0, which have been reported to lead to a disc height reduction of 10-30% (Elliott et al., 2008),

and an incision and partial discectomy has been shown to reduce disc height by approximately 20% (Varma et al., 2018).

3.1.2. Loading

Overloading the disc under cyclic or static conditions have both been used to initiate disc degeneration. Uniaxial methods, designed to damage the endplates, include ramp loading (Alkhatib et al., 2014; Wade et al., 2014) and impact loading (Dudli et al., 2012). These loading methods cause CEP fracture via an increase in the intradiscal pressure, which is transferred to the CEPs and AF, with the CEP generally failing prior to the AF in axial compression. A reduction in GAGs (≈29 %) and an increase in the expression of proinflammatory cytokines associated with disc degeneration have also been reported after axial impact (causing endplate fracture) in a whole organ IVD culture model (Dudli et al., 2012). These studies suggested that endplate damage can initiate degenerative changes in the IVD, though it should be noted that the magnitude of the applied load to create such damage is generally hyperphysiological, so may not represent normal degenerative mechanisms in the absence of traumatic loading.

Axial loading applied to specimens positioned in a flexed position has been used to initiate regional damage to the IVD. Flexion of the IVD in vivo leads to compression in the anterior AF and tension in the posterior AF. The addition of compressive loading will increase the NP pressure, particularly in the posterior region as the flexion of the IVD will push fluid from the compressed anterior region to the posterior region. This combination of flexion and compression can lead to increased stress and tearing in the posterior AF. Static compression of 0.2 MPa combined with 15° flexion, which is at the general physiological limit of IVD flexion range of motion, applied for 7 days in a whole organ IVD culture model has been shown to lead regional differences (Walter et al., 2011), with the compressed side of the AF of the wedge loaded IVDs exhibiting a reduction in aggrecan content, an increase in aggregate modulus, increased apoptosis, and the cell viability was also lower on the

compressed side of the AF (13%) compared to the extended side (78%) or control IVDs (84 %). However, as the effects of wedge loading were generally limited to the more highly stressed compressed region of the IVD, alternative loading may be more suitable to replicate clinically relevant damage such as that associated with disc bulging or herniation, or to replicate degeneration across the entire IVD. This may be achieved through more dynamic loading; positioning motion segments into a flexed position (10°) and subjecting them to a slow axial ramp load (2 mm/min) has been used to initiate damage at the posterior mid-outer AF border, and a high load rate (40 mm/min) provoked endplate fracture (Wade et al., 2014).

Studies conducted under cyclic compression in the neutral (Gregory and Callaghan, 2012; Wade et al., 2015; Xing et al., 2020) and flexed postures (Wade et al., 2016; Żak and Pezowicz, 2021) have also been used to provoke IVD damage. Cyclic compression of 0.5 MPa at 1 Hz for 1.5 h a day for 7 days in a murine IVD culture model has shown to reduced cell viability in the AF compared to unloaded controls, along with a reduction in PG content, and a reduction in loaded disc height of approximately 25 % (Xing et al., 2020). These changes are consistent with mild disc degeneration, though no biomechanical testing was completed. However, similar axial loading (0.2-1 MPa at 1 Hz, or 0.2-2.5 MPa at 1 Hz) using bovine tail IVDs in a whole organ culture model for 6 days suggests that the dynamic loading does not compromise mechanical properties, disc height, water content, or PG content (Korecki et al., 2008b). Studies have shown that a change from a neutral to a flexed posture of 10° when applying cyclic compression leads to significantly increased damage (Wade et al., 2016; Wilke et al., 2016). Additionally, these studies both demonstrated that the degree of damage is dependent on the number of loading cycles and frequency. In agreement with the flexed ramp loading described above, the damage initiated from flexed cyclic loading occurred predominantly in the posterior region of the AF (Wade et al., 2016).

More complex experimental loading methods have also been designed, in which specimens have not only been subjected to compressive loading (static or cyclic) but also to conditions that simulate physiological movement such as flexion-extension (FE), lateral bending (LB)

and axial rotation (AR) (Berger-Roscher et al., 2017; Gregory and Callaghan, 2011; Wilke et al., 2016). Although significant reductions in disc height or changes in stiffness were not measured after testing porcine FSUs under both cyclic axial compression and FE loading periods, damage in the AF fibres was observed (Gregory and Callaghan, 2011). Similarly, combining flexion-extension, lateral bending and axial rotation with static axial compression caused AF and CEP damage, which eventually led to IVD herniation (Berger-Roscher et al., 2017; Wilke et al., 2016). Herniation is a consequence of annular rupture and NP tissue migration, and would generally be considered as a separate pathology to IVD degeneration (Lama et al., 2013). However, CEP damage may lead to disc degeneration (Adams et al., 2000; Dudli et al., 2012), and it is possible that such complex loading could be modified to lead to the lower levels of AF damage more representative of IVD degeneration.

3.2. Biochemical methods of IVD degeneration

For the purposes of this review, protocols where the degenerative initiator is a chemical compound introduced into the IVD are defined as biochemical models (Table 3). These agents are used to replicate the degradation of the components of the ECM during human disc degeneration.

The breakdown of collagen, PGs aggregates and GAGs of the ECM has been achieved by injecting digestive enzymes such as trypsin (Jim et al., 2011; Roberts et al., 2008; Rosenzweig et al., 2018), papain (Chan et al., 2013; Malonzo et al., 2015; Newton et al., 2018; Roberts et al., 2008), chymopapain (Chen et al., 2009), or inflammatory cytokines such as TNF-alpha (Purmessur et al., 2013) into the IVD. The severity of the resulting tissue damage depends on the concentration, exposure time, and presence of loading. Exposing bovine discs to papain (Chan et al., 2013; Malonzo et al., 2015), and porcine discs to chymopapain (Chen et al., 2009), in unloaded conditions, results in cavities in the NP. In contrast, whilst fissures in the NP were identified in specimens subjected to physiological loading during digestion using papain or trypsin, cavities were not (Alsup et al., 2017; Newton

et al., 2018; Roberts et al., 2008). The injection of trypsin and papain also reduce the compressive (≈40%) and rotational stiffness (>50%) of the IVD (Alsup et al., 2017; Chan et al., 2013; Newton et al., 2018).

Biochemical models of IVD degeneration have also employed collagenase and elastase (Antoniou et al., 2006; Barbir et al., 2011, 2010). Collagenase degrades the collagen fibres of the ECM, altering the collagen/GAG content ratio in the IVD (Antoniou et al., 2006; Barbir et al., 2010; Rustenburg et al., 2020), and causing tissue compaction, which increases the compressive stiffness by approximately 10 %. Moreover, the disc height of collagenase-treated samples was significantly reduced (≈40 %) with respect to the control group (Barbir et al., 2010). The breakdown of elastin due to the injection of elastase reduces tensile (25%) and torsional (65%) stiffness and disc height (≈30%) (Barbir et al., 2011, 2010). The loss of GAGs was also identified in samples treated with elastase, and this led to a corresponding increase in the free amine content (Barbir et al., 2010).

A bovine disc cell culture model has been used to demonstrate that the injection of TNF-alpha (inflammatory cytokine) triggers the activation of gene expressions associated with nerve ingrowth common in severe cases of IVD degeneration (Purmessur et al., 2013), which may provide a valuable model to understand how IVD degeneration may develop into DDD, and the corresponding pain that can differentiate asymptomatic IVD degeneration with DDD.

3.3. Hybrid methods of IVD degeneration

Hybrid models of disc degeneration are defined as those which combine both mechanical and biochemical initiators (Table 4). While many biochemical models introduce the degenerative compound via an injection into the IVD, we did not consider these a hybrid method unless they aimed to cause depressurisation through the needle puncture. If the injection was combined with additional initiators such as physical disruption to the IVD or mechanical loading then it was be regarded as a hybrid model.

Puncturing the disc components (AF and NP) 100 times to cause depressurisation and annular tearing, combined with the injection of an enzyme (collagenase or MMP-3) to degrade collagen and PGs has been used (Growney Kalaf et al., 2017, 2014). It was found that there was a reduction in disc height that was approximately 25 % greater in both the 0.5 % and 1.0 % collagenase groups compared to the intact group after 30k cycles, whereas the IVDs injected with PBS or MMP-3 resulted in an increase in disc height (Growney Kalaf et al., 2014). Similarly, there was an order of magnitude or greater reduction in the NP Young's modulus in the collagenase groups compared to the PBS injection group and MMP3 injection groups (0.005 % and 0.0025 % concentration). The authors concluded that the 0.5 % collagenase group provided the closest resemblance of natural IVD degeneration, with the higher concentration (1.0 % collagenase) resulting in excessive destruction of the IVD. However, the same hybrid model comprising 100 disc punctures and with an injection of 1 % collagenase was subsequently used alongside a 100 puncture model to evaluate various alginate hydrogel implants (Growney Kalaf et al., 2017). While the large variation in the results led to few statistical differences being observed, it may have been the use of an unrepresentative model of IVD degeneration that meant that the subsequent introduction of hydrogel treatments did not restore the mean Young's Modulus (15 MPa) to that of the intact IVD (23 MPa).

To overcome the severe ECM digestion and cavities in the NP caused trypsin in unloaded conditions (Jim et al., 2011), Unloaded-trypsin samples showed lower levels of GAGs when compared with loaded-trypsin samples after 7 days of testing in a whole organ IVD culture model (Gawri et al., 2014). The NP of loaded-trypsin samples have also been shown to have significantly reduced swelling pressure (≈80 % reduction) and compressive modulus (≈80 %) when compared with loaded-buffer samples (Mwale et al., 2008), and significantly greater reduction in disc height compared with intact samples (0.43 mm compared to 0.1 mm) (Varma et al., 2018). These, effects replicate important aspects of degeneration, but the addition of physiological loading has been adopted (Kuo et al., 2014; Mwale et al., 2008;

Périé et al., 2006), which replicates the same characteristics of degeneration without the cavities associated with unloaded specimens.

Hybrid models IVD organ cultures have also combined loading, a biochemical agent, and altered nutrition to provoke degeneration (Lang et al., 2018; Li et al., 2020). Overloading and a low glucose environment led to significantly greater reduction in disc height (≈20%) compared to physiologically loaded controls (≈10%) relative to the IVD height after dissection, and the overloading also led to a significant reduction in cell viability after 11 days. However, the combination of overloading, a low glucose environment, and injecting the NP with TNF-alpha, also increased proinflammatory cytokines associated with IVD degeneration, and GAG content released from the IVD into the culture medium. These results suggest that the combination of loading, culture environment, and introduction of a biochemical initiator of degeneration may allow different aspects of the degenerative process to be simulated.

4. Discussion

IVD degeneration is a complex phenomenon where the biomechanics, ECM structure, cellular activity, and nutrient supply can all be detrimentally altered, and all these aspects of are linked in what has been described as a vicious circle of IVD degeneration (Vergroesen et al., 2015). Imitating all the characteristics of degeneration in the same model is extremely challenging, and to critically assess the degenerative models described above, models were compared with the characteristics and grading of IVD degeneration observed in the clinical setting, and the morphological and biomechanical changes reported in the literature based on laboratory studies (Table 1). Linking the models of degeneration with such grading (Table 2-4) provides a clearer understanding of how different models might be used to understand degenerative mechanisms, and to evaluate treatments and therapies for DDD that may be indicated for use at different stages of the degenerative process.

A commonly used method to initiate degeneration is via one or more needle punctures, which leads to a loss in PGs and a reduction in IDP. However, the acute damage this leads to is not representative of normal degeneration. Therefore, these models cannot be easily graded according to clinical and biomechanical observations of degeneration (Table 2). The reduction in IDP in many puncture models is representative of moderate/severe degeneration (grade III-IV), and while these models have generally reported a reduction in stiffness associated with moderate degeneration (Elliott et al., 2008; Korecki et al., 2008a; Torre et al., 2019), it is often accompanied by a limited reduction in disc height even with a relatively large needle (needle diameter/disc height ratio of 0.5-1.0) (Elliott et al., 2008), which is more representative of mild degeneration (Grade II). Furthermore, aside from the localised damage at the site of the puncture, and the localised inflammation and apoptosis that this causes in organ culture or in-vivo puncture models, these models do not replicate the structural damage to the CEPs or AF of moderate/severe degeneration. Therefore, puncture models should be regarded as an injury model rather than a model of IVD degeneration. However, despite such limitations in puncture models, which may include biochemical models that puncture the IVD for the subsequent injection of a biochemical agent, they have the advantage that they can be used both in-vitro and in-vivo, which is more challenging with loading-based models. This translation from in-vitro models to in-vivo pre-clinical studies (Malonzo et al., 2015; Rosenzweig et al., 2018) is valuable in the development and evaluation of treatments for DDD (Kotani et al., 2002), and may justify the use of such models. The use of relatively small needles does avoid the depressurisation and reduction in disc height and maintains mechanical properties compared to intact IVDs. However, these models do not change water or GAG content, or any qualitative measures from histology, radiology, or MRI (Elliott et al., 2008). Therefore, they are not suitable as a degenerative model without the added introduction of a biochemical agent.

The loss in PGs, more fibrous tissue in the NP and more irregular CEPs observed in mild degeneration (Grade II) is less likely to be linked with DDD than more severe degeneration,

as there is limited interruption to the IVD structure, and though there may be a slight reduction in disc height, the stiffness is similar to healthy IVDs (Grade I). Therefore, whilst in-vitro models of mild degeneration may be valuable in the investigation of the mechanisms associated with the early stages of degeneration, they may be less useful in the evaluation of treatments for DDD.

All but two biochemical models (Barbir et al., 2011, 2010) included in this review introduced the biochemical agent via an injection. Therefore, although a large number of these models were developed without including biomechanical outcome measures such as stiffness or IDP, and whilst using small needle may limit the mechanical disruption to the IVD, it has been shown that there is slow depressurisation of the IVD following injection (Varden et al., 2019). Therefore, although some of these models were graded as mild degeneration to reflect the specific aspects of degeneration that the biochemical agent caused (Table 2), these models are generally less likely to be suitable models of mild degeneration compared to those that do not disrupt the IVD with a needle. Furthermore, the use of biochemical models without any loading has been shown to lead to cavities in the NP, which do not reflect normal IVD degeneration (Chan et al., 2013; Chen et al., 2009; Malonzo et al., 2015; Roberts et al., 2008), so the application of physiological loading should be considered for all degenerative models, even if the loading itself is not applied as an initiator of degeneration.

A degenerative loading model (0.32-0.5 MPa at 5 Hz for 2 hours a day) has been shown to result in a slight reduction in disc height, and reduced cell viability after 11 days of culture compared to physiologically loaded IVDs (Lang et al., 2018). Although this model does not lead to an increase in GAG content in the surrounding culture media, or the upregulation of proinflammatory cytokines associated with IVD degeneration that an additional injection of TNF-alpha causes, it avoids the depressurisation of the IVD that is associated with puncture and injection models. The development of this model did not undertake biomechanical analyses, but a similar loading protocol comprising combined cyclic compression at 5 Hz (Gregory and Callaghan, 2011). This loading protocol was specifically designed to provoke

herniation, by combining FE with axial compression, and even under the same FE loading with a static compressive load, herniation was observed in 4 out of 10 IVDs. Therefore, limiting a cyclic overloading to axial compression-extension (Lang et al., 2018) may provide the most suitable model for mild degeneration (Grade II).

Moderate degeneration (Grade III) is characterised by a reduction in IDP and stiffness, and further reduction in disc height compared to mild degeneration. On a microstructural level, moderate degeneration is where the CEP irregularities progress to CEP defects, and there is a loss in the demarcation of the NP-AF boundary. Low-frequency (0.5 Hz) cyclic compressive loading (1525±1425 N) combined with neutral-flexion (0-7°) has been shown to lead to annular tears and infolding of the inner AF compared to control specimens, and such loading has a dose-response, with greater disruption observed at 10,000 and 30,000 cycles compared to 5,000 cycles (Schollum et al., 2018). The low-frequency, high magnitude loading was suggested to be the reason for the occurrence of more infolding and distortions in the AF, and the variable flexion from 0-7° through the compression cycle thought to be why NP material did not penetrate into the AF compared to lower magnitude loading (1300±500 N) at a higher frequency (5 Hz) in a fixed flexion posture of 7° (Wade et al., 2016). Both these models (Schollum et al., 2018; Wade et al., 2016) have potential as models for moderate degeneration, and lead on from the suggested model for mild degeneration (Lang et al., 2018) in terms of being initiated through a cyclic loading protocol with the potential for the dose-response to be used to tune the level of degeneration as required. However, neither of these studies completed biomechanical analyses, and disc height loss may be limited (Schollum et al., 2018). The substantial disc height loss observed in moderate degeneration of the IVD is a key factor in the reduction in stiffness; the reduced water content, and the infolding of the AF mean that collagen fibres are less likely to be loaded tension under normal ranges of motion compared to a healthy IVD, and the reduction in disc height also leads less stability due to laxity in the spinal ligaments. Therefore, while the above cyclic loading protocols successfully replicate microstructural damage of moderate

degeneration, further research into the dose-response to replicate the biomechanical changes associated with moderate degeneration is required.

The use of biochemical initiators of degeneration combined with the above cyclic loading protocols may offer the potential to combine the degradation of the ECM, microstructural damage, and reduction in disc height and stiffness associated with moderate degeneration. The immersion of rat IVDs in biochemical agents (elastase, collagenase, genipin) has been shown to lead to significant changes in the biochemical content of the IVD, as well as affecting the tensile stiffness and the neutral zone under cyclic loading, and cumulative disc height under creep loading (Barbir et al., 2010), with a similar study showing that elastase immersion significantly reduces torsional stiffness (Barbir et al., 2011). However, further research is needed to understand if this immersion technique will scale-up to larger in-vitro animal models or whether it is necessary to inject the biochemical agent. The injection of papain as a degenerative initiator in a whole-organ culture model has been shown to reduce both compressive and rotational stiffness to ~50-85 % of control specimens depending on the loading frequency and papain concentration (Chan et al., 2013), but these specimens were not subject to any loading, which led to cavities in the NP. The injection of trypsin has also been shown to reduce compressive stiffness compared to an injection of a buffer, and this was similar whether specimens were unloaded or loaded (Mwale et al., 2008).

No biochemical models were identified that achieve severe (IV) or very severe (V) degeneration (Table 3). While some biochemical models, such as the introduction of collagenase, trypsin or papain in high concentration or over long time periods, will severely disrupt the IVD, the damage is not representative of normal disc degeneration. This may limit the value of such models for investigating degeneration and evaluating the efficacy of DDD treatments. A variety of the techniques discussed above have been used to create aspects of moderate to severe degeneration (Grades III-IV), including hybrid methods that combine multiple IVD punctures with biochemical injection and cyclic testing (Growney Kalaf et al., 2014), but the majority of models achieving this level of degeneration rely upon

hyperphysiological ramp loading, often in a flexed posture (Table 2). It is noteworthy that only one model was identified as achieving very severe degeneration (Grade V) and this also employs an injurious ramp load to specimens in a flexed posture (Adams et al., 2000). These high-magnitude ramp loads can cause defects and fractures in the CEPs, leading to a loss of PGs, IVD depressurisation, and a reduction in disc height, though no current models replicate the dramatic height loss of associated with severe degeneration. However, as with the puncture models discussed above, ramp and impact loading are more representative of trauma than degeneration.

More research is therefore needed to refine severe models of degeneration. There is potential that the magnitude and number of cycles in cyclic loading could be increased to induce severe degeneration. The varied levels (Grades II-IV) of degeneration that cyclic loading has been used to achieve (Table 2) (Gregory and Callaghan, 2011; Schollum et al., 2018; Wade et al., 2016), and the dose-response that such loading provides, offers promising opportunities to provide a standardised and tunable in-vitro model of degeneration. However, such developments should include both morphological and biomechanical analyses to increase their impact on the spinal and biomechanical research communities. Additionally, a hybrid model that combines cyclic loading of a magnitude sufficient to provoke CEP damage, as well as tears, delamination, and distortion of the AF, with a biochemical agent such as trypsin or papain introduced through immersion or injection via a small needle to degrade the ECM, particularly within the NP, may provide the macro- and micro-structural degeneration representative of severe degeneration, including a reduction in disc height and increase in stiffness compared to the healthy IVD.

The effect of mechanical loading on the cellular activity in the IVD remains unclear, with conflicting reports of whether loading leads to degeneration or improved disc health. This may be the result of a wide variety of loading conditions being used, methods that are challenging to replicate across laboratories due to the complexity of combining mechanical loading systems with cell culture protocols, and the potential for scaling issues between small

and large animal models. Mechanical loading affects IVD nutrition (Gullbrand et al., 2015; Zhu et al., 2012), which is an important factor in both degeneration and regeneration (De Geer, 2018; Huang et al., 2014). Therefore, it is critical that nutrition is considered in culture models investigating the effect of degeneration on cell activity and viability, and in the evaluation of regenerative therapies for DDD, which may require viable cells to be effective, or the maintenance of non-native cells through sufficient IVD nutrition.

5. Conclusion

There is no one in-vitro model that can replicate all the features and stages of human IVD degeneration. Most models included in this review achieve mild to moderate degeneration in terms of biomechanics, ECM composition, or microstructural damage. Methods that initiate degeneration with cyclic loading provide the best potential to reproduce structural failure and biomechanical changes similar those of natural degeneration, and have a dose-response that enables researchers to tune the level of degeneration (Schollum et al., 2018; Wade et al., 2016). There is potential that such loading can be combined with a biochemical agent to degrade the ECM, which can also provide a dose-response based on concentration and exposure time.

There are several areas where further research is warranted. Cyclic compression loading models frequently include flexed postures (Wade et al., 2016; Żak and Pezowicz, 2021) or cyclic flexion (Gregory and Callaghan, 2011; Schollum et al., 2018), but there is a lack of standardisation across tests., Further research into posture and combined loading, and the dose-response would be valuable, as certain magnitudes of combined compression and flexion can lead to herniation rather than degeneration (Berger-Roscher et al., 2017; Gregory and Callaghan, 2011; Wilke et al., 2016). Further research into the immersion of IVD specimens in a biochemical agent (Barbir et al., 2011, 2010) is needed to understand if it can degrade the ECM of the NP in large animal models, without the damage associated with a needle puncture to inject the agent directly into the IVD. Finally, there are limited models that

replicate the characteristics of severe degeneration without subjecting the IVD to traumatic loading. Hybrid models combining cyclic overloading and a biochemical agent have not yet been used extensively but offer potential, and more research is needed to understand if they can replicate severe and very severe degeneration, which represents the likely level of degeneration that would indicate surgical/therapeutic intervention in the clinical setting.

6. Conflicts of interest

The authors declare no conflicts of interest.

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8. References

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