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REVIEW

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Controversies in spine research: Organ culture versus in vivo models for studies of the intervertebral disc

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Abstract

Intervertebral disc degeneration is a common cause of low back pain, the leading cause of disability worldwide. Appropriate preclinical models for intervertebral disc research are essential to achieving a better understanding of underlying pathophysiology and for the development, evaluation, and translation of more effective treatments. To this end, in vivo animal and ex vivo organ culture models are both widely used by spine researchers; however, the relative strengths and weaknesses of these two approaches are a source of ongoing controversy. In this article, members from the Spine and Preclinical Models Sections of the Orthopedic Research Society, including experts in both basic and translational spine research, present contrasting arguments in support of in vivo animal models versus ex vivo organ culture models for studies of the disc, supported by a comprehensive review of the relevant literature. The objective is to provide a deeper understanding of the respective advantages and limitations of these approaches, and advance the field toward a consensus with

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respect to appropriate model selection and implementation. We conclude that complementary use of several model types and leveraging the unique advantages of each is likely to result in the highest impact research in most instances.

KEYWORDS
in vivo, intervertebral disc, models, organ culture, spine

1 | INTRODUCTION

The prevalence of musculoskeletal conditions is growing worldwide, and low back pain (LBP) is significant among these as the leading cause of disability.[1] It is estimated that approximately 80% of adults are affected by LBP at some point in their lifetime.[2] LBP impacts individuals in both developed and developing countries alike, affects all age groups from children to the elderly,[2,3] and thus represents a significant burden for patients, health care systems, and the economies of many countries. Approximately 40% of LBP cases are attributable to degeneration of the intervertebral discs (IVDs), making this the most common cause of chronic LBP.[4] Intervertebral disc degeneration (IVDD) is a progressive, cell-mediated cascade involving each of the IVD's three main anatomical regions: the central, proteoglycan-rich nucleus pulposus (NP); the peripheral, fibrocartilaginous annulus fibrosus (AF), and the two cartilage endplates (CEPs) that interface with the adjacent vertebrae. The earliest manifestations of IVDD commonly occur in the NP, where proteoglycan loss compromises the distribution of loads leading to structural and mechanical derangement of the entire spinal motion segment. While IVDD commonly occurs with increasing age, risk factors for accelerating its progression include genetics, smoking, lifestyle, obesity, trauma, and mechanical stress.[5–7] IVDD may lead to LBP through direct compression of adjacent neural elements or by innervation of the IVD structures themselves, which combined with increased nerve sensitizing agents leads to increased pain.[8–14]

The complexity of IVDD pathophysiology poses great challenges for effective long-term treatment of associated LBP.[15] Current clinical treatments are predominantly focused on managing symptoms (e.g., alleviation of pain) rather than addressing underlying causes. These treatments may involve medications such as nonsteroidal anti-inflammatory drugs (NSAIDs), which can address acute symptoms but carry the risk of increased internal bleeding during long-term use.[16] For more severe symptoms, opioid-based medications may be prescribed,[17] but they pose a serious risk of addiction, exacerbating the opioid epidemic and associated morbidities.[18–21] Where conservative treatments do not appear to modify disease progression, surgical interventions such as spinal fusion or total disc arthroplasty may be employed, but these fail to preserve disc structure or mechanical function long-term and may result in progression of IVDD in adjacent levels.[15] Therefore, there is a significant clinical need for improved treatment options for patients suffering from IVDD and LBP that directly target the underlying causes.

The successful development, evaluation, and translation of new treatments for IVDD require the use of appropriate preclinical models that recapitulate the structural, functional, and biological characteristics of the clinical condition as closely as possible. Therefore, a deeper understanding of the benefits and limitations of various approaches to implementing currently available preclinical models is critical for advancing investigations of IVDD pathophysiology and treatment. Despite wide-ranging attempts to develop both in vivo (large and small animal) and ex vivo (organ culture of viable postmortem tissue) models, controversies remain regarding the selection of appropriate models for IVD research.

The objective of this article is to contrast and debate the respective advantages and limitations of in vivo animal models versus ex vivo organ culture models for studies of IVDD and its treatment. To achieve this, we have leveraged the broad expertise of the members of two leading groups focused on basic and translational spine research—the Spine and Preclinical Models Sections of the Orthopedic Research Society (ORS)—coupled with a comprehensive review of the current scientific literature. We begin with arguments in support of in vivo animal and ex vivo organ culture models, respectively, for studies of IVDD and its treatment, and conclude with recommendations for incorporating these models into experimental designs to address specific research questions most effectively, with an emphasis on the complementary use of multiple models in order to generate the highest impact results.

2 | ARGUMENTS IN SUPPORT OF IN VIVO MODELS

2.1 | Introduction

Preclinical research studies are commonly conducted on the cervical, thoracic, lumbar, and caudal spines of research animals. Animal models have played a critical role in advancing understanding of the temporal evolution of IVDD, including how constitutive, environmental, or biomechanical risk factors may initiate, promote, or otherwise regulate degenerative changes, and how therapeutic strategies may ameliorate, resolve, or prevent IVDD.[22] Currently, in vivo studies of IVDD are conducted in small animals such as mice, rats, and rabbits; as well as larger animals such as dogs, pigs, goats, sheep, cows, and nonhuman primates.[23] However, given the complexity of human IVDD, a perfect animal model does not exist.[24] In this section, we outline key advantages that in vivo models have over ex vivo organ culture models,

including pain evaluation, nutrition and blood supply, systemic effects related to the immune system, crosstalk with surrounding tissues, imaging, the requirements from regulatory agencies for clinical translation of new treatments for IVDD, and as prerequisites for clinical translation.

2.2 | Pain evaluation

Pain can be defined as cortical interactions that initiate changes in behavior.[25] Pain behaviors may be influenced by physiological and immunological factors, cognition, and conduct. In human patients, LBP as a result of IVDD results in significant morbidity, preventing patients from completing their daily routine, removing individuals from the workforce, and resulting in stress, anxiety, and depression.[26] This pain is the main driver for patients seeking care, and a paramount factor in the diagnosis of IVDD. Importantly, studies have shown that IVDD does not always directly correlate with pain, and that IVDD may often be present in asymptomatic individuals.[27]

While the direct connections between IVDD and pain remain complex, animal models have been and continue to be essential research tools for understanding physical and metabolic pathways of symptomatic IVDD (discogenic pain), and in the development of new therapeutics aimed at mitigating and preventing the onset of degeneration and pain. Put simply, only in vivo models can recreate the complex processes of pain resulting from disc degeneration, and permit assessments of behavioral and functional changes as outcome measures. This is not without its challenges, as each species has unique physical and behavioral manifestations of pain, and species-specific, repeatable, and standardized pain scores must be used.[28] Among large animals, dogs provide an interesting model for discogenic pain as distinct and appreciable behavioral changes make these animals particularly valuable when assessing analgesics.[29–33] Nonetheless, the optimal way to measure pain in both preclinical models (and patients) is still the subject of extensive debate. Important aspects such as the nociceptive response generators, pain thresholds, and clinical and behavior manifestations need to be contemplated before selecting an animal model.[28] Validated methods of pain measurement include physical performance (e.g., grimace scales, lameness examinations, gait measurements),[34] behavioral changes (e.g., decreased burrowing and rearing),[35,36] and response to mechanical stimuli (e.g., hind-paw mechanical hyperalgesia test). A recent study has shown that the Grimace scale (a subjective pain assessment method based on facial expressions) is highly reliable in mouse and rat models, and moderately reliable in rabbits, piglets, and sheep.[37]

Large animal models have also led to the identification of molecular biomarkers of discogenic pain.[38] Biomarkers not only represent potentially powerful, noninvasive diagnostic tools for evaluating IVDD progression and response to therapeutic intervention, but also provide mechanistic insights into how local pathophysiological changes lead to the manifestation of clinical symptoms, informing the development of new therapies. This simply cannot be accomplished using ex vivo models where clinical manifestations of IVDD (e.g., pain) cannot be measured.

2.3 | Nutrition and blood supply

The IVDs are largely avascular structures. During human development, blood vessels penetrate deep into the lamellar structure of the AF from around 35 weeks gestation.[39,40] Vessels then recede, and by the second decade of life remain only at the margins. At no point do blood vessels penetrate the central NP; instead, blood vessels terminate within the subchondral bone adjacent to the CEP. These locations—the AF margins and the vertebral endplates—are the sole sources of nutrition for cells within the IVD itself, with the latter considered the most important.[41] Physiological nutrition via these routes is therefore critical for IVD cell survival, and alterations to the adjacent vasculature that disrupt nutrient supply are considered to play an important role in the onset and progression of IVDD. Importantly, the role of vasculature in IVDD can only be investigated using in vivo animal models with an intact circulatory system and cannot be achieved using ex vivo organ culture models.

At a fundamental level, in vivo models have been used to establish mechanisms of nutrient flow into the IVD. For example, historically, in vivo large animal models were used to establish that vertebral endplate vasculature is the primary nutrient diffusion pathway into the IVD.[42,43] More recently, a rabbit model was used to demonstrate how alterations in microvasculature that occur with degeneration affect nutrient supply to the IVD.[44] In vivo models have also been essential for studies investigating how certain drugs impact the vasculature supplying nutrients to the IVD. For example, in vivo models have been used to show how vasoactive agents such as acetylcholine and nicotine, as well as cigarette smoking itself, may alter vasculature and nutrient supply to the IVD, implicating smoking in the etiology of IVDD.[45–48] In vivo models are also essential for evaluating the efficacy of therapeutic agents administered systemically to treat IVD pathologies, such as intravenous stem cells and antibiotics.[49,50]

2.4 | Long-term evaluation

Irrespective of the factors initiating or driving IVDD, it is most often a long-lasting process with changes in the cellular environment and the different structures of the IVD occurring over months or years, before leading to the gross structural and functional alterations that are associated with the manifestation of clinical symptoms.[51] As such, in vivo models have been important tools for elucidating the long-term natural history of IVDD.[52] Furthermore, in vivo models are crucial for evaluating the long-term efficacy of novel treatments for IVDD.[53] The primary goal of IVDD treatments is to both restore IVD function and structure, and alleviate painful symptoms. Acute toxicity and initial structural (e.g., an increase in cellularity and extracellular matrix [ECM] or IVD height) and functional changes can be assessed ex vivo and in vivo; however, potential therapeutic agents may have either a short half-life or may diffuse out of the IVD, so their long-term effects must be determined. Furthermore, initial treatment success may be diminished by the unfavorable degenerative environment of IVDD. In vivo models allow an observation period of several weeks (small animal

models) to months or even years (large animal models), facilitating confirmation of sustained or permanent therapeutic effects. Furthermore, the same animal may be assessed over time using noninvasive, gold-standard imaging techniques such as magnetic resonance imaging (MRI), radiographs, or computed tomography, increasing the clinical relevance of findings and reducing the number of experimental animals required. In addition, it is crucial to ensure both acute (i.e., toxicity) and long-term safety (e.g., tumorgenicity) of novel biological treatments, which is only possible using in vivo models.

2.5 | Systemic factors

A major advantage of using in vivo animal models for IVDD and LBP research is the ability to assess the contributions of systemic biological processes such as the immune system, or co-morbidities such as diabetes or obesity on IVDD progression and treatment. Immune cell infiltration of mast cells, macrophages, neutrophils, and T lymphocytes has been identified in the painful human degenerate IVD following rupture of the AF or CEP[54–57]; however, the mechanisms underlying the roles of these cells in IVDD are underexplored. The healthy IVD is largely avascular and immune-privileged, yet with degeneration, there is evidence that these immune cells can infiltrate the disc from the bone marrow via lesions in the vertebral endplate and/or via aberrant blood vessel ingrowth into the endplate and AF.[58] In vivo animal models of IVDD and LBP are valuable tools with which to investigate the recruitment, invasion, and function of immune cells in pathological environments, which cannot be readily investigated ex vivo. For example, transgenic mice over-expressing the pro-inflammatory cytokine TNFα demonstrate increased infiltration of Tryptase-expressing (mast) cells or CD68+ (macrophage) cells in IVD tissue regions associated with higher risks of herniation.[59] The increased presence of immune cells, specifically macrophages in herniated IVD tissue, has been corroborated using a novel in vivo mouse model of IVD herniation-induced radiculopathy.[60] Green Fluorescent Protein (GFP) transgenic bone marrow chimeric mouse models of IVD injury have been used to determine the origin of M1 macrophages and demonstrated that following IVD injury, M1 macrophages are recruited specifically from outside the IVD.[61] A subsequent study verified these findings by demonstrating increased recruitment of macrophages to the dorsal region of the IVD together with neo-innervation in an IVD injury model for up to 12 months.[62] These studies highlight the importance of in vivo models for investigating the role of the immune system in IVDD.

Systemic inflammatory diseases such as obesity and diabetes demonstrate a strong association with IVDD and back pain,[63,64] and animal models (rodents in particular), demonstrating these disease phenotypes are useful tools to conduct mechanistic and therapeutic studies in which changes in whole IVD joint structure/function and pain behaviors can be investigated. Obesity and diabetes co-exist and can be readily investigated simultaneously using in vivo animal models. Male and female leptin receptor-deficient mice fed with a control (low fat) or high-fat diet to mimic the effects of obesity and diabetes on disc health have been used to examine the effects of obesity and type-2 diabetes on healthy intervertebral IVDs.[65,66] Sex-dependent effects have been described, with only females developing diabetes and the most pronounced changes in IVD and bone structure, pointing toward a sex-dependent role for leptin in the spine.[65]

In a type-2 diabetic rat model, several changes have been identified in the IVD joint compared to healthy control and obese rats. Specifically, decreases in the glycosaminoglycan (GAG) and water contents of the IVD, increases in mechanical stiffness, advanced glycation end-products (AGEs) and catabolic markers, as well as increases in vertebral endplate thickness and decreased porosity were found, suggesting a reduction in nutrition to the IVD.[67] Similarly, AGE-fed mice demonstrated age-accelerated IVDD together with ectopic calcification of the spinal tissues and insulin resistance, highlighting a role for AGEs in promoting diabetes-induced IVDD.[68] To further validate the role of AGEs in diabetes-induced IVDD, diabetic mice were treated with oral anti-inflammatory and anti-AGE drugs. These drugs mitigated pathological effects observed on disc height, GAG content, and catabolic markers in diabetic mouse models, demonstrating broad clinical applications of anti-AGE drugs on spinal health.[69] Together, these studies highlight the critical role of in vivo animal models in evaluating the effects of systemic co-morbidities on IVDD progression and response to treatment.

2.6 | Crosstalk with surrounding tissues

Investigating crosstalk with surrounding tissues is essential for a comprehensive understanding of IVDD progression and the development of LBP, and this is best achieved with the biological complexity inherent to in vivo models. For example, tissue crosstalk is important to consider when studying nociception. The dorsal root ganglion (DRG) has been suggested to interact with the NP in IVD herniation to elicit pathological consequences. This involves induction of pro-inflammatory signaling pathways,[70,71] activation of microglia,[72] and modulation of the AMPK-mTOR axis[73] in the DRG. Ex vivo co-culture models may be able to model some tissue interactions. For example, a gene-editing study using ex vivo co-culture systems suggested that inflammatory signals from degenerative IVDs can sensitize nociceptive neurons,[74] and such sensitization can be manifested under mechanical stress,[75] suggesting that IVDD may play a role in pain sensitization.[76] However, even in ex vivo work, different results can be obtained depending on the study design. For example, differential effects of hypoxic stress on neurite outgrowth in DRGs were reported between the single cell and tissue levels.[77] Therefore, the study of neural activity in context of IVDD in vivo may yield contrasting results from those obtained ex vivo.

There are numerous examples of the importance of tissue crosstalk in IVDD pathophysiology. In a rabbit cornea implantation model, cartilaginous endplate explants may inhibit neovascularization while AF explants may promote it.[78] This implies compartmental crosstalk can craft the nutritional pathway of the discs. On the other hand, loss of vertebral bone integrity, for example, due to vertebroplasty[79] or

bone loss in ovariectomized mice[80] may affect IVD health. Schmorl's nodes, an endplate defect, have been associated with IVDD,[81] and is consistent with findings that experimental injury to the endplate can initiate disc degeneration in large animal models.[82] IVD herniation may initiate at the endplate-annulus interface in aged rats[83] and involves systemic TNF-α upregulation.[59] These studies support that endplate and vertebral bone have major influences on IVD tissue homeostasis. At the cellular level, IVDD is associated with remodeling of the NP, which transitions into a fibrocartilaginous tissue composed of chondrocyte-like and fibroblastic cells. NP ECM remodeling may in part be mediated by cell types originating in adjacent tissues.[84–87] Thorough interrogation of such dynamic cellular exchange among tissue compartments/systems requires in vivo models.

2.7 | Physiologically relevant imaging

To ensure the physiological relevance of IVD imaging findings, it is important to consider tissue interaction. Imaging of whole IVD motion segments in live animals can better reflect the physiological status of the IVDs. For example, when performed in vivo, radiographic assessments of disc height (an important surrogate of IVDD progression and response to treatment) can be normalized to adjacent vertebral dimensions to account for variation across spine levels and individual animals. Moreover, in vivo imaging accounts for the mechanical constraints of para-spinal tissues such as muscles and ligaments when evaluating IVD geometry. Sedation or anesthesia can be used to ensure proper positioning and muscle relaxation.[28] Animals with altered muscle activity such as GDF-8 mouse mutants and botulinum toxin-treated monkeys exhibit reduced IVD height.[88] Lastly, in vivo imaging permits long-term, longitudinal imaging evaluations that cannot be achieved ex vivo.

2.8 | Regulatory requirements and prerequisites for clinical translation

In vivo animal models provide superior preclinical platforms to address regulatory requirements and accelerate clinical translation by answering critical questions regarding both the safety and efficacy of novel IVDD treatments. Regulatory agencies such as the U.S. Food and Drug Administration (FDA) oversee the approval process of any drug or medical device aimed at IVDD treatment, with the exception of human-derived, minimally manipulated tissues. Preclinical studies must demonstrate that the benefits of the treatment outweigh its risks before approval for clinical use. Although animal testing is not required by the FDA, it is the most effective way to demonstrate the biological response in a living system and, therefore, is rarely excluded from the Investigational New Drug (IND) application process. The FDA has recognized this and has issued draft guidance to ensure such studies are rigorously conducted.[89] Also recognized is the need to refine, reduce and replace animal models in device and therapeutic testing where possible.[90] In most cases, preclinical animal study results are used to support an IND application and are followed by human clinical trials prior to FDA approval; however, in some specific instances, animal study results alone may be used for approval. This type of approval is covered by the FDA Animal Rule in situations where human efficacy trials may not be ethical or feasible.[91]

Several preclinical in vivo animal models may be utilized in combination to satisfy regulatory requirements. For example, initial discovery of pathological mechanisms and screening of therapeutic targets may be carried out in rodent models that permit genetic manipulation, while subsequently, large animal models provide platforms for long-term evaluation of safety and efficacy where IVD size and geometry are closer to that of humans. While organ culture models may also play a role in this process, ex vivo models are largely supportive of in vivo studies.

Intermixed with the FDA approval process is the concept of and strategy surrounding commercialization and translation to the clinic. Commercialization of a drug or device for the prevention or treatment of IVDD relies heavily on acceptance by medical physicians such as spine surgeons. A therapy could be groundbreaking with a high impact on affected patients but never realize its potential as a gold standard treatment if it is not considered sufficiently clinically relevant or if efficacy data is unconvincing. Preclinical in vivo animal models, and large animal models, in particular, are vital to the commercialization process of any groundbreaking therapy by more closely recapitulating the human condition, anatomy, IVD size and geometry, and life span. With respect to novel device development, large animal models mimic the surgical application requirements of such devices, providing practical feedback in the development of instrumentation and delivery systems, which may be as impactful to the overall success of the therapy as the therapy itself. If a surgeon cannot safely or consistently instrument an implant or deliver a therapy, then said therapy is irrelevant. Organ culture models do not provide this realistic, clinically relevant scenario. Additionally, advanced diagnostic imaging, specifically MRI, has grown to be the gold standard modality for assessing IVDD severity. As such, clinicians rely on MRI as an essential diagnostic tool for IVDD patients. Unlike organ culture models, MRI can be utilized in in vivo animal models to follow IVDD progression as well as to assess treatment efficacy, which is highly impactful with respect to the goal of achieving acceptance of therapies by clinicians and eventual commercialization. Ultimately, for a device or therapy to be useful, it must integrate seamlessly into the clinical environment, and leveraging clinically relevant in vivo animal models throughout the product development and translational process is the best way to achieve this.

Unfortunately, no model of IVDD mimics the human condition in all aspects. Despite their important role in the assessment for a new device or therapy, ethical considerations also impact the choice and use of in vivo models. For example, dog and primate models with spontaneously occurring IVDD closely translating to clinical findings in humans undergo increased public scrutiny making these models less accessible and more expensive. On the other hand, preclinical models utilizing livestock animals such as sheep, goats, and pigs are more widely accepted by the general public, although some are more limited for investigating human IVDD due to the retention of notochordal cells (pigs). There is evidence that animals that retain notochordal

cell-rich NPs, such as nonchondrodystrophic dogs, exhibit different biomechanical properties to animals that do not retain notochordal cells.[92] While organ culture models carry little ethical stigma, it is currently unusual for a therapy to move from benchtop to the affected patient via solely the use of organ culture models. Even if organ culture models were acceptable by regulatory agencies to provide safety and efficacy, it would be challenging to translate those results into the clinical situation without additional analysis in living systems.

3 | ARGUMENTS IN SUPPORT OF ORGAN CULTURE MODELS

3.1 | Introduction

Organ culture models are distinguished by the culture of whole multitissue organs, under sterile conditions, over various periods of time from short-term (hours or days) to longer-term (weeks or months). In IVD research, organ culture models have been used for basic and translational studies for several decades.[93] In 1998, one of the first reports on long-term IVD culture described the maintenance of entire rabbit IVDs embedded in alginate to preserve their structure and prevent excessive swelling.[94] In the ensuing years, methods have been advanced by the introduction of organ-specific culture systems and bioreactors, with cultured IVDs originating from several different species including rodents, rabbits, large animals (e.g., sheep, goat, bovine), and humans.[93,95–101]

Organ culture models for IVD research are popular for several reasons. First, the interaction between the IVD's tissue components is crucial for the functionality of the IVD, thus the culture of the whole organ is important for the study of the IVD in both healthy and diseased states. Second, whole organ culture means that the cells of the IVD, especially those of the NP, are naturally exposed to physiological nutrition, oxygen, pH, and hydrostatic pressure. Moreover, IVD tissues are characterized by a low cell density within an extensive ECM. Isolating the cells from this unique environment may alter their phenotype and behavior. Single-cell cultures are therefore reduced from the true physiological environment, while three-dimensional cell cultures and the use of specially tailored culture media are somewhat more representative in this respect. Third, the IVD with intact AF and CEP is considered a largely avascular, immune-privileged organ; blood vessels and infiltrating immune cells are minimally present in the healthy IVD, and thus isolated whole organ studies are appropriate. Fourth, most of the existing in vivo animal models of IVDD still do not entirely recapitulate the pathophysiology of human IVDD, and their limitations must therefore be taken into account for addressing certain translational research questions.[23,52] Organ culture models can be precisely controlled in terms of the biochemical and biomechanical environment; they are flexible with respect to study design and, depending on the throughput of the specific model, are suitable as a screening platform. Moreover, the biological response, such as the production of cytokines, local inflammation, and structural changes,[102] can be directly attributed to the experimental variables with the appropriate control groups, due to fewer covariates compared to in vivo models. They avoid unnecessary use of animals by utilizing surplus tissue from donor animals or human cadavers.

Finally, organ culture models have the advantage of a favorable cost–benefit profile. The design, development, manufacturing, and set-up of custom organ culture systems and bioreactor devices may be initially cost-intensive; however, once the method is established, numerous different studies can be performed in a standardized manner, ensuring reproducibility. For example, it has been estimated that the expenses for the set-up of an IVD bioreactor system capable of culturing and loading four large animal IVDs simultaneously, are approximately equal to the costs of one typical large animal (e.g., sheep) study, involving 10 animals in total in Switzerland.[103] Moreover, in vivo studies, especially large animal studies, require a significant contribution from highly trained professionals (e.g., veterinary surgeons) and specialized animal facilities (e.g., surgical suites, animal care, and monitoring) that necessitate significantly more specialized infrastructure investment than organ culture models. These factors make in vivo models less accessible to diverse sets of researchers worldwide. Given the vast burden of LBP due to IVDD, rapid and rigorous research can be more easily achieved with organ culture models.

3.2 | Addressing the "3Rs": Reduce, Refine, Replace

Importantly, IVD organ culture models address the 3Rs principle (Reduce, Refine, Replace) of animal testing, especially if the IVDs originate from animals that are not specifically euthanized for research purposes. The number of live animals in preclinical research can be reduced by evaluating new therapies, such as molecular,[104] cellular,[105] or biomaterial-based approaches,[106] under organ culture conditions prior to planning an in vivo study. In this respect, prescreening of treatment formulations in an organ culture model may help to rule out sub-optimal or ineffective methods, thereby avoiding unnecessary live animal studies.[107] Many questions on the interaction between the treatment and the host tissue can reliably be addressed with organ cultures,[103] given the avascular nature of the IVD. As such, only an optimized method with satisfactory organ culture results would ultimately be studied in vivo to provide the safety data required for regulatory approvals, which is currently required. With the continuous advancement of complexity of organ culture models, the complete replacement of live animals in preclinical IVD research may be possible in the future. The implementation of physiological mechanical loading in specific bioreactors, and the co-culture of IVDs with other cell types further expand the application of IVD culture models. In addition, the possibility of using whole human IVDs for research, which are naturally degenerated, reflects a model environment of unequaled physiological relevance,[108] as species differences are a well-known shortcoming when working with animal IVDs,[23] and methods of IVDD induction do not fully mimic the pathophysiology of human IVDD.

In relation to the 3R principles of animal research, the institutional regulatory processes for obtaining study permission are negligible if IVDs for organ culture are obtained from animals that are euthanized for other purposes, such as porcine, bovine, caprine, or ovine IVDs that are sourced from animals used as a source of meat. Accordingly, there are no further regulatory requirements for the ethical use of such tissues for research. The administrative and veterinary efforts required for approval of an in vivo study are thus not necessary for organ culture experiments, which saves significant time and institutional resources. On the other hand, the availability of human whole IVDs is still very limited and subject to ethical regulations. Access to whole human IVDs is rarely obtainable from surgical specimens and thus cadaveric materials are required; however, given the avascular nature of the IVD and in the experience of the authors, living IVDs can be sourced from cadaveric material for prolonged periods after death for up to 1 week.

Organ culture models allow for higher throughput analysis of disease-simulating or therapeutic agents, including crosstalk between the disease state and therapy. One major advantage of organ culture models is the ability to examine ECM-related changes (integrity and content) sooner than through in vivo models. This is of paramount importance given that the IVDD phenotype is often defined by ECM degradation. For example, organ culture models exposed to inflammatory cytokines exhibit GAG loss within 1–2 weeks.[109] Such effects in animal models require evaluation over weeks and months,[23] in part because the severity of the degenerative stimuli in vivo is limited by the number of injections and volume (e.g., injection of catabolic enzymes or cytokines). The allocation of IVD tissue from multiple spinal levels to organ culture groups facilitates increased sample size per group, the inclusion of both positive and negative controls, and evaluation of factors at multiple time points while bypassing the use of extensive live animals. These advantages are also paramount for enhancing rigor and reproducibility of experiments using organ culture models.

Thus, in this section, we present arguments outlining key features that make organ culture models more advantageous compared to in vivo animal models for IVD research, including the capability to use both human and animal IVDs, controllable physical and biochemical environments (i.e., nutrition, mechanical loading, and immune and inflammatory factors), flexible model types (i.e., diabetes, rapid degeneration, etc.), the ability to study IVDD mechanisms and crosstalk between tissue structures, the ability for both short and long-term evaluation with numerous time points, and improved imaging outcomes compared to in vivo imaging. Furthermore, we address regulatory concerns, and question the need for in vivo models as a prerequisite for clinical translation.

3.3 | Species differences

Organ culture models can employ either nonhuman animal *or* primary human tissues. Several species differences that differentiate human versus animal IVDs are highlighted below, such as size limitations when using small rodent models, and the presence of notochordal cells in some animals (i.e., porcine, mouse), whereas notochordal cells are not present in the skeletally mature adult human IVD.[52,110] As the clinical prevalence of LBP is in humans, the use of human tissue in organ culture may offer the most immediately relevant insights compared to animal models.

3.4 | Molecular mechanisms of pain evaluation

Evaluation of pain as an outcome measure in studying therapeutics for IVDD is critical. While in vivo models may be useful for studying behavioral characteristics, the translatability of pain behaviors assessed in animal models, especially small rodents, to the human condition requires further validation.[111] Furthermore, the induction of IVDD using AF puncture in animal models does not necessarily recapitulate the initiating mechanisms of IVDD in humans. Nevertheless, there are many similarities in the degenerative changes in IVD structure and chronicity of inflammatory and pain-associated cytokines.[36,62,102] In humans, LBP in the presence of an intact degenerate IVD is associated with nerve ingrowth and neurotrophic factor release. In other cases, following AF or CEP rupture, exposure of local nerves to disc material, released factors, and induction of inflammatory responses become important. These pathophysiological mechanisms of pain are not fully replicated in all in vivo models of LBP, which, combined with limited validated methodologies to accurately measure pain in such models, limits the relevance of investigation in vivo. Meanwhile, pain-related molecular factors can be studied in organ culture models; for example, neurotrophic factor expression, which reduces the need to provoke pain behavior in animal models, in alignment with the 3Rs.[77] In addition, these cellular and signaling mechanisms in organ culture models can be deterministically attributed to the IVD, and the results are specific to the biology of the IVD.

3.5 | Biochemical environment

Due to its largely avascular nature, the environment of the IVD is characterized by hypoxia, acidic pH, and low nutrient supply. Additionally, the consumption of glucose and oxygen, and the production of lactate by the IVD cells are interdependent. There is, however, great variation in the reported intra-discal oxygen and nutrient concentrations in vivo. The reason for this variation is the complex regulation of metabolites as a combination of nutrient supply, access, and demand, whereby the latter depends on the individual IVD cell density and activity. In an experimental study, oxygen concentrations were measured in IVDs of patients during discography or spine surgery.[112] The levels ranged from 5 to 150 mmHg (~0.7%–20% O_2) in the center of the NP, whereby no correlation with age or degeneration state was found. While the in situ measurements are challenging, different numerical models have calculated the concentration gradients of oxygen, lactate, and glucose within the IVD. Most studies estimate oxygen concentrations between 0.3 and 1.1 kPa (~0.3%–1.1% O_2) in the

center of the IVD[113,114]; while glucose concentrations of around 1–2 mM were predicted for the IVD center, with levels of less than 1 mM in degenerated IVD or due to endplate calcification.[113,115,116] Finally, high lactate levels are correlated with a low intradiscal pH. There are only a few reports on in vivo pH levels; pH values of ~6.7 and ~6.9 were measured in lumbar IVDs from patients with severe and moderate LBP, respectively.[117] Interestingly, these values lie between the values for IVDs with impermeable endplates and IVDs with 50% permeable endplates as predicted from numerical models,[118] stressing the importance of the endplate permeability for IVD metabolism.

Organ culture models should mimic in vivo human conditions as closely as possible. Studies show that physiological glucose, oxygen, and pH levels can be reproduced in organ culture systems to simulate healthy and degenerate IVD conditions. This implies a balance between sufficient nutrition to maintain cell viability and activity, while avoiding supra-physiological levels of nutrients and oxygen. Interestingly, around 70% of previous organ culture experiments have been carried out under high glucose (4.5 g/L or 25 mM) medium conditions.[119] Computational and experimental models show that high glucose media results in glucose levels between ~5–15 mM in the center of an organ-cultured bovine caudal IVD, depending on the size of the IVD.[119] In general, these high glucose conditions are referred to as a "physiological" culture environment. Indeed, a significant drop in cell viability by 40%–50% has been observed in both NP and AF of ovine IVDs cultured in low glucose media containing 2 g/L (11 mM) glucose compared to the standard high glucose (4.5 g/L) condition.[120] The reduction in cell viability was evident after 7 days and was stable until 21 days of culture under simulated physiological loading conditions in a bioreactor. Moreover, limited glucose culture can be implemented as a degeneration organ culture model, simulating compromised nutrition in combination with high-frequency loading, which showed additive effects on cell death.[121] Studies with bovine IVDs confirmed the findings from ovine explants, demonstrating a decrease in AF and NP cell viability under low glucose (2 g/L) medium and high-frequency loading conditions.[104,122] Meanwhile, low glucose concentration is viable for culturing human cells due to the low cell concentration, further contributing to the clinical advantage of human organ culture.[123–126]

In view of the physiological blood glucose level of approximately 5.5 mM, the level of 25 mM necessary to keep the IVD cells viable seems highly supra-physiological. In fact, high blood glucose levels in vivo have been shown to be detrimental to IVD homeostasis. Similarly, the predicted physiological intradiscal in vivo glucose levels are 5–10 times lower than the computed and measured ex vivo levels (see above).[113,115,116,119] This discrepancy may result from differences between the ex vivo and in vivo situations, such as the absence of capillaries in the IVD explants, the different mechanical loading, and osmotic pressure conditions.

Several studies have shown that low oxygen concentrations of 1%–5% are beneficial for the maintenance of the NP cell phenotype.[127,128] Most reported IVD organ culture experiments have been conducted under normal oxygen conditions externally, implying 20%–21% oxygen tension to the outer regions of the disc. According to computed or experimental data, this would correspond to an approximate oxygen tension of 1%–5% in the center of a bovine IVD,[119] which is similar to the in vivo oxygen tension. The removal of the CEP significantly alters the diffusion into the center of the IVD. Therefore, oxygen levels of 1%–5% are in line with the physiological levels that are known to promote the phenotype and function of IVD cells, and this can be reproduced using organ culture models that retain the CEP.

The experimentally determined and predicted pH values of standard cultured bovine IVD organ cultures have been reported to range between ~6.7 and ~6.9; hence, they are quite consistent with measured values from patients.[117] An increase in oxygen concentration and pH level was however predicted in a numerical model when dynamic axial compression was applied to the disc,[118] emphasizing the importance of mechanical bioreactors for culture of whole IVD organs.

IVD cell nutrition equally depends on the diffusion of nutrients through the CEP and/or the AF. In most ex vivo organ cultures, the vertebral bone part is removed, whereas the CEP is maintained. Care should be taken to clean the CEP from blood clots and debris to facilitate the diffusion of molecules into and out of the IVD,[93] since the central endplate region has been recognized as the major area of nutrient exchange.[129] In this context, the species- and age-related differences in the CEP thickness and the presence of a growth plate in young animals need to be considered, as these parameters can markedly influence the diffusion rate. There are also organ culture systems where the bony endplate and some vertebral bones are maintained as well. These cultures require a special preparation that ensures the preservation of both the bony structure and long-term IVD cell viability.[130] Furthermore, it has been suggested that nutrient exchange through the AF plays a more prominent role in organ-cultured IVDs, because of the increased lateral surface area surrounding the AF which permits more nutrient transport through the periphery compared to the in vivo situation.[119]

Taken together, by varying the glucose concentration, oxygen tension, pH, and nutrient transport, various metabolic states can be induced in organ-cultured IVDs, which may represent different degrees or types of degeneration. Current numerical models provide a relevant indication of the intra-discal nutrient gradients under defined circumstances. More experimental and clinical data are required to adjust each organ model to a particular clinical situation. Importantly, however, in ex vivo organ culture, there is consistency and control over all these biochemical influences, which are poorly controlled in in vivo models: levels can be measured and maintained in a predictable fashion, removing confounding factors from studies.

3.6 | Mechanical loading

Another major advantage of organ culture models over in vivo animal models is the ability to control mechanical loading at the tissue level, and even present models with the desired mechanical properties and

level of tissue damage to mimic physiological or disease conditions and relevant forces. Most animal models, with the exception of primates, are quadrupeds, which may differ in load transfer throughout the spine compared to bipedal humans. In addition, the sizes and geometries of animal IVDs exhibit differences compared to human IVDs as highlighted previously, which may confound the ability to study IVDD under physiological human conditions. The use of organ culture permits researchers precise control of the mechanical forces presented to the IVD, including physiological and injurious loading similar to that experienced by the human spine. The human IVD is normally exposed to multimodal loading (compression, tension, shear, HP, and osmotic pressure) ranging up to 4× body weight.[131–138] Organ culture models have provided significant insights into the response of the IVD to loading. Zonal biological responses have been observed that depend on tissue location, magnitude, and frequency of loading.[118,139–145] A maintenance stimulus of approximately 0.1–0.5 Hz applied at moderate stress levels (e.g., 0.2–0.5 MPa) promotes steady-state IVD metabolic responses. Compressive loading above this level (e.g., high-frequency loading) or below this level (e.g., static loading) typically results in remodeling or degeneration. Occupational exposures to high-frequency vibration can also cause LBP[146] and IVDD.[147,148] Lying in recumbency promotes rehydration, increasing IVD height and volume, and normalization of intradiscal hydrostatic pressure,[149] which can be simulated in organ culture with diurnal loading profiles. Exercise can be beneficial for the IVD, with specific moderate-frequency exercise protocols providing the greatest improvement in IVD material properties.[150] These loading factors can be simulated in organ cultures with the use of dynamic mechanical loading profiles. Indeed, dynamic loading is favorable for promoting mechanotransduction in IVD cells and for maintaining physiological nutrition, whereby at least a diurnal cycle, representing daily IVD compression and decompression (recovery, or swelling) can be applied to organ cultures of isolated whole IVDs.[93,118,151] Advanced bioreactor systems will allow researchers to apply controlled multiaxial loading to the IVD under long-term culture conditions.[152] In contrast, the application of controlled, physiological loading using in vivo models is extremely challenging, and has only been successfully accomplished in rodents and rabbits.[153–155]

3.7 | Systemic effects

Since the healthy IVD is a largely avascular, immune-privileged organ, unless structural defects expose it to the systemic environment of the body, infiltrating immune cells generally do not penetrate the intact healthy IVD, and thus isolated studies within an organ culture setting are physiologically appropriate. Furthermore, the influence of systemic co-morbidities such as diabetes can be investigated at a mechanistic level, for example by identifying influences of increased glucose or the presence of damage-associated molecular patterns (DAMPS) without compounding factors such as obesity and poor circulation, which occur in vivo and manifest differently in different animal models. Thus, where systemic effects such as inflammation and diabetes are described as advantages of in vivo models in the presence of immune cell migration/infiltration, in an intact IVD, this has little relevance or appropriateness; however, organ culture models have a unique advantage with respect to assessing specific mechanisms, by controlling the presence of specific immune cells to simulate interactions of the local or systemic immune system with rupture or disease.[156,157] Other specific soluble factors such as catabolic enzymes and cytokines, and DAMPs, can also be simulated with organ culture models,[158–160] in addition to environmental factors (e.g., glucose) which allows for a more precise mechanistic evaluation than in vivo.

3.8 | Tissue-specific responses and cross-talk

A key argument for in vivo, as opposed to organ culture studies, is that tissue cross-talk cannot be investigated in organ culture studies. On the contrary, organ culture models allow for the disambiguation of different tissue types within the IVD and surrounding bone structures and muscles, providing the capability for studying tissue-specific responses. They can also be co-cultured in the presence of multiple associated tissues, enabling carefully controlled tissue crosstalk investigations to be undertaken. The dissection of tissue-specific roles and interactions cannot be studied easily in in vivo models. While using co-culture systems, specific cross-talk investigations can be investigated, where IVDs complete with CEPs can be maintained within a loaded bioreactor improving nutrient flow and maintenance of IVD and bone cell viability. IVDs could also potentially be co-cultured with muscle, ligament, nerve, and fat to investigate tissue cross-talk in a controlled environment, enabling mechanistic interactions between these tissues to be understood.

3.9 | Rapid degeneration models

In vivo models generally require long-term time points (anywhere from weeks to months) in order to generate IVDD comparable to the human condition. In comparison, rapid degeneration can be induced in organ culture models, which allows the study of IVDD under accelerated conditions, thus reducing the time needed for respective studies. For example, using enzyme induction of degeneration in a large animal goat or sheep model, 3 months is required for induction of degeneration,[161] while a similar degeneration process can be induced within 1 week using organ cultures of enzyme degradation followed by physiological loading.[162]

3.10 | Imaging

While imaging, such as micro-CT (with the use of contrast agents) and MRI can be conducted in vivo or ex vivo; the resolution and fidelity of the acquired data are typically superior in the ex vivo scenario where the surrounding tissues are removed and thus do not obscure the IVD. Additional advantages of ex vivo imaging include the ability to

conduct longer imaging sessions (thereby improving the signal-to-noise ratio), the lack of motion artifacts from breathing, and not having to handle and administer anesthesia. The improvement in resolution and imaging quality enables more sophisticated biochemical and detailed structural analyses of the IVD and enables more mechanistic studies to be conducted. Likewise, parallel, clinically relevant imaging parameters, such as IVD hydration, IVD height index, and bone parameters, can also be obtained from the higher-resolution ex vivo imaging.[97,163–165] Another advantage in terms of imaging organ culture models is the application of molecular imaging to track changes in the biological activity of cells in organ culture over time (e.g., cell metabolism). One example of this is the use of fluorescence molecular tomography (FMT) which is capable of retrieving the 3D bio-distribution of fluorescent molecular markers noninvasively, thus offering higher molecular sensitivity than microCT or MRI.[166] One key feature of FMT is the use of near-infrared (NIR) fluorescence probes, which have been shown to be the most effective for deep tissue imaging. In the NIR spectral range, the attenuation of living tissue is minimal, allowing the use of sufficient laser power for fluorescence excitation and detection without causing tissue damage under prolonged illumination. Moreover, molecular-based imaging findings can also be coupled with analyses of changes in the culture media, to inform coupled in situ and surrounding microenvironmental changes.

3.11 | Regulatory requirements and clinical translation

A major argument put forward by in vivo model proponents involves the regulatory requirements for in vivo animal evaluations prior to human clinical trials. However, numerous studies have shown critical differences between animals and humans, and not just solely in the spine field. With the further development of highly functional and systemically controlled organ culture systems, the use of animals could be reduced, and regulatory pathways limited to more ethical and clinically relevant ex vivo human organ culture testing.

4 | APPROPRIATE MODEL SELECTION

It is clear that the selection of a model system for any project must be driven by the research question. Just as an inappropriate sample size can invalidate the results of a project, so too can the use of an inappropriate model system. Therefore, an understanding of the strengths and weaknesses of the various ex vivo organ culture and in vivo models available is a critical step in study design. The relative strengths of in vivo animal and ex vivo organ culture models, as outlined in the preceding sections and summarized in Figure 1, are not necessarily universal; again, they are driven by the question that is being asked and the outcome measures that best answer that question. A particularly obvious example would be that it would not be possible to test a new spinal implant intended for human use in a rat or rabbit, but it would be achievable in a sheep, pig, or calf model. When selecting among the available options, in this case, outcome measures are particularly important: what do you need to sample, how often, and over what period of time?

Both organ culture and in vivo models have their limitations, but both also play a vital role in the overall successful understanding of the disease process and the development of potentially life-changing therapies for human patients. The types of available in vivo animal models used for spine research have ranged from non-mammalian vertebrates (such as zebrafish) to small mammals (such as rodents) to large mammals (such as dogs and livestock). With the increasing complexity of the model, there is more likely to be translation to human disease; however, the increased complexity may complicate the mechanistic understanding across multiple tissues. Furthermore, the cost of larger models is higher, both in dollars and potentially in negative public perception. In general, single-cell organisms, invertebrates, and non-mammalian vertebrates have the most utility in investigating the cellular or molecular basis of disease. Non-mammalian vertebrates and some rodents are amenable to genetic manipulation, allowing for the creation of genetic models that display a particular phenotype (which may include susceptibility or resistance to disease). This type of manipulation is not currently possible in most large mammalian models, but these species are highly useful for the study of naturally occurring and induced models of disease. It should be noted that modern gene-editing technology is making genetic manipulation of larger animals more feasible.[167] No animal model can perfectly recapitulate human disease, and the ability to use cadaveric human tissue in organ culture must be considered a potential advantage of that approach. Organ culture models may offer the benefit of systematic control of the biomechanics and metabolics of the experimental system that more closely mimic the human condition.

There are many advantages to naturally occurring models of disease. Because they are closest to the "real mechanism," they can give the best insight into disease biology and the best evaluation of diagnostics and therapeutics. Furthermore, if companion animals can be used—for example, when studying IVDD in dogs—then researchers may be able to recruit client-owned cases, which may reduce costs and reduce unnecessary animal usage. Such investigations are also of dual benefit, with advances made for the treatment of the species being studied as well as potential translational benefits to humans. However, there are also possible disadvantages to studying naturally occurring diseases, including the fact that variables beyond the researchers' control may affect results (such as genetic diversity within highly outbred species), and appropriate cases may be difficult to find.

In contrast, experimentally induced models have the advantage of enhanced reproducibility of the intervention/injury, in as many animals as needed and when they are needed. The downside is that induced disease may not exactly recapitulate natural disease and therefore response to therapeutics might not translate perfectly. Furthermore, there are significant costs and ethical concerns to navigate. When considering induced models of disease for spine research, surgical models are most common; however, other methods of inducing disease may be considered, including genetic manipulation, dietary manipulation, and chemically induced disease.[23]

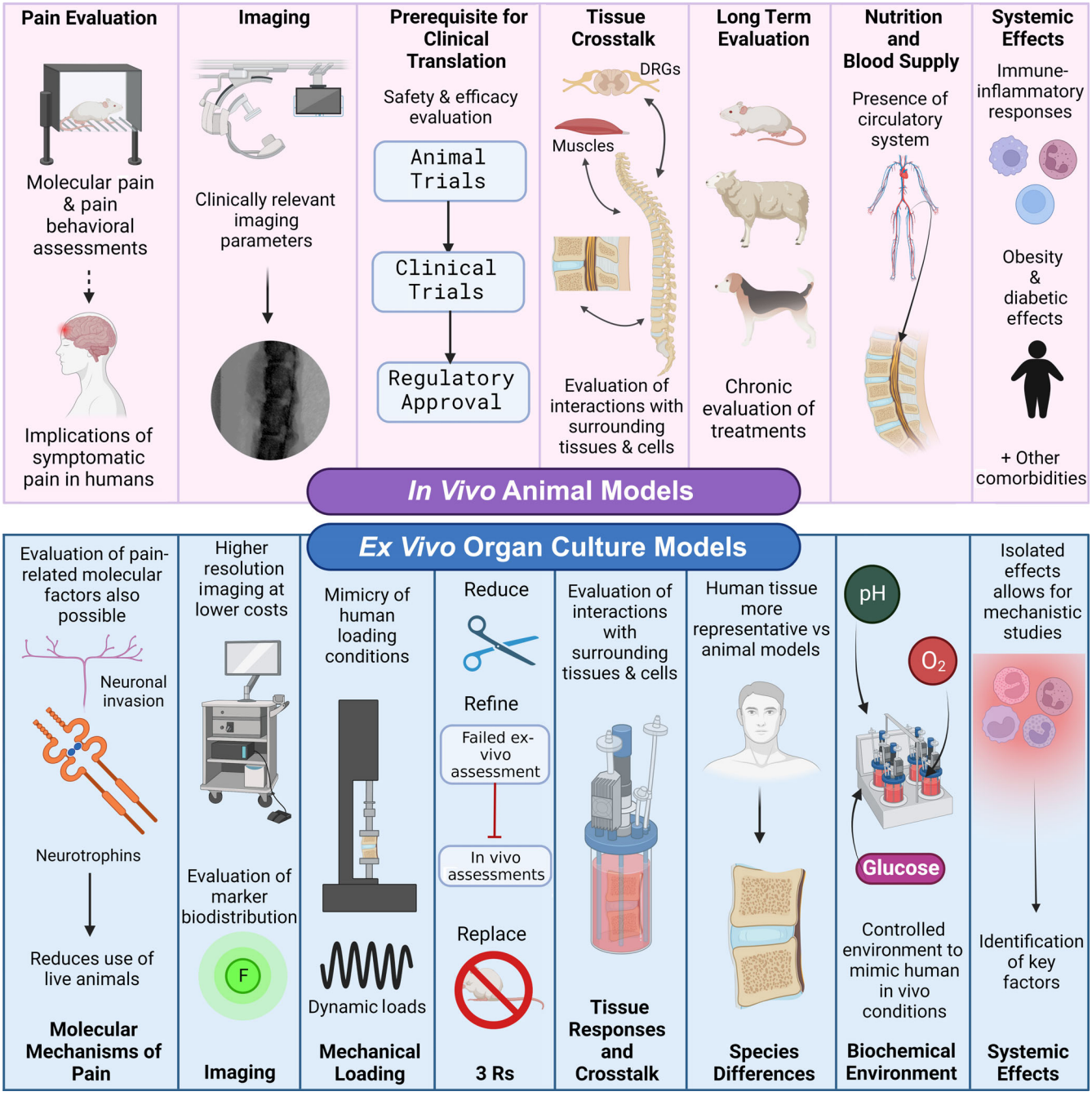

FIGURE 1 Summary of the respective advantages of in vivo animal versus ex vivo organ culture models for studies of the intervertebral disc. Figure created using BioRender.com by Shirley N. Tang with license to publish

There is no single "gold standard" model for IVD research precisely because different research questions lend themselves to different approaches. Thus, the path to selecting the right model starts with the research question. This will lead to the outcomes of interest, and the selection of the methods that will be used to measure them. These, in turn, will drive the selection of a specific model. In some cases, the elimination of clearly unsuitable models may be the easiest first step. From there, the strengths and weaknesses of potentially suitable options can be weighed. It may turn out that two models could answer your question equally well, and in this case, other factors such as cost and convenience will certainly play a role. It is possible (even likely) that a broad research question cannot be answered effectively by a single model, and that multiple models must be used in sequence or simultaneously to address different aspects of the question. Indeed, complementary use of several IVD model types and leveraging the unique advantages of each is likely to result in the highest impact research in most instances. For example, taking the development of a novel biologic for IVDD treatment as a general case study, a study may commence by establishing and characterizing an IVDD phenotype in a naturally occurring or transgenic rodent model,

and identifying a putative therapeutic target. Subsequently, potential therapeutic agents could be screened in organ culture models under controlled experimental conditions and utilizing cadaveric human discs to confirm relevance to the human condition. Short-term safety and efficacy studies could then be undertaken in rodent or rabbit models, followed by longer-term studies in large animal models using gold-standard clinically relevant outcome measures. As access to such a wide array of model systems may be beyond the capabilities of a single laboratory, financially and/or logistically, such studies could be undertaken through collaborations across laboratories and institutions.

In conclusion, in this article, we debate the relative advantages of in vivo animal and ex vivo organ culture models for studies of the IVD. In doing so we also identify their respective limitations, and the continued need to strive for improved experimental platforms in order to achieve the best possible treatment outcomes for LBP patients. Many reviews of different IVD model systems are available in the published literature,[23,93,168–171] and these can serve as valuable resources for researchers seeking the best model system for their research question. Consideration should also be given to the development and use of standardized outcome measures for various models,[172,173] which makes comparing results across studies easier and more valuable.

AUTHOR CONTRIBUTIONS

Lachlan J. Smith conceived the original idea for the manuscript. Andres F. Bonilla and Shirley N. Tang led the drafting of the in vivo and organ culture subsections respectively. All authors provided conceptual input, drafted sections of the manuscript, and reviewed and approved the completed document prior to submission.

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CONFLICT OF INTEREST

Lachlan J. Smith, Sibylle Grad, Victor Leung, Christine L. Le Maitre, and Devina Purmessur are members of the JOR Spine Advisory Review Board. The authors have no other relevant conflicts to declare.

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