

In vitro evaluation of freeze-dried bone allografts combined with platelet rich plasma and human bone marrow stromal cells for tissue engineering

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Abstract Freeze-dried bone allograft (FDBA) might be more effective in combination with platelet rich plasma (PRP) and bone marrow stromal cells (BMSC) in accelerating bone healing. The isolation of BMSC through density gradient (pBMSC) is not extensively applicable in clinical practice, because it increases the risk of infection. Alternatively, BMSC can be concentrated by simple centrifugation (wBMSC) directly in the operating room. However, we do not know if wBMSC act in the same way as pBMSC. BMSC from 10 donors were tested whether, in the presence of a combination of FDBA and autologous PRP, the osteogenic differentiation of the cells concentrated by simple centrifugation (wBMSC + FDBA + PRP) was similar to that of pBMSC. Cell-associated alkaline phosphatase, osterix and fibroblast growth factor-2 were higher in wBMSC + FDBA + PRP. In conclusion, the combination of FDBA and PRP had a favouring effect on the differentiation towards osteoblasts and allowed BMSC concentrated by simple centrifugation to differentiate as fast as BMSC purified by density gradient.

1 Introduction

Synthetic or biological substitutes are currently used for the treatment of bone defects and non-unions, and generally when inadequate repair reaction may adversely affect the success of surgery. A fresh graft of autologous cancellous bone is usually considered to be the most effective treatment for large bone defects. However, the clinical application of autologous bone grafts is sometimes limited, but they can be replaced by bone substitutes, e.g. freeze-dried bone allografts (FDBA). It was recently demonstrated that in vivo bone formation was improved by the seeding of bone marrow stromal cells (BMSC) on bovine bone-derived scaffolds before implantation [1].

A further improvement could be made by supplementation with recombinant or autologous growth factors (GFs). Until now, the safety and effectiveness of recombinant GFs for clinical use have not been fully demonstrated. Activated platelet rich plasma (PRP) represents an alternative source of GFs. In fact, platelet α -granules contain GFs favouring bone repair, such as transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF), which are released during activation [2]. Autologous PRP has been successfully used in autogenous mandible bone grafting [3], periodontal defects [4] and mandible reconstructions [5]. However, the efficacy of PRP is controversial: other studies have suggested that its effect is very limited or even non-existent [6–8].

BMSC and osteoblasts are also able to produce osteogenic growth factors by themselves, such as bone mineral proteins, TGF- β , fibroblast growth factor-2 (FGF-2) and others [9]. An osteogenic treatment might also induce the synthesis of GFs by the patient's own cells, so as to support their proliferation and differentiation.

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In orthopaedic and maxillo-facial surgery, bone allografts are frequently used in combination with PRP to improve bone regeneration [10]. It has been shown that bone scaffolds might be more effective in combination with PRP in accelerating the bone healing process [11], particularly if BMSC are also added [12]. However, in a recent case-report no increase in the growth of new bone was observed in humans [13]. Besides, the exact mechanism of action of bone allografts in combination with PRP is unclear.

To improve further the repair-promoting effect, the combination of PRP and bone allograft could be enriched with autologous BMSC. However, the isolation of BMSC through a density gradient solution, which is the method usually employed for *in vitro* and *in vivo* research, is not extensively applicable in clinical practice, because it increases manipulation, affects sterility, and increases the risk of infection. The concentration of bone marrow cells by simple centrifugation without density gradient could be an alternative procedure, to be directly performed in the operating room. However, we do not know if BMSC concentrated by simple centrifugation have a similar osteogenic differentiation potential as BMSC isolated through density gradient.

In this study, we tested whether, in the presence of a combination of PRP and FDBA, the osteogenic differentiation of BMSC concentrated by simple centrifugation was similar to that of cells isolated by density gradient, with special focus on the effects on cell-associated alkaline phosphatase (ALP), calcium deposition, core binding transcription factor (Cbfa1/Runx2), osterix (Osx), ALP and osteocalcin (OC) gene expressions, and FGF-2 production.

2 Materials and methods

The study protocol was approved by the Institutional Ethical Committee on human research and was performed in compliance with human studies according to the Helsinki Declaration of 1975, as revised in 1996. Informed consent was given by all the patients involved.

2.1 Preparation of platelet rich plasma

Whole blood was drawn from 10 patients undergoing high tibial osteotomy for *genu varum* (7 men and 3 women; age 25 to 59), with citrate-phosphate-dextrose (CPD) as anticoagulant (blood/CPD ratio 7:1), was centrifuged at 1000g for 15 min to remove erythrocytes, then at 3000g for 10 min at 20°C to obtain a platelet concentrate. By this separation technique, platelet counts of $1,399 \pm 372 \times 10^3$ platelets/ μl (min–max range $919\text{--}2,033 \times 10^3$ platelets/ μl) were obtained. Autologous thrombin was generated by the addition

of 330 μl calcium gluconate (100 mg/ml) to 10 ml of plasma. Cryoprecipitate was prepared from autologous fresh frozen plasma by freeze–thaw precipitation of proteins and subsequent resuspension in plasma. PRP was obtained through the activation of 16 ml of platelet concentrate with 6 ml of autologous thrombin and 4 ml of calcium gluconate.

2.2 Isolation and culture of human bone marrow stromal cells *in vitro*

Bone marrow samples were obtained from the iliac crest of the same patients until a final volume of 100 ml, and 500 U of heparin in 10 ml of saline solution was employed as anticoagulant. The bone marrow was centrifuged at 3800g for 10 min; 50 ml of buffy coat was harvested and washed again by adding 300 ml of saline solution and centrifuging at 4000g for 5 min to reduce the heparin inhibition on thrombin. Thus, concentrated bone marrow was obtained containing $59.1 \pm 22 \times 10^3$ cells/ μl (min–max range: $28.3\text{--}90.0 \times 10^3$ cells/ μl). The concentrated bone marrow was divided into three aliquots. An aliquot was directly seeded 1×10^6 cells per well in 24-well plates (whole BMSC or wBMSC). The second aliquot (20 ml) before seeding was mixed with 8 ml of cryoprecipitate, 3 cm^3 of FDBA, produced by the Musculoskeletal Tissue Bank of our Institute [14], and 16 ml of PRP (wBMSC + FDBA + PRP). The third aliquot was diluted with phosphate buffered saline (PBS) (1:1 ratio), purified on Ficoll-Hystopaque gradient (Sigma) at 800g for 30 min, washed with PBS and then seeded in a 24-well plate, 1×10^6 cells per well (purified BMSC or pBMSC). All the cultures were incubated at 37°C with a 5% CO_2 humidified atmosphere in a α -modification of Eagle's Medium (α -MEM) (Sigma), supplemented with 10% foetal bovine serum (FBS) (Mascia Brunelli), 100 $\mu\text{g/ml}$ ascorbic acid, 100 units ml^{-1} penicillin (Gibco), 0.1 mg ml^{-1} streptomycin (Gibco) and 2 mM L-glutamine (Sigma). After 4 days, non-adherent cells and cell fragments were removed and the medium was changed with the aforementioned medium supplemented with 10 nM dexamethasone. Osteogenic markers were determined at settled times.

2.3 Cell-associated alkaline phosphatase and calcium deposition

After 25 days, the cells were detached with a solution of 0.5% trypsin–0.2% EDTA (Mascia Brunelli). Two replicates for each culture condition were analysed. After neutralization of trypsin with 10% FBS-added medium, an aliquot of the suspension was stained with trypan blue and the cells were counted in duplicate in a Burkler chamber. The cells in the suspension were lysed with 0.1% Triton-X 100 (Sigma) in PBS, for 10 min at 37°C. Cell-associated

alkaline phosphatase (ALP) activity was determined by *p*-nitrophenyl phosphate reaction (Sigma). After 15 min at 37°C, the product was measured at 405 nm, and cell-associated ALP was expressed as μM *p*-nitrophenol formed/cells.

After 21 days, 10 mM β-glycerophosphate (Sigma) was added to the culture medium. After 17 more days, the cells were fixed for 30 min with glutaraldehyde at 37°C, rinsed with PBS and stained with alizarin red S (Sigma) for 60 min at room temperature. The samples were examined by microscopy to detect the formation of calcium nodules, then the stain was solubilized with a 10% cetylpyridinium chloride solution (p/v) (Aldrich) and the optical density was measured at 570 nm.

2.4 Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene expression was determined after 15 days of culture for *Cbfa1/Runx2*, after 15 and 21 days for *Osx* and *OC*; after 15, 21 and 33 days for ALP. RNA was extracted using RNeasy Mini Kit (Qiagen). RNA concentration and purity in the samples was determined at 260 and 280 nm. Total RNA (1 μg) was reverse transcribed into cDNA using the kit Advantage[®] RT-for-PCR kit (Clontech), for 2 min at 70°C, 60 min at 42°C and 5 min at 95°C. PCR was performed using Taq, MgCl₂, dNTPs and PCR Buffer from Sigma. Primers are described in Table 1. RT-PCR

reactions were performed using iCycler (Bio-Rad) (Table 2). Products were separated by electrophoresis using 2% agarose gel stained with ethidium bromide (0.5 μg/ml). The DNA Ladder (100 base pairs) was run in parallel as a molecular weight marker (New England Biolabs). The pictures of the gel were transferred to the computer by a camcorder, and quantified by dedicated software for the densitometry evaluation of the bands (Quantity One, Biorad Laboratories). The expression level of each target gene was normalized to the housekeeping gene β-actin and the results were expressed as target gene/β-actin ratio.

2.5 Fibroblast growth factor-2 enzyme immunoassay

After 4, 11, 15, 21 and 25 days from the seeding, the conditioned culture media were collected, centrifuged at 3000 r.p.m. for 5 min, aliquoted and stored at -80°C. Negative controls consisted of non-conditioned culture medium, PRP alone, and FDBA alone in culture medium. They were incubated at 37°C; the supernatants were treated as the cell cultures at the same end-points. FGF-2 levels were determined by enzyme immunoassay (Kit DuoSet Human FGF-basic, R&D Systems). All the procedures were performed following the manufacturer’s instructions. A microplate was coated with a monoclonal anti-human FGF-2 antibody (2 μg/ml). Mouse biotinylated anti-human FGF-2 (0.5 μg/ml) was used to detect the presence of

Table 1 Oligonucleotides of 5' and 3' primers

Gene bank sequence	5' primer	3' primer	Position	Product length (bp)
β-actin (NM_001101)	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC	326–1164	838
<i>Cbfa1/Runx2</i> (NM_004348)	ATG GCG GGT AAC GAT GA	GAC AGC GGG GTG GTA G	427–924	497
<i>Osterix</i> (AF477981)	GAG GGG AGA AGG ACT GGA A	AAG ACA GGG GAC GAC AGA G	2370–2852	482
ALP (NM_000478)	ACAAGGTGGTGGGCGGTGA	AGGTGGGCGGCAGACTTTG	1508–1792	464
Osteocalcin (NM_199173)	AGCGAGGTAGTGAAGAGA	AGGGGAAGAGGAAAGAAG	164–414	250

Table 2 RT-PCR protocols

Gene	Initial denaturation	Amplification				Final extension
		Denaturation	Annealing	Extension	Number of cycles	
β-actin	94°C 5 min	94°C 30 s	65°C 45 s	72°C 30 s	30	72°C 10 min
<i>Cbfa1/Runx2</i>	94°C 5 min	94°C 30 s	58°C 30 s	72°C 30 s	30	72°C 7 min
<i>Osterix</i>	94°C 5 min	94°C 30 s	60.6°C 30 s	72°C 30 s	30	72°C 7 min
ALP	94°C 5 min	94°C 30 s	60.6°C 30 s	72°C 30 s	30	72°C 7 min
Osteocalcin	94°C 5 min	94°C 30 s	55°C 30 s	72°C 30 s	30	72°C 7 min

FGF-2 in each sample. The optical density was determined using a 450 nm wavelength filter, with 540 nm correction. The concentration of FGF-2 in the samples was extrapolated from a standard curve, prepared with scalar dilutions of recombinant FGF-2, from 0 to 1000 pg/ml, in PBS with 1% bovine serum albumin.

2.6 Statistical analysis

Results are reported as the arithmetic mean and the standard error of the mean. The influence of the different treatments was evaluated through the Kruskal-Wallis test. The differences between groups were analysed using the Mann-Whitney U test (StatView™ 5.0.1 software for Windows (SAS Institute Inc., Cary, NC, U.S.A.)). The level of significance was set at a *P*-value of 0.05.

3 Results

3.1 Cellular assays

Significantly higher levels of cell-associated ALP were shown in wBMSC + FDDBA + PRP in comparison with pBMSC ($P = 0.0190$) and higher, but not significantly, in comparison with wBMSC (Fig. 1a). However, after 38 days the calcium deposition was similar in the three culture conditions (Fig. 1b).

3.2 Molecular results

After 15 days, *Cbfa1/Runx2* expression was not significantly different in the three culture conditions (Fig. 2a). *Osx* expression in wBMSC + FDDBA + PRP after 15 days was significantly higher than in wBMSC ($P = 0.0172$); there were no significant differences between wBMSC + FDDBA + PRP and pBMSC. At this end-point, wBMSC had a significantly lower expression of *Osx* not only than that of wBMSC + FDDBA + PRP but also than that of pBMSC ($P = 0.05$). After 21 days, *Osx* decreased both in pBMSC and in wBMSC + FDDBA + PRP; any significant difference was no more found among the three culture conditions (Fig. 2b). At both 15 and 21 days, *OC* expression was not significantly different among the different culture conditions. In wBMSC + FDDBA + PRP, as well as in pBMSC, *OC* expression showed a decreasing trend (Fig. 2c). No significant difference was shown in ALP mRNA expression among the cultures at any end-point (Fig. 2d).

3.3 Fibroblast growth factor-2 enzyme immunoassay

FGF-2 in the non-conditioned culture medium with 10% FBS was constantly lower than 3 pg/ml. The range of FGF-2 in

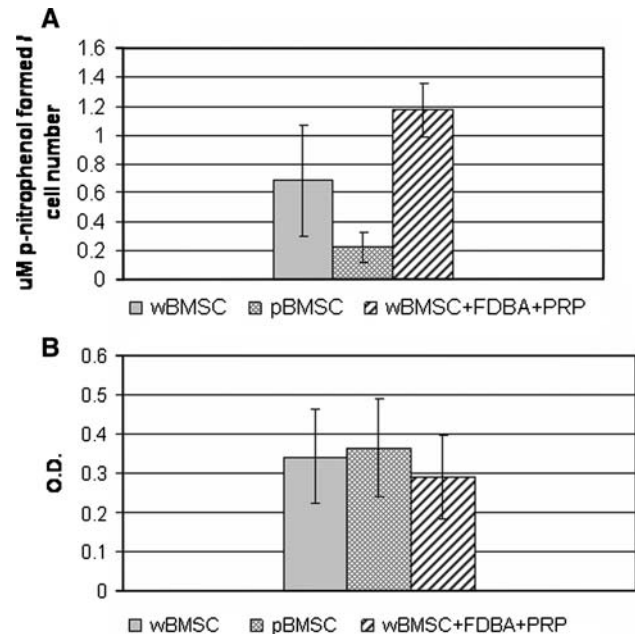


Fig. 1 Arithmetic mean and standard error of the mean of cellular assays. (a) Cell-associated alkaline phosphatase (microM *p*-nitrophenol formed/cell number) after 25-days culture. (b) Calcium deposition (O.D.) after 38 days of culture. wBMSC = BMSC directly seeded without isolation on density gradient, pBMSC = BMSC purified on Ficoll-Hystopaque gradient, wBMSC + FDDBA + PRP = BMSC mixed with FDDBA and PRP. Cell-associated alkaline phosphatase in wBMSC + FDDBA + PRP was significantly higher than pBMSC ($P = 0.0190$); there were no significant differences between wBMSC + FDDBA + PRP and wBMSC. There were no significant differences in calcium deposition among the samples

PRP varied from less than 3 to 13.8 pg/ml immediately after preparation and was constantly lower than 3 pg/ml after 11 days. FDDBA alone released less than 3 pg/ml after 4 and 11 days. No more FGF-2 was released by FDDBA after 15, 21, and 25 days. In all the culture conditions of BMSC, FGF-2 levels were lower than 3 pg/ml after 4 days. Then FGF-2 increased, but with different trends. In the cultures of wBMSC + FDDBA + PRP, FGF-2 levels on days 11 ($P = 0.0117$), 15 ($P = 0.0173$) and 25 ($P = 0.0464$) were significantly higher than on day 4. In pBMSC and in wBMSC, no significant difference was found between day 4 and the other end-points. FGF-2 levels in wBMSC + FDDBA + PRP was constantly higher than in wBMSC and pBMSC. In particular, wBMSC + FDDBA + PRP produced significantly more FGF-2 than wBMSC after 11 days ($P = 0.05$) (Fig. 3).

4 Discussion

In this study, the effects of FDDBA combined with PRP on human BMSC were studied *in vitro*. The differentiation of BMSC towards osteoblasts was evaluated by mRNA expression for *Runx2*, *Osx*, ALP and *OC*, as well as by

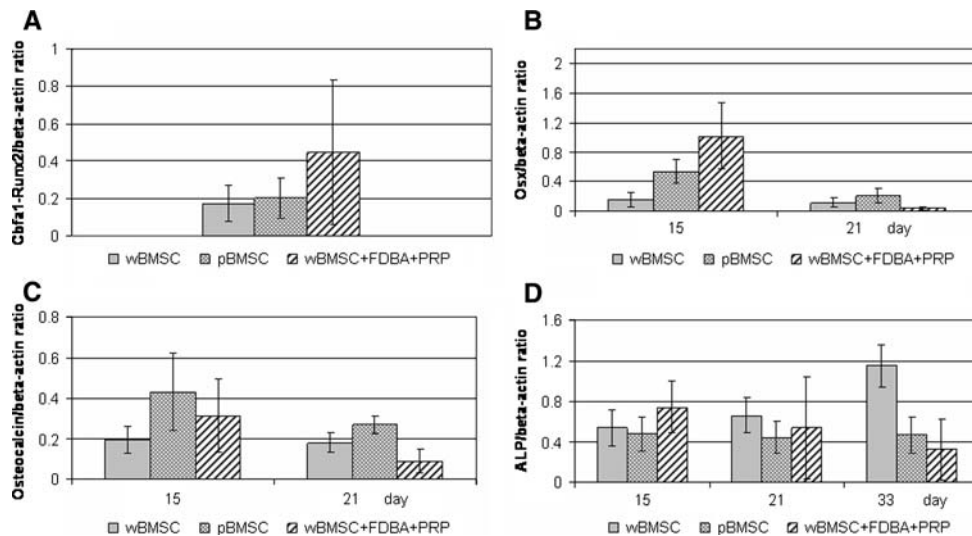


Fig. 2 Arithmetic mean and standard error of the mean of molecular results. The expression of mRNA specific for (a) Cbfa1/Runx2 after 15 days, (b) Osx and (c) osteocalcin after 15 and 21 days, and (d) ALP after 15, 21 and 33 days is reported as gene of interest/ β -actin ratio. wBMSC = BMSC directly seeded without isolation on density gradient, pBMSC = BMSC purified on Ficoll-Hystopaque gradient, wBMSC + FDDBA + PRP = BMSC mixed with FDDBA and PRP. At

15 days wBMSC + FDDBA + PRP expressed significantly more Osx than wBMSC ($P = 0.0172$) and pBMSC expressed more Osx than wBMSC ($P = 0.05$). No significant differences were observed between wBMSC + FDDBA + PRP and pBMSC. At 21 days, there were no significant differences among the samples. There were no significant differences among the samples for Cbfa1/Runx2, osteocalcin and ALP

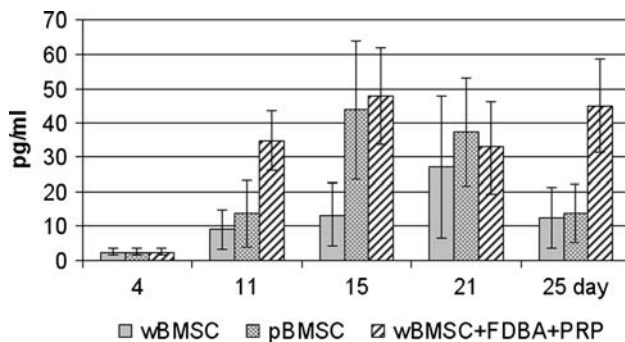


Fig. 3 Mean and standard error of FGF-2 levels (pg/ml) in the supernatants of the cultures after 4, 11, 15, 21 and 25 days. wBMSC = BMSC directly seeded, without isolation on density gradient, pBMSC = BMSC purified on Ficoll-Hystopaque gradient, wBMSC + FDDBA + PRP = BMSC mixed with FDDBA and PRP. In wBMSC + FDDBA + PRP, FGF-2 levels on days 11 ($P = 0.0117$), 15 ($P = 0.0173$) and 25 ($P = 0.0464$) were significantly higher than on day 4. In pBMSC and in wBMSC, no significant difference was found between day 4 and the other end-points. wBMSC + FDDBA + PRP produced significantly more FGF-2 than wBMSC after 11 days ($P = 0.05$)

cell-associated ALP and calcium deposition. Cbfa1/Runx2, belonging to the runt family of tumour suppressor genes, is essential for inducing osteoblast differentiation of BMSC. Cbfa1/Runx2 binds an osteoblast-specific cis-acting element in the OC promoter [15]. Osx acts down-stream Cbfa1/Runx2. Cbfa1/Runx2 and Osx modulate the expression of osteoblast-specific genes, including ALP and OC, during osteogenic differentiation [16]. OC is the most

abundant non-collagenous protein of the bone matrix and is secreted by osteoblasts. It is mainly incorporated into the bone matrix where it is bound to hydroxyapatite. Because Cbfa1/Runx2 is expressed early, it was assessed in the samples extracted only at 15 days, whereas Osx, ALP and OC genes were tested at 15 and 21 days. ALP mRNA was also evaluated after 33 days, to explain the results of cell-associated ALP. The latter was assayed after 25 days, which is generally considered the time of the maximal ALP activity in BMSC [17].

FGF-2 was investigated because it is a well-known mediator of bone cell proliferation, differentiation and functions [18], and is stored in the extracellular matrix [19]. Recombinant FGF-2 was employed by some authors as a supplement to the culture medium of BMSC [20]. BMSC cultured in FGF-2-added medium and seeded on an artificial scaffold showed an accelerated calcium deposition [21]. FGF-2, in addition to the osteogenic effect, also favours angiogenesis [22]: this effect can be useful in bone tissue engineering, where an adequate vascular contribution is required. However, the production of FGF-2 by differently stimulated BMSC has not been reported in the literature.

In our research, BMSC isolated through density gradient, which concentrates osteoblast precursors, showed an earlier expression of the genes related to the osteoblast differentiation. Without density gradient separation, the expression of these genes was generally low. The

combination of FDBA and PRP determined a significantly higher *Osx* expression of BMSC at 15 days, and a trend towards an earlier expression of ALP mRNA. FGF-2 in the conditioned medium of wBMSC + FDBA + PRP was constantly higher than wBMSC.

In comparison with pBMSC alone, BMSC stimulated by platelet gel and FDBA, even if not isolated through a density gradient, showed a similar gene expression, but had a significantly higher cell-associated ALP after 25 days. FGF-2 concentration in the conditioned medium was higher than that in pBMSC at 11 and 15 days, albeit not significantly, and remained high also after 21 days, when the production from pBMSC decreased.

Therefore, the combination of PRP and FDBA promoted osteogenic differentiation of the BMSC concentrated by simple centrifugation but not previously separated on density gradient, practice which is not easily feasible in the operating room. However, after 38 days mineralization was evident in all culture conditions. Therefore, it could be argued that BMSC are able to differentiate into osteoblasts under each culture condition, but the differentiation is accelerated in the presence of the combination of PRP and FDBA.

The higher FGF-2 levels in wBMSC + FDBA + PRP cultures were dependent neither on FDBA nor on platelet release reaction. In fact, only low levels of FGF-2 were found in FDBA or in PRP at the same end-points. Therefore, we could suppose that FGF-2, in the conditioned culture medium, was exclusively produced by BMSC. PRP is not only a concentrate of growth factors, but, at least in combination with FDBA, also induces the production, by BMSC themselves, of osteogenic factors such as FGF-2. Probably FGF-2 production from BMSC treated with PRP and FDBA was determined by TGF- β released by platelet α -granules. It has been demonstrated, at least in mouse osteoblastic MC3T3-E1 cells, that TGF- β induced FGF-2 expression [23].

5 Conclusions

The combination of FDBA and PRP had a favourable effect on the differentiation towards osteoblasts and allowed BMSC concentrated by simple centrifugation to differentiate as fast as BMSC purified by density gradient. Therefore, if BMSC without density gradient separation are added intraoperatively to FDBA and PRP, the end result is predictably the same as after the time-consuming *ex vivo* procedures of cell enrichment, which also affects sterility and increases the risk of infection.

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