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EX VIVO INTERVERTEBRAL DISC CULTURES: DEGENERATION-INDUCTION METHODS AND THEIR IMPLICATIONS FOR CLINICAL TRANSLATION

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Abstract

Because low back pain is frequently a result of intervertebral disc degeneration (IVDD), strategies to regenerate or repair the IVD are currently being investigated. Often, *ex vivo* disc cultures of non-human IVD organs or tissue explants are used that usually do not exhibit natural IVDD. Therefore, degenerative changes mimicking those reported in human IVDD need to be induced. To support researchers in selecting *ex vivo* disc cultures, a systematic search was performed for them and their potential use for studying human IVDD reviewed. Five degeneration induction categories (proinflammatory cytokines, injury/damage, degenerative loading, enzyme, and other) were identified in 129 studies across 7 species. Methods to induce degeneration are diverse and can induce mild to severe degenerative changes that progress over time, as described for human IVDD. The induced degenerative changes are model-specific and there is no "one-fits-all" IVDD induction method. Nevertheless, specific aspects of human IVDD can be well mimicked. Currently, spontaneously degenerated disc cultures from large animals capture human IVDD in most aspects. Combinatorial approaches of several induction methods using discs derived from large animals are promising to recapitulate pathological changes on several levels, such as cellular behaviour, extracellular matrix composition, and biomechanical function, and therefore better mimic human IVDD. Future disc culture setups might increase in complexity, and mimic human IVDD even better. As *ex vivo* disc cultures have the potential to reduce and even replace animal trials, especially during preclinical development, advancement of such models is highly relevant for more efficient and cost-effective clinical translation from bench-to-bedside.

Keywords: Disc culture, organ culture, explant culture, 3R, low back pain

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List of Abbreviations

2D	2-dimensional
3D	3-dimensional
3R	refine, reduce and replace
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AF	annulus fibrosus
AGE	advanced glycation end product
BMP	bone morphogenetic protein
CEP	cartilaginous endplate
chABC	chondroitinase ABC
Delamin.	delamination
DMEM	Dulbecco's modified Eagle's medium
DOF	degrees of freedom
ECM	extracellular matrix
FBS	foetal bovine serum
G	gauge
HTRA1	HtrA serine peptidase 1
IFN	interferon
IL	interleukin
ITS	insulin-transferrin-selenium
IVD	intervertebral disc
IVDD	intervertebral disc degeneration
LBP	low back pain
LPS	lipopolysaccharide
macroph.	macrophages
micrograv.	microgravity
MMP	matrix metalloproteinase
n.p.	needle perforation
NC	notochordal cell
NEAA	non-essential amino acids
NO	nitric oxide
NP	nucleus pulposus
NPC	nucleopulpocyte
nr	not reported
pen/strep	penicillin/streptomycin
PGE2	prostaglandin E2
sGAG	sulphated glycosaminoglycan
SPL	simulated physiological loading
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumour necrosis factor
TWEAK	tumour necrosis factor-like weak inducer of apoptosis
VEP	vertebral endplate

Introduction

LBP is a global health problem with a high socio-economic burden and growing prevalence (Hartvigsen *et al.*, 2018; Manchikanti *et al.*, 2014). It is frequently associated with IVDD (Ravindra *et al.*, 2018). IVDs are load-bearing joints located between the vertebral bodies of the spine. They consist of the central NP, the outer circumferential AF, and the cranial and caudal CEPs (Cramer, 2013).

IVDD causing LBP is a disease, where several interconnected and cell-mediated processes, of both mechanical and biological origin, can trigger and amplify each other and finally induce degenerative changes that progress in severity over time (Vergroesen *et al.*, 2015). Degenerative changes in the human IVD occur earlier in life than in comparable tissues such as the articular cartilage. First degenerative changes start already in the second decade of life and progress with age (Boos *et al.*, 2002; Sakai *et al.*, 2012; Urban and Roberts, 2003). The large vacuolated NC morphology is lost and smaller, non-vacuolated, chondrocyte-like NPCs become the predominant resident cells in the NP. This cellular transition is accompanied by a catabolic shift, in which NPCs actively produce proinflammatory cytokines (Johnson *et al.*, 2015; Le Maitre *et al.*, 2005) that mediate inflammatory and catabolic protein expression (Bermudez-Lekerika *et al.*, 2022; Vo *et al.*, 2013) while reducing anabolic protein expression (Fusellier *et al.*, 2020; Hunter *et al.*, 2003; Risbud and Shapiro, 2014). The resulting matrix breakdown is detectable early during degeneration by a proteoglycan reduction in the NP (Antoniou *et al.*, 1996). Together with the concomitant sGAG reduction, the fixed charge density, and the osmotic pressure decrease. As the disc still encounters the same compressive loads, dehydration occurs, resulting in loss of disc height and biomechanical function (Neidlinger-Wilke *et al.*, 2014; Salzer *et al.*, 2022; Urban and Maroudas, 1979). With ECM breakdown, tissue stiffness changes as the fluid pressure decreases while the solid stress increases (Zhou *et al.*, 2021a). Once degenerated, loading can additionally enhance IVDD, *e.g.* biomechanical forces are re-distributed from the NP towards the AF, overloading it (Adams *et al.*, 1996; Adams *et al.*, 2015). This can lead to lamellar disorganisation, *i.e.* physical damage such as radial fissures, tears, or rupture of the AF, as well as endplate damage, frequently leading to herniations *via* the AF or CEP (Adams *et al.*, 2015; Grignon *et al.*, 2000; Lama *et al.*, 2021). Vertebral body sclerosis and osteophyte formation as well as blood vessel and nerve neo-formation and ingrowth into the inner AF and NP are frequently described at later stages of degeneration (Binch *et al.*, 2015; Freemont *et al.*, 1997; Klaassen *et al.*, 2011; Luoma *et al.*, 2000; Wade *et al.*, 2020). Traumatic events can also lead to or accelerate degenerative changes (Alkhatib *et al.*, 2014), or *vice versa*, degenerated discs or parts thereof might be more prone to injury (Sitte *et al.*, 2016).

Treatments for LBP are scarce and sophisticated testing is necessary before they can be applied in the clinic. If LBP cannot be managed by conservative treatment and IVDD progresses (*e.g.* herniation or nerve impingement), surgery may become necessary but comes with considerable morbidity and questionable patient outcome (Eisenstein *et al.*, 2020). To avoid this, non-surgical therapies for IVDD are being developed, and need to be tested for their safety and effectiveness in preclinical settings. A logical route to study IVDD and potential treatments, even though laborious, is: 1) 2D *in vitro* cell cultures; 2) 3D *in vitro* cell cultures; 3) tissue and organ tests

without *ex vivo* culture (*e.g.* biomechanical evaluation with thawed discs); 4) ***ex vivo* (animal) tissue and organ derived disc cultures**; 5) small and large animal studies *in vivo* (Thorpe *et al.*, 2018). However, in many studies, the multiple possible steps between 2D cell culture and *in vivo* animal experiments are skipped (Kamali *et al.*, 2021; Thorpe *et al.*, 2018). Although *in vivo* animal studies are considered essential by the regulatory authorities for end-stage safety and proof-of-principle studies (Lee *et al.*, 2021), the 3R principles to refine, reduce and replace *in vivo* studies can be achieved by using *ex vivo* disc cultures for preclinical development.

In this review *ex vivo* disc cultures are discussed, *i.e.* IVD tissue and organ derived *ex vivo* cultures (Gantenbein *et al.*, 2015; Pfannkuche *et al.*, 2020). *Ex vivo* cultures are advantageous over *in vitro* cultures as the IVD cells are already in their natural environment with its structural integrity (McDonnell and Buckley, 2021; Thorpe *et al.*, 2018; Urban, 2002). While human disc cultures most closely mimic the situation found in patients, availability of such discs is limited. The many advantages of *ex vivo* cultures, such as a controlled laboratory environment, ethical acceptance, moderate throughput, flexibility, and comparatively low cost, compensate for their limitations like limited culture duration and the lack of a systemic response compared to animal models (Cramer *et al.*, 2021; Pfannkuche *et al.*, 2020; Tang *et al.*, 2022). The history of IVD cultures was recently extensively discussed by Pfannkuche *et al.* (2020). Previously, stable culture systems that support the culture of healthy control discs were developed, which is a prerequisite for disc cultures. For example, IVD cultures retaining VEPs from large animals failed after 1-2 d in culture and removing the CEP led to immense swelling. The limited cell survival in organ cultures was solved by rinsing the endplates, as blood clots and debris prevented nutrient supply by reducing glucose diffusion (Grant *et al.*, 2016), or by culturing with CEPs only (Chan and Gantenbein-Ritter, 2012; Jim *et al.*, 2011). Likewise, early explant cultures were proven difficult as swelling of the NP needs to be accounted for by a counterforce (Urban and Maroudas, 1981). Nowadays, *ex vivo* setup complexity can be high when *in vivo* loading situations are mimicked with dynamic bioreactor systems, *i.e.* SPL. SPL consists of one or several mechanical loading modes, such as compression, strain, or torsion (Chan *et al.*, 2011; Gantenbein *et al.*, 2015; Pfannkuche *et al.*, 2020), which has been described to support cell viability and overall cell concentration compared to no loading (Paul *et al.*, 2012). Degenerative loading occurs when SPL is exceeded (described below).

IVDD can be detected and categorised in severity. As IVDD is multifactorial and can affect all three regions of the IVD as well as the adjacent vertebrae, there is a large collection of destructive and non-destructive methods to evaluate IVDD in *ex vivo* disc cultures. All methods evaluate the composition and function of the ECM and/or the cells and their behaviour. IVDD can be scored by gross morphology, *e.g.* Thompson grading (Thompson *et al.*, 1990), histologically (Le Maitre *et al.*, 2021), or radiographically *e.g.* from X-rays (Kettler *et al.*, 2006; Wilke *et al.*, 2006) or MRI Pfirrmann (Pfirrmann *et al.*, 2001). Discs can also be evaluated biomechanically, *e.g.* by non-destructive testing (Lee *et al.*, 2021; Newell *et al.*, 2017). The biochemical composition is often studied, *e.g.* assaying sGAG content (Farnadale *et al.*, 1982). On a biomolecular level, gene, and protein expression, and immunohistology are often performed. All of the above-mentioned measurements are oftentimes accompanied by experiment-specific analysis methods. Various evaluation and grading methods are usually combined. The clinical evaluation is even more complex, as the analysis methods are limited to non-invasive techniques and degenerated discs can be asymptomatic, *i.e.* pain free.

Disc cells reside in a harsh microenvironment. With respect to modelling IVDD during preclinical studies in disc cultures, disturbed molecular transport to and from the disc has been hypothesised as a potential mechanism initiating IVDD and can also hinder regeneration (Urban *et al.*, 2004; Urban and Roberts, 2003). The lumbar IVD is the largest avascular structure of the adult human body; in the healthy disc, nerves, lymphatics, and blood vessels do not reach into the disc (Kirnaz *et al.*, 2021; Yuan *et al.*, 2009). Vital nutrients and toxic metabolites are transported almost exclusively *via* diffusion to and from the NP cells, mainly through the CEP (Holm *et al.*, 1981; Urban *et al.*, 2004; Zhu *et al.*, 2016). Calcifications of the CEP during IVDD and ageing reduce the transport surface of the CEP, which can decrease the nutrient flux (Benneker *et al.*, 2005; Nachemson *et al.*, 1970). As a result, cells in the NP and inner AF experience conditions that impede cellular survival and metabolism, as glucose concentrations and pH are low in the disc centre (Urban *et al.*, 2004).

Researchers are additionally confronted with many different animals that can act as sources of IVDs. There are many differences between the discs of animals that can influence their selection to study IVDD, such as whether they contain an NP (Bruggeman *et al.*, 2012), availability, predominant cell type in the NP (Alini *et al.*, 2008; Cappello *et al.*, 2006), geometry and diffusional distances (Fusellier *et al.*, 2020), age, cellular activity (Cappello *et al.*, 2006; Pereira *et al.*, 2014; Seguin *et al.*, 2004), and many others. Especially, NC containing discs may not be representative of the mature human disc containing smaller chondrocyte-like NPCs (Bach *et al.*, 2022). However, NC-containing models may be suitable to study the onset of NC phenotype switch to NPCs or comparisons to non-NC containing discs. While AF repair and regeneration can also be studied in NC-containing disc models, the presence of NCs and their secretome might influence AF regeneration.

Finally, as skeletally mature animals normally have healthy discs, degeneration must be induced. Nevertheless, with growing interest in disc cultures, it becomes increasingly difficult to understand the various induction methods that have become available. While inducing IVDD is frequently seen as "a means to an end", careful characterisation of the treatment effects and appropriate control groups are necessary to better understand the models and their limitations. Therefore, the aim of this review is to summarise existing methods to induce degenerative changes in non-human *ex vivo* disc cultures and compare them to human IVDD.

In this review we provide an overview of current degeneration-induction methods, so that researchers can choose the most suitable model for the specific research question in mind in future disc studies.

Materials and Methods

A systematic literature search without date restriction was performed on 01/01/2022 within PubMed and Web of Science with the following search terms: "intervertebral disc* OR nucleus pulposus OR anulus fibrosus OR annulus fibrosus OR cartilage* endplate AND *ex vivo* OR *in vitro* OR *ex situ* OR organ OR tissue OR explant AND degenerate* OR degrad*". Full text screens were performed based on the following inclusion criteria: English literature from a peer-reviewed journal with full text available, non-human IVD organ or explant without a freezing step, culture in medium for at least 24 h at 37 °C, and only if degenerative changes were studied using any method. Human disc cultures were excluded as availability of non-degenerated discs is limited which makes the induction of IVDD redundant. Quality standards were not pre-defined, *i.e.* minimal criteria of reported information to be included, as such standards are difficult to define in an objective manner. Of 4074 potential articles, 129 studies were included. Full articles were screened for culture conditions, degeneration-induction method, and resulting degenerative changes and summarised in this review.

Results and Discussion

First studies were reported in 2000 and since 2013, approximately 10 studies were reported annually (Fig. 1**a**). The median experiment duration was 10 d (range 1-56), the 25 % percentile 7 d, and the 75 % percentile 14 d (Fig. 1**b**). Discs were used in descending frequency from cows, mice, rabbits, rats, pigs, goat, and sheep (Fig. 1**c**). While early disc cultures often made use of discs derived from mice and rabbits, discs derived from other animals have only been used for the last 10 to 15 years. In recent years, cow tail discs are used most frequently. Most of the discs were from caudal (50 %) or lumbar (29 %) regions (Fig. 1**d**). Approximately 10 % of the studies were conducted with explants, one study with a mouse spine, and the IVD organ cultures either with

Table 1. Parameters to consider reporting when publishing an IVD organ or explant culture where degenerative changes were induced (where applicable and known). This table was derived from the authors' experiences and after interpretation of frequently missing information of the included articles.

Topic	Parameters to report
Organ or explant	Animal, life stage of animal, species, strain, age, weight, sex, disc level(s), NP cell type, disc geometries (*e.g.* height), harvest method, precise description of the explant/organ (*e.g.* nucleus pulposus explant, IVD with cartilage endplates (*i.e.* the vertebrae were removed up to the cartilage endplates), IVD with vertebral endplates (*i.e.* part of the vertebra on top of the cartilage endplates).
Degeneration method	Method used, tools with exact description (*e.g.* needle gauge, blade size etc.), location of treatment, number of interventions, *e.g.* stabs, *etc*., duration/ frequency of treatment, enzyme/cytokine used, dosage (*e.g.* activity in units), injection volume, media changes.
Culture conditions	Basal medium used (including catalogue number of medium and supplier), glucose concentration, adjustments (*e.g.* pH, osmolarity and concentrations), supplements (*e.g.* growth factors, foetal bovine serum, ascorbic acid, antimicrobial ingredients *etc*. and concentrations), incubator settings (oxygen, temperature, CO_2), media changes and media volume, culture chamber, mechanical loading parameters.
Control groups	Day 0 control (fresh disc, not cultured) – native healthy disc. Control (cultured without any induction) – stable disc culture. Degenerated (disc treated with degeneration induction) – effect of degeneration. Sham (degenerated disc with sham treatment) – effect of treatment method to repair or regenerate the disc. Optionally: degenerated and sham can be done together. Treatment groups according to study design.

remaining vertebral body (17 %), VEP (58 %), or CEP (14 %). Based on the results obtained, a set of basic information that can be considered for reporting *ex vivo* studies in the IVD field is proposed (Table 1).

Culture conditions

Generally, concentrations of, *e.g.* FBS, oxygen, or glucose, are expected to be lower in the core of the disc than in the surrounding medium. Most studies applied 5 % CO_2 (Fig. 2**a**). In 90 % of the studies, O_2 concentration was either nr or reported to be atmospheric, *i.e.* 20 % (Fig. 2**b**). Only a limited number of studies employed physiologic culture conditions; the IVD is the largest avascular structure of the human body and oxygen concentration *in vivo* is reduced to < 5 % (Holm *et al.*, 1981; Thorpe *et al.*, 2018; Urban *et al.*, 2004). In line with this, cellular metabolism of NPCs is best maintained at hypoxic conditions, *e.g.* 5 % O_2 (Feng *et al.*, 2018; Holm *et al.*, 1981; Horner and Urban, 2001; Neidlinger-Wilke *et al.*, 2012; Risbud *et al.*, 2006). Loading was applied in 56 % of the studies and in over 30 % of all studies dynamic axial compression was applied (Fig. 2**c**). This is not surprising, as loading, especially axial loading, is necessary for IVD health and homeostasis in *ex vivo* disc cultures (Gantenbein *et al.*, 2015; Pfannkuche *et al.*, 2020).

DMEM and DMEM/Nutrient Mixture F12 combinations were used in 89 % of the studies (Fig. 2**d**). The FBS concentration ranged from 0-20 %; 5-10 % FBS was added to more than half of the media compositions (Fig. 2**e**). The glucose concentration was nr in 49 % of the studies, while in *circa* a third of the studies 4.5 g/L glucose was used (Fig. 2**f**). This supraphysiological glucose concentration is around five times as high as normal blood glucose concentrations (Röder *et al.*, 2016). Penicillin and streptomycin were added in more than 70 % of the studies and antimycotics in *circa* 50 %. Sodium pyruvate and L-glutamine are typical components in the reported basal media compositions and 42 % of the studies added ascorbic acid (Fig. 2**g**). Cell survival is orchestrated by an interplay of [glucose], [oxygen], pH and their cellular consumption/production that can be further modulated by physical loading (Salzer *et al.*, 2023). Nutrient demand increases with higher cell density and an increasing disc size is correlated to a lower cell density with smaller rodent discs having a higher cell density compared to sparsely populated bovine discs; even more so compared to larger human discs (Urban *et al.*, 2004). Thus, cell density should be accounted for when adapting medium components.

With regard to the study design, 30 % of the studies made use of a native control, *i.e.* a freshly harvested healthy control disc before culture, whereas most studies include a sham/untreated control and/or a degenerated control. Most studies investigated regenerative therapies (Fig. 2**h**). The proposed set of basic information for reporting future disc cultures can be found in Table 1.

Methods for inducing degeneration

The studies were divided into five different methods for inducing degenerative changes in healthy IVDs: proinflammatory cytokines (31 %), injury/damage (25 %), degenerative loading (17.5 %), enzyme (15.5 %), and other (11 %) (Fig. 3). Degenerative loading was the only category that was applied to all 7 species, whereas the largest category, proinflammatory cytokines, was mainly applied to discs from small animals (Fig. 4). Due to the large variability and the small dataset for certain methods or in certain categories, caused by, *e.g.* methodology, specific research question, animal species, experiment duration, or incomplete reports, quantitative meta-analysis leading to specific suggestions and recommendations remain difficult. Nevertheless, using this review will allow the reader to funnel down the overwhelming variability of methodologies to find the most suitable method for the research question in mind.

Proinflammatory cytokines

The most frequently used cytokines which expose cells to a pro-catabolic environment are IL-1β and TNF, which have been found to be elevated in human degenerated discs (Johnson *et al.*, 2015; Le Maitre *et al.*, 2005). Note that cytokines such as TNF or IL-1β are referred to as proinflammatory due to their important role in the immune system; however, they frequently fulfil a pro-catabolic function in the disc rather than an immunological one, as they are often increased in absence of infection, *i.e.* a so-called sterile inflammation (Bermudez-Lekerika *et al.*, 2022; Krock *et al.*, 2017). Nevertheless, to distinguish them from other catabolic factors, we refer to them as proinflammatory. Cytokines or other factors stimulate downstream cellular cytokine production when injected or added, as cells adapt rapidly by producing proinflammatory factors themselves (Pelle *et al.*, 2014; Zhao *et al.*, 2020). Note that it remains unclear as to how long is the active period of cytokines and whether cytokine treatment induces a transient or a permanent switch of cellular phenotype. Therefore, they are often refreshed with every medium exchange (Du *et al.*, 2020; Kamali *et al.*, 2021). TNF has been shown to induce a cellular catabolic shift that is not recoverable (Purmessur *et al.*, 2013) and after removal, TNF was still found in the medium as it was released by the affected cells (Walter *et al.*, 2015). Nevertheless, when injected intradiscally, there appears to be a maximum effective concentration, as *e.g.* TNF did not show further degenerative changes in calf discs above an injection of 40 µL of 100 ng TNF/cm^3 tissue (Du *et al.*, 2020). Furthermore, dynamic loading should be accounted for as molecular transport of these cytokines is influenced by dynamic loading (Lang *et al.*, 2018). Preliminary data from the authors' labs (not published) furthermore indicate, that there may be differences in the effect of cytokines derived from different species and that species specific cytokines can potentially be more potent.

Therefore, species-specific cytokine variants should preferably be applied (personal communication Prof. Marianna Tryfonidou). Nevertheless, this makes the comparison of dosage between studies more complex.

The cytokines IL-1β (10-100 ng/mL), TNF (1-200 ng/mL), and TWEAK (10-300 ng/mL) were primarily studied in small NC-containing discs. In these discs, these cytokines induce a switch in cellular activity similar to that reported in humans, *i.e.* a catabolic shift with an upregulation of proinflammatory and catabolic markers with a concomitant decrease in anabolic and anti-catabolic markers. In small NC-containing discs, proteoglycan intensity was additionally frequently reduced (Ellman *et al.*, 2012a; Fujita *et al.*, 2012; Huang *et al.*, 2019; Liu *et al.*, 2021; Ni *et al.*, 2020; Ohba *et al.*, 2008; Pelle *et al.*, 2014; Takayama *et al.*, 2018; Wako *et al.*, 2007; Wako *et al.*, 2008; Wang *et al.*, 2018; Yu *et al.*, 2021; Zhao *et al.*, 2020). In rabbit IVDs, IL-1β additionally triggered apoptosis at low doses and cell death at higher doses (Duan *et al.*, 2007; Ellman *et al.*, 2012b; Kim *et al.*, 2013), whereas there were no changes in the ECM of cow AF explants but cells were metabolically more active (Neidlinger-Wilke *et al.*, 2021). Similarly, lower doses of TNF had little effect on cow IVDs, but higher doses led to matrix breakdown and even loss of the AF/NP demarcation and AF bulging after 21 d (Arkesteijn *et al.*, 2015; Du *et al.*, 2020; Lang *et al.*, 2018; Purmessur *et al.*, 2013; Walter *et al.*, 2015; Walter *et al.*, 2016). Additionally, TNF can increase IL-1β production and *vice versa* (Zhao *et al.*, 2020).

Interestingly, IL-1β and TNF combined (both 100 ng/mL), added on day 0 and on day 7 to a cow NP explant culture, had no influence on gene expression on day 3 and day 7. However, on day 14, proinflammatory and catabolic gene expression were increased, but anabolic markers and the ECM composition remained unchanged (Krupkova *et al.*,

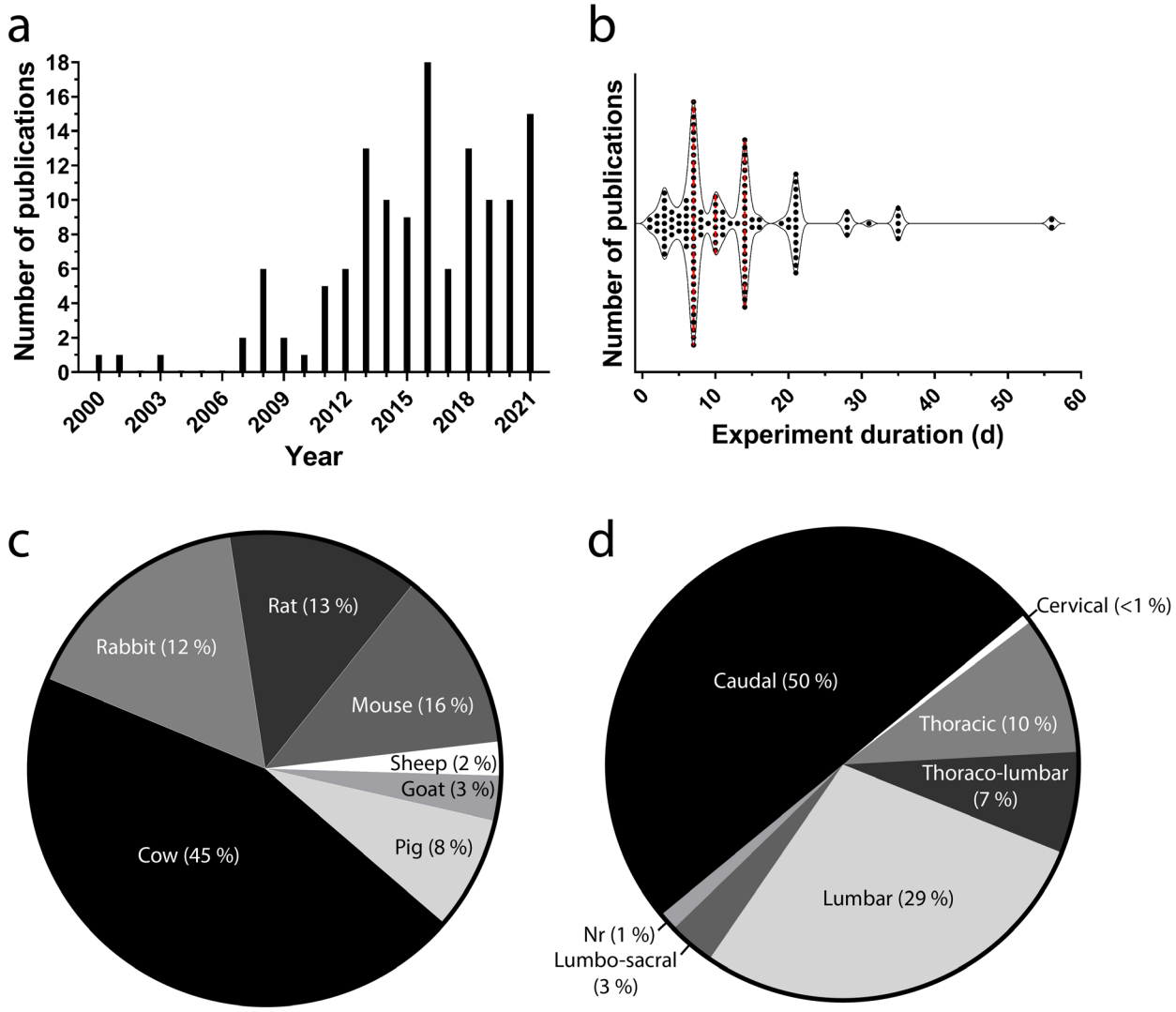

Fig. 1. Overview of *ex vivo* IVD studies where disc degeneration was induced. (**a**) After the first studies were performed in the early 2000s, there were around 5-15 studies reported each year since 2013 indicating that disc cultures are well established. (**b**) The median experiment duration was 10 d; longer durations are frequently necessary to study IVD regeneration. (**c**) Cow discs were used in almost half of the studies and are the preferred model in recent years. (**d**) Caudal and lumbar disc levels were the most frequently used disc levels.

2016). In small NC containing discs, the combination resulted in effects comparable to using the single cytokines (Huang *et al.*, 2018; Ponnappan *et al.*, 2011). More studies using discs derived from larger animals are necessary to better understand the effect of cytokine treatment. Still, both cytokines create a pro-catabolic environment and lead to cellular adaptations typically found in human cells of degenerated discs and are therefore recommended for induction of a cellular catabolic shift.

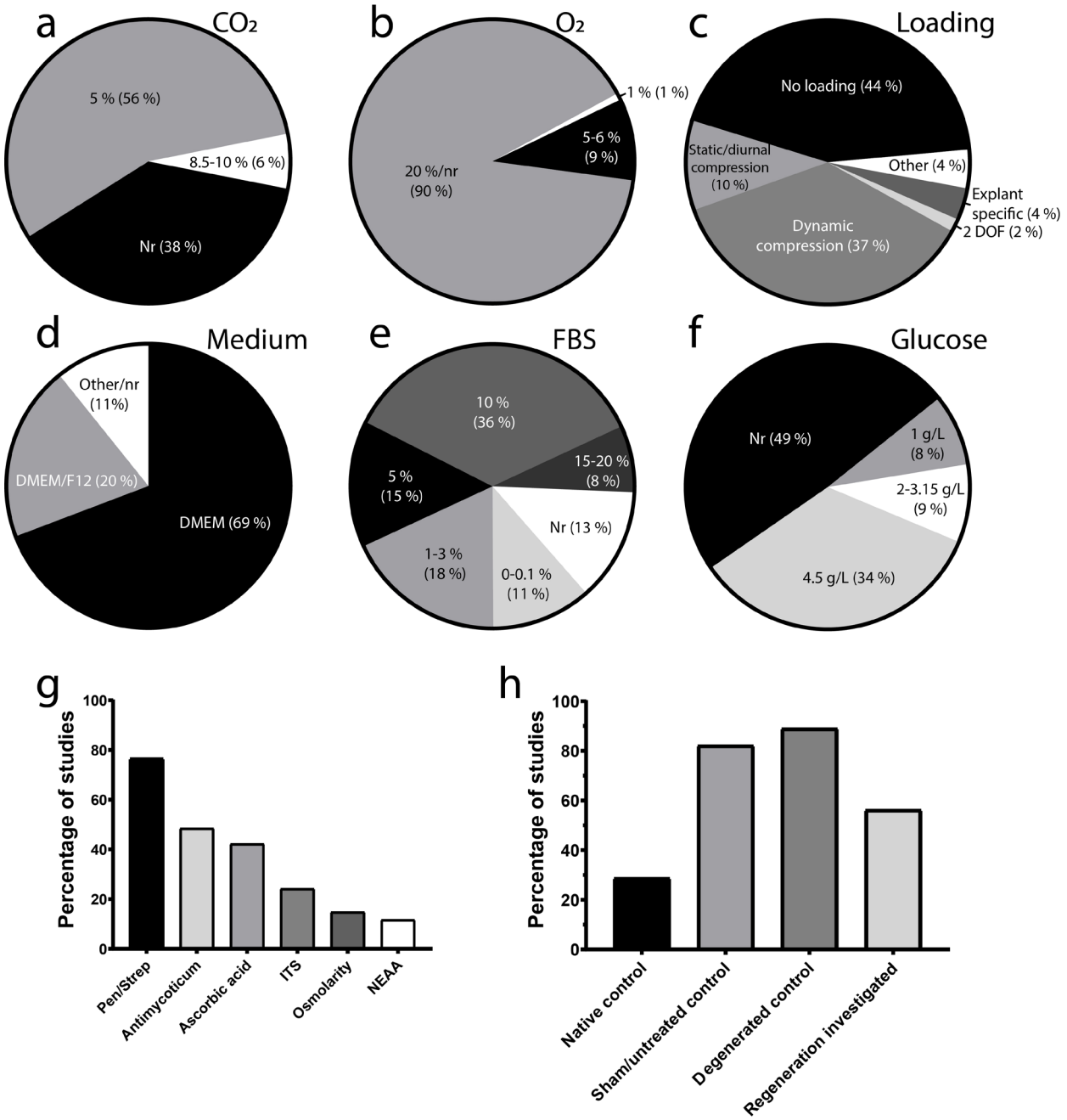

Fig. 2. Culture conditions in IVD organ and explant cultures. (**a**) Most studies applied 5 % CO_2 (**b**) and atmospheric O_2 (*i.e.* 20 %). However, there is a trend towards 5 % O_2, which better mimics the *in vivo* situation. (**c**) Most of the studies applied loading with almost no studies applying no load in recent publications; frequently dynamic axial compression was used. (**d**) The basal medium consisted in most studies of DMEM or a combination of DMEM with Nutrient Mixture F12 (F12), which was already established early in disc cultures. (**e**) A FBS concentration of 5-10 % was used in 50 % of the studies; however, it is highly dependent on the species used and several groups recently started to reduce the FBS concentration. (**f**) The most frequently mentioned glucose concentration was 4.5 g/L (*i.e.* 25 mmol/L), in half of the studies it was nr. Recently, there have been trends towards mimicking the *in vivo* situation of human discs, which have a lower glucose concentration (**g**) Almost 80 % of the studies reported the addition of pen/strep to the culture medium. The medium was frequently enriched with other components, such as ascorbic acid, ITS, or NEAA, especially when low concentrations of FBS were used. (**h**) A sham/untreated an/or a degenerated control was used in most studies and regenerative interventions were frequently studied.

Compared to IL-1β and TNF, stimulation by means of other cytokines are less frequently studied and the induction of a cellular catabolic shift is often less pronounced. Nevertheless, such models might be better suited to study the cellular development during IVDD. In mouse IVDs co-cultured with macrophages, which can release various cytokines, proteoglycan staining intensity was subsequently reduced for 2 d; however, the cellular response was not investigated (Haro *et al.*, 2000). Other cytokines such as IL-6, IFN-γ, or TGF-β (all 10 ng/mL) but also the factors TGF-β3 or BMP-2 (both 1 µg/mL) did not

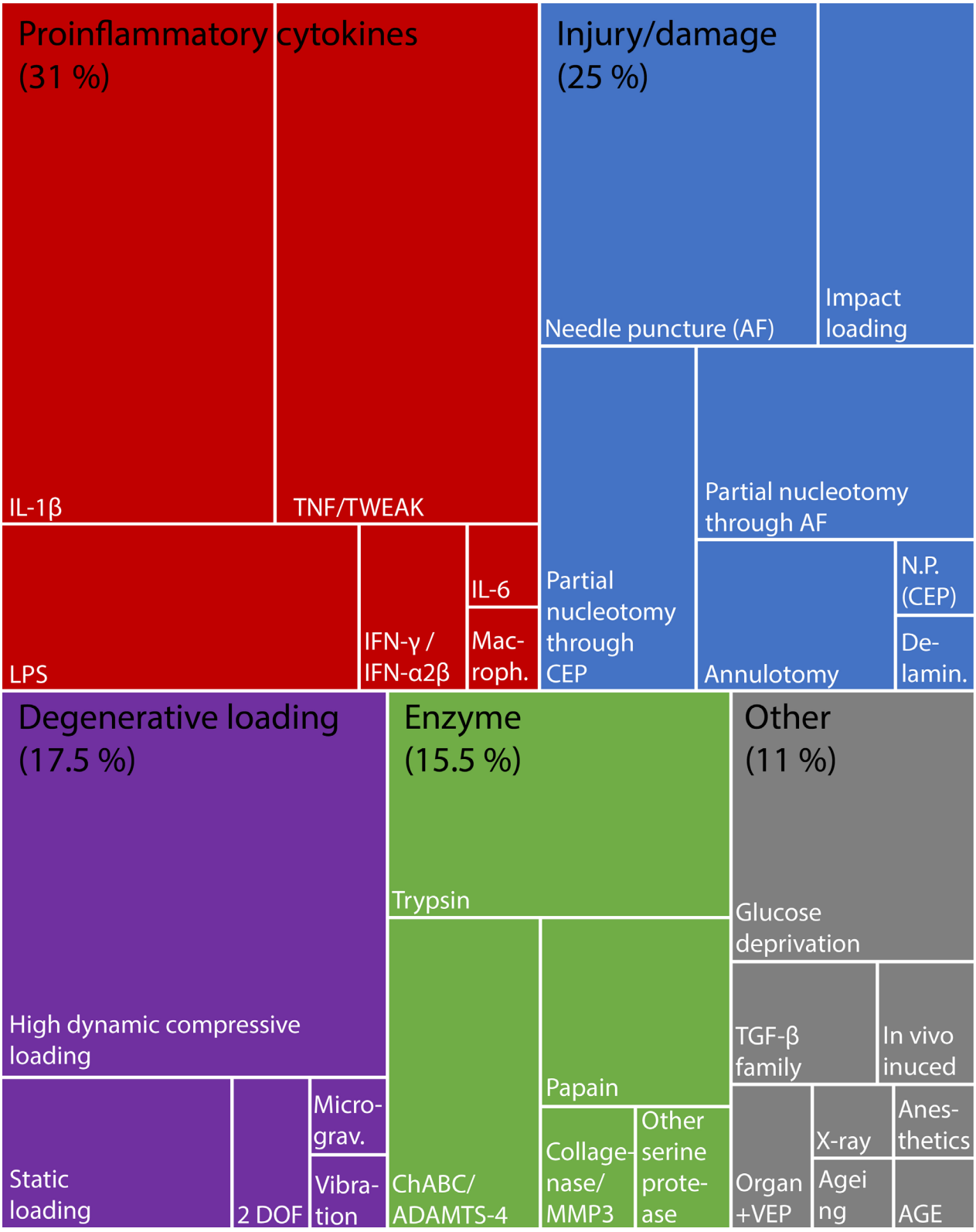

Fig. 3. A tree map of methods to induce degenerative changes in disc cultures. The 5 categories were proinflammatory cytokines (red), injury/damage (blue), degenerative loading (violet), enzyme (green), and other (grey). 2 DOF: 2 degrees of freedom (shear and compression).

induce a proinflammatory or catabolic response in mouse IVDs (Ohba *et al.*, 2008; Wako *et al.*, 2008). Of note, however, is that TGF-β3 and BMP-2 induced AF ossification (Haschtmann *et al.*, 2012). LPS is an endotoxin derived from gram-negative bacteria that binds to TLR-4, a receptor which is also present on disc cells, which triggers cytokine production (Rajan *et al.*, 2013; Teixeira *et al.*, 2016a). LPS was only used in small NC containing discs (10 ng/mL-10 μg/mL), but a catabolic switch as described by IL-1β or TNF was not reported. Furthermore, the cell viability was not affected, while ECM degradation was frequently found, with up to 50 % sGAG reduction when high doses were used. Furthermore, collagen degradation and AF cleft formation was described (Kim *et al.*, 2013; Li *et al.*, 2015a; Li *et al.*, 2015b; Li *et al.*, 2016b; Li *et al.*, 2016a; Ohba *et al.*, 2008; Wako *et al.*, 2008; Xiao *et al.*, 2019). The mechanism of ECM degradation due to LPS treatment are not well understood yet but could be caused (partially) by TLR activation. TLRs activation by alarmins (ECM fragments) or specific TLR agonists in human IVDs led to matrix degradation, protease secretion, and proinflammatory cytokine production after 28 d of culture. Nevertheless, TLR sub-type activation is species-dependent, which should be taken into account when choosing receptor agonists for new disc cultures and for translation to human discs (Krock *et al.*, 2017). Additionally, LPS might be suitable to mimic bacterial infection of the disc, *e.g.* to study Modic changes (Dudli *et al.*, 2016).

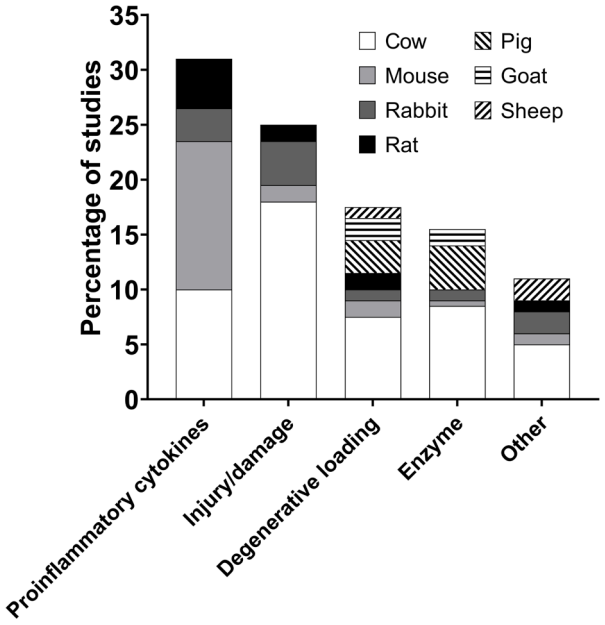

Fig. 4. Distribution of degenerative stimuli across species. The most frequently applied method to induce degenerative changes, proinflammatory cytokines, was mainly applied to discs from small animals. Injury/damage was mainly applied to cow discs. Degenerative loading is the only common category among the 7 species. Enzymatic treatment was mainly tested using discs from large animals.

Injury/damage

Various methods of physically disrupting the tissue have been used to induce IVDD, with the main advantage that they can be relatively quickly applied compared to other approaches. Frequently, scalpels or needles are used to extract or damage tissue with different degrees of severity. Additionally, impact loadings, *i.e.* a single event of high instantaneous force or displacement, have frequently been studied. Injury/damage models can damage any three regions of the disc in all possible combinations, making such induction methods especially interesting when specific structures need to stay unaffected.

All injury/damage models induce NP decompression; however, to various degrees of severity and by different mechanisms, depending on the chosen method. IVDs with NC-rich NP, *e.g.* pig discs, especially tend to easily extrude following injury. The induced NP decompression starts a degenerative cascade that progresses over time. Depending on the severity of the damage, the ECM composition and structure are affected, ranging from small reductions in proteoglycan content up to AF lamella disorganisation and cleft formation in the NP and AF. Typical effects of NP decompression are a reduction of cell viability in the NP and biomechanical changes such as decreased stiffness and height recovery, but increased creep and disc height loss (Dudli *et al.*, 2012; Dudli *et al.*, 2013; Dudli *et al.*, 2015b; Dudli *et al.*, 2015a; Frauchiger *et al.*, 2018; Guillaume *et al.*, 2015; Haschtmann *et al.*, 2008; Korecki *et al.*, 2008a; Li *et al.*, 2021; Likhitpanichkul *et al.*, 2015; Peroglio *et al.*, 2012; Pirvu *et al.*, 2015; Sun *et al.*, 2022; Teixeira *et al.*, 2016a; Zhou *et al.*, 2021b). Additionally, cells often react immediately with an increased NO release, apoptosis, oxidative stress marker release and the adaptation of their energy metabolism (Croft *et al.*, 2021; Dudli *et al.*, 2015a; Dudli *et al.*, 2015b; Li *et al.*, 2021; Sun *et al.*, 2022).

Annulotomy or annular fenestration damages the AF, but NP herniation has already been reported to occur as a result of the creation of 2 mm diameter holes in calf discs (Guillaume *et al.*, 2015; Heuer *et al.*, 2008; Likhitpanichkul *et al.*, 2015; Peroglio *et al.*, 2012; Pirvu *et al.*, 2015), similar to human discs (Wilke *et al.*, 2013; Zengerle *et al.*, 2021). Annulotomy was compared to an endplate delamination, where a 4 mm deep incision was made close to the endplate in cow discs. The delamination led to an increased expression of catabolic genes and a concomitant downregulation of anabolic genes (Alexeev *et al.*, 2020). This novel route to study herniations takes into account that most discs rupture at the endplate due to AF damage (Berger-Roscher *et al.*, 2017; Rajasekaran *et al.*, 2013; Wade *et al.*, 2022; Wilke *et al.*, 2016). Similarly, the perforation of the CEP of a rabbit IVD (18 gauge (G) needle) led to NP herniation *via* the CEP; catabolic and proinflammatory signalling was increased in the NP (Dudli *et al.*, 2013).

The most frequently applied injury model is the needle puncture *via* the AF, with needle gauges

ranging from 14 to 27 G, depending on the disc size and, as such, the species (Abraham *et al.*, 2016; Hibino and Tang, 2017; Korecki *et al.*, 2008a; Liu *et al.*, 2017; Teixeira *et al.*, 2016a; Vaudreuil *et al.*, 2019). For example, needle puncture in mouse discs (27 G) led to a reduction of proteoglycans, cell viability, and stiffness, and an increase of proinflammatory markers (Abraham *et al.*, 2016; Hibino and Tang, 2017; Liu *et al.*, 2017), but in rabbit discs (16 G) no differences in gene expression or proteoglycans were found (Vaudreuil *et al.*, 2019). In cow IVDs (14-25 G), cell viability was only reduced next to the needle track (Korecki *et al.*, 2008a; Teixeira *et al.*, 2016a).

Impact loadings of up to 50 % displacement that lead to pressures of around 35 MPa in rabbit and cow discs are commonly used to fracture the VEP through which the NP can extrude (Dudli *et al.*, 2012; Dudli *et al.*, 2013; Dudli *et al.*, 2015a; Dudli *et al.*, 2015b; Haschtmann *et al.*, 2008; Li *et al.*, 2021; Sun *et al.*, 2022; Zhou *et al.*, 2021b). Notably, anabolic markers were decreased, whereas catabolic and proinflammatory markers were increased and, all in all, gene regulation in a cow disc after insult was affected in both, NPCs (167 upregulated, 85 downregulated) and in AF cells (119 upregulated, 89 downregulated) (Cui *et al.*, 2021).

The most obvious way for NP decompression is a (partial) nucleotomy *via* the CEP (Leite Pereira *et al.*, 2018; Li *et al.*, 2014; Li *et al.*, 2016c; Li *et al.*, 2016d; Li *et al.*, 2017; Pereira *et al.*, 2014; Pereira *et al.*, 2016; Peroglio *et al.*, 2013) or AF (Illien-Jünger *et al.*, 2014; Li *et al.*, 2016d; Long *et al.*, 2018; McKee *et al.*, 2020; Naqvi *et al.*, 2019; Raines *et al.*, 2020; Richards *et al.*, 2019; Stannard *et al.*, 2015), after which the defect is frequently closed. In partial nucleotomies *via* the AF, the remaining NP often herniates during culture or even a cavity is formed in the NP, with the exception of nucleotomies *via* needles, where the decompression often leads to AF bulging indicating stress redistribution (Heuer *et al.*, 2007; Illien-Jünger *et al.*, 2014; Li *et al.*, 2016d; Long *et al.*, 2018; Naqvi *et al.*, 2019; Richards *et al.*, 2019). Therefore, if herniations need to be avoided, the induced defect needs to be small.

When damage models are combined with proinflammatory stimulation, cells frequently react by producing more proinflammatory and catabolic markers. For example, when needle puncture was combined with IL-1β treatment or LPS, proinflammatory and catabolic markers were increased, and anabolic signals were reduced in cow discs (Ferreira *et al.*, 2021; Silva *et al.*, 2019; Teixeira *et al.*, 2016b; Teixeira *et al.*, 2016a; Teixeira *et al.*, 2018). Similarly, in a combined method with annulotomy and IFN-α2β treatment, anabolic markers were decreased, whereas apoptotic and proinflammatory markers were increased in a cow disc (Kazezian *et al.*, 2016). Even though scarce, such combinatorial approaches are promising models for future disc studies to induce degenerative changes on multiple levels.

Degenerative loading

As summarised by Chan *et al* (Chan *et al.*, 2011), magnitude, duration (per day and total), and frequency are crucial factors during dynamic compressive loading. Generally, axial compressive loading above 1 Hz, for more than 1 h, or above 1 MPa may be considered as excessive mechanical loading that can induce degenerative changes, *i.e.* would constitute degenerative loading for *ex vivo* disc cultures. Nevertheless, *in vivo*, intradiscal pressures can reach magnitudes above 1 MPa in humans (Wilke *et al.*, 1999) and sheep (Reitmaier *et al.*, 2013), leading to high hydrostatic pressures (Neidlinger-Wilke *et al.*, 2006). Therefore, the complex interaction of amplitude, frequency, and duration needs to be adjusted for the specific culture setup and disc model. Especially disc geometry and correlated cell density among the different species is important to consider. For example, in young NC containing pig IVDs, intermediate loading values (≤ 0.4 MPa, ≤ 1 Hz, ≤ 4 h) are anabolic, whereas high loading parameter values (0.8-1.3 MPa, or 3-5 Hz, or > 4 h) induce a catabolic shift (Kanda *et al.*, 2021; Kurakawa *et al.*, 2015; Xu *et al.*, 2016). However, when skeletally mature NPC containing cow IVDs were loaded with up to 2.5 MPa (1 Hz, 1 h/day), no changes of IVDD were observed after 5 d (Korecki *et al.*, 2008b). Controversially, Haglund *et al.* (2011) reported reduced cell viability and increased aggrecan fragmentation when cow discs were loaded for 10 d at relatively lower loads (≥ 0.1-0.6 MPa, 0.1 Hz, 4 h/d), indicating the model and loading parameter dependent effects of compressive loading. It has been noted by several investigators (*e.g.* personal communication Dr. Sybille Grad, Prof. James Iatridis and others), that when excessive loading regimes are used to induce degeneration, that this is typically accompanied by height reductions of more than *circa* 15-20 %. Additionally, IVD height loss is frequently permanent and not restorable to the height at culture start. Therefore, relative displacement might be used as an indicator of degenerative compressive loading.

When degenerative loading is applied, cell viability is frequently reduced due to increased apoptotic activity (Illien-Jünger *et al.*, 2010; Paul *et al.*, 2013; Paul *et al.*, 2017; Xu *et al.*, 2016; Zhang *et al.*, 2018). Note that cell viability measurements only give a current snapshot of the proportion of viable cells, while cell density measurements allow quantitative temporal comparisons. Additionally, proteoglycan content in the NP is often reduced together with changes in the macroscopic appearance and tissue damages (Han *et al.*, 2017; Kanda *et al.*, 2021; Kurakawa *et al.*, 2015; LePage *et al.*, 2021; McCann *et al.*, 2013; Paul *et al.*, 2013; Paul *et al.*, 2017; Peroglio *et al.*, 2017; Xu *et al.*, 2016). When dynamic axial compression was combined with cyclic torsion, *i.e.* complex loading with 2-DOF, cell viability was even reduced to 0-10 % in the NP at stress levels of 0.4-0.8 MPa and ± 2° torsion at 0.05-0.2 Hz for 4-8 h/d in cow discs (Chan *et al.*, 2013a; Croft *et al.*, 2021).

For future setups, a 6-DOF bioreactor will allow the application of complex loading to motional segments (Costi *et al.*, 2008; Šećerović *et al.*, 2022; Wilke *et al.*, 2016). However, static loading between 0.5-1 MPa also led to a reduction of cell viability and density due to apoptosis and matrix disorganisation in mouse, rabbit, or goat discs (Ariga *et al.*, 2003; Paul *et al.*, 2013; Paul *et al.*, 2017; Zhan *et al.*, 2016; Zhan *et al.*, 2021). A static load can also be applied with a wedge, leading to apoptosis in the concave AF, whereas the convex AF had an increase of proinflammatory and catabolic markers (Walter *et al.*, 2011). Under conditions that mimic space flight, *i.e.* microgravity by culturing a mouse disc in a rotating wall vessel bioreactor, DNA and proteoglycan content were reduced and apoptosis increased in mice discs after 56 d in culture (Jin *et al.*, 2013). Two further studies cultured whole rat IVDs in microgravity; however, there were no degenerative changes observed but they concluded that a stable culture platform was obtained (Raines *et al.*, 2020; Stannard *et al.*, 2015).

Loading has also been combined with other induction stimuli. The combination of high frequency loading (5-10 Hz) with glucose deprivation or needle puncture, led to an increase of proinflammatory markers in sheep or cow discs (Illien-Jünger *et al.*, 2010; Illien-Jünger *et al.*, 2012; Lang *et al.*, 2018; Navone *et al.*, 2018; Pattappa *et al.*, 2014; Wangler *et al.*, 2019) and cell viability was reduced when glucose was deprived. However, the matrix composition often remained unchanged in these combinatorial models at early timepoints of < 11 d (Illien-Jünger *et al.*, 2010; Lang *et al.*, 2018; Navone *et al.*, 2018). Nevertheless, the combination of glucose deprivation and degenerative loading had stronger effects than the respective conditions alone (Illien-Jünger *et al.*, 2010; Jünger *et al.*, 2009). When TNF injection was combined with high frequency loading and glucose deprivation, the cellular phenotype adapted to a more degenerative type with increased proinflammatory and anabolic gene markers but reduced catabolic gene expression in cow discs (Lang *et al.*, 2018; Li *et al.*, 2020; Saravi *et al.*, 2021). Also, tensile strain on cow AF explants (6-12 %, 1 Hz, 3 h/d) combined with IL 1β treatment led to PGE2 release and proinflammatory marker deposition within the translamellar bridging network (Saggese *et al.*, 2019). In pig IVDs, trypsin injection together with degenerative loading led to a more severe degeneration type (Hsu *et al.*, 2013; Kuo *et al.*, 2014; Nikkhoo *et al.*, 2018). Therefore, future studies investigating combinatorial approaches are very promising for mimicking human IVDD on several levels.

To note, in many of the load induced IVDD cultures, the loading conditions were often changed back to physiological loading conditions after treatment initiation, *e.g.* cellular injections, as otherwise applied agents would also be subjected to degenerative loading conditions.

Enzyme

By injecting a proteolytic enzyme, the ECM is degraded, and with matrix breakdown, the biomechanical behaviour and cellular microenvironment change. Even though some enzymes are very specific, most ECM proteins are interconnected, and digestion of a specific element can, over time, loosen many ECM components. Enzymes can either be injected into the IVD or added to the culture medium. However, a slow injection of small volumes *via* small gauge needles (dependent on disc size) is suggested, to avoid large shear forces and physical injury that might induce degenerative changes itself, especially to the AF (Elliott *et al.*, 2008; Lee *et al.*, 2021; Mao *et al.*, 2011). Nevertheless, NPCs morphology and viability usually remain unchanged following enzyme treatment, indicating that stronger cellular reactions may need to be provoked by means other than enzymes (Chan *et al.*, 2013b). Enzymatic activity is dependent on the environment, *e.g.* pH and temperature, but also on enzyme-type, dose, time, and loading, thereby creating mild to severe progressive degeneration. Additionally, molecular transport out of the disc but also into other disc regions *via* diffusion and convection contribute to the enzymatic induction of disc degeneration. Dynamic loading can increase the convective transport of larger molecules (> 1 kDa) into/out off the disc, *e.g.* TNF (around 17 kDa, approximately the same size as IL-1β and IL-6) had an increased concentration in a dynamically-loaded IVD (Walter *et al.*, 2015). Furthermore, it was recently shown that chABC-induced sGAG reduction is affected by diurnal loading (Salzer *et al.*, 2022).

Two frequently used enzymes, papain and trypsin, have been reported to lead to NP cavity formation following injection in a dose-dependent manner in miniature-pig discs (Chen *et al.*, 2009). Additionally, collagenase was reported to lead to cavity formation and NP destruction in goat discs, indicating that a breakdown of the collagenous network may be needed for cavity formation (Rustenburg *et al.*, 2020). For papain, NP cavity formation was reported in all studies and often extended to the CEP and AF (Chan *et al.*, 2013b; Gryadunova *et al.*, 2021; Malonzo *et al.*, 2015; Roberts *et al.*, 2008; Schmocker *et al.*, 2016). However, it is hypothesised that damage to the CEP and AF can also arise from NP decompression that leads to overloading of other disc parts that initiates a progressive degenerative cascade, as described above. Additionally, trypsin, led to NP cavity formation starting at a dose of around 1,240 U in cow discs, similar to papain treatment; however, without extending to the AF after 3 weeks. An additional common observation was reduced sGAG content (AlGarni *et al.*, 2016; Gawri *et al.*, 2014; Hsu *et al.*, 2013; Jim *et al.*, 2011; Mwale *et al.*, 2014; Nikkhoo *et al.*, 2013; Nikkhoo *et al.*, 2017; Roberts *et al.*, 2008; Wangler *et al.*, 2019). Interestingly, this was not the case when cow discs were loaded with SPL following trypsin injection (Gawri *et al.*, 2014).

chABC has been injected into cow NP explants, pig IVDs and a goat IVD in doses of 0.02-0.4 U. The injection led to a dose- and time-dependent sGAG reduction and a less homogenous proteoglycan distribution. Therefore, chABC also leads to NP depressurisation and progressive IVDD like papain and trypsin, but with less severe damages on the ECM network (Krupkova *et al.*, 2016; Li *et al.*, 2018; Paul *et al.*, 2018; Salzer *et al.*, 2022). This method might therefore be more suitable to mimic the early loss of fixed charge density that reduces the osmotic pressure (Urban and Maroudas, 1979). The combination of chABC with collagenase type II led to more severe degeneration than each enzyme alone, with cell cluster formation, reduced anabolic activity, and increased catabolic and proinflammatory activity. Furthermore, AF fissures, demarcation between NP and AF, loss of pericellular matrix and changed biomechanics occurred (Rustenburg *et al.*, 2020). When chABC was combined with IL-1β in a rabbit NP or AF explant, an increase of collagenase and NO activity was found in the AF explant, but no changes in the inflammasome and MMP activity was found in NP explants (Sakuma *et al.*, 2002).

Other enzymes used are ADAMTS4, HTRA1, MMP-3, or thrombin. Thrombin (100 nmol/L) added to culture medium led to a catabolic and proinflammatory response and reduced proteoglycan staining intensity in mice AF, CEP, and NP (Takayama *et al.*, 2018). MMP3, HTRA1, and ADAMTS4 (all 10 µg/mL, (Furtwängler *et al.*, 2013)) were studied in cow discs and might be considered rather mild compared to papain, trypsin, and chABC. While cells adapted their catabolic/anabolic activity, sGAGs remained unchanged and there were no macroscopic differences compared to healthy controls. Only in the ADAMTS-4-treated group was a trend of proteoglycan content reduction observed (Furtwängler *et al.*, 2013). Thus, these enzymes might take an extended time to induce IVDD but resulting degenerative changes may better mimic the slower progressive human IVDD.

Unfortunately, the combinatory effect of cytokines and enzymes remains scarcely investigated even though it is very powerful and useful. Long-term cultures could elucidate what the combined effects would be and whether that would be comparable to human disc degenerative processes. Most studies also did not report the use of an enzymatic inhibitor to control/stop enzymatic activity. Additionally, some enzymes can be inhibited by factors secreted by resident cells. However, the side effects, dose response, and the level of inhibition of various molecules are mainly unknown and need further investigation in future studies.

Other methods

Several groups have used disc cultures so study the side-effects of commonly used clinical methods, *e.g.* ionising X-ray radiation experienced during radiotherapy (Liu *et al.*, 2020), but also to test clinical treatments such as disc analgesia or spinal fusion (Haschtmann *et al.*, 2012; Iwasaki *et al.*, 2014). Although they have not been used to induce disc degeneration *per se*, such treatments can induce IVDD and, therefore, their further investigation as induction methods for IVDD models may be fruitful. Similarly, co-morbidities such as diabetes or ageing, often studied to better understand the aetiology of IVDD, may also be useful as disease-specific induction methods, *e.g.* AGE, when added to rat IVDs, led to collagen disruption and loss (Hoy *et al.*, 2020). Another approach to consider would be to carry out the induction *in vivo* and then to investigate treatments in culture. This was done in two studies (Hamamoto *et al.*, 2012; Ura *et al.*, 2019); however, there does not seem to be much gained in terms of ethical considerations and 3R principles with this approach, thus other methods should be considered first.

Finally, the most interesting induction method may be that of using spontaneously degenerated IVDs. In the pioneering work of Frapin *et al.* (2020), sheep discs degenerated by ageing (3-7 years old) were cultured *ex vivo*. Pfirrmann scoring (Pfirrmann *et al.*, 2001) was grade 1 for 1 year old compared to grade 2-3 in 3-7 year-old sheep. This approach might be considered as the most realistic model of human IVDD, as ageing sheep were previously described to develop IVDD with features similar to those found in human IVDD, *e.g.* by changes observed through imaging and histology. (Alini *et al.*, 2008; Bouhsina *et al.*, 2021; Bouhsina *et al.*, 2022; Lee *et al.*, 2021; Nisolle *et al.*, 2016). Similarly, mouse models have also been used to study disc ageing where more degenerative changes were found in aged mice compared to younger mice, indicating the importance of animal age (Fujita *et al.*, 2012; Liu *et al.*, 2020). Nevertheless, neither mouse nor sheep models of spontaneous IVDD are used frequently, potentially due to the manyfold limitations, *e.g.* the relatively high costs (*e.g.* due to trained personnel and animal housing), low availability, time, ethical considerations and the 3Rs (*e.g.* compared to slaughterhouse material), and the necessity of grading – which requires special equipment and trained personnel. Furthermore, more in-depth research is necessary on spontaneously degenerated animal discs and their comparison to human IVDD to ensure that the mechanisms of disease are similar at the tissue-, cell- and molecular-level. Additionally, not only differences in cell density due to ageing but also between species should be considered, as the intrinsic regeneration potential could be relatively higher in such discs. Finally, companion animals like dogs are frequently patients, which means that they can and should benefit from IVD research. There are two major types of breed groups with different common clinical entities, the acute NP thoracolumbar herniation (in chondrodystrophic dog breeds) or chronic building lumbosacral disc (in non-chondrodystrophic breeds). Both share similarities with human IVDD (Bergknut

et al., 2013; Kranenburg *et al.*, 2013; Smolders *et al.*, 2013). Furthermore, experimental dogs and client-owned dogs suffering from chronic LBP have been used to test regenerative therapies. As such, disc cultures from, *e.g.* beagle dogs, that are used as experimental animals for non-spine related studies, are currently underutilised; notwithstanding the use of donor material from diseased client-owned dogs of older age. But also, discs obtained from other species from spine-unrelated studies, *e.g.* aged mice or diabetic rats, are currently underutilised and might be good models, *e.g.* to study co-morbidities.

What can and cannot be mimicked with current *ex vivo* IVDD models?

In this review the emerging field of disc cultures to study IVDD were summarised with the aim of supporting researchers to choose the most suitable model for their research questions but also develop new models and further adapt existing methods. As induced degenerative changes typically progress within days to weeks, various severity degrees can be studied with most models. Furthermore, this allows for the study of a treatment at several degrees of severity using the same model. Nevertheless, the direct comparison of consistency in between studies is difficult due to large variations in methodology. However, the most important question when developing an IVDD model is, which overall strategy is best to induce degenerative changes: **1) mimicking the pathophysiological processes of human IVDD; or 2) mimicking one or several hallmark(s) of IVDD?** While the first option might be more relevant for translation to human IVDD but with the drawback of high complexity, the second option is faster, relatively less complex, and perfectly suitable to answer a specific research question. Independent of the chosen degeneration method, the culture should – in best case – be representative of the situation found in human degenerated discs, *e.g.* by applying dynamic loading conditions and adaptations of the culture medium to reduced glucose, pH, osmolarity and O_2 concentrations.

For the first strategy, the models that mimic human IVDD best are spontaneously degenerated discs. These are derived from larger animals and caused by ageing, and human IVDD is a slowly progressive disease over the course of years to decades in humans. While this model is the most sophisticated, which hampers a broad application, more studies are necessary to understand the specific differences compared to human IVDD and spontaneously degenerated discs of various large animals, *e.g.* due to sex, or anatomical differences (Lee *et al.*, 2021). The most promising strategy for a simplified version of strategy one is a combinatorial model of proinflammatory cytokine stimulus *via* TNF or IL-1β (or potentially even more complex formulations) together with enzymatic or mechanical degenerative stimuli. Alternatively, TLR activation, with the appropriate agonist for each species, might be further investigated as a trigger of IVDD (Krock *et al.*, 2017). By applying such methods, researchers will be able to replicate many hallmarks of human IVDD, especially if the biochemical environment of the degenerated IVD is additionally imitated.

However, many research questions do not necessarily require mimicking human IVDD in as many aspects as possible and the second strategy can be applied. Various factors are easy to apply and thereby add to already existing culture setups, such as a lower glucose concentration to reduce the cell density. Another suggested and easy addition is a cytokine stimulus such as TNF, as many of the current methods do not induce cellular changes found in human IVDD. Disc cultures are also suitable as preparation for preclinical *in vivo* trials, *e.g.* to determine required enzyme-dose and to find the most suitable readout parameters and assay methodologies. To investigate anti-inflammatory therapeutics, proinflammatory cytokine treatment such as TNF or IL-1β are good stimuli that can even induce a catabolic shift after several weeks of culture in cow discs (Purmessur *et al.*, 2013; Walter *et al.*, 2015). Methods that create a large void in the central NP, *i.e.* NP decompression due to enzymes or injury/damage, allow for injection of large amounts of a biomaterial, even with an intact AF and CEP to prevent the material from migrating out of the disc. Alternatively, AF or CEP defects can not only be used to mimic herniations and to test repair/regeneration strategies, but also for extrusion of injected biomaterials, *e.g.* for NP augmentation (Schmitz *et al.*, 2020). In NP decompression models with dynamic loading, degenerative changes due to the redistribution of the applied force from the NP to the other disc regions will additionally occur. Another hallmark of IVDD that can be easily mimicked is sGAG reduction *via* chABC injection. This method allows for the study of the effect of a reduced fixed charge density and the resulting reduced osmotic pressure that leads to reduced biomechanical function. Using this method, therapies that can increase the swelling pressure can be studied, *e.g.* using swelling hydrogels or by cellular injections.

Future disc cultures may be used to study discogenic pain, co-morbidities such as diabetes, or the role of the CEP on IVDD. Maybe even dynamically loaded whole spinal segments derived from large animals will be cultured in future setups once technical challenges are overcome. Other approaches to study IVDD that are not discussed in this review are tissue engineered IVDs (Gullbrand *et al.*, 2018; Hamilton *et al.*, 2006) and microfluidic-based systems (Mainardi *et al.*, 2022).

Conclusion

Non-human IVD organ and tissue explant cultures can be used to mimic mild to severe human IVDD; degeneration can be induced with several methods

affecting all parts of the IVD and progressing over time. Disc culture models are especially suitable for studying novel techniques to augment the NP or regenerate the disc and its substructures with biomaterials, cells, or biologically active ingredients. Overall, most degeneration methods only mimic some aspects of human IVDD as that is often sufficient when answering a specific research question. Additionally, there is a clear trade-off between mimicking human IVDD as close as possible, to the applicability, availability, costs, and complexity of the model. The model that most completely mimics human IVDD in many aspects are disc cultures derived from spontaneously degenerated sheep IVDs, and dog-disc cultures are promising for this application. Unfortunately, such models are not expected to become broadly available. However, future applications using combinatorial degeneration induction methods can induce pathological changes on multiple levels affecting cellular behaviour, ECM composition, and biomechanical behaviour, and therefore more closely recapitulate aspects of human IVDD. With increasing complexity and specificity of disc culture systems, their potential to substitute animal trials is expected to steadily increase, making them a powerful tool for a better clinical translation from bench-to-bedside.

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Conflicts of interest

The authors declare no conflicts of interest.

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Author contribution statement

ES, MAT, and KI planed the review. Data were collected by ES and analysed by all authors. The manuscript was drafted by ES and KI and critically revised by all authors.

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Discussion with Reviewers

James Iatridis: Please discuss the very large difference in cellularity between for example bovine and human IVDs and how this will affect the glucose and oxygen concentrations needed to maintain physiological conditions in different model systems.

Authors: The cell density in the nucleus pulposus decreases with increasing disc size, so that rodents have a much higher cell density than, for example, bovine discs or human discs. A higher cell density increases the nutrient demand (Urban *et al*., 2004) to maintain them. However, regardless of the cell density, dynamic loading increases the metabolic activity of nucleus pulposus cells and can lead to a reduction of the cell density if the demand exceeds supply or transport capacity (Salzer *et al*., 2023). Furthermore, the nutrient supply is different in rodents compared to large animals (Alini *et al*., 2008). Therefore, physiological and pathophysiological conditions, for example adjusted *via* the glucose and pH in the medium, should be adjusted to the specific model and the research question in mind. In this respect, discs derived from larger animals better mimic the cell density of human discs; but even the most frequently used model of discs derived from young cow tails have a higher cellularity than human discs, which can influence the intrinsic regeneration potential. Finally, cellular phenotypes and changes thereof with degeneration need to be taken into consideration, as cells from animals are typically in a healthy state at the initiation of culture.

Lisbet Haglund: Please discuss how the oxygen consumption rate varies with glucose concentration and the degenerative state of the tissue. Please also discuss how this may relate to IVD size and the blood glucose level in different species.

Authors: In the healthy and degenerated IVD, the NPCs reside in a hypoxic environment. Under such conditions, glucose is metabolised to lactate. However, it has been reported that even under 21 % O_2 conditions (hyperoxia), the metabolism of NPCs is still anaerobic (Horner *et al.*, 2021, additional reference). Moreover, it has been reported that such high oxygen tensions can lead to reactive oxygen formation (Feng *et al.*, 2018, additional reference). Interestingly, under 21 % O_2 conditions, the oxygen consumption rate of human degenerated NP, AF, and CEP cells was found to be highest at low glucose (1 mmol/L) compared to higher (5 and 25 mmol/L) glucose concentrations, and overall higher than in healthy cells. Glucose concentration did not influence the oxygen consumption of healthy NP, AF, and CEP cells. However, neither healthy nor degenerated NPCs did show a different oxygen consumption compared to CEP and AF cells (Cisewski *et al.*, 2019, additional reference). NCs have a higher proteoglycan production rate than NPCs (Miyazaki *et al.*, 2009, additional reference) and a higher oxygen consumption than AF cells (Guehring *et al.*, 2009, additional reference), indicating a different energy metabolism, which has not been well characterised. This interplay between the transport of basic nutrients and the effect on NP cells metabolism both in the physiological and pathopysiological state would be of interest to study further in well defined *ex vivo* cultures.

In general, blood glucose levels are relatively comparable between all the species mentioned, whereas the size of their disc and there cellularity are not (see response to Iatridis above). However in addition, there are other anatomical differences that are important to consider. Mice, rats, and *e.g.* sandrats don't have vascular buds in their vertebral endplates, and it is speculated that due to their small size, nutrient supply through the AF is sufficient (Gruber *et al.*, 2005, additional reference; Alini *et al.*, 2008). Contrary, in larger animals, approximately starting at the size of rabbits, vascular buds terminate in the vertebral endplates and small molecules are transported almost exclusively by diffusion through the cartilage endplate. With increasing disc size, the diffusion distance increases to the central region of the NP (Urban *et al.*, 2004). Finally, with ageing and degeneration, calcifications can block vascular buds at the BEP/CEP interface (Benneker *et al.*, 2005).

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