Effect of Activated Platelet-Rich Plasma on Chondrogenic Differentiation of Rabbit Bone Marrow-Derived Mesenchymal Stem Cells

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Research

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Abstract

Background

Platelet-rich plasma (PRP) has revealed benefits in tissue repair and regeneration, however, the effect of PRP on proliferation and chondrogenesis of mesenchymal stem cells remains controversial. This study is to evaluate the effect of different concentration PRP on proliferation and chondrogenic differentiation of rabbit bone marrow-derived mesenchymal stem cells (BMSCs).

Methods

PRP was obtained by centrifugation and activation, in which growth factors and cytokines were detected. BMSCs were isolated from rabbit bone marrow and characterized by flow cytometry. About $5 \times 10^3$ BMSCs were cultured in high glucose Dulbecco’s modified Eagle’s medium (HG-DMEM) with 4 different compositions: 10% fetal bovine serum, 5%PRP, 10%PRP, and 15%PRP for consecutive 7 days. Cell counting assays were performed on days 1, 3, 5, and 7 to evaluate the BMSCs proliferation. In chondrogenic differentiation, high-density cell pellets composed of $5 \times 10^5$ BMSCs were induced in 4 conditions: commercial chondrogenic medium (control), 5%PRP (HG-DMEM + 5%PRP), 10%PRP (HG-DMEM + 10%PRP), and 15%PRP (HG-DMEM + 15%PRP) for 21 days. The gene expression levels of aggrecan (ACAN), collagen type II (COL2A1), and SRY-Box Transcription Factor 9 (SOX9) in pellets were detected. Histological assessments were performed by morphologically observation and pathological stain. Independent-samples $t$-test and one-way analysis of variance were used in statistical analyses.

Results

The concentrations of growth factors and cytokines were elevated in PRP. BMSCs proliferation was enhanced in all groups, and 10% PRP revealed more obvious outcome than the others from day 5. In chondrogenic differentiation, the levels of ACAN, COL2A1, and SOX9 were lower in 3 PRP groups than control, but ACAN and SOX9 was higher in 10% PRP group than 5% and 15%. Histological examinations showed 10% PRP-treated pellets showed more regular appearance, larger size, and abundant extracellular matrix than 5% and 10% groups, but still inferior to commercial chondrogenic medium.

Conclusions

PRP may enhance the proliferation of rabbit BMSCs, while with limited effect on chondrogenic differentiation compared with commercial chondrogenic medium in pellets culture. Whether optimizing and modifying PRP components would lead to satisfying chondrogenesis of BMSCs, it is deserved furthermore study.
1 Introduction

Clinically, repairing massive articular cartilage defects remains a challenging issue. Current available surgical techniques include osteochondral transplantation and autologous chondrocyte implantation\(^1,2\). Despite of relative satisfying results, these techniques have been limited by morbidity of the donor sites and loss of the chondrogenic phenotype during \textit{ex vivo} expansion\(^3\). Alternatively, whether tissue-engineered cartilage can repair massive articular cartilage defects effectively, it has been a novel focus in this field.

Bone marrow-derived mesenchymal stem cells (BMSCs) have the potential for chondrogenic differentiation, self-renewal, and proliferation with less loss of phenotype than chondrocytes, and have been considered as an ideal cell source for tissue-engineered cartilage formation\(^4\). Previous studies have showed that BMSCs can be induced into hyaline-like cartilage tissue with high expression of aggrecan (ACAN), collagen type II (COL2), and SOX9 in high-density pellets or scaffold \textit{in vitro} culture\(^5,6\). However, the extremely low percentage of BMSCs in the bone marrow means that \textit{in vitro} expansion is the first step to obtain enough cell numbers before chondrogenic differentiation. Besides, the suitable medium containing growth factors is also an essential factor for tissue engineering. So far, the most widely applied media to date are almost commercially synthesized reagents, which have shown excellent chondrogenic-differentiation capacity in tissue-engineered cartilage formation\(^6,8\). While these media contain kinds of ectogenic growth factors and cost expensive, whether some autologous biomaterials with similar induction effects can be an alternative to the synthetic reagents, it is still worthy of further study.

Platelet-rich plasma (PRP) is the plasma separated from autologous whole blood, with a high platelet proportion. After activation, PRP releases large amounts of growth factors, including transforming growth factor-beta (TGF-\(\beta\)), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF), which can enhance tissue repair and regeneration. Clinically, PRP has been shown to inhibit inflammation and induce cell proliferation and differentiation, resulting in pain relief and functional improvement in some musculoskeletal regenerated diseases\(^9,10\). Meanwhile, PRP has also demonstrated effects on the proliferation of chondrocytes and mesenchymal stem cells (MSCs)\(^12,13\), and induced chondrogenic differentiation of MSCs \textit{in vitro} culture\(^16,17\). However, are there any differences of chondrogenic differentiation between autologous PRP and commercially synthesized medium, or whether PRP may replace these medium or not, limited articles have been reported.

The current study aimed to evaluate the effects of different PRP concentrations on the proliferation and chondrogenic differentiation of BMSCs cultured in 3D pellets \textit{in vitro}. We also determined if PRP alone was sufficient to induce the chondrogenesis of BMSCs compared with traditional chondrogenic differentiation medium.

2 Materials And Methods
2.1 Experimental animals

The animal experiments were approved by the Yangzhou University Medical School Animal Ethics Committee (YZMC/IACUC 2019101801, Yangzhou, China).

Six mature rabbits weighting 1.5–2kg (Qinglong Mountain Experimental Animal Center, Nanjing, China) were used in this study. The animal experiments were approved by the Yangzhou University Medical School Animal Ethics Committee (YZMC/IACUC 2019101801, Yangzhou, China).

2.2 PRP preparation

Under anesthetized condition, 50 ml of whole blood was aspirated via heart puncture from each rabbit using 10-ml vacutainer tubes containing 1 ml sodium citrate (Nigale, Chengdu, China). PRP was prepared by a two-step method in room temperature according to our previous report\textsuperscript{19}. Briefly, the first centrifugation was performed at 250 $\times$ $g$ for 7 minutes, then the upper plasma layer and middle buffy coat were gathered into another sterile coring tube to the second centrifugation at 350 $\times$ $g$ for 10 min. About 2 ml of sediment and plasma were collected from the bottom to obtain PRP. All the products were activated with one tenth volume of 10% CaCl\textsubscript{2} and incubated overnight at 37°C, then centrifugated at 1000 $\times$ $g$ for 10 minutes to collect the supernatant to get activated PRP. The activated PRP samples were divided stored at $-70^\circ$C and used within one month for subsequent experiments.

2.3 Assay of growth factors and cytokines

One milliliter of activated PRP and plasma of whole blood from each animal were used for the assay of growth factors and cytokines. The levels of TGF-β3, PDGF, IGF-1, and VEGF in samples were detected by enzyme-linked immunosorbent assay kits (Cusabio, Wuhan, China), according to the manufacture’s instruction.

2.4 Isolation and characterization of BMSCs

About 5 ml bone marrow samples were obtained from rabbit bilateral iliac crests and washed twice with high glucose Dulbecco’s modified Eagle's medium (HG-DMEM, Gibco, Waltham, MA, USA), then resuspended in HG-DMEN with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After 5 days of culture in an incubator with 5% CO\textsubscript{2} at 37°C, non-adherent cells were removed, then the medium was changed. Remaining adherent cells were further cultured until 80% confluence, then passaged at a ratio of 1:3 in new flasks. Culture medium was exchanged twice weekly. The phenotype of the passage 3 (P3) cells was characterized by detecting surface expression of CD34-phycoerythrin (PE) (GTX75414), CD45-PE (GTX01462-08), CD44-fluorescein isothiocyanate (FITC) (GTX76381), and CD105-FITC (GTX11415) (Genetex, CA, USA) by flow cytometry, according to the manufacturer's instructions.

2.5 Cell proliferation assay

To observe Cell proliferation in different medium, P3 BMSCs were enzymatically harvested and continued to culture in HG-DMEM + 10% FBS (v/v), HG-DMEM + 5% PRP (v/v), HG-DMEM + 10% PRP, and HG-DMEM
+ 15% PRP, respectively. Cells in 6-well culture plates containing $5 \times 10^3$ cells/100 µl per well were assigned to each group, and the medium were changed every 3 days. Cell proliferation was detected using a cell counting kit-8 (CCK8; Abcam, Cambridge, MA, USA) on days 1, 3, 5, and 7, as described previously$^{20}$.

### 2.6 BMSC pellets culture

Chondrogenesis was evaluated using high-density pellet cultures. About $5 \times 10^5$ P3 BMSCs were pelleted by centrifugation at $150 \times g$ for 5 minutes, and then cultured in 4 different groups as follows: control (Ctrl), commercial chondrogenic medium (Cyagen, Guangzhou, China); 5% PRP (HG-DMEM + 5% PRP (v/v)); 10% PRP (HG-DMEM + 10% PRP); and 15% PRP (HG-DMEM + 15% PRP). Pellets were cultured for 21 days consecutively and the media were changed three times a week. Eight duplicates were assigned for each group.

### 2.7 Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The pellets were harvested at day 21 and 2 samples from each group were homogenized, then total RNA extraction with the RNAiso Plus Kit (Takara, CA, USA). Complementary DNA (cDNA) was synthesized from RNA using the PrimeScript™ RT reagent Kit (Takara, CA, USA). Gene expression levels of aggrecan (ACAN), Collagen type II (COL2A1) and SOX-9 were detected by RT-PCR, as described previously$^{21}$. The primer sequences were designed and synthesized by General Biol (Hefei, Anhui, China) (Table 1). The products of RT-PCR were quantitatively analyzed by the comparative CT method ($\Delta \Delta CT$) and data was presented as target gene expression normalized to GAPDH.

| Gene   | Primer                  | Product (bp) |
|--------|-------------------------|--------------|
| ACAN   | F- AACAGCCCAAGAAGCAGAA  | 103          |
|        | R- TGGGTCCAGAAATCCAGAATG|              |
| COL2A1 | F-CAAGTCCCTCAACAACCAGAT | 124          |
|        | R-TATCCAGTAGTCACCGCTCTT |              |
| SOX9   | F- GGAGGAAGTGGTGAAGAATG | 91           |
|        | R- TGCAGCGCCTTGAAAGAT  |              |
| GAPDH  | F- AATCCACTGCCGTCTTCAC  | 117          |
|        | R- TCACGCCCATCAAAACA   |              |

F, forward; R, reverse.

### 2.8 Morphological observation and histological examination
At the end of 3-week chondrogenic differentiation culture, followed by gross observation, all the pellets from 4 groups were harvested and fixed in neutral-buffered formalin overnight at 4°C, embedded in paraffin, and 5-µm sections were cut and stained with safranin-O and Alcian Blue to evaluate the production of extracellular matrix (ECM).

2.9 Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS 18.0 (SPSS, IBM Software Inc., Armonk, NY, USA). Independent-samples t-tests were used to evaluate the differences of growth factor levels in plasma and PRP. One-way analysis of variance (ANOVA) was used in cell proliferation and gene expression levels assays, followed by the LSD test to assess multiple comparisons. \( P < 0.05 \) was considered statistically significant.

3 Results

3.1 Growth factor levels

The concentrations of TGF-β3, PDGF, IGF-1, and VEGF were significantly increased in activated PRP compared with plasma (\( P < 0.05 \); Fig. 1): TGF-β3: 30.49 ± 2.22 vs. 6.62 ± 1.76 ng/ml; PDGF: 1134.00 ± 27.26 vs. 371.42 ± 56.24 pg/ml; IGF-1: 5.24 ± 0.42 vs. 2.72 ± 0.32 ng/ml; and VEGF: 76.22 ± 6.23 vs. 28.10 ± 6.05 pg/ml, respectively.

3.2 Characteristics of isolated BMSCs

After isolation and culture in vitro, P3 cell populations were characterized by flow cytometry to determine the expression of MSC-associated surface markers. The percentages of CD105(+)/CD34(−) and CD44(+)/CD45(−) cells were 96.5% and 92.9%, respectively (Fig. 2).

3.3 BMSC proliferation in PRP

The results of BMSC proliferation assays in 10% FBS and 5%, 10%, and 15% PRP groups after 1, 3, 5, and 7 days are shown in Fig. 3. Although proliferation increasing with time was observed in all the groups, the effect was significantly weaker in the 5% PRP compared with the 10% FBS group (\( P < 0.05 \)). Cell proliferation was enhanced with the increasing PRP concentration, stronger effects revealed in the 10% and 15% PRP groups compared with the 10% FBS group from day 5 (\( P < 0.05 \)). Furthermore, proliferation assay appeared to be more obvious in 10% PRP group than 15% PRP group from day 5, though no significant difference was observed.
3.4 Expression of cartilage-specific genes

Gene expression levels of ACAN, COL2A1, and SOX9 in cell pellets under the four different chondrogenic differentiation conditions after 21 days of culture are shown in Fig. 4. ACAN and SOX9 expression levels were significantly higher in 10% PRP group than 5% and 15% groups, and COL2A1 expression was increased in 10% and 15% PRP groups compared with 5% PRP group ($P < 0.05$). However, the expression levels of these cartilage-specific genes were lower in PRP-treated groups compared with Ctrl group ($P < 0.05$).

3.5 Gross morphology and histological examination

After 3-week *in vitro* culture, all the cell pellets revealed spheroids appearance, but with different sizes (Fig. 5). Generally, the pellets in Ctrl group revealed larger volume than that in PRP-treated groups. However, the pellets sizes did not increase in line with PRP concentration. The diameter of pellets cultured in 15% PRP was similar to 5% PRP, pellets exposed to 10% PRP were obviously larger than those in 5% and 15% PRP groups, but still less than the Ctrl.

Contents of cartilage-specific ECM, including ACAN and COL2, were staining with Safranin O, Alcian Blue, and immunohistochemistry (Fig. 6). More abundant ACAN and COL2 deposition was observed in Ctrl group than PRP-treated groups, pellets exposed to 10% PRP showed stronger ACAN and COL2 staining compared with the other 2 PRP groups, but still inferior to the Ctrl.

4 Discussion

PRP is an autologous blood products containing a variety of bioactive components with anti-inflammatory and tissue repair- and regeneration-promoting functions, which has been widely used in the treatment of chronic degenerative diseases$^{9-11}$. *In vitro* experiments have confirmed the ability of PRP to enhance the proliferation of chondrocytes and MSCs$^{12-15}$. However, the effect of PRP on cartilage differentiation of BMSCs in tissue engineering remains controversial. Although recent studies$^{17,18,22}$ demonstrated the effects of different concentrations of PRP on chondrogenic differentiation of MSCs, they did not compare them with traditional commercial cartilage induction medium. The present study, therefore, aimed to compare the effects on cartilage differentiation of BMSCs between different PRP concentrations and commercial cartilage induction medium, and to evaluate the role of PRP in tissue-engineered cartilage formation.

In this study, concentrations of TGF-$\beta$3, IGF-1, PDGF, and VEGF were significantly increased in activated PRP compared with plasma. The addition of high concentrations of PRP to basic medium promoted the expansion of BMSCs *in vitro* compared with 10% FBS, revealing a concentration-time dependent effect. Higher growth factors and cytokines concentrations in PRP may induce this result. In addition, we also found that 10% PRP enhanced chondrogenic differentiation in cultured BMSC pellets, but still inferior to traditional commercial cartilage induction medium.
Commercial MSC chondrogenic differentiation media from different manufacturers have been widely used in chondrogenic differentiation experiments. These synthetic reagents contain not only basic cell culture medium, but also additional ingredients, such as TGF-β3, insulin, transferrin and selenium supplement, ascorbate, sodium pyruvate, proline, and dexamethasone. Among these, TGF-β3 is a vital regulatory factor promoting chondrogenic differentiation of MSCs, while the other components also improve cell metabolism, promote cell proliferation, and inhibit cell aging. The concentration of TGF-β3 in the cartilage differentiation medium used in this study was 10 ng/ml, which was higher than that in 10% PRP (3.05 ng/ml), while PRP itself may not contain any other additives. We therefore considered that basic cell culture medium with 10% PRP might have a limited effect on BMSC cartilage differentiation compared with commercial cartilage differentiation medium.

Whether the increased concentration of PRP in medium would enhance chondrogenic differentiation in BMSCs? Krüger et al. have reported that human PRP enhanced the migration and stimulated the chondrogenic differentiation of human BMSCs derived from spongy bone of the tibia or femur head. Liou et al. investigated the effects of different concentrations of PRP on adipose-derived stem cells and found that increasing the PRP concentration did not enhance chondrogenic differentiation. Amaral et al. also observed cartilage differentiation medium containing different PRP concentrations on chondrogenesis of BMSC pellets and showed that increasing the PRP proportion from 1–10% reduced the expression levels of cartilage-specific genes and proteoglycans in pellets. In addition, some previous studies reported that commercial induction medium containing 10% PRP did not enhance chondrogenic differentiation compared with induction medium alone. The similar results were also observed in this study. Though certain PRP concentration induced chondrogenic differentiation in BMSCs pellets, this effect was not enhanced with the increasing of PRP contents.

In this study, we observed that 10% PRP promoted the chondrogenic differentiation of BMSCs pellets by analysis of gene expression levels, gross morphological observation, and pathological staining of cartilage-specific proteins. We supposed that the biological mechanism of PRP in chondrogenic differentiation of MSCs may be complicated and Intricate. Furthermore, whether MSC derived from different sources respond consistently to PRP in induction chondrogenesis, further studies are deserved.

Activated-PRP includes cytokines such as TGFβ, PDGF, VEGF, and epidermal growth factor (EGF), but the beneficial effects of these growth factors in chondrogenesis remain unclear. The TGFβ family is known to induce proliferation and chondrogenesis of BMSCs during cartilage formation, meanwhile, PDGF supports chondrocytes to maintain the hyaline-like chondrogenic phenotype and induces proteoglycan synthesis. However, VEGF showed poor chondrogenic effects on muscle-derived stem cells in a rat model, and an EGF receptor ligand promoted chondrocyte catabolic activity and inhibited anabolic activity in an osteoarthritis mouse model. VEGF and/or EGF may therefore weaken the chondro-inductive effects of PRP. Further studies are needed to determine if specific depletion of such anti-chondrogenic factors and optimization of other stimulatory components in PRP might enhance BMSC chondrogenic differentiation.
In addition to optimizing the differentiation induction medium, it is also necessary to improve the seed cells to facilitate the construction of tissue-engineered cartilage. Some researchers have constructed a co-culture system composed of autologous chondrocytes and MSCs in order to observe the efficiency of cartilage differentiation and to inhibit chondrocyte hypertrophy and osteogenic differentiation by the paracrine effect of different cells\textsuperscript{26,27}. Although the results were not consistent, they provided a novel research direction for the construction of tissue-engineered cartilage. Recent studies has reported that human placental\textsuperscript{28}, umbilical cord blood\textsuperscript{29}, and amnion-derived MSCs\textsuperscript{30} all reveal high proliferative ability, multipotency, and low immunogenicity, and are thus becoming novel seed cells in tissue engineering. Whether PRP could enhance chondrogenic differentiation in these stem cells or not, further studies are deserved.

There are some limitations in this study. Firstly, we only observed the end-stage chondrogenic differentiation of BMSCs after 21 days of culture, and the continuous effect of PRP were not examined. Secondly, limited number of PRP-treated groups were designed, the details of concentration-dependent effect of PRP on chondrogenesis were limited. Additionally, due to commercial chondrogenic differentiation media from different manufacturers may contain various components and efficacy, the reagents used in this experiment are of limited representativeness.

5 Conclusion

Activated-PRP contains abundant growth factors and cytokines, which can enhance BMSC proliferation \textit{in vitro} and induce chondrogenic differentiation in 3D-pellet culture. However, the effects of PRP alone used were limited compared with traditional commercial MSC chondrogenic differentiation media. Future studies aimed at optimizing and modifying the composition and concentration of PRP may have satisfying results in tissue-engineered cartilage formation.

Abbreviations List

PRP: Platelet-rich plasma

BMSCs: Bone marrow-derived mesenchymal stem cells

HG-DMEM: High glucose Dulbecco's modified Eagle's medium

ACAN: Aggrecan

COL2A1: Collagen type II Alpha 1

SOX9: SRY-Box Transcription Factor 9

TGF-β: Transforming growth factor-beta

PDGF: Platelet-derived growth factor
Declarations

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Ethics declaration

The animal experiments were approved by the Yangzhou University Medical School Animal Ethics Committee (YZMC/IACUC 2019101801, Yangzhou, China).

Consent for publication
Not applicable.

**Competing interests**

All the authors declare that they have no competing interests. Co-authors, their families and institutions did not receive any financial payments or benefits from any commercial entities related to the subject of this article.

**Availability of data and material**

According to the contract requirement of Military Medical Scientific Research Foundation of China, the data in this project will not be shared.

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Figures
Figure 1

Growth factor levels in plasma and activated PRP. Concentrations of TGF β3, PDGF, IGF-1, and VEGF were significantly increased in PRP compared with plasma (*P < 0.05, n = 6).
Figure 2

Surface marker profiles of isolated and cultured BMSCs indicated high expression of CD105(+)/CD34(−) (A) and CD44(+)/CD45(−) cells (B).

![Surface marker profiles](image)

Figure 3

Cell counting kit-8 for BMSCs proliferation assays in the different media. *P < 0.05 compared with 10% FBS, +P<0.05 compared with 5% PRP. OD, optical density; FBS, fetal bovine serum; PRP, platelet-rich plasma.
**Figure 4**

Expression of cartilage-specific genes in the four groups following 21-day culture. Ctrl, commercial chondrogenic differentiation medium. *P < 0.05 compared with Ctrl, +P < 0.05 compared with 5% PRP, #P < 0.05 compared with 15% PRP.

**Figure 5**

Gross morphology of BMSC pellets in four different media after 3-week culture in vitro.
Figure 6

Histological evaluation of aggrecan (Safranin O and Alcian Blue staining) and Collagen type II (immunohistochemical staining) deposition in BMSC pellets from four different groups after 3-week culture in vitro. Scale bar = 500 µm.