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Effects of Platelet-Rich Plasma & Platelet-Rich Fibrin with and without Stromal Cell-Derived Factor-1 on Repairing Full-Thickness Cartilage Defects in Knees of Rabbits

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Abstract

Background:

The purpose of this study was to create biomaterial scaffolds like platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) containing stromal cell-derived factor-1 (SDF_1) as a chemokine to induce hyaline cartilage regeneration of rabbit knee in a full thickness defect.

Methods:

We created a full thickness defect in the trochlear groove of thirty-six bilateral knees of eighteen mature male rabbits. The knees were randomly divided into six groups (group I: untreated control, group II: PRP, group III: PRF, group IV: Gelatin+SDF_1, group V: PRP+SDF_1, and group VI: PRF+SDF_1). After four weeks, the tissue specimens were evaluated by macroscopic examination and histological grading, immunofluorescent staining for collagen type II, and analyzed for cartilage marker genes by real-time PCR. The data were compared using statistical methods (SPSS 20, Kruskal-Wallis test, Bonferroni post hoc test and $P<0.05$).

Results:

Macroscopic evaluations revealed that international cartilage repair society (ICRS) scores of the PRF+SDF_1 group were higher than other groups. Microscopic analysis showed that the ICRS score of the PRP group was significantly lower than other groups. Immunofluorescent staining for collagen II demonstrated a remarkable distribution of type II collagen in the Gel+SDF_1, PRP+SDF_1 and PRF+SDF_1 groups compared with other groups. Real-time PCR analysis revealed that mRNA expression of SOX9 and aggrecan were significantly greater in the PRF+SDF_1, PRP+SDF_1, Gel+SDF_1 and PRF groups than the control group ($P<0.05$).

Conclusion:

Our results indicate that implantation of PRF scaffold containing SDF1 led to the greatest evaluation scores of full-thickness lesions in rabbits.

Keywords: Platelet-rich plasma, Platelet rich fibrin, Chemokine CXCL12, Cartilage, Knee

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What's Known

- Implantation of a combination of collagen I scaffold and ultrapurified alginate gel containing stromal cell-derived factor-1 (SDF_1) improved the repairing process by conferring better support for the migration of cartilage mesenchymal stem cells (C-MSCs) and synovial membrane mesenchymal stem cells (SM-MSCs).
- Adding platelet-rich fibrin (PRF) to bone allograft in sinus elevation surgery can reduce the healing time.
- Some studies have shown that if platelet-rich plasma (PRP) is applied in chondral lesions, signs of healing will be observed.

What's New

- The effects of PRF+SDF_1 and PRP+SDF_1 were not studied so far.
- We used PRP and PRF scaffolds and SDF_1 to magnify their effects and reconstruct injured sites. These scaffolds are rich in slow-releasing growth factors and are easily harvested from autologous blood.
- There is similarity between PRF fibrin network and the natural one.
- SDF_1, which is expressed by BMSCs, regulates the trafficking, homing, and migration of stem cells into the injury site.

Introduction

Adult articular cartilage has insufficient ability to repair after either erosion or damage and it is unlikely to be reformed to normal condition once it has been impaired.[1] Functional repair of articular cartilage defects encountered a major challenge to cartilage tissue engineering.

Articular cartilage repair has been based on finding and resolving the primary reason for the injury, but it has been recognized that only treating the damage site is not adequate to gain good long-term functionality.[2] The matrix environment of hyaline cartilage is different from that of subchondral bone in biological, physical and chemical characteristics.[3] This led to the proposal that in order to gain positive results in tissue repair, the 'microenvironment' in which the repair process takes place should

be retouched, such as those elements that play a pro-angiogenic, conductive, cell differentiation and chemotactic role.[2] This tendency has been the reason behind a growth in using genetic and tissue engineering in the examination of articular cartilage repair.[4]

A number of natural and artificial biomaterials have been used as scaffolds for cartilage reformation, but their biological effectiveness as well as safety remains unresolved. Therefore, scaffold biomaterials extracted from the patients' own body have long been a tempting choice.[5] Platelet-rich plasma (PRP), which can be easily harvested from autologous blood, has been used in clinical experiments for decades.[6] PRP is rich in growth factors, containing those that help the proliferation of chondrogenic cells and discharging of cartilaginous matrix, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-b), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF).[6,7]

A new member in platelet concentrated products is platelet-rich fibrin (PRF), which can be prepared easily without biochemical blood handling. PRF is similar to PRP except in biochemical architectures because of slow polymerization during PRF preparation.[8] The PRF fibrin network is similar to the natural one. Such a network leads to a more competent cell mobilization and repopulation and consequently healing.[8] Stromal cell-derived factor-1 (SDF_1), a chemokine factor expressed by BMSCs, regulates the trafficking, homing and migration of stem cell by mediating chemokinesis and chemotaxis into the injury site.[9] Bone marrow, which is adjoining the chondro-osseous junction, discharges SDF_1.[10]

We believe that autologous PRP and PRF could be used alone as a 3-D scaffold capable of cartilage tissue regeneration because of releasing endogenous growth factors. SDF_1 stimulates cartilage mesenchymal stem cells (C-MSCs), synovial membrane mesenchymal stem cells (SM-MSCs), and bone marrow stromal cells (BMSCs) to migrate to the subchondral bone in full-thickness defects to make cartilage. The aim of the study was to evaluate tissue repair along with PRP and PRF or without treatment. The secondary objective was to evaluate the efficacy of PRP and PRF used with SDF_1.

Materials and Methods

PRP Gel Preparation

Blood samples were taken from the hearts of six rabbits. The 9 mL of whole blood collected in a tube containing 1 mL an anticoagulant (3.8% sodium citrate solution) was centrifuged for 5 minutes at 2,000 rpm and the supernatant was placed in a new tube. The supernatant was centrifuged for 10 minutes at 3,000 rpm. The plasma at the top was discarded and the remaining 1mL of liquid PRP precipitate was collected. After mixing PRP (200 µL/defect) and 40 mmol/L calcium chloride (CaCl2), a gel was formed. The PRP gel bonded to the adjacent cartilage about 10 minutes after implantation.[11]

PRF Preparation

The 10 mL blood samples collected from the hearts of six rabbits without anticoagulant were immediately centrifuged at 3,000 rpm for 10 minutes. In the middle of the tube, a fibrin clot was formed between supernatant acellular plasma and the lower red corpuscles.[8]

SDF_1 Incubation

Before transplantation, 600 µL of rhSDF$_1$ alpha (120 ng/mL Peprotech) was incubated with PRP gel, PRF and gelatin separately for 2 hours at 37ºC.[12]

Study Design and Animal Experimentation

A total of 18 adult male white Japanese rabbits (weighing 2.5-3.0 kg) were used in this study. The animals were housed in metal wire cages, in a temperature-controlled room under a 12:12 h light-dark cycle at 22–24ºC and 50–60% relative humidity. They were fed ad libitum with standard laboratory chow and tap water. Animal interventional experiments were carried out at Animal Experiments of Tehran University of Medical Sciences under the rules and regulation of the Animal Care and Use Committee of Shiraz University School of Medicine.

The 36 bilateral knees of the 18 rabbits were randomly divided into 6 experimental groups of 6 knees per group. Each animal was sedated by intramuscular injection of ketamine hydrochloride 60 mg/kg and xylazine 6 mg/kg. In sterile conditions, a medial para-patellar arthrotomy was made in both knees. A full-thickness cylindrical cartilage defect of 4 mm in diameter and 3 mm in depth was created in the patellar groove using a standard size stainless biopsy punch. The joints were thoroughly rinsed with sterile saline solution before transplantation. In group I, the defects were left unfilled (control group). In group II, the defects were filled with platelet-rich plasma only (PRP group). In group III, the defects were filled with platelet-rich fibrin only (PRF group). In group IV, the defects were filled with gelatin containing stromal cell-derived factor-1 (Gel+SDF$_1$ group). In group V, the defects were filled with platelet-rich plasma containing stromal cell-derived factor-1 (PRP+SDF$_1$ group). In group VI, the defects were filled with platelet-rich fibrin containing stromal cell-derived factor-1 (PRF+SDF$_1$ group) in a randomized manner. The rabbits were accommodated in separate cages and allowed unrestricted activity after surgery.[9,11,12]

Gross Morphology

Four weeks after implantation, the rabbits were sacrificed with an overdose of anesthesia. The distal parts of the femurs were excised, photographed and graded for cartilage repair, according to the international cartilage repair society score (ICRS) macroscopic assessment scores[11] (table 1).

Table 1

ICRS macroscopic evaluation of cartilage repair

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Histological and Immunohistochemical Analysis

After gross evaluation, samples were fixed in 4% paraformaldehyde for 7 days, decalcified in 10% EDTA for 3 weeks and then embedded in paraffin and cut perpendicularly into 5 µm sections. Then, the sections were stained for general histology with hematoxylin and eosin (H&E) and with Toluidine blue to estimate the cartilaginous matrix distribution. The re-created tissue was scored blindly according to the ICRS scale[9,13] (table 2).

Table 2

ICRS visual histological assessment scale

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Immunofluorescent analysis was performed using a monoclonal antibody against Collagen II (Cat number: MA1-37493). The sections were deparaffinized by xylene and rehydrated by decreased concentrations of ethanol solutions. Heat-induced epitope retrieval was done in citrate buffer (sodium citrate, 10 mM; pH 6.0) in a pressure cooker for 4 minutes at full pressure. Subsequent to cooling, endogenous peroxidase was blocked using a 3% hydrogen peroxide solution for 20 minutes. The slides were then washed with phosphate buffer solution (10 mM; pH 7.4) and incubated with primary antibodies overnight at 4°C. The slides were washed again three times with PBS and subsequently incubated with an FITC-conjugated secondary antibody (Cat number: 11555100) according to the respective manufacturer's instructions. The slides were washed three times with PBS and then covered with cover slips. Finally, samples were analyzed by florescent microscope.[9]

Real-time PCR Analysis

Total RNA was extracted from the regenerated tissues in the defect area. Total RNA extraction was performed using RNX-plus kit (CinnaGen) according to the protocol. The RNA samples were resuspended in 30 µL of nuclease-free water. The concentration and quantification of total RNA were measured with spectrophotometer, with the OD260/OD280 ratio of all RNA samples 1.9–2.0 and OD260/OD230 ratio up to 2. The first strand cDNA was synthesized with the first-strand cDNA synthesis kit (Bioneer kit, K-2101). For each reaction, 1 µg (1 µL) RNA was used for reverse transcription, in a mixture of 20 pmoles (1 µL) random primer, and 18 µL DEPC-D.W with a final volume of 20 µL. The mixture was incubated at 15ºC for 1 min, 50ºC for 60 minutes and heated at 95ºC for 5 minutes to terminate the reaction. The cDNA was subsequently stored at -20ºC. qPCR was performed in a volume 1 µL of primer and 1 µL from template also add 3 µL DEPC.D.W with 5 µL Master Mix (AccuPower® 2X GreenStarTMqPCR Master Mix, Bioneer kit). Real-time PCR was done by ABI (Applied Biosystems). All PCR reactions were in the following condition: initial 95ºC for 15 minutes, followed by 35 cycles at 95ºC for 15 s and 60ºC for 30 s. After completing the qPCR, their products run on agarose gels for quality check and real-time data analyzed by Livak rules.[14] Beta actin was used for the internal control. The PCR primers for each gene are displayed below. Each sample was tested in duplicate. The sequences of primers used in real-time PCR analyses were as follows: Aggrecan forward GAGACCAAGTCCTCAAGCCC; reverse CTCTGTCTCCTGGCAGGTTC. Sox9 forward GCTCCGACACCGAGAATACA; reverse TTGACGTGGGGCTTGTTCTT.[13]

Data Analysis

Data are expressed as mean±SD (standard deviation). Significance was considered at the 0.05 level. Outcome variables were macroscopic evaluation scores, histological scores, and genes expression. A comparison between groups for each outcome was carried out with the Kruskal-Wallis test. Bonferroni post hoc tests were used for the comparison analysis of each two groups. Data were analyzed using SPSS (version 20) software.

Results

Macroscopic Observation

Four weeks after surgery, the regenerated regions of all groups were studied. The macroscopic evaluation of the treated areas in the PRF+SDF_1 group revealed complete repair and integrated well with the surrounding cartilage as compared with other groups (figure 1). A post-hoc analysis showed that ICRS scores of all experimental groups were significantly higher than those in the control group and the scores of the PRF+SDF_1 group were greater than those of the other groups ($P<0.001$) (figure 2).

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Figure 1

Gross appearance of defects in the trochlear groove at four weeks after surgery, respectively. A) Control group: Transplantation was not performed. The defect is partially vacant and clearly noticeable from the surrounding cartilage. B) PRP group: Healing tissue is thinner than normal surrounding cartilage and shows a large fissure. C) PRF group: Repaired tissue covers defect with a minor concavity. D) Gel+SDF_1 group: Repaired tissue covers the defect almost completely, but is thin and distinguishable from the surrounding cartilage. E) PRP+SDF_1 group: Healing tissue covers defect with smooth white tissue and distinguishable from the surrounding cartilage. F) PRF+SDF_1 group: Repaired tissue covers the defect completely and no obvious margin was notable.

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Figure 2

A) ICRS macroscopic and B) ICRS microscopic scores in the regenerated cartilage of defects in the trochlear groove at four weeks postoperatively. Data are presented as mean±SD. *Significant difference with non-treated control ($P<0.05$); **Significant difference with RPP group ($P<0.05$).

Microscopic Observation

Four weeks after surgery, the regenerated regions were evaluated by H&E and Toluidine blue staining. The regenerated regions of the PRF+SDF_1 group were entirely filled by the repaired tissue and showed better results than the other groups (figures 3 and 4). A post-hoc analysis showed that ICRS scores of all groups, except for the PRP group, were significantly higher than the control group ($P<0.001$). The scores of the PRF+SDF_1 group were greater than the other groups, but were not significant when compared with the Gel+SDF_1 and PRP+SDF_1 groups. The scores of the PRP group were significantly lower than the other groups except for the PRF group, which was not significant (figure 2). The

qualitative study of immunofluorescent staining of collagen II demonstrated that neo-cartilages were minimally positive in the control, PRP, and PRF groups. In contrast, the Gel+SDF_1, PRP+SDF_1 and PRF+SDF_1 groups exhibited a remarkable distribution of type II collagen (figure 5).

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Figure 3

The microscopic appearance of H&E staining of defects in the trochlear groove at four weeks after surgery, respectively (Original magnification=200×). A) Control group: The defect is concave and a noticeable thin layer of non-cartilaginous tissue. B) PRP group: The defect is filled by fibrous tissue with a crack. C) PRF group: The defect is covered by fibrous tissue. D) Gel+SDF_1 group and (E) PRP+SDF_1 group: The defects in both groups are filled with a repaired tissue, which are thinner than the normal surrounding cartilage. F) PRF+SDF_1 group: The defect is entirely filled by the repaired tissue, resembling the healthy cartilage surrounding the tissue.

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Figure 4

Histological appearance of Toluidine blue staining of defects in the trochlear groove at four weeks after surgery, respectively (Original magnification=200×). A) Control group: The defect is filled by no cartilage matrix. B) PRP group, and C) PRF group: The defects in both groups have poor staining fibrocartilagous tissues and poorly organized ECM. D) Gel+SDF_1 group, and E) PRP+SDF_1 group: The repaired tissues show a good staining in both groups. F) PRF+SDF_1 group: The defect has an intense staining.

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Figure 5

Immunofluorescent staining appearance of defects in trochlear groove at four weeks after surgery. A) Control group, B) PRP group, C) PRF group, D) Gel+SDF_1 group, E) PRP+SDF_1 group, and F) PRF+SDF_1 group. The neo-cartilages are minimally positive in A, B and C. The neo-cartilages are positive in D and E. A remarkable distribution of type II collagen is seen in F.

Real-Time PCR Analysis

Four weeks after surgery, a real-time PCR analysis revealed that the mRNA expression of SOX9 was significantly greater in the regenerated tissue of all groups than the untreated control group ($P<0.001$). The level of gene expression of aggrecan in all groups was significantly higher than the control group,

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except for the PRP group that was not significant (P<0.001) (figures 6 and 7).

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Figure 6

The amount of gene expression of SOX9 and aggrecan in the regenerated cartilage of defects in the trochlear groove at four weeks postoperatively. Data are presented as mean±SD. *Significant difference with non-treated control (P<0.05).

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Figure 7

The quality checking of RT-PCR for SOX9 and aggrecan from the regenerated cartilage of defects in the trochlear groove at four weeks after surgery. Lane A: Control group where transplantation was not performed; Lane B: PRP group; Lane C: PRF group; Lane D: Gel+SDF_1 group; Lane E: PRP+SDF_1 group, and Lane F: PRF+SDF_1 group.

Discussion

This study considered the effects of PRP and PRF with or without SDF_1 in full-thickness lesions in rabbits' knees. A literature review indicated that the effects of PRF+SDF_1 and PRP+SDF_1 were not studied so far. We demonstrated that the implantation of PRF+SDF_1 led to the greatest yield scores of full-thickness lesions in rabbits' knee. Therefore, it can be concluded that the scaffolds containing SDF_1 have a better result on cartilage repair. Zhang W. et al.[12] implanted a combination of collagen I scaffold to mimic the subchondral bone matrix environment containing SDF_1 in order to repair partial-thickness defects in rabbits' knee. The results demonstrated that collagen I scaffold containing SDF_1 improved the repairing process by a better support for C-MSCs and SM-MSCs migration and adhesion six weeks after transplantation. Some researchers used ultra-purified alginate gel containing SDF_1 to improve osteochondral lesions in rabbits' knee. According to their studies, the repairing effect of osteochondral defects is elevated, because SDF_1 increases the migration of host cells (mainly BMSCs) to the site of injury, and this makes SDF_1 as a potential factor in cartilage repair.[15] Wei L. et al. used SDF_1 to induce chondrocyte hypertrophy. They studied the length of the hypertrophic zone by immunohistochemical analysis of tibia cartilaginous growth plates of chicken embryos, which were incubated with SDF_1 for 2, 4, and 6 days in organ culture. The results showed the length of the hypertrophic zone was significantly elevated after 4 and 6 days.[3] In injured tissue with the permissive matrix environment, endogenous stem cells can participate in corrective process. Some studies have introduced populations of stem/progenitor cells in the synovial membrane[16,17] and articular cartilage,[18-20] which show characteristic of stem cells and have the potential for cartilage repair.[21,22] The findings of the above studies are in agreement with our results that show SDF_1 can facilitate the

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migration of C-MSCs and SM-MSCs to full-thickness defect sites. Although SDF_1 has been notified to promote inflammatory cell migration,[23] we could not find inflammatory cells in the newly formed tissue in RPF, PRP and Gel containing SDF_1 groups. In parallel with our data, a study on tendon repair by exogenous SDF_1 showed improvement of injury site and diminishing accumulation of inflammatory cells.[9] In PRF, the fibrin formation meshes, which are similar to biochemical architectures of the natural one,[8] can trap a significant amount of circulating cytokines. This is what renders PRF as a distinctive treatment in injury repair. This method increases the lifespan of these cytokines (as a long-term effect), without which, it will be only released and used at the time of the primary phase of matrix remodeling. As a result, when the cells begin extracellular matrix remodeling to reconstruct the injured site, the cytokines will be available in the affected area for the required longer period. Cytokines are important factors in the delicate balance of tissue homeostasis.[9]

In a pilot study, Haleem A.M. et al. showed that all patients with osteochondral defects who received BM-MSCs transplanted on platelet-rich fibrin glue (PR-FG) had a significant improvement in their functional knee scores and MRI findings after 6 months and remained stable 12 months post operation.[1] In agreement with this clinical study, Choukroun J. et al. revealed that filling a tooth socket by PRF can enhance the healing process without any complication.[24] In a supplementary study, they reported that adding PRF to bone allograft in sinus elevation surgery can lead to 4-month reduction in healing time by increasing the speed of bone allograft maturation.[25] However, in contrast to our results, an in-vivo study by Shao X.X. et al. on osteochondral defects in rabbits' knee showed that BMSCs seeded in fibrin glue matrix forms a poor-quality cartilage-like tissue.[26] It can be concluded that the reason for varying results can be due to the differences in biomechanical conditions of defect types. Kelly D.J. et al. reported that differentiation of bone-marrow derived stem cells can be affected by mechanical signals.[27] Moreover, suitable recurring compressive stress significantly increases chondrocyte proliferation in addition to aggrecan and collagen synthesis by chondrocytes.[28] Some researchers reported that cell adhesion and migration can facilitate by some elements such as different cell type and substrate stiffness.[29,30] Most importantly, in a separate study, Wang H. et al. had compared the amount of bounded bovine chondrocytes on various surfaces of middle deep zone bone or calcified cartilage or hyaline cartilage. They reported that different contact surface stiffness give different ability to cells for migration and attachment.[31]

Opinions about autologous PRP usage in terms of its composition is controversial.[32,33] Activated PRP can be used in different forms like gel as a scaffold, liquid form injection or can be infiltrated in the tissue thickness.[34] The selection of each form relies upon the type of tissue and the existing problem.[35,36] By degradation of the fibrin skeleton of PRP, it can release endogenous growth factors without modifying genes or cytokine delivery technologies. Growth factors play a significant role in tissue repair, especially in the regenerative process of tissues with low cellular density and vascularization, such as cartilage.[6,37,38] A study showed that if PRP was applied in full-thickness chondral lesions, the signs of healing will be observed, but it did not show healthy or functional behavior.[4] Wu W. et al. reported cartilage formation into the PRP scaffold after implanting PRP gel as a carrier for cultured autologous chondrocytes in the subcutaneous tissue of rabbits' knees.[39] Another study suggested that transplantation of PRP gel along with synovial membrane derived mesenchymal stem cells (SDSCs) can facilitate the repair of osteochondral lesions in rabbits' knees.[11] Perhaps the underlying reason for different results is various compositions of this plasma fraction in different species, including rabbits.[32,34,40]

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It should be noted that our study had a limitation. PRF was harvested from an autologous blood sample that had a limited volume and PRF tissue banks were not achievable.

Conclusion

In the present study, in addition to using PRP and PRF as scaffolds for cell seeding, we used a mixture of SDF1 and PRP and PRF scaffolds to enhance the migration of host cells. In our research, the effects of PRP and PRF alone on the full-thickness defects were also studied separately. We found that the added SDF_1 stimulated cell or the combination of PRP, PRF, and SDF_1 stimulated the observed improvement.

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Conflict of Interest: None declared.

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