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Osteogenic potential of platelet-rich plasma in dental stem-cell cultures

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Abstract

The purpose of this study was to analyse the potential of platelet-rich plasma (PRP) culture media to induce osteogenic differentiation of periodontal ligament stem cells and dental pulp stem cells compared with four other methods of culture. Both types of cell were collected from 35 healthy patients and cultured in five different media (Dulbecco's modified eagle's medium (DMEM); DMEM and melatonin; DMEM and PRP; DMEM and ascorbic acid 200 μmol; DMEM and L-ascorbate 2-phosphate 50 μmol). Cells were characterised by flow cytometry. Alizarin Red stain, alkaline phosphatase stain, and the expression of collagen type 1 (Col-1), runt-related transcription factor (RUNX2), osteoprotegerin, and osteopontin (quantified by qRT-PCR) were used to detect the osteogenic profile in each culture. Flow cytometry showed that both types of stem cell were a homogeneous mixture of CD90(+), CD105(+), STRO-1(+), CD34 (−), and CD45 (−) cells. Dental pulp stem cells that were cultured with PRP showed the best osteogenic profile (RUNX2 $p = 0.0002$; osteoprotegerin $p = 0.001$). The group of these stem cells that showed the best osteogenic profile was also cultured with PRP (osteoprotegerin $p = 0.001$). Medium five (with L-ascorbate 2-phosphate 50 μmol added) showed an increase in all osteogenic markers for periodontal ligament stem cells after PRP, while the best culture conditions for osteogenic expression of dental pulp stem cells after PRP was in medium four (ascorbic acid 200 μmol added). These results suggested that culture in PRP induces osteogenic differentiation of both types of stem cell, modulating molecular pathways to promote bony formation.

Keywords: Bone regeneration; dental pulp stem cells; periodontal dental ligament stem cells; Platelet-rich plasma; cell culture

Introduction

Treatment with stem cells is one of the most promising strategies for the repair and regeneration of tissues and organs.[1] Although adult stem cells are not totipotent, they can renew themselves for the lifetime of the organism, and can differentiate into many types of mature cells.[2]

Mesenchymal stem cells (MSC), which are present in bone marrow and in adipose and connective tissue,[3] have been identified in dental pulp and periodontal ligaments. Stem cells interact with signal molecules to differentiate into specific line cells. Cells derived from dental pulp and periodontal ligament express MSC markers including CD105, CD73, CD90, CD146, and STRO-1 but do not express CD45 and CD34 cell surface markers, and can also differentiate into osteoblasts and odontoblasts.[4]

Stem cells from dental pulp and periodontal ligaments are a good option for bony regeneration in the treatment of periodontitis and for the reconstruction of deficient alveolar bone for dental implants.[5] They have also been used for reconstruction of bony defects with autologous platelet-rich

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plasma (PRP) but their efficacy for improving tissue regeneration is controversial.[6] MSC derived from umbilical cord, adipose tissue, and bone marrow, combined with PRP induce osteogenesis in vitro,[7] and in vivo, dental pulp stem cells with PRP showed osteogenic potential around dental implants. However, to our knowledge, the response of both types of cell cultured in a PRP medium for osteogenic differentiation in vitro has not been studied. Furthermore, they have the potential for mineralisation in response to appropriate pharmacological induction, but we do not yet know which specific media will influence appropriate tissues for bony regeneration.

The purpose of this study therefore was to analyse the potential of PRP culture media (compared with four other methods of culture) to induce the osteogenic differentiation of stem cells from dental pulp and periodontal ligaments.

Material and methods

Cell isolation

Sixty human impacted third molars were collected from 35 healthy patients (aged between 18 and 30 years) after they had given written informed consent according to the protocol approved by the Ethics Committee of Dentistry, Pontificia Universidad, Javeriana, Bogotá.

The teeth were immersed in Dulbecco's modified eagle's medium (low-glucose DMEM, Sigma-Aldrich, St Louis, USA) and immediately transported to the cell culture laboratory for processing. Periodontal ligament was gently removed from the middle third to the apical segment, by washing the teeth with 5.25% sodium hypochlorite. To obtain the dental pulp, we used a zekrya bur in a high-speed handpiece to section the teeth (while irrigating them with saline solution) and a sterile endodontic excavator.[8] Both types of stem cell were then dissolved separately in a solution containing Collagenase/Dispase® 4 mg/ml (Roche Diagnostics, Indianapolis, USA) for 30 minutes at 37 °C.

Flow cytometry

We used flow cytometry to characterise the stem cells. A total of 1×10^5 dental pulp stem cells were suspended in PBS 300 μl for analysis in FACS (Calibur™, BD Bioscience, San Jose, USA). The same procedure was applied to periodontal ligament stem cells. The commercial monoclonal antibodies CD90, CD105, CD45, CD34, and STRO-1 (Rochem Biocare, Bogotá, Columbia) were used to characterise the cells. Background staining for antibodies was used to show negative cell lines.

Cell culture

The cells were grown in DMEM at 37 °C in 5% carbon dioxide supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 g/ml; Invitrogen, Carlsbad, USA) and 10% fetal bovine serum (Gibco™, ThermoFisher Scientific, USA). The cells were then treated with 0.3% trypsin (Invitrogen) and cultured. The medium was changed every four days until the cells reached confluence. Each culture was analysed in passages three to four.

Osteogenic differentiation

Both types of stem cells (1×10^4 cells/cm^2) were seeded on 90-well culture plates, and five different media for osteogenic differentiation were used (Table 1). PRP was obtained from 25 healthy patients using a technique described by Gonshor in 2002.[9] The cultured flask of each type of cell was randomly divided for osteogenic differentiation into five groups at 14 days and five groups at 21 days. The negative control group was cultured in low-glucose DMEM (Sigma-Aldrich), and the Clonetics™ Normal Human Osteoblasts System (Lonza, Walkersville, USA) was used for positive control. Reverse transcription quantitative real-time PCR (qRT-PCR) was used for differentiation.

Cultures were stained with Alizarin Red (Sigma-Aldrich) on days 14 and 21 to show deposits of calcium phosphate. Alkaline phosphatase activity was measured by a colorimetric assay using an assay kit (104-LS, Sigma-Aldrich) according to the manufacturer's instructions.

Table 1
Composition of media used for osteogenic differentiation.

Medium	Composition
1 (negative control medium)	Dulbecco's modified Eagle's medium (low-glucose DMEM, Sigma-Aldrich)
2	Low-glucose DMEM (Sigma-Aldrich); 10% bovine serum (Gibco™); 1% antibiotic (Invitrogen); dexamethasone 100 nmol (Sigma-Aldrich), ascorbic acid 200 μmol (Sigma-Aldrich), β-glycerophosphate 10 mmol (Sigma-Aldrich); melatonin 50 μmol (Sigma-Aldrich)
3	DMEM and 10% platelet-rich plasma (PRP)
4	Low-glucose DMEM (Sigma-Aldrich); 10% bovine serum (Gibco™); 1% antibiotic (Invitrogen); dexamethasone 100 nmol (Sigma-Aldrich); ascorbic acid 200 μmol (Sigma-Aldrich); β-glycerophosphate 10 mmol (Sigma-Aldrich)
5	Low-glucose DMEM (Sigma-Aldrich); 10% bovine serum (Gibco™); 1% antibiotic (Invitrogen); dexamethasone 100 nmol (Stemcell Technologies); L-ascorbate-2 phosphate 50 μmol (A2-P)(Stemcell Technologies); β-glycerophosphate 10 mmol (Stemcell Technologies)

Table 2
Primers and annealing temperatures for qRT-PCR analysis.

Gene	Temp (°C)	Primers
Collagen 1 (Col-1)	62	R:GCTGGGGAGGGGGTTAGTGT F:TCGTCGGAGCAGACGGGAGTT
Osteoprotegerin (OPG)	62	R:ACGCGGTTGTGGGTGCGATT F:AAGACCGTGTGCGCCCCTTG
β-actin	63	R:AGGGGCCGGACTCGTCAT F:GCCCTGGCACCCAGCACAAT
Runt-related transcription factor (RUNX2)	63	R:TGGCTGGTAGTGACCTGCGGA F:AGAGAACCACAACTGCGGTGCAA
Osteopontin (OP)	66	R:CCTTGGTCGGCGTTTGGCTGA F:GCAGACCACAGCATCGTCGGG

Quantitative real-time PCR

The total cellular RNA from all cultures was isolated by adding proteinase K 10 ul (Sigma-Aldrich) at 55 °C for four hours, then Trizol reagent 750 ul (Invitrogen) for 15 minutes, and finally chloroform 250 ul for 15 minutes.

The samples were centrifuged at 14000 rpm for 10 minutes at 4 °C. For every 100 uL of PCR product we added 3 M sodium acetate 20 uL and isopropanol 80 uL, which was centrifuged for 20 minutes at 14000 rpm. We then detected qRT-PCR at 4 °C, the relative expression of the osteogenic markers type-I collagen (Col-1), runt-related transcription factor 2 (RUNX2), osteoprotegerin, and osteopontin, using DNase I, Amplification Grade (Invitrogen 18068-015) and SuperScript™ III Reverse Transcriptase (Invitrogen 18080-093). After initial denaturation at 95 °C for 15 minutes, a two-step cycle was used (denaturation at 95 °C for 10 seconds, annealing and extension at 60 °C for 45 seconds) for 40 cycles. qRT-PCR were measured in triplicate using the Platinum® SYBR® Green Super Mix with ROX (Invitrogen) according to the manufacturer's instructions. Gene expression was normalised according to the amount of β-actin expressed (ΔCt).

The ΔCt of the test sample was normalised to the ΔCt of the controls (ΔΔCt). The expression ratio was calculated with the $2-\Delta\Delta Ct$ method (2-ΔΔCT = Ct target gene − CT). The list of primers and annealing temperatures are shown in Table 2.

Statistical analysis

Data were expressed as mean (SD), and the significance of differences between the groups was assessed using the two-way ANOVA and Tukey's test as appropriate. Probabilities of less than 0.05 were accepted as significant.

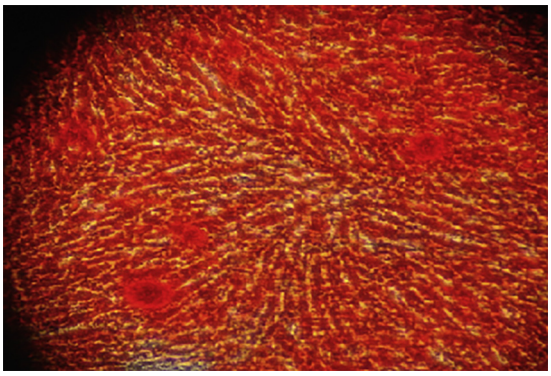

Fig. 1. Dental pulp stem cells stained with Alizarin Red.

Results

Isolation and proliferation of cells

There were differences in cellular migration between the pulp and ligament cultures. At the end of day nine, cells showed confluence of 90% in 38% of the pulp and 13% of the ligament cultures.

Characterisation of stem cells by flow cytometry

The expression of CD90, CD105 and STRO-1 and the low level of CD45, and CD34 confirmed the phenotype of MSC for both types of cell, and they had the same immunophenotype.

Osteogenic differentiation

Both types of stem cell produced mineralised deposits under osteogenic conditions as detected by autonomous replicating sequence (ARS) and alkaline phosphatase (ALP) activity (Fig. 1).

mRNA expression of osteogenesis-specific markers

Different expressions for osteogenic markers (RUNX2, osteopontin, osteoprotegerin, Col-1) were shown between all groups. All osteogenic markers expressed at days 14 and 21 for both types of cell in all media were similar. Pulp stem cells cultured in medium three with PRP had the highest expression of all osteogenic markers compared with the other experimental groups, although osteoprotegerin was the single significant value ($p = 0.006$), and the culture medium that contained ascorbic acid 200 μmol showed the second highest expression (Fig. 2).

Periodontal ligament stem cells cultured with PRP showed the best osteogenic profile. The expression of RUNX2 in PRP was higher than in medium four ($p = 0.0002$), five ($p = 0.001$), and two ($p = 0.009$). Similarly, expression of osteoprotegerin was significantly higher in these cells cultured with PRP than in those cultured in the other media ($p = 0.001$). Cells cultured

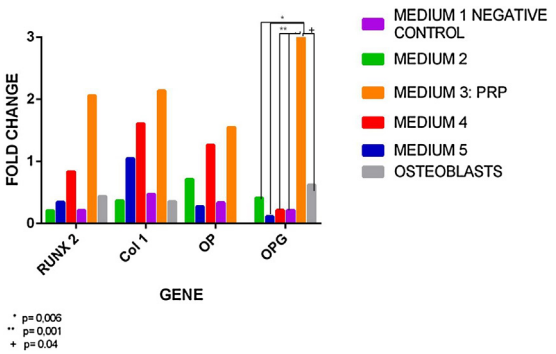

Fig. 2. Osteogenic markers expressed for dental pulp stem cells in different methods of culture. The best conditions for osteogenic differentiation were shown in PRP. Osteoprotegerin (OPG) showed the highest expression when these cells were cultured in PRP medium (RUNX2 = runt-related transcription factor; Col 1 = collagen type 1; OP = osteopontin).

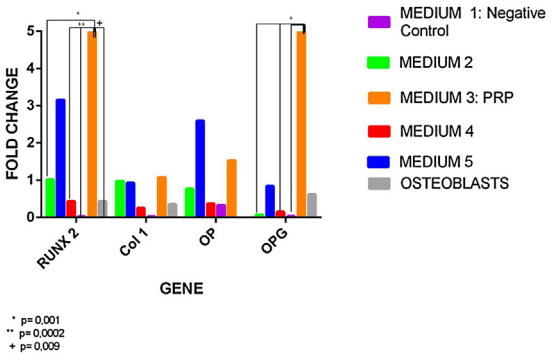

Fig. 3. Osteogenic markers expressed for periodontal ligament stem cells in different methods of culture. The best conditions for osteogenic differentiation were shown in PRP. RUNX2 and osteoprotegerin (OPG) showed the highest expression when these cells were cultured in PRP medium (RUNX2 = runt-related transcription factor; Col 1 = collagen type 1; OP = osteopontin).

in medium five (with 50 μmol of A2-P) showed increased expression of all osteogenic markers, but these were not significant. Medium two with melatonin showed osteogenic differentiation in both types of cell (Fig. 3).

Discussion

The presence of stem cells in dental pulp and periodontal ligament was confirmed by flow cytometry. Analysis showed a similar mean (SD) pattern of expression in both types of cell: CD90: 90 (4.9); CD105: 74.1 (23); STRO-1: 15.2 (9.8); and CD34: 7.1(6.2); CD 45: 0.7 (0.1). These results are similar to those of previous reports, which confirmed the presence of MSC in these tissues,[10] but they contrast with the findings of Hakki et al,[11] which showed differences in the expression of CD105.[12]

In the present study, stem cells cultured in several media expressed osteogenic markers, but the best condition of cell niche was different for each type, as has been described for other MSC cultured for osteogenic induction.[13] PRP gave the best conditions for osteogenic differentiation from both types of cell. Osteoprotegerin showed the highest expression in the culture of pulp cells and PRP ($p = 0.001$), while the highest osteogenic marker expressed in ligament cells cultured with PRP was shown for RUNX2 ($p = 0.0002$). However, osteoprotegerin in ligament cells also increased significantly ($p = 0.001$). Col-1 and osteopontin were increased in cultures of both types of cell in PRP, but this was not significant. In contrast with the findings of Hakki et al,[11] we found that the expression of Col-1 was similar for both types of cell, but RUNX2 and osteoprotegerin showed the highest expression in ligament cells, and osteoprotegerin showed the highest expression in pulp cells. These differences could be explained by the different methods of culture used in both studies. Although PRP has been used for bony augmentation in humans because of the amount of growth factors that are released around PRP in tissues after an operation,[14] there is a lack of evidence to support the increase of osteogenic potential of pulp and ligament stem cells when they are cultured with PRP.

Osteoprotegerin or TNFRSF11B is a member of the TNF-receptor superfamily, and is produced by osteoblasts that participate in bony modelling.[15] RUNX2 is a transcriptional factor that regulates critical genes during osteoblastic differentiation and skeletal morphogenesis. Cultures of MSC derived from diverse tissues such as human exfoliated deciduous teeth (SHED) increased the expression of RUNX2 when they were cultured with PRP.[16,17] Parsons et al suggested that RUNX2 activates pathways that lead MSC to become lineage-specific tissue-forming cells.[18] It has also been reported that MSC treated with osteoprotegerin increased the expression of osteocalcin and improved osteogenesis.[18]

The other culture media were used concurrently with conventional osteogenic culture conditions, various doses and presentations of ascorbic acid (media four and five), or with melatonin (medium two). The best conditions for osteogenic induction were PRP and medium five for ligament stem cells, and PRP and medium four for pulp stem cells. Previous studies have reported that both pulp and ligament stem cells have the potential for osteogenic differentiation. The difference between medium four and medium five was the type and concentration of ascorbic acid (200 μmol for medium four and 50 μmol A2-P for medium five).

The importance of ascorbic acid in the maintenance of normal extracellular function is well known. It is required as a cofactor for prolyl-4-hydroxylase, which is necessary for the secretion of procollagen to form stable triple-helical collagen in connective tissues. Additionally, ascorbic acid induces MSC to differentiate into osteoblasts,[19] and A2-P, a stable form of ascorbic acid, has been shown to improve cell proliferation and induce osteogenic differentiation.[20] We found that the standard cultures for osteogenic induction seemed to work better when ascorbic acid was added to cultures of pulp cells and when A2-P was added to ligament cells, but the effects of ascorbic acid in these cultures need further investigation.

Medium two worked well for osteogenic differentiation in both types of cell, although it did not show the highest expression for osteogenic differentiation. It was enriched with melatonin because melatonin has been reported to improve osseointegration around implants.[21] In vitro and in vivo studies have suggested that melatonin affects the terminal differentiation of osteoblasts, but promoted expression of RUNX2 improves osteogenic differentiation in the early stages of osteogenesis of MSC.[22] Dexamethasone inhibits osteoblastic differentiation through the repression of BMP-2 expression,[23] and melatonin promotes osteoblastic differentiation and mineralisation through the BMP/ERK/Wnt pathways.[24] Dexamethasone and melatonin may have the same signalling pathway for bony metabolism, and this may be the reason why the osteogenic markers in this medium did not get the highest values.

Our results have confirmed the capacity of dental-pulp and periodontal-ligament stem cells to differentiate into osteogenic cells, but this capacity depends on the stem-cell niche and molecular interactions. The importance of the cell niche and the growth factors used to induce the regeneration of specific tissues has been argued in numerous studies. PRP and autologous platelet-rich fibrin (PRF) have increased the cellular proliferation of dental stem cells and their osteogenic activity in vitro and in vivo in endodontic treatments, tooth regeneration, and around dental implants.[25] However, to the best of our knowledge the effects of PRP have not previously been compared with different osteogenic protocols on the capacity for osteogenic differentiation in dental stem cells.

It is therefore important to find the best conditions for osteogenic differentiation in periodontal-ligament and dental-pulp cultures. The clinical implications of the present work lie in the development of culture media with supplementation that is ideal for obtaining higher differentiation and mineralisation rates with specific application for dental ligament stem cells for bony regeneration and dental implants. This knowledge might enable us to understand the physiological mechanisms of osteogenic differentiation by dental MSC for use in tissue engineering.

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Conflict of interest

We have no conflicts of interest.

Ethics statement/confirmation of patients' permission

This work was approved by the Ethics Committee of Dentistry of the faculty of the University. All patients signed informed consent approved by the Ethics Committee of Dentistry of faculty of the University.

References

1. Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* 2005;**85**:635–78.
2. Fortier LA. Stem cells: classification, controversies and clinical applications. *Vet Surg* 2005;**34**:415–23.
3. Malgieri A, Kantzari E, Patrizi MP, et al. Bone marrow and umbilical cord blood human mesenchymal stem cells: state of the art. *Int J Clin Exp Med* 2010;**3**:248–69.
4. Xiao L, Nasu M. From regenerative dentistry to regenerative medicine: progress, challenges, and potential applications of oral stem cells. *Stem Cells Cloning* 2014;**7**:89–99.
5. Ito K, Yamada Y, Nakamura S, et al. Osteogenic potential of effective bone engineering using dental pulp stem cells, bone marrow stem cells, and periosteal cells for osseointegration of dental implants. *Int J Oral Maxillofac Implants* 2011;**26**:947–54.
6. Del Fabbro M, Corbella S, Taschieri S, et al. Autologous platelet concentrate for post-extraction socket healing: a systematic review. *Eur J Oral Implantol* 2014;**7**:333–44.
7. Qi Y, Niu L, Zhao T, et al. Combining mesenchymal stem cell sheets with platelet-rich plasma gel/calcium phosphate particles: a novel strategy to promote bone regeneration. *Stem Cell Res Ther* 2015;**6**:256.
8. Caviedes-Bucheli J, Ariza-García G, Restrepo-Méndez S, et al. The effect of tooth bleaching on substance P expression in human dental pulp. *J Endod* 2008;**34**:1462–5.
9. Gonshor A. Technique for producing platelet-rich plasma and platelet concentrate: background and process. *Int J Periodontics Restor Dent* 2002;**22**:547–57.
10. Patil R, Kumar BM, Lee WJ, et al. Multilineage potential and proteomic profiling of human dental stem cells derived from a single donor. *Exp Cell Res* 2014;**320**:92–107.
11. Hakki SS, Kayis SA, Hakki EE, et al. Comparison of mesenchymal stem cells isolated from pulp and periodontal ligament. *J Periodontol* 2015;**86**:283–91.
12. Vasandan AB, Shankar SR, Prasad P, et al. Functional differences in mesenchymal stromal cells from human dental pulp and periodontal ligament. *J Cell Mol Med* 2014;**18**:344–54.
13. Honda Y, Ding X, Mussano F, et al. Guiding the osteogenic fate of mouse and human mesenchymal stem cells through feedback system control. *Sci Rep* 2013;**3**:3420.
14. Zhong W, Sumita Y, Ohba S, et al. In vivo comparison of the bone regeneration capability of human bone marrow concentrates vs platelet-rich plasma. *PLoS One* 2012;**7**:e40833.
16. Jurado S, Garcia-Giralt N, Díez-Pérez A, et al. Effect of IL-1beta, PGE(2), and TGF-beta1 on the expression of OPG and RANKL in normal and osteoporotic primary human osteoblasts. *J Cell Biochem* 2010;**110**:304–10.
17. Wen J, Li HT, Li SH, et al. Investigation of modified platelet-rich plasma (mPRP) in promoting the proliferation and differentiation of dental pulp stem cells from deciduous teeth. *Braz J Med Biol Res* 2016;**49**:e5373.
18. Parsons P, Butcher A, Hesselden K, et al. Platelet-rich concentrate supports human mesenchymal stem cell proliferation, bone morphogenetic protein-2 messenger RNA expression, alkaline phosphatase activity, and bone formation in vitro: a mode of action to enhance bone repair. *J Orthop Trauma* 2008;**22**:595–604.
19. Leon ER, Iwasaki K, Komaki M, et al. Osteogenic effect of interleukin-11 and synergism with ascorbic acid in human periodontal ligament cells. *J Periodontal Res* 2007;**42**:527–35.
20. Yu J, Tu YK, Tang YB, et al. Stemness and transdifferentiation of adipose-derived stem cells using L-ascorbic acid 2-phosphate-induced cell sheet formation. *Biomaterials* 2014;**35**:3516–26.

21. Tresguerres IF, Clemente C, Blanco L, et al. Effects of local melatonin application on implant osseointegration. *Clin Implant Dent Relat Res* 2012;**14**:395–9.
22. Luchetti F, Canonico B, Bartolini D, et al. Melatonin regulates mesenchymal stem cell differentiation: a review. *J Pineal Res* 2014;**56**:382–97.
23. Hong D, Chen HX, Xue Y, et al. Osteoblastogenic effects of dexamethasone through upregulation of TAZ expression in rat mesenchymal stem cells. *J Steroid Biochem Mol Biol* 2009;**116**:86–92.
24. Park KH, Kang JW, Lee EM, et al. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signaling pathways. *J Pineal Res* 2011;**51**:187–94.
25. Chen YJ, Zhao YH, Zhao YJ, et al. Potential dental pulp revascularization and odonto-/osteogenic capacity of a novel transplant combined with dental pulp stem cells and platelet-rich fibrin. *Cell Tissue Res* 2015;**361**:439–55.