



**HAL**  
open science

## Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor.

Bart van Dijk, Esther Potier, Maarten van Dijk, Marloes Langelaan, Nicole  
Papen-Botterhuis, Keita Ito

### ► To cite this version:

Bart van Dijk, Esther Potier, Maarten van Dijk, Marloes Langelaan, Nicole Papen-Botterhuis, et al.. Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor.. Journal of Orthopaedic Research, 2015, 33 (11), pp.1724-1731. 10.1002/jor.22946 . hal-01758572

**HAL Id: hal-01758572**

**<https://hal.science/hal-01758572>**

Submitted on 4 Apr 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor

**Authors :** B. van Dijk<sup>1</sup>, E. Potier<sup>1</sup>, Maarten van Dijk<sup>2</sup>, Marloes Langelaan<sup>3</sup>, Nicole Papen-Botterhuis<sup>3</sup>, Keita Ito<sup>1</sup>

**Affiliations:**

1 Orthopaedic Biomechanics, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, the Netherlands

2 DSM Biomedical B.V., Geleen, the Netherlands

3 TNO The Netherlands Organization for Applied Scientific Research, Eindhoven, the Netherlands

**Key words:** explant culture; disc herniation; inflammation; regenerative therapy; sustained release

*To cite this article:* van Dijk B, Potier E, van Dijk M, Langelaan M, Papen-Botterhuis N, Ito K (2015). *Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor.* J Orthop Res, 33(11): 1724-31. doi: 10.1002/jor.22946

*Document Version:* Accepted manuscript including changes made at the peer-review stage

**Abstract:** In intervertebral disc herniation with nucleus pulposus (NP) extrusion, the elicited inflammatory response is considered a key pain mechanism. However, inflammatory cytokines are reported in extruded herniated tissue, even before monocyte infiltration, suggesting that the tissue itself initiates the inflammation. Since herniated tissue swells, we investigated whether this simple mechanobiological stimulus alone could provoke an inflammatory response that could cause pain. Furthermore, we investigated whether sustained-release cyclooxygenase-2 (COX2) inhibitor would be beneficial in such conditions. Healthy bovine NP explants were allowed to swell freely or confined. The swelling explants were treated with Celecoxib, applied either as a bolus or in sustained-release. Swelling explants produced elevated levels of interleukin-6 (IL-6) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) for 28 days, while confined explants did not. Both a high concentration bolus and 10 times lower concentration in sustained release completely inhibited PGE<sub>2</sub> production, but did not affect IL-6 production. Swelling of NP tissue, without the inflammatory system response, can trigger cytokine production and Celecoxib, even in bolus form, may be useful for pain control in extruded disc herniation.

## Introduction

Low back-related leg pain, or radicular pain, is a common variation of low back pain. Its main cause is extruded lumbar disc herniation (1), during which the central core of the intervertebral disc, the nucleus pulposus (NP), pushes through the outer ring, the annulus fibrosus (AF). This extruded NP tissue can cause inflammation of nerve roots, which has been recognized as a key factor in painful extruded herniated discs (2).

Presence of inflammatory factors, interleukin-1 $\beta$  (3,4) and 6 (5,6) (IL-1 $\beta$  and IL-6), tumor necrosis factor alpha (3,4,7) (TNF $\alpha$ ), and prostaglandin-E<sub>2</sub> (5,6) (PGE<sub>2</sub>), is reported in herniated tissue. This is mainly attributed to the body's immune response: monocyte infiltration (8), macrophage maturation, and resorption of extruded tissue (9). But, production of several cytokines have been measured, albeit in lower quantities, in protruded NP tissue, which is not exposed to the body's immune response (6). Furthermore, Yoshida et al. (4) have observed IL-1 $\beta$  and TNF $\alpha$  positive cells, before infiltration of monocytes. Thus, NP tissue itself may initiate

the inflammatory response through an unknown mechanism. We hypothesize that when NP tissue is extruded, it is exposed to a lower osmolarity and this osmotic shock in turn stimulates the native NP cells to produce inflammatory factors.

Radicular pain caused by herniation is generally treated conservatively, for example, oral analgesics, and 50% of patients recover spontaneously (10). However, in many patients, analgesia is insufficient (11), and they are treated with epidural steroid injections or surgery. Long-term results of surgery are not different from conservative treatment for radicular pain (12), and although epidural injections can be effective in 80% of cases, patients often need four to five injections within a year (13). Moreover, steroids might slow down the natural resorption of extruded NP tissue as shown in a rabbit tissue model (14). Reducing pain, while not inhibiting resorption, would be a promising approach to treat herniation.

One of the inflammatory factors in herniation is PGE<sub>2</sub> which can sensitize nerves and induce pain (15). Two enzymes are involved in PGE<sub>2</sub> production, cyclooxygenase 1 and 2 (COX1 and

2). Contrary to COX1, COX2 is inducible and, therefore, COX2 inhibitors are used in pain management, and have been shown to reduce pain in rat models of disc herniation (16). Celecoxib (Cxb) is a COX2-specific inhibitor and a candidate for treating herniated discs whose cells can produce PGE<sub>2</sub>, when stimulated by macrophages (2). However, because the half-life of Cxb is only 7.8 h (17), a biodegradable Cxb sustained release option is likely to be more successful than a single Cxb injection.

We have previously cultured bovine NP tissue explants in an artificial annulus, to prevent swelling and provide a near in vivo environment for the NP cells up to 6 weeks (18). In this study, we allow the tissue to swell, as in extruded herniation, and investigate if this simple mechanobiological stimulus can stimulate the tissue to produce cytokines, in the absence of the inflammatory system response. Two injectable sustained release biomaterials loaded with Cxb were then tested in this model and compared to a single Cxb injection.

## Materials and Methods

### Tissue

Tissue NP explants (150-350 mg) were isolated with an 8 mm biopsy punch (Kruuse, Sherburn, UK) from the center of fresh caudal discs of 24-month-old cows, obtained from a local abattoir in accordance with local regulations. After weighing, free swelling samples were immediately placed in 6 ml of culture medium (DMEM; Gibco Invitrogen, Carlsbad, CA), with 4 mM L-glutamine (Lonza, Basel, Switzerland), 1% penicillin/streptomycin (Lonza), 50 mg/l ascorbic acid (Sigma), and 5% fetal bovine serum (Gibco). Control samples (non-swelling) were cultured in an artificial annulus system (18) in 6 ml of medium (Fig. 1). In this system, a jacket knitted from UHMWPE fibers (Dyneema, DSM, Heerlen, the Netherlands) lined with a 100 kDa molecular weight cut off (MWCO) semi-permeable membrane (Spectrum Laboratories, Breda, the Netherlands) prevents swelling.

### Injectable gel

A hybrid thermo-reversible biodegradable hydrogel (Fig. 2) was used as one controlled release platform (TNO, Eindhoven, the Netherlands) (19). The hydrogel consists of a network of biodegradable nanoparticles linked to Lower Critical Solution Temperature (LCST) polymers. At room temperature, they are dispersed in water, injectable through a 32-gauge needle. However, at 37°C, they gel by crosslinks arising from hydrophobic interactions of the LCST polymers. The hydrogel was mixed with Cxb (LC Laboratories, Woburn, MA) in three different concentrations (12, 120, and 1200 mg/ml gel). Previous *in vitro* experiments, showed that 0.5 ml gel, loaded with these concentrations, resulted in average releases of 0.1, 1, and 10 mM, respectively, when added to 6 ml of medium.

### Microspheres

Biodegradable microspheres (DSM, Fig. 2) were prepared with an average particle diameter of 40 μm (20). During production, microspheres were loaded with 8.5%w/w Cxb. Previous *in vitro* experiments showed that 2.7mg of microspheres (loaded with 22.9 mg Cxb) in 6 ml of medium resulted in an average release of 10 mM. Therefore 2.7, 0.27, and 0.027 mg microspheres were added to a well to release 10, 1, and 0.1 mM Cxb.

### Treatment conditions

Six NP tissue samples from independent donors were cultured in every experimental group (n = 6/group). Control artificial

annulus samples remained untreated. Samples in free swelling condition were (1) not treated; (2) treated with a bolus (10 mM Cxb in medium for 3 days); (3) treated with a sustained release control (1 mM Cxb in medium for 28 days); and (4-11) treated with the microspheres or gel, loaded for an aimed release of: 0, 0.1, 1, or 10 mM Cxb (Table 1). Explants were cultured in 12-well deep-well insert plates (Greiner) with cell culture inserts (0.4mm pore size, Greiner) to hold the sustained release biomaterials. Samples were cultured for 28 days at 37°C, 5% O<sub>2</sub>, and 5% CO<sub>2</sub> and medium was changed twice a week. During medium changes, temperature was kept at 37°C to prevent hydrogel liquefaction.

### Biochemical content

Samples were weighed at the beginning and end of culture, and the percent increase in sample wet weight (ww) was calculated. Subsequently, they were stored frozen at -30°C, lyophilized overnight (Freezone 2.5; Labconco) and the dry weight (dw) was measured. The water content was calculated from ww-dw/ww. The samples were then processed as described earlier and used to determine their content of sulfated glycosaminoglycans (sGAG), hydroxyproline (HYP), and DNA, as well as the fixed charge density (FCD) (18). The amounts of sGAG, HYP, and DNA were expressed as percentage of the sample dw.

### Cytokine release into the media

At every medium change, samples were collected and stored at -80°C. Release of cytokines into the medium was measured with ELISAs specific for bovine IL-1β and IL-6 (both Thermo Fisher Scientific, Waltham, MA), specific for bovine TNFα (R&D Biosystems, Minneapolis, MN), and PGE<sub>2</sub> (Enzo Life Sciences, Farmingdale, NY) following manufacturer's instructions, and normalized to the original sample ww. All standard curves were dissolved in fresh culture medium, to account for any effects of the media on the analysis. Release was measured for all groups at days 3 and 28 as well as on days 14 and 21 for the best performing concentration of both loaded biomaterials and control groups.

### Cxb concentration

At every medium change, samples were stored at -80°C, before Cxb concentration analysis by InGell Pharma (Groningen, the Netherlands). Samples were pre-treated as described before with slight modifications, that is concentration using liquid-liquid extraction with ethyl acetate, and mefenamic acid (Sigma) used as internal standard (21). The samples were analyzed with UPLC (1290 Infinity, Agilent, Santa Clara, CA) where the detection-limit was 10 ng/ml Cxb. The medium concentration of Cxb was measured for bolus treatment at days 14 and 28 and the best performing concentration of both loaded biomaterials at days 3, 14, 21, and 28.

### Statistics

Matlab (Mathworks, Inc., Natick, MA) was used for statistical analysis. For all biochemical data, one-way analysis of variance (ANOVA) was performed, followed by Dunnett's test for differences compared to artificial annulus. For the cytokine release data of the selected groups, Kruskal-Wallis analysis was performed at each time point, followed by Bonferroni corrected Mann-Whitney post hoc tests for differences compared to artificial annulus. To investigate the difference in Cxb release between the two biomaterials, two-way ANOVA was performed, followed by Bonferroni corrected post hoc t-tests. Statistical significance in all cases was assumed for p<0.05.

## Results

The ww of the artificial annulus group decreased 10% during culture, while free swelling groups increased between 100 and 150% ww (Fig. 3A). In all free swelling groups, the water content increased significantly (Fig. 3B), and the sGAG content (Fig. 3C) and FCD (Fig. 3D) decreased significantly compared to the artificial annulus group. In addition, the DNA content increased five-fold compared to the artificial annulus group (Fig. 3E). There were no differences in hydroxyproline content (Fig. 3F).

With both biomaterials, 1 mM aimed release was the lowest dosage that completely inhibited PGE<sub>2</sub> at day 28 (Fig. S1). These were analyzed at the intermediate time points (days 14 and 21), together with the artificial annulus, free swelling, sustained control, and bolus groups (Fig. 4). In artificial annulus samples, high levels of PGE<sub>2</sub> were produced during the first 3 days, but were ameliorated from day 14 onwards (Fig. 4A). Free swelling samples produced PGE<sub>2</sub> throughout culture, with maximal levels from days 14 to 28. Due to the large variance in response, these levels were only significantly different from artificial annulus at day 28. Bolus, sustained control and both biomaterial samples were significantly different from the artificial annulus at day 3, with a complete inhibition of PGE<sub>2</sub> production. From day 14 onwards, these groups were not different from the artificial annulus group, indicating continuous inhibition of PGE<sub>2</sub> production. In artificial annulus samples, very low or undetectable levels of IL-6 were produced throughout culture (Fig. 4B). In free swelling explants, significantly higher levels of IL-6 were produced, from day 14 onwards, which were maximal at days 14 and 21. In all treated groups, high levels of IL-6 were produced throughout the culture, except for gel 1 mM where IL-6 production was partially reduced at days 14 and 21 and not significantly different from artificial annulus. Very low or undetectable levels of IL-1 $\beta$  and TNF $\alpha$  were produced in all groups and at all time points, and there were no differences between groups (Fig. S2).

Both biomaterials, loaded for an aimed release of 1 mM, released Cxb for 28 days (Fig. 5). The average release, determined from these four time points, was 0.84 and 0.68 mM for the microspheres and gels, respectively, that is, close to the aimed concentration of 1 mM Cxb release in the gel was stable around 0.7 mM for 28 days. A burst release was observed in microspheres during the first 3 days, which was significantly higher compared to the gel. Thereafter, the release decreased in the microspheres and was significantly lower at each subsequent time point. Cxb release at days 21 and 28 was significantly lower in the microspheres compared to the gel. Cxb was still measured in the culture medium of the bolus group at day 14 and even at day 28, and was significantly larger than 0.

## Discussion

The artificial annulus prevented swelling, kept NP tissue stable (18, 22) and showed no long-term inflammatory response. Free swelling of NP tissue induced a sustained inflammatory response, of the inflammatory cytokine IL-6 and the nociceptive stimulus PGE<sub>2</sub>. This supports our hypothesis that even without interaction with the immune system, the extruded NP may alone initiate and/or contribute to the inflammation seen in herniation. With its high tonicity, extruded NP tissue will absorb water and swell (22), causing a hypo-osmotic stress to the cells. Such a stimulus has been shown to also stimulate amnion derived cells in a COX2 dependent manner resulting in production of PGE<sub>2</sub> (23). NP cells

regulate daily changes in osmolarity through NFAT (5, 24) which is produced more in hypertonic conditions. Although NP cells in monolayer stimulated with hypo-osmotic stress demonstrated MEK/ERK and AKT involvement (25), further research is needed, to clearly understand the involved biological pathway(s), which may be exploited for alternative treatment strategies. Nevertheless, this simple mechanobiological stimulus, similar to the initial phase of extruded herniation, did consistently elicit an inflammatory response although the variance was large between samples. This could have been due to the differences in osmotic pressure between samples, but this will be even more variable in patients with their different states of disc degeneration.

PGE<sub>2</sub> is involved in painful herniated discs (15); thus, the observation that NP tissue itself is able to produce PGE<sub>2</sub> shows the promise of COX2-specific treatment for radicular pain. The role of IL-6 in extruded herniation is not clear. It may be beneficial to alleviate the negative effects of herniation by contributing to resorption of herniated tissue via upregulation of matrix metalloproteases (26), inhibiting proteoglycan production (26), and stimulating macrophage maturation (27). On the other hand, as it can induce hyperalgesia in rats (28), it may be detrimental as well. However, IL-6 can induce PGE<sub>2</sub> (29) so, it is possible that hyperalgesic effects of IL-6 were not direct but due to increased PGE<sub>2</sub> production. This ambiguous role of IL-6 in herniated disc disease should be further investigated, but if it is mostly beneficial, treating painful herniated discs with Cxb could be a promising alternative to currently used steroids that have been reported to slow down the natural resorption of extruded NP tissue in a rabbit tissue model (14).

Interestingly, swelling healthy bovine NP tissue did not directly produce effective doses of TNF $\alpha$  and IL-1 $\beta$ , although both have been detected in herniated human tissue (3,7) and rabbits (4). However, similar to our results, culture of such specimens did not lead to presence of either cytokine in the medium, even after lipopolysaccharide stimulation (5). This could be because TNF $\alpha$  detected in homogenates of human herniated NP tissue was only membrane bound and no soluble TNF $\alpha$  was found (7). Furthermore, expression of these cytokines is associated with increasing degree of disc degeneration (31), but can also be produced by activated macrophages (32) neither of which were present in our system. Low levels of TNF $\alpha$ , though, can have already strong effects in NP-like tissues (33) and TNF $\alpha$  inhibitors have the potential to stop radicular pain in patients (34). Thus, the roles of TNF $\alpha$  and IL-1 $\beta$  in painful herniation remain pertinent.

The DNA content in free swelling samples increased five-fold compared to artificial annulus. However, the PGE<sub>2</sub> and IL-6 production at day 28 increased 30-90-fold, respectively, thus, cannot be explained by the increased cell number alone. In a previous study (22), increase in DNA was attributed to cell cloning and formation of a fibrous layer at the sample periphery. In the present study, we did not investigate the contribution of this fibrous layer to the production of IL-6 and PGE<sub>2</sub>. As this fibrous layer most likely contains active dedifferentiated cells, its production of inflammatory factors might be considerable. However, the increased PGE<sub>2</sub> and IL-6 production in the first 3 days, during absence of the fibrous layer, indicate that NP cells within the tissue produce inflammatory cytokines as well. Furthermore, *in vivo* this layer will probably also be formed (35) and may initiate the formation of the granulation tissue seen in sequestered and extruded surgical samples (30). Any contribution

of this layer to the production of cytokines in this study will occur *in vivo* as well.

Artificial annulus samples also increased PGE<sub>2</sub> production but only for the first 3 days. This is most likely a result of the shrinkage step needed before placing samples in the system. In this step, samples received a strong hyperosmotic stimulus (50% PEG in PBS (w/v) for 100 minutes), which has been shown to induce COX2 in renal cells (36). Besides this temporal production of PGE<sub>2</sub>, no other cytokines were produced, showing that healthy bovine NP explants do not produce inflammatory cytokines, unless they are triggered.

Cxb was able to completely inhibit elevated PGE<sub>2</sub> levels for 28 days, when delivered continuously at a concentration of 1 mM in the sustained control group. This result was expected, as this is in the order of the therapeutic plasma concentration of Cxb (17). Both biomaterials were able to release Cxb continuously for 28 days and, thus, inhibit PGE<sub>2</sub>. However, the release kinetics from the two biomaterials were significantly different, that is, microspheres showed a burst release, and a slowly declining release afterwards as observed earlier (20), while the gels showed constant release for 28 days.

To our surprise, the 10 mM bolus was also able to inhibit PGE<sub>2</sub> production of swelling NP explants for 28 days. This dosage is 10 times higher than the biomaterials and 3 times higher than maximum serum levels (17), but as this is a local deposit it will not lead to high serum concentrations in patients. We did not test a lower bolus; thus, we do not know if the bolus' success is because of the higher dose. What was most surprising is that we still measured Cxb in the medium at days 14 and 28, although all initial culture medium was removed at day 3. If Cxb was not degraded and distributed evenly throughout the fluid in tissue and medium, only approximately 70% of Cxb was removed at each medium change. Therefore, the bolus is likely to provide an effective dose longer than 3 days, possibly even until day 14, but not until day 28. Cxb only decreased 50% between days 14 and 28, where a 99% decrease was expected, so it is probable that Cxb is binding to the tissue. In blood serum samples, 97% of Cxb is bound to serum albumin (17), which is also a component of our culture medium. Furthermore, albumin is detected in osteoarthritic cartilage tissue, bound to keratan sulfate, one of the main sGAGs in the disc, through disulfide bonds (37). If this proposed mechanism of albumin binding to the tissue is correct, this will have clinical implications. In 80% of extruded herniation samples, blood vessels are observed (38) so, Cxb injected adjacent to extruded NP tissue can diffuse in and bind to pre-bound albumin, lengthening the therapeutic effect. Possibly, this might explain the relative success of epidural steroid injections, as the NP tissue can prolong the effect of a single drug injection. Nevertheless, more research needs to be done to determine the validity of this mechanism. It is also possible that Cxb is binding to other proteins in the NP. This interesting finding shows the value of using our tissue model for preclinical evaluation of therapies for disc herniation, as in cell culture this property of Cxb would be missed. However, a limitation of this model is the absence of the body immune response. Repeating this study in a suitable animal model will truly show if a single injection of Cxb will be as successful as a sustained release biomaterial. Nevertheless, treatment with a COX2 inhibitor like Cxb has clinical potential, as not only the swelling NP tissues in this study, but also infiltrated samples, produce PGE<sub>2</sub>.

Another interesting finding is that the gel alone was able to reduce IL-6 production to some extent, especially at day 14. This reduction was also observed at day 14 with the 10 mM loaded gels but also with the empty gels (Fig. S3), indicating that the biomaterial itself affected IL-6 production. As we used Transwell® systems (pore size 0.4 mm) in this experiment, there was no direct contact between tissue and biomaterials, but degradation products could have reached the tissue. When the gels degrade, magnesium ions (Mg<sub>2</sub>) leach out, which can affect IL-6 production (39). Furthermore, a relatively large amount of biomaterial was used in the gel groups (500 ml per sample in all concentrations). Nevertheless, any direct effect of the gel on IL-6 production is only partial and transient, and any benefits thereof remain to be investigated.

## Conclusion

Exposing NP tissue explants to lower tonicity conditions, without the inflammatory system response, increased PGE<sub>2</sub> and IL-6. These cytokines are interesting candidates when treating acute herniation, as they are produced before infiltration of macrophages, and involved in pain and inflammation. Cxb could successfully stop PGE<sub>2</sub>, but not IL-6, production for 28 days when supplied by sustained release biomaterials. Interestingly, a bolus was able to achieve the same result, revealing that NP tissue itself can function as a carrier for sustained release of Cxb.

## Acknowledgments

This research forms part of the Project P2.01 IDiDAS of the research program of the BioMedical Materials institute, a Dutch public-private partnership. We would like to acknowledge Paul van Midwoud at InGell Pharma for celecoxib release measurements, Irene Arkesteijn at TU/e for the valuable contribution to the artificial annulus model, Renz van Ee and Klaas Timmer at TNO, and Detlef Schumann at DSM for supplying us with the biomaterials.

## Disclosure Statement

The academic partners did not receive direct funding from the commercial partners and only non-commercial authors interpreted the data.

## References

1. Gibson JN, Waddell G. 2007. Surgical interventions for lumbar disc prolapse: updated Cochrane Review. *Spine (PhilaPa 1976)* 32:1735-47.
2. Takada T, Nishida K, Maeno K, et al. 2012. Intervertebral disc and macrophage interaction induces mechanical hyperalgesia and cytokine production in a herniated disc model in rats. *Arthritis Rheum* 64:2601-10.
3. Takahashi H, Suguro T, Okazima Y, et al. 1996. Inflammatory cytokines in the herniated disc of the lumbar spine. *Spine (PhilaPa 1976)* 21:218-24.
4. Yoshida M, Nakamura T, Sei A, et al. 2005. Intervertebral disc cells produce tumor necrosis factor alpha, interleukin-1beta, and monocyte chemoattractant protein-1 immediately after herniation: an experimental study using a new hernia model. *Spine (PhilaPa 1976)* 30:55-61.
5. Burke JG, Watson RWG, Conhyea D, et al. 2003. Human nucleus pulposus can respond to a pro-inflammatory stimulus. *Spine (Phila Pa 1976)* 28:2685-93.
6. Kang JD, Georgescu HI, McIntyre-Larkin L, et al. 1996. Herniated lumbar intervertebral discs spontaneously produce matrix

- metalloproteinases, nitric oxide, interleukin-6, and prostaglandin E2. *Spine (Phila Pa 1976)* 21:271-7.
7. Andrade P, Visser-Vandewalle V, Philippens M, et al. 2011. Tumor necrosis factor- $\alpha$  levels correlate with postoperative pain severity in lumbar disc hernia patients: opposite clinical effects between tumor necrosis factor receptor 1 and 2. *Pain* 152:2645-52.
  8. Doita M, Kanatani T, Harada T, et al. 1996. Immunohistologic study of the ruptured intervertebral disc of the lumbar spine. *Spine (PhilaPa 1976)* 21:235-41.
  9. Komori H, Shinomiya K, Nakai O, et al. 1996. The natural history of herniated nucleus pulposus with radiculopathy. *Spine (PhilaPa 1976)* 21:225-9.
  10. Frymoyer JW 1988. Back pain and sciatica. *N Engl J Med* 318:291-300.
  11. Croft PR, Macfarlane GJ, Papageorgiou AC, et al. 1998. Outcome of low back pain in general practice: a prospective study. *BMJ* 316:1356-9.
  12. Jacobs WCH, van Tulder M, Arts M, et al. 2011. Surgery versus conservative management of sciatica due to a lumbar herniated disc: a systematic review. *Eur Spine J* 20:513-22.
  13. Manchikanti L, Singh V, Falco FJ, et al. 2010. Evaluation of the effectiveness of lumbar interlaminar epidural injections in managing chronic pain of lumbar disc herniation or radiculitis: a randomized, double-blind, controlled trial. *Pain Physician* 13:343-55.
  14. Minamide A, Tamaki T, Hashizume H, et al. 1998. Effects of steroid and lipopolysaccharide on spontaneous resorption of herniated intervertebral discs. An experimental study in the rabbit. *Spine (Phila Pa 1976)* 23:870-6.
  15. Samad TA, Moore KA, Sapirstein A, et al. 2001. Interleukin-1 $\beta$ -mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 410:471-5.
  16. Kawakami M, Matsumoto T, Hashizume H, et al. 2002. Epidural injection of cyclooxygenase-2 inhibitor attenuates pain-related behavior following application of nucleus pulposus to the nerve root in the rat. *J Orthop Res* 20:376-81.
  17. McCormack PL 2011. Celecoxib. *Drugs* 71:2457-89.
  18. van Dijk BGM, Potier E, Ito K. 2013. Long-term culture of bovine nucleus pulposus explants in a native environment. *Spine J* 13:454-63.
  19. Craenmehr EGM, ten Cate AT. 2011. Liquid composition comprising polymer chains and particles of an inorganic material in a liquid. (US 2011/0068289 A1).
  20. Yang H-Y, van Dijk M, Licht R, et al. 2015. Applicability of a newly developed bioassay for determining bioactivity of anti-inflammatory compounds in release studies-celecoxib and triamcinolone acetate released from novel PLGA based microspheres. *Pharm Res* 32:680-90.
  21. Zarghi A, Shafaati A, Foroutan SM, et al. 2006. Simple and rapid high-performance liquid chromatographic method for determination of celecoxib in plasma using UV detection: application in pharmacokinetic studies. *J Chromatogr B Analyt Technol Biomed Life Sci* 835:100-4.
  22. van Dijk BGM, Potier E, Ito K. 2011. Culturing bovine nucleus pulposus explants by balancing medium osmolarity. *Tissue Eng Part C Methods* 17:1089-96.
  23. Lundgren DW, Moore RM, Collins PL, et al. 1997. Hypotonic stress increases cyclooxygenase-2 expression and prostaglandin release from amnion-derived WISH cells. *J Biol Chem* 272:20118-24.
  24. Tsai TT, Danielson KG, Guttapalli A, et al. 2006. TonEBP/ OREBP is a regulator of nucleus pulposus cell function and survival in the intervertebral disc. *J Biol Chem* 281:25416-24.
  25. Mavrogenatou E, Kletsas D. 2010. Effect of varying osmotic conditions on the response of bovine nucleus pulposus cells to growth factors and the activation of the ERK and Akt pathways. *J Orthop Res* 28:1276-82.
  26. Studer RK, Vo N, Sowa G, et al. 2011. Human nucleus pulposus cells react to IL-6: independent actions and amplification of response to IL-1 and TNF- $\alpha$ . *Spine (PhilaPa 1976)* 36:593-9.
  27. Mitani H, Katayama N, Araki H, et al. 2000. Activity of interleukin 6 in the differentiation of monocytes to macrophages and dendritic cells. *Br J Haematol* 109:288-95.
  28. DeLeo JA, Colburn RW, Nichols M, et al. 1996. Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res* 16:695-700.
  29. Crowl RM, Stoller TJ, Conroy RR, et al. 1991. Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response. *J Biol Chem* 266:2647-51.
  30. Koike Y, Uzuki M, Kokubun S, et al. 2003. Angiogenesis and inflammatory cell infiltration in lumbar disc herniation. *Spine (PhilaPa 1976)* 28:1928-33.
  31. Le Maitre CL, Freemont AJ, Hoyland JA. 2005. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis Res Ther* 7:R732-45.
  32. Haro H, Shinomiya K, Komori H, et al. 1996. Upregulated expression of chemokines in herniated nucleus pulposus resorption. *Spine (PhilaPa 1976)* 21:1647-52.
  33. Seguin CA, Pilliar RM, Roughley PJ, et al. 2005. Tumor necrosis factor- $\alpha$  modulates matrix production and catabolism in nucleus pulposus tissue. *Spine (PhilaPa 1976)* 30:1940-8.
  34. Korhonen T, Karppinen J, Malmivaara A, et al. 2004. Efficacy of in- $\alpha$ iximab for disc herniation-induced sciatica: one-year follow-up. *Spine (PhilaPa 1976)* 29:2115-9.
  35. Specchia N, Pagnotta A, Toesca A, et al. 2002. Cytokines and growth factors in the protruded intervertebral disc of the lumbar spine. *Eur Spine J* 11:145-51.
  36. Moeckel GW, Zhang L, Fogo AB, et al. 2003. COX2 activity promotes organic osmolyte accumulation and adaptation of renal medullary interstitial cells to hypertonic stress. *J Biol Chem* 278:19352-7.
  37. Mannik M, Person RE. 1993. Immunoglobulin-G and Serumalbumin isolated from the articular-cartilage of patients with rheumatoid arthritis or osteoarthritis contain covalent heteropolymers with proteoglycans. *Rheumatol Int* 13:121-9.
  38. Virri J, Gronblad M, Savikko J, et al. 1996. Prevalence, morphology, and topography of blood vessels in herniated disc tissue - A comparative immunocytochemical study. *Spine (PhilaPa 1976)* 21:1856-1863.
  39. Nowacki W, Malpuech-Brugere C, Rock E, et al. 2009. Highmagnesium concentration and cytokine production in human whole blood model. *Magnes Res* 22:93-6.

*Corresponding author:*

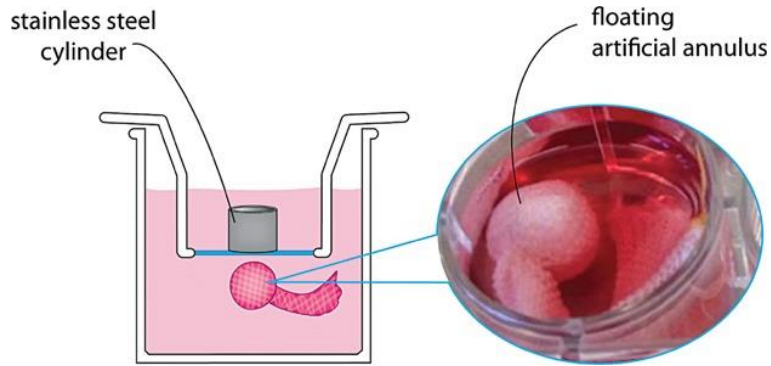
Keita Ito  
T: 31 40 247 4350  
F: 31 40 247 3744  
E-mail: k.ito@tue.nl

**Table 1. Overview of the culture groups.**

<b>Group description</b>	<b>Swelling</b>	<b>Treatment duration (Days)</b>	<b>Delivery</b>
(1) Artificial annulus	Contained by artificial annulus	-	-
(2) Free swelling	Free swelling	-	-
(3) Bolus 10 $\mu\text{M}$	Free swelling	3	Added to media
(4) Sustained release control 1 $\mu\text{M}$	Free swelling	28	Added to media
(5) Microspheres empty	Free swelling	-	Microspheres
(6) Microspheres 0.1 $\mu\text{M}$	Free swelling	28	Microspheres
(7) Microspheres 1 $\mu\text{M}$	Free swelling	28	Microspheres
(8) Microspheres 10 $\mu\text{M}$	Free swelling	28	Microspheres
(9) Gel empty	Free swelling	-	Injectable gel
(10) Gel 0.1 $\mu\text{M}$	Free swelling	28	Injectable gel
(11) Gel 1 $\mu\text{M}$	Free swelling	28	Injectable gel
(12) Gel 10 $\mu\text{M}$	Free swelling	28	Injectable gel

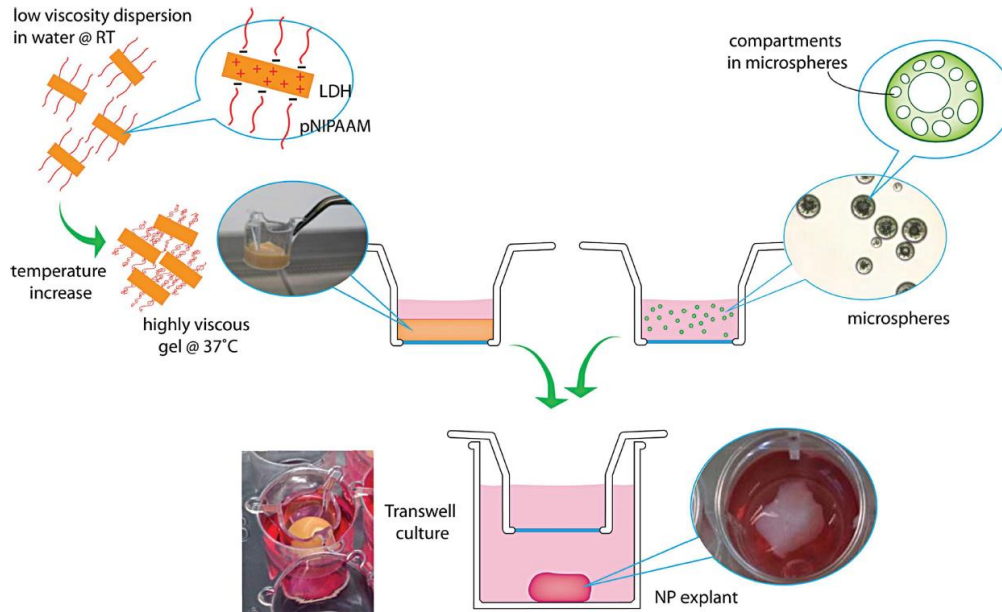
**Figure 1. Tissue NP explant culture system.**

Image of nucleus pulposus (NP) explants in artificial annulus culture. Artificial annulus samples (right) were cultured in a deep-well Transwell® plate with 6 ml of culture medium. As the artificial annulus samples might be buoyant, a stainless steel cylinder was added to the culture insert (left) to keep samples submerged (drawn by Anthal Smits).



**Figure 2. Schematic image of NP explants in free swelling culture.**

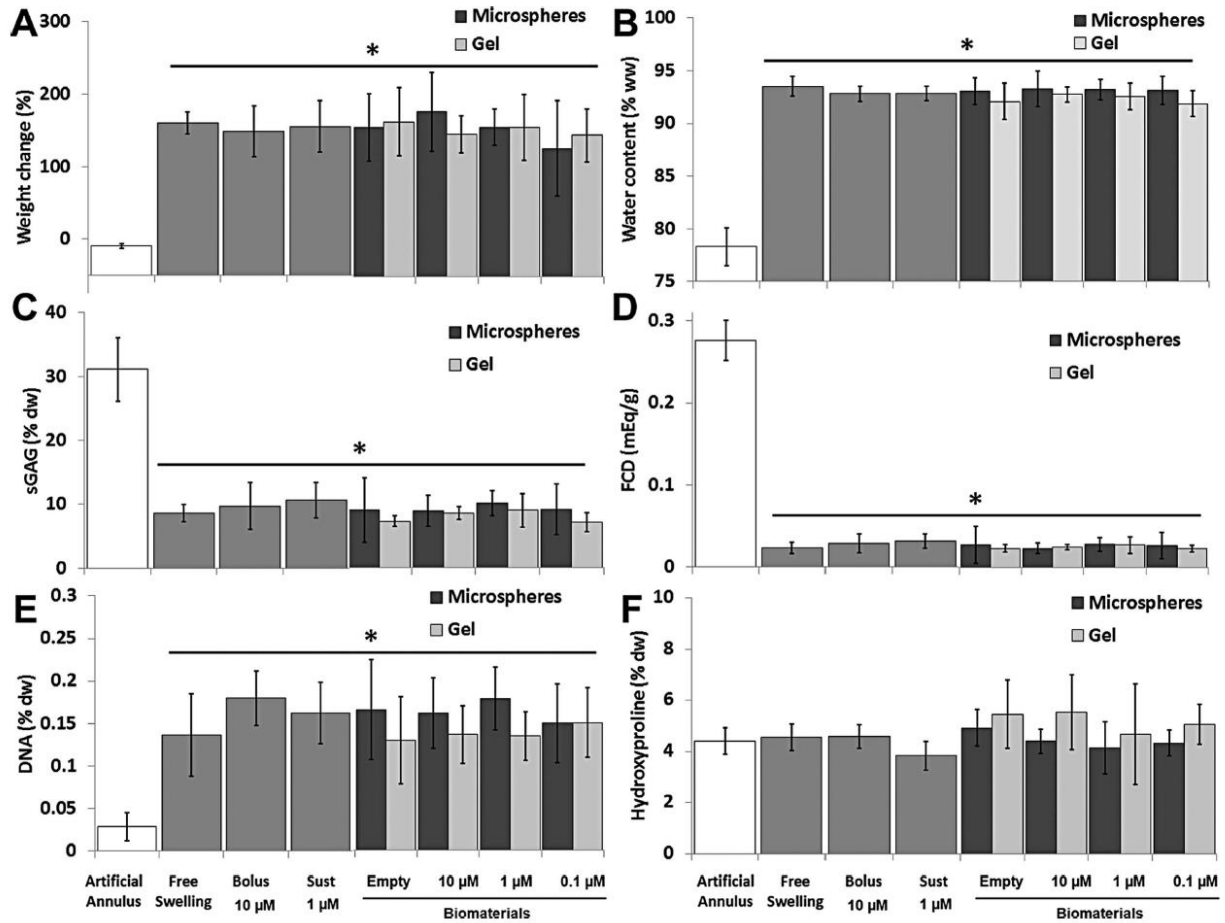
Explants were cultured in a deep-well insert plate (bottom). In some groups, celecoxib (Cxb) was added to the medium directly. Both the injectable gel (top left), and the microspheres (top right) were added using a culture insert. LDH, layered double hydroxide; pNIPAAm, poly(N-isopropylacrylamide); RT, room temperature (drawn by Anthal Smits).





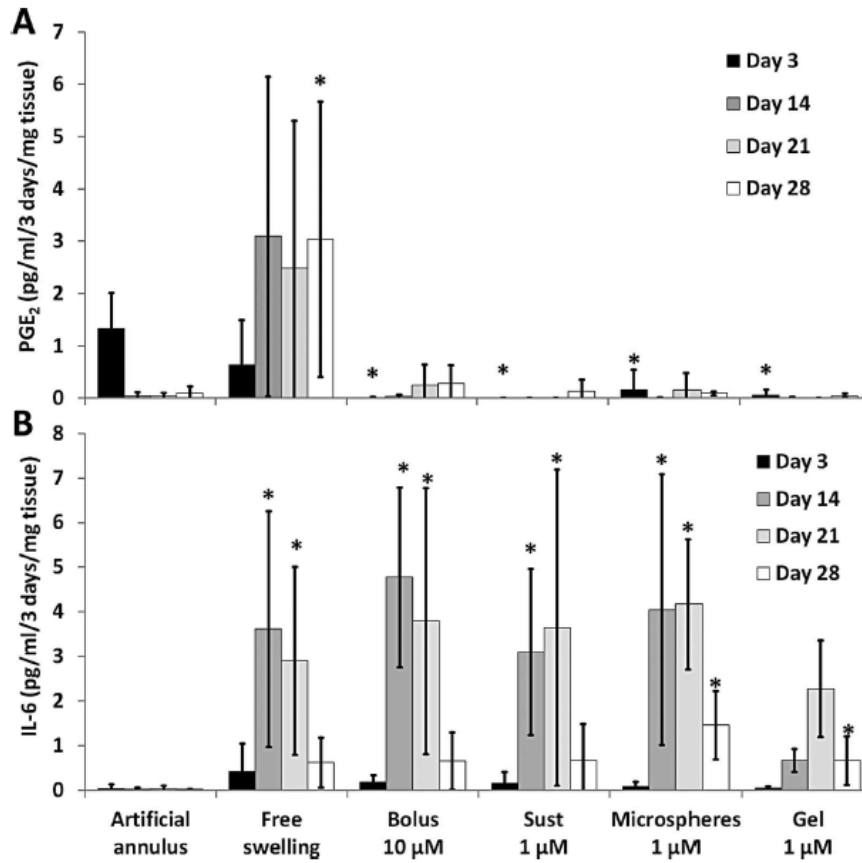
**Figure 3. Biochemical content of NP explants after 28 days.**

Weight change (A), water content (B), sulfated glycosaminoglycan (sGAG) content expressed per dry weight (dw) (C), fixed charge density in mEq/g (D), DNA content expressed per dw (E) and hydroxyproline content expressed per dw (F). Values are means ± standard deviation, n = 6. \* Different from artificial annulus; p < 0.05.



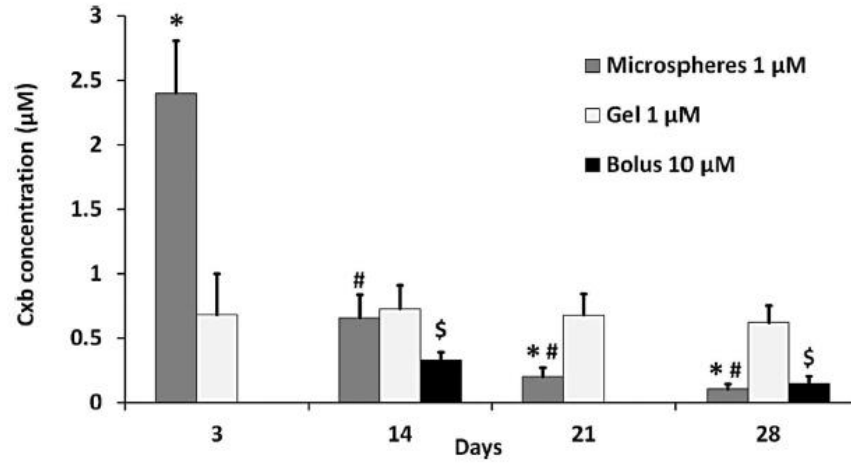
**Figure 4. Cytokine release into the medium over time.**

Release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>, **A**) and interleukin-6 (IL-6, **B**), in pg/ml, during 3 days, normalized to original sample wet weight (ww). Values are means +/- standard deviation, n = 6. \* Different from artificial annulus at same time point, p < 0.05.



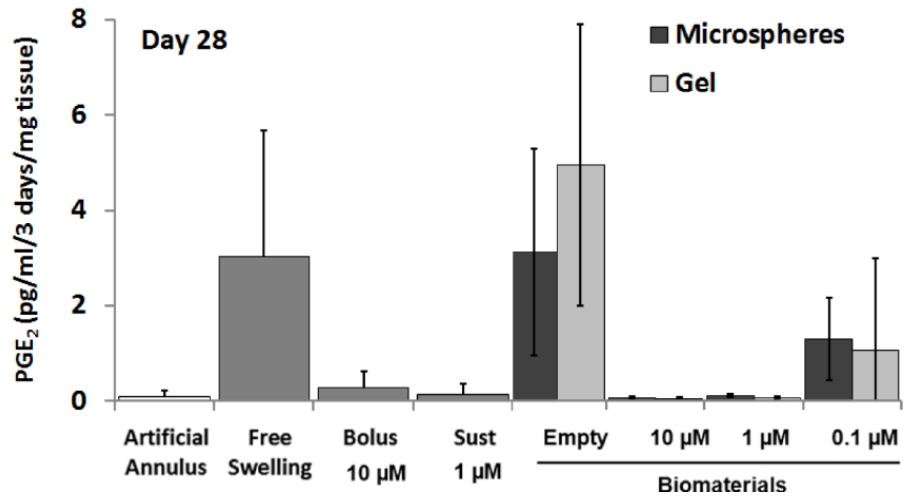
**Figure 5. Cxb concentration measured in the medium over time.**

Concentration in mM at days 3, 14, 21, and 28 for both biomaterials aimed for 1mM release, and at days 14 and 28 for bolus. Values are means + standard deviation, n = 6. \* Different from gel at same time point, # different from previous time point of the same biomaterial, \$ different from 0 mM, p < 0.05.



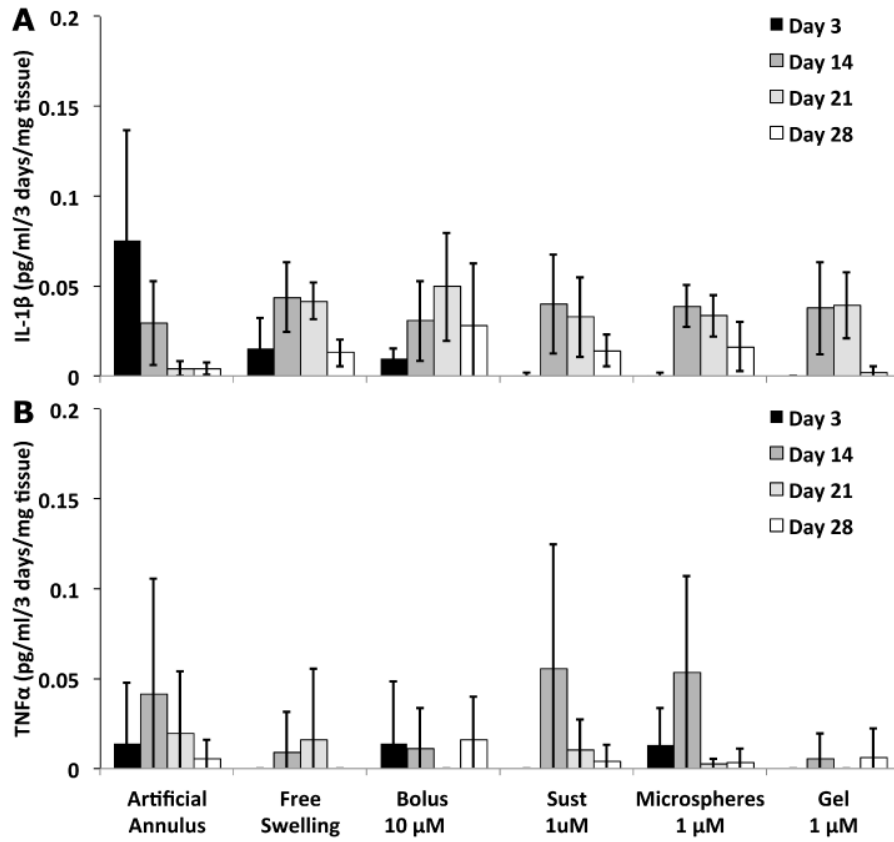
**Figure S1. PGE<sub>2</sub> release into the media.**

Release at day 28, in pg/ml, during 3 days, normalized to original sample ww. Values are means  $\pm$  standard deviation, n = 6.



**Figure S2. Cytokine release into the medium over time.**

Release of interleukin-1 $\beta$  (IL-1 $\beta$ , a) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ , b), in pg/ml, during 3 days, normalized to original sample ww. Values are means  $\pm$  standard deviation, n = 6.



**Figure S3. IL-6 release into the media.**

Release at day 14, in pg/ml, during 3 days, normalized to original sample ww. Values are means  $\pm$  standard deviation, n = 6.

