# Original article

## Preliminary separation of the growth factors in platelet-rich plasma: effects on the proliferation of human marrow-derived mesenchymal stem cells

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***Keywords:*** *platelet-rich plasma; gel chromatography; mesenchymal stem cell*

**Background** Platelet-rich plasma (PRP) as a storage vehicle of growth factors has been successfully used in clinical applications, but in most cases the platelets were autologous. However, the large volume of blood withdrawn has detrimental effects on patients with anemia or poor general health. To overcome these limitations, this study was designed to separate the growth factors in homologous platelet-rich plasma.

**Methods** The gel chromatography with Superdex-75 column was applied to separate PRP supernatants into 4 major fractions. Then the four fractions were vacuumed freeze-dried and re-dissolved in phosphate buffered saline. Proteins concentrations in PRP and in four fractions were detected by bicinchoninic acid protein assay; platelet derived growth factor-AB (PDGF-AB) and transforming growth factor β1 (TGF-β1) levels were determined by sandwich enzyme-linked immunosorbent assays. The effects of fractions on the proliferation of human marrow-derived mesenchymal stem cells (MSCs) were determined by 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

**Results** PRP supernatants were separated into four major fractions by gel chromatography. The proteins recovery was 96.72%. Of the four fractions, fraction B contained the highest TGF-β1 and PDGF-AB levels, and the highest proteins concentrations. Cell proliferation curves of MSC demonstrated that fraction B and C induced a remarkable increase of MTT values compared to the untreated culture ($P$ <0.05), and the effects of fraction B and C showed no significant difference compared to the PRP group ($P$ >0.05). Fraction A and D showed no significant difference to the negative control group ($P$ >0.05).

**Conclusions** The growth factors in PRP supernatants could be preliminarily separated into four fractions by gel chromatography, and the freeze-drying fractions retained the biological activity of growth factors. The growth factors were mostly presented in fraction B and C, and they promoted cell proliferation effectively.

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Wound healing is a complex process, involving a mechanism of complex cascading regulatory events at both the molecular and cellular levels.[1,2] Growth factors (GFs) are secreted by a wide variety of cells to regulate the wound healing process in an orderly manner.[3,4] Over the last decade, various GFs, including platelet-derived growth factor (PDGF), and transforming growth factor-beta (TGF-β), have been used to accelerate the healing process.[5-9]

Platelet-rich plasma (PRP), as a storage vehicle of growth factors, is a new application of tissue engineering which was considered for the application of growth factors. PRP is a concentration of platelets in plasma developed by gradient density centrifugation.[10] It contains many growth factors, such as PDGF, TGF-β, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), etc.[11,12] And it has been successfully used in a variety of clinical applications for improving hard and soft tissue healing.[13-17]

PRP has been successfully used to improve clinical outcomes, but in most cases the platelets were autologous, and thus, patient blood had to be drawn and processed to yield the platelet products required. Moreover, the large volume of blood withdrawn has detrimental effects on patients with anemia or poor general heath. In addition, PRP can not be stored for long periods of time. To overcome these limitations, some authors have tried homologous PRP to treat chronic wounds and have concluded that they are as effective as autologous equivalents.[18,19] But that brought out new problems about pathogen contamination and immunological rejection. Therefore we hypothesized that if we could separate the GFs fractions in homologous platelet-rich plasma, and reduce the immunogenicity and other defects, then make the GFs fractions product using the vacuumed freeze-drying technique, this should benefit the clinical application of homologous PRP.

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The purpose of this study was to preliminarily separate the GFs fraction in homologous PRP, and to observe the effects of fractions on proliferation of human marrow-derived mesenchymal stem cells (MSCs).

## METHODS

### Collection and preparation of PRP

PRP was obtained from the venous blood of 5 healthy volunteers from the Central Blood Bank, Guangzhou, China. At the Blood Bank, PRP was collected from each donor by plateletpheresis with the automated instruments (CS-3000plus, Baxter Biotech, USA) at a ratio of 10:1 volume of blood to anticoagulant citrate dextrose A (ACD-A, Red Cross Blood Center, Guangzhou, China). Approximately, 260 ml PRP was obtained from each donor. The platelets present in the whole blood and in PRP were also counted by an automated hematology analyzer (Nikon Kohden, Japan). The PRP was activated by bovine thrombin and calcium chloride at a ratio of 10 ml PRP to 1000 IU bovine thrombin (Sigma, USA) and 1 ml 10% calcium chloride solution. After incubated at room temperature for 1 hour for platelets to release enough GFs, the PRP gel was centrifuged at 4°C for 10 minutes at 3000 *g* and the supernatants were harvested. Finally, 15–17 ml of activated PRP supernatants was obtained from 20 ml PRP. Five-milliliter sample of supernatants was immediately stored at –80°C until further experiment.

### Gel filtration

Superdex-75 pregrade (GE Healthcare, USA) gel slurry was packed into a 2.6 cm in diameter, 100 cm high column. The gel column was equilibrated previously with 25 mmol/L phosphate buffered saline (PBS) and 0.1 mol/L NaCl buffer. Each time 10 ml of activated PRP supernatants were loaded into the column. The proteins or proteins complex were separated in the column according to their molecular weight by 0.01 mol/L PBS with the linear velocity of 0.5 cm/min. Thirteen tubes of liquid were collected after gel filtration and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). According to the result of gel filtration, 13 tubes of liquid were merged into four fractions coded as A, B, C, and D. Then the four fractions were immediately freeze-dried at –4°C for more than 16 hours with HETO drier (Denmark). Before the next experiment, the freeze-drying fractions were re-dissolved with 2.5 ml of 0.01 mol/L PBS. Proteins concentrations of PRP gel supernatants and 0.1 ml sample of four fractions were determined by bicinchoninic acid (BCA) protein assay kits (Pierce, USA).

### Measurement of PDGF-AB and TGF-β1 levels

A quantitative sandwich enzyme-linked immunosorbent assays (ELISA) (Quantikine ELISA kits, R&D Systems, USA) was used for examining the amount of TGF-β1 and PDGF-AB in PRP supernatants and in the four fractions. The immunoassays were performed following the manufacturer′s instructions. Triplicate measurements were performed for all assays.

### Isolation and cultures of human MSCs

Human marrow-derived MSCs were isolated and cultured using the method described by Lucarelli et al.[20] A total of 8 ml bone marrow aspirates was collected from the iliac crest of 3 Asian adult male donors who had provided informed consent. Nucleated cells were isolated with a density gradient and resuspended in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) containing 10% fetal calf serum (FCS, Hyclone, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L glutamine (Gibco BRL, USA). The nucleated cells were plated in three 25 $cm^2$ culture flasks and incubated in a humidified atmosphere of 95% air, 5% $CO_2$ at 37°C. Non-adherent cells were discarded after 2 days and adherent cells were cultured for further expansion. When cultured cells became near confluent, cells were detached by mild trypsinization (Gibco-BRL) and reseeded onto new plates at 1/3 density for continued passage. The medium was changed every 2 or 3 days.

### Cell proliferation assays

3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used for the quantitative determination of cellular proliferation. The confluent second-passage cells were trypsinized and re-suspended. The cells were counted and seeded at a density of 2000 cells per well in 96-well plates. The cells were cultured for 24 hours. After the 24 hours adhesion period, the medium was removed and replaced with DMEM in combination of 10% FCS (negative control), 10% PRP supernatants (positive control) and 10% four fractions respectively. Medium was replaced every 3 days. MTT was assayed at day 2, 3, 4, 5 and 6 after treating to establish a growth curve of cells cultivated. The cells were incubated with 5 mg/ml of MTT in the last 4 hours of culture period tested. The medium was removed and formazan salts were dissolved with 150 μl of dimethylsulphoxide, and the absorbance was determined at 570 nm/630 nm with an ELISA reader. Each experiment was repeated 3 times with 5 samples for each group.

### Statistical analysis

All of the results were expressed as mean±standard deviation (SD). A comparative study of means was performed using the analysis of variance (ANOVA). The results were considered significantly different when $P<0.05$.

## RESULTS

### Platelet concentrations of whole blood and PRP

The platelet counts in the whole blood had a mean value of $(188.75\pm32.74)\times10^9$/L, and platelet counts in PRP had a mean value of $(1240.01\pm159.72)\times10^9$/L. The mean platelet density increased by 656.96% when compared to the whole blood.

**Gel filtration**

PRP supernatants were separated into 3 major peaks and collected as 4 fractions by gel filtration (Figure 1). Thirteen tubes of liquid were collected and merged into four fractions. Tube 1–2: merged as fraction A; Tube 3–4: merged as fraction B; Tube 5–9: merged as fraction C; Tube 10–13: merged as fraction D. We split the first peak into two fractions A and B for detailed analysis, because it contains high molecular weight proteins, which may serve as protein carriers in growth factor binding. The second and third peaks were named as fraction C and fraction D respectively, which contained medium and low molecular weight proteins.

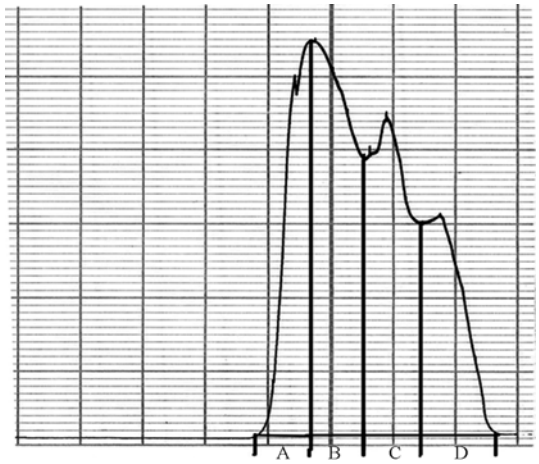


**Figure 1.** Representative gel filtration chromatogram. Ten milliliters of platelet-rich plasma supernatants applied to a Superdex-75 column. Linear flow rate: 0.5 cm/min. UV detection: 280 nm.

Thirteen tubes of samples were analyzed by native SDS-PAGE (Figure 2). SDS-PAGE results showed high molecular weight proteins were presented in tube 1–4 (fraction A and B); tube 3 and 4 (fraction B) contained the most high molecular weight proteins, however, these tubes still contained the low molecular weight proteins. Albumin protein (66 kD) was mainly distributed in fraction 5–11. High molecular weight and low molecular weight proteins were presented in all tubes and were not separated completely.

**Amounts of total proteins and growth factors in different fractions**

Growth factors levels and proteins concentrations in PRP and in different fractions were shown in Table. In four fractions, B contained the highest TGF-β1 and PDGF-AB levels as (5205.71±1219.25) pg/ml and (29214.58±8765.74) pg/ml, also with the highest proteins concentrations as 81.43 mg/ml. TGF-β1 and PDGF-AB were mainly collected in fraction B. Proteins concentrations in fraction C was second higher as (58.60±18.07)mg/ml, but TGF-β1 level was lower than fraction B ($P$ <0.01) and A ($P$ <0.05). PDGF-AB concentrations in fraction A, B and D showed no significant difference ($P$ >0.05). TGF-β1 was not detected in fraction D. The proteins, TGF-β1 and PDGF-AB recovery was 96.72%, 19.70% and 16.00%

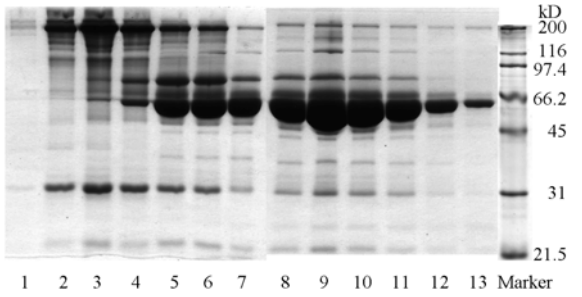


**Figure 2.** Native SDS-PAGE analysis of 13 tubes from four peaks in chromatography collection. Lane 1–13: Tube 1–13 of samples.

**Table.** Growth factors and proteins concentration in each fraction ($n$=5)

| Groups | TGF-β1 (pg/ml) | PDGF-AB (pg/ml) | Proteins (mg/ml) |
|---|---|---|---|
| A | 835.80±168.76 | 8435.25±2710.85 | 18.74±4.43 |
| B | 5205.71±1219.25 | 29214.58±8765.74 | 81.43±19.61 |
| C | 474.44±85.16 | 9623.91±2553.51 | 58.60±18.07 |
| D | N/A | 8064.59±2092.38 | 25.34±7.76 |
| PRP | 8269.67±1742.61 | 86449.48±23834.94 | 47.59±9.99 |

respectively. Recovery=(concentration of A+B+C+D) ×2.5 ml/(concentration of PRP×10 ml)×100%

**Cell proliferation**

Cell proliferation was sensitive to PRP supernatants, fraction B and fraction C. They all induced a remarkable increase of MTT values compared to the untreated culture ($P$ <0.05), and the effects of fraction B and C on cell proliferation showed no significant difference compared to the PRP group ($P$ >0.05) (Figure 3). Fraction A promoted cell proliferation with the lower proliferation effects in duration of 4 to 6 days, but there was no significant difference to the negative control group ($P$ >0.05). Fraction D demonstrated no cell proliferation function.

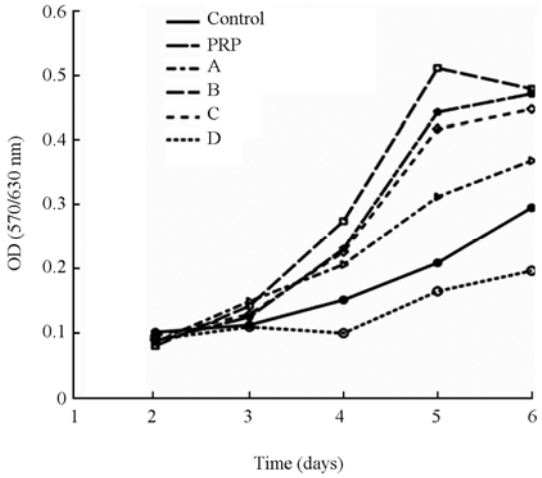


**Figure 3.** Cell proliferation curves of MSC cultured in PRP and each fraction. The result represents the mean of triplicate cultures of one representative experiment of 5 performed.

## DISCUSSION

In the present study, the PRP was obtained from the

Blood Bank by plateletpheresis procedure. The normal plateletpheresis procedure collected above $2.5\times10^{11}$ platelets per bag containing a total of less than $5\times10^{6}$ white blood cells (Quality control of component blood transfusion in China). The platelet density in the Blood Bank PRP was $(1240.01\pm159.72)\times10^{9}$ platelets/L and met requirement of working PRP presented by Marx ,[21] which states that PRP should be more than $1000\times10^{9}$ platelets/L. The PDGF and TGF-β1 levels in it were also similar to previous study.[10,20] Moreover, the Blood Bank PRP is a reliable source of homologous PRP and obviates the time and effort required to locate a suitable donor.

Gel filtration chromatography separates proteins, peptides, and oligonucleotides on the basis of molecular size. Molecules move through a bed of porous beads, diffusing into the beads to greater or lesser degrees. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter less or not at all and thus move through the bed more quickly. Therefore, proteins components in a mixture can be separated according to their different molecular weight. This method was convenient to operate with high reproducibility and provide a mild environment to protect the activity of proteins or peptides.

PRP releases many GFs at high concentration after activation with thrombin and calcium.[11,22,23] The major GFs in PRP include PDGF, TGF-β1, VEGF, EGF and IGF with the molecular weight of 28–35, 25, 34–45, 6, 7.5 kD respectively. So far, we selected Superdex-75 as matrix with the exclusive molecular weight ranged from 3 kD to 70 kD. The four fractions were separated from PRP gel supernatants by gel filtration. The proteins recovery was 96.72%. SDS-PAGE results showed high molecular weight proteins contained the low molecular weight proteins. It was suggested that low molecular weight proteins may bind to the high molecular weight proteins via protein interaction.

Although PRP contains many growth factors functioned as active mitogens in cell proliferation,[24] PDGF and TGF-β1 are the majority and the most important factors in PRP, so many reports focus their attention on PDGF, TGF-β1, and IGF[25-28] in particular. Study of Ogino et al[29] demonstrated PDGF and TGF-β1—not IGF-I contributed to the proliferation of osteoblast-like cells significantly. Thus, we selectively detected the concentration of PDGF-AB and TGF-β1 in four fractions and in PRP by ELISA. In the present study, fraction B contained the highest concentration of TGF-β1 and PDGF-AB, also the highest proteins concentrations. Fraction D contained the lowest PDGF-AB and no TGF-β1 was detected in it. Although the proteins recovery is 96.72%, the PDGF-AB and TGF-β1 recovery is 16.00% and 19.70% respectively. This result may be caused by the absence of additives in vacuumed freeze-drying to protect the proteins, which then lead to GFs degradation.

MSCs can be stimulated to differentiate toward lineages of the mesenchymal tissue, including bone, cartilage, fat, muscle, and tendon.[30] Thus, MSCs are employed widely in tissue engineering. In the present study, the effects of the four fractions on the growth of human MSCs were also determined. Although the concentration of PDGF-AB and TGF-β1 in fraction B was lower than PRP supernatants, especially the PDGF-AB concentration was 33.79% of that in PRP, it was a surprise to see the effects of fraction B on the growth of MSCs similar to PRP; so we hypothesized that PDGF and TGF-β1 affected the growth of MSCs in a concentration saturated pattern, which means in a certain high concentration shred GFs showed no increased stimulation of cell proliferation. In addition, there was an interesting phenomenon, the PDGF-AB concentrations in fraction C was not significant compared to fraction A, and the TGF-β1 concentrations was lower than fraction A, but the effects on MSC growth were that fraction C was significant compared to fraction A. So we suggested some low molecular weight GFs presented in fraction C and may have played an important role in HMSC growth, it accelerated MSCs growth and collaborated with PDGF and TGF-β1. Further studies are needed to identify components of fraction C. Moreover, compared with its high PDGF-AB amounts, fraction D showed a lower proliferation curve compared with the negative control, thus we hypothesized that GFs in fraction D may have lost biological activity or only fragments of GFs were recognized by ELISA.

In conclusion, the GFs in PRP supernatants can be preliminarily separated by gel filtration, and the freeze-drying fractions retain the biological activity of GFs. The GFs mostly are presented in fraction B and C, and they promoted cell proliferation effectively. Theoretically, removing large molecular weight proteins can reduce immunogenicity in fraction B and C, but this needs further study for identification. Our pilot research suggested that PRP supernatants were successfully separated and retained the most of effective GFs in fractions. This provided the fundamental data to develop the GFs product from homologous PRP. The further study should focus on the composition of fraction B and C, especially detection of other growth factors in B and C.

## REFERENCES

1. Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. Cardiovasc Res 2001; 49: 507-521.
2. Koveker GB. Growth factors in clinical practice. Int J Clin Pract 2000; 54: 590-593.
3. Murakami S, Takayama S, Ikezawa K, Shimabukuro Y, Kitamura M, Nozaki T, et al. Regeneration of periodontal tissues by basic fibroblast growth factor. J Periodontol Res 1999; 34: 425-430.
4. Murakami S, Takayama S, Ikezawa K, Shimabukuro Y, Kitamura M, Nozaki T. Bone growth factors. Orthop Clin North Am 2000; 31: 375-388.

5. Stefani CM, Machado MA, Sallum EA, Sallum AW, Toledo S, Nociti FH Jr. Platelet-derived growth factor/insulin-like growth factor-1 combination and bone regeneration around implants placed into extraction sockets: a histometric study in dogs. Implant Dent 2000; 9: 126-31.
6. Lind M, Overgaard S, Glerup H, Soballe K, Bunger C. Transforming growth factor-beta1 adsorbed to tricalciumphosphate coated implants increases peri-implant bone remodeling. Biomaterials 2001; 22: 189-193.
7. Vuola J, Bohling T, Goransson H, Puolakkainen P. Transforming growth factor beta released from natural coral implant enhances bone growth at calvarium of mature rat. J Biomed Mater Res 2002; 59: 152-159.
8. Jiang D, Dziak R, Lynch SE, Stephan EB. Modification of an osteoconductive anorganic bovine bone mineral matrix with growth factors. J Periodontol 1999; 70: 834-839.
9. Sun Y, Zhang W, Ma F, Chen W, Hou S. Evaluation of transforming growth factor beta and bone morphogenetic protein composite on healing of bone defects. Chin Med J 1997; 110: 927-31.
10. Whitman DH, Berry RL, Green DM. Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. J Oral Maxillofac Surg 1997; 55: 1294-1299.
11. Weibrich G, Kleis WK, Hafner G, Hitzler WE. Growth factor levels in platelet-rich plasma and correlations with donor age, sex and platelet count. J Craniomaxillofac Surg 2002; 30: 97-102.
12. Landesberg R, Roy M, Glickman RS. Quantification of growth factor levels using a simplified method of platelet-rich plasma gel preparation. J Oral Maxillofac Surg 2000; 58: 297-300.
13. Ouyang XY, Qiao J. Effect of platelet-rich plasma in the treatment of periodontal intrabony defects in humans. Chin Med J 2006; 119: 1511-1521.
14. Nikolidakis D, van den Dolder J, Wolke JG, Jansen JA. Effect of platelet-rich plasma on the early bone formation around Ca-P-coated and non-coated oral implants in cortical bone. Clin Oral Implants Res 2008; 19: 207-213.
15. Schaaf H, Streckbein P, Lendeckel S, Heidinger K, Görtz B, Bein G, et al. Topical use of platelet-rich plasma to influence bone volume in maxillary augmentation: a prospective randomized trial.Vox Sang 2008; 94: 64-69.
16. Chang T, Liu Q, Marino V, Bartold PM. Attachment of periodontal fibroblasts to barrier membranes coated with platelet-rich plasma. Aust Dent J 2007; 52: 227-233.
17. Kitoh H, Kitakoji T, Tsuchiya H, Katoh M, Ishiguro N. Distraction osteogenesis of the lower extremity in patients with achondroplasia/hypochondroplasia treated with transplantation of culture-expanded bone marrow cells and platelet-rich plasma. J Pediatr Orthop 2007; 27: 629-634.
18. Crovetti G, Martinelli G, Issi M, Barone M, Guizzardi M, Campanati B, et al. Platelet gel for healing cutaneous chronic wounds. Transfus Apher Sci 2004; 30: 145-151.
19. Han SK, Kim DW, Jeong SH, Hong YT, Woo HS, Kim WK, et al. Potential use of blood bank platelet concentrates to accelerate wound healing of diabetic ulcers. Ann Plast Surg 2007; 59: 532-537.
20. Lucarelli E, Beccheroni A, Donati Dl, Sangiorgi L, Cenacchi A, Del Vento AM, et al. Platelet-derived growth factors enhance proliferation of human stromal stem cells. Biomaterials 2003; 24: 3095-3100.
21. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant Dent 2001; 10: 225-228.
22. Marineau I, Lacoste E, Gagnon G. Effects of calcium and thrombin on growth factor release from platelet concentrates: kinetics and regulation of endothelial cell proliferation. Periodontol 2003; 74: 1498-1507.
23. Carlson NE, Roach RB. Platelet-rich plasma: clinical applications in dentistry. J Am Dent Assoc 2002; 133: 1383-1386.
24. Nomi M, Miyake H, Sugita Y, Fujisawa M, Soker S. Role of growth factors and endothelial cells in therapeutic angiogenesis and tissue engineering. Curr Stem Cell Res Ther 2006; 1: 333-343.
25. Weibrich G, Kleis WK, Hafner G. Growth factor levels in the platelet-rich plasma produced by 2 different methods: curasan-type PRP kit versus PCCS PRP system. Int J Oral Maxillofac Implants 2002; 17: 184-190.
26. Zhang CQ, Yuan T, Zeng BF. Experimental study of the effect of platelet-rich plasma on osteogenesis in rabbit. Chin Med J 2004; 117: 1853-1855.
27. Weibrich G, Kleis WK, Buch R, Hitzler WE, Hafner G. The Harvest Smart PRePTM system versus the Friadent-Schutze Platelet-rich plasma kit. Clin Oral Implants Res 2003; 14: 233-239.
28. Weibrich G, Kleis WK, Hafner G, Hitzler WE, Wagner W. Comparison of platelet, leukocyte, and growth factor levels in point-of-care platelet-enriched plasma, prepared using a modified Curasan kit, with preparations received from a local blood bank. Clin Oral Implants Res 2003; 14: 357-362.
29. Ogino Y, Ayukawa Y, Kukita T, Koyano K. The contribution of platelet-derived growth factor, transforming growth factor-beta1, and insulin-like growth factor-I in platelet-rich plasma to the proliferation of osteoblast-like cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006; 101: 724-729.
30. Pittenger MF. Mesenchymal stem cells from adult bone marrow. Methods Mol Biol 2008; 449: 27-44.

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