Effect of Coflex interspinous stabilization on the prevention of progression of adjacent segment degeneration after single level and skipped level spinal fusion in a canine model

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**SUBJECT AREAS**
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**KEYWORDS**
- Coflex, interspinous spacer, adjacent segmental degeneration, spinal fusion, canine model
Abstract

Background

Interspinous spacer (ISPs) was a promising treatment method for adjacent segment degeneration (ASD) after spinal fusion. Coflex, one of ISPs, has been deceived to prevent or decelerate ASD after spinal fusion, while the proof of the effectiveness of such device is still very limited. The purpose of this study was further to investigate the protection role of Coflex in vivo after spinal fusion, when implanted in adjacent segment and middle segment.

Methods

Three groups of beagles were allocated as follows (n=6): (1) L4-5 lumbar interbody fusion (IF). (2) L4-5 lumbar interbody fusion + L5-6 interspinous Coflex implantation (Cof1). (3) L4-5 and L6-7 interbody fusion + L5-6 interspinous Coflex implantation (Cof2). In all animals, L5-6 discs were punctured to generate degeneration, and the intact L2–3 disc served as a noninjuries control (Con group). The effectiveness of Coflex on the prevention or deceleration of the progression of ASD was determined by magnetic resonance imaging, gross anatomical observation, histological and immunohistochemically analysis, and Real-time PCR analysis of gene expression.

Results

The objective disc in every group showed degeneration, however, the degeneration was more significant in IF group than Cof1 and Cof2 groups. MRI and histologic assay demonstrated that the discs of Cof1 and Cof2 groups maintained a relatively well-preserved structure as compared to the discs of IF group. Furthermore, immunohistochemistry analysis and real-time PCR demonstrated that the indicators of disc degeneration, TIMP1, BMP2, Col I, were up-regulated and disc matrix gene, Col II was down-regulated in IF group significantly

Conclusions

Coflex could decelerate the progression of ASD after spinal fusion, and it holds the same value not only at adjacent segment after single level spinal fusion, but also dose at the middle segment after “skipped” level (nonconsecutive) fusion

Background

From now on, the rapidly aging population has become a most common situation in global society,
and the increasingly prevalence of lumbar spine disorders have paralleled this trend, unfortunately, most of these clinical conditions affecting lumbar spine cannot exempt from surgery treatment [1, 2]. Spinal fusion has been employed to treat a variety of spinal disorders, including degenerative disc disease, segmental instability, spondylolisthesis, trauma, and scoliosis for decades [3]. Due to spinal fusion surgery has significantly facilitated patient comfort and mobility, fusion is regarded as a gold standard treatment for degenerative lumbar spine disease. However, we cannot ignore the fact that many researchers have demonstrated that fusion can accelerate the degeneration of adjacent lumbar segment, due to it can lead to excessive stress at unfused adjacent levels [4, 5]. Therefore, society of spine surgery holds the promise to develop an effective method to prevent or slow down the progress of adjacent segmental degeneration (ASD), after surgical treatment of lumbar degenerative disorders [6].

Many risk factors for ASD have been reported, such as pre-existing degeneration of adjacent discs, facet tropism, post-operative sagittal alignment and so on. Among those risk factors, the elimination of fixed segment motion are thought to play the most important role, as it can increase unfused adjacent segment motion, and then accelerates the degeneration of adjacent segments[7].

Implantation of an interspinous spacer(ISPs) device in adjacent segment is a known feasible technique to tackle with ASD, because it can facilitate to decelerate the degenerative process by providing intervertebral dynamic stability for adjacent segment [6]. Coflex is a dynamic ISP, it is a U-shaped, compressible device which can be implanted between the spinous processes after decompression[8]. As far as we know, there are few studies have focused on the effectiveness of such device to prevent or decelerate the progress of adjacent segment degeneration after spinal fusion.

In this study, we postulated that the implantation of Coflex between the spinous processes in adjacent segment after spinal fusion could prevent or decelerate ASD. And it can also play the same important role in the middle segment as a “transition” area in the treatment of “skipped” level (nonconsecutive) disc degeneration (SLDD) after fusion were delivered at two “skipped” level, when used in a canine spinal fusion model.

**Methods**

**Animals and Groups**

Eighteen healthy and skeletal mature beagles (19 months old on average), weight 16 to 21 kg and average weight 18kg, were used in this study. The experimental animals were provided by the animal experimental center of the Shanghai Jiao Tong University Affiliated Sixth People’s Hospital. And experiments of animals were approved by the Animal Research Committee of Sixth People’s Hospital,
Shanghai Jiao Tong University School of Medicine. All animals were healthy and free of infection, and were magnetic resonance imaging (MRI) scanned of the spine to assure the absence of Intervertebral disc (IVD) degeneration related diseases before the study. 18 beagles were bred and randomly allocated into the following study groups: (1) L4-5 lumbar interbody fusion I (2) L4-5 lumbar interbody fusion + L5-6 interspinous Coflex implantation Cof1. (3) L4-5 and L6-7 interbody fusion + L5-6 interspinous Coflex implantation Cof2. In all animals, L5-6 discs were punctured to generate degeneration and the intact L2-3 disc served as a noninjuries control (Con group). The animals were followed up for 6 months after the initial operation. The changes in the L5/L6 lumbar discs were assessed using gross anatomical observation, MRI, histology, and gene expression analysis at 3 and 6 months respectively. At each point 3 months and 6 months postoperatively, 3 beagles were randomly selected from each group to be killed with an excess dose of ketamine hydrochloride and xylazine hydrochloride injection after MRI scan.

Preparation of Autologous Iliac Bone Graft
24 hours abstinence for food and 12 hours abstinence for water was maintained before surgery, and Surgical procedures were performed under general anesthesia by intramuscular administration of ketamine hydrochloride injection (0.1 ml/kg) and xylazine hydrochloride (0.08 ml/kg). After anesthetization, the dogs were placed in the lateral position. The incision was 4 cm along the lateral border of the spina iliaca anterior superior. After exposure of spina iliaca anterior superior, an approximately 3 × 2-cm autologous iliac bone graft was obtained by bone drill. The autologous iliac bone graft was prepared for anterior lumbar interbody fusion.

Anterior lumbar interbody fusion and implantation of Coflex
When the harvesting of iliac bone graft finished, beagles for experiment were placed in a supine position, median abdominal incision were delivered, then lateral incision approach of the rectus abdominis was achieved, once an opening through the muscle was obtained, carefully protect the peritoneum and reflect it anteriorly by blunt dissection. After retraction of the peritoneum and its contents using a wide retractor, the appropriate involved vertebrae were identified. Separate the intervertebral disc and annulus from the cartilaginous endplates of the vertebrae with a thin osteotome, and then the disc was removed by pituitary rongeurs, Kerrison rangers and curets. When interbody space was cleaned and prepared for fusion, predisposed grafts were used to complete interbody fusion. After completion of the fusion, close all layers with absorbable sutures. When the interbody fusion was delivered, the Coflex groups underwent additional implantation of the Coflex (Paradigm Spine, LCC, New York) at the involved level. The beagles were placed in prone position, surgery was performed through a standard posterior midline approach, and then the interspinous
ligament was dissected and excised. The device was inserted between the adjacent spinous processes and the flanges were crimped follow the manufacturer's instructions, so that it was seated fitly. Appropriate placement of the implant and adequate segmental sagittal alignment were identified under C-arm machine.

Generation of L5-6 disc degeneration
After all of surgical procedures described above were done, the beagles were placed in prone position, L5–6 discs were then punctured with 18-gauge needles HuaYi Bio-technology, Shanghai, China along the outside of the facet joints under C-arm fluoroscopic guidance until the needle tips reached contralateral side of the discs both in anterior-posterior and lateral radiographs. Following the operation, antibiotic was intravenously delivered in five consecutive days. Beagles were fed in cages, and food and water were placed at a relatively high place, which would force them in erect position more than 8 hours per day. The animals were monitored daily for potential complications or abnormal behavior. Important surgical procedures were shown in Fig. 1.

Magnetic resonance imaging
MRI (Achieva 3.0T. Philips, Holland) scans were administered to evaluate signal changes in T2-weighted (T2-W; TR 2270 ms, TE 126 ms) images at baseline, 3 and 6 months after the operation in all groups. The subjects were imaged at the time of follow-up using the same scanner and examination protocol, and the slice thickness and interslice gap were 4 mm and 0.4 mm for sagittal. Lumbar IVD degeneration was graded on T2-weighted MR images according to the Pfirrmann classification system on a scale of I–V, where grade V denotes the most degenerate category [9]. All radiological assessments were made independently by two observers, including one radiologist and one orthopedic spine surgeon. when disagreement occurred with respect to the radiological grade, a consensus opinion with involvement of a third observer was sought.

Gross anatomical observation
At 3 and 6 months, after MRI scan, 3 beagles of each groups were killed with an excess dose of ketamine hydrochloride and xylazine hydrochloride injection, and the spines were harvested. Then the L2-3 and L5–6 discs were isolated intact, bilateral cartilage endplate and some vertebral body were preserved. The discs from each dog were cut coronally at the center of the disc for Gross anatomical observation.

Histological and immunohistochemically analysis
All L5-6 discs of 18 dogs were cut transversally at the center of the nucleus pulposus (NP). One
half of every disc was used for histological studies, and the other half was used for Realtime PCR analysis of gene expression. The NP tissues were isolated immediately, and fixation were done in 10 % neutral-buffered formalin for 72 hours and then processed for paraffin embedding and cut into transversal sections (6 μm thick) using a microtome. The sections were stained with hematoxylin and eosin for evaluation. Immunohistochemical detection of Col I, Col II performed using formalin fixed sections obtained as described above. Briefly, NP tissue sections were maintained at room temperature for 60 minutes and dewaxed by xylene twice (10 minutes each time). The tissues were then rehydrated by a series of 5-minute washed in 100 %, 95 %, 80 %, and 70 % ethanol, followed by 5-minute washed in distilled water and three consecutive 3-minute washed with PBS (PBS; Gibco Grand Island, New York, USA). Incubation in 3 % hydrogen peroxide for 10 minutes were delivered in purpose of inactivating the endogenous peroxidase, after that, antigen retrieval was performed by heating the samples at 95 °C for 20 minutes in 10 mM sodium citrate (pH 6.0). Nonspecific binding was blocked by incubating with 10 % normal goat serum (Gibco Grand Island, New York, USA) for 20 minutes, then the NP tissue sections were labeled overnight at 4 °C with primary antibody: anti-Col I (1:200dilution), anti-Col II (1:200 dilution), polyclonal antibodies (immunoglobulin G) (Hua An Biotech, Hangzhou, China). The NP sections were then incubated for 60 minutes each with a horseradish peroxidase-labeled secondary antibody and then streptavidin-peroxidase (Hua An Biotech). After washing with PBS, the sections were incubated with 3,30-diaminobenzidine substrate until a brown color generated. Finally, the sections were counterstained with hematoxylin. Dehydration was performed by using a series of 2-minute washes in 50 %, 70 %, 95 %, 95 %, and 100 % ethanol. After two consequent 2-minute washes with xylene, the slides were sealed with coverslips. To quantify the immunohistochemical results, staining intensity was analyzed using the Image-Pro Plus 6.0 (Media Cybernetics, Rockville, Maryland, USA). The area of interest in all sections was analyzed, and the mean density was calculated by integrated optical density divided by the area.

Real-time PCR analysis of gene expression

Total RNA was extracted from the NP using the Trizol reagent (Life Technologies) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using AMV reverse transcriptase (Life Technologies). After the cDNA had been obtained by reverse transcription, relative gene expressions of COL1, COL2, TIMP-1 and BMP2 were determined by real-time PCR and normalized to the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. These primers were designed using Primer Premier 6.0 software (PREMIER Biosoft, palo alto, California, USA) (Table 1). The Mini Opticon™ Detector System (Life Technologies) and the SYBR Green PCR kit (Life Technologies) were used for Realtime PCR analysis. The real-time PCR consisted of an initial enzyme activation step at 95 °C for 20 seconds, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 20 seconds. A cycle
threshold (Ct) value was obtained for each sample, and triplicate sample values were averaged. The 2-ΔΔCt value was then used to calculate relative expression of each target gene [10]. The data presented (mean) were from three independent experiments in which both sample sets were analyzed in triplicate.

Statistical analysis
All data were statistically analyzed with GraphPad Prism (v6.0). Error bars in graphical data represent mean ±s.d. Statistical significance was determined using a Mann–Whitney U test, in which p values of p 0.05 were considered statistically significant. Variance was similar between the groups that were statistically compared.

Results
MRI assessment
The Pfirrmann classification results at the 3 time points are shown in Fig 2. At 3 and 6 months after surgery, the signal intensities on T2-weighted images of punctured discs in IF group were significantly degenerated as compared to before surgery (Fig. 2b.c). At 3 months after surgery, the degree of degeneration in the punctured discs was mainly grade III according to the Pfirrmann classification and grade IV to V at 6 months (Fig 3). The MRI results showed a gradual increase in Pfirrmann grade after surgery. In Cof1 and Cof2 groups at 3 months, the signal intensities on T2-weighted images of punctured discs were similar as before surgeryFig. 2e.h, and at 6 months there are only a slight decrease, no prominent degeneration or disc herniation were observed (Fig. 2f.i). For these two groups, Pfirrmann grade were mainly grade I to II at 3 months after surgery, and the degree of degeneration at 6 months were mainly grade II to III (Fig 3).

Gross anatomical findings
The control discs (L2-3) in all groups demonstrated no degenerative changes at 6 months, as anatomical morphology showed Gel-like nucleus pulposus, discrete fibrous lamellas and there were no cleft or anular disorganization can be identified. The L5-6 discs in IF group showed that tissue defects happened in the nucleus, and fibrous tissue are indistinguishable from annulus. Besides, anular disorganization, cleft extended throughout NP and anulus fibrosus (AF) were also can be seen (Fig. 4a). The Cof1 and Cof2 groups showed slight degeneration happened in NP with NP appeared viscous and suffered peripheral fibrous infiltration. Nucleus and anulus were distinguishable, anulus were well-organized and half ring-shaped and no identified cleft in NP and AF, no prominent degeneration
were seen, except for some tiny osteophytes generated at the margin of anterior region of the vertebrae (Fig. 4b,c).

Histological and immunohistochemical analysis
To verify the occurrence of disc degeneration, H & E staining were performed to examine the morphology of the NP and AF. As shown in Fig 5, the control discs had a normal boundary between the AF and the NP, the cells of the nucleus pulposus were normal, the NP were mixed of large, vacuolated (notochordal) cells and smaller, chondrocyte-like cells, and the AF was well organized parallel.

When Compared with controls, noteworthy degenerative morphological changes in the IVDs in IF group were observed after surgery, cracks and ruptures of collagen fibers could be seen in the annulus fibrosus, most contents of normal nucleus pulposus were lost, and there were very few chondrocyte-like cells. In addition, the boundary between the NP and AF was not exist (Fig. 5b).

Though histologic results showed degeneration in all objective discs, the degenerative changes of the NP in the IF groups were more apparent than those in the Cof1 and Cof2 groups, as shown in (Fig. 5c,d) To further demonstrate the protection effect of Coflex on the objective disc, the NP sections were stained by Col ICol II antibodies at 6 months. As shown in Fig 6, the immunohistochemical staining indicated that the NP in the IF discs was significant strongly positive for Col I, compared to the control and Cof1 and Cof2 groups. When compared to the control and other two groups, the staining intensity of Col II decreased significantly in IF group. Immunohistochemical staining intensity analysis were performed by Image-Pro Plus 6.0, the results showed that the staining intensity of Col I was significantly higher and Col II was significantly lower in the IF group than that in the control and other two groups (p <0.05).

Gene expression analysis
To confirm the protection effect of Coflex on the objective disc, real-time PCR was used to measure the levels of TIMP1, BMP2, Col I, Col II, in the control, IF, Cof1 and Cof2 groups at 3 and 6 months. As shown in (Fig 7), the levels of a TIMP1, BMP2, Col I was increased in the IF, Cof1, Cof2 groups at two observe time point, and the Col II expression was decreased. At 3 month, TIMP1 mRNA expression markedly increased and Col 2 decreased in discs from the IF group compared with the Cof1 and Cof2 groups p0.05, while the increase of BMP2 and COL I were not significant. Noteworthily, at 6month, remarkably increase of Col I, TIMP1, BMP2 and decrease of Col 2 were recorded in IF group (p <0.01).

In IF group, when comparison was delivered chronologically, the result showed that the TIMP1, BMP2 and Col I increased and Col II decreased more prominently at 6 months compared to those at 3 month (p0.01), however, such significant difference were not seen in Cof1 and Cof2 groups. Moreover, as we
assumed, no conspicuous difference between Cof1 and Cof2 groups were detected. Together, the results show that adjacent segment inter-body fusion increased the expression of TIMP1, BMP2, Col I and decreased Col II, and such tendency has become more significant with the pass of time. Moreover, the implantation of Coflex can attenuate this effect.

Discussion
Rigid spinal fusion is the most popular surgical procedure in the management of lumbar instability or lumbar disk herniation (LDH) [11]. Though rigid fusion has been proved to be an effective way to restore the disc space height, reconstruct the stability and alignment of spine, a variety of complications had emerged such as adjacent segment disease with the change of spinal mechanical activities [12]. The rigid fusion decreased the flexibility and mobility of the entire lumbar spine, leading to the center of rotation of vertebral body shifts over the disc, consequently, the stress on the facets and/or disc of the adjacent mobile segment significantly increased [4]. The increased stress will lead to a significant effect on intersegmental mobility and the increase in intradiscal pressure, overall, it accelerates degenerative changes at adjacent unfused segment, especially at the cranial level [13]. Moreover, several researchers have demonstrated that rigid fusion not only play an important role in the initiation of adjacent segment degeneration, but would also make pre-existing degenerative changes in the adjacent level deteriorate [14].

Recently, a long time follow up remarked that adjacent segment disease is a severe problem that causes refractory low back pain, which would cause patients’ as well as surgeons’ dissatisfactions, and revision laminectomy and extension of fusion may become unavoidable in some cases [15]. Long segment lumbar arthrodesis would become a mandatory procedure in the treatment of such refractory pain, which leads to spinal deformities and deliver a heavy burden both on individual and nations [16].

For the purpose of avoiding or ameliorating these adverse effects, the appearance of dynamic stabilization devices such as Coflex has brought new hope to spine society, such device is considered to reduce the stress on adjacent segments to achieve relatively ideal mobility, thereby avoid the harmful effects of rigid fusion [17]. Hybrid surgery such as Interspinous device+ interbody fusion has become a promising method to delay the adjacent segment degeneration, and some researchers have investigated its efficacy in clinical use [18]. Though many preliminary evidences have showed its protection role in prevention or deceleration of ASD, there are still some controversy yet to be
In our study, we established an L5-6 disc degeneration model in beagles by annular puncture from the posterior approach using 18-gauge needles under C-arm fluoroscopic guidance and observed the role of Coflex in the objective segment after rigid fusion was performed at adjacent segment. We firstly showed that rigid fusion could accelerate ASD, and further demonstrated that Coflex could not completely prevent the deterioration of ASD but can significantly decelerate the progressive of ASD. Moreover, Coflex can demonstrate its protection role both in adjacent segment after one level spinal fusion and in the middle segment as a “transition” area after "skipped" level (nonconsecutive) interbody fusion.

Beagles exhibited gradually increasing degenerative changes in IF group as demonstrated by both Pfirrmann classification and histological evaluation after surgery. Moreover, gene expression analysis showed an increase in the mRNA expression levels of Col I, TIMP1 and BMP2, three major indicators of disc degeneration, and a decrease of Col II, a major component of normal disc [20]. These results were consistent with the matrix breakdown observed in human degenerative disc associated with decrease of Col II expression, fibrosis associated with up-regulation of Col 1, and matrix degradation and disc remodeling associated increase of TIMP1 and BMP2 [21]. Interestingly, our data showed that the progress of degeneration was more rapidly in later-3 months than it in pre-3 months, and among the genes expression we investigated, only the change of TIMP1 and Col 2 were significant at 3 months. One explanation to this divergence is that animals were lack of activity due to the pain and feebleness after surgery at first several months, consequently, motion and load bearing of spine were very limited in these periods. When they have rehabilitated and normal activities were restored, significant degeneration were exhibited in IF group at 6 months compared to other groups. Though progressive degeneration can be found in all experimental groups, a significant prevention or deceleration effect was exhibited by Coflex.

A variety of animal models have been established for the exploration of intervertebral disc degeneration and treatment [22]. IVD could successfully developed in many models, however, the pathological and molecular changes are different, according to the varied anatomical and physiological structures of different animals. Rodents model is the most popular one among those models, nevertheless, the different structure and components of disc between rodents and humans has limit its accuracy in the study of IVD and treatment [23]. In this study, we chose beagles as animal model because the beagle’s disc is similar with human both in physiology and morphology, in addition, though beagles are quadrupeds animals, they like squatting for most time, such mechanical property was of the same status in human [22, 24]. We force beagles stay in erect position more than 8 hours per day after surgery by place food and water in a high place to reach, which would facilitate the progression of degeneration. A variety of methods have been conceived to achieve the...
establishment of the model of IVD degeneration, scalpel blade and gauge needle were used in those classic models [21]. According to these previous study, the velocity of the progression of disc is determined by the extent of damage to the annulus pulposus, using scalpel blade can cause an acute disc degeneration within 2 weeks, while a 16 or 18-gauge needle would make it happen in a gradually, relatively slow pattern, which is similar to how its work in human IVD degeneration [25, 26]. As the purpose of this study is to clarify the ability of Coflex to decelerate or prevent ASD, which is considered as a slow progressive pathological change, a 16-gauge needle was used to develop the IVD degeneration model. In this study, we arranged control and experimental segments in the same dog, which can eliminate individual differences such as age, weight, pre degeneration status. The present study has some limitations. It has been reported that the IVD of beagles have a tendency of degeneration at an earlier age, which might cause difference of existed among objective IVDs to some extent. Therefore, this might bring potential bias to the present study. In addition, Pfirrmann classification is a non-quantitative analysis, quantitative evaluation of MRI was not performed, and the number of animals for histological and immunohistochemically analysis was small. Nevertheless, the study demonstrated the interspinous spacer device Coflex can protect adjacent segment after spinal fusion and decelerate the progression of adjacent IVD degeneration. The protect role can both exhibited well in adjacent segment and the middle segment in the treatment of “skipped" level (nonconsecutive) disc degeneration (SLDD) after fusion were delivered at two “skipped” level.

Conclusions
The present study demonstrated that the implantation of Coflex could decelerate the progression of adjacent IVD degeneration after spinal fusion. The results support the premises that Coflex is an effective interspinous device in the prevention or deceleration of ASD after spinal fusion, both in adjacent segment and middle segment.

Abbreviations
ISPs: Interspinous spacer;
ASD: adjacent segment degeneration;
IF: interbody fusion;
Cof1: L4-5 lumbar interbody fusion +L5-6 interspinous Coflex implantation
Cof2: L4-5 and L6-7 interbody fusion+ L5-6 interspinous Coflex implantation
Con group: noninjuries control
SLDD: "skipped" level (nonconsecutive) disc degeneration
MRI: magnetic resonance imaging
IVD: Intervertebral disc
NP: nucleus pulposus
AF: anulus fibrosus
LDH: lumbar disk herniation

Declarations
Ethics approval and consent to participate
Experiments of animals were approved by the Animal Research Committee of Sixth People’s Hospital, Shanghai Jiao Tong University School of Medicine.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions
XJG conceived the initial idea and the conceptualization, chose the animal model and assisted in the surgery. LJM, LFQ and DJN conceived and participated in its design, searched databases, participated in the operation, extracted and assessed studies, participated in the data extraction and drafted the manuscript. LJM wrote and revised the manuscript. All authors read and approved the final manuscript.
Consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests
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Tables

| Table 1 Primers designed using Primer Premier 6.0 software |
|----------------------------------------------------------|
| Gene | Gene bank | Forward Primer | Reverse Primer |
|------|-----------|----------------|----------------|
| TIMP1 | NM_001003182.1 | 5'-ACCTATGCTGCTGGCTGTG-3' | 5'-GGTTTCCAGAGCCGCCAC-3' |
| BMP2 | XM_534351.5 | 5'-AACTCCACTAACCACGCCATTG-3' | 5'-GGTTGTGGAGGGTTGTGGGT-3' |
| Col 1 | NM_001003090.1 | 5'-GTAGACACCACCCTCAAG-3' | 5'-GGAAGAGCGGAGGAATACT-3' |
| Col 2 | NM_001006951.1 | 5'-AGCAGCAAGAGCAGGGACAAG-3' | 5'-CCTTCCTCCGCTGCTGT-3' |
| GAPDH | NM_001003142.2 | 5'-ATTCCACGGCAGCAAGTCAG-3' | 5'-GGTGATGCTGGTGCTGAG-3' |
Primer Premier 6.0 software from PREMIER Biosoft (palo alto, California, USA)

TIMP1 inhibitors of matrix metalloproteinases 1, BMP2 Bone morphogenetic protein 2

Col 1 type I collagen, Col 2 type II collagen, GAPDH glyceraldehyde-3-phosphate dehydrogenase

Figures
Figure 1

Surgical procedure. a. Anterior lumber interbody fusion use prepared iliac bone graft. b. Posterior implantation of Coflex. c.d. Development of the disc degeneration model. Needle tips reached contralateral side of the discs both in anterior-posterior and lateral radiographs.
Figure 2

before surgery

3month

6month

IF  Cof1  Cof2
Sagittal T2-weighted images by MRI at 3 observed time points. Pre-operative images (a, d, g) at 3 groups showed hyperintense signal and the homogeneous structures of the L5-6 discs.

At 3 months post-operation (b, e, h), MRI T2-weighted image showed inhomogeneous structure of the disc with an intermediate gray signal intensity in the L5-6 disc in IF-group (b.), while in Cof1 and Cof2 groups (e, h.), hyperintense signal were still seen. At 6 months post-operation (c, f, i.), in IF group (c.), the structure of the disc is inhomogeneous, with a hypointense black signal intensity. The distinction between nucleus and anulus is lost, and the disc collapse even herniation could be seen, however, Cof1 and Cof2 groups (f, i.) only showed high intensity slightly decreased, any further degeneration were not seen.
| Grade | IF  | Cof1  | Cof2  |
|-------|-----|-------|-------|
| I     | ★★★★★★★★★★★ |        |       |
| II    | ★    | ★     |       |
| III   |       | ★     |       |
| IV    |       |       |       |
| V     |       | a     |       |

| Grade | IF  | Cof1  | Cof2  |
|-------|-----|-------|-------|
| I     | ★    | ★★★★★ | ★★★★★|
| II    | ★★★★ | ★★★★ | ★     |
| III   | ★★★ | ★★★ | ★★★  |
| IV    | ★★★ | ★★★ |       |
| V     | ★    | b     |       |

Figure 3
Analysis of signal changes in MRI images with Pfirrmann classification. a. T2-weighted images showed no degeneration in all groups before surgery. b. There was significantly decreased grading of MRI scans in the IF group compared with Cof1 and Cof2 groups at 3 months after surgery ($p<0.5$). c. MRI grading in the IF was more significantly decreased at 6 months compared with Cof1 and Cof2 after surgery ($p<0.001$). MRI grading were compared between 3 months and 6 months (b.c.), significant decrease was seen in IF group at 6 month ($p<0.001$), however, such decrease were not seen in Cof1 and Cof2 groups ($p>0.05$).
Figure 4

Gross anatomical views of L5/6 disc at 6 months after surgery. a. Disc in the IF group showed nucleus defected, fibrous tissues are indistinguishable from annulus. Anular disorganization, cleft extended throughout NP and AF were also can be seen. b. Disc in the Cof1 and Cof2 groups showed nucleus and anulus were distinguishable, annuls were well-organized, and some tiny osteophytes generated at the margin of anterior region of the vertebrae.
Histological images of the NP in 3 groups and the control at 6 months. The paraffin sections of the NP tissues were stained by hematoxylin and eosin. a. Showed intact annulus fibrosus and cell-enriched nucleus pulposus. b. Disc in IF group showed chondrocyte-like cells in nucleus pulposus were approximately disappeared, and cracks among the layers of collagen fibers of the annulus fibrosus emerged. c.d. Discs in Cof1 and Cof2 groups showed intact annulus fibrosus, while the number of chondrocyte-like cells in nucleus pulposus were relatively reduced.
Figure 6

Typical immunohistochemical images at 6 months. The immunohistochemical micrographs showed the images of the NP sections which were stained by an antibody against type I collagen (Col I), and type II collagen (Col II). Staining of the Col I for the IF group was stronger than that for the Cof1 and Cof2 groups and the control, while the staining of the Col2 was much weaker.
Gene expression analysis

Figure 7
Real-time PCR analysis. Real-time PCR was used to analyze the levels of the disc matrix components Col I, Col II, and anticatabolic factors such as TIMP1 and growth factors such as BMP2 from the Control, IF, and Cof1, and Cof2 groups. Expression was normalized to the average of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase). The results showed that TIMP1 mRNA expression markedly increased and Col 2 decreased in discs from the IF group compared with the Cof1 and Cof2 groups at 3 months. *statistical significance (p<0.05). Excepted for the existed differences became more significant, BMP2, and Col I significantly increased at 6 months, **statistical significance (p<0.01).