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

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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-inflammatory 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 IDD 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.
ANIMAL STUDY

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 collagen fibers, and Safranin O stain for 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.

**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 was calculated according to the formula.

\[
\text{DHI} = \frac{2 \times (\text{DH1} + \text{DH2} + \text{DH3})}{(\text{LB1} + \text{LB2} + \text{LB3}) + (\text{UB1} + \text{UB2} + \text{UB3})}
\]

\[
\%\text{DHI} = \frac{\text{postoperative DHI}}{\text{preoperative DHI}} \times 100\%
\]

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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, 90.0±8.44 at 6 weeks; B: 75.3±6.35% 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.

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<sup>b</sup>          | 70.83±6.35<sup>b,c</sup>       | 121.999 | <0.001 |
| C     | 100        | 74.67±5.53<sup>b</sup>          | 69.92±6.16<sup>b,c</sup>       | 137.446 | <0.001 |
| F     | /          | 65.213                          | 31.247                          | /    | /   |
| P     | /          | <0.001                          | <0.001                          | /    | /   |

<sup>a</sup> vs. before IDD, P<0.01; <sup>b</sup> vs. A group, P<0.01; <sup>c</sup> vs. subgroup 1, P<0.01.

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   |
|-------|------------|--------------------------------|--------------------------------|-----|-----|
| A1/A2 | 12         | 0 0 0 0 | 11 1 0 0 | 9 3 0 0 | 3.828 | 0.147 |
| B1/B2 | 12         | 0 0 0 0 | 0 0 0 0 | 3 9 0 0 | 4 8  0 0 | 30.640 | <0.001 |
| C1/C2 | 12         | 0 0 0 0 | 2 9 1 0 | 0 3 9 3 | 30.117 | <0.001 |
| H     | /          | 26.970                      | 26.847                          | /   | /   |
| P     | /          | <0.001                      | <0.001                          | /   | /   |
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).
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

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  | 4  | 6  | 2  |      |     | 3.235 | 0.001 |
| C1/C2 | 0  | 4  | 7  | 1  | 0  | 0  | 0  | 3  | 6  | 3  |      |     | 3.382 | 0.001 |
| H     | 25.217 |      | 24.909 |      |     |     |     |     |     |     |     |     |
| P     | <0.001 |      | <0.001 |      |     |     |     |     |     |     |     |     |

Figure 6. Histological examination at different time points (HGC ×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.
ANIMAL STUDY

ported that DH and water content in the PRP (embedded in
treatment. In 2009, based on MRI images, the same group re
embedded in gelatin microspheres may be an effective IDD
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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 cellu-
lar 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, im-
proving 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 pro-
liferation and extracellular matrix metabolism. This was sig-
nificantly 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, pro-
moted mucopolysaccharide aggregation, and participated in the
engineering of nucleus pulposus differentiation into car-
tilage 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 therapeu-
tic role in IDD via promoting the proliferation and differentia-
tion of nucleus pulposus cells and tissue engineering to form
the nucleus pulposus. In 2007, Nagae et al. [22] initially re-
ported in vivo experiments in which a partial nucleus pulpo-
sus suction-induced IDD rabbit model was used and autolo-
gous PRP was embedded in gelatin microspheres and injected
into nucleus pulposus. Eight weeks later, immunohistochem-
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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 re-
ported that DH and water content in the PRP (embedded in
gelatin microspheres) injection group were significantly high-
er than in other groups with corresponding increases in pro-
teoglycan core protein and mRNA expression of type II colla-
gen, 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
disks 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 fibro-
sus-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 injec-
tions could significantly inhibit IDD, which is in line with the
findings of Obata et al. [12]. We also noticed that thrombin
and CaCl₂, often used as PRP activators, could stimulate the
release of growth factors by PRP. Only Obata et al. [12] used
CaCl₂ 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 stimu-
lating 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 ob-
served satisfactory results, more experiments are needed to
verify whether PRP intervention alone has good efficacy on
severely degenerated IVD. In addition, although radiograph-
ic and histological examination revealed that PRP can signif-
icantly inhibit IDD, it is unclear if the treatment could allevi-
ate low back and lower limb pain due to IDD. In addition, the
mechanisms by which PRP play a role need to be further clar-
ified. 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 pre-
clinical data were needed and are still necessary before per-
forming 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 inter-
vention 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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