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ORIGINAL ARTICLE • SPINE - EXPERIMENTAL SURGERY

Autologous platelet-rich plasma (PRP) effect on intervertebral disc restoration: an experimental rabbit model

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Abstract

Purpose Platelet-rich plasma (PRP) treatment for intervertebral disc (IVD) repair and tissue engineering technologies have been the target of intense research with promising results. The purpose of this study was to investigate the effect of only one intradiscal injection of PRP in the degenerated rabbit IVD and to assess the restoration process over a 6-week follow-up period.

Methods The L3–L4 and L4–L5 discs of 18 adult female rabbits were injured, according to an established degenerative model, with an 18-gauge needle, and classified into two groups: In the discs of group A rabbits, after needle puncture, an intradiscal injection of autologous PRP growth factors was performed, using a 27-gauge needle, and in the discs of the control group (group B), the same procedure was followed by intradiscal injection of normal saline. The PRP preparation was carried out aseptically, after blood collection from the same rabbit.

Results During the 6 weeks, there was a noteworthy progression of degeneration process in group B, whereas the grade of degeneration was significantly lower in group A, both for annulus fibrosus (AF) and for nucleus pulposus (NP). The intervertebral disc regeneration and reversal process of the lesions are obvious on 45 days after the injury, in group A. The hematoxylin and eosin histology grading score and the expression of collagen type II in NP and inner layer of AF were the markers better mirroring the degeneration and restoration process.

Conclusion PRP intradiscal treatment in degenerative disc disease provokes the maintenance of the disc's basic morphological characteristics with restoration being evident early after injury.

Keywords Intervertebral disc degeneration · Platelet rich plasma (PRP) · Annulus fibrosus · Nucleus pulposus

Abbreviations

AF-inner	Annulus fibrosus inner
AF-outer	Annulus fibrosus outer
NP	Nucleus pulposus
PRP	Platelet-rich plasma
TNF-α	Tumor necrosis factor-α
TGF-β	Transforming growth factor-β
H–E	Hematoxylin and eosin

Introduction

Intervertebral disc (IVD) disorders are common health problems resulting in potentially serious complications and disabilities. Research efforts have been made to find a disc-healing enhancer and prevent further degeneration [1–4]. A clinically promising method is the injection of platelet-rich plasma (PRP) into injured–degenerated discs. The method although still experimental has successfully been applied on collagenous structures: rotator cuff tears, elbow epicondylitis, anterior cruciate ligament and others [5, 6].

Three tissue regions are recognized in IVD: the vertebral endplates, annulus fibrosus (AF) and nucleus pulposus (NP). NP is the central gelatinous region consisting of a sparse cell population within a complex hydrated extracellular matrix (ECM) of collagen fibers and hydrophilic proteoglycans. IVD degeneration is accompanied by structural changes compromising its biomechanics [7, 8]. Biological therapies

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for IVD repair and tissue engineering technologies have been the target of intense research, and results are promising [1, 3]. Recently, PRP has received attention as an advantageous technique, mainly because of the potential autologous preparation in clinical settings. PRP contains biological growth factors capable of stimulating cellular growth and proliferation. In vitro experiments have demonstrated that PRP can effectively promote animal intervertebral disc cell proliferation and extracellular matrix metabolism [9]. Animal models have been recruited to experiment with, and the artificially degenerated discs in the rabbit annular needle puncture model are considered as a satisfactory material to show the healing effect of PRP [1, 3]. There are most promising results, but questions about restoration evolution through time, the role of PRP cytokines and the mode of injury and injection remain [10–12]. The purpose of the present study was to investigate the effect of only one intradiscal injection of PRP in the degenerated rabbit IVD and to assess the restoration process over a 6-week follow-up period.

Materials and methods

The study protocol was reviewed and approved by the institutional review board and ethics committee. The animal study was also approved by the institutional review board and animal care committee.

The rabbit annular puncture model of disc degeneration has been well established [1]. Eighteen female New Zealand white rabbits, ranging from 2.9 to 3.4 kg in body weight, were used in this study. The test subjects were divided into 2 groups of 9. In the first team, group (A), after inducing disc degeneration via an 18-gauge needle, autologous platelet-rich plasma (PRP) growth factors were injected in the same disc. In the second team, group (B), disc degeneration was induced by the same manner, and then, the corresponding saline solution was injected (control group). Animals were killed at 2, 4, and 6 weeks, into groups of 3. The three vertebrae from L2 to L5 were taken, and the intervertebral discs L3–L4 and L4–L5 were prepared for a histological examination.

The histological examination assessed disc morphology, and the degeneration was graded. The grades of degeneration were assessed regarding both the annulus fibrosus and the nucleus pulposus of each intervertebral disc.

Experimental surgery

All the experimental surgeries were performed at the laboratory of Biomedical Engineering, Department of Orthopaedics, University of Ioannina. Intramuscular injections of 10 mg/kg ketamine and 0.5 mg/kg midazolam were used for anesthesia of the rabbits, followed by 0.3 mg/kg midazolam and 4 μg fentanyl per hour. Under sterile surgical conditions, the rabbits were prepared for surgery in a lateral prone position. With a posterolateral retroperitoneal approach, three lumbar intervertebral discs L2–L3, L3–L4, and L4–L5 levels were exposed, and the discs were punctured and prepared with great care. An 18-gauge needle was used to puncture the discs. The punctures were 5 mm deep. The needle was inserted twice for 5 s in the nucleus pulposus of the L3–L4 and L4–L5 discs according to the described technique by Masuda et al. [13]. The puncturing was performed for the rabbits of both groups A and B.

In the rabbits of group A, after the puncture an intradiscal injection of 0.2 cc autologous platelet-rich plasma (PRP) growth factors was performed using a 27-gauge needle. The PRP preparation was carried out after the blood collection, aseptically, from the same rabbit.

In the rabbits of group B, 0.2 cc of normal saline was injected using a 27-gauge needle. Skin and fascia were sutured. The rabbits were returned to their cages with no constraints.

Preoperatively 80 mg/kg of ceftriaxone sodium was administered intramuscular. Post-op, the rabbits were housed individually with free access to water. The rabbits tolerated general anesthesia well, and no mortalities from complications caused by anesthesia or infections were found among the animals. Weight, food intake and sleeping habits were recorded.

Preparation of platelet-rich plasma

Whole blood was collected by using RegenTM DCT-D1 tubes. After gently inverting the tubes to mix, 1 ml of ethanol 95% and 0.4 ml of CaCl2 were transferred into the Regen Lab ET tube. The tubes were centrifuged for 5 min at 3000 rpm with the Regen Lab centrifuges.

The supernatant plasma (PPP) was carefully removed, and the remaining PPP (approximately 200 μl) and precipitated platelets were designated as PRP. The number of platelets of each whole blood and PRP fraction was counted by using a hemocytometer.

Histological evaluation

The specimens were fixed with 4% paraformaldehyde for 24 h at 4 °C, then transferred to a sealed vial containing a solution of 70% ethanol and decalcifying agent for 30 days. After washed with water, the specimens were sequentially dehydrated, split down the midsagittal plane and embedded in paraffin for histology sectioning. Serial sections were cut in the transverse plane at 5 μm with a microtome, and then, sections stained with hematoxylin and eosin (HE) for morphological changes were qualitatively analyzed by a pathologist, who was blinded to the different treatments between

Table 1 Classification scale described by Masuda et al. [12]

	Collagen type II (the proportion of cells staining within a given area)	TNF-α	TGF-β
0	None expression	Little or no stain in the majority of cells	Little or no stain in the majority of cells
1	< 25%	Minimal stain in the majority of cells	Minimal stain in the majority of cells
2	25–75%	Moderate stain in the majority of cells	Moderate stain in the majority of cells
3	> 75%	Strong stain in the majority of cells	Strong stain in the majority of cells

groups, using a digital image analysis system, according to the classification scale described by Masuda et al. [12], which is presented in Table 1. The histological grading score is the sum of the scores of the four individual parameters and ranged from 4 to 12, where normal is 1 point for each of the 4 categories listed above, for a total of 3 points. A total of 12 points is representative of severe degeneration. For immunohistochemistry detection, sections were incubated with primary antibody specified to collagen type II (Monosan, Sanbio B.V.), TGFB (Leica, Biosystems, Newcastle Ltd.) and TNF-α (Santa Cruz Biotechnology, Inc.), which were also evaluated with semiquantitative methods.

Statistics

To assess time series of disc restoration/degeneration, the repeated-measures analysis was used with Friedman test, as appropriate for small samples without normal distribution. To detect differences between different time points (post hoc analysis), Student–Newman–Keuls (SNK) test was used. Differences between controls and experiment group at different time points were assessed with Mann–Whitney *U* test. The scale 0–3 was also used for the semiquantitative methods (Table 1). Statistical significance was set at $p = 0.05$. SPSS (Statistical package for Social Science) 22.0 was used.

Results

The control group exhibited clear degenerative changes, progressively maximized on day 45, as shown by repeated-measure analysis. HE histology grading score and the expression of collagen type II (NP and AF inner) were the markers better mirroring the degeneration and restoration process. Despite collagen index improvement over the follow-up period, values did not reach statistical significance, although results could be considered indicative of statistical significance ($p = 0.135$ for HE and $p = 0.104$ in the case of the expression of collagen type II (AF inner). On the other hand, the time-dependent deterioration in degenerating discs is prominent and statistically evidenced. Moreover, in the case of HE summary score the two groups differed statistically significant for 30 days and

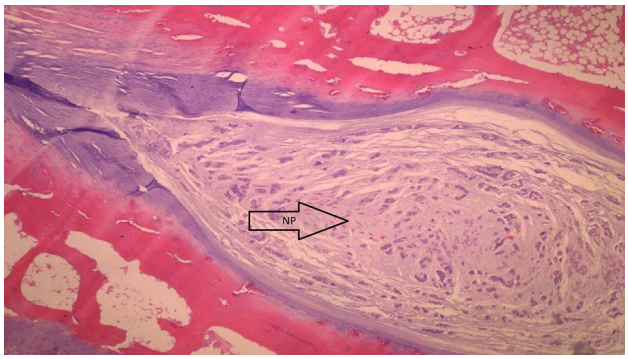

Fig. 1 Control group B with gradual degenerative changes, depending on the time point, of killing. Hematoxylin–eosin-stained sections at 15 days after injection in group B (control). Almost a normal appearance, normal pattern of fibrocartilage lamellae with U-shaped, and normal cellularity, with large vacuoles in the gelatinous structure of the matrix

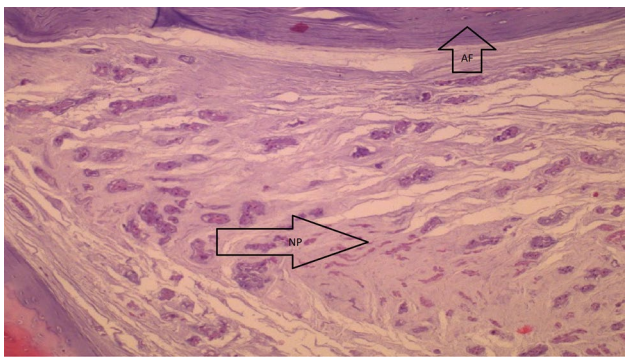

Fig. 2 NP and AF 15 days after the injection in the control group

45 days, respectively. Regarding the expression of collagen type II, difference was statistically evident on 45 days in NP. In controls, collagen fiber (inner layer of AF) content decreased by 82%, while in study groups increased by approximately 10%, comparing to initial values. Decrease in collagen type II (NP) reached 71% in controls, while no substantial difference was observed in the PRP group (Figs. 1, 2, 3, 4, 5, 6, 7). TGF-β increased in both groups; values did not reach statistical significance, even though below 0.1 level. Regarding TNF-α, this factor was steadily

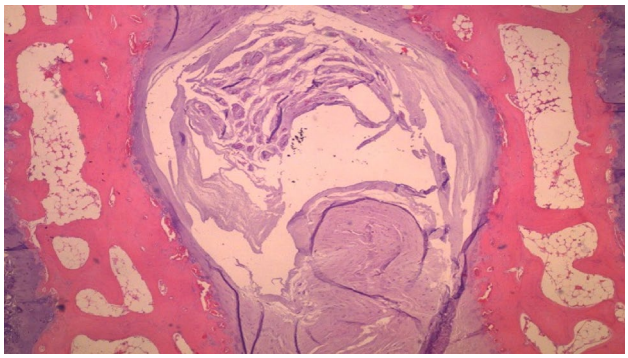

Fig. 3 30 Days after the injection in the control group. Moderately degenerated disc

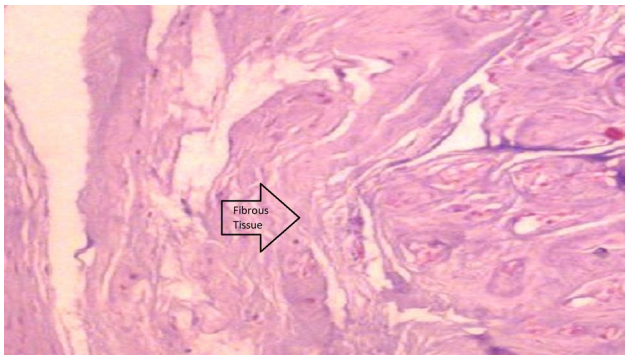

Fig. 4 Extensive fibrous tissue 30 days after injection in the control group

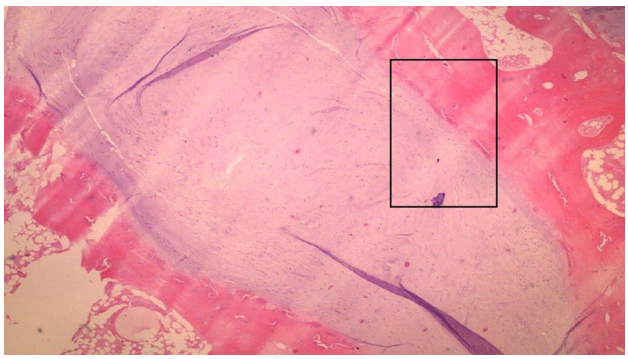

Fig. 5 45 Days after the injection in the control group. Severe degenerative disc, loss of characteristic visible border between nucleus pulposus and annulus fibrosus, and their replacement of fibrous tissues. The disappeared border is within the box

elevated in PRP group, differing largely from controls (Table 2). All other parameters (TGF-β outer/inner, TNF outer and collagen outer) did not show any significant variation between different time points or between study group and controls ($p > 0.15$).

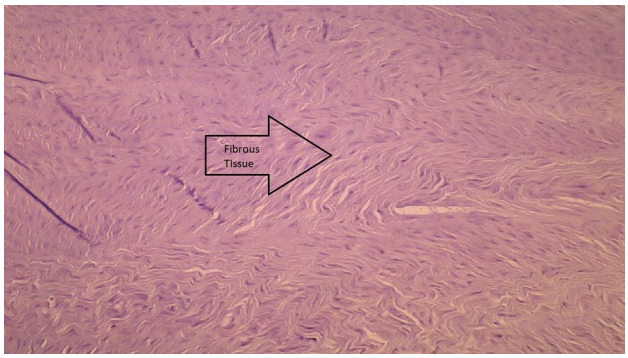

Fig. 6 Extensive fibrous tissue 45 days after the injection in the control group

Discussion

The present study examined the disc regeneration process with PRP injection through time in the well-established rabbit annular needle punctuation model. According to our findings, during the 6 weeks, there was a noteworthy progression of the degeneration in group B, whereas the grade of degeneration was significantly lower for group A, both for the annulus fibrosus and for the nucleus pulposus. The intervertebral disc regeneration and the reversal of the lesions that triggered artificially are obvious on 45 days after the injury, thus demonstrating not only the efficacy of PRP effusion, but also the course of repairing through time. The regeneration was most evident in terms of NP integrity, cellularity collagen II fibers. In controls, the degeneration score had already passed the 9-unit cutoff point after a month from injury, whereas in the study group values remain stable throughout the study period. However, a closer look at histological grading and its components revealed significant restoration of matrix and cellularity, the improvement being evident even from day 30. Collagen II fiber (AF inner), on the other hand, follows a distinctly reverse course in the two groups, being apparently enhanced in the study group (despite lacking statistical significance, 45-day mean score exceeds the initial score by 10%, in the study group). In controls, degeneration is obvious on 45 days, as collagen fibers have been decreased to a 1:5.5 ratio (45-day score/initial score). Regarding NP collagen, About 75% reduction was noted in control group Results on TNF-α and TGF-β1 reveal up-regulated concentration in the study of PRP group. These findings enlighten our knowledge about degeneration and restoration evolution through time, demonstrate an evident regeneration with autologous PRP and allow further questioning on the role of cytokines and PRP synthesis in vertebral disc regeneration.

Our findings confirm the efficacy of PRP administration, as already reported in previous studies, and give further information on the healing process through time. Indeed, in the study of Gullung et al. [3], including sham and PRP

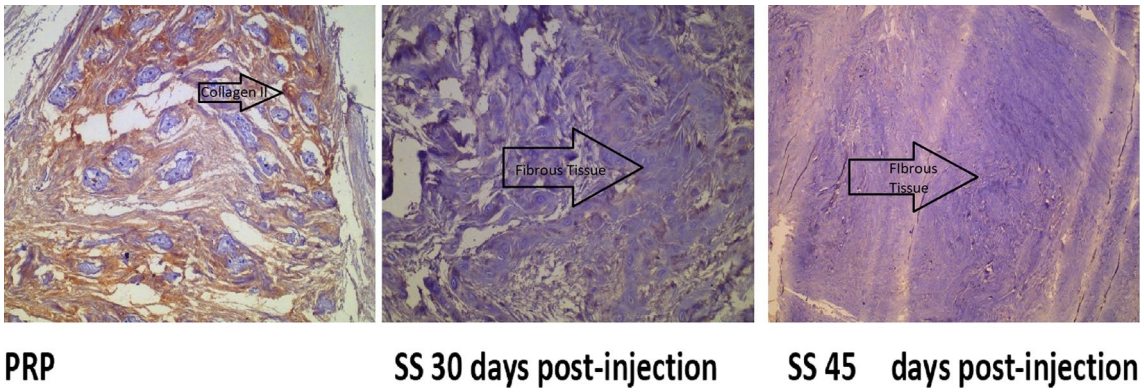

Fig. 7 Immunohistochemical expression of type II collage in disc tissue derived from different time points after injection. PRP group detected almost the same intensity of expression mostly to the nucleus pulposus, for the three subgroups (×100). In solution saline (SS) group, there was a gradual replication of tissue-like fibrocartilage, with corresponding decrease in the type II collage expression (×100)

Table 2 Significant differences between time point and groups

N = 6	Study group Mean ± SD	Controls Mean ± SD	*p***
HE-summary15d	6.00 ± 6.00	6.00 ± 0.00	1.000
HE-summary30d	6.67 ± 6.67	8.83 ± 0.98	**0.015**
HE-summary45d	6.67 ± 6.67	12.00 ± 0.00	**0.002**
*p**	0.135	**0.002*****	
Collagen type II-inner15d	1.67 ± 1.67	1.83 ± 0.75	0.665
Collagen type II-inner30d	1.17 ± 1.17	1.33 ± 0.52	0.523
Collagen type II-inner45d	1.83 ± 1.83	0.33 ± 0.52	0.523
*p**	0.104	**0.022*****	
Collagen type II-NP15d	2.33 ± 2.33	2.33 ± 0.52	0.699
Collagen type II-NP30d	2.00 ± 2.00	1.67 ± 0.52	0.589
Collagen type II-NP45d	2.17 ± 2.17	0.67 ± 0.82	**0.007**
*p**	0.584	**0.008*****	
TNFα-inner15d	0.33 ± 0.58	0.00 ± 0.00	0.548
TNFα-inner30d	0.80 ± 0.45	0.00 ± 0.00	**0.030**
TNFα-inner45d	0.67 ± 0.52	0.00 ± 0.00	0.565
*p**	0.14	N/a	
TNFα-NP15d	1.75 ± 1.26	0.33 ± 0.82	0.114
TNFα-NP30d	1.67 ± 1.21	0.20 ± 0.45	**0.052**
TNFα-NP45d	1.67 ± 0.52	0.00 ± 0.00	**0.004**
*p**	0.58	0.61	
TGF-β-NP15d	1.17 ± 1.17	0.50 ± 0.84	0.368
TGF-β-NP30d	1.67 ± 1.67	1.75 ± 0.50	0.879
TGF-β-NP45d	2.00 ± 2.00	2.00 ± 0.00	1.000
*p**	0.10	0.09	

Bold indicates statistical significance

*Friedman test, post hoc analysis (SNK): values for 45d differed statistically significantly ($p < 0.05$) from 30d and 15d

**Mann–Whitney–*U* test

***Post hoc analysis with SNK test (45d vs 15d & 30d)

groups, one immediately treated with PRP and another with delayed—2 weeks after injury, the PRP groups exhibited fewer inflammatory cells and higher fluid content and maintained NP structure, despite limited lesions. Even the delayed treatment with PRP also resulted in further degeneration prevention. In the present study, PRP injection took place immediately after injury, and the last specimens were harvested at 6 weeks, while PRP specimens in the study of Gullung [3] were not available at week 6; thus, comparison is not possible despite the similarity in the results. In 2006, Chen et al. [14] tried to evaluate the proteoglycan accumulation in human NP cells which were treated with PRP. It was shown that the proliferation of human NP was increased due to the use of PRP. Similarly, in our study 45 days after the injection the difference in collagen type II expression in NP was obvious in favor of the PRP group. One year later, Nagae et al. [15] after comparing PRP–gelatin–hydrogel microspheres with PRP alone and their results advocated for the PRP–gelatin–hydrogel microspheres which proved to provoke less severe lesions in the NP and in the AF. In 2012, Hu et al. [16] examined the effect of PRP injection in the intervertebral disc at 1 and 2 weeks after the induction of degeneration. The authors concluded that the degeneration of the intervertebral disc was reduced with the use of PRP and that there was promotion of extracellular matrix production.

Obata et al. [1], experimenting with rabbits, reported that the administration of an autologous PRP compound induced a reparative effect on degenerated discs, as estimated by MRI and histology. The number of chondrocyte-like cells in the inner layer of AF or in the NP of the PRP-treated discs was significantly higher than in controls. In that study, besides the controls a platelet-poor plasma groups have also been included; however, the results, despite being better than the controls, were rather disappointing. Differences between

controls and PRP groups were evident at 4 weeks after the injection, while in our study findings reveal a rather delayed pattern of restoration, noting, however, that restoration may be histologically present even earlier.

Regarding cytokines TNF and TGF concentrations, they were significantly up-regulated in the study group, a rather unexpected finding as these cytokines are traditionally considered as markers of inflammation and degeneration. TGF in particular may also have a deleterious effect on degenerated cartilage tissue [17–19]. The fact that PRP contains these cytokines, together with the qualitative method of determining their presence in the tissue, should make one cautious in interpreting our results, given that differences through time did not reach statistical significance, although an increase trend was detected in both groups in the case of TGF-β.

PRP treatment has been considered as an advantageous technique for disc restoration, more promising than other techniques using purified growth factors, mainly because PRP can be isolated from autologous source, thus eliminating immune reaction worries [9, 20, 21]. However, platelet activation manner and cells or cytokines concentration may vary, and results might be obscure, especially if PRP is rich in leukocytes [1]. Additionally, the annular punctual model has been shown to effectively detect the healing effect. In the present study, the PRP content of leukocytes and blood cells was not determined, while PRP injected immediately after injury, a pattern surely not reflecting the course of human disc degeneration. The latter is not the case even in models including one-month degeneration or any delayed injection, where healing might be less prominent. In any case, restoration process seems to be a time-consuming one, and models should probably include longer duration of follow-up. Another limitation present in all animal studies is the extent of vertical loading of the spine in an upright model; the effect of PRP may be not as noticeable as forces on disc increase. The present study supports previous findings that immediate PRP-treated discs maintain their basic morphological characteristics, increase their cellularity and preserve matrix metabolism. Moreover, our results suggest that early PRP injections are able to inhibit intervertebral disc degeneration. Future studies with more specimens and longer follow-up period may further help researchers acquire information about disc restoration process. Additionally, the vast majority of the studies examined the effect of PRP in early stage of degeneration, and future research should focus in more late stages of degeneration.

Despite the promising results, the present study has certain limitations. Most of these limitations are those encountered in most animal model studies. First of all, the correlation of animal model studies can achieve up to 70% with human models [22]. Moreover, the specimens included in the study were sex-restricted without any possible comorbidities which may be present in humans. The number of the experimental animals is also a limitation of the study and the evaluation of the results only is in a short postoperative period. Another limitation is the short time between the degeneration of the disc and the PRP injection.

PRP injections consist of a biological treatment approach for degenerative disc disease. The cellular mechanism of how growth factors or PRP affect the endogenous progenitor/stem cells within the discs needs to be further studied. In addition, future directions concerning this field of research include the use of tissue-engineered disc implantation in combination with PRP as well as the use of viral vector gene therapy, RNA interference and micro-RNAs.

Conclusion

The use of PRP for degenerative disc disease presents a significant improvement concerning after approximately 30 days, which is mostly depicted the expression of collagen type II and collagen fibers from the inner layer of AF. On the other hand, despite the promising results more research is needed with longer follow-up period in order to be able to exclude safer conclusions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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