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Inhibitory Effects of Platelet-Rich Plasma on Intervertebral Disc Degeneration: A Preclinical Study in a Rabbit Model

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 Keke Gui*
ABCDEF 2 Weimin Ren*
ABCDF 3 Yonglin Yu
BC 4 Xin Li
BC 1 Jiachun Dong
BC 1 Wangping Yin

1 Department of Orthopedics, Jinshan Hospital, Affiliated to Fudan University, Shanghai, P.R. China
2 Department of Pathology, Jinshan Hospital, Affiliated to Fudan University, Shanghai, P.R. China
3 Department of Orthopedics, Huashan Hospital, Affiliated to Fudan University, Shanghai, P.R. China
4 Department of Radiology, Jinshan Hospital, Affiliated to Fudan University, Shanghai, P.R. China

* These authors contributed equally to this study

Corresponding Authors: Keke Gui, e-mail: guikekemedsci1@163.com; Yonglin Yu, e-mail: yuyonglin@fudan.edu.cn
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Background: Platelet-rich plasma (PRP) contains multiple growth hormones that may stimulate tissue repair. This study aimed to assess the effects of PRP in a rabbit model of IDD (annulus fibrosus puncture).

Material/Methods: Thirty-six adult New Zealand white rabbits were randomly divided into 3 groups: 0.1 mL PRP (group A), 0.1 mL phosphate-buffered saline (group B), and control (group C) (n=12/group). Annulus fibrosus puncture was performed to establish L4/5 and L5/6 IDD models. Two and 4 weeks later, 6 rabbits from each group were given an IVD injection at L4/5 and L5/6. Two or 4 weeks after injection, rabbits were scanned with X-ray and MRI before being sacrificed. IVDs were collected for hematoxylin and eosin, Masson's trichrome, and Safranin O staining, and type II collagen immunohistochemistry.

Results: Over time, IVD height and disc imaging signal intensity decreased gradually in groups B and C, but only slightly in group A (baseline: 100% for all groups; A: 95.9±4.2% at 4 weeks, 90.1±8.4 at 6 weeks; B: 75.3±5.7% at 4 weeks, 70.8±6.4% at 6 weeks; C: 74.7±5.5% at 4 weeks, 69.9±6.2% at 6 weeks; all $P<0.001$, $P<0.01$ between A *vs.* B and C). Degenerative histological changes in IVDs in groups B and C were more severe compared with group A.

Conclusions: Platelet-rich plasma interventions can effectively attenuate the IDD process in rabbits.

MeSH Keywords: Intervertebral Disc Degeneration • Platelet-Rich Plasma • Rabbits • Therapeutics

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Background

Degenerative changes are inevitable in aging humans. Intervertebral disc (IVD) degeneration (IDD) play a role in almost all lumbar diseases [1]. Lower back pain is a common health issue in modern societies and IDD is one of the main causes [2]. However, there is no generally accepted effective clinical treatment for this condition. Recently, some studies provided some clues about the molecular mechanisms and treatment of IDD [3,4]. Molecular mechanisms of IDD involve genetic factors (such as polymorphisms in vitamin D receptor, aggrecan, type IX collagen and interleukins [5]), cell senescence, reduced production of extracellular matrix, increased synthesis of degradative enzymes, expression of pro-inflammation cytokines, apoptosis, and neurovascular ingrowth [6]. IDD results in increased synthesis of proteoglycans, increased aggrecan fragment accumulation, decreased type II collagen synthesis, and increased type I collagen synthesis [7]. A number of inflammatory mediators such as nitric oxide, interleukin-1, matrix metalloproteinases, prostaglandin E2 and tumor necrosis factor α are also involved in IDD [7].

Insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), and transforming growth factor β (TGF-β) can promote matrix synthesis and cell proliferation of the IVD, and that epidermal growth factor (EGF) can also promote cell proliferation [8]. Many studies have confirmed that growth factors promote matrix synthesis and cell proliferation [7]. However, maintaining the homeostasis of the discs requires the interaction of a variety of growth factors, and the use of 1 growth factor alone may not produce satisfactory IVD repair and regeneration.

It was proposed that using a combination of a variety of growth factors could be appropriate to treat IDD, and platelet-rich plasma (PRP) has been proposed as the source of these growth factors [9]. As an autologous derivative of whole blood containing a supraphysiological concentration of platelets, PRP is rich in growth factors and cytokines. PRP has attracted attention in the fields of plastic surgery and orthopedics [10]. Indeed, PRP is able to promote the migration and proliferation of many types of cells, which then provide a good microenvironment for the repair and regeneration of bone, cartilage, tendon, and muscle [10]. Akeda et al. [11] have reported *in vitro* experiments showing that PRP can effectively promote pig IVD cell proliferation and extracellular matrix metabolism. Obata et al. [12] have shown in a rabbit model of IDD (using anular puncture) that PRP could induce a repair effect on degenerated IVDs. However, data on the effects of PRP on IVDs *in vivo* are still scarce, and more studies are still necessary to be able to perform clinical trials.

In the present study, we used the annulus fibrosus puncture rabbit model, a well-known model of IDD [12–14], to evaluate the efficacy of PRP IVD injection on IDD using imaging and histological analysis.

Material and Methods

Experimental animals

Thirty-six healthy adult male and female New Zealand white rabbits (2.5±0.5 kg) were provided by the experimental animal center of Fudan University. The rabbits were scanned with X-ray and MRI to exclude those with congenital vertebral malformation or IVD diseases. They were randomly divided into 3 groups: PRP intervention group (group A), phosphate-buffered saline (PBS) group (group B), and control group (group C) (n=12/group). The experiments were approved by the Animal Care and Use Committee of Fudan University. Animals were kept in standard conditions with free access to food and water. Animal handling and experiments complied with animal ethics standards.

IDD model

Animals were fixed with limbs in the left lateral position and then injected with 0.2 g ketamine and 10 mg diazepam. Fur was removed and the skin was disinfected and covered with sterilized towels. An 8-cm vertical incision parallel to the spine was made from the edge of the 12th rib to the iliac crest. The external oblique and dorsal muscles were cut and blunt dissected to the transverse process of the spine, taking care to avoid damage to the peritoneum. Since the iliac crest and L6 vertebra are roughly at the same level, the iliac crest can be used as a reference to locate and expose the L4/5 and L5/6 IVDs. Annulus fibrosus puncture was performed using a 16G needle in a direction parallel to the disc side of the front end plate. Using mosquito forceps to control the depth of penetration, the needle was inserted 5 mm and the position of the needle was maintained for 5 s before it was gently withdrawn without bringing out nucleus pulposus. The wound was sutured after being washed with saline. All animals were injected intramuscularly with 800,000 U penicillin before and after the surgery. An Elizabethan collar was applied to prevent licking and biting the incision. Animals were housed under standard conditions with free access to food and water.

Preparation of PRP

Approximately 11 mL of central ear artery blood was collected from animals in group A. Of this blood, 0.5 mL was used for platelet counting. The remaining 10.5 mL was added to a tube containing sodium citrate and mixed. According to the method reported by Landesberg et al. [15], blood was centrifuged (ALC, Cologna Monzese, Italy) for 10 min at 200 g.

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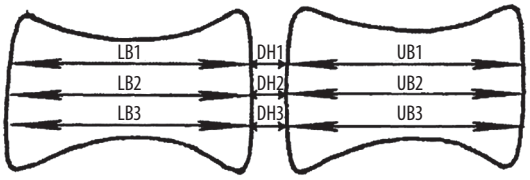

$$DHI = \frac{2(DH1 + DH2 + DH3)}{(LB1 + LB2 + LB3) + (UB1 + UB2 + UB3)}$$

$$\%DHI = \text{postoperative DHI/preoperative DHI} \times 100\%$$

Figure 1. Estimation of disc height index. X-ray images were used for measurement of lateral disc height (DH), upper vertebral height (UB), and lower vertebral height (LB). Disc height index (DHI) and%DHI was calculated according to the formula.

Supernatant and pellet within 1 mm below the interface (a total of 4.5 mL) was centrifuged for another 10 min at 200 g. The bottom one-fourth of the pellet (about 1.1 mL) was collected as PRP. Platelets were counted using 0.5 mL of PRP. Finally, 0.06 mL thrombin (Sigma, St Louis, MI, USA, concentration 1000 μ/mL) was added to the remaining 0.6 mL of PRP to obtain a PRP gel for IVD injection.

Intervertebral disc injection

Two weeks after the IDD surgery, 6 animals (subgroup 1) from each group were randomly selected for IVD injection. In group A1, L4/5, and L5/6 IVDs were exposed and injected with 0.1 mL autologous PRP. The wound was closed after injection. In group B1, L4/5 and L5/6 IVDs were exposed and injected with 0.1 mL PBS buffer (pH 7.4). The wound was closed after the injection. In group C1, L4/5 and L5/6 IVDs were exposed without injection. The wound was closed afterwards. At 4 weeks after the IDD surgery, the same procedures were done on the remaining 6 animals (subgroup 2) of each group (A, B, and C).

X-ray examination

Before IDD surgery and 2 weeks after IVD injection, lateral plain digital radiographs (DR; Siemens, Erlangen, Germany) of the lumbar spine were taken. The images were analyzed for the corresponding lateral disc height (DH), upper vertebral height (UB), lower vertebral height (LB), disc height index (DHI), and%DHI (postoperative DHI/preoperative DHI ×100%; Figure 1).

MRI examination

Before IDD surgery and 2 weeks after IVD injection, all animals underwent sagittal T2WI MRI in the supine position with SE sequence, TR/TE 3500/100 ms, thickness 1.5 mm, and spacing 0 mm using a 1.5T superconducting MRI scanner (Siemens, Erlangen, Germany). According to the modified Pfirrmann grading [16] criteria, T2WI signal IVD intensity (representing the degeneration) was graded using 5 levels. I: even, bright white nucleus pulposus structure; distinct border of annulus fibrosus; strong MRI signal; and normal height of IVD. II: uneven nucleus pulposus structure with visible horizontal band; indistinct border of annulus fibrosus; strong MRI signal; and normal height of IVD. III: grey, uneven nucleus pulposus structure; indistinct border of annulus fibrosus; low to medium MRI signal; and decreased height of IVD. IV: grey or black uneven nucleus pulposus structure; lost annulus fibrosus border; low to medium MRI signal; and decreased height of IVD. V: black uneven nucleus pulposus structure; lost annulus fibrosus border; low MRI signal; and significantly decreased height of IVD.

Histological examination

At the end of the X-ray and MRI examinations, animals were sacrificed by injecting 10 mL of air into an ear vein. Samples of nucleus pulposus of IVDs were collected and sliced into thin sections. Sections were stained with hematoxylin and eosin (H&E) for histological changes, Masson's trichrome stain for changes in collagen fibers, and Safranin O stain for changes in proteoglycan. To observe the expression of type II collagen, sections were stained with goat anti-rabbit antibody (primary antibody, Amresco, Solon, OH, USA; 1:200 dilution), donkey anti-goat antibody (secondary antibody, Amresco, Solon, OH, USA; 1:200 dilution), and visualized with DAB (Dako, Glostrup, Denmark). Based on H&E and Safranin O staining, and with Nomura's standard as references, we graded nucleus pathological changes as the following [17]: grade 0, normal structure; grade 1, extracellular matrix had a honeycomb structure, no connective tissue hyperplasia; grade 2, less than 25% of the nucleus pulposus was replaced by proliferated connective tissue; grade 3, 25% to 50% of the nucleus pulposus was replaced by proliferated connective tissue; grade 4, more than 50% of the nucleus pulposus was replaced by proliferated connective tissue; grade 5, original nucleus pulposus was completely replaced by proliferated connective tissue.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Continuous quantitative data are presented as mean ± standard deviation. Differences between different time points within the same group and differences between different groups at the same time point were compared using analysis of variance (ANOVA) and the Student-Newman-Keuls post hoc test. Differences among multiple grades were compared with the Kruskal-Wallis test and differences between two grades were compared with the Mann-Whitney U test. Two-sided P-values <0.05 were considered statistically significant.

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Table 1. %DHI at different time points (mean ±SD, *n*=12).

Group	Before IDD	4 weeks after IDD (subgroup 1)	6 weeks after IDD (subgroup 2)	*F*	*P*
A	100	95.92±4.19	90.08±8.44	10.080	<0.001
B	100	75.33±5.69[a,b]	70.83±6.35[a,b,c]	121.999	<0.001
C	100	74.67±5.53[a,b]	69.92±6.16[a,b,c]	137.446	<0.001
F	/	65.213	31.247	/	/
P	/	<0.001	<0.001	/	/

[a] *vs.* before IDD, P<0.01; [b] *vs.* A group, P<0.01; [c] *vs.* subgroup 1, P<0.01.

Results

General observations

All animals survived to the end of the experiment. All wounds healed within 2 weeks without infection, effusion, or dehiscence. All animals had normal functions of the lower extremities without paralysis after the puncture.

X-ray examination

X-ray examination (Table 1) revealed that at 4 weeks (subgroup 1) and 6 weeks (subgroup 2) after IDD, group A (PRP injection), group B (PBS injection), and group C (without injection) had significantly decreased DH and%DHI (A: 95.9±4.2% at 4 weeks, 90.1±8.4 at 6 weeks; B: 75.3±5.7% at 4 weeks, 70.8±6.4% at 6 weeks; C: 74.7±5.5% at 4 weeks, 69.9±6.2% at 6 weeks; all P<0.001). Compared with groups B and C, group A had a significantly better%DHI (P<0.01); there was no difference between groups B and C (P>0.05).

MRI examination

When comparing the sagittal T2WI MRI images at different time points, groups B and C had a gradual decrease in IVD nucleus pulposus signal strength, nucleus pulposus area, and DH after IDD (Figure 2). There was no decrease in group A.

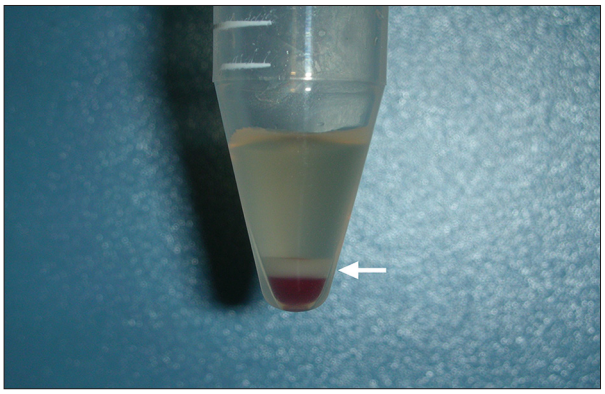

Figure 2. MRI images at different time points. Arrows indicate the L4/5 and L5/6 IVDs. Subgroups A1 and A2 did not show significant degeneration in the IVD; subgroups B1, B2, C1, and C2 had significant IVD degeneration after surgery, as shown by decreases in nucleus pulposus signal strength, nucleus pulposus area, and DH after IDD.

IVD degeneration grading at different time points after IDD were similar in time in group A (P=0.147). Groups B and C were

Table 2. IDD MRI images graded at different time points (n=12, analyzed with Mann-Whitney U test).

Group	Before IDD				4 weeks after IDD subgroup 1				6 weeks after IDD subgroup 2				H	*P*
	I	II	III	IV	I	II	III	IV	I	II	III	IV		
A1/A2	12	0	0	0	11	1	0	0	9	3	0	0	3.828	0.147
B1/B2	12	0	0	0	0	3	9	0	0	0	4	8	30.640	<0.001
C1/C2	12	0	0	0	0	2	9	1	0	0	3	9	30.117	<0.001
H	/				26.970				26.847				/	/
P	/				<0.001				<0.001				/	/

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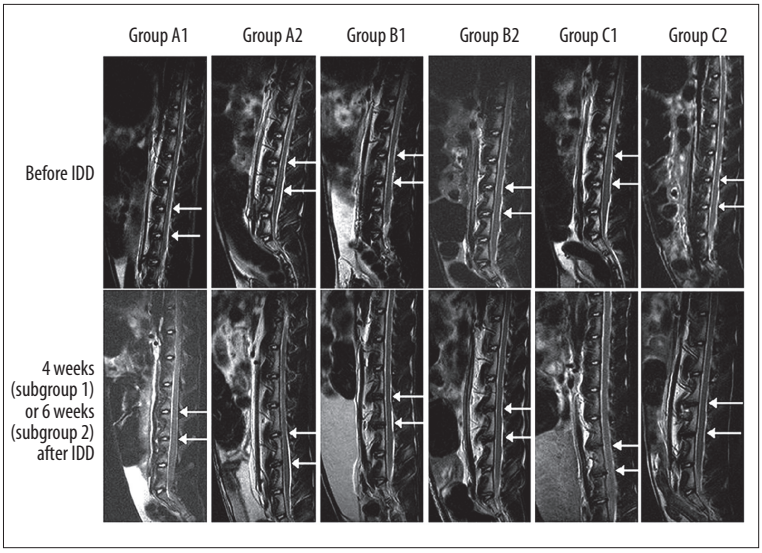

Figure 3. Histological examination at different time points (H&E, ×100). Nucleus pulposus sections of IVD sections were stained with H&E. Subgroups A1 and A2 did not show significant degeneration in the IVDs; subgroups B1, B2, C1, and C2 had significant IVD degeneration after surgery, as demonstrated by nucleus pulposus chondrocyte degeneration and necrosis, irregular shape, and uneven distribution.

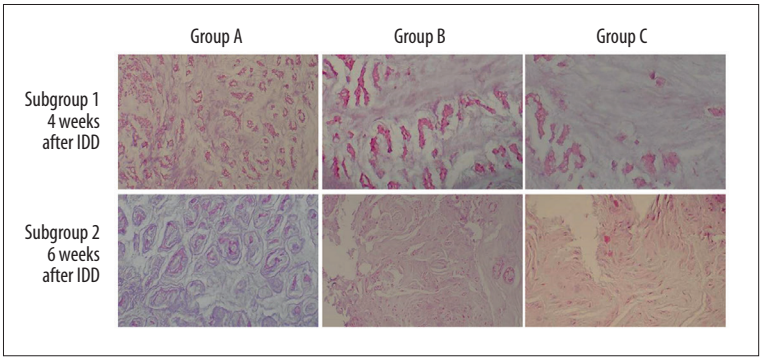

Figure 4. Histological examination at different time points (Masson,×100). Nucleus pulposus sections of IVD sections were stained with Masson's trichrome stain. Subgroups A1 and A2 did not show significant degeneration in IVDs; subgroups B1, B2, C1, and C2 had significant IVD degeneration after surgery, as demonstrated by cartilage matrix being gradually replaced by fiber bundles.

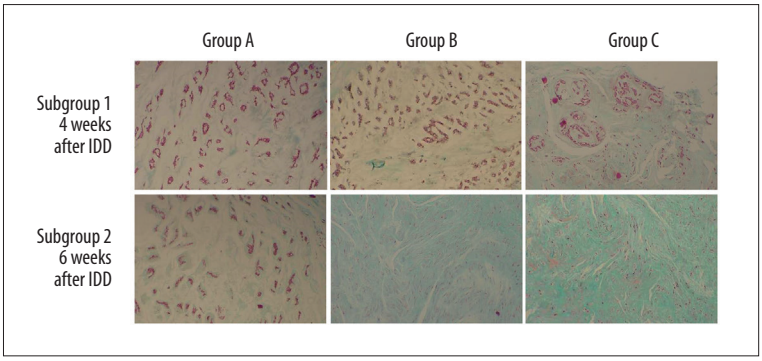

Figure 5. Histological examination at different time points (Safranin O, ×100). Nucleus pulposus sections of IVD sections were stained with Safranin O. Subgroups A1 and A2 did not show significant degeneration in IVDs; subgroups B1, B2, C1, and C2 had significant IVD degeneration after surgery, as demonstrated by reduction in proteoglycan.

significantly different at 4 and 6 weeks after IDD (P<0.001). At 4 and 6 weeks after IDD surgery, IDD grading of groups B and C were significantly different than that of group A (P<0.001), while there was no significant difference between groups B and C (P>0.05; Table 2).

Histological examination

Over time, the IVD tissue in groups B and C showed nucleus pulposus chondrocyte degeneration and necrosis, irregular shape and uneven distribution, and cartilage matrix being gradually replaced by fiber bundles. Immunohistochemistry revealed reduction in type II collagen. Safranin O staining revealed changes in proteoglycan. IVD tissue morphology in group A did not change significantly (Figures 3–6).

IDD was not graded differently at different time points in group A (P=0.09), but there were significant differences (P<0.001) in groups B and C. At 4 and 6 weeks after IDD surgery (subgroups 1 and 2, respectively), groups B and C were significantly different compared with group A (P<0.001), while there were no significant differences between groups B and C (P>0.05; Table 3).

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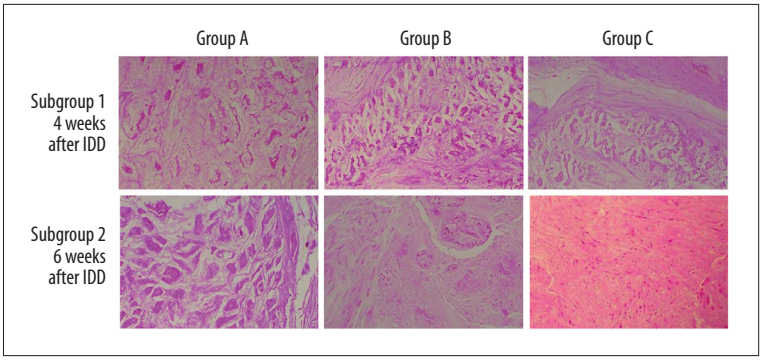

Figure 6. Histological examination at different time points (IHC ×100). Nucleus pulposus sections of IVD sections were stained for collagen using immunohistochemistry. Subgroups A1 and A2 did not show significant degeneration in IVD; subgroups B1, B2, C1, and C2 had significant IVD degeneration after surgery, as demonstrated by reduction in type II collagen.

Table 3. IDD histology (H&E staining) grading at different time points (n=12) (analyzed with Mann-Whitney U test).

Group	4 weeks after 1st operation						6 weeks after 1st operation						Z	P
	0	1	2	3	4	5	0	1	2	3	4	5		
A1/A2	5	7	0	0	0	0	2	8	2	0	0	0	–1.687	0.092
B1/B2	0	0	5	6	1	0	0	0	0	4	6	2	–3.235	0.001
C1/C2	0	0	4	7	1	0	0	0	0	3	6	3	–3.382	0.001
H	25.217						24.909						/	/
P	<0.001						<0.001						/	/

Discussion

Masuda et al. [14] proposed establishing the IDD rabbit model by using annulus fibrosus puncture with an ordinary needle, which subsequently has become widely used because of its merits of being simple, having a short experimental period, and a high success rate. Puncture can directly damage the annulus fibrosus, thereby reducing the hydrostatic pressure within the disc, which induces changes in proteoglycan and collagen content that decrease the load-bearing capacity and exacerbates degeneration. This degenerative process is quite similar to the IDD process that occurs in humans. In previous experiments, we confirmed that annulus fibrosus puncture can successfully establish an IDD model [18]. We therefore used the same approach in this study and it resulted in gradual reduction of imaging signal intensity of the IVD nucleus pulposus; gradual reduction in the nucleus pulposus area; gradually reduced disc height; nucleus pulposus chondrocyte degeneration; necrosis, irregular shape, and uneven distribution; and cartilage matrix being gradually replaced by fiber bundles, indicating reduction in type II collagen and proteoglycan, which is consistent with the process of IDD.

Results showed that over time, IVD height and disc imaging signal intensity decreased gradually in groups B and C, and that these changes were slight in group A. Compared with groups B and C, group A showed significantly different disc height index percentage and MRI image grading at 2 and 4 weeks after PRP injection. Degenerative histological changes in IVDs in groups B and C were more severe compared with group A. These results are supported by a previous study performed in a rabbit model of IDD [12].

X-rays can provide data on disc height reduction, osteophytes, and calcification, and DHI [14], an important indicator for quantitative analysis of the degree of IDD. MRI is a valuable and objective tool that can provide a clear picture of the disc to assess IDD. MRI can reveal changes in IDD by detecting reduction of T2 signal intensity (representing water content) and IVD space narrowing [19]. The results of the present study showed that in groups B and C, the disc height and IVD signal intensity significantly decreased over time, which is consistent with the process of IDD, while disc height and signal intensity in the PRP injection group did not change significantly, suggesting IDD inhibition in this group of animals.

Histologically, H&E staining can clearly show the structure of different cellular layers. Masson's trichrome allows the evaluation of the changes in collagen fibers. Safranin O staining mainly reflects the proteoglycan content and distribution. Immunohistochemistry reveals the changes in collagen type II. A normal IVD nucleus pulposus is mainly composed of notochord cells and cartilage-like cells, and the fibrous ring is mainly composed of cartilage-like cells in the inner layer and

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fiber-like cells in the outer layer. Notochord cells play a vital role in maintaining the integrity of the IVD and stability of the disc matrix. In the early stage of IDD, cartilage-like cells proliferate to repair the degeneration caused by the reduction in notochord cells. Once this repair process fails, matrix and cartilage-like cells decrease as degeneration progresses, and the nucleus pulposus is replaced by fibrocartilage accompanied by widely spread osteophytes [20]. In the present study, over time, the PBS injection and control groups had nucleus pulposus chondrocyte degeneration and necrosis, irregular shape, and uneven distribution, and cartilage matrix being gradually replaced by fiber bundles, whereas in the PRP injection group the IVD tissue morphology did not change. The PBS injection and control groups compared with the PRP group had significantly increased proteoglycans and type II collagen, indicating that PRP intervention can inhibit the IDD process.

In recent years, some uses of PRP have been demonstrated for plastic surgery and orthopedics because of its effects on bone, cartilage, tendon, and muscle regeneration [10]. Current research on PRP for repairing IDD remains limited to the cellular and small animal levels, and the mechanisms are unclear. This may be due to the repair mechanisms involving a variety of growth factors and cytokines regulating cell function, improving the microenvironment, and promoting regeneration. Akeda et al. [11] reported that with pig IVD cells cultured in alginate beads, PRP can effectively promote pig IVD cell proliferation and extracellular matrix metabolism. This was significantly more effective than using bovine serum stimulation and platelet-poor plasma, and its effects on annulus cells were stronger than on nucleus pulposus cells, therefore providing a preliminary theoretical basis for using local PRP injection to promote IVD repair or as a supplement for tissue engineering. Chen et al. [21] co-cultured PRP and human nucleus pulposus cells and found that PRP significantly up-regulated Sox9 and mRNA expression of type II collagen and proteoglycan, promoted mucopolysaccharide aggregation, and participated in the engineering of nucleus pulposus differentiation into cartilage via formation of collagen scaffolds, probably mediated by the Smad signaling pathway. Therefore, PRP, as a complex mixture of a variety of growth factors, can play a therapeutic role in IDD via promoting the proliferation and differentiation of nucleus pulposus cells and tissue engineering to form the nucleus pulposus. In 2007, Nagae et al. [22] initially reported *in vivo* experiments in which a partial nucleus pulposus suction-induced IDD rabbit model was used and autologous PRP was embedded in gelatin microspheres and injected into nucleus pulposus. Eight weeks later, immunohistochemical staining for nucleus pulposus and inner annulus fibrosus proteoglycan was enhanced, indicating that injection of PRP embedded in gelatin microspheres may be an effective IDD treatment. In 2009, based on MRI images, the same group reported that DH and water content in the PRP (embedded in gelatin microspheres) injection group were significantly higher than in other groups with corresponding increases in proteoglycan core protein and mRNA expression of type II collagen, and with significantly reduced apoptotic cells in nucleus pulposus [23]. Gullung et al. [24] evaluated the therapeutic potential of PRP IVD injection using MRI and histological tests in a percutaneous annulus fibrosus puncture-induced rat IDD model. Their study showed the protective effect of PRP on discs damaged by IDD, and the effect was more significant with earlier intervention. Obata et al. [12] studied an annulus fibrosus puncture-induced rabbit IDD model with high-speed centrifugal preparation of autologous PRP IVD injections, and using MRI and histological tests confirmed the repair effect of PRP on IDD. In the present study, we used the annulus fibrosus-induced IDD model, which has a lesser degree of injury compared with the nucleus pulposus aspiration model used by Nagae et al. [22]. We demonstrated that early PRP injections could significantly inhibit IDD, which is in line with the findings of Obata et al. [12]. We also noticed that thrombin and $CaCl_2$, often used as PRP activators, could stimulate the release of growth factors by PRP. Only Obata et al. [12] used $CaCl_2$ as PRP in the *in vivo* experiments described above. The thrombin and PRP gel used in the present study might play an active role in the inhibition of the IDD process via stimulating the release of growth factors.

One of the limitations of this study was the relatively short time between IDD surgery and PRP injection. Although we observed satisfactory results, more experiments are needed to verify whether PRP intervention alone has good efficacy on severely degenerated IVD. In addition, although radiographic and histological examination revealed that PRP can significantly inhibit IDD, it is unclear if the treatment could alleviate low back and lower limb pain due to IDD. In addition, the mechanisms by which PRP play a role need to be further clarified. Finally, the results of the present study are not entirely new and confirmed the results of a previous study [12], but with further histological analyses. Nevertheless, additional preclinical data were needed and are still necessary before performing a clinical trial.

Conclusions

In conclusion, PRP intervention significantly restored disc height and signal intensity, and increased the expression of proteoglycan and type II collagen. IVD tissue morphology was close to normal. These results strongly suggest that PRP intervention can be used to effectively suppress the IDD process.

Conflict of interest

The authors declare that they have no conflict of interest.

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