An intervertebral disc whole organ culture system to investigate proinflammatory and degenerative disc disease condition

Gernot Lang¹ | Yishan Liu¹,² | Janna Geries¹,² | Zhiyu Zhou²,³,⁴ | David Kubosch¹ | Norbert Südkamp¹ | R. Geoff Richards¹,² | Mauro Alini² | Sibylle Grad² | Zhen Li²,⁴

¹ Department of Orthopedics and Trauma Surgery, University Medical Center Freiburg, Albert-Ludwigs-University of Freiburg, Freiburg im Breisgau, Germany
² AO Research Institute Davos, Davos, Switzerland
³ Guangdong Provincial Key Laboratory of Orthopedics and Traumatology/Orthopedic Research Institute, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China
⁴ Shenzhen Key Laboratory of Anti-aging and Regenerative Medicine, Department of Medical Cell Biology and Genetics, Health Sciences Center, Shenzhen University, Shenzhen, China

Correspondence
Zhen Li, AO Research Institute Davos, Clavudelerstrasse 8, 7270 Davos Platz, Switzerland.
Email: zhen.li@aofoundation.org

Funding information
Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing (SET), Grant/Award Number: 59 InflamoDisc; National Natural Science Foundation of China, Grant/Award Number: 81772333; German Research Foundation (DFG); Sino-Swiss Science and Technology Cooperation, Grant/Award Number: EG 04-032015; China Scholarship Council; Natural Science Foundation of Guangdong Province, Grant/Award Number: 2014A030310466

Abstract
The aim of this study was to compare the effect of different disease initiators of degenerative disc disease (DDD) within an intervertebral disc (IVD) organ culture system and to understand the interplay between inflammation and degeneration in the early stage of DDD. Bovine caudal IVDs were cultured within a bioreactor for up to 11 days. Control group was cultured under physiological loading (0.02–0.2 MPa; 0.2 Hz; 2 hr/day) and high glucose (4.5 g/L) medium. Detrimental loading (0.32–0.5 MPa, 5 Hz; 2 hr/day) and low glucose (2 g/L) medium were applied to mimic the condition of abnormal mechanical stress and limited nutrition supply. Tumour necrosis factor alpha (TNF-α) was injected into the nucleus pulposus (100 ng per IVD) as a proinflammatory trigger. TNF-α combined with detrimental loading and low glucose medium up-regulated interleukin 1β (IL-1β), IL-6, and IL-8 gene expression in disc tissue, nitric oxide, and IL-8 release from IVD, which indicate a proinflammatory effect. The combined initiators up-regulated matrix metalloproteinase 1 gene expression, down-regulated gene expression of Type I collagen in annulus fibrosus and Type II collagen in nucleus pulposus, and reduced the cell viability. Furthermore, the combined initiators induced a degradative effect, as indicated by markedly higher glycosaminoglycan release into conditioned medium. The combination of detrimental dynamic loading, nutrient deficiency, and TNF-α intradiscal injection can synergistically simulate the proinflammatory and degenerative disease condition within DDD. This model will be of high interest to screen therapeutic agents in further preclinical studies for early intervention and treatment of DDD.

KEYWORDS
degenerative disc disease, inflammation, intervertebral disc, organ culture, TNF-α, detrimental loading

1 INTRODUCTION

Low back pain is one of the most prevalent musculoskeletal conditions and a leading cause of disability in western societies, resulting in enormous socio-economic burden (Hoy et al., 2010). To date, it is believed that degeneration of the intervertebral disc (IVD) is a major contributor to low back pain (Freemont, 2009; Pye et al., 2004). The IVD contains the soft and gelatinous nucleus pulposus (NP), which is surrounded by the fibrocartilaginous annulus fibrosus (AF), and the cartilaginous endplates, which connect the IVD to the adjacent vertebrae. Degenerative disc disease (DDD) is characterized by extracellular matrix (ECM) degradation, release of proinflammatory cytokines, altered spinal biomechanics, and neovascularization, which can lead to an increased pain sensation (Le Maitre, Hoyland, & Freemont, 2007b; Podichetty, 2007). DDD can be induced, triggered, and exacerbated by multiple factors, including mechanical stress, infection, trauma, genetic predisposition, and inflammation (Rannou, Revel, & Poiraudeau, 2003; Stirling, Worthington, Rafiq, Lambert, & Elliott, 2001). Recently, it was suggested that the “first hit” (initial damaging event—i.e., trauma and infection) in the course of DDD is followed...
by an inflammatory response within the IVD (Risbud & Shapiro, 2014). Tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) are key proinflammatory cytokines contributing to disc degeneration, inflammation, and discogenic pain. Both cytokines regulate the expression of major catabolic enzymes for ECM deterioration, such as A Disintegrin and Metalloprotease with Thrombospondin Motifs (ADAMTS) and matrix metalloproteinases (MMP), through activation of mitogen-activated protein kinase and NF-κB signalling pathways, which subsequently leads to tissue degeneration (Tian et al., 2013). TNF-α has been shown to be highly expressed in degenerated human IVD tissue (Weiler, Nerlich, Bachmeier, & Boos, 2005). The inflammatory and degenerative cascade of DDD is further accelerated through chemokine-promoted recruitment of T- and B-cells, macrophages, neutrophils, and mast cells causing neovascularization and chronic pain (Hayashi et al., 2008; Kokubo et al., 2008; Murata et al., 2006; Olmarker & Larsson, 1998; Olmarker & Rydevik, 2001; Redford, Hall, & Smith, 1995; Shamji et al., 2010).

Because the IVD does not possess a sufficient self-repair capacity, current treatment options for DDD range from conservative treatments to invasive therapies for severe and symptomatic courses of DDD, such as spinal fusion or total disc replacement, although the long-term benefit compared to conservative therapies remains questionable and complications are common (Ghiselli, Wang, Bhatia, Hsu, & Dawson, 2004; Lurie et al., 2014). Additionally, none of the current invasive techniques address the underlying pathogenesis. Numerous patients with mild to moderate symptomatic disc degeneration who failed conservative therapies and at the same time do not qualify for surgery still face the lack of a sufficient therapeutic option that relieves pain while preserving motion without the adverse effects associated with conventional approaches.

Therapies that inhibit the expression and function of proinflammatory cytokines might be a promising therapeutic approach. The aim of this strategy is to reduce the inflammatory micro-environment of the IVD in order to slow down the progressive degenerative cascade, preserve the IVD’s mechanical and biological function, and relieve pain at early to moderate stages of degeneration.

IVD whole organ culture bridges the gap between in vitro and in vivo assays. Establishment of a proinflammatory and degenerative IVD organ culture model can be instrumental in deciphering the role of therapeutic agents in a relevant and controlled environment. A whole organ culture system, which is capable of inducing degeneration via high frequency dynamic loading and limited nutrition, was introduced utilizing ovine caudal IVD by our group (Illien-Junger et al., 2010); although the proinflammatory effect of this degenerative culture condition has not been investigated. In recent years, bovine IVD organ culture models have gained popularity because the dimensions and biological composition of bovine IVD is relatively equivalent to human discs (Alini et al., 2008; Gawri et al., 2014; Li, Lang, et al., 2016; Purmessur et al., 2013). However, previous studies investigating the interplay between inflammation and DDD lack the influence of dynamic loading besides an proinflammatory environment and nutrition deficiency (Ponnappan et al., 2011; Purmessur et al., 2013; Teixeira et al., 2016; Walter, Purmessur, et al., 2015).

The aim of the current study was to compare the effect of different initiators of DDD within an IVD organ culture system. Specifically, the interplay of detrimental loading, limited nutrition, and the proinflammatory micro-environment was assessed by analysing the cellular and tissue response of the IVD to provide novel insights on the pathophysiology of DDD.

2 | MATERIALS AND METHODS

2.1 | IVD dissection and organ culture

Bovine caudal spines (7–12 months old) were obtained from local abattoirs. After removing the soft tissue, IVDs were dissected with a band saw (Exakt Apparatebau, Norderstedt, Germany), and the endplates of each IVD were cleaned with a Pulsavac jet-lavage system (Zimmer, IN, USA; Li, Lang, et al., 2016). Hereafter, the IVDs were washed with phosphate buffered saline (PBS) containing 10% Pen/Strep (Penicillin/Streptomycin) for 15 min, then transferred to a six-well plate and incubated at 37 °C, 85% humidity and 5% CO2. IVDs were cultured in Dulbecco’s Modified Eagle Medium supplied with 2% fetal calf serum, 1% Pen/Strep, 1% ITS+ Premix (Discovery Labware, Inc., Bedford, USA), 50 μg/ml ascorbate-2-phosphate (Sigma–Aldrich, St. Louis, USA) and 50 μg/ml Primocin (InvivoGen, San Diego, USA). The experiments were performed using IVDs from 12 bovine tails, with five IVDs from each tail. The disc height of bovine IVDs for this study was 10.31 ± 1.47 mm, and the disc diameter was 16.03 ± 1.54 mm. IVDs from each tail were randomized among four experimental groups (Phy, Phy + TNF-α, Deg, Deg + TNF-α, see definition in 2.2) and Day 0 control group to achieve equivalent mean disc size and distribution of disc levels for each group.

2.2 | IVD organ culture under different dynamic loading and nutrient conditions

Bovine IVDs were cultured for 4 or 11 days within a bioreactor system (Illien-Junger et al., 2010) under four culture conditions: (a) Phy—physiological loading (0.02–0.2 MPa; 0.2 Hz; 2 hr/day) and high glucose (4.5 g/L) medium, (b) Phy + TNF-α—physiological loading and high glucose medium, with TNF-α injection, (c) Deg—degenerative loading (0.32–0.5 MPa; 5 Hz; 2 hr/day) and low glucose (2 g/L) medium, and (d) Deg + TNF-α—degenerative loading and low glucose medium, with TNF-α injection. Our previous study (Illien-Junger et al., 2010) has shown that culture of IVDs under either limited glucose at 2 g/L (AF 65%, NP 75%) or high frequency loading conditions (AF 61%, NP 55%) led to a significant drop in cell viability. Combined treatment with limited glucose and high frequency loading resulted in an additive increase in cell death in both the AF (alive cells 39%) and NP (alive cells 57%), and in an increase in gene expression of catabolic marker MMP13. Therefore, in the current study, low glucose at 2 g/L combined with high frequency high amplitude loading was selected as detrimental culture condition. To investigate if the induced degenerative and inflammatory effect is reversible, a group of IVDs were cultured first under degenerative loading and low glucose medium with TNF-α injection for 4 days, followed by physiological loading and high glucose medium for 7 days. During loading, IVDs were positioned in custom-made chambers containing 5-ml IVD culture medium. Between dynamic loading, IVDs were cultured in six-well plates with 7-ml IVD culture...
medium for free swelling recovery overnight. It has been reported that the diurnal disc height loss of human lower lumbar IVDs was 11.1% (Botsford, Essex, & Ogilvie-Harris, 1994). Therefore, a 10% diurnal disc height loss was considered as physiological for organ cultured discs. Within the current study, our physiological loading protocol applied on bovine caudal IVDs has shown to be physiologically relevant, as indicated by disc height loss and recovery at physiological level.

2.3 | Intradiscal injection of TNF-α

Recombinant human TNF-α (R&D systems, Zug, CH) was injected one-time with a 30-gauge insulin needle (Braun, Melsungen, GE) into the NP tissue, after the first dynamic loading cycle on Day 1, at a concentration of 100 ng TNF-α within 70 μl PBS/IVD. This concentration was selected based on literature, which has shown that same amount of TNF-α injected into porcine lumbar IVD induced early stage disc degeneration (R. Kang et al., 2015). Injection test with PBS containing trypan blue dye revealed reproducible and even distribution of injected liquid in the NP tissue, and macroscopic evaluation of the IVD after free swelling showed that no back leakage was observed with this injection method (Figure S1A). To investigate the effect of PBS injection with the insulin needle, a pilot experiment (n = 4) was performed to measure the gene expression of NP and AF tissues after 4 days of culture. For none of the gene expression levels, PBS injection with insulin needle showed any dysregulation effect (Figure S1B–E). Therefore, IVDs cultured under Phy or Deg culture condition without PBS injection were used as control groups in the following experiments.

2.4 | Disc height change

To assess whether detrimental loading and/or intradiscal injection of TNF-α caused biomechanical alterations within cultured IVDs, the total amount of creep and recovery was assessed by measuring the disc height with a caliper at the following time points: Day 0 after dissection, then after free swelling culture overnight and after dynamic load daily. Each IVD was measured at two positions, and the average value was used to calculate the percentage of disc height change. Disc height change was normalized to the initial dimension after dissection.

2.5 | Analysis of gene expression and protein expression

Disc tissue was collected on Day 4 or Day 11 for gene expression measurement. Cartilaginous endplates of each IVD were removed, and NP and AF tissues were harvested by using a biopsy punch and a scalpel blade. Approximately 150 mg of each AF and NP tissue was used for RNA extraction; 50 mg of each AF and NP tissue was digested with 0.5 mg/ml proteinase K at 56 °C overnight for glycosaminoglycan (GAG), collagen, and DNA content measurement. For RNA extraction, tissue samples were digested with 2 mg/ml pronase for 1 hr at 37 °C, flash frozen, pulverized in liquid nitrogen, and homogenized using a TissueLyser (Qiagen, Venlo, Netherlands; Lee et al., 2007; Peroglio et al., 2017). Total RNA was extracted with TRI Reagent (Molecular Research Center), and reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA). Quantitative real-time polymerase chain reaction was performed using the Step-One-Plus instrument (Life Technologies). The sequences of custom designed bovine primers and TaqMan™ probes are shown in Table S1. For amplification of ribosomal protein large P0 (RPLP0, Bt03218086_m1), IL-8 (Bt03211906_m1), and β-catenin (CTNNB1, Bt02667775_m1), gene expression assays from Applied Biosystems (Life Technologies) were used. Comparative Ct method was performed for relative quantification of target mRNA with RPLP0 as endogenous control (Lopa et al., 2016).

The GAG content in the NP and AF tissues was determined by using the 1,9-dimethylmethylene blue dye (DMMB) method (Farndale, Buttle, & Barrett, 1986). Quantification of the total amount of hydroxyproline (OHP) in NP and AF samples was performed as described previously (Li, Lang, et al., 2016). DNA content was measured spectrophotometrically using Hoechst (33258) dye.

Conditioned medium was collected for analysis of released matrix components and mediators. The GAG content in the conditioned medium was determined using the DMMB method (Farndale et al., 1986). Levels of nitric oxide (NO) production in the conditioned medium of IVDs were determined as the concentration of its stable oxidation product, nitrite (NO2−), using the Griess Reagent Kit (Promega, Madison, USA; J. D. Kang et al., 1996). IL-1β, IL-6, and IL-8 content was measured with bovine IL-1β, IL-6, and IL-8 Do-It-Yourself ELISA kits (Kingfisher Biotech, St. Paul, USA).

2.6 | Histology

After free swelling recovery on Day 11, whole IVDs were frozen in cryo-embedding compound (Sismex, Horgen, CH) after removal of the intact endplate from one side. Transverse sections (10 μm) were cut with a microtome (Microm, Germany). Sections were stained with 0.1% Safranin-O and 0.02% Fast Green to reveal proteoglycan and collagen deposition, respectively, and counterstained with Weigert’s Haematoxylin to reveal cell distribution.

Cell viability was determined using lactate dehydrogenase and ethidium homodimer-1 staining as described previously (Li, Lezuo, et al., 2016). Staining was performed with ethidium homodimer (1 μg/ml) and lactate dehydrogenase in 40% polypep solution (Sigma-Aldrich, Buchs, Switzerland; Li, Lezuo, et al., 2016). Blue or blue/red staining indicates a living cell, whereas red only staining indicates a dead cell. The cell viability at the NP, inner AF, and outer AF regions of IVDs were quantified with the Axiovision software. Two IVDs per group were analysed, from which four randomly taken images in each region of each IVD were counted.

2.7 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). D’Agostino-Pearson omnibus normality test was used to define whether the data were normally distributed. For data that were normally distributed, unpaired t test was used to determine differences between two groups; one-way analysis of variance was used to determine differences between three or more groups. For data that were not normally distributed, Mann-Whitney U test was used to determine differences between two groups; Kruskal Wallis test was used to determine differences
between three or more groups; \( p < .05 \) was considered statistically significant.

3 | RESULTS

3.1 | Gene expression

After 4 days of culture with dynamic load, gene expression levels of NP and AF tissues were compared to the IVD tissues from respective bovine tails before starting the organ culture on Day 0 (Figures 1 and 2). Deg culture condition down-regulated COL2 gene expression in the NP, under the conditions with \(( p = .0321)\) or without \(( p = .0150)\) TNF-\( \alpha \) injection (Figure 1a). Compared with Phy culture condition, Deg culture condition combined with TNF-\( \alpha \) injection further reduced COL2 gene expression \(( p = .0049,\) Figure 1a). 

TNF-\( \alpha \) down-regulated COL1 gene expression in the AF under Phy culture condition \(( p = .0229,\) Figure 2a). Deg culture condition \(( p = .0518)\) and combined condition \(( p = .0549)\) also showed a trend of down-regulation for the COL1 gene expression (Figure 2a). Gene expression level of ACAN was neither altered by detrimental culture conditions nor by the inflammatory micro-environment. Furthermore, MMP1 a major catabolic enzyme showed a trend of increase under Deg culture condition \(( p = .0743)\) and was significantly

FIGURE 1  Gene expression level of NP tissue after 4 days of culture under Phy or Deg culture condition, with or without TNF-\( \alpha \) intradiscal injection, \( n = 8, * p < .05, ** p < .01, \) min to max (bottom and top bar) with interquartile range (middle box). NP = nucleus pulposus; ADAMTS = A Disintegrin and Metalloprotease with Thrombospondin Motifs; MMP = matrix metalloproteinases; TNF-\( \alpha \) = tumour necrosis factor alpha [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2  Gene expression level of AF tissue after 4 days of culture under Phy or Deg culture condition, with or without TNF-\( \alpha \) intradiscal injection, \( n = 8, * p < .05, \) min to max (bottom and top bar) with interquartile range (middle box). AF = annulus fibrosus; ADAMTS = A Disintegrin and Metalloprotease with Thrombospondin Motifs; MMP = matrix metalloproteinases; TNF-\( \alpha \) = tumour necrosis factor alpha [Colour figure can be viewed at wileyonlinelibrary.com]
overexpressed in the NP tissue due to TNF-α injection combined with Deg culture condition ($p = .0284$, Figure 1b), revealing the strong degenerative stimulus even after short-term culture. Other catabolic enzymes ADAMTS4, ADAMTS5, and MMP13 did not present altered gene expression.

In addition to the induction of disc catabolism, detrimental culture condition and TNF-α intradiscal injection resulted in a strong inflammatory response by means of an up-regulation of proinflammatory gene expression markers. Deg culture condition alone induced an increase of IL-6 ($p = .0339$, Figure 1c) and IL-8 ($p = .0489$, Figure 1c) gene expression in NP. TNF-α injection combined with Deg culture condition further enhanced the up-regulation of IL-6 ($p = .0044$) and IL-8 ($p = .0024$) in NP. In AF, TNF-α injection combined with Deg culture condition up-regulated IL-1β gene expression ($p = .0315$, Figure 2c).

### 3.2 GAG, NO, IL-1β, IL-6, and IL-8 content in conditioned medium

Conditioned medium was collected daily during free swelling period and loading period for the measurement of released molecules from IVD. Under Deg culture condition, TNF-α injection up-regulated GAG release during the free swelling period on Day 3 ($p = .0280$), as well as the NO release during the free swelling period on Day 4 ($p = .0281$). Intradiscal TNF-α injection combined with Deg culture condition enhanced GAG as well as NO release of IVD compared to Phy culture condition in the conditioned media during the free swelling period from Day 3 on (Figure 3a,b).

According to the gene expression results, most significant differences in inflammatory marker expression were only observed between

![Figure 3](https://via.placeholder.com/150)

**FIGURE 3** (a) GAG, (b) NO, (c) IL-6, and (d) IL-8 release in IVD culture medium during 20 hr of free swelling culture (FS) and 2 hr of dynamic loading (load), (e) disc height change normalized to the original dimension after dissection. Means $\pm$ 95% confidence interval, (a–d) $n = 8$, (e) $n = 10$, *$p < .05$, **$p < .01$, ***$p < .001$. GAG = glycosaminoglycan; NO = nitric oxide; IVD = intervertebral disc.
the Phy group and the Deg + TNF-α group. Therefore, IL-1β, IL-6, and IL-8 content in the conditioned medium from these two groups were measured to investigate the protein expression levels. IL-1β concentration was below the detection limit within the medium samples. Deg culture condition and TNF-α injection did not alter IL-6 release (Figure 3c), but significantly up-regulated IL-8 release on Day 2 during loading (p = .0070) and on Day 4 during free swelling (p = .0047, Figure 3d).

3.3 Disc height change

After repetitive dynamic loading, the degenerative loading protocol caused significantly higher disc height loss (~20%) compared to the physiological loading protocol (~10%, p < .001; Figure 3e). TNF-α intradiscal injection did not further induce disc height loss after loading. After free swelling recovery, all the IVDs recovered to the initial disc height before load. This diurnal disc height change pattern was observed throughout the entire period of 4 days repetitive dynamic load (Figure 3e). The same pattern was maintained throughout 11 days of prolonged culture (data not shown).

3.4 Biochemical analysis of disc tissue

GAG/DNA (μg/μg) ratio and OHP/DNA (μg/μg) ratio of NP and AF tissue measured on Day 0 and Day 4 are shown in Table 1. Neither GAG nor OHP content was affected by Deg culture condition and/or TNF-α injection after 4 days of culture.

3.5 Cell viability

Cell viability in disc tissue after 11 days of culture was assessed via lactate dehydrogenase and ethidium homodimer-1 staining. Representative images from the NP, inner AF, and outer AF regions of IVDs are shown in Figure 4a. Compared with Phy culture condition, TNF-α injection did not influence the cell viability significantly, whereas Deg culture condition with/without TNF-α injection markedly reduced cell viability (Figure 4b, p < .05).

3.6 Histology

Images of Safranin O/Fast Green stained sections of IVDs cultured for 11 days are shown in Figure 5. The staining intensity of proteoglycan (red) in NP tissue and collagen (green) in AF tissue was comparable among the four groups.

### TABLE 1 GAG/DNA (μg/μg) ratio and OHP/DNA (μg/μg) ratio of NP and AF tissue on Day 0 and Day 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 4 Phy</th>
<th>Day 4 Phy + TNF-α</th>
<th>Day 4 Deg</th>
<th>Day 4 Deg + TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>GAG /DNA</td>
<td>1241 ± 896</td>
<td>963 ± 685</td>
<td>1096 ± 666</td>
<td>1111 ± 692</td>
</tr>
<tr>
<td></td>
<td>OHP /DNA</td>
<td>81.34 ± 49.56</td>
<td>70.43 ± 40.17</td>
<td>72.01 ± 29.59</td>
<td>83.08 ± 35.92</td>
</tr>
<tr>
<td></td>
<td>OHP /DNA</td>
<td>62.30 ± 39.3</td>
<td>60.72 ± 51.88</td>
<td>96.80 ± 75.6</td>
<td>65.89 ± 51.41</td>
</tr>
</tbody>
</table>

Means ± 95% confidence interval, n = 8. GAG = glycosaminoglycan; NP = nucleus pulposus; AF = annulus fibrosus; TNF-α = tumour necrosis factor alpha.

3.7 Effect of physiological culture condition following induced degeneration and inflammation

To investigate if the induced degenerative and inflammatory effect is reversible or not, a group of IVDs were cultured with TNF-α injection under Deg condition culture for 4 days, then cultured under Phy condition for another 7 days. Gene expression levels in disc tissue between Day 4 and Day 11 were compared (Figure 6). The IL-1β (p = .0167), IL-6 (p = 0.0238), and IL-8 (p = .0238) gene expression decreased significantly from Day 4 to Day 11, whereas COL2, COL1, and MMP1 stayed at the same level compared with Day 4.

4 DISCUSSION

DDD is a multifactorial disease, which can be caused by different initiating factors. Within the complex pathology of DDD, it is mostly unclear which initiator is causing a certain effect. Therefore, there is a need of relevant translational models to investigate the molecular cascades of different initiators in DDD. In the current study, an IVD organ culture model was used to address this question. We applied single or combined disease initiators, one being detrimental mechanical loading and limited nutrition, defined as degenerative culture condition in our previous study (Iljin-Junger et al., 2010), the other being proinflammatory trigger. The effect of these initiators on the cellular response and tissue degradation within the IVD was investigated.

Recent research shows that the expression of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, is associated with the severity of DDD in both human and animal models (Andrade et al., 2016; Bachmeier et al., 2007; Le Maitre, Freemont, & Hoyland, 2005; Le Maitre, Hoyland, & Freemont, 2007a; Sutovsky et al., 2017). In the current study, intradiscal injection of TNF-α combined with degenerative culture conditions caused a marked up-regulation of proinflammatory cytokine expression equivalent to the above-mentioned findings in degenerated IVD. Specifically, IL-1β was up-regulated in the AF, whereas IL-6 and IL-8 were up-regulated in the NP. A previous porcine animal study also showed that TNF-α intradiscal injection induced IL-1β expression in the AF but not NP (R. Kang et al., 2015). These findings indicate a spatially different effect on the regulation of interleukin expression within the disc tissue. TNF-α injection alone triggered an up-regulation of these inflammatory cytokines, though the difference was not significant. Degenerative culture condition only induced IL-6 and IL-8 gene expression in NP tissue. These results indicate a stronger and additive proinflammatory effect of combined degenerative culture condition and TNF-α injection, compared with the single initiators. The IL-6 and IL-8 protein concentrations in the conditioned media did not show as pronounced differences
between the Phy and Deg + TNF-α groups as the gene expression levels, which may be due to the fact that some of the synthesized protein was entrapped inside the tissue, and only part of the protein was released into the medium. Further analysis of the protein expression within the tissue using immunohistochemistry or western blot will be needed to reveal the difference at the total protein level. The elevated expression of IL-1β, IL-6, and IL-8 significantly decreased after another 7 days of culture under physiological condition, which indicates an acute inflammatory effect. Gene expression level of TNF-α was neither affected by the degenerative culture condition nor by the TNF-α injection, which indicates that up-regulated TNF-α within DDD may originate from other stimuli or within another time frame. Additionally, an elevated NO release into conditioned medium was observed due to TNF-α injection, which is consistent with previous findings in bovine articular cartilage (Stevens, Wheeler, Tannenbaum, & Grodzinsky, 2008). Our results showed that the NO release was further enhanced by degenerative culture condition combined with TNF-α treatment. These conditions mimic the multifactorial initiators in DDD, which have shown to increase the levels of NO in the conditioned media from herniated lumbar disc tissue (J. D. Kang et al., 1996). In cartilage, NO acts as a proinflammatory mediator, stimulates MMPs, and suppresses ECM anabolism (Vuolteenaho, Moilanen, Hamalainen, & Moilanen, 2002). We hypothesize that NO may have equivalent effects in the IVD. These results also corroborate a stronger and additive proinflammatory effect of combined degenerative culture condition and TNF-α injection.

Here, we demonstrate that TNF-α injection and short-term degenerative loading altered the IVD phenotype towards catabolism and ECM breakdown. Exclusive application of TNF-α induced a down-regulation of COL1 in AF cells. Degenerative culture condition down-regulated the COL2 gene expression in NP cells. Whereas intradiscal injection of TNF-α in combination with degenerative culture condition enforced a catabolic effect on bovine IVDs, as revealed by significant upregulation of MMP1 (NP) and down-regulation of major ECM components such as COL2 (NP) expression. Similarly, another study showed that high mechanical strain on AF cells exposed to...
IL-1β induced more severe catabolic gene expression compared to high mechanical strain alone (Sowa et al., 2011). The proteolytic response of the IVD due to the harsh degenerative and inflammatory environment could also be observed by means of increased cleavage of GAG into conditioned medium. Nevertheless, gene expression of other catabolic enzymes, such as ADAMTS4, ADAMTS5, and MMP13, were not significantly altered. These results partially confirm previous findings, which found that MMP1, 3, 13, and ADAMTS4 expression increased with disease severity, whereas ADAMTS5 levels were not altered during the course of DDD (Le Maitre, Freemont, & Hoyland, 2004). ECM gene expression markers, such as COL2, COL1, and MMP1, remained on their dysregulated levels after another 7 days of culture under physiological condition, suggesting that the IVDs underwent a nonreversible catabolic shift without any sign of recovery—even after decline of the inflammatory and degenerative micro-environment. Equivalent findings have been reported in a bovine ex vivo study, which showed that TNF-α reduced anabolism was not able to recover (Purmessur et al., 2013).

In this study, the mechanical response of IVDs faced with a degenerative proinflammatory stimulus was assessed via disc height change. Results indicate that a short-term application of degenerative loading...
increased temporary disc height loss of bovine IVDs, whereas TNF-α injection alone had no effect. However, the disc height recovery capacity after free swelling did not differ among the four groups. This is due to the high intrinsic content of GAG within the young bovine discs, which did not show a reduction during the culture period. The negatively charged GAG within the IVDs could still absorb water during free swelling culture, enabling the disc height to recover. These results indicate that the current model may mimic a “degenerative”, while not “degenerated” IVD condition. To simulate a degenerated IVD with loss of ECM content, longer term culture or enzyme injection (Chan, Burki, Bonel, Benneker, & Gantenbein-Ritter, 2013; Jim, Steffen, Moir, Roughley, & Haglund, 2011; Krupkova et al., 2016) would be needed to deplete the ECM components.

Consistent with previous findings in organ cultures where TNF-α was supplemented in the culture medium (Purmessur et al., 2013; Walter, Likhitpanichkul, et al., 2015), TNF-α injection did not significantly affect the cell viability. Our results showed that degenerative culture condition markedly reduced cell viability, which indicates that detrimental loading and limited nutrition supply may play a major role on cell death in DDD.

Several proinflammatory degenerative culture systems have been developed using explant/organ culture (Walter, Likhitpanichkul, et al., 2015; Krupkova et al., 2016; Ponnappan et al., 2011; Purmessur et al., 2013; Teixeira et al., 2016). Though, many studies lack the 3D micro-environment of the IVD and/or cyclic dynamic loading condition, and therefore might fail to represent relevant biological and biomechanical alterations. Bovine NP tissue has been utilized as a degenerative/proinflammatory explant culture model, with TNF-α or IL-1β supplementation within the medium (Krupkova et al., 2016; Teixeira et al., 2016). One major drawback of this model is the lack of the endplate and AF tissue. Purmessur et al. cultured bovine IVDs under free swelling condition with TNF-α supplement in the medium and demonstrated that TNF-α induced catabolic activities after 7 and 21 days of culture (Purmessur et al., 2013). Walter et al. further applied dynamic loading on this model and demonstrated that dynamic loading facilitated the transport of TNF-α from medium into the disc tissue, primarily through the cartilage endplate but not the AF, which led to a higher TNF-α expression in the NP compared with the AF (Walter, Likhitpanichkul, et al., 2015). These results support our TNF-α intradiscal injection model, with a TNF-α gradient higher in the NP than in the AF. Ponnappan et al. used a rodent organ culture model, 100 ng/ml TNF-α, 10 ng/ml IL-1β, and nutritional deficiency to induce inflammation and a catabolic phenotype (Ponnappan et al., 2011), while lacking dynamic loading condition and comparable disc size compared with human IVD. As far as we are aware, this is the first IVD whole organ culture system simulating the early stage of DDD by application of combined initiators, detrimental dynamic loading, nutrient deficiency, and proinflammatory cytokine injection, which may better mimic the multifactorial condition in DDD. Bovine IVD for whole organ culture represents a reasonable ex vivo model because size, composition, and metabolism of bovine IVDs are relatively equivalent to human lumbar IVDs (Demers, Antoniou, & Mwale, 2004). A significant advantage of our organ culture model is that the IVDs can be maintained viable under dynamic load in a bioreactor for several weeks (Junger et al., 2009).

5 | CONCLUSION

The combination of detrimental dynamic loading, nutrient deficiency, and TNF-α intradiscal injection work synergistically to simulate the proinflammatory and degenerative condition within DDD, in an IVD whole organ culture system. This model will be of high interest to develop effective treatments to combat early stage DDD.

ACKNOWLEDGEMENTS

The current study was funded by the Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing (SET) under the project InflamoDisc (Project 59) and the National Natural Science Foundation of China (81772333). Gernot Lang was funded by the German Research Foundation (DFG). Zhiyu Zhou was funded by China Scholarship Council, Sino-Swiss Science and Technology Cooperation (EG 04-032015) and Natural Science Foundation of Guangdong Province (2014A030310466). We would like to acknowledge Nora Goudsouzian and Robert Peter (AO Research Institute Davos, Davos, Switzerland) for technical support.

CONFLICT OF INTEREST

This article does not contain information about medical device(s)/drug(s). No benefits in any form have been or will be received from a commercially party directly or indirectly related to the subject of this article. The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Gernot Lang: Substantial contributions to study design, interpretation of data, drafting the article, revising it critically, and final approval. Yishan Liu and Janna Geries: Substantial contributions to acquisition of data, analysis, interpretation of data, revising the article critically, and final approval. Zhiyu Zhou: Substantial contributions to acquisition of data, revising the article critically, and final approval. David Kubosch, Norbert Südkamp, R. Geoff Richards, and Mauro Alini: Substantial contributions to study design, revising the article critically, and final approval. Sibylle Grad: Substantial contributions to study design, interpretation of data, revising the article critically, and final approval. Zhen Li: Substantial contributions to study design, acquisition, analysis, interpretation of data, drafting the paper, revising it critically, and final approval. Zhen Li takes responsibility for the integrity of the work as a whole, from inception to finished article.

ORCID

R. Geoff Richards http://orcid.org/0000-0002-7778-2480
Sibylle Grad http://orcid.org/0000-0001-9552-3653
Zhen Li http://orcid.org/0000-0002-9754-6389

REFERENCES


