Comparative evaluation of platelet-rich fibrin, platelet-rich fibrin + 50 wt% nanohydroxyapatite, platelet-rich fibrin + 50 wt% dentin chips on odontoblastic differentiation - An in vitro study-part 2

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Abstract

Aim: The purpose of this study was to investigate the effects of platelet-rich fibrin (PRF) modified with bioactive radiopacifiers—nanohydroxyapatite (nHA) and dentin chips (DC) on odontoblastic differentiation in human dental pulp cells (HDPCs).

Subjects and Methods: PRF was modified with 50 wt% of nHA (G bone-SHAG31, Surgiwear Company) and 50 wt% of DC. HDPCs differentiation and mineralization by the groups ([Group A - Control (Dimethyl sulfoxide), Group B - PRF, Group C – PRF + nHA, Group D – PRF + DC]) were assessed. ELISA was done to quantify the interleukin (IL)-6 and IL-8 cytokine expression. The odontoblastic differentiation was determined by the expression of odontogenesis-related genes and the extent of mineralization using alizarin red S staining.

Statistical Analysis Used: One-way ANOVA with post hoc Tukey-honestly significant difference tests were applied to assess the significance among various groups.

Results: The level of inflammatory cytokines (IL-6 and IL-8) expression by Group D (PRF + 50 wt% DC) was higher compared to Group B (PRF) and Group C (PRF + 50 wt% DC). Group C (PRF + 50 wt% nHA) induced more mineralization nodules compared to other groups. The integrated density value for the DSPP and DMP-1 protein expression by Group C (PRF + 50 wt% nHA) and Group D (PRF + 50 wt% DC) was higher compared to Group B (PRF).

Conclusions: The results suggest that the addition of bioactive radiopacifiers into PRF has a synergistic effect on the stimulation of odontoblastic differentiation of HDPCs, hence inducing mineralization.

Keywords: Cytokines expression, dentin chips; human dental pulp cells; nanohydroxyapatite; odontoblastic differentiation; platelet rich fibrin

INTRODUCTION

The modern concept of tissue engineering came in the late 1980s and one of the key components of this modern concept is the utilization of synthetic biodegradable materials as a scaffold to hold ex vivo expanded tissue cells.[1] The scaffold provides a three-dimensional environment for cells to attach and grow, therefore mimicking the in vivo condition. Current studies have been focused on the use of an autogenous material like platelet concentrates, which provides an osteoconductive scaffold along with growth factors to stimulate the patient’s own cells toward a regenerative response.[2] The recent reports have shown

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that the use of platelet-rich fibrin (PRF), an autologous fibrin matrix along with bioceramic materials, is beneficial for the endodontic management of an open apex.\textsuperscript{[3,4]} The study conducted by Woo et al. revealed that the combination of bioactive material (mineral trioxide aggregate [MTA]) and PRF promotes the differentiation of human dental pulp cells (HDPCs) into odontoblast like cells and the effect of combination is synergic compared with the effect of PRF or MTA alone.\textsuperscript{[4]}

One of the clinical success of apexification/regenerative procedure is the precise placement of biomaterial inside the root canal, which allows cell migration into the scaffold when contact with live tissue or blood at the apical interface.\textsuperscript{[5]} Our previous study demonstrated that the addition of bioactive material (tricalcium phosphate [TCP] and DC) to PRF offers an interesting and potential clinically useful modality to the clinician by making it as traceable material.\textsuperscript{[6]} Along with that, these bioactive materials have been proven to be noncytotoxic when tested along with L929 mouse fibroblast cell lines.\textsuperscript{[6]}

This original research is the continuation of our previous study and purpose is to investigate the effects of PRF modified with bioactive radiopacifiers–nHA and dentin chips (DC) on odontoblastic differentiation in HDPCs by quantifying proinflammatory cytokines, expression of odontoblastic genes, and mineralization potential.

**SUBJECTS AND METHODS**

The study was approved by the authorities of the Institutional Ethical Committee (R.C.No: 0420/DE/2016) and followed the guidelines of the National research committee (ICMR).

**Preparation of samples**

From 1 mg/ml stock solutions of PRF extract, nHA (G bone-SHAG31, Surgiwear company) and DC extracts, further concentrations (100 μg/ml, 10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml) were prepared by serial dilution.

- Group A – dimethyl sulfoxide (Control)
- Group B – PRF
- Group C – PRF + nHA
- Group D – PRF + DC.

**Primary human dental pulp cells culture**

Freshly extracted, impacted human third molars were collected from patients between the ages of 18 and 25 years who were treated in the Department of Oral Surgery. Immediately after extraction, the molars were stored in phosphate-buffered saline. The extracted teeth were split open, and the pulp tissues were removed under sterile conditions, minced and transferred to 60-mm culture dishes for enzymatic cell isolation in a solution of 3 mg/ml collagenase type I and II containing medium for 60 min at 37°C in an incubator. The suspended cells were centrifuged at 1500 rpm for 10 min. The single-cell suspensions were resuspended in 100-mm culture dishes containing Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C under 5% CO2 in air. After confluence, HDPCs in the 3–6 passages were used in this study.

**Evaluation of expression of inflammatory cytokines**

Cells were incubated overnight at 4°C with interleukin (IL)-6 and IL-8. After the contents from ELISA plate was disposed and dried, then exposed to1 mg/ml of samples (Group A, Group B, Group C, and Group D). Further, these were incubated for 2 h at room temperature. The levels IL-6 and IL-8 were determined using enzyme-linked immunosorbent assay kits (R&D Systems Inc, Minneapolis, MN). In all cases, a standard curve was constructed from the standards provided by the manufacturer. The absorbance was measured at 450 nm within 20–30 min.

**Evaluation of expression of odontogenic protein**

Cell lysates (5 ul) were placed in an NP-40 lysis buffer (Thermo Fisher Scientific, Waltham, MA), and protein concentrations were detected using a Bradford method.\textsuperscript{[7]} Proteins were separated by 12% polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane (Santa Cruz, San Diego, CA) according to the standard procedures. The membrane was blocked in 5% nonfat dry milk for 1 h and was incubated with 20 ml of primary antibodies of 1:2000 dilution (Anti-DMP1, Anti-DSPP, Anti-STRO-1, and β-Actin antibody) for 4 h at room temperature. After incubation with the specific peroxidase-coupled secondary antibodies (Thermo Scientific) for 1 h, the blotted bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Evaluation of mineralization activity by Alizarin red staining**

For the mineralized nodule assay, HDPCs were cultured with 1 mg/ml of samples and incubated overnight. Then the cells were fixed in 10% (v/v) formaldehyde at room temperature for 30 min. The monolayers were then washed with de-ionized water before the addition of 10 ml of 100 mM ARS (pH 4.1) per well and were incubated at room temperature for 45 min. The cells were then washed with distilled water and allowed to dry. Calcified nodules, which appeared bright red in color, were identified through light microscopy.

**RESULTS**

The values were recorded for each group, and values were analyzed statistically by one-way analysis of variance (for
Expression of inflammatory cytokines
IL-6 cytokine expression was significantly greater in Group B (PRF), Group C (PRF + nHA), Group D (PRF + DC) compared to Group A (Control) \( (P < 0.05) \), among which the IL-6 score was highest for Group D (PRF + DC). For IL-8 cytokine expression scores, there was no significant difference among Group B (PRF), Group C (PRF + nHA) compared to Group A (Control), except for Group D (PRF + DC) [Graph 1 and Table 1].

**Effect on odontoblastic differentiation**
The addition of bioactive radiopacifiers up-regulated the protein of DSPP and DMP-1 (Group D [PRF + DC] followed by Group C [PRF + nHA]) to a greater extent than the increase in expression seen with PRF alone (Group B [PRF] and Group A [Control]) [Figure 1 and Graph 2].

**Characterization of stem cell**
The addition of PRF and modified PRF were able to induce proliferation of STRO-1 positive immature HDPCs.

**Evaluation of mineralization activity by Alizarin red staining**
Alizarin red staining for calcium showed a highly positive stained mineralized nodule in Group B (PRF), Group C (PRF + nHA), and Group D (PRF + DC) when compared with the untreated HDPCs. Group C (PRF + nHA) recorded with greater number of positively stained mineralized nodules [Figure 2].
## DISCUSSION

Regenerative endodontics proved to have a significant effect on clinical practice where the primary focus is on providing effective therapies for regenerating functioning pulp tissue and, ideally, restoring lost dentinal structure.\(^8\) For accomplishing this differentiation of native stem cells into odontoblast-like cells is essential. The present study investigated the biological effect of the combination of nHA and DC along with PRF on odontoblastic differentiation and mineralization of HDPCs *in vitro*, respectively.

The PRF was prepared according to choukrons protocol, from which extract was prepared.\(^9,10\) The use of PRF addresses two parts of the triad for tissue regeneration growth factors and a scaffold. HDPCs were cultured in medium-containing bioactive radiopacifiers (nHA and DC) and PRF, without differentiation-inductive factors (ascorbic acid and b-glycerophosphate). nHA (G-Bone, nHA) is synthetic hydroxyapatite, derived from the bovine bone, which is available as granules and blocks.\(^11\) Synthetic apatite demonstrates good biological properties, including biocompatibility, bioactivity, lack of toxicity and a relatively high bioreosorbability.\(^12\)

DC obtained from freshly extracted tooth, composed of 35% of organic materials and 65% of mineral.\(^13\) It is considered to be a suitable material for use in bone tissue engineering, since it can serve as a scaffold and a rich source of growth factors which is capable of vascularization and angiogenesis.\(^14\) It has been used in several forms, which includes extracted noncollagenous dentin proteins, dentin particles or regular dentin (tooth ash, RD), deproteinized dentin (DepD) and demineralized dentin (DemD).\(^15\) Since the radiopacifiers used were alloplastic materials, the inflammation produced by samples was evaluated by the level of IL-6 and IL-8, which was quantified through ELISA. The primary cultured HDPCs treated with PRF and PRF in combination with radiopacifiers increased the release of IL-6, and IL-8, which are well-known to be key proinflammatory mediators in the pathogenesis of inflammatory condition. Group D (PRF + DC) showed maximum IL-8 cytokine expression compared to other three groups (Group A-control, Group B-PRF, Group C-PRF + nHA). The dentin used for the study was without any modifications like deproteinization. The organic component of dentin, which contains acidic proteins, might have caused the inflammation of HDPCs. This result was in accordance with a study conducted by Brandell *et al.*, where demineralized dentin, hydroxyapatite and DC used as a filling material in the apical 2 mm of canals with perforated apexes.\(^16\) After 6 months, the samples with apical plugs of hydroxyapatite had more hard tissue formation and less inflammation than the others.

The differentiation from dental pulp cells into odontoblasts was evaluated from the expression of genes associated with odontoblastic differentiation, i.e; DSPP and DMP-1, and mineralization formation. The density of protein bands formed was measured by an Image J Software [Java-based image processing program developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin) in 2010]. When measured the integrated density value to quantify the amount of proteins (DSPP and DMP-1) expressed, the results showed that Group C (PRF + nHA) and Group D (PRF + DC) increased the protein expression of DSPP and DMP-1 of HDPCs compared when PRF used alone as in Group B (PRF).

This might be due to their hydrophilicity together with the alkaline pH and calcium ion released as a result of their hydration reaction as explained by Mohamed and Fayad who studied the effect of different bioactive materials (nHA, MTA, calcium-enriched mixture) on the odontogenic differentiation potential of dental pulp stem cells.\(^17\) Similarly, a study conducted by Liu *et al.* observed mineralized tissue formation with the DPSC and demineralized dentin matrix (DDM) & DPSC and hydroxyapatite–tricalcium phosphate combination.\(^18\) The mineralized tissue of the DPSC + DDM combination stained more positive for DSPP, similar to the dentin tissue.

### Table 1: Intergroup comparison of Mean IL 6 and IL 8 Values

| Group | IL-6 Mean | IL-6 Std. Deviation | IL-6 F value | IL-6 P value | IL-8 Mean | IL-8 Std. Deviation | IL-8 F value | IL-8 P value |
|-------|-----------|---------------------|--------------|--------------|-----------|---------------------|--------------|--------------|
| A     | 0.111     | 0.007               | 227.07       | <0.001       | 0.110     | 0.007               | 27.98        | 0.004        |
| B     | 0.164     | 0.005               |              |              | 0.176     | 0.001               |              |              |
| C     | 0.182     | 0.008               |              |              | 0.188     | 0.007               |              |              |
| D     | 0.267     | 0.001               |              |              | 0.315     | 0.045               |              |              |
in vitro and concluded that its expression was characteristic of less well-differentiated cells.[23]

Alizarin red assay results showed highly positive stained mineralized nodules for all groups except the control group. Group C (PRF + nHA) had more positively stained mineralized nodules compared to Group D (PRF + DC). The increased differentiation potential of Group C (PRF + nHA) might be attributed to the difference in their chemical composition and surface topography. Evidence shows that surface properties of grafts play an important role in their biological properties and function under in vitro and in vivo conditions.[22] nHA is similar to the natural mineral phase in dental hard tissue building unit (calcium and phosphates) and have special biological and physicochemical properties.[23]

The hydration reaction of nHA with physiological fluids results in appropriate Ca$^{2+}$ concentrations and alkaline pH (10–12) that have been shown to favor cell proliferation and differentiation, forming strong mineralized interface.[24] Wang et al. developed a hybrid composite of MSC sheets with nanoscale hydroxyapatite (nHA) and autologous PRF granules for enhanced bone formation within a critical-sized rabbit cranial defect.[25] The percentage of new bone in the MSC/PRF group (35.7 ± 5.1%) was found to be significantly higher than that in the MSC (18.3 ± 3.2%; P < 0.05) and empty defect groups (4.7 ± 1.5%; P < 0.05).

Furthermore, the osteoinductive and osteoconductive properties vary according to the size and shape of dentin particles.[19] DepD and DemD samples had a smoother and more homogenous surface than RD samples.[15] This is especially important because smoother surfaces are more suitable for proliferation and differentiation of osteoblasts, while rougher surfaces can trigger a response by macrophages and subsequent bone loss.[19]

CONCLUSIONS

The combination of these bioactive materials (nHA and DC) with PRF has got synergistic effect than when PRF used alone and has promising properties to serve the best for the future. Considering the limitations of this study, further studies must be carried out with different methods of preparation of these radiopaque materials (nHA and DC) for the better properties. And also, experimental studies are needed to elucidate the anti-inflammatory effect of PRF on the regulation of odontoblastic differentiation.

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Conflicts of interest

There are no conflicts of interest.

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