Injection of a polymerized hyaluronic acid/collagen hydrogel matrix in an in vivo porcine disc degeneration model

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Received: 21 August 2011 / Revised: 12 February 2012 / Accepted: 25 March 2012 / Published online: 25 April 2012
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Abstract

Introduction Disc degeneration and re-herniation after nucleotomy procedures are common problems. Simultaneous application of hyaluronic acid (HA)-based matrix has been proposed to limit disc degeneration. This, however, is hampered by loss of the substituted matrix out of the disc. Hence, in situ polymerization of the injected matrix with ultraviolet light (UVL) directly used after injection may be useful. Therefore, this study evaluates a new HA/collagen hydrogel matrix with in situ polymerization after implantation in an established porcine nucleotomy model.

Materials and methods 12 mature minipigs were used. A total of 60 lumbar discs were analyzed. 36 discs underwent partial nucleotomy with a 16G biopsy needle. Of those, 24 discs received matrix (porcine nucleus pulposus

collagenous scaffold component and chemically modified HA) which was in situ polymerized using UVL immediately after transplantation. 12 nucleotomized discs and 24 non-nucleotomized discs served as controls. After 24 weeks, animals were killed. X-rays, MRIs, histology, and gene expression analysis were done.

Results Disc height was reduced equally after sole nucleotomy and nucleotomy with HA treatment and in MRIs signal intensity decreased. For both nucleotomy groups, the nucleus histo-degeneration score showed a significant increase compared to controls. In histology, HA treatment resulted in more scarring and inflammation in the annulus. Gene expression of catabolic MMPs was up-regulated, whereas IFN-gamma, IL-6, and IL-1b were unchanged.

Conclusion Although nucleotomy and administration of the implant material did not cause generalized inflammation of the disc, localized annular damage with annulus inflammation and scarring resulted in detrimental degenerative disc changes. As a result, therapeutic strategies should strongly focus on the prevention of annular damage or techniques for annular repair to remain disc integrity.

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Keywords Disc degeneration · Nucleotomy · Porcine animal model · Ultraviolet light polymerization · Hyaluronic acid hydrogel matrix

Introduction

Several studies of disc cell transplantation, growth-factor injection, or gene therapy [1] raised hopes to revitalize a degenerated intervertebral disc (IVD). In the last decade, regenerative approaches focused on the transplantation of culture-expanded, autologous, disc-derived cells or stem cells. First clinical trials have been established on humans

with 2-year follow-up results after disc cell therapy [2]. However, at present such approaches to humans are still rare and limited due to remaining concerns about safety and biological potency. Although tremendous efforts have been made to improve disc cell therapy, today's results are rather disappointing with early loss of implanted cells caused by annular damage at the injection site and cell death due to the harsh intradiscal environment characterized by insufficient nutrition, accumulation of waste products and high intradiscal pressures [3–7]. Additionally, cell-based strategies are expensive, time demanding, and invasive with potentially more complications, since autologous cells have to be collected in a first operation, with the need for processing and cultivation, and then application to the disc in a second operative procedure.

Cell-free intradiscal matrix implantation with hyaluronic acid (HA) has been proposed as a less sophisticated alternative to improve disc repair and limit disc degeneration after nucleotomy procedures [8]. Promising results have been reported 24 weeks after nucleotomy with additional intradiscal application of different HA formulations in monkeys [9]. Latest studies focused on cell-free disc implants and found promising results for HA-based biomaterials [10, 11]. Cell-free implants made of polyglycolic acid (PGA) and HA induced the regeneration of articular cartilage in a sheep model [12]. In a rabbit model of disc degeneration, a cell-free PGA/HA nucleus implant facilitated the formation of nucleus pulposus repair tissue and limited the loss of disc height [13]. In a recent rabbit study of disc degeneration, cell-free PGA hyaluronan implants accelerated the healing process and were able to induce disc regeneration with cell migration into the defect [14]. Another rabbit study found promising results after application of a novel cross-linked hyaluronate hydrogel and cross-linked chondroitin sulphate hydrogel [15]. However, all these disc studies were limited using small and/or young animals with immature notochordal discs that generally offer a high regenerative potential. In bovine samples, a cell-free PGA/HA disc implant found restoration of spinal stability and prevention of implant herniation due to sealing of the annulus defect, but analysis of disc regeneration and biology was not possible due to the design of a biomechanical ex vivo study [16]. The idea of treating the nucleus with a liquid cell-free HA-based implant that is able to fill irregularly shaped defects and then do in situ polymerization to transform the liquid matrix into a solid form that is stable enough to withstand disc loading was also addressed in several studies [17–19]. Though, in vivo evaluation in large animal models with anatomical and biomechanical properties close to humans is still lacking.

Therefore, the present study evaluates a new HA/collagen-based cell-free matrix after implantation in partially nucleotomized aged porcine IVDs using an established

large porcine animal model. The implant material is a composite hydrogel matrix consisting of a collagenous scaffold component and chemically modified HA providing the capacity for in situ polymerization by long wave ultraviolet light (UVL) in order to fix the matrix inside the disc and prevent matrix extrusions. The specific objectives of this study were to compare radiographic, histological and gene expression changes of nucleotomized discs receiving HA hydrogel matrix against discs only nucleotomized and against exposed but not-nucleotomized discs.

Materials and methods

Animal population and operative procedure

A recently described porcine nucleotomy model was used [20]; here 12 Goettingen minipigs (age 2 years, skeletally mature, weight 50–70 kg) were treated using an anterolateral retroperitoneal approach to the spine. The animals underwent partial nucleotomy in general anaesthesia by a 16G biopsy cannula (Bard Angiomed, Karlsruhe, Germany). Five lumbar IVDs (L2–L6) were surgically exposed, of those three discs underwent partial nucleotomy (removal of 0.04–0.12 g jelly-like nucleus), whereas two other discs served as untreated control. Fibrin glue was used after nucleotomy to superficially seal the annular defect. Two randomized discs of the three nucleotomized discs received additional HA hydrogel matrix through the 16G nucleotomy needle immediately after nucleotomy (0.1–0.2 ml). Then, the UVL probe was inserted to polymerize the fluid HA hydrogel matrix that had filled the nucleus defects inside the remaining nucleus tissue. The survival time was 24 weeks. At the study endpoints, animals were killed, and discs were harvested aseptically.

Implant material

The implant materials are a composite hydrogel matrix consisting of a collagenous scaffold component and chemically modified HA. The chemical modification of HA in the hydrogel matrix provides the capacity for in situ polymerization using long wave UVL. The constituents of the hydrogel in saline consist of photo-oxidized porcine nucleus pulposus, glycidyl methacrylate (GM)–HA (HA modified by glycidyl methacrylate), Irgacure 2959, and vinyl caprolactam. Porcine nucleus pulposus tissue is processed and cross-linked by photo-oxidation using a proprietary technique (PhotoFix). Photo-oxidized porcine collagenous tissue is sterilized with ethanol. HA is reacted with GM in the presence of specific catalysts to produce GM–HA. GM is removed from the reaction mixture by precipitation with acetone. GM reacts with the hydroxyl or acetamido group on

HA producing an ester or tertiary amine linkage. The acrylate group added onto the HA molecule contains a carbon = carbon double bond which supplies the site to cross link GM–HA. GM–HA is polymerized into a solid gel by free radical polymerization in the presence of UVa light (365 nm), using an UVL probe which is inserted into the nucleus through the injection hole immediately after injection. Irgacure 2959 provides the free radical initiator. Vinyl caprolactam acts as accelerant and co-monomer in the chemical reaction. Pre-experiments on the photo-initiating system showed that the lowest effective dose of Irgacure 2959 (1,000 ppm) was adequate for use in short exposure regimens to initiate polymerization chemistry, with negligible immediate or residual cytotoxicity.

In vivo examinations

Each animal underwent lumbar spinal X-rays and MRIs preoperatively, 12 weeks postoperatively, and 24 weeks postoperatively. Disc thickness was calculated in 4 equally sized areas in anteroposterior and lateral radiographs by measuring longitudinal distances between adjacent endplates. Endplate and bone structures were evaluated by radiographs and T1- and T2-weighted sagittal MRIs (1.0 Tesla imager, Philips, Einthoven, The Netherlands) in supine animal position. IVDs were rated according to a modified Thompson classification from 1 (normal) to 4 (severe decrease of SI). Quantitative signal intensity (SI) was calculated by 1.8.3TM-Merge-eFilm-station-software (Toronto, Canada). Hence, SI of IVDs was normalized to sacral bone; for each disc, the mean from three sagittal planes was calculated. Detailed methods have been described earlier [20].

Ex vivo examinations

Histology

Analysis was done in nucleotomized discs ($n = 6$), control discs ($n = 12$), and nucleotomized discs treated with HA ($n = 12$) according to an established protocol [20] based on the scoring system from Boos et al. [21, 22].

Specimens underwent paraffin embedding and vertical 5 μ m sections were obtained in coronal plane from the central part of the disc including the mid nucleus pulposus, the surrounding annulus and the endplates. Haematoxylin-eosin and Alcian-blue stained sections were evaluated for the histo-degeneration score (HDS). The collagen fibre network was additionally evaluated under polarized light. For the HDS, ratings for cellular formation (0–5 points), tears and clefts (0–3), granular matrix changes (0–3), mucoid matrix changes (0–3) and necrosis (0–1) were used. Higher HDS scores (0–15 points) indicate more degenerated IVDs.

For endplate evaluation, the semi-quantitative endplate score was used with ratings for sclerosis (0–3), cellular formation (0–3), and necrosis (0–3). A higher total score (maximum 9 points) indicates more endplate degeneration. Discal scarring was evaluated with ratings from 0 to 3 (0 = no fibrosis; 3 = severe fibrosis) and evaluated separately for nucleus and annulus. Inflammation was evaluated with a yes or no judgment.

All histological slides were first investigated by one pathologist. Subsequently, a subset of 20 randomly selected histological slides was reviewed by a second observer. The interobserver concordance rate was high at 79 % in agreement with a kappa value of 0.83, suggesting excellent concordance rates.

Gene expression analysis

Realtime reverse transcriptase (RT) polymerase chain reaction gene expression analysis was done in control discs ($n = 12$), nucleotomized discs ($n = 6$), and HA-treated discs ($n = 12$) 24 weeks after surgery. mRNA isolation protocols, cDNA generation, primers, and the Lightcycler protocol (Roche Diagnostics, Mannheim, Germany) have been described earlier [20]. Porcine-specific primers for an additional selection of inflammation markers (IFN-gamma, IL-1b, IL-6) were designed.

Statistical analysis

Statistical analysis was performed using the primary outcome measures “disc height” and “SI diff” for MRI, “histo-degeneration-score“, “scarring”, “inflammation”, and “endplate-score” for histology and “gene expression relative to house keeping gene” for PCR. Median, range and standard deviations were calculated to show the empirical distributions of the measured values. For pre-post comparisons within one group the Signed-Rank test was used. The Kruskal–Wallis test was performed as a global test to calculate between group comparisons. In case of a statistical difference between the groups, pair-wise comparisons with the Mann–Whitney U test were calculated applying the closed testing procedure [23]. A two-tailed p value less than 0.05 was considered significant. Data analysis was performed with SPSS for Windows 12.0 (SPSS Inc., USA).

Results

All animals completed the study to the follow-up endpoint. No local or systemic toxic or anaphylactic reactions were seen.

Radiology

Preoperative disc height was reduced from 7.1 mm (range 5–10) to 5.3 mm (range 4–7) and 4.8 mm (range 3–7) 12 and 24 weeks after nucleotomy alone [reduction 25 % ($p < 0.0001$) and 32 % ($p < 0.0001$), respectively]. After nucleotomy with HA hydrogel matrix administration, disc height was also reduced from 7.7 mm (range 5–11) to 5.4 mm (range 2–7) and 5.7 mm (3–8) after 12 and 24 weeks, respectively [reduction 30 % ($p < 0.0001$) and 26 % ($p < 0.0001$), respectively]. Between nucleotomized discs and discs with additional HA hydrogel matrix treatment, no statistical difference was found at each time point. The intraobserver precision error was 0.015. X-rays found no disc calcifications. Minor osteophyte formations were only found after 24 weeks at discs with nucleotomy alone (4 from 12) or nucleotomy with HA hydrogel matrix administration (7 from 24).

Thompson scores found a deterioration from grade 1 preoperatively to grade 3 and 4 in discs with nucleotomy alone (after 12 weeks $n = 6$ grade 3, $n = 6$ grade 4, after 24 weeks $n = 4$ grade 3, $n = 8$ grade 4). All discs with HA treatment deteriorated to grade 4 and did not further change 24 weeks postoperatively. MRIs did not show significant endplate irregularities or protrusions of nucleus material into the spinal canal in all groups. In HA-treated discs, no dislocations of HA material out of the nucleus were found.

Calculated SI after nucleotomy—compared with preoperative SI—decreased to 57 % ($p < 0.0001$) after 12 weeks and 60 % ($p < 0.0001$) after 24 weeks with no statistical difference between 12 and 24 weeks. With UVL HA hydrogel matrix treatment, SI decreased to 30 % after 12 weeks and 26 % ($p < 0.0001$) after 24 weeks [vs. nucleotomy $p = 0.024$ (12 weeks) and $p = 0.018$ (24 weeks)]. The intraobserver precision error was 0.017.

Histology

The histological analysis of complete mid-sagittal disc specimens revealed major differences between the three groups: All controls showed typical histological disc features including a central nucleus pulposus with a myxoid matrix, low cell number without evidence for cell proliferation (such as cluster formation) nor disruption of the matrix. The glycosaminoglycans were evenly distributed (Figs. 1a, 2a). The inner annulus revealed increasing fibre content and a densely packed connective tissue of the outer annulus. Nucleotomy caused an increase of fibrous tissue in the inner annulus and especially the nucleus pulposus, often associated with cell clustering and focal disruption of the matrix, as well as mucoid matrix transformation (Figs. 1b, 2b). Those animals with HA treatment after nucleotomy also revealed tissue scarring, cell cluster

Fig. 1 Representative overview of mid-sagittal histological features: in controls (a), the low power view shows a well-arranged central myxoid nuclear matrix which is framed by dense hyaline/fibrous cartilage of the end plates and the inner annulus. 24 weeks after nucleotomy (b), the myxoid structure of the nucleus is almost lost and dense fibrous tissue is detectable. The endplates have a scar-like appearance, the inner annulus is hardly discernable from the nucleus. Those animals with additional HA application (c) show a pattern comparable to samples after nucleotomy alone. Areas with higher magnification particularly show the matrix and cell changes between the three groups: In normal discs (a), moderate cellularity is present in a moderately dense collagenous matrix. In the nucleotomy group (b) and HA group (c), enhanced cell density is visible

formation, disruption of the matrix and mucoid degeneration, but no considerable inflammation (Figs. 1c, 2c). Focal necrosis was present in the nucleus and inner annulus, but not in the outer annulus. Inflammatory changes were

Fig. 2 Alcian-blue staining of glycosaminoglycan matrix also reveals differences between the three groups. Normal discs (a) contain a fairly homogenous non-collagenous matrix with interspersed cells. Focal mucoid cystic changes are present in the nucleotomy group (b) and HA group (c)

restricted to the outer annulus in areas of surgical intervention and UV light exposure in HA-treated discs. The HA material was not detectable by histological stains, due to the fact that the solid particles were lost during

sectioning of the specimens. However, the presence of optically “empty” spaces in those discs suggests that at least part of the HA matrix had been retained in the treated discs.

In addition to these more descriptive aspects of disc histology, the extent of tissue changes was ranked using semi-quantitative scores with the following observations:

Nucleus and annulus HDS

Compared to controls, a statistically significant increase in HDS was found in the nucleus both for discs treated with UVL HA hydrogel matrix ($p = 0.02$) and discs treated with nucleotomy only ($p = 0.004$), indicating increased disc degeneration. In the nucleus, HDS was lower after UVL HA hydrogel matrix (6) compared with discs only nucleotomized (8). However, this difference was not statistically significant, although there was a trend towards a reduced HDS in the HA hydrogel matrix group ($p = 0.066$), indicating less degeneration.

In the annulus, a significant increase of HDS was not found.

Detailed results are depicted in Fig. 3a, b.

Nucleus and annulus scarring score

In the nucleus, no statistical difference was found whereas in the annulus, discs treated with UVL HA hydrogel matrix showed more annular scarring ($p = 0.009$). Detailed results are depicted in Fig. 3c, d.

Nucleus and annulus inflammation

In the nucleus no considerable inflammation was found in all groups. In the annulus, considerable inflammation was only found in HA-treated animals beneath the annular lesion, where nucleotomy was performed and the UVL probe was inserted.

Endplate degeneration score

Compared to controls, a statistically significant increase was found both in discs treated with UVL HA hydrogel matrix ($p = 0.006$) and discs treated with nucleotomy only ($p = 0.027$). No significant difference was found between HA and nucleotomy only ($p = 0.93$). Detailed results are depicted in Fig. 3e.

Gene expression

Extracellular matrix genes such as Col1, Col2, and Decorin were up-regulated in HA-treated discs as well as catabolic MMP13 and MMP3. Anabolic TIMP1 was also

Fig. 3 Results of nucleus (a) and annulus (b) histo-degeneration-score (HDS), nucleus (c) and annulus (d) discal scarring score, and endplate degeneration score (e) with median score values (y axis) and range (bars). Three columns are depicted for the three groups:

controls (white), nucleotomy (grey), nucleotomy with UVL HA hydrogel matrix (dark grey). * $p < 0.05$ versus controls; + $p < 0.05$ versus nucleotomy

up-regulated, whereas anabolic BMP2 m-RNA levels were equally expressed both after sole nucleotomy and nucleotomy with additional HA hydrogel matrix. Inflammation markers (IFN-gamma, IL-1b and IL-6) did not show statistically significant regulations. Detailed results are depicted in Fig. 4a–d.

Discussion

Spinal surgery for neural decompression after disc herniation with predominant radicular leg pain is a common and successful procedure. However, in the long-term, major problems develop due to painful disc degeneration of the treated segment as well as re-herniation [24]. Thus, as a possible strategy against developing disc degeneration, the administration of UVL HA hydrogel matrix during primary surgery was analyzed in an established porcine nucleotomy model with aged discs and biomechanical properties close to the human situation [20].

In the present study, both treatment groups showed significantly more degenerative signs in the HDS than non-operated control segments 24 weeks after surgery. Additional application of UVL HA hydrogel matrix only

resulted in a histological trend towards less degeneration compared with nucleotomy alone and disc height and disc hydration was not improved. In MRIs, no matrix herniations or dislocations were found and SI was reduced as a specific characteristics of the HA hydrogel matrix which was in situ polymerized and hence aggregated with the remaining nucleus tissue, resulting in a stable fixation in the nucleus centre.

Histological signs of disc degeneration in both the treatment groups can be attributed to the detrimental effect of both partial nucleotomy and annular damage with the 16G needle and results were compatible with recent data on the used porcine animal model [20].

However, results after UVL HA hydrogel matrix application have to be discussed further. Compared to nucleotomy alone, additional needle and UVL probe manipulations with increased annular damage will have progressed disc degeneration, which is supported by localized annular inflammation and scarring in this group as well as increased gene expression levels for MMP3, MMP13 and Col1 (see Fig. 4a, b). Discs with nucleotomy only were not exposed to additional sham manipulations to simulate hydrogel application or UVL probe insertion. In a clinical setup, additional manipulations with more annular

Fig. 4 Median mRNA levels with standard deviations (bars) in % normalized to controls and relative to housekeeping gene. For each gene, three columns are depicted for the three groups: controls

(white), nucleotomy (grey), nucleotomy with UVL HA hydrogel matrix (dark grey). * $p < 0.05$ versus controls; # $p < 0.05$ versus nucleotomy

damage would also be necessary. Nevertheless, histological disc degeneration was not higher in the UVL HA hydrogel matrix group and in histology there was a trend towards less degeneration in the nucleus compared to nucleotomy alone without inflammatory changes or foreign body reactions (no giant cells). This is compatible with m-RNA levels of the inflammation markers IFN-gamma, IL-1b and IL-6 (see Fig. 4d) which did not show statistically significant regulations 6 months after nucleotomy alone, or nucleotomy and HA hydrogel treatment, compared to internal controls. These results may have several reasons: First, analysis was done late (6 months) after acute disc injury, when m-RNA levels of inflammatory factors seem

to be down regulated again. This supports the concept that treatment options targeting inflammatory factors or interleukins may be rather effective immediately after acute disc trauma but not at later stages of disc degeneration. Second, in the literature inflammatory mediators were mostly elevated in herniated or painful discs [25–29]. Hence, our gene expression data suggest that 6 months after disc injury, generalized painful disc inflammation was not apparent. As a limitation of this study, only a small number of individuals were analyzed with rather heterogeneous m-RNA levels. Consequently, only very pronounced differences will reach statistical significance whereas less pronounced differences cannot be detected.

As control discs were “internal controls” with surgery to induce disc degeneration in adjacent discs, m-RNA levels of those control discs might be slightly elevated as well, causing less pronounced differences between the groups.

Apart from the evident additional annular damage with local annular inflammation and scarring, other contributing factors for the degenerative changes in HA-treated discs could be detrimental cell effects caused by UVL exposure and the photo-initiating system as well as biomechanical changes of the disc properties.

If the HA hydrogel matrix is capable of preventing re-herniation of the disc after nucleotomy, procedures cannot be sufficiently answered in this animal model. Annular damage by the 16G needle is moderate in this model and consequently, no disc herniations were found after the partial nucleotomy procedure.

Limitations of the animal model have to be mentioned, as to the artificial setup of an acute disc injury model of partial nucleotomy. In general, animal models show limitations in size, biomechanics and tissue properties. Especially, the existence of notochordal cells in animal discs in opposite to human discs is important. However, the used porcine model seems to be appropriate and offers biomechanical conditions compatible to the human situation [20]. Early changes after implantation were not analyzed. The hydrogel was not particularly visible in the histological sections since the material was obviously removed during sectioning. However, optically “empty” spaces with a surrounding more or less concentric fibre arrangement were found highly suggestive for HA-particle spaces. MRIs did not reveal extruded material after 12 or 24 weeks. At tissue preparation no evidence for matrix extrusions was found. Since analysis was not done immediately after implantation, however, a partial dislocation of the HA matrix cannot be excluded.

Whereas most implants are designed to perform a nucleus replacement with the original nucleus being almost completely removed [30], the intention of the present study was different. Smaller defects of the nucleus developing both in the natural course of disc degeneration as well as after traumatic disc injury or after disc herniation may be treated only with small amounts of hydrogel matrix. Under these circumstances, only the defect areas (~10 % of nucleus volume) were filled homogenously by the fluid hydrogel and most of the original nucleus tissue was remained. Since the nucleus was filled gently without much pressure, severe biomechanical alterations are not expected and even pressures should result over the endplates. To avoid material extrusion through the annulus defect, UVL was finally administered for in situ polymerization. However, as a serious limitation, biomechanical and intradiscal pressure analysis was not performed.

Although the annular lesion was closed with fibrin glue and, from a macroscopical view, seemed to close

completely through retraction forces of the annulus lamellae, microscopic analysis revealed considerable annular damage with increased postoperative scarring and localized inflammation maintaining the degeneration process. Injected cells and fibrin matrix (without UVL polymerization) get lost easily through this annular lesion [3, 4, 6, 7]. Consequently, the nucleus is exposed to the immune system and to detrimental degenerative cascades with up-regulation of MMPs [31, 32]. These mechanisms seem to be more pronounced in heavily loaded discs of large adult animal models offering higher human like body weights. Smaller and younger animals achieve better results in regenerative disc therapies due to biomechanical, anatomical, and cellular differences offering higher regenerative potential [1, 10, 12–15]. However, portability to the human situation seems to be more doubtful in small animal models and therapeutic strategies should finally be evaluated on large models with biomechanical properties similar to humans.

Disc herniation treatment by nucleotomy procedures should avoid further damage to the annulus as much as possible. Key and Ford analyzed annular healing in a dog model with 20G needle puncture and found sufficient annular healing after 22 weeks [32]. Newer studies focused on the very limited regenerative capacity of the annulus fibrosus resulting in thin layers of biomechanical inferior fibrous tissue [6], and Korecki et al. [33] recently described detrimental effects of needle puncture injury on disc mechanics and biology even by small needles up to 25G. New sealing and barrier techniques as well as biological annulus repair are currently being developed, but promising results are still missing [6].

In conclusion, the management of annular damage seems crucial for new disc therapies. The administration of UVL HA hydrogel matrix in an established large adult porcine animal model of partial nucleotomy was not sufficient to regenerate the disc and caused further annular scarring and localized annular inflammation. In further studies, more efforts should be taken to seal the annular lesion with biomechanically appropriate methods. Under such circumstances, potency of polymerized hydrogel implants should be re-judged.

Acknowledgments We thank Bernhard Pieta for helping with animal acquisition and surgical assistance.

Conflict of interest None.

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