Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62100R1
Full Title:	A proinflammatory, degenerative organ culture model to simulate early-stage intervertebral disc disease
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
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TITLE:

A proinflammatory, degenerative organ culture model to simulate early-stage intervertebral disc disease.

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KEYWORDS:

intervertebral disc, inflammation, spine, 3R, organ culture, experimental model, disc degeneration, bioreactor

SUMMARY:

This protocol presents a novel experimental model of proinflammatory, degenerative bovine organ culture to simulate early-stage intervertebral disc degeneration.

ABSTRACT:

Symptomatic intervertebral disc (IVD) degeneration (IDD) is a major socioeconomic burden and is characterized by inflammation and tissue degradation. Due to the lack of causative therapies, there is an urgent need for innovative experimental organ culture models to study the mechanisms involved in the progression of the disease, find therapeutic targets, and reduce the need for animal models. We here present a novel, three-dimensional organ culture model protocol mimicking the proinflammatory and catabolic microenvironment, which is present during IDD.

Initially, bovine caudal IVDs were dissected, cleaned, and cultured in the tissue culture medium. Dynamic physiologic or pathologic loading was applied in a custom-made bioreactor for 2 hours

per day. IVDs were assigned to a control group (high glucose medium, physiological loading, phosphate-buffered saline injection) and a pathological group (low glucose medium, pathological loading, tumor necrosis factor-alpha injection) for four days. Gene expression analysis from collected nucleus pulposus cells of the IVDs and enzyme-linked immunosorbent assay of the conditioned organ culture media was performed.

Our data revealed a higher expression of inflammatory markers and reduced disc heights after loading in the pathological group compared to the control group. This protocol is reliable to simulate IVD inflammation and degeneration and can be further expanded to broaden its application scope.

INTRODUCTION:

Low back pain (LBP) can affect individuals of all ages and is a leading cause for disability worldwide[1–3]. The total cost associated with LBP exceeds $100 billion per year[4,5]. Symptomatic intervertebral disc (IVD) degeneration (IDD), a condition characterized by inflammation and tissue degradation, is a major cause of LBP[6,7]. Specifically, IDD is characterized by a gradually evolving breakdown of the IVD's extracellular matrix (ECM), induced and triggered by multiple factors that lead to an accelerated pathology, neurological disorders, and eventually disability. Furthermore, IDD is associated with the release of proinflammatory cytokines, altered spine biomechanics, angiogenesis, and nerve ingrowth, which increases pain sensation, altogether causing chronic LBP (active discopathy)[6,8]. To date, treatment options include discectomy and subsequent fusion of the adjacent vertebrae, implantation of an IVD prosthesis, or non-surgical approaches, such as non-steroidal anti-inflammatory drugs, opioids, and muscle relaxants for patients with IDD[9]. Both current standard therapeutic options, surgical and non-surgical, are only partly effective and fail to address the underlying biological problem[9,10]. Early-stage degenerative disc disease is characterized by an initial inflammatory tissue response, especially an increase in tumor necrosis factor-alpha (TNF-alpha) expression[11]. These early disc changes primarily occur at the cellular level without disrupting the disc architecture and could previously be mimicked by nutritional deficiency under pro-inflammatory conditions[12]. Therefore, precise simulation of the in vivo situation to investigate these degeneration mechanisms and find suitable therapeutic targets is crucial. Additionally, to these simulations of molecular properties, the mechanical loading environment of the discs plays a key role in pathological and physiological changes of IVD. Consequently, combining these approaches would bring us one step forward to mimic the complex microenvironment of IVDs in vivo. There are currently no studies considering the aspect of dynamic loading along with the pro-inflammatory and nutritional setting to the best of our knowledge.

Although large animal models allow the investigation of potential relevant in vivo interactions, they are costly and work intensive. Moreover, as the use of animal models in research has long been a matter of controversy, the reduction of the number of animals needed to answer important research questions is of great interest. Finally, there is currently no ideal animal model to mimic IDD in IVD research[13,14]. Therefore, it is necessary to establish a cost-effective and reliable replacement, such as an organ culture model to simulate IDD and associated inflammatory and degenerative processes. Recently, the application of the present protocol on

the establishment of a proinflammatory and degenerative organ culture model to simulate early-stage intervertebral disc disease allowed us to investigate the effect of anti-inflammatory drugs in the IDD organ culture[15].

Here, we describe how to obtain bovine intervertebral discs and induce the state of early-stage IDD via a catabolic and proinflammatory microenvironment caused by direct intradiscal injection of tumor necrosis factor-alpha (TNF-α) and degenerative loading in a bioreactor under low nutritive medium conditions. **Figure 1** illustrates the experimental model and shows the bioreactor used to simulate degenerative and physiological loading conditions.

[Place **Figure 1** here]

PROTOCOL:

Experiments were performed using bovine tails obtained from local abattoirs. The biological materials used in the current study are taken from the food chain and require no ethical approval in Swiss and European law.

1. Dissection of the bovine intervertebral disc

1.1. Rinse the whole tail thoroughly with tap water to remove dirt and hair on the surface.

NOTE: With intact, distal ends, a maximum of 9 IVDs (coccygeal 1-9) per tail can be used for the experiments depending on the desired size of the IVDs. Considering the desired diameter between 15-20 mm, we used 12 bovine tails with 5 IVDs per tail for the experiments.

1.2. Immerse the whole tail in a box containing 1% betadine solution for 10 min. Briefly dry the tail with sterile gauze and place it on a sterile drape.

NOTE: During dissection of the disc, humidify the tails with Ringer's solution wetted gauze to prevent dehydration. Store the tails (or left-over segments) wrapped in wet gauze until the whole dissection procedure is completed.

1.3. Use a scalpel (No. 20) to remove the soft tissue as completely as possible from the caudal spine to facilitate the identification of the IVDs. Remove the spinous and transverse processes of the vertebrae with bone removal pliers.

NOTE: Select IVDs with the desired diameter. IVDs with a diameter range of 15-20 mm were used in the current study.

1.4. Cut transversely with bone pliers through the middle of each vertebral body to obtain individual motion segments. Put motion segments in a Petri dish with gauze wetted with Ringer's solution.

1.5. Locate the IVD and vertebra by palpation and by moving the motion segments gently. Make two parallel cuts with the band saw in the growth plate of the IVDs, one on each side of the IVD. Identify the location of the growth plate by touching and finding the convex site of the bony endplate part (hard) adjacent to the disc (soft) with a safety distance of approximately 0.5-1 mm from the IVD towards the vertebra. Ensure that the blade of the band saw is cooled with Ringer's solution while cutting the vertebrae.

1.6. Transfer IVDs in a clean Petri dish with clean gauze wetted with Ringer's solution.

NOTE: The gauze should be moistened and not too wet to prevent swelling of the IVDs,

1.7. Use the scalpel blade to scrape off the vertebral body (red/pink bone), growth plate (white cartilage), leave the endplate intact (yellow-pink). Make the two surfaces flat and parallel for the loading procedure. Transfer scraped IVD's to a fresh Petri dish with gauze wetted with Ringer's solution.

NOTE: Wear a chainmail glove to protect the hand while holding the IVD and scraping.

1.8. Measure the disc height and diameter with a caliper. Clean the blood clots in the vertebrae bone with Ringer's solution using a jet lavage system.

1.9. Transfer the IVDs to 50 mL plastic tubes, one IVD per tube. Add 25 mL of Phosphate-Buffered Saline (PBS) + 10% Penicillin/Streptomycin (P/S) per IVD and leave it shaking for 15 min on an orbital shaker at room temperature.

1.10. Aspirate the supernatant and add 10 mL of PBS + 1% P/S per IVD for 2 min to rinse the IVDs.

2. IVD culture and loading

2.1. Transfer discs to IVD chambers and add IVD culture medium (Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/L high glucose DMEM for the physiological group and 2 g/L low glucose DMEM for the pathological group) + 1% P/S + 2% fetal calf serum + 1% ITS (contains 5 µg/mL insulin, 6 µg/mL transferrin, and 5 ng/mL selenious acid) + 50 µg/mL ascorbate-2-phosphate + 1% non-essential amino acid + 50 µg/mL antimicrobial reagent for primary cells) and place in an incubator at 37 °C, 85% humidity and 5% CO_2.

2.2. Culture the discs for 4 days within a bioreactor system according to experimental groups[16]. In the pathologic group, maintain degenerative loading conditions at 0.32-0.5 MPa, 5 Hz for 2 h/day. In the physiological control group, use a loading protocol of 0.02-0.2 MPa, 0.2 Hz for 2 h/day.

NOTE: Position the IVDs in chambers containing 5 mL of IVD medium during the loading procedures. The volume depends on the size of the bioreactor's loading chambers. Between the loading procedures, place the IVDs in six-well plates with 7 mL of IVD culture medium for free-

swelling recovery.

2.3. For analyzing the changes in disc height during the experimental period, measure the disc height with a caliper after IVD dissection (baseline) and then daily after the free swelling period and after dynamic loading for the experimental duration.

3. Intradiscal tumor necrosis factor-alpha (TNF-α) injection

3.1. Directly after the first dynamic loading cycle on day 1, place the IVDs in a Petri dish in a vertical position and stabilize the IVDs with a tweezer.

3.2. Inject recombinant TNF-α (100 ng in 70 µL of PBS per IVD) with a 30-gauge insulin needle into the nucleus pulposus tissue of the pathological group[17]. Inject slowly at a speed of approximately 70 µL in 1 min.

3.3. After injecting, pull the syringe halfway back within the IVD and pull the syringe plunger to create a vacuum that prevents the injected solution from leaking back, before removing the needle and syringe completely from the IVD.

NOTE: Perform a pilot experiment by injecting PBS containing trypan blue dye to evaluate the distribution of the injected solution after loading and overnight culture.

4. Gene expression

4.1. Harvest the IVDs on day 4. Collect the nucleus pulposus (NP) tissue (gelly part in the middle of IVD) with a biopsy punch. Collect the outer annulus fibrosus (AF) with a scalpel blade (No.20).

NOTE: For the baseline reference at day 0, collect reference tissues immediately after dissection for RNA extraction.

4.2. Use the amount of NP or AF tissue needed for gene expression analysis, depending on the experimental design.

NOTE: For the present experiments, approximately 150 mg tissue was used. The ratio of RNA isolation solution to tissue mass should be at least 2 mL per 100-150 mg tissue for efficient extraction.

4.3. Digest the NP or AF tissue with the digestion solution (0.2% pronase in DMEM, filter sterilized) and incubate for 1 h at 37 °C with magnetic stirring[18].

4.4. Flash-freeze the tissue samples using liquid nitrogen and pulverize to a fine powder. Divide the pulverized tissue powder equally into two 2 mL tubes each containing 1 mL of guanidine thiocyanate and phenol in a monophase solution (RNA isolation solution).

4.5. Perform the homogenization in 2 mL tubes containing the RNA isolation solution and the pulverized tissue powder. Homogenize the tissue powder 5x with an 8 mm stainless steel ball and a tissue-lyzer at 30 Hz for 3 min. Centrifuge at 12,000 x *g*, 4 °C for 10 min and transfer the supernatant to a fresh tube. The supernatants can be stored at -80 °C for at least one month.

4.6. Add 0.1 mL of 1-bromo-3-chloropropane (BCP) per 1 mL of RNA isolation solution and shake vigorously for 15 s. Store the resulting mixture at room temperature on an orbital shaker for 15 min and centrifuge at 12,000 x *g* for 15 min at 4 °C.

NOTE: The RNA remains exclusively in the upper aqueous phase.

4.7. Transfer the aqueous phase into a fresh tube and precipitate RNA with 0.25 mL of isopropanol and 0.25 mL of high salt precipitation solution per 1 mL of RNA isolation solution used for initial homogenization. Store the samples at room temperature for 15 min on an orbital shaker and centrifuge at 12,000 x *g* for 8 min at 4 °C.

NOTE: Alternatively, use the column-based RNA extraction method which generally leads to higher RNA purity but lower RNA yield.

4.8. Remove the supernatant and wash the RNA pellet with 1 mL of 75% ethanol per 1 mL of RNA isolation solution used for initial homogenization. Centrifuge at 7,500 x *g* for 5 min at 4 °C.

4.9. Remove the ethanol wash and briefly air-dry RNA pellet for 3-5 min. Dissolve the RNA in 20 µL of diethylpyrocarbonate (DEPC) treated water by passing the solution a few times through a pipette tip and incubating for 10-15 min at 55-60 °C.

4.10. Measure the absorbance at 230 nm, 260 nm, and 280 nm (A230, A260 and A280 respectively). A260 of 1.0 corresponds to 40 µg/mL RNA. An A260/A280 ratio of 1.6-1.9 is expected, whereas contamination results in a A260/A280 ratio of <1.6.

4.11. Prepare a reverse transcriptase (RT) reaction mix for a 20 µL reaction volume. The mix contains 10x solution (2.0 µL, contains RT enzyme mix and ribonuclease inhibitor, and helper protein), 5x solution (4.0 µL, contains primers, dNTPs and $MgCl_2$), RNase free water (14.0 µL- RNA sample volume), and RNA sample (0.4 µg of total RNA).

4.12. Briefly centrifuge the RT tubes to mix all the components at the bottom of the tube.

4.13. Place the samples in the thermocycler instrument. Select the appropriate program for the RT. Run the RT for 10 min at 25 °C, followed by the reverse transcription step for 120 min at 42 °C and inactivation of the reverse transcriptase for 5 min at 85 °C, cooling it down to 4 °C at the end.

4.14. Dilute the resulting cDNA with Tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) (TE) buffer (10 mM Tris with 1 mM EDTA) to a final

concentration of 0.4 µg RNA used for RT per 100 µL cDNA solution. Store the cDNA samples at -20 °C.

4.15. Perform real-time polymerase chain reaction (PCR) using a 10 µL reaction volume. The reaction volume contains the master mix (containing DNA polymerase, uracil-DNA glycosylase, dNTPs with dUTP, passive reference and optimized buffer components), forward primer 45 µM, reverse primer 45 µM, probe 12.5 µM (containing a reporter dye linked to the 5' end of the probe, a minor groove binder at the 3' end of the probe, and a nonfluorescent quencher at the 3' end of the probe), 2 µL cDNA and DEPC-treated water.

4.16. Run an endogenous control (RPLP0) for relative quantification with the $2^{-\Delta\Delta CT}$ method[19].

4.17. Add samples in duplicates and run a no template control by adding TE-buffer instead of cDNA. Run PCR at standard conditions (2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C).

4.18. Perform relative quantification of mRNA targets following the comparative CT method. The amount of mRNA normalized to the baseline sample is calculated as $2^{-\Delta\Delta CT}$, whereas ΔΔCT is the difference between the ΔCT (CT target- CT endogenous control) of sample and ΔCT (CT target and CT endogenous control) of the baseline sample.

5. Quantification of protein content in the IVD medium

5.1. Collect the medium conditioned by the IVD samples to measure the protein content in the medium. Perform enzyme-linked immunosorbent assay (ELISA) according to the protocol of the target protein.

5.2. For quantifying interleukin-8 (IL-8), dilute the capture antibody to the working concentration with PBS and immediately coat a 96-well ELISA plate with 100 µL of the diluted capture antibody. Seal and incubate the plate overnight at room temperature.

5.3. Aspirate each well and wash the wells 3x with wash buffer (0.05% polysorbate 20 in PBS, pH 7.2-7.4; 400 µL per well).

5.4. Block the plates by adding 300 µL of the block buffer (1% bovine serum albumin (BSA), in PBS, pH 7.2-7.4, 0.2 µm filtered sterile) to each well. Incubate at room temperature for a minimum of 1 h.

5.5. Repeat the wash steps as in **step 5.3** and add 100 µL of samples or standards diluted in reagent diluent (0.1% BSA, 0.05% polysorbate 20 in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.2-7.4, 0.2 µm filtered sterile). Standard concentrations in this protocol were 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL. Seal and incubate 2 h at room temperature.

5.6. Repeat the washing steps as in **step 5.3**. Add 100 µL of the working dilution (using reagent diluent) of streptavidin-horseradish peroxidase (HRP) to each well. Avoid placing the plate in direct light. Incubate for 20 min at room temperature.

5.7. Repeat the washing steps as in **step 5.3**. Add 100 µL of the substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine). Incubate the plate for 20 min at room temperature in the dark by covering it with aluminum foil.

5.8. Add 50 µL of stop solution (2 N H_2SO_4) to each well and ensure good mixing by gently tapping the plate a few times before insertion into the microplate reader.

5.9. Measure the optical density of each well immediately using a microplate reader at a wavelength of 450 nm with the corrected wavelength set at 570 nm.

REPRESENTATIVE RESULTS:

Degenerative loading in low glucose medium combined with TNF-α injection caused a significant increase of the gene expression of proinflammatory markers interleukin 6 (IL-6) and interleukin 8 (IL-8) compared to the physiological control group in NP cells after 4 days of culture (**Figure 2**). In contrast, we did not observe significant changes for the proinflammatory genes interleukin 1β (IL-1β) and TNF-α in NP cells (data not shown). Furthermore, degenerative culture conditions did not alter the gene expression of IL-6 and IL-8 in AF cells.

[Place **Figure 2** here]

Consistent with the gene expression findings, the IL-8 protein content in the medium showed a marked increase after 2 days and 4 days compared to the physiological condition (**Figure 3**). However, the measurements at day 3 (after free-swelling or loading) revealed no significant differences between the study groups, although the results indicated higher values for the degenerative group.

[Place **Figure 3** here]

The disc height (DH) changes normalized to the DH after dissection are shown in **Figure 4**. Whereas DH reductions after loading revealed higher values (i.e., more height reduction) for the degenerative group compared to the physiological groups, no differences in disc height gains after the free-swelling period were seen between the study groups. This indicated that the difference in disc height changes between the pathological and physiological group was higher after the loading procedure. Furthermore, the differences were less pronounced after 1 day compared to the measurements on days 2 and 3, indicating a progressive effect of the degenerative and inflammatory conditions.

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of the experimental setup. A: bovine tail; **B**: dissected bovine intervertebral discs; **C**: transfer of the disc to a well-plate with culture medium; **D**: loading the simulation in a bioreactor; **E**: intradiscal injection technique; **F**: IVD after injection of PBS/trypan blue dye to reveal distribution. IDD: intervertebral disc degeneration.

Figure 2: Gene expression levels in the nucleus pulposus (NP) tissue and annulus fibrosus (AF) tissues. These were measured after 4 days of culture under physiological or pathological culture condition, normalized to baseline (day 0). Means ± 95% confidence interval n=8, **p<0.01. This figure has been modified from[20].

Figure 3: Quantification of the pro-inflammatory protein IL-8 release in the IVD culture medium after free swelling culture (FS) and dynamic loading (Load). The results are shown as the original concentrations in ng/mL in the medium without normalization. Mean ± 95% confidence interval, n=8, **p<0.01. This figure has been modified from[20].

Figure 4: Disc height changes normalized to the baseline values (after dissection). Mean ± 95% confidence interval. FS: free-swelling. N=10, ***p<0.001. This figure has been modified from[20].

DISCUSSION:

We here provided a detailed protocol to simulate degenerative and inflammatory IVDD. This protocol can be applied for detailed examinations of inflammatory pathways leading to the destructive effects on the disc. Moreover, the protocol can help to determine promising therapeutic targets involved in the progression of the disease.

We recently showed that human recombinant TNF-alpha could induce inflammation in both bovine and human NP cells[21], which is in accordance with other studies in the field confirming that TNF-alpha can be used for inflammation simulation in IVD cells[22,23]. In the present study, a dose of 100 ng TNF-α per IVD was used to induce inflammation. This dose showed effective induction of inflammatory markers when applied in combination with degenerative loading and limited nutrition[15,20]. In a recent study using TNF-α as the sole initiation factor of IVD degeneration, a threshold of 100 ng TNF-α / cm^3 disc volume has been determined as an effective dose for the induction of inflammatory and degenerative changes in the IVD[21]. It is suggested that the TNF-α dose used to induce inflammation should be normalized to the volume of the disc for reproducible effects. Furthermore, it should be ensured that there is a good distribution of the injection material in the IVD and that this injection will be adequately reproducible in subsequent experiments. As injection pressure may vary between individuals, there might be different distributions of the material among the same study groups in different experiments. One approach we chose to examine the equal distribution was using PBS diluted trypan blue injections. It is recommended to standardize the injection technique, for example, with injection pumps and predefined and reproducible injection rates. As shown previously, one single needle puncture with 22-gauge, 25-gauge, or 30-gauge needles into the IVD did not cause an effect that interacted with study outcomes[20,21,24,25]. It is recommended to consider the disc size for the volume of the substance to be injected and for the needle size used for injection. Further, it is recommended to use a single injection to avoid inducing potential degenerative effects caused

by multiple annular injections[21,26].

Some limitations of the present model need to be addressed. The loading of the IVDs requires access to bioreactors, which are currently not widely available in many labs working on IDD. However, the need for preclinical IVD degeneration models is rising. Organ culture models bring ethical benefits of reducing the need for animal models, and the one-time investment on bioreactor costs could be affordable considering the reproducibility of the degenerative stimulation, and the reductions in the costs associated with animal experiments. Nevertheless, some in vivo interactions, such as the role of the immune system, cannot be simulated with the provided organ culture model. Moreover, the measurement of nerve signals and the evaluation of pain that would be possible in animal models can currently not be considered with the current model. Some concerns were raised whether the mechanical properties of tail IVDs could be comparable with human IVDs as the upright spine position is unique in humans[28]. Anatomical and molecular properties of bovine tail IVDs, such as cell density, lack of notochordal cells, biochemical composition[29,30], and mechanical properties of bovine caudal IVDs, such as range of motion in flexion, extension, torsion, and bending[31] are very similar to human IVDs. Notably, human IVDs gain more and more attention recently for organ culture models[28]. Human cadaveric IVDs in ex vivo models are considered highly efficient as they can avoid species differences which are more clinically relevant[32]. In contrast to bovine IVD tails obtained from the abattoirs, this would require transplantation of human IVDs 24 h post mortem and, thus, ethical approval following local organ transplantation laws[28]. The present method can also be easily adapted to other species.

One major advantage of the technique compared to other methods of ex vivo cultures is the combination of intradiscal injection, pathological medium conditions, and detrimental loading to simulate the early stage of degenerative discs better. Walter et al.[23] used TNF-alpha in the medium of dynamically loaded bovine IVDs instead of injection into the IVDs and showed increased transport of TNF-alpha from the medium into the nucleus pulposus compared to the annulus fibrosis, which is in accordance with our results. As we aimed to simulate the early stages of IDD, which occurs at the cellular level without major disruptions of the discs architecture[12], we chose the TNF-alpha concentrations based on previous organ culture studies using TNF-alpha in the medium to simulate the early stages of IDD[12,22,23]. The use of other inflammatory stimulants and higher concentrations of TNF-alpha could be tested to simulate the desired degenerative disc state depending on the study question. Higher concentrations of TNF-alpha may better compensate the lack of systemic immune regulatory feedbacks present during disc degeneration as proposed by Ponnappan et al.[12] The use of detrimental medium conditions with low-glucose was based on previous work showing that nutritionally limiting media and exposure to TNF-alpha mimics molecular change characteristics which are available in early disc degeneration state[12, 27]. Thus, the approach combines the evidence provided by previous studies in one ex vivo organ culture model of early degenerative disc disease.

This protocol can be further improved in several ways. The duration and extent of the loading and free-swelling period can be chosen based on the desired study plan. Long-term effects of inflammatory or degenerative stimulations on disc degeneration are of high clinical interest and

can be accomplished with the current protocol. We used only selected genes considered highly relevant for the early state of IDD[11]. Additional validation of this model could include a widened assessment of genes and proteins, such as omics approaches. Consequently, other inflammatory factors could be investigated in combination with the degenerative loading, depending on the desired degenerative state. For example, lipopolysaccharide injections have been shown to stimulate inflammation in the IVD[33]. A comparison of different inflammatory stimulants can be accomplished with the present protocol to find the most appropriate inflammatory stimulant for the desired study question. We have evaluated changes of selected inflammatory markers (IL-6, IL-8), anabolic markers (aggrecan, collagen), and catabolic markers (matrix metallopeptidases, a disintegrin and metalloproteinases with thrombospondin motifs) with the present protocol[20]. As the whole gene expression profile might change, future examinations could include next-generation RNA sequencing techniques to determine novel biomarkers for disc degeneration diagnostics and therapeutic targets for early biological intervention. Furthermore, other methodologies, such as histology, immunohistochemical staining, biochemical measurements of extracellular matrix components (such as glycosaminoglycans, GAGs), and dynamic compressive tests can be performed to further analyze the biological, biochemical, and biomechanical properties of IVDs with the present model. Another possible analytical way would be to separately analyze the impact on outer AF and NP tissue by using bCol1a1, Col1a2, CD146, SM22α, and MKX as gene expression markers for the outer AF[34,35]. The Spine Research Interest Group at the 2014 Annual ORS Meeting in New Orleans recommended following healthy NP phenotypic markers: HIF-1alpha, GLUT-1, aggrecan/collagen II ratio >20, Shh, Brachyury, KRT18/19, CA12, and CD24[36]. The NP marker genes bBrachyury/T and bSecreted frizzled-related protein 2 were the most convincing separating NP from inner and outer AF tissue in the bovine IVD[34]. Furthermore, the sGAG content of the NP was reported to be significantly higher compared to the outer AF[34].

In conclusion, this novel proinflammatory and degenerative IVD organ culture model provides relevant conditions to simulate early-stage IDD in a highly relevant 3D microenvironment. Certainly, the present protocol can be further modified according to the investigator's objectives. Furthermore, our model is able to reduce the number of needed study animals and thus totally reflects the 3R principles of reduce, replace, refine.

ACKNOWLEDGMENTS:

The current study was funded by the German Spine Society (DWG), the German Osteoarthritis Foundation (DAH), AO Foundation, and AOSpine International. Gernot Lang was supported by the Berta-Ottenstein-Programme for Advanced Clinician Scientists, Faculty of Medicine, University of Freiburg, Germany. Gernot Lang was funded by the German Research Foundation (DFG).

DISCLOSURES:

The authors have nothing to disclose.

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Figure1

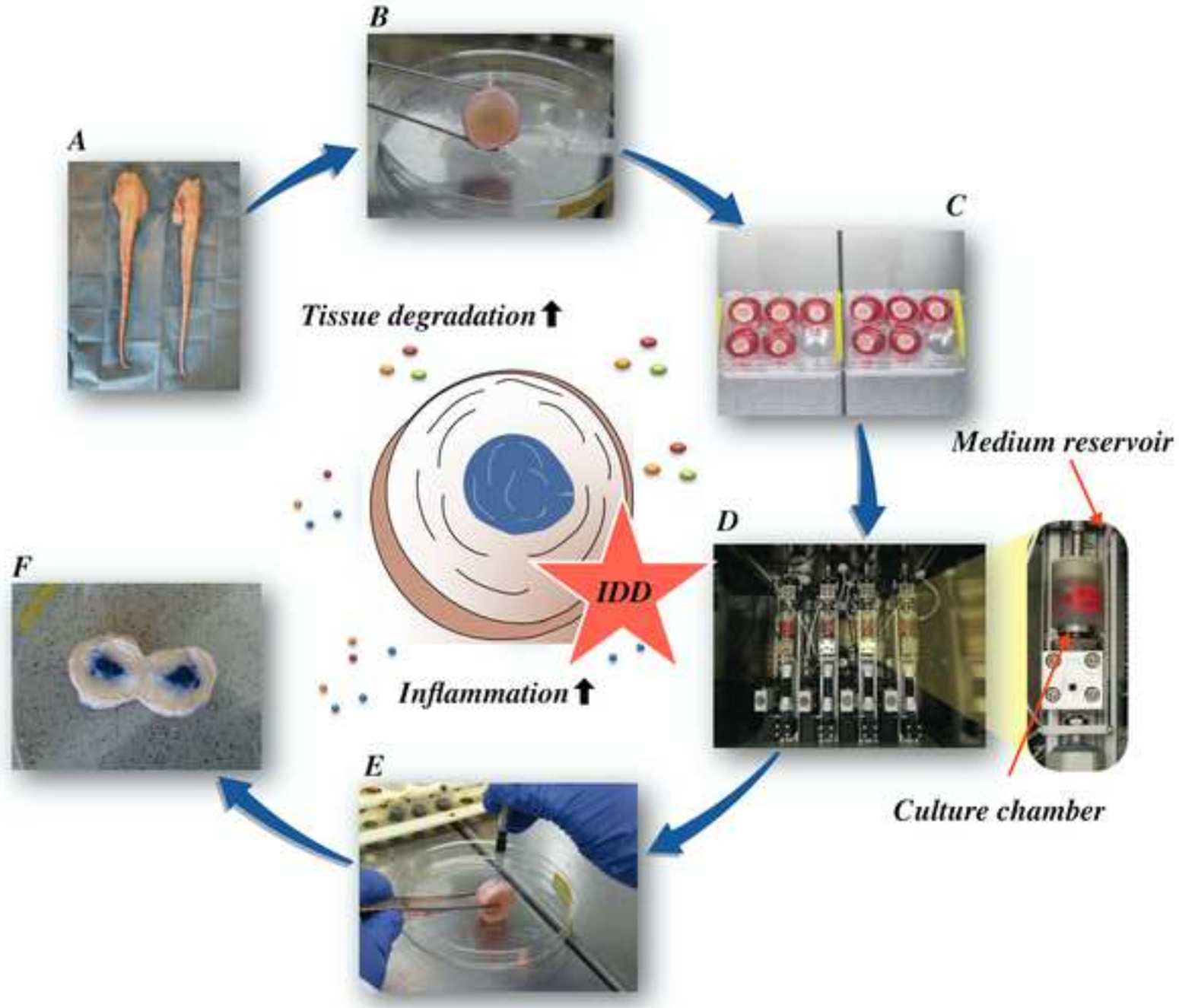


Figure2

Click here to access/download;Figure;Figure2.jpg

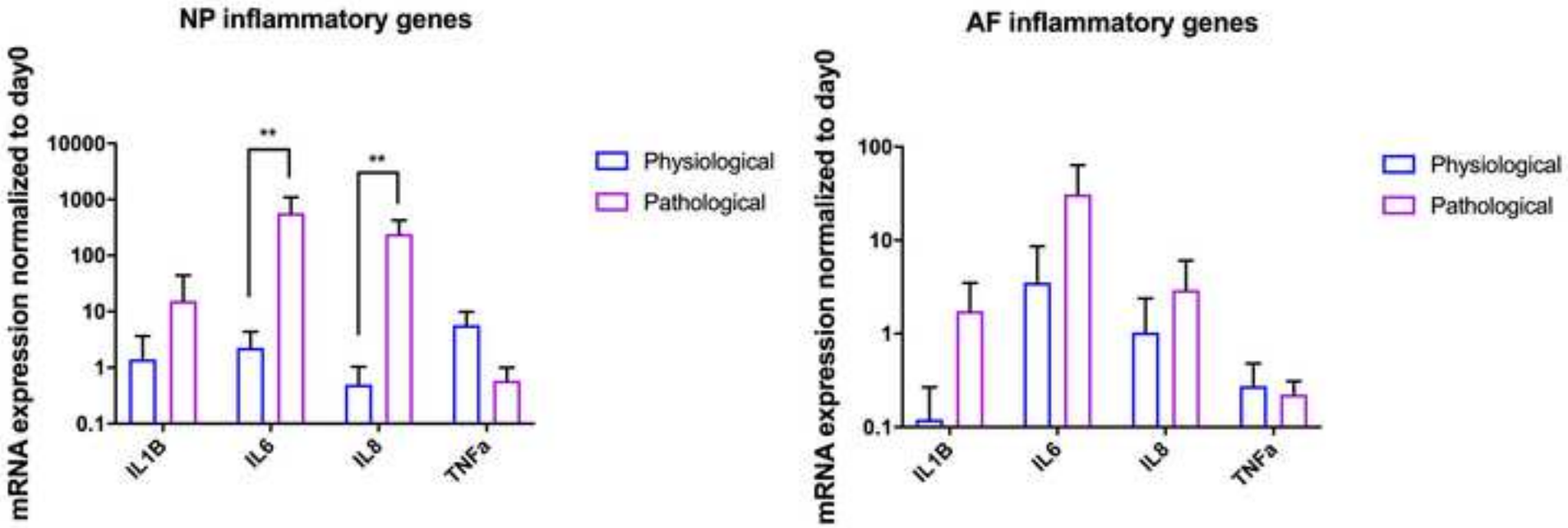


Figure3

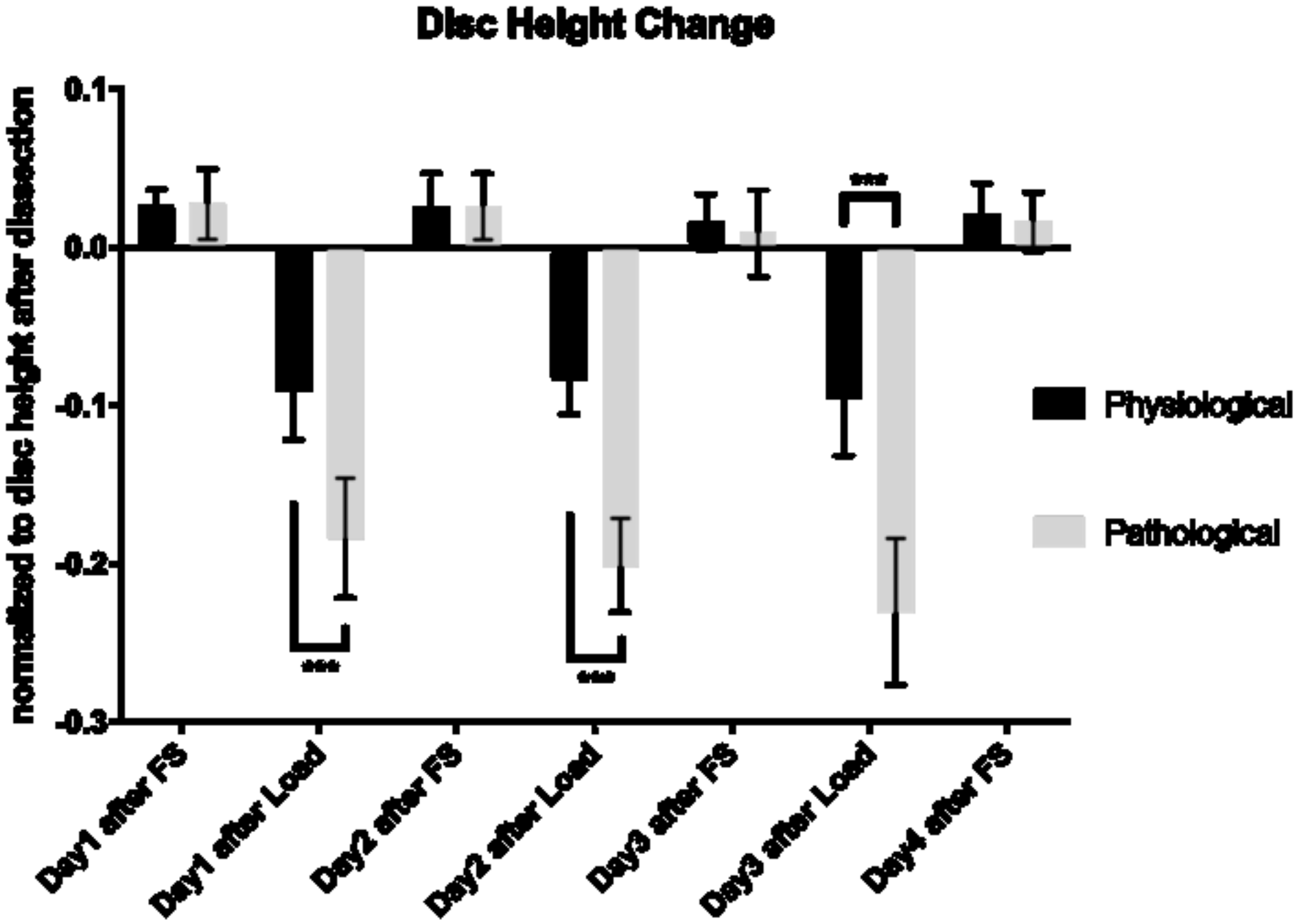
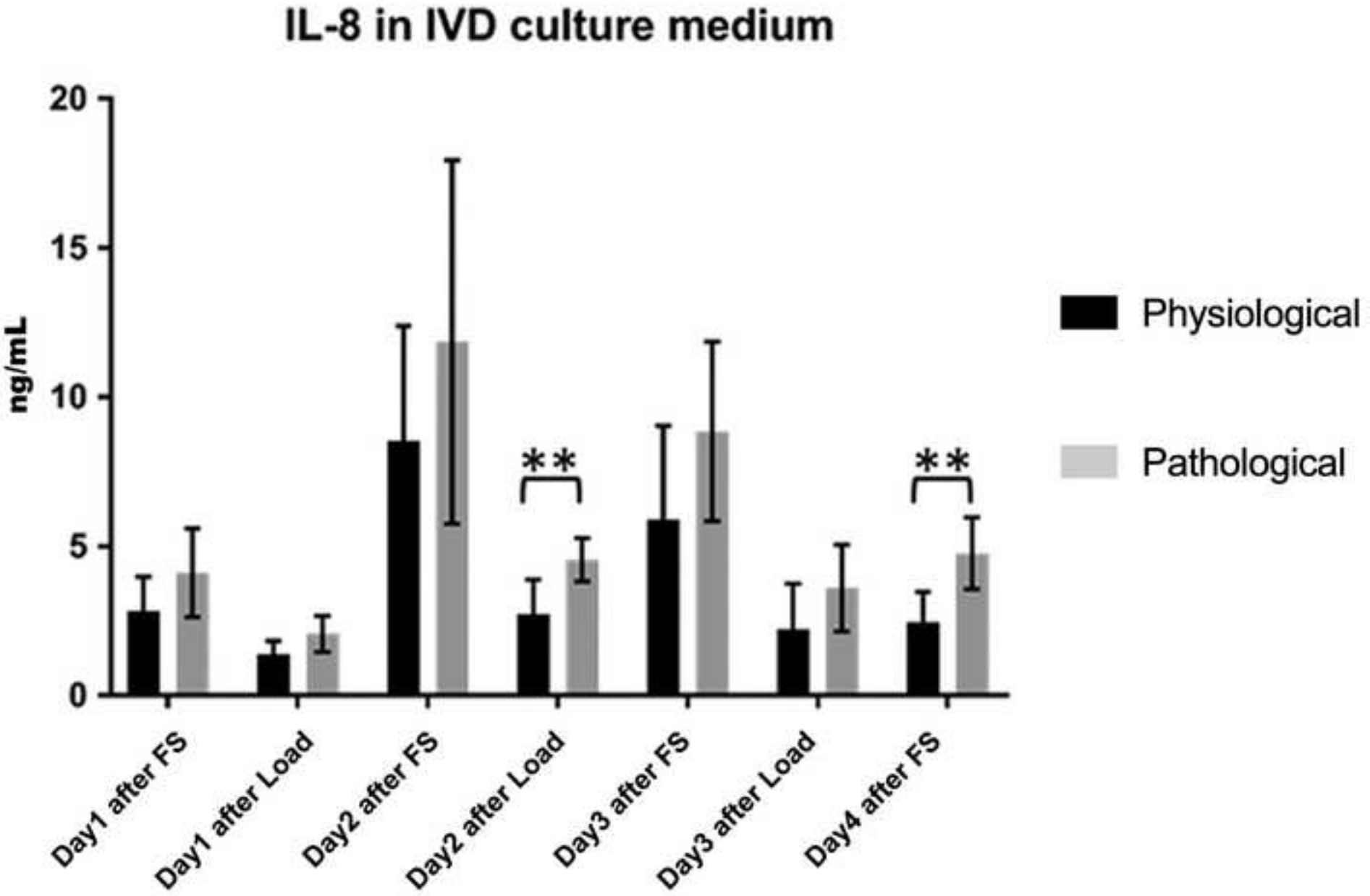


Figure4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-Bromo-3-chloropropane(BCP)	Sigma-Aldrich, St. Louis, USA	B9673	
Ascorbate-2-phosphate	Sigma-Aldrich, St. Louis, USA	A8960	
Band saw	Exakt Apparatebau, Norderstedt, Germany	model 30/833	
Betadine	Mundipharma, Frankfurt, Germany		
Bovine IL-8 Do.it-Yourself ELISA	Kingfisher Biotech, St. Paul, USA	DIY1028B-003	
Corning ITS Premix	Corning Inc., New York, USA	354350	
DMEM high glucose	Gibco by life technologies, Carlsbad, USA	10741574	

DMEM low glucose	Gibco by life technologies, Carlsbad, USA		11564446
Ethanol for molecular biology	Sigma-Aldrich, St. Louis, USA	09-0851	
Fetal Bovine Serum (FBS)	Gibco by life technologies, Carlsbad, USA	A4766801	
Non-essential amino acid solution	Gibco by life technologies, Carlsbad, USA		11140050
Penicillin/Streptomycin(P/S)	gibco by life technologies, Carlsbad, USA		11548876
Phosphate Buffer Solution, tablet	Sigma-Aldrich, St. Louis, USA	P4417	

Pronase	Sigma-Aldrich, St. Louis, USA	10165921001	
Primocin	InvivoGen, Sandiego, USA	ant-pm-05	
Pulsavac Jet Lavage System	Zimmer, IN,USA		
TissueLyser II	Quiagen, Venlo, Netherlands		85300
Streptavidinn-HRP	Kingfisher Biotech, St. Paul, USA	AR0068-001	
Superscript VILO	Invitrogen by life Technologies, Carlsbad, USA		10704274
cDNA Synthesis Kit	Applied Biosystems by life technologies		10400745

TaqMan Universal Master Mix	Applied Biosystems by life technologies			
TNF-alpha, recombinant human protein	R&D systems, Minnesota, USA	210-TA-005		
TRI Reagent	Molecular Research Center, Cincinnati, USA	TR 118		
Tris-EDTA buffer solution	sigma-Aldrich, St. Louis, USA		93283	
Gene bIL-6 g	Applied Biosystems by life technologies	Custom made probes		Primer fw (5'–3')TTC CAA AAA TGG AGG AAA AGG A Primer rev (5'–3')TCC AGA AGA CCA GCA GTG GTT Probe (5'FAM/3'TAMRA)CTT CCA ATC TGG GTT CAA TCA GGC GATT

Gene bIL8	Applied Biosystems by life technologies		Bt03211906_m1
Gene bTNF-alpha	Applied Biosystems by life technologies	Custom made probes	Primer fw (5'–3')CCT CTT CTC AAG CCT CAA GTA ACA A Primer rev (5'–3')GAG CTG CCC CGG AGA GTT Probe (5'FAM/3'TAMRA)ATG TCG GCT ACA ACG TGG GCT ACC G
GENE bIL1beta	Applied Biosystems by life technologies	Custom made probes	Primer fw (5'–3')TTA CTA CAG TGA CGA GAA TGA GCT GTT Primer rev (5'–3')GGT CCA GGT GTT GGA TGC A Probe (5'FAM/3'TAMRA)CTC TTC ATC TGT TTA GGG TCA TCA GCC TCA A
RPLP0	Applied Biosystems by life technologies	Bt03218086_m1	

Reviewer Number / Comment number	Original comments of the Reviewer	Changes made/reply by the authors **Thank you for your constructive comments. We highly appreciate the time and effort you have invested in our manuscript. We have tried to address each of your recommendations as precisely as possible.**	Changes made
Q1	*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.*	Dear Editor, thank you very much for your efforts. We proofread the manuscript and defined all abbreviations at first use as advised.	**Authors reply**
Q2	*2. Please provide an email address for each author.*	Email addresses have been provided.	**Title Page**
Q3	*3. Please revise the following lines to avoid overlap with previously published work: 264 (incubate for…)-271*	The mentioned lines were revised as advised.	**P6L299-306**
Q4	*4. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.*	Dear Editor, thank you very much for your efforts. We added the ethics statement as advised.	**P2L100-102**
Q5	*5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please*	Dear Editor, thank you very much. We removed the mentioned names and revised them with more generic terms.	**Authors reply**

	remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. *For example: Falcon tube; Corning ITS Premix; TRI reagent; Mastercycler; TaqMan etc*		
Q6	*6. As we are a methods journal, please add limitations of technique to the Discussion and also address why you chose low-glucose medium for the pathological group.*	Dear Editor, thank you very much. We added a limitation section, discussing the limitations of the present method and addressed why we chose a low-glucose medium for the pathological group.	**Discussion P8L391-P9L428**
Q7	*7. Please ensure that the highlighted section of the protocol is no more than 3 pages (and no less than 1 page) including headings and spacings.*	Dear Editor, thank you very much. We ensured that the steps follow your mentioned guidelines. The steps can be expanded or shortened in the production process if required.	**M&M**
Q8	*8. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the Editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to*	Dear Editor, thank you very much for your help. We added copyright permission for the figures and cited the figures appropriately in the legends.	**Authors reply Figure 2-4 (legends)**

	your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."		

Reviewer Number / Comment number	Original comments of the Reviewer	Changes made/reply by the authors	Changes made
Q1	Manuscript Summary: This is a very interesting and well thought out methods. Harvesting and culturing a whole intervertebral disc has certain challenges with the authors have identified clearly. Swelling is a considerable issue with disc culture which the authors have identified and incorporated into the model. They have correctly identified that needle injection can lead to a prior inflammatory cascade as well and have used the smallest needle possible and injected at a very slow controlled rate. This manuscript is ready for publication as there with no changes. The authors should be commended on their excellent job. Major Concerns: None Minor Concerns: None	Dear Reviewer, thank you very much for your kind judgment. We highly appreciate the time and effort you have invested in our manuscript.	**Authors reply**

Reviewer Number / Comment number	Original comments of the Reviewer	Changes made/reply by the authors **Thank you for your constructive comments. We highly appreciate the time and effort you have invested in our manuscript. We have tried to address each of your recommendations as precisely as possible.**	Changes made
Q1	*The background should give more information on existing organ culture models and what type of pro-inflammatory and degenerative conditions occur with this model. How does this model mimic human degeneration?*	Dear Reviewer, thank you very much for your insightful comment. We added a section to answer your question in the introduction section, emphasizing the improvement compared to existing research on this topic.	**Introduction P1L66-77**
Q2	*An important aspect of this protocol is the loading. What role does that play in this model? Can this model be adapted at institutions without access to a bioreactor set up.*	Dear Reviewer, thank you very much for mentioning this point. We addressed your question in the limitation section of the manuscript. To provide relevant conditions simulating physiological or degenerative loading, a bioreactor model is needed. However, we believe that more and more researchers will use bioreactors in research as it has some significant benefits. We discuss them along with the answer to your question in the limitation section	**Discussion P8L391-P9400**
Q3	*Why was bovine species chosen for this model? Can this method be adapted to other species, like human or porcine?*	Dear Reviewer, thank you very much for your helpful comment. We answered this critical question in our discussion section.	**Discussion P9L400-410**
Q4	*1.1 Is one tail donor used for these experiments? How many IVDs can be isolated per tail?*	The experiments were performed using IVDs from 12 bovine tails with 5 IVDs from each tail. 9 IVDs per tail can be used if the tail is intact. The number per tail depends on the desired size of the IVDs. We added information in the methods section.	**M&M 1.1**
Q5	*1.6. This step says to "Perform two parallel cuts in the growth plate of the disc".* *Each growth plate is subject to one cut.*	Dear Reviewer, thank you very much for your thorough review. We revised this part to be more clearer as advised. The bony part adjacent to the more "soft" part representing the IVDs was palpated, and the tail gently moved to locate the parts before the cuts. The identification of the growth plate was conducted afterward by visualization (white cartilage) (see step 1.8). We added this part to the video section filmed by the JOVE team as we agree that this part should be visualized to the reader for accurate repetition.	**M&M 1.6**

	There are two growth plates to be cut; one on either side of the disc. Therefore, this step should be re-phrased to be clearer. How is the location of the growth plate identifiable before cutting?		
Q6	*1.8. Pictures of vertebral body bone, growth plate, and endplates at each step of scraping should be provided.*	Dear Reviewer, thank you for mentioning this important point. As mentioned in Q5, we added this part to the video part filmed by the JOVE team. We agree with you that this should be thoroughly visualized with voice guidance to allow other institutions to perform this critical part adequately.	**Authors reply**
Q7	*1.11 and 1.12 are redundant considering the same composition of buffer and P/S are recommended. Perhaps 1.11 should read a higher concentration of P/S, like 10%.*	Dear Reviewer, thank you very much for pointing the mistake. We indeed used 10% P/S for 1.11.	**Authors reply**
Q8	*2.1 For pathological group, low glucose DMEM should be used for culture medium. For physiological group, high glucose DMEM should be used for culture medium. This differentiation needs to be made before medium preparation, and thus should be described in step 2.1.*	Dear Reviewer, we transferred this step in 2.1 as advised to make this better structured.	**M&M 2.1**
Q9	*2.2. It is stated that 5mL of medium should be used in the loading chambers. However, this depends on the size of*	Dear Reviewer, thank you very much. You are right. We added the information to this part to make clear that the volume depends on the bioreactor chamber size.	**M&M 2.2**

	the loading chambers, which may vary per institution. It may also depend on IVD size and volume.		
Q10	*3.2. Assuming TNF-alpha injection is done only in the pathological group, this should be specified in the procedure. Also, in step 3.2, the verbs shift to past tense after the second sentence. Verb tense should be kept consistent throughout the procedure. "vacumm" should be changed to "vacuum".*	Dear Reviewer, thank you very much for your help. We revised 3.2 as advised and added the missing information.	**M&M 3.2**
Q11	*4.1. Harvest IVDs at day 4. For baseline reference, should gene expression also be performed on tissue immediately after dissection? If so, this should be mentioned in the procedure. Otherwise, is baseline refence the physiological condition?*	Dear Reviewer, thank you very much for your precise attention regarding this. We added the information as the baseline reference for gene expression analysis was Day0 immediately after tissue dissection.	**M&M 4.1**
Q12	*4.1. The procedure says that tissue should not be sampled from the transition zone between the NP and AF. Gene expression for certain markers may vary across the IVD, especially with*	Dear Reviewer, we agree with your point and omitted this part from the procedure.	**M&M 4.1**

	the diffusion of TNF-alpha across the disc with loading. Depending on the needs of the researcher, the transition zone may be sampled and thus, this sentence should be omitted from the procedure.		
Q13	*4.2 The amount of tissue isolated from the NP or AF depends on the needs of the researcher, like the number of gene expression markers to be analyzed. Therefore, rather than specify an absolute value for the mass of tissue that should be isolated from the IVDs, the procedure should give a ratio of tissue mass to TRI reagent volume needed for efficient extraction.*	Dear Reviewer, thank you very much for your constructive comment. We revised this part to clarify that this depends on the experimental design and provided the recommended ratio of tissue mass to TRI reagent volume for efficient extraction as advised.	**M&M 4.2**
Q14	*4.4 What size metal ball should be used? Multiple types of tissue lyzers can be purchased. This step should specify the type of tissue lyzer used.*	Dear Revier, thank you very much for your help. We agree and added information regarding your comment to section 4.5.	**M&M 4.4**
Q15	*4.7 Since the IVD is a GAG-rich tissue, can the authors comment on the need for columns (RNeasy Mini Kit) for extraction of RNA with high purity? It is*	Dear Reviewer, thank you very much for your attention regarding this point. The current method with isopropanol and high salt precipitation solution for RNA precipitation worked well for IVD tissue containing high GAG content. Alternatively, the Qiagen RNeasy Kit could be used, which generally leads to higher RNA purity but lower RNA yield. This has been added as a note in 4.7.	**M&M 4.7**

	mentioned in the table of materials, but not in the procedure.		
Q16	*4.10 What absorbance values are expected and needed for PCR analysis?*	Dear Reviewer, we provided information regarding the expected and needed absorbance for accurate PCR analysis as advised.	**M&M 4.10**
Q17	*4.14. What is the concentration of TE buffer used for dilution? Should be fully defined as "Tris EDTA" in the procedure before shortening to TE.*	Dear Reviewer, thank you very much for your help. We added the concentrations used and defined the term TE before shortening.	**M&M 4.14**
Q18	*Can the authors comment on other useful analyses that can be done on the bovine IVDs to characterize the model, such as histology or biochemical composition?*	Dear Reviewer, thank you very much for your constructive comment. We expanded the section considering other useful analyses and improvements that can be done with the experimental approaches you mentioned, as these are very useful for the analysis of degenerative IVD changes.	**Discussion P9L430-P10L457**
Q19	*Are different genetic markers recommended for analysis for NP versus outer AF?*	Dear Reviewer, thank you very much for this interesting question. We provided this information in the discussion section of the manuscript. Finally, we would like to thank you for your tremendous efforts in improving the quality of this manuscript. We believe that your comments will help readers to interpret better and implement the knowledge transferred with the present manuscript.	**Authors reply And P10L449-457**

Reviewer Number / Comment number	Original comments of the Reviewer	Changes made/reply by the authors **Thank you for your constructive comments. We highly appreciate the time and effort you have invested in our manuscript. We have tried to address each of your recommendations as precisely as possible.**	Changes made
Q1	*Thanks to the Authors of this interesting methodological paper for having developed an organotypic culture model of intervertebral disc degeneration, potentially of great use to bypass in vivo studies, allowing both to satisfy ethical requirements, with a view to saving animal sacrifice, and a potential experimental versatility.* *There are only a few points that, in my opinion, could be discussed in more depth to give even more strength to this valuable work.*	Dear Reviewer, we would like to thank you for the kind judgment on our manuscript and your insightful comments. We believe that your efforts have significantly strengthened our manuscript and will help readers interpret and implement the provided experimental approach better.	**Authors reply**
Q2	*-Please, better define what the authors mean with "early-stage intervertebral disc disease"..*	Dear Reviewer, thank you very much for your advice. We added information on this part to make this more clear.	**Introduction P1L66-77**
Q3	*-How the pathological conditions were selected? Why do the Authors considered low glucose maintenance a pathological condition for disc? For what concern the*	Dear Reviewer, thank you very much for your constructive comment. We provided a section in our discussion answering your valuable comments.	**P9L412-428**

	pro-inflammatory stimulus, why TNFα (and not IL-1β or both the stimuli) was chosen and how the concentration/duration of the pro-inflammatory stimulus was selected? Please, introduce a sentence and references about this choice, in addition to the one already present referring to your work (13), with particular mention to the tissue physiology.		
Q4	*-Given that the loading conditions experienced by a human disc are different from those experienced by a bovine disc, and since loading is a fundamental component in defining the physiological/pathological model, do you think there can be substantial differences in the model response using human discs? Even in this case, references concerning the selected stimulus would be needed, in addition to the one already present referring to your work (13).*	Dear Reviewer, thank you very much for mentioning this important point. We discussed the question of whether the bovine intervertebral discs could be replaced by human intervertebral discs in our discussion section separately to your comment in Q3 pointing to the selection of the stimulus as advised.	**Discussion P9L400-P9L410**

Q5	*-No rationale for the choice of selected genes/cytokines used for validation of the model was reported. Please, add some sentences to explain why an approach based on a panel of few molecules instead of "omics" approaches was used to validate the model.*	Dear Reviewer, thank you very much for your thorough review. We agree that a more widened analysis would deliver more information about the changes in IVDs associated with our provided model. However, this model could be expanded in different ways, depending on the researcher's aim. For example, the degenerative disease state could be varied through other loading settings, concentrations of inflammatory stimulants, or other inflammatory stimuli. We believe that this model could function as a baseline model for other researchers who can expand it according to their desires. We stated this possible expansion of methods and the lack of the omics approach in the discussion section to make this clear.	**Discussion P9L430-P10L457**
Q6	*-Only in the discussion section, in paragraph reporting the study limitations (lines 355-368), there is a reference to almost all the above requested issues. In my opinion, a clear explanation of the selected experimental conditions and of the selected validation markers/strategy should be present since the beginning of the manuscript, especially in a methodological paper. As a consequence, I strongly suggest to improve the introduction, aims and methods with some information concerning all the experimental choices above mentioned, not only to reporting them as limitations in the Discussion.*	Dear Reviewer, thank you very much for your thorough review and your constructive comments. We added information about why we chose the present experimental approach with an expansion in the introduction and discussion section as advised. This approach can be expanded based on the researcher's need. It could function as a baseline methodology to study IVD degeneration. There are multiple ways for other researchers to expand the genes of interest, protein analyses, or combine other methodological techniques such as histology, with the provided methodology.	**Introduction P1L66-77 Discussion P9L412-L428 P9L430-P10L457**

Q7	*-no sample size was present throughout the work, only in figure legends. Please specify the "n" for each experimental evaluation (i.e. gene expression, protein quantification).*	Dear Reviewer, thank you very much for your advice. We added the missing information on the number of IVDs used. The exact sample sizes per group depend on the need of the researcher and the experimental design. Thus, we only provided them in the respective figures following other protocol manuscripts published in JOVE.	**M&M 1.1**
Q8	*-please, specify the glucose concentration in culture medium, line 139.*	Dear Reviewer, thank you very much. We added the missing information as advised.	**M&M 2.1**
Q9	*-Gene expression: please insert a table with the genes/probes used in gene expression experiments.*	Dear Reviewer, thank you very much for mentioning this point. We added information in the Materials Table of the Manuscript.	**Authors reply**
Q10	*-please insert a reference for the endogenous control RPLP0 selected for gene expression relative quantification, line 226.*	Dear Reviewer, thank you very much. We added a reference for the selection of the endogenous control RPLP0.	**M&M 4.16**
Q11	*-Quantification of protein content in the IVD medium: why IL-8 alone is mentioned (line 241)? Which cytokines were evaluated and why did you selected only these cytokines? How the single protein content was normalized in each sample, i.e. total protein content?*	Dear Reviewer, thank you very much for your thorough review. We added information in the figure legend to make clear that absolute protein concentrations were shown. These are representative results showing the model's impact on an important pro-inflammatory protein, IL-8, in IVDs. We used this method to date with different inflammatory cytokines, but the provided results were part of a previously conducted experiment evaluating IL-8. We added information on the section to make this representative choice more clear. However, the researchers using this technique could use various other markers, depending on their study aims. The method of protein analysis in the medium with ELISA would stay the same.	**Authors reply and 5.2 and Figure 3**
Q12	*Representative Results: -please show every analyzed time-points,*	Dear Reviewer, thank you very much for your constructive comment. We revised Figures 2-4 according to your comment, with additional information. There was one time point for the gene expression analysis, as gene expression analysis was conducted after 4 days of exposure,. We revised the time points shown in Figures 3,4 to maintain consistency.	**Authors reply and Figures 2-4**

	also data not showing significant changes, line 278. Moreover, Figures 2, 3 and 4 should be consistent, showing all the analyzed time-points for a better understanding of the readers. Please modify them accordingly.	Finally, we would like to thank you for your tremendous efforts to improve our manuscript's quality. We believe that your comments helped us a lot in presenting readers our model in the best possible way.	

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