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Research Article

Exploring the Therapeutic Potential of Curcumin for Periodontal Regeneration

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Abstract

Periodontitis is a common inflammatory condition that results in destruction of tooth supporting tissues. Studies have demonstrated the regenerative potential of mesenchymal stem cells and biomaterials that induce healing and promotion of repair of the periodontal tissue.

Recently, natural compounds have been identified as good candidates for drug development due to their immunomodulatory and healing properties. Specifically, curcumin has been shown to have anti-cancer, antibiotic and anti-inflammatory functions and has been reported safe and efficient for treatment of many diseases. In this study we investigate curcumin and ones of its metabolites, tetrahydrocurcumin, to identify a potential natural candidate that can contribute to the healing of periodontal tissue and promote its regeneration.

In this study, human PDL and Gingival epithelial cells were treated with curcumin and tetrahydrocurcumin. Using RNA sequencing we investigated the pathways that were affected in these cell types. We show that curcumin and tetrahydrocurcumin promote Wnt signaling pathway, upregulate TGF-beta regulation of extracellular matrix. Curcumin promotes organization of extracellular matrix in PDL cells and cell migration, differentiation, and angiogenesis in gingival epithelial cells.

The results of this study suggest that curcumin and its metabolite exhibit anti-inflammatory and have regenerative potential on PDL and gingival epithelial cell. Particularly, curcumin can be a low-cost, well-tolerated and effective natural candidate for therapeutic approaches in periodontal tissue. Further *in vivo* screening of this compound is required to confirm the optimum biological activity and to harness its full potential in clinical application.

Keywords: Tissue regeneration, Periodontitis, PDL, Gingival epithelial cells, Curcumin, Tetrahydrocurcumin

Introduction

Periodontitis, the inflammation of the supporting tissues of the dentition, is one of the most predominant diseases affecting the oral cavity. The goal of periodontal treatment is to control inflammation and restore structure and function of periodontal tissues including alveolar bone, gingiva, and the Periodontal Ligament (PDL). An efficient treatment has long been one of the main aims of dentistry since disease progression can lead to tooth morbidity and loss.

Therapeutic potential of mesenchymal stem cells has been shown in regeneration of periodontal tissues [1-6]. Recognition of tissue heterogeneity and various populations of resident stem cells in periodontal tissue has opened new avenues for effective regenerative treatment for periodontitis [7-12]. Various advanced biomaterials can assist in this process by serving as delivery vehicles or scaffolds [13-15]. Despite their many advantages, use of stem cell-based therapies has numerous issues including immune rejection, long term storage, transport and overall cost [16]. Low toxicity, availability and affordability of natural compounds has rendered them an alternative to stem cell therapies in regenerative medicine. Additionally, natural compounds have anti-inflammatory, wound healing and antioxidant activity *in vivo* which contributes to their application in tissue regeneration [16-19]. Studies have shown effective use of herbal medicines in treatment of various conditions. For example, *Ithonia diversifolia* extract can be used for treatment of diabetes and wound healing

[20]. Plant-based antimicrobials can be applied against oral bacteria [21]. Acacia arabica, a blend of calcium, magnesium, and potassium has antibacterial activity in gingivitis [22-25].

Curcumin, a traditional natural herbal medicine has been proven to be a novel treatment to promote wound healing. Curcumin-derived compounds have shown antioxidant, anti-inflammatory, anticancer, antiviral, neurological, and immunological characteristics. Independent studies have shown that these compounds can promote regeneration in various tissues [26–28]. Tetrahydrocurcumin is one of the major metabolites of curcumin that exhibits similar pharmacological activities to curcumin but targets different signalling pathways and cellular responses [29-31].

Novel stem cell technologies and bioactive scaffolds have been promising to enhance the periodontal regeneration however, few studies have identified the signalling pathways affected following treatment with natural compounds in periodontal tissue.

In this study we investigate the effect of curcumin and tetrahydrocurcumin on human PDL and gingival epithelial cells to find candidates that can stimulate regenerative pathways in PDL and Gingival epithelial cells and be used as effective natural candidates in treatment of periodontitis.

Materials and methods

Cell culture

Primary Human PDL Cells (hPDL) used in these assays were a kind donation from Dr. Ana Angelova in King's College London. Original cell harvest experiments were undertaken with the understanding and written consent of each subject and in full accordance with World Medical Association Declaration of Helsinki (version 2002). The study was approved and followed the guidelines set by the Ethical Committee for human studies at King's College Hospital, King's College University of London. hPDL cells were used at passage [2-3]. These cells were expanded in DMEM high glucose, 10%FBS, 1% L Glutamine and 1% Penicillin/Streptomycin.

Primary Human Gingival Cells (HGEP) were purchased from CELLnTEC, advanced cell systems (CnT Gingival Epithelium Progenitors, Adult, Pooled). HGEP were used at passage [2-3]. These cells were maintained and expanded in specialized epithelial media by CELLnTEC, CNT-PRIME (#CNT-PR).

Viability

For each cell type 20000 cells/cm^2 were plated in triplicate 96 well plates and incubated in cell culture incubator (37°C, 5% CO_2) for 24h. We used a range of concentrations of curcumin and tetrahydrocurcumin (0.5-10 µM) to treat hPDL and hGEP cells. Dimethylsulfoxide (DMSO) was used as vehicle-only control in all the experiments. Cell viability was assessed by MTS Assay Kit (Cell Proliferation, Colorimetric, ab197010) after 24 hr following manufacturers instruction. A colorimetric plate reader (Thermo Multiskan Ascent 354 microplate reader) was used to read the absorbance at 490 nm. Normal distribution of results was tested with Shapiro-Wilk test. Statistical significance against 100% viability in Media was reported using one-way ANOVA and Dunnett's multiple comparisons in GraphPad Prism 8.3.0. Adjusted P value is reported in graphs according to New England Journal of Medicine guidelines: P<0.001 (***), P<0.002 (**), P<0.033 (*), P>0.12 (ns).

QPCR

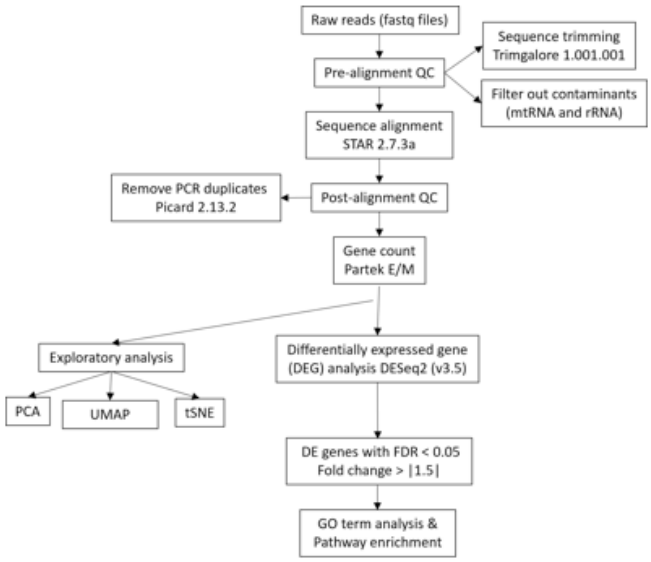
For each cell type 50000 cells were plated in triplicate in 24-well plates and incubated for 24 hrs (37°C, 5% CO_2/95% air, 100% humidity) using standard culture medium. Media only, DMSO (vehicle only) and Bio 50 nM (positive control for Wnt signalling pathway) from Sigma, St. Louis, MO, USA were used as controls. Following 24 hr treatment, cells were lysed with Trizol for extraction of RNA. RNA was reverse transcribed using random primers (M-MLV Reverse Transcriptase kit, Promega, Madison, WI, USA) according to the manufacturer's instructions. Gene expression was then assayed by real-time qPCR using Syber Green (Roche, Basel, Switzerland) on a Rotor-Gene Q cycler (Qiagen, Hilden, Germany) system. Beta-actin was used as housekeeping gene (Forward-GGCTGTATTCCCCTCCATCG, Reverse-CCAGTTGGTAACAATGCCTGT) and Axin2 as the read-out for Wntpathwayactivity(Forward-TGACTCTCCTTCCAGATCCCA, Reverse-TGCCCACACTAGGCTGACA. Reactions were performed in triplicate and relative changes to housekeeping gene expression were calculated by the 2−ΔΔC T method where CT is the threshold cycle. Groups were then analysed with one-way ANOVA followed up with multiple comparison tests in GraphPad Prism 8. Adjusted P value is reported in graphs according to New England Journal of Medicine guidelines: P<0.001 (***), P<0.002 (**), P<0.033 (*), P>0.12 (ns).

Bulk sequencing

PDL and gingival epithelial cells were seeded at the density of 50000 in triplicate and treated with freshly made curcumin and tetrahydrocurcumin for 24hr to assess the differentially expressed genes. RNA was isolated with Trizol and Qaigen Mini kit and after assessment of quality and quantity was sent for bulk RNA sequencing. We used the Partek RNA sequencing pipeline. All algorithms to analyse the data eres run with default settings, unless otherwise indicated. The quality of the sequencing reads was examined using Fast QC (v0.11.4) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw sequencing reads (100-nt, paired-end) were trimmed using Trimgalore (v1.001.001). (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Traces of ribosomal RNA and mitochondrial RNA were removed using the Bowtie2 (v2.2.5) 32. Reads were aligned to the human reference genome GRCh38 using STAR (v2.7.3a) aligner with multi-sample setting 33. Mapping and alignment quality were examined using FastQC. Duplicate reads were removed using

the Mark Duplicates function of the Picard tools (v2.17.11 (http://broadinstitute.github.io/picard/). Reads were annotated using the Partek E/M with Ensembl Transcripts release 104. Samples were then visualized and explored using unsupervised methods. Samples were clustered based on principle Component Analysis (PCA), UMAP, tSNE and Hierarchical clustering. Differentially Expressed Genes (DEGs) between controls vs. different components using DESeq 2 (v3.5)34. DEGs with FDR value<=0.05, Fold change=>1.5 were filtered out for further analysis. The analysis pipeline can be seen in the chart below. For gene ontologies Enrichr biological function and bioplanet pathways were used. For biological function, we used -log10 p value for the selected GO terms and graphed those with -log10 pvalue.1.5. For functional enrichment analysis G profiler was used with significance threshold set at Bonferroni correction and the term size set at 50.

Data will be deposited in the portal specified by the journal.



Results

Effect of curcumin and tetrahydrocurcumin on the viability of human PDL cells

To evaluate the impact of curcumin and tetrahydrocurcumin on PDL cells, we first investigated the effect of these compounds on viability of PDL cells. MTS viability assays were used after treatment of PDL cells with a range of curcumin and tetrahydrocurcumin concentrations (10 µM, 5 µM, 1 µM and 0.5 µM) for 24hours. Dimethylsulfoxide (DMSO) was used as vehicle-only control in all the experiments. At 10µM, 5µM and 1µM, treatment with tetrahydrocurcumin resulted in lower viability of PDL cells than the same concentrations of curcumin. Treatment with 0.5µM of both compounds resulted in more than 90% viability of PDL cells. As treatment with 1µM concentration with curcumin and tetrahydrocurcumin resulted in more than 75% viability of PDL cells, this was selected as the optimum concentration for further downstream analysis (Figure 1).

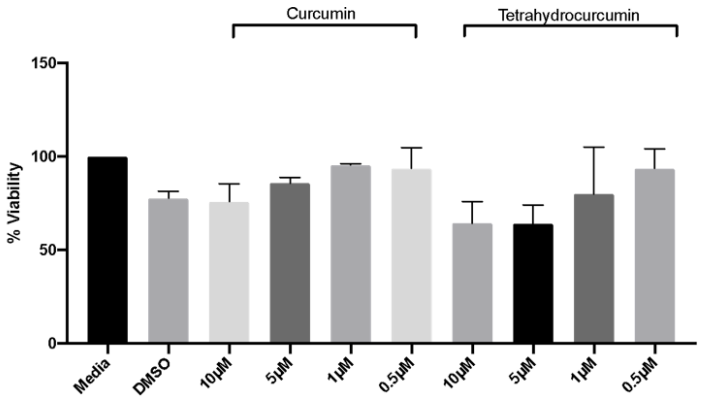


Figure 1: Viability of PDL cells after treatment with different concentrations of curcumin and tetrahydrocurcumin. MTS assay of PDL cells shows a reduction in cell viability with 10 µM Curcumin and an increase in cell viability with at 5 µM. Treatment with 1 µM and 0.5 µM results in the highest % viability. Treatment with 10 µM and 5-µM tetrahydrocurcumin results in the lowest level of viability in PDL cells. Treatment with 1 µM and 0.5 µM tetrahydrocurcumin results in increased cell viability.

Curcumin and tetrahydrocurcumin promote matrix organization and exhibit anti-inflammatory properties in PDL cells

After determining the suitable concentration of curcumin and tetrahydrocurcumin for the treatment of PDL cells, 1 µM of these compounds were used to investigate the Differentially Expressed Genes (DEG). Media and DMSO treatments were used as controls (supplementary). At FDR value<=0.05, Fold change=>1.5, treatment with curcumin as well as tetrahydrocurcumin resulted in more than 100 DEGs.

Curcumin showed a bigger impact on PDL cells with a higher number of upregulated genes. We used gene ontologies to investigate the biological functions and pathways that were associated with upregulated genes in PDL after treatment with curcumin and tetrahydrocurcumin. We found that some of the biological functions upregulated after curcumin treatment of PDL cells were regulation of extracellular matrix, regulation of apoptosis, membrane trafficking and interferon signalling. Biological functions associated with upregulated genes after treatment of PDL with tetrahydrocurcumin were regulation of extracellular matrix, regulation and ECM receptor interaction, angiogenesis, and prostaglandin biosynthesis.

There were 22 shared upregulated genes between treatment of PDL cells with curcumin and tetrahydrocurcumin. These shared genes were enriched in development, morphogenesis of anatomical structure, collagen fibril organization, cell adhesion and vasculature development. Gene ontologies for upregulated

genes are shown in Figure 2.

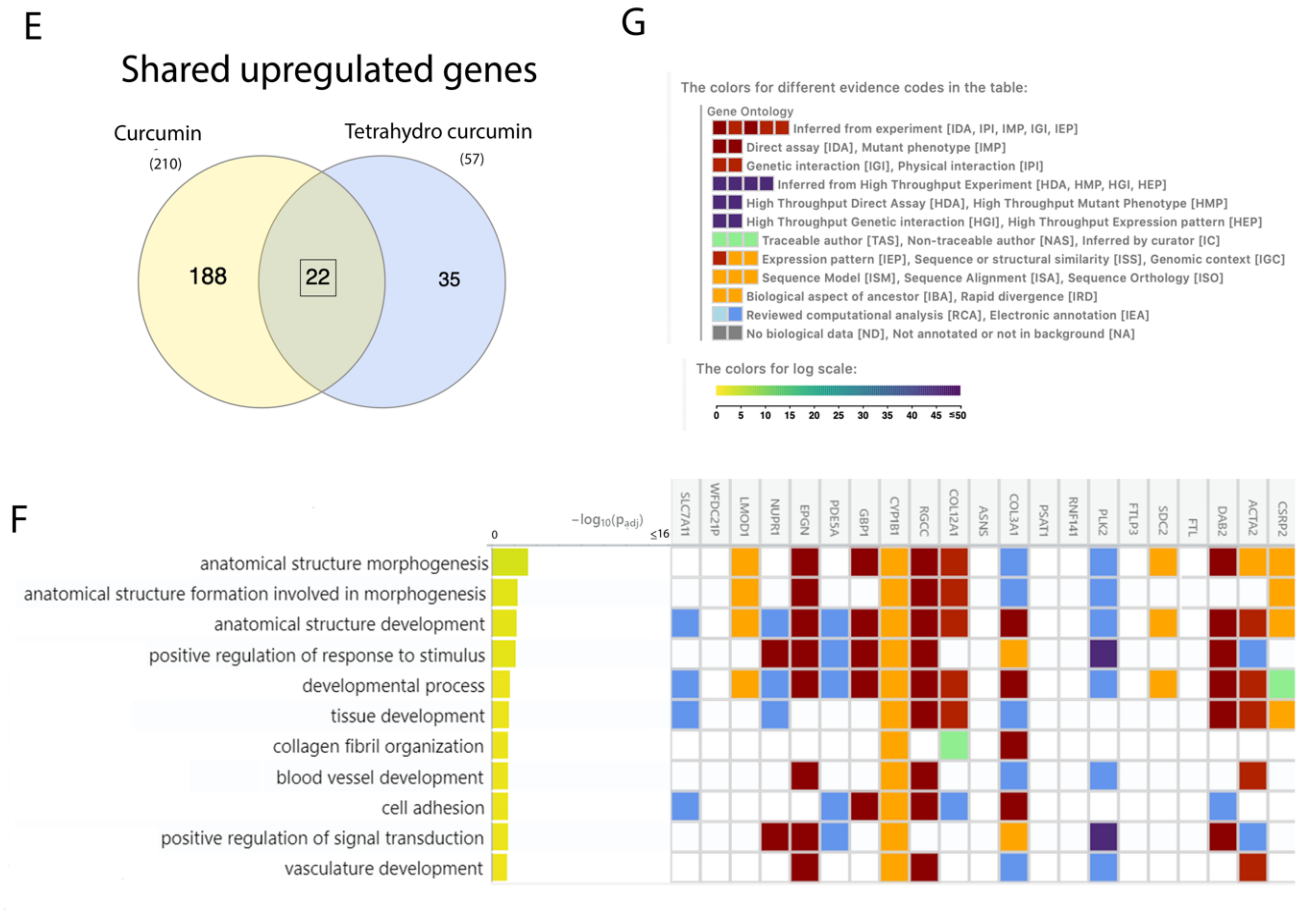


Figure 2: Differential gene expressions following treatment of PDL cells with curcumin and tetrahydrocurcumin. A) Volcano plots of differentially expressed genes after treatment of PDL cells with curcumin. B) Volcano plots of differentially expressed genes after treatment with tetrahydrocurcumin. C) Gene ontology of selected biological function in curcumin treatment at FDR value<= 0.05, Fold change=>1.5. D) Gene ontology of selected biological function in Tetrahydrocurcumin treatment at FDR value<=0.05, Fold change=>1.5. E) Venn diagram of number of unique and shared upregulated genes upon treatment of PDL cells with curcumin and tetrahydrocurcumin. F) GO enrichment analysis by g: Profiler of the shared upregulated genes after treatment with curcumin and Tetrahydrocurcumin. G) Graphical illustration guide.

Curcumin and Tetrahydrocurcumin promote Wnt signalling pathway in PDL cells

The Wnt signalling pathway is crucial in development and regeneration of many tissues in the oral cavity. Axin2 is a negative regulator and a down-stream target of this pathway and its expression is commonly used as a read-out of the level of Wnt signalling pathway activation [35,36]. Therefore, we asked if treatment with curcumin and tetrahydrocurcumin would affect the Wnt signalling pathway in PDL cells. Treatment with 1 μM curcumin as well as tetrahydrocurcumin resulted in upregulation of Axin2 in PDL cells. We used DMSO as a negative control and For Wnt signaling pathway, inhibitor 6-Bromoindirubin-3-Oxim (BIO) as a positive control [37].

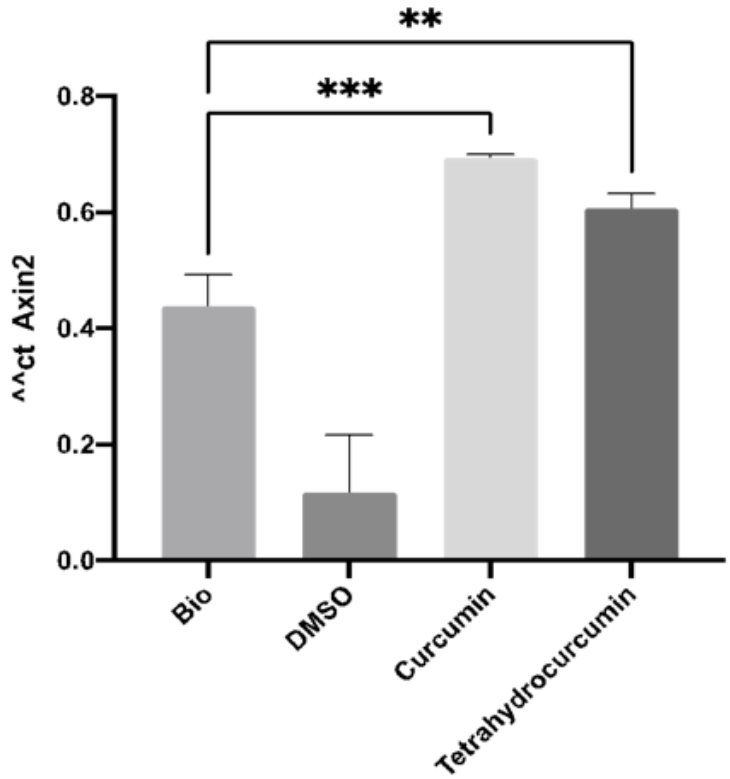


Figure 3: Effect of curcumin derived compounds on Wnt signalling pathway in PDL cells. PDL cells were treated with DMSO, 50 nM Bio and 1μM concentration of curcumin and tetrahydrocurcumin were used for the treatment of PDL cells. Increased Axin2 expression was detected after treatment with both compounds (*P* value<0.0001)

Curcumin and tetrahydrocurcumin promote cell migration and stem cell differentiation in gingival epithelial cells

Gingival Epithelial Cells (hGEP) serve as the barrier against pathogens and foreign bodies in periodontal tissue. We thus investigated the effect of curcumin compounds on these cells. To ensure that 1μM concentration used for PDL cells was not toxic for these cells, viability of hGEP was confirmed after treatment with selected concentrations of curcumin and tetrahydrocurcumin.

Similar to PDL cells, treatment of gingival epithelial cells with 1μM curcumin resulted in higher level of Axin2 induction in comparison to tetrahydrocurcumin. However, the impact of these compounds on differentially expressed genes was substantially different compared to PDL cells.

At FDR value<=0.05, Fold change=>1.5, treatment with curcumin and tetrahydrocurcumin resulted in only a few differentially expressed genes in gingival epithelial cells. There were 3 shared upregulated genes between treatment of hGEP cells with curcumin and tetrahydrocurcumin. These shared genes were enriched in positive regulation of stem cell differentiation and regulation of morphogenesis.

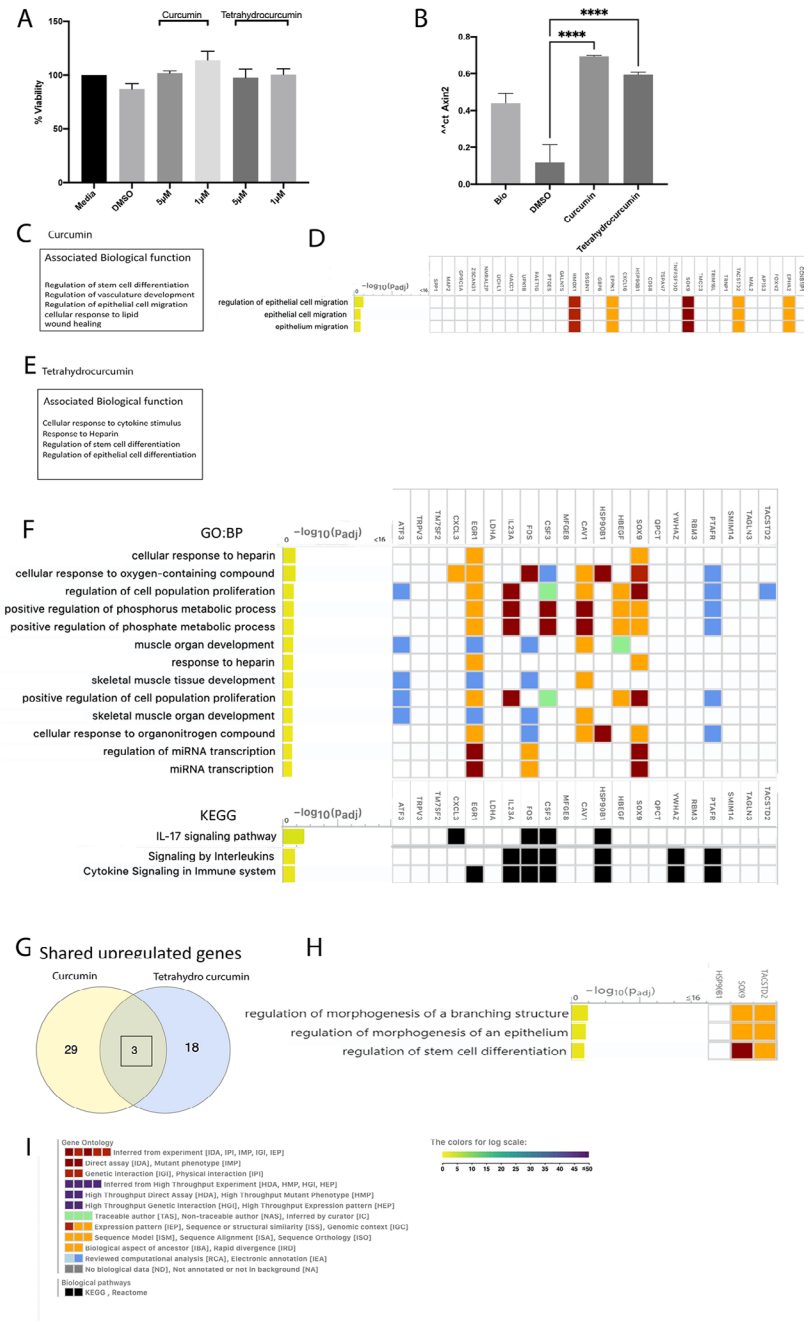


Figure 4: Impact of curcumin and tetrahydrocurcumin on gingival epithelial cells. A) MTS assay of hGEP cells after treatment with 5 μM and 1 μM of curcumin and tetrahydrocurcumin. B) Treatment of HGEPs with 1 μM of curcumin and tetrahydrocurcumin results in upregulation of Axin2. C) Enrichr GO Biological functions associated with upregulated genes after treatment of HGEP with curcumin. D). GO enrichment analysis by g: profiler of upregulated genes in curcumin treatment. E) Enrichr GO Biological functions associated with upregulated genes after treatment of HGEP cells with tetrahydrocurcumin. F) GO enrichment analysis by g: profiler of upregulated genes with tetrahydrocurcumin treatment. G) Venn diagram of unique and shared upregulated genes upon treatment of HGEP cells with Curcumin and tetrahydrocurcumin. H) GO enrichment analysis by g: profiler of the shared upregulated genes after treatment with curcumin and tetrahydrocurcumin. I) Graphical illustration guide. FDR value<= 0.05, Fold change=>1.5.

Shared upregulated genes in PDL and Gingival epithelial cells after treatment with curcumin and tetrahydrocurcumin

Since treatment with curcumin compounds resulted in differentially expressed genes in both PDL and gingival epithelial cells, we looked at the shared and unique upregulated genes between these cells with each compound treatment. Upregulated genes after treatment with curcumin and Tetrahydrocurcumin in both cell types were selected and reflected in Venn diagrams. This was done at FDR value<=0.05, Fold change=>1.5 and showed that both compounds result in higher numbers of upregulated genes in PDL. Only two upregulated genes, UCHL1 and TRIM16L, were shared between PDL and hGEP with curcumin treatment.

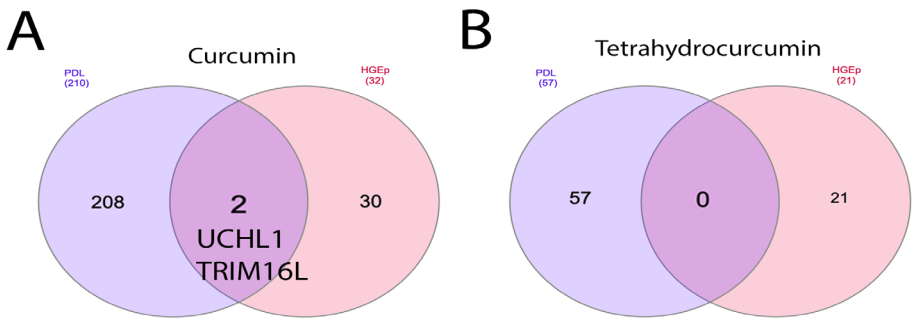


Figure 5: Shared and unique upregulated genes in PDL and Gingival cells. A) Venn diagram showing shared and unique upregulated genes after treatment of PDL and HGEP cells with curcumin. UCHL1 and TRIM16L are upregulate in both cell types. B) Venn diagram showing shared and unique upregulated genes after treatment of PDL and HGEP cells with tetrahydrocurcumin. Treatment with Tetrahydrocurcumin did not result in any shared upregulated gene.

Discussion

In this study we investigated the regenerative potential of curcumin and tetrahydrocurcumin on gingival epithelial and periodontal ligament cells. Assessment of cell toxicity showed that 1 µM of both compounds was safe to be used with these cell types. We show that the Wnt signalling pathway which is associated with an early response to damage in many tissues, is promoted in these two cell types upon treatment with curcumin and tetrahydrocurcumin [35-40]. At 1 µM, curcumin shows promotion of Axin2 expression in both PDL and hGEP cells, although this level of induction was not very significant, higher concentrations could be further tested to determine the optimum dosage for induction of Wnt pathway in hGEP and PDL cells.

Our RNA sequencing results demonstrate that curcumin and tetrahydrocurcumin can promote cell matrix organization. TGF-beta regulation of extracellular matrix was the most prominent biological function affected in gingival epithelial and PDL cells in these treatments. In PDL cells, treatment with curcumin upregulated regulation of apoptosis, RAGE and FRA pathways, Gap junction, TP53 network and interferon signalling. FRA and RAGE pathways are associated with immune response and inflammation [41,42]. Their upregulation suggests a pronounced anti-inflammatory role of curcumin in PDL. Treatment with Tetrahydrocurcumin however, results in upregulation of Syndecan 1 pathway, collagen biosynthesis, prostaglandin biosynthesis and angiogenesis in PDL. Syndecan pathway is known to regulate signalling of growth factors and morphogens and affects cell adhesion [43]. Syndecan1 also has a significant role in cell-cell and cell-matrix interactions and has been shown to promote axon regeneration [44,45]. This suggests a pronounced matrix remodelling role for Tetrahydrocurcumin in PDL cells, a process that is required in wound healing and regeneration. Interestingly, some upregulated genes were shared in treatment of PDL cells with curcumin and Tetrahydrocurcumin. These genes are enriched in collagen fibril organization, response to stimulus, development of anatomical structure and vasculature. This suggests these natural compounds can exert anti-inflammatory and pro-healing properties in PDL cells.

Gingival epithelial cells exhibit a different transcriptional landscape and are subsequently differently impacted by treatments with curcumin and Tetrahydrocurcumin. Cell differentiation and cell-cell adhesion were the main biological functions associated with treatment of these natural compounds. More specifically, treatment with curcumin resulted in upregulation of genes associated with cell migration, stem cell differentiation and vascular development in gingival epithelial cells whereas treatment with Tetrahydrocurcumin, upregulated genes enriched in cell proliferation, response to heparin and interleukin signalling pathways. Heparins modulate inflammation and interleukin bioactivity [46-48]. This in addition to upregulation of cell

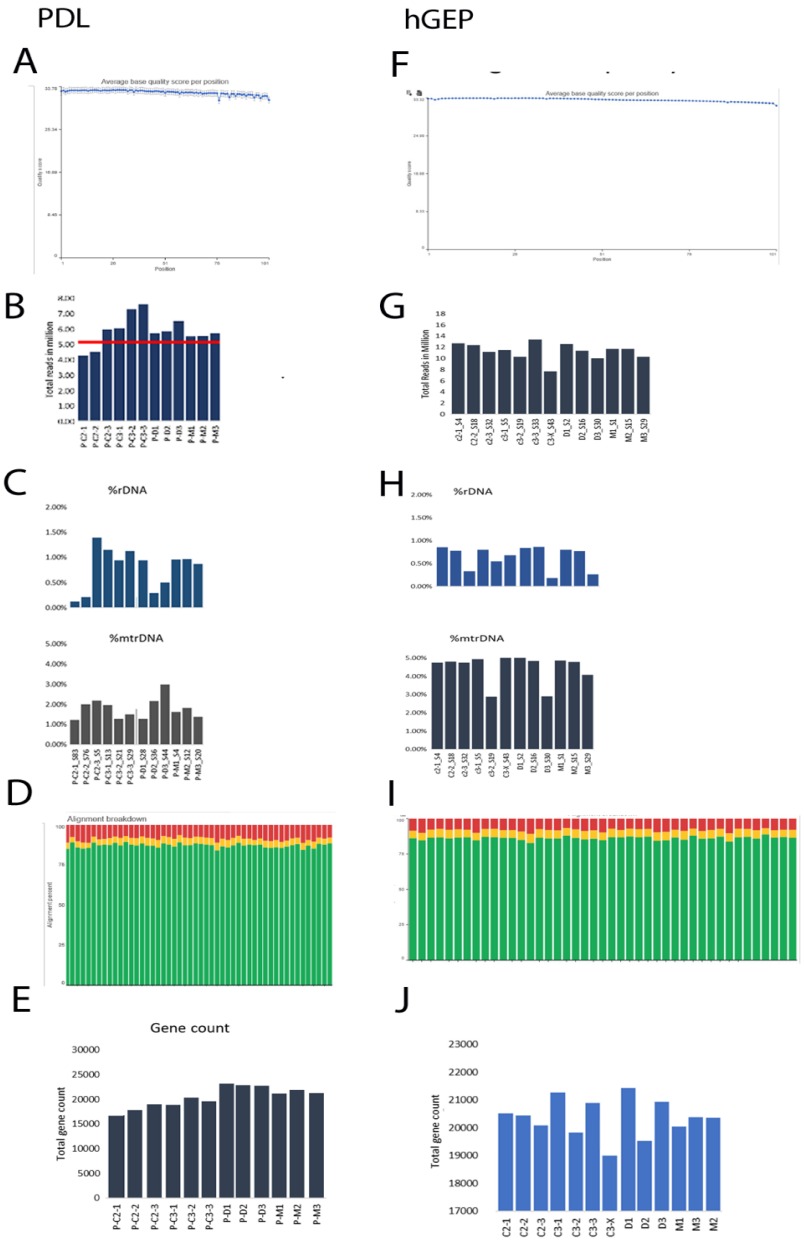
migration and stem cell differentiation suggests a regenerative potential of Tetrahydrocurcumin on gingival epithelial cells. Although in our study curcumin and Tetrahydrocurcumin appeared more potent on the PDL cells with higher numbers of upregulated genes, our data suggests that both compounds have anti-inflammatory properties and can promote healing and tissue regeneration in these periodontal cells. This can be through regulation of extracellular matrix and angiogenesis in PDL cells and via epithelial morphogenesis and stem cell differentiation in gingival epithelial cells.

Wound healing, anti-inflammatory and antioxidant properties have been reported in other tissues. Our current study highlights the regenerative potential of this natural compound in treatment of periodontal diseases. Curcumin nanoemulsions are effective against oral squamous cell carcinoma through inhibition of cell proliferation [49]. Regenerative potential of curcumin has been shown in peripheral nerve regeneration through induction of neural stem cell proliferation [50,51]. Curcumin can also induce regeneration of Beta cells in diabetic mice and contribute to accelerated muscle recovery [52,53]. In dental follicle cells, curcumin can regulate proliferation, senescence, and osteogenic differentiation and subsequently act as anti-senescence therapeutic [54]. Some of the anti-inflammatory properties of curcumin are through induction of apoptosis in inflammatory cells and shortening of the inflammatory phase to promote healing. Curcumin has also been shown to promote cutaneous would healing [55] and has anti-inflammatory roles in human dental pulp stem cells [56]. Other reported curcumin properties are collagen synthesis, fibroblasts migration, and differentiation [57-62]. These properties were also seen on treatment of PDL cells with curcumin in our study. Similar to previous studies, we demonstrate that Tetrahydrocurcumin can target different signalling pathways and cellular responses. Anti-inflammatory and neuroprotective properties of Tetrahydrocurcumin have been reported in other cell types. Tetrahydrocurcumin can inhibit cell cycle arrest and apoptosis through Ras/ERK signalling pathway and prevent sepsis and oxidate stress Tetrahydrocurcumin exerts neuroprotective effects in hippocampal cells and angiogenesis and vascular protection in brain endothelial cells [63-66]. Additionally, independent studies have shown that Tetrahydrocurcumin exerts anti-inflammatory, antiangiogenic, and neuroprotective properties in ocular disease and can improve insulin sensitivity and high blood pressure in kidney injury [67-69]. Anti-inflammatory, antiangiogenic properties associated with treatment of Tetrahydrocurcumin in our study is in line with the properties identified in previous studies.

Amongst our studied compounds, curcumin was more potent on both PDL and gingival epithelial cells. Additionally, treatment with curcumin results in upregulation of TRIM 16 and UCHL1 in both PDL and gingival epithelial cells. UCHL1 is a mitochondrial 10-formyltetrahydrofolate dehydrogenase that contributes to recovery after axonal injury and can regulate the immunosuppressive capacity and survival of mesenchymal stem cells in inflammatory diseases [70,71]. TRIM16 is a protein coding gene that acts as a tumour suppressor, affecting differentiation and cell migration. Interestingly, TRIM16 has been shown to promote differentiation of PDL stem cells and protect PDL from oxidative stress-induced damage [72,73]. Upregulation of TRIM16 and UCHL1 in both PDL and gingival epithelial cells demonstrates that curcumin has the potential to promote wound healing and tissue remodelling in both PDL and gingival epithelial cells. This together with upregulation of Wnt signalling, which is associated with tissue regeneration, emphasises the regenerative potential of this compound on periodontal cells. Clinical trials have demonstrated that curcumin can reduce gingival inflammation when used with scaling and oral gel containing Curcuma longa extract is efficient in treating initial infective inflammatory periodontal disease [74]. Animal studies have shown that natural curcumin can inhibit the inflammatory process and reduction in alveolar bone loss in experimentally induced periodontitis [75-78]. However, the need of human studies to better evaluate properties of curcumin in treatment of periodontitis has been highlighted [79].

Our current study provides evidence of cellular benefits of curcumin on human PDL and gingival epithelial cells. Investigation of differentially expressed genes in PDL and gingival epithelial cells after treatment with curcumin and Tetrahydrocurcumin provides the basis for a screening method to select potential candidates for natural therapeutic approaches in periodontal tissue. Additionally, this method allows detection of any adverse impact that these compounds may inflict on the cells. The results of this study suggest that curcumin and Tetrahydrocurcumin exhibit anti-inflammatory and regenerative potential on PDL and gingival epithelial cells. In particularly, curcumin can be a low-cost, well-tolerated and effective natural candidate for therapeutic approaches in periodontal tissue as it promotes organization of extracellular matrix in PDL cells and cell migration, differentiation, and angiogenesis in gingival epithelial cells. Further *in vivo* testing of this compound is required to confirm the optimum biological activity and to harness its full potential in clinical application.

Supplementary figure



Supplementary Figure 1: Bulk Sequencing of PDL and hGEP cells after treatment with curcumin and Tetrahydrocurcumin. Pre-alignment quality control of different compounds used for PDL and hGEP treatment (A, F), Total reads per sample (B, G), Ribosomal and mitochondrial RNA contamination with <3% ribosomal and mitochondrial RNA suggesting good quality samples (C, H). Alignment quality control where >80% of the reads (D, I) Number of genes that were detected per samples (E, I)

Author Contribution

AAB: Designed the study, conducted the experiments, created manuscript draft, and critically revised it. PS designed the study and critically revised the manuscript.

Acknowledgments

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Conflict of Interest

Authors declare no conflict of interest.

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