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Regenerative Capacity Of Resolvin D2 On Stem Cells Of Apical Papilla

Research Article

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Abstract

Introduction: Regenerative endodontics is based on the triad of tissue engineering which has three components; stem cells, biomimetic scaffold and biomimetic growth factors which are introduced in to the canal space so as to induce pulp tissue regeneration and root maturation. Resolvins are lipid mediators that are released during the resolution phase of inflammation and regulate tissue repair. Thus the present study was conducted with the aim to evaluate whether resolvin D2 (RvD2) is capable of inducing hard tissue formation by its action on stem cells of apical papilla and compare it to concentrated growth factor (CGF).

Materials and Methods: The root apical papilla tissues were carefully isolated from the root apex. Enzymatic separation was used for the cells of the primary apical papilla. The cells were subjected to the three groups namely RvD2, CGF and a combination of CGF and RvD2. Mineralized nodule formation was analyzed by alizarin red staining and dentin matrix protein 1 secretion was analyzed using ELISA test.

Result: The combination of RvD2 and CGF had the highest values for mineralized nodule formation and DMP1 secretion followed by CGF. These were statistically more significant than the control group. However the results for RvD2 alone did not exceed that of CGF.

Conclusion: Although RvD2 possesses regenerative potential and is capable of inducing stem cells of apical papilla for formation of mineralized hard tissue, its potential by itself does not surpass that of CGF. However the combination could be a promising new strategy in the management of immature necrotic permanent teeth.

Keywords: Concentrated Growth Factor; Lipid Mediators; Regeneration; Resolvin D2; Stem Cells.

Introduction

Traditionally, immature permanent teeth with necrotic pulp were treated by apexification using calcium hydroxide which helped in generating a hard tissue barrier or by placing an apical plug using MTA, biodentine or calcium hydroxide followed by root canal filling [1, 2]. However, apexification does not restore the vitality of the tooth and neither does it help in maturation of the root by thickening of the walls or closure of the apex. It was in 2001 that Iwaya et al introduced the term revascularisation [3]. Regenerative endodontics is based on the triad of tissue engineering which has three components; stem cells, biomimetic scaffold and biomimetic growth factors which are introduced into the canal space so as to induce pulp tissue regeneration and root maturation [4].

Stem cells from the apical papilla (SCAP) are related to developing roots and have been shown to be a promising tool with the ability for self-renewal, multilineage differentiation and regenerative potential [5]. They play a pivotal role in regenerative endodontics which is currently based on the cell homing approach [6].

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Growth factors are proteins that bind to the receptors present on the target cells and induce the migration, proliferation and differentiation of stem cells [7]. Scaffold is a three dimensional matrix that helps in cell adhesion, interaction and deposition of the extracellular matrix. These scaffolds can either be biological such as Platelet derived factors or artificially synthesized [8].

Resolvins are lipid mediators that are released during the resolution phase of inflammation and regulate tissue repair. Resolvin D2 (RvD2) has been shown to enhance post ischaemic revascularization while resolving inflammation by promoting apoptosis of polymorphonuclear neutrophils (PMSs), controlling bacterial sepsis as well as promoting arteriogenesis [9]. It has also been shown to have defensive properties against P. gingivalis which causes periodontal bone loss. Studies have shown that RvD2 is capable of regulating the RANKL/OPG ratio [10]. RvD2 is also known to be capable of inhibiting transient receptor potential channels present in sensory neurons and is thus capable of reducing postoperative pain [11]. Siddiqui et al in a study in 2019 showed that RvD2 induced resolution of periapical inflammation and promoted periapical healing in rats. Further, calcified canals apices were observed in RvD2 treated canals with apex closure [12]. However in their study the regenerative capacity of RvD2 was observed against a placebo control.

Previously our team has a rich experience in working on various research projects across multiple disciplines [13-27]. Now the growing trend in this area motivated us to pursue this project.

Thus the present study was conducted with the aim to evaluate whether resolvin D2 is capable of inducing hard tissue formation by its action on stem cells of apical papilla and to compare it to the regenerative potential of concentrated growth factor (CGF) and to evaluate whether the combination of concentrated growth factor and RvD2 has any synergistic effect.

Materials and Methods

Chemicals

The test drug RvD2 was purchased from Santa Cruz BioTech and CGF was obtained commercially. All media, broth and other chemicals required for the project were obtained from Himedia Laboratories (Anna Nagar, chennai).

Stem Cell Separation

The root apical papilla tissues were carefully isolated from the root apex. Enzymatic separation was used for the cells of the primary apical papilla. SCAPs were seeded on 100mm plates at 20,000 cells/cm2. The medium was changed to a growth medium after analyzing that it reached the 90% confluence. After six days, the cells were washed five times with phosphate-buffered saline (PBS) and cultured in a serum-free medium for 24 hours. The media was collected after centrifugation at 1,000rpm for 10 mins to remove the cellular debris and filtered through a 0.22µm filter. The samples were concentrated, air-dried, re-dissolved in triethylammonium bicarbonate, and reduced with dithiothreitol at 55°C for one hour. Next, iodoacetamide was added to the samples, which were maintained for one hour at room temperature in the dark. The protein concentration was determined.

Cell Culturing

The separated cells were cultured in Dulbecco's modified Eagle's medium (Himedia Laboratories), supplemented with 10% fetal bovine serum and 100 U/ml penicillin-G and streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. The characterization of SCAPs was analyzed for other assays.

Grouping

The SCAPs were treated with different drugs.

Group 1: Cells treated with concentrated growth factor (1µg/ml).

Group 2: Cells treated with Resolvin D2 100nM (1µg/ml).

Group 3: Cells treated with the combination of concentrated growth factor and resolvin D2 100 nM.

Group 4: Negative control group (NC).

For alizarin red staining to check the mineralized nodule formation and to quantify the DMP-1 levels the cells were incubated with inducing medium for three weeks.

Alizarin Red Staining (ARS)

The culture medium was removed from each well and gently washed the cells three times with 1xPBS. The cells were fixed in 4% formaldehyde for 15 minutes at room temperature. The fixative was removed and the cells were washed three times with distilled water. The distilled water was removed completely and 1mL of 40 mM Alizarin red stain was added per well. It was incubated at room temperature for 20-30 min with gentle shaking followed by removal of the dye and washing of the cells five times with distilled water. The test plates were stored at -20°C prior to dye extraction. 800µL of 10% acetic acid was added to each well of a 6-well plate and incubated at room temperature for 30 minutes with shaking (200µL per well for a 24-well plate). The cells were collected using a cell scraper and transferred in 10% acetic acid to a 1.5-mL microcentrifuge tube and vortexed for one min. The samples were sealed with parafilm heated at exactly 85°C for 10 minutes and then incubated on ice for five minutes. The centrifugation was done at 20,000g for 15 minutes. After centrifugation, 500µL of the supernatant was transferred to a new tube and 200µL of 10% ammonium hydroxide was added to neutralize the acid. 150µL of the sample was added per well in a 96-well plate and the absorbance was read at 405 nm with a plate reader.

Quantification Of Dentin Matrix Protein -1 (DMP 1)

The adherent cells were detached with trypsin and then collected by centrifugation and washed three times in PBS. Cells were resuspended in PBS and subjected to ultrasonication for three times. Alternatively, freezing and thawing the cells was done and repeated for three times. Centrifugation was done at 1000×g (or 3000rpm) for 15 minutes at 2-8°C to remove cellular debris. The cell were coated into the wells at a density of 2x104 and then 100µL of standards or samples was added to the appropriate well in the antibody pre-coated Microtiter Plate. Next 100µL of PBS (pH 7.0-7.2) was added in the blank control well and 100µl of

primary Ab (DMP-1) 1:1000 dilution was added to each well and after 1-1.5 hours the primary antibody was removed, and the wells were washed 3 times with PBS. Then 100µL of secondary antibody (anti-rabbit horseradish protein) was added to each well and incubated for 1-1.5 hours. The secondary antibody was discarded and washed three times with PBS. After washing for 5 times, 50 µL of tetramethylbenzidine (TMB) substrate was added to each well including blank control well, subsequently. It was covered and incubated for 10-15 minutes at 20-25°C followed by addition of 50µL of 1M sulphuric acid Solution to each well including blank control well. The Optical Density (O.D.) at 450nm was read using a microplate reader immediately.

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference test. Intercomparison groups analysis was done using the SPSS software version 22.0.

Results

It was observed that RvD2 induced the stem cells for the deposition of mineralized nodules (Table 1 and fig 1 and 2) (0.34 ± 0.027) and DMP-1 secretion (Table 2 and Fig 2) (8.267 ± 1.305). This was significantly higher than the negative control group. However, the highest values were observed for the group of cells treated by the combination of CGF and RvD2 followed by the group of cells treated only by CGF. These values were significantly higher when compared to the RvD2 treated group. However, there was no significant difference seen between the CGF group and the group of cells treated by the combination of two.

Discussion

Our institution is passionate about high quality evidence based research and has excelled in various fields [17, 28-37].

In the present study, the regenerative capacity of RvD2 was tested by identifying the ability to form mineralized hard tissue. This was demonstrated by alizarin red staining and DMP1 secretion by stem cells of apical papilla.

Dentin matrix protein 1 is involved in maturation of odontoblasts and osteoblasts, biomineralization of bone and dentin as well as hydroxyapatite formation [38, 39]. DMP1 is a key protein that induces odontogenesis. Over expression of DMP1 by pluripotent cells acts as a signal for differentiation of cells [40]. Hence DMP1 expression by SCAP was analysed. The concentration of RvD2 was set at 100nM based on the results by siddiqui et al. The results of the present study are in accordance to the study conducted by siddique et al where it was demonstrated that RvD2 increased expression of DMP1 in dental pulp stem cells. However

Table 1. Quantification of Alizarin red staining expressed as mean and standard deviation.

Groups	N	Mean	Std. Deviation
NC	3	0.134	0.021
CGF	3	0.587	0.022
RvD2	3	0.34	0.027
CGF+RvD2	3	0.637	0.02
Total	12	0.424	0.212

Table 2. Quantification of DMP1 secretion by SCAP expressed as mean and standard deviation.

Groups	N	Mean	Std. Deviation
NC	3	6.567	0.833
CGF	3	8.833	0.586
RvD2	3	8.267	1.305
CGF+RvD2	3	10.733	1.159
Total	12	8.6	1.777

Figure 1. ARS quantification of SCAPs cells in control and experimental groups. The results expressed as Mean ± SEM (n = 3). ***p<0.001 statistically significant as compared with NC. RvD2 is statistically significant (ap<0.01) as compared with the CGF treated group. CGF+RvD2 statistically significant (#p<0.05) as compared with RvD2.

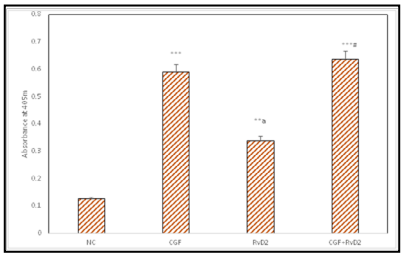


Figure 2. DMP-1 quantification of SCAPs cells in control and experimental groups. The results expressed as Mean ± SEM (n = 3). *P<0.05;P<0.01; ***P<0.001 statistically significant as compared with NC. RvD2 is statistically significant (ap<0.05) as compared with the CGF treated group. CGF+RvD2 statistically significant (#P<0.05) as compared with RvD2.**

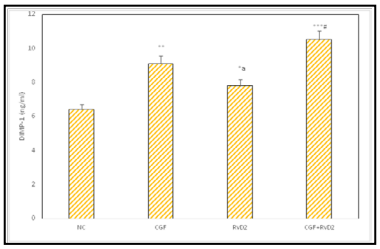
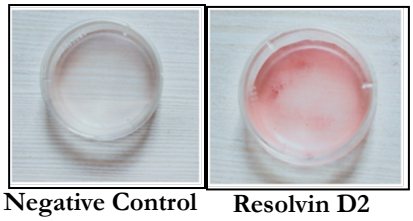
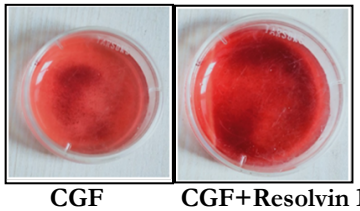


Figure 3. Alizarin red staining of SCAP cells.



Negative Control **Resolvin D2**



CGF **CGF+Resolvin D2**

in the present study, CGF showed better results than RvD2 and the two of them had a synergistic effect. Similarly observations were made with respect to mineralized tissue formation [12]. This could be due to the diversity of growth factors released by CGF. CGF has a complex three□dimensional structure with a high density of platelets and nucleated cells and factors such PDGF□BB, IGF□1, Transforming growth factor TGF□β1, fibroblast growth factor bFGF and vascular endothelial growth factor VEGF [41].

Derived from omega 3 fatty acids namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as well as docosapentaenoic acid (DPA); resolvins are specialised pro resolving mediators which restore normal cellular function after tissue injury [42]. It was from the self resolving exudates of murine during the resolution phase of acute inflammation, that RvD2 was first isolated [43]. The biosynthesis involves 17-lipoxygenation of DHA to 17S-hydroperoxy-DHA (17S-HpDHA). This is then further transformed to a 7(8) epoxide-containing intermediate in leukocytes via 5-lipoxygenase (LOX) enzymatically, followed by enzymatic hydrolysis to form RvD2. RvD2 can be endogenously found in human serum, plasma [44], adipose tissue [45], placenta [46], breast milk [47], sepsis patients [48] and lung [49].

Resolution at cellular level involves cessation of PMN entry in to the tissue and elevated phagocytosis of apoptotic PMN by macrophages. In human macrophages, RvD2 stimulates phagocytosis and efferocytosis in a DRV2-dependent manner. Resolvin acts by binding to a specific G protein coupled receptor GPR18/DRV2 which activates the cyclic AMP-PKA pathway and phosphorylation of STAT3 and increases phagocytosis mediated bacterial clearance [50].

Mizraji et al found that RvD2 prevented alveolar bone loss in murine periodontitis and prevented destructive immunity. It prevented osteoblast-mediated and T-cell-mediated signaling of osteoclast formation by RANKL leading to alveolar bone loss [10]. Proresolving mediators have shown promising results in the healing of apical periodontitis with reduction in the size of the periapical lesion and recalcification of bone [12, 51]. Resolvins can be administered in active inflammatory lesions without any deleterious effects, the bacterial load is reduced and there is no increase in disease activity [52].

One of the possible reasons for the regenerative potential of RvD2 is because of sustained vitality of migrated cells that could be a direct effect of reduction in inflammation, control of bacterial sepsis and stimulation of angiogenesis. The present study leaves a future scope for experimenting with higher concentrations of RvD2 to analyse if there is any increase in the regenerative potential and to determine weather RvD2 could be incorporated in to a scaffold which would induce vascularized tissue formation in immature necrotic permanent teeth.

In 2019, shi et al customised a polycaprolactone PCL graft with aspirin triggered RvD1 by electrospinning for vascular regeneration [53]. A similar concept can be adopted for generating synthetic scaffolds for regenerative endodontics that incorporate RvD2.

Conclusion

Although resolvin D2 possesses regenerative potential and is capable of inducing stem cells of apical papilla for formation of mineralized hard tissue, its potential by itself does not surpass that of CGF. However, owing to its good antimicrobial effect and anti-inflammatory properties, RvD2 can be used in combination

with CGF or as a component of a synthetic scaffold in regenerative endodontics to enhance their bioactivity. This combination could be a promising new strategy in the management of immature necrotic permanent teeth if it translates from preclinical studies in to successful clinical trials in future.

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