Proteoglycan-depleted regions of anular-injury promote nerve ingrowth in a rabbit disc degeneration model

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Research

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Abstract

Background

To assess the effects of proteoglycan-depleted regions of disrupted annulus on nerves ingrowth into the injury site in Vivo. Our study was designed to provide a test group of polymeric carriers loaded with chABC, using to enzymatically digest CSPGs in a controlled manner at the site of injury. And 2 control groups, one loaded with PBS buffer alone or the other created annular defect.

Methods

New Zealand white rabbits (n=18) received annular injuries at L3/4, L4/5, and L5/6. The experimental discs were randomly assigned to four groups: a) annular defect was created(1.8 mm diameter; 4 mm depth); b) annular defect implanted PLGA/fibrin/PBS plug; c) annular defect implanted with a PLGA/fibrin/chABC plug; d) uninjured L2/3 disc (control). Disc degeneration was evaluated by radiography, MRI, histology, and analysis of proteoglycan content. And immunohistochemical detection of nerve fibers and chondroitin sulfate was respectively performed with PGP9.5 and CS-56.

Results

The radiographic, MRI, histological and biochemical changes demonstrated that the injured discs produced progressive and reliable disc degeneration. In the defect discs, lamellated appearance of AF has been replaced by extensive fibrocartilaginous-like tissue formed outside the injured sites. In contrast to be distributed along small fissures within newly formed tissue accompanied with small blood vessels appeared in the outer part of disrupted area in the PLGA/fibrin/PBS discs. Following chABC effectively enzymic deglycosylation, the residual scaffolds were surrounded by newly formed tissues, where more sprouting nerve fibers grew further into the depleted annulus regions in the PLGA/fibrin/chABC discs than control discs and those receiving PLGA/fibrin/PBS. In addition, innervations scores in the PLGA/fibrin/chABC discs showed significantly higher than those of PLGA/fibrin/PBS discs and defected discs.

Conclusions

ChABC-based PLGA/fibrin gel achieved the bio-integration with native annulus tissue and providing a local source for sustained release of active chABC. Disc-derived proteoglycan inhibited nerves and blood vessels ingrowth were evidently abrogated by chABC enzymic deglycosylation in an annular-injured rabbit disc degeneration model. The inhibitory effects of Neural and vascular ingrowth are inversely associated with the proteoglycan content. CS secreted from discs are potential candidates that could be useful to reduce neurite growth associated with discogenic pain.

Background

Low back pain is an extremely common socioeconomic problems in orthopaedics, although the pathophysiology of this condition remains uncertain, degeneration of the intervertebral disc(IVD) is believed to be a major cause of low back pain[1–3]. The degenerative change ultimately results in increased matrix degradation, pro-inflammatory cytokine expression and inferior mechanical properties [4–6]. More recently, progressive neurovascular growth in the annular fissures and sensitization of nerve fibers has been observed in the degenerated IVD[7, 8]. Ingrowth of nociceptive nerve fibers deep within disrupted intervertebral discs are believed to be one of the potential sources of discogenic pain in clinical cases [9–12].

It has been shown that aggrecan, a proteoglycan found in the disc, may act as a barrier to inhibit nerve fiber growth in vivo/vitro [13–16]. However, the precise inhibitory mechanism remains poorly understood. In previous work we showed that a minimally invasive annulotomy-induced rabbit model was well established, an orientated growth-permissive nerves growing into annulus defects during the degenerative process. Also, loss of proteoglycan at the injury site involved in IVD degeneration have been observed to facilitate the infiltration of blood vessels and nerve fibers into disrupted disc tissue[17, 18].

Chondroitinase ABC (chABC) is a well-studied bacteria-derived enzyme for being able to specifically depolymerize the glycosaminoglycan (GAG) side chains for the induction of mild disc degeneration and favor axonal regeneration by reduction of proteoglycan in the injury site [19–24]. According to our previous works, PLGA/fibrin constructs as delivery system have good performance on biocompatibility, safety, and biodegradability[18, 25], also in promoting in tissue repair without compromising the function of incorporating proteins [26–30]. Therefore, as a potential platform for the carrier of chABC, it should be a simple and desirable alternative to achieve local delivery in a controlled manner as to cleavage of GAGs via digestion overcome CSPG-mediated inhibition at the injured site of the degenerated IVD.

In the present study, we hypothesized that proteoglycan-depleted regions of disrupted annulus could facilitate nerves and blood vessels ingrowth into the deeper parts of degenerated disc in a rabbit animal model. Here, fibrin-based polymeric carriers loaded with chABC for its sustained local delivery in vivo were implanted into injured annular site, to evaluate the effects of reduction of proteoglycan on nerves ingrowth during the IVD degeneration.

Methods

Preparation of PLGA-coated fibrin gel/ChABC constructs

The PLGA sponges were fabricated by a porogen leaching method as reported previously[31]. The PLGA/fibrin/chABC constructs were prepared as follows. The fibrinogen was isolated from fresh human plasma and final concentration used in all the experiments as previously established protocols [25, 32]. ChABC (2U, Sigma, St. Louis, MO) was dissolved in buffer with a final concentration of 0.10 U/ml according to the product instructions and previous reported[19, 33]. Thereafter, the fibrinogen (20 mg/mL) and chABC (0.10 U/ml) solutions were homogenized and sterilized by filtering through syringe filters. The

PLGA plugs of 1.8 mm diameter and 4 mm length were immersed into the homogeneous fibrinogen/chABC solution (1 mL) under reduced pressure. The composite constructs were lyophilized and imaged using a SEM, and subsequently stored at -20 °C until further use.

Evaluation Of chABC Release From PLGA/fibrin In Vitro

To obtain the release profiles of each PLGA/fibrin gel loaded with chABC was suspended in 100 μ L of PBS solution (pH, 7.4) in polypropylene tubes placed in a shaker bath (37 °C) at 100 rpm. 10 μ L of supernatant was collected from each PLGA/fibrin sample at predetermined time intervals (1, 2, 3, 4, 5, 7, 9, 12 and 14 days) and individually stored at -20 °C. According to previously reported protocol[34], the solution was tested for enzymatic activity by measuring the formation of unsaturated disaccharides by degrading the chondroitin sulfate over time (Sigma enzymatic assay-EC 4.2.2.4 per manufacturer instructions). Finally, the percentage of sample absorbance relative to the standard was calculated, the composite construct's accumulated release kinetic curve was obtained.

Animal Surgery

A total of 18 New Zealand rabbits (age 8.60 ± 1.25 months, weight 3.42 ± 0.16 kg) were supplied by the Laboratory Animal Center of Zhejiang province. All aspects of the study were approved by our Institutional Animal Care and Use Committee. The rabbits were randomly allocated to 1, 3, and 6-month survival groups ($n = 6$ in each group). The procedure of surgical implantation was used as described previously[17]. The consecutive levels of the rabbit IVD including L3/4, L4/5, and L5/6 were exposed by an anterolateral retroperitoneal approach. Annular injuries were randomly allocated to 4 disc levels: (1) Annular defects group: annular defects (diameter 1.8 mm; 4 mm depth) were created by a mini-trephine, as previously described[17, 18]; (2) PLGA/fibrin/PBS group: annular defects were filled with a PLGA/fibrin gel plug loaded phosphate buffered saline; (3) PLGA/fibrin/chABC group: annular defects were filled with a PLGA/fibrin gel plug loaded chABC; (4) Intact group: the L2/3 disc served as uninjured control. Finally, the wound was closed in layers. Following surgery, the rabbits were permitted free cage activity and food and water ad libitum.

Radiographic Analyses And Magnetic Resonance Imaging (MRI)

X-rays were obtained under general anesthesia (sodium pentobarbital, 30 mg/kg) at 1, 3, and 6 months after surgery ($n = 6$ per time point). Comparison of disc height index (DHI) was calculated as a ratio of the injured disc height to the sum of the height of the two adjacent vertebral bodies, as previously described[35, 36]. The DHI% therefore were expressed as DHI normalized to the baseline preoperative measurement (postoperative DHI/ baseline DHI $\times 100$). In vivo MRI of the lumbar spine examinations were performed using a 1.5-T MRI system (Signa, General Electric, Milwaukee, Wisconsin). Midsagittal

T2-weighted images were obtained for analysis as previously described by the Pfirrmann's classification scores[37] based on changes of degree and area of signal intensity. All measurements were done using the picture archiving and communication system (PACS) routinely used in the local hospital.

Tissue Harvesting

At 1,3 and 6 months after surgery (n = 6), the rabbits were euthanized by intravenous sodium pentobarbital overdose for histology and immunohistology analysis. The experimental IVDs (L2/3, L3/4, L4/5, and L5/6) were removed from each lumbar spine under sterile conditions. The specimens were then dissected sagittally and divided into two symmetric parts. From one half of each disc, the nucleus pulposus (NP) was bluntly separated from the AF and then snap-frozen in liquid nitrogen, with subsequent storage at -80 °C in preparation for s-GAG analysis. The other half was used for histological analysis.

Sulfated-glycosaminoglycan Content Measurement

Samples of the NP were isolated from each level discs at 6 months after surgery (n = 6). The amount of proteoglycan content was quantified using the DMMB assay[38, 39]. Briefly, each lyophilized sample was digested with 125 µg/mL papain (Sangon Inc, ShangHai; PRC) in sterile PBS, 5 mM EDTA, and 5 mM cysteine·HCl at pH 6.8 and 60 °C overnight. After complete digestion, 20 µL papain digest were added to 200 µL of DMMB reagent, with absorbance detected at 520 nm. Total sGAG in the disc for each group was normalized according to the tested DNA amount, and then the sGAG/DNA ratio was measured and reported. The s-GAG content of the nucleus was measured and expressed as a percent of dry disc weight.

Histology And Immunohistochemistry

The specimens were fixed in 10% formalin, decalcified in ethylenediamine tetraacetic acid (EDTA), and processed for paraffin sectioning. Blocks of tissue were paraffin-embedded and sectioned at a thickness of 5 µm. Sections of IVD samples were stained with hematoxylin/eosin (HE) to observe degenerative changes, or with safranin-O staining for the assessment of the proteoglycan content. Alternatively, the sections were subjected to immunohistology for the nerve marker Protein gene product 9.5 (PGP9.5). CS-56 immunostaining was used to identify intact chondroitin sulfate (CS). All stained sections were analyzed under an optical microscope (Leica Microscope, Wetzlar, Germany). Briefly, the epitopes for PGP9.5 immunohistology were first heat-induced retrieved. The sections were then blocked with hydrogen peroxide and 25% normal bovine serum albumin (BSA)/tris buffered saline (TBS), and incubated overnight at 4 °C with the primary antibody to mouse monoclonal antibody against human protein gene product 9.5 (diluted 1:80, Abcam, Cambridge, GB). For CS56 staining, the sections were subjected to blocking of the endogenous peroxidase activity with 0.5% hydrogen peroxide for 1 h and then washed three times in TBS. Then, blocking with 25% normal BSA for 1 h (both at RT), and overnight incubation at

4 °C with a primary antibody to mouse anti CS56 (diluted 1:100, Sigma, St. Louis, MO). The sections were then washed again and incubated in biotinylated goat anti-mouse IgG antibody (1:200; Vector Laboratories) overnight. The sections were then processed with the avidin–biotin amplification method with conjugated peroxidase (Vectastain ABC Elite Kit; Vector) and visualized with diaminobenzidine (DAB; Sigma). Sections were then counterstained with hematoxylin and mounted with Aquatex for light microscopy. Control immunoglobulins consistently yielded negative results. The ingrowth of immunoreactive nerve fibers in the specimen was scored using a previously described grading scale [18, 40].

Statistical analysis

Results were expressed as means $\pm$ standard error of the mean. Statistical analysis was performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Significant differences in the radiograph measurements were analyzed by repeated-measurement analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) test. The effect of time after surgery was analyzed with the Kruskal-Wallis test. Mann-Whitney U tests were used to analyze the MRI score, innervation, and biochemical data. The level of significance was set at $P < 0.05$.

Results

PLGA/fibrin/ChABC morphology and ChABC release kinetics

The fabricated PLGA sponges filled with fibrin gel and chABC-coating of the PLGA/fibrin construct demonstrated by SEM, are shown in Fig. (1a) and (1b). SEM micrographs showed that interconnected micropores with a mean pore size of 350 μm . The profiles of chABC release from PLGA/fibrin scaffold over 14 days are shown in Fig. 1(c). The curve of chABC from fibrin-based carrier showed a significant initial burst release during the first five days, and the chABC release continued steadily thereafter. Overall, the cumulative release rate of active chABC was found to be reached about 80% after 8 days.

Radiographic And MRI Assessment

Compared with the normal control group, disc height of the injured groups showed a slow but progressive decrease, sustaining for up to 6 months. At 6 months, narrowing disc space and bridging osteophyte were apparently observed in the two scaffold groups (Fig. 2a-b). The DHI% of the two scaffold groups and the AF defect group were significantly lower than that of normal control groups at all postoperative periods ($p < 0.05$), whereas no significant difference was observed between PLGA/fibrin/PBS and PLGA/fibrin/chABC group ($p > 0.05$). Notably, DHI% in the defect group demonstrated a significant decrease overtime by 1,3,6 months after surgery ($p < 0.01$) (Fig. 3).

Serial MRI scans of rabbit lumbar were showed that MRI appearance of the normal control discs remained relatively constant. Progressive decreases of signal intensity in NP area were apparent for each of the injured discs at all time points in Fig. 2c-d. The grade of MRI in the injured groups were progressively higher than that in the normal control groups at the postoperative time point ($p < 0.01$). Additionally, both the MRI grade of AF defect and PLGA/fibrin/chABC discs were significantly higher than that of the PLGA/fibrin/PBS or control discs after surgery($p < 0.05$). However, no significant differences in the MRI grade was found between the PLGA/fibrin/chABC and the AF defect group at 1, 6 months after surgery($p > 0.05$) (Fig. 4).

Sulfated-glycosaminoglycan Content Of Nucleus

The s-GAG content of three injured groups decreased significantly compared to control group at postoperative different time-point ($p < 0.01$). And the s-GAG content of PLGA/fibrin/chABC group showed a significant decrease when compared with that of the PLGA/fibrin/PBS and AF defect groups after surgery ($p < 0.05$). The decrease of sGAG/DNA ratio observed in the AF defect group was significantly more pronounced than in the PLGA/fibrin/PBS group at 1,6 months after surgery($p < 0.05$) (Fig. 5).

Histologic Assessment

At 6 months, HE staining demonstrated that the uninjured, healthy discs (control group) were characteristically well-organized intact AF with its concentric lamellae. On safranin-O staining, uninjured discs showed minimal disruption of the proteoglycan matrix within the annulus (Fig. 6a, b). In the AF defect group, HE staining showed that loss of the lamellated appearance of AF, replacement of the annular defect by extensive fibrocartilaginous-like tissue formed outside the injured sites. Some blood vessels and small fissures at a limited depth were typically distributed in the outer scar tissue and AF, but not extending to the inner AF(Fig. 6c). The safranin-O staining indicated the presence of proteoglycan-rich content in the fibrocartilaginous tissue (Fig. 6d). In the PLGA/fibrin/PBS discs, HE staining showed small residues of PLGA scaffold with their naturally irregular form were enfolded by newly-formed tissue and well-integrated within the inner AF (Fig. 6e). In addition, safranin-O staining showed open concave cavity increased severe loss of proteoglycan content (Fig. 6f). In contrast, advanced degeneration was observed in the PLGA/fibrin/chABC group, including NP fibrosis, disorganization of the AF and clusters of newly tissue extended further into the deeper inner AF along the fissures. Meanwhile, some blood vessels and small fissures were observed relatively common compared with that in PLGA/fibrin/PBS group (Fig. 6g). The proteoglycan content was markedly reduced in the injured region (Fig. 6h).

CS-56 Immunolabeling For CS-GAG Digestion

CS-56 immunolabeling in the lesion site indicated that chondroitin sulfate was degraded following treatment with chABC. In the control group, the outer lamellar AF was weakly stained with the CS-56

antibody (Fig. 7a). In the defect discs, scar tissue was formed on the surface of the injury site. A fuzzy CS56-positive deposit was distributed in the scar tissue (Fig. 7b). In the PLGA/fibrin/PBS group, small residues of PLGA scaffold were surrounded by newly repair tissue penetrating deeper toward the NP. CS56-positive deposits were relatively negligible in the clusters of new regenerated tissue (Fig. 7c). In contrast, in the chABC/PLGA/fibrin-treated groups, CS-56 immunolabeling was high in the region of newly generated tissue at the lesion site but was intense reactivity overall (Fig. 7d).

Innervation Of The Intervertebral Disc

PGP9.5-positive nerves were seen sparsely in the lamellae and adjacent connective tissue of the outer AF (Fig. 8a). In the AF defect group, small nerves in the vicinity of the outer AF were distributed along the fissures, but barely seen to invade the deeper inner AF (Fig. 8b). In the PLGA/fibrin/PBS group, nerve fibers reactive for PGP9.5 were identified within the newly generated tissue of inner AF (Fig. 8c). In contrast, more sprouting PGP9.5-immunoreactive fibers in the PLGA/fibrin/chABC group were localized predominantly in the vicinity of vascularized repair tissue extended further into the inner AF as well (Fig. 8d).

In terms of semi-quantitative innervation scores, three injured groups increased slightly over the follow-up period. Notably, Innervation grade of the PLGA/fibrin/chABC group was significantly higher than that in the AF defect or PLGA/fibrin/PBS group at 1,6 months after surgery ($p < 0.05$). The innervation score in the PLGA/fibrin/PBS group was significantly higher than that of AF defect group at 1 and 6-month time point ($p < 0.05$), but no significant differences among the different time points ($p > 0.05$; Fig. 9).

Discussion

In our previous study[17, 18, 41], an AF injury model was established by creating a defect, resulted in reproducible and degenerative MRI, radiograph, and histologic changes. Interestingly, degenerative changes occurred, characterized mainly by formation of the regenerated tissue and ingrowth of nerves and blood vessels into disrupted disc tissue. More recent studies have shown that aggrecan derived from both the AF and NP has inhibitory effect on nerve ingrowth into the IVD[13, 18, 42]. Thus, this experiment was conducted in an effort to evaluate the effects of degrading chondroitin sulphate (CS)-PG on neurite ingrowth by use of PLGA/fibrin gel incorporated with/without chABC was anchored in the annular defects during the degenerative process of the IVD. The results showed that fibrin-based PLGA loaded chABC exhibited to integrate well with native annulus tissue and provided a local source for sustained release of active chABC. Importantly, more nerve fibers were orientated conductively to grow into the deeper proteoglycan-depleted regions of disrupted annulus following chABC enzymic deglycosylation. To our best knowledge, there were few reports involving in vivo testing to assess the effects of proteoglycan-depleted site in the annular wall on nerves ingrowth in a rabbit disc degeneration model.

It is well known that the action of chABC on proteoglycans was more specific degrades the chondroitin sulfate side chains of proteoglycans[24, 43]. Indeed, the use of proteolytic enzyme for the induction of

mild disc degeneration has been successfully defined one of the modalities to achieve both in rabbits[44, 45] and in large animal sheep[19, 33]. Furthermore, reduction of proteoglycan using fibrin-based delivery system achieving the enzymatic digestion depends on its dosage and activity[25, 46–48]. Based on the promising preliminary results, a dose of 0.1 U/mL chABC incorporated into PLGA/fibrin gel was determined in the present study. In line with previous studies[34, 49], the rate and duration of in vitro chABC release from the fibrin-based polymer was characterized by an initial burst (70–80%) and presented in sustained manner up to 8 days. Thereafter, a small amount of chABC was still being released from the PLGA/fibrin gel, which achieved a delayed-release effect, as indicated in Fig. 1c. In addition, CS56-immunolabeling in the lesion site revealed that local distribution of chondroitin sulfate was degraded following treatment with chABC. Taken together, the results indicated that fibrin-based polymeric carriers in the injured site achieved the sustained, localized release of chABC, the activity of chABC in vivo was maintained after surgery, as shown by continuous significant decrease in the s-GAG content of PLGA/fibrin/chABC discs in comparison to 2 control discs. Conceivably, the present study rather undertakes a detailed analysis of the actual process of the glycosaminoglycan reduction and its activity, as it is very unlikely that the in vitro release kinetics would accurately reflect the more relevant in vivo release kinetics in the lesion site.

We observed PGP9.5-immunoreactive fibers in the defect group only in the superficial area of scar tissue, similar findings have been described in previous study[18]. However, increased nerve ingrowth into PG-depleted discs which sealed with PLGA/fibrin/chABC showed a significantly higher innervation score compared to PLGA/fibrin/PBS or empty defect discs. In addition, it indicated that advanced degeneration with small fissures and vascularization became more significant and penetrated deeper into the PLGA/fibrin/chABC discs in early postoperative period (see Fig. 6g). Eventually, degenerative procession appeared increasingly innervated in the chABC-treated discs following reduction of proteoglycan. Several factors potentially influence nerve ingrowth into the injured IVD: (i) more extensive proteoglycan reduction in the depleted annulus regions conducive to neural and vascular ingrowth following chABC enzymic deglycosylation; (ii) loss of structural integrity resulting in early leakage of NP with depletion of proteoglycan and increasing their attractiveness to ingrowing nerves; (iii) disruption of the tight collagen network that entraps the PGs and eventually more substantial newly tissue (e.g.,periannular innervated and vascularized granulation tissue) ingrowth along the local AF deficiency; (iv) ingrowth possibly inhibited by scar tissue due to less proteoglycan reduction than newly regenerated tissue in the AF injured track. A further limitation of using enzyme degradation in vivo model is that there could be still be local undegraded islands of CSPGs and be healed with the cells synthesizing and replenishing the lost GAGs. Thus, they do not completely mimic a natural 'degenerative' process. Additionally, further study is necessary to evaluate in the degenerated IVD for neural ingrowth in the application of the control enzyme (eg., hyaluronidase, Matrix Metalloproteinases) as a treatment option in a large animal model, with longer follow-up times and dose-related deglycosylation.

Conclusion

Our results indicate that chABC-based PLGA/fibrin gel showed promising results with regard to achieving the bio-integration with native annulus tissue and providing a local source for sustained release of active chABC. Disc proteoglycan inhibited nerves and blood vessels ingrowth were evidently abrogated by deglycosylation in an annular-injured rabbit disc degeneration model. Moreover, reduction of proteoglycan may play a direct role in nerves and blood vessels ingrowth into degenerated IVDs. Intact GAGs such as CS secreted from discs are potential candidates that could be useful to reduce neural and vascular ingrowth associated with discogenic pain in degenerated IVDs.

List Of Abbreviations

chABC chondroitinase ABC

PBS phosphate buffered saline

PLGA poly (lactic-co-glycolic acid)

PG proteoglycan

PGP9.5 protein gene product9.5

AF annulus fibrosus

IVD intervertebral disc

GAG glycosaminoglycan

MRI magnetic resonance imaging

DHI disc height index

NP nucleus pulposus

PACS picture archiving and communication system

DMMB 1,9-dimethylmethylene blue

EDTA ethylenediamine tetraacetic acid

HE hematoxylin/eosin

CS chondroitin sulfate

TBS tris buffered saline

ANOVA analysis of Variance

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving animals were approved by the institutional review board and animal care committee of Tongde Hospital. Protocols were conducted in accordance with the Guidance for the Care and Use of Laboratory Animals, as formulated by the Ministry of Science and Technology of the People's Republic of China, as well as the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed.

Consent for publication

Individual person's data is not applicable in the study.

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors were equally involved in conception and design of the study. LX, JL and WXX performed the surgical treatment and animal care. FY, JW and WW performed the acquisition, analysis and interpretation of the data. LX, YY and SWF finished the drafting of the manuscript and revised it critically for important intellectual content. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 1

SEM micrographs of (a) PLGA/fibrin gel sponge; (b) the spongy composite construct filling fibrin gel and chABC with different magnification and in vitro cumulative release profile of chABC/PLGA/fibrin(c).

Figure 2

Figure 2

Representative lateral radiographs (a-b) and MRI(c-d) of rabbit lumbar spine taken 6 months after surgery. White arrows indicate the injured disc level.

Figure 3

Figure 3

Changes in disc height index after surgery. There was a slow, progressive decrease in the disc height of operated discs over the follow-up period. The DHI in both two scaffold groups and the AF defect group were significantly lower than that of the control group after surgery (*, # $p < 0.05$, vs. the control group). DHI% in the defect group demonstrated a significant decrease overtime by 1,3,6 months after surgery (+ $p < 0.01$, vs. the two scaffold groups).

Figure 4

Figure 4

The grade of MRI in the injured groups were progressively higher than that in the normal control groups at the postoperative time point (* $p<0.01$, vs. the control group). Both the MRI grade of AF defect and PLGA/fibrin/chABC discs were significantly higher than that of the PLGA/fibrin/PBS or control discs after surgery (# $p<0.05$, vs. the control group or PLGA/fibrin/PBS group).

Figure 5

Figure 5

Changes in proteoglycan content of the NP at 6 months after surgery. The s-GAG content of three injured groups decreased significantly compared to control group at postoperative different time-point (* $p < 0.01$, vs. the control group). The PLGA/fibrin/chABC group showed a significant decrease of the sGAG/DNA ratio compared with the PLGA/fibrin/PBS or AF defect groups after surgery (# $p < 0.05$ vs. the PLGA/fibrin/PBS group or AF defect group). The decrease of sGAG/DNA ratio observed in the AF defect group was significantly more pronounced than in the PLGA/fibrin/PBS group at 1,6 months after surgery(+ $p < 0.05$)

Figure 6

Figure 6

HE (a, c, e, g) and safranin O (b, d, f and h) staining of the lesion site at 6 months after surgery. The uninjured AF displayed a multilamellar structure rich in proteoglycans, as shown by strong safranin O staining (a, b). loss of the AF structural integrity, replacement by extensive fibrocartilaginous-like tissue in the injured sites. The safranin-O staining indicated the presence of proteoglycan-rich content in the fibrocartilaginous tissue (c, d). Small irregular remnants of PLGA scaffold were well-integrated within the inner AF. Safranin-O staining showed open concave cavity increased severe loss of proteoglycan content (e, f). advanced degeneration was observed in the PLGA/fibrin/chABC group, including NP fibrosis, disorganization of the AF and reparative tissue extended further into the deeper inner AF along the fissures (g). The proteoglycan content was markedly reduced in the injured region (h). White arrows indicate areas of some blood vessels and small fissures.

Figure 7

Figure 7

Immunohistochemical staining of CS56 in the lesion site. (a) the outer lamellar AF was weakly stained with the CS-56 antibody in the control group. (b) In the defect discs, scar tissue was formed on the surface of the injury site. A fuzzy CS56-positive deposit was distributed in the scar tissue. (c) In the PLGA/Fibrin/PBS discs, newly formed tissue penetrated deeper, toward the NP. CS56-positive deposits were relatively negligible in the reparative tissue. (d) In the PLGA/Fibrin/chABC discs, CS56 positive-staining showed intense reactivity at the site of injury following treatment with chABC.

Figure 8

Figure 8

Immunohistochemical staining of PGP 9.5 at 6 months after surgery. The intact AF showed sparsely PGP 9.5–positive nerves distributed sparsely in the lamellae and adjacent connective tissue of the outer AF(a). Small nerves in the vicinity of the outer AF were distributed along the fissures, but barely seen to invade the deeper inner AF in the AF defect group (b). Nerve fibers reactive for PGP9.5 were present within the newly generated tissue of inner AF (c). In the PLGA/fibrin/chABC discs, more sprouting nerve fibers were predominantly identified in the vicinity of vascularized repair tissue extended further into the inner AF as well (d).

Figure 9

Figure 9

Changes in innervation scores at 6 months after surgery. The innervation score in the PLGA/fibrin/chABC group was significantly higher than that in the AF defect or PLGA/fibrin/PBS group at 1,6 months after surgery (1 and 6 months, * $p < 0.01$). The innervation score of the PLGA/fibrin/PBS discs was significantly higher than that of AF defect discs at 1 and 6-month time point. ($\dagger p < 0.05$ vs. the AF defect group).

Supplementary Files

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