Gene Expression during Osteo/Odontogenic Differentiation of Mesenchymal Stem Cells with Platelet Rich Plasma and Mineral Trioxide Aggregate

Amit Anandswarup Vanka¹, Shanthi Vanka², Sandeep Vishwakarma³, Manohar Bhat⁴, Dinesh Rao⁵, Othman Wali⁶ and Aleem Khan³

¹Pacific Academy of Higher Education and Research University, Udaipur – 313003, Rajasthan, India; amitvanka18@gmail.com
²Department of Preventive Dental Sciences, Ibn Sina National College for Medical Studies, Jeddah – 22421, Kingdom of Saudi Arabia
³Central Laboratory for Stem Cell Research and Translational Medicine, CLRD, Deccan College of Medical Sciences, Kanchanbagh, Hyderabad – 500 058, Telangana, India
⁴Department of Pedodontics and Preventive Dentistry, Jaipur Dental College, Jaipur – 302015, Rajasthan, India
⁵Department of Pedodontics and Preventive Dentistry, Pacific Dental College and Hospital, Udaipur – 313011, Rajasthan, India
⁶Ibn Sina National College for Medical Studies, Jeddah – 22421, Kingdom of Saudi Arabia

Abstract

Platelet Rich Plasma (PRP) has the potential to regenerate pulp in immature pulpless teeth. Mineral trioxide is commonly used to seal the PRP into the pulp canal space. We investigated the effect of PRP and MTA individually and combined on osteo/odontogenic differentiation potential and the phenotype of tissue formed. MSCs were cultured in vitro with MTA, 5% PRP, 10% PRP, MTA with 5% PRP and MTA with 10% PRP. Osteo/odontogenic differentiation was assessed and quantified with alizarin red staining. Relative expression of Alkaline Phosphatase (ALP) activity, type 1 collagen (COL1A1), Dental Sialo-Phospho Protein (DSPP), Dentin Matrix Protein (DMP-1), Bone Gamma-carboxyglutamate Protein (BGLAP), Runt-related transcription factor 2 (Runx2), Osterix (Osx) and TGF-β1 was identified by RT-q PCR. 10% PRP with MTA displayed significantly higher calcium deposition during differentiation and high ALP levels. Significantly enhanced levels of DSPP, DMP1, COL1A1, BGLAP, Runx2, and Osx and TGF-β1 transcripts were observed. Within limitations of the in vitro environment, results imply enhanced osteodentin formation, on combining PRP with MTA.

Keywords: Differentiation, Mesenchymal Stem Cells, Mineral Trioxide Aggregate, Odontogenic, Platelet Rich Plasma

1. Background

Revascularization of the pulp in immature pulpless teeth has garnered considerable attention in recent times. The colonization of pulp space can occur by mesenchymal stem cells from various sources in the pulp-periodontal complex that allows a vascular supply to be re-established. The procedure requires...
introducing a disinfectant into the root canal followed by inducing bleeding to form a clot. This procedure of blood clot formation suffers from the drawback of having an unpredictable number and population of cells. Histological evaluation of the tissue formed with a blood clot has been described as an extension of the periodontal ligament\(^5\). Animal studies have reported that the hard tissue formed to increase root length and internal diameter thickness, resembles cementum\(^3\) or bone (bony islands)\(^4\). These reports have prompted some researchers to believe that pulp regeneration is different from revascularization. While pulp revascularization indicates the presence of angiogenesis into the root canal system, a true regenerative procedure would involve establishment of a blood supply and restoration of functional cells\(^5\).

Post natal stem cell therapy has the potential to overcome some of the disadvantages of revascularization by blood clotting. The ability to differentiate into the cell population found in adult pulp, namely odontoblasts, is a critical prerequisite for the success of therapy with post natal stem cells. The growth factors required for proliferation and differentiation of these cells without contamination, that may occur during processing in the lab, may be provided by Platelet Rich Plasma (PRP)\(^6\). Mineral Trioxide Aggregate (MTA) is a biocompatible material used in several clinical applications in dentistry, including sealing the orifice during revascularization\(^7\). A study has demonstrated that the combination of PRP and MTA is capable of enhancing the differentiation of cells towards an osteo/odontogenic lineage\(^8\). Nevertheless, the phenotype of the cells and mineralized deposits formed during the differentiation process has not been analyzed. Hence the present study was undertaken to assess the expression of several markers associated with osteo/odontogenic differentiation of MSC’s by combination of PRP with MTA.

### 2. Materials and Methods

Approval from institutional review board was taken for the study. Signed informed consent forms were collected from each volunteer enrolled in the study. A double centrifugation method was used for PRP preparation modified from a previous study\(^9\). The concentration of the platelets within the PRP was assessed, and standardized to 1200 X 10\(^3\) platelets / ml. The process of isolating MSCs from human bone marrow blood has been described elsewhere\(^10\). MSC’s were cultured at 37°C temperature and 5% CO\(_2\) in sterile tissue culture plates (Corning, USA). MTA was mixed as per manufacturer’s instructions (DENTSPLY, Endodontics) and coated onto the cell culture plates.

### 3. Osteo/Odontogenic Differentiation Potential of MSCs Cultured in Different Conditions

Dulbecco’s Modified Eagle’s Medium (DMEM, Cat#: AT068, Himedia, India) was supplemented with 5% PRP, 10% PRP, 5% PRP with MTA and 10% PRP with MTA to prepare 5 experimental groups. DMEM with 10% Fetal Calf Serum (FCS) was used as control. MSC’s thus derived were induced to osteo/odontogenic differentiation using osteogenic differentiation supplements (MesenCult™ XF osteogenic stimulatory kit, human, Cat#: 05434, Stem Cell Technologies, Canada). Cells were allowed to differentiate for 14 days and characterized for their differentiation potential.

### 4. Alizarin red Staining

Staining with 10% alizarin red solution (Cat#: 40-1009-5-500ML-J, Sigma, USA) was carried out at 14 days after washing differentiated cells from each group thrice with 1X PBS. Alizarin red staining was used to assess the mineralization and presence of calcium deposits. Calcium content was quantified using the calcium colorimetric assay (Sigma Aldrich, USA).

### 5. Biochemical Evaluation of Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase colorimetric assay kit (Cat#: ab83369, Abcam, Cambridge, UK) was used to measure intracellular ALP activity in differentiating MSCs at day 3, 7 and 14. A micro-plate reader measured the absorbance at 405nm.
6. Quantitative Gene Expression Analysis

Isolation of total Ribonucleic Acid (RNA) was performed as described elsewhere\textsuperscript{11} at day 3, 7 and 14 from cells differentiated in the presence of MTA, 5% PRP, 10% PRP, MTA with 5% PRP, and MTA with 10% PRP and 10% FCS (control). 1 microgram RNA was converted into complementary Deoxyribo-Nucleic Acid (cDNA) as per the manufacturer’s instructions, using the first strand cDNA synthesis kit (Amersham Bioscience Europe; Freiburg, Germany). 5 nanograms of cDNA were used for RT-qPCR analysis using SYBR green chemistry in StepOne Real-Time PCR (Applied Biosystems, Carlsbad, CA, USA). Fold changes in gene expression of associated markers; type 1 Collagen (COL 1A1), Dental Sialo-Phospho Protein (DSPP), Dentin Matrix Protein (DMP-1), Bone Gamma-carboxyglutamate Protein (BGLAP), Runx-related transcription factor 2 (Runx2), Osterix (Osx) and TGF-\(\beta\)1 was identified by RT-qPCR at day 3, 7 and 14. Glyceraldehydes 3-Phosphate De-Hydrogenase (GAPDH) gene was used for normalization. Primer sequences are listed in (Table 1). The 2\(^{-\Delta\Delta CT}\) method was used to calculate the fold difference using StepOne software (ver. 2.2).

7. Statistical Analysis

Mean and standard deviation was calculated for all samples. Statistical tests were performed in GraphPad Prism software (Version 5). Two way Analysis Of Variance (ANOVA) was applied to compare means. \(p\) value < 0.01 was considered to be statistically significant. RT-qPCR efficiency was calculated using StepOne (ver. 2.2) software.

8. Results

8.1 Induced Mineralization during Osteo/Odontogenic Differentiation

MSC’s demonstrated alizarin red positive staining at 14 days in all 5 experimental and control groups. The amount of staining was highest in MTA with 10% PRP (**\(p\) < 0.001). Calcium deposition was quantified and similarly, the highest calcium deposition was observed in MTA with 10% PRP group (**\(p\) < 0.001).

8.2 Measurement of Intracellular ALP Activity

Differentiating cells showed enhanced ALP activity compared to control at day 14(**\(p\) < 0.001) for MTA, at day 7 and 14 (**\(p\) < 0.0001) for MTA with 5% PRP and at day 3, 7 and 14(**\(p\) < 0.0001) for MTA with 10% PRP. Moreover, MTA with 10% PRP was found to have the highest ALP level as compared to control and other test groups (**\(p\) < 0.0001) (Figure 1).

8.3 Relative Gene Expression of Odontogenic/Osteogenic Markers

The relative expression of all the tested genes exhibited significantly higher levels (\(p\) < 0.01) in MTA with 5%

---

| Gene name     | Forward primer (5’-3’)       | Reverse primer (5’-3’)       |
|---------------|------------------------------|------------------------------|
| COL1A1        | AAGTCTTCTGCAACATGGAG         | TACTCGAACTGGAATCCTCA        |
| DSPP          | GAAGATGCTGGGCTGGGATAA        | TCTTCTTTCCATGGTCTGCT       |
| DMP-1         | CCCTTGAGAGAGAGCTGTAGGC      | CTCCTTTGTCCATGGGTCTCAG     |
| OCN/BGLAP     | GTGCAGAGTGCCGCAAGGGT        | TCAGCCAACTGCTGACAGTC       |
| TGF-\(\beta\)1 | TCCTGCTTCTGCAAGGGC         | CTCAGGCTCAGGTGAGTAG         |
| Runx2         | CCGGACGACAAACCGACCAC        | CGCCTCGACGCGCAGGATC        |
| GAPDH         | ACATCAAGAGGAGGTTGAGG        | AAATGAGCTTGACAAAGGTG        |
PRP and MTA with 10% PRP groups as compared to control at day 14 (Figure 2). The fold change for all genes except Runx2 were significantly higher for MTA at day 14 (p < 0.01).

8.4 TGF-β1 Expression during Osteo/Odontogenic Differentiation

The relative expression analysis of TGF-β1 in this study showed significantly higher levels in 10%PRP, MTA with 5% PRP and MTA with 10% PRP groups during osteo/odontogenic differentiation (***p<0.0001) (Figure 3).

Figure 1. Intracellular ALP activity normalized to DNA content in differentiating MSCs cultured in different induction medium showed significant up regulation in MTA, MTA with 5% PRP and MTA with 10% PRP at day 14. The highest ALP activity was observed in MTA with 10% PRP group at all time points.

Figure 2. Relative fold difference for mRNA transcripts of genes: collagen type I (COL1A1), Dentin Sialo-Phospho-Protein (DSPP), Dentin Matrix Protein 1 (DMP1), Bone Gamma-carboxyglutamate Protein (BGLAP), Runx-related transcription factor 2 (Runx2) and Osterix (Osx). Graphs showing differentially expressed gene levels normalized to GAPDH and compared to control at day 3, 7 and 14 during MSCs osteo/odontogenic differentiation in medium supplemented with MTA, 5% PRP, 10% PRP, MTA with 5% PRP and MTA with 10% PRP and control.

Figure 3. Fold difference for mRNA expression of TGF-β1 at day 3, 7 and 14 of osteo/odontogenic differentiation in different combinations of medium supplements showed highly significant up regulation in MTA with 10% PRP at all time points.
9. Discussion

Several methods are currently available for the preparation of PRP. We used the double centrifugation method since it has been reported to enhance platelet recovery, enhance platelet activity and prevent loss of growth factors. The process utilized for preparation of PRP in the current study allowed leukocytes to be retained and can therefore be classified as a PRP (L). Standardization of the concentration of platelets in PRP is an essential prerequisite for activity and could be achieved in the current study. Results indicated that all the groups of PRP and MTA were capable of differentiating MSC's into osteo/odontogenic lineage. Positive staining of the cells with alizarin red indicates the presence of calcium deposition, and the quantity of mineralization was much higher in the PRP and MTA combined group. These results were similar to findings from a previous study. MTA is a biocompatible material which has been shown to increase the release of angiogenic factors such as vascular endothelial growth factor. Additionally, MTA has been investigated and reported to affect several signaling pathways that may help induce mesenchymal stem cell proliferation and differentiation. PRP is also known to have high concentrations of growth factors stored in ‘alpha granules’ the release of which has a modulatory effect on MSC's.

The phenotype of the differentiating MSCs was further investigated in terms of gene expression of mineralization associated genes. Both bone and dentin have an extracellular organic matrix. Several proteins present in this extracellular matrix, termed as the Non-Collagenous Proteins (NCP) play an important role during the process of mineralization and hence the overall process of regeneration. Due to the structural similarity of dentin and bone, NCPs found in dentin are also expressed in bone, albeit in concentrations different than those in dentin.

The most frequently used markers of the osteogenic/odontogenic differentiation process are alkaline phosphatase (ALP) and type I collagen (COL1A1). ALP has been known to be associated with different calcifying tissues, including odontoblasts and enamel. Type I collagen is the most abundant protein in connective tissue to be expressed during mineralization. An increased ALP activity, as observed with PRP and MTA together, coupled with increased expression of COL1A1 was observed in our study. These results suggest that when combined, PRP and MTA promote a higher degree of active mineralization.

Runx2 and Osx are both known to play a role in the differentiation of MSC’s to osteoblasts. Runx2 is considered to be critical for regulating the differentiation of progenitor cells into preosteoblasts. Additionally, Runx2 is also responsible for the expression of other non-collagenous proteins. Osx gene has been reported to act downstream of Runx2 and may be considered a marker for osteoblast differentiation. Similarly, BGLAP is a component of bone extracellular matrix, produced exclusively by osteoblasts and is considered as a specific indicator of latter stages of osteoblastic differentiation. In the current study, Runx2, Osx and BGLAP were all highly upregulated at 14 days when 10% PRP and MTA were combined. The enhanced expression was more than PRP or MTA individually. These observations indicate that the combination enhances the differentiation of MSC’s towards osteogenic lineage with the formation of bone-like structure.

DSPP is believed to be a specific marker for the odontogenic differentiation process. Although DSPP has also been found in bone, but the levels of DSPP in dentin are significantly higher than those detected in bone. Dentin matrix protein 1 (DMP1) is another Non-Collagenous Protein (NCP) reported to play a role in the process of mineralization by facilitating the expression of the osteo/odontogenic differentiation marker genes. In the current study, highly upregulated levels of DSPP and DMP1 were observed at 14 days of culture. These levels were again highest when 10% PRP and MTA were combined. Thus elevated levels of DSPP and DMP1 indicate enhanced odontogenic differentiation by the combination with formation of dentin matrix. Thus, progressively up-regulated expression of genes in culture collectively is an indicator of enhanced osteo/odontogenic differentiation. Together with increased expression of osteo/odontogenic genes, the mineralization nodules indicate the presence of osteodentin. Nevertheless, further research is needed to determine the histological structure of the tissue formed.

In our study, significantly enhanced gene expression for TGF-β1 was observed during differentiation, in presence of MTA with 10% PRP. TGF-β1 plays an important role in cell differentiation and reportedly facilitates osteoblastic differentiation, while inhibiting myogenesis and adipogenesis. The expression of NCP’s such as DSPP and DMP-1 is up-regulated by TGF-β1 and by extension it is involved in the synthesis of extracellular proteins.
matrix synthesis. These findings reaffirmed our belief that combining these two materials led to an enhanced effect of pushing MSCs towards osteo/odontogenic lineage.

10. Conclusion

Overall, this study shows that, differentiation of cells into osteo/odontogenic lineage with highly up regulated differentiation markers was observed with 10% PRP and MTA together. Within the limitations of the invitro environment results suggest an increased osteodentin deposition by the combination rather than a passive effect on PRP by MTA. Future studies may be required but the combination appears to provide a dual advantage of both expanding the existing cell population simultaneously producing tissue such as osteodentin in the canals which may be more desirable than cementum or bony islands.

11. Acknowledgement

We acknowledge the services of Varanasi Satyavani, English Language Instructor, English Language Institute, King Abdul Aziz University, Jeddah, Saudi Arabia for the English language revision.

Conflict of interest: None declared

12. References

1. Thomson A, Kahler B. Regenerative endodontics—biologically-based treatment for immature permanent teeth: a case report and review of the literature. Aust Dent J. 2010; 55(4):446-52. https://doi.org/10.1111/j.1834-7819.2010.01268.x. PMid: 21133946.
2. da Silva L, Nelson-Filho P, da Silva R, et al. Revascularization and periapical repair after endodontic treatment using apical negative pressure irrigation versus conventional irrigation plus triantibiotic intracanal dressing in dogs' teeth with apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010; 109:779-87.
3. Wang X, Thibodeau B, Trope M, Lin L, Huang G. Histologic characterization of regenerated tissues in canal space after the revitalization/revascularization procedure of immature dog teeth with apical periodontitis. J Endod. 2010; 36:56-63. https://doi.org/10.1016/j.joen.2009.09.039. PMid: 20003936.
4. Yamauchi N, Yamauchi S, Nagaoaka H, et al. Tissue engineering strategies for immature teeth with apical periodontitis. J Endod. 2011; 37:390-97. https://doi.org/10.1016/j.joen.2010.11.010. PMid: 21329828.
5. Mao JJ, Kim SG, Zhou J, Ye L, Cho S, Suzuki T, Fu SY, Yang R, Zhou X. Regenerative endodontics: Barriers and strategies for clinical translation. Dental Clinics. 2012 Jul 1; 56(3):639-49. https://doi.org/10.1016/j.jcldent.2012.05.005. PMid:22835543. PMCid: PMC4093795.
6. Hwang YJ, Choi JY. Addition of mesenchymal stem cells to the scaffold of platelet-rich plasma is beneficial for the reduction of the consolidation period in mandibular distraction osteogenesis. J Oral Maxillofac Surg. 2010; 68:1112-24. https://doi.org/10.1016/j.joms.2008.08.038. PMid: 20223574.
7. D'antìo V, Di Caprio MP, Ametrano G, Simeone M, Rengo S, Spagnuolo G. Effect of mineral trioxide aggregate on mesenchymal stem cells. J Endod. 2010; 36:1839-43. https://doi.org/10.1016/j.joen.2010.08.010. PMid: 20951297.
8. Vanka A, Sandeep KV, Shanthi G, Manohar KB, Wali O, Khan AA. Osteo/odontogenic differentiation of human mesenchymal stem cells with Platelet Rich Plasma (PRP) and Mineral Trioxide Aggregate (MTA). J Contemp Dent Pract (accepted for publication).
9. Baussot O, Giraudo L, Veran J. Formulation and storage of platelet-rich plasma homemade product. Biore Open Access. 2012; 1:115-23. https://doi.org/10.1089/biores.2012.0225. PMid: 23516761, PMCid: PMC3559222.
10. Vanka A, Sandeep KV, Shanthi G, Manohar KB, Khan AA. In vitro proliferation of MSCs using mineral trioxide aggregate: A most recent material for in situ stem cells mobilization. Int J of Adv Res. 2014; 2:561-67.
11. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987; 162:156-59. https://doi.org/10.1016/0003-2697(87)90021-2, https://doi.org/10.1016/0003-2697(87)90021-2. PMid: 10099990.
MAPK signalling pathways. Cell proliferation. 2014 Jun; 47(3):241-48. https://doi.org/10.1111/cpr.12099. PMid: 24635197.

16. Perut F, Filardo G, Mariani E, Cenacchi A, Pratelli L, Devescovi V, Kon E, Maracci M, Facchini A, Baldini N, Granchi D. Preparation method and growth factor content of platelet concentrate influence the osteogenic differentiation of bone marrow stromal cells. Cytotherapy. 2013 Jul 1; 15(7):830-39. https://doi.org/10.1016/j.jcyt.2013.01.220. PMid: 23731763.

17. Smith AJ, Scheven BA, Takahashi Y, Ferracane JL, Shelton RM, Cooper PR. Dentine as a bioactive extracellular matrix. Arch Oral Biol. 2012; 57:109-21. https://doi.org/10.1016/j.archoralbio.2011.07.008. PMid: 21855856.

18. Huang W, Yang S, Shao J, Li YP. Signaling and transcriptional regulation in osteoblast commitment and differentiation. Frontiers in Bioscience: A Journal and Virtual Library. 2007 May 1; 12:30-68. https://doi.org/10.2741/2296. PMid: 17485283, PMCid: PMC3571113.

19. Linde A, Granstro¨ MG. Odontoblast alkaline phosphatases and Ca^2þ transport. J Biol Buccale. 1978; 6:293-308.

20. Couchourel D, Aubry I, Delalandre A. Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production. Arthritis and Rheumatology. 2009; 60:1438-50. https://doi.org/10.1002/art.24489. PMid: 19404930, PMCid: PMC350342.

21. Miron RJ, Zhang YE. Osteoinduction: a review of old concepts with new standards. Journal of Dental Research. 2012 Aug; 91(8):736-44. https://doi.org/10.1177/0022034511435260. PMid:22318372.

22. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, De Crombrugge B. The novel zinc-finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002 Jan 11; 108(1):17-29. https://doi.org/10.1016/S0092-8674(01)00622-5.

23. Nakamura A, Dohi Y, Akahane M, Ohgushi H, Nakajima H, Funaoka H, Takakura Y. Osteocalcin secretion as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells. Tissue Eng Part C Methods. 2009; 15:169-80. https://doi.org/10.1089/ten.tec.2007.0334. PMid: 19191495.

24. Qin C, Brunn JC, Cadena E, Ridall A, Tsujigiiwa H, Nagatsuka H, Nagai N, Butler WT. The expression of dentin sialophosphoprotein gene in bone. J Dent Res. 2002; 81:392-94. https://doi.org/10.1177/154405910208100607. PMid: 12097430.

25. Massa LF, Ramachandran A, George A, Arana-Chavez VE. Developmental appearance of dentin matrix protein 1 during the early dentinogenesis in rat molars as identified by high-resolution immunocytochemistry. Histochem Cell Biol. 2005; 124:197-205. https://doi.org/10.1007/s00418-005-0009-9. PMid: 16049693.

26. Teti G, Salvatore V, Ruggeri A, Manzoli L, GESI M, Orsini G, Falconi M. In vitro reparative dentin: A biochemical and morphological study. Eur J Histochem. 2013; 57:e23. https://doi.org/10.4081/ejh.2013.e23. PMid: 24085272, PMCid: PMC3794354.

27. Bakopoulou A, Leyhausen G, Volk J, Tsifisoglou A, Garefis P, Koids P, Geurtsen W. Comparative analysis of in vitro osteo/odontogenic differentiation potential of human Dental Pulp Stem Cells (DPSCs) and Stem Cells from the Apical Papilla (SCAP). Arch Oral Biol. 2011; 56:709-21. https://doi.org/10.1016/j.archoralbio.2010.12.008. PMid: 21227403.

28. Zhao L, Hantash BM. TGF-β1 regulates differentiation of bone marrow mesenchymal stem cells. Vitam Horm. 2011; 87:127-41. https://doi.org/10.1016/B978-0-12-386015-6.00042-1. PMid: 22127241.

29. Jadlowiec JA, Zhang X, Li J, Campbell PG and Sfeir C. Extracellular matrix-mediated signaling by dentin phosphophoryn involves activation of the Smad pathway independent of bone morphogenetic protein. The Journal of Biological Chemistry. 2006; 281(9):5341-47. https://doi.org/10.1074/jbc.M506158200. PMid: 16326713.