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Chrysin-Mediated Promotion of Odontoblastic Differentiation: A Pathway Towards Dental Pulp Regeneration

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ABSTRACT

Dental pulp, a critical component in tooth development and maintenance, plays a vital role in dentin formation, nutrient supply, and signal transmission. When damaged, pulp can become infected and necrotic, leading to pain and a decline in the quality of life. Current clinical treatments, such as root canal therapy, though effective, result in the loss of pulp vitality and associated sensory and defensive functions. Recognizing the potential of regenerative approaches, our study explored the odontogenic differentiation effects of Chrysin, a natural flavonoid, on human dental pulp cells (hDPCs). Our investigations revealed that Chrysin, particularly at a concentration of 10 μM, exhibited no cytotoxic effects on hDPCs over a 5-day period. Notably, this concentration demonstrated optimal effects on alkaline phosphatase (ALP) activity and mineralization capacity, indicating its potential as an agent for odontogenic differentiation. Further analyses at the molecular level confirmed increased expression of odontogenic markers, including dentin matrix protein 1 (DMP-1), dentin sialophosphoprotein (DSPP), and ALP, supporting the pro-odontogenic effects of chrysin. In conclusion, our study underscores the positive effects of Chrysin on odontogenic differentiation in hDPCs, offering a promising avenue for further exploration in dental regenerative medicine. Future research should delve into in vivo applications and clinical assessments to validate the translational impact of Chrysin in preserving the vital pulp and advancing dental tissue regeneration.

Keywords: Human dental pulp, Cellsosteolectinodontoblastic, Differentiation

1 Introduction

Dental pulp, a fragile connective tissue containing minute blood vessels, nerves, and lymphatics, plays a crucial role in tooth development by contributing to dentin formation, nutrient supply, and signal transmission [1,2]. Its significance extends to fully mature teeth, but when the pulp undergoes damage, it becomes susceptible to infection and necrosis, leading to pain and a diminished quality of life [3,4]. The conventional clinical approach for treating pulp-involved teeth involves root canal treatment. However, the loss of pulp vitality not only results in a decline in the sensory and defensive functions of teeth but also induces changes in color and structure. Preserving the vital pulp is particularly essential in this context [5,6].

As dental materials and technology continue to advance, and our understanding of pulpitis deepens, there is a growing possibility of considering pulp regeneration as an alternative to traditional root canal therapy. The concept of regenerative endodontic treatment was introduced in 1952 by Dr. B. W. Hermann, who employed calcium hydroxide in endodontic procedures. The primary objective of regenerative endodontic treatment is to facilitate pulp regeneration, specifically in the formation of the dentin-pulp complex. The key elements involved in this approach include stem cells, biomaterials, and growth factors [7,8].

Chrysin is a natural flavonoid found in various plants, and it has been studied for its potential health benefits, includ-

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ing anti-inflammatory and antioxidant properties [9,10]. While there is some research on the effects of Chrysin on bone health and cellular processes related to osteogenesis, the specific odontoblastic (related to tooth dentin formation) properties of Chrysin may not have been extensively studied. A study investigated the biocompatibility and osteogenic properties of the Copper (II)-Chrysin complex (C/Cu) 1 [11]. The study found that C/Cu promoted mineralization in osteoblastic cultures by increasing ALP activity. At the molecular level, C/Cu significantly promoted the mRNA levels of osteoblast differentiation marker genes such as runt-related transcription factor 2 (Runx2), Type 1 collagen and ALP. In addition to this, secretory proteins, osteonectin (ON) and osteocalcin (OC) levels were also stimulated. The study concluded that C/Cu exhibited enhanced osteogenic properties and can be used as an osteogenic agent in bone tissue engineering. In another study, Huo and et al explored the impact of chrysin, a flavonoid from oroxylum seeds, on dental pulp stem cells (DPSCs) for bone regeneration [12]. Chrysin enhanced osteogenic differentiation, bone formation, and Smad3-related pathways, suggesting its potential in bone defect repair. However, there is no information on the effect of Chrysin on odontoblastic differentiation of human dental pulp cells (hDPCs). Here, we assessed the odontoblastic differentiation ability of osteolectin in hDPCs and further explored its internal mechanism through the signaling pathway.

2 Materials and Methods

2.1 *Cytotoxicity Assay*

For the cytotoxicity assessment, hDPCs at passage 3 were placed in 96-well plates (Corning, NY) at a seeding density of 1 × 104 cells/well. Subsequently, they were exposed to 100 μl of culture medium per well containing varying concentrations of Chrysin (0, 1, 10, 100, 1000, 10000 μM) for a duration of 24 hours. The impact of Chrysin on the cytotoxicity of hDPCs was determined using the MTT assay. The optical density (OD) was measured at a wavelength of 450 nm employing a microplate spectrophotometer (Thermo Scientific, Waltham, MA).

2.2 *Alkaline Phosphatase and Alizarin Red S Staining Assays*

hDPCs were planted in 48-well plates (Corning) at a density of 1.5 × 104 cells per well and cultured in a standard medium. Odontoblastic differentiation medium (OM), consisting of the standard medium with the addition of 10 mM β-glycerophosphate (Stata Cruz Biotechnology Inc, Dallas, TX) and 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), was also used. Additionally, OM was supplemented with various concentrations of Chrysin. Following a 10-day incubation period, the medium was removed, and the cells underwent washing with DPBS for alkaline phosphatase (ALP) staining.

Subsequently, the cells were fixed with 70% ethanol, rinsed with distilled water, and stained with 200 μl of ALP staining reagent (BCIP/NBT Liquid Substrate System; Sigma-Aldrich) for 15 minutes. The staining was dissolved in 150 μl of 10% (w/v) cetylpyridinium chloride solution (CPC, pH = 7.0) for 30 minutes, and the intensity of staining was quantified by measuring the optical density using a microplate spectrophotometer reader (Thermo Scientific) at 562 nm.

For Alizarin red S staining, after 14 days of odontogenic induction, calcium deposits formed on hDPCs were stained with 2% Alizarin red S. The cells were observed under an inverted optical microscope (Olympus IMT-2, Tokyo, Japan).

2.3 *Quantitative Real-time Polymerase Chain Reaction Analysis*

The cells were planted in 6-well plates and subjected to treatment with culture medium, OM with or without osteolectin at a concentration of 10 μM for durations of 3 and 7 days. Following the incubation period, cells were collected, and RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). Subsequently, the total RNA concentration was determined using a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL), and 2000 ng of RNA was utilized for the synthesis of complementary DNA. The resulting cDNA samples underwent further analysis through quantitative RT-PCR, employing the Rotor-Gene 6000 instrument (Corbett, Australia). The 2-ΔΔCt method was employed to assess the expression levels of the target genes.

2.4 *Statistical Analysis*

GraphPad Prism 8 (GraphPad Software Inc, San Diego, CA) was utilized for all statistical analyses. Each assay was independently replicated a minimum of three times with distinct samples. The presented data indicate the mean ± standard deviation (SD). The results were normalized against the control and subjected to one-way analysis of variance, followed by Tukey's multiple post hoc tests for evaluation. A significance level of $P < .05$ was deemed statistically significant.

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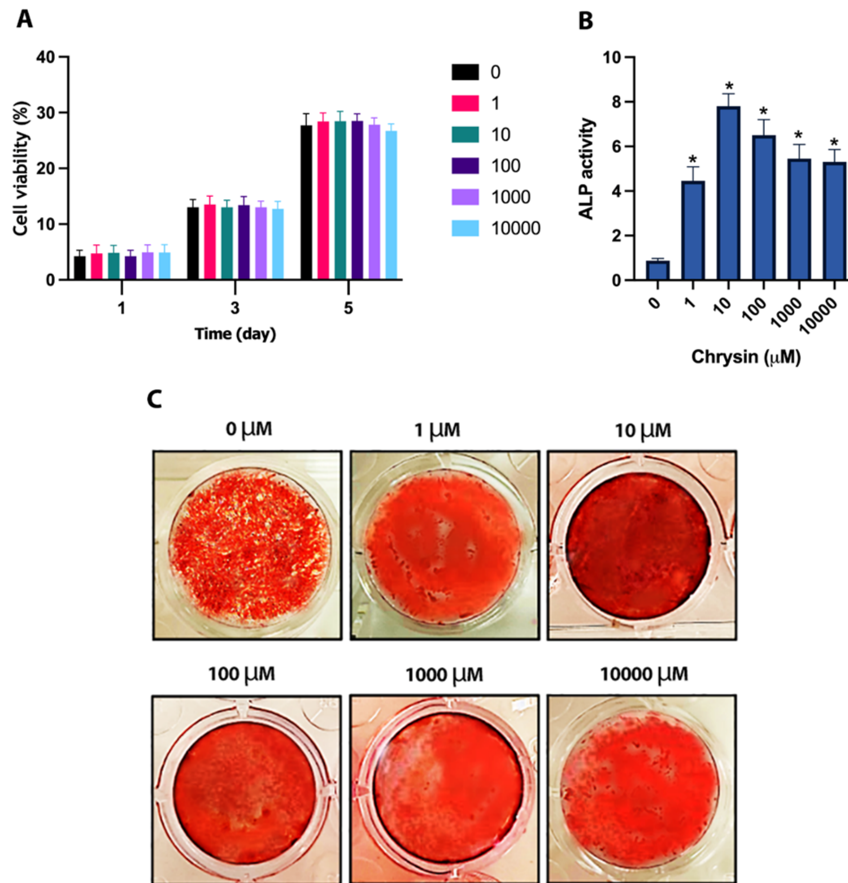

Fig. 1. The effect of osteolectin on odontogenic differentiation of hDPCs. (A) Viability of DPSCs cultured in osteogenic medium and treated with different concentrations (1, 10, 100, 1000 and 10000 μM) of Chrysin for the indicated days. (B) ALP staining was used for 10 days of hDPCs osteogenic induction under different concentration of Chrysin treatment. (C) Calcium spots deposition was evaluated by Alizarin red S staining for 14 days of hDPCs osteogenic induction with or without Chrysin. ∗ Significant difference compared to other groups ($P < .05$, $n = 3$).

3 Result

Effect of Osteolectin on Cytotoxicity ALP Activity, and Mineralization Ability of hDPCs We initially examined the impact of varying concentrations of Chrysin on the viability of DPSCs. DPSCs were cultured in osteogenic differentiation medium and exposed to Chrysin for a duration of 5 days. The assessment of cell viability revealed no changes, indicating the absence of cytotoxic effects exerted by Chrysin on DPSCs (Fig. 1A).

Additionally, the highest ALP activity in DPSCs was observed after treatment with 10 μM Chrysin for 7 days (Fig. 1B). Mineralization capacity, determined by Alizarin red S staining to detect the formation of mineralized spots, showed a notable increase in calcium deposits in the osteogenic differentiation medium group with 10 μM at 14 days compared to other Chrysin concentrations (Fig. 1C). Collectively, these findings suggested that the optimal concentration for Chrysin-induced odontogenic differentiation of DPSCs was 10 μM, and this concentration was selected for subsequent experiments.

3.1 *Effect of Chrysin on the odontogenic markers of hDPCs*

We proceeded to confirm the impact of Chrysin on the odontogenic differentiation of hDPCs at the mRNA level. The mRNA expression levels of DSPP, DMP-1, and ALP exhibited a significant increase in the group induced by Chrysin at 7 days (Fig. 2). In summary, these results affirm the influence of 10 μM Chrysin on the odontogenic differentiation of hDPCs.

4 Discussion

In this study, we investigated the impact of Chrysin on the odontogenic differentiation of hDPCs by examining cell viability, ALP activity, mineralization ability, and the expression of odontogenic markers. Our results provide valuable insights into the potential application of Chrysin in promoting odontogenic differentiation, which is crucial for dental tissue engineering and regenerative medicine.

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The initial focus of our investigation was to assess the cytotoxic effects of Chrysin on hDPCs. We found that Chrysin, at concentrations ranging from 1 to 10000 μM, did not induce any significant changes in cell viability over a 5-day period, suggesting that Chrysin has no cytotoxic effects on hDPCs. This finding is crucial for the safety profile of Chrysin, making it a potential candidate for further exploration in dental regenerative applications [13].

Subsequent analyses revealed a concentration-dependent effect of Chrysin on ALP activity in hDPCs. The highest ALP activity was observed after treatment with 10 μM Chrysin for 7 days. This concentration was further validated as the optimum for promoting odontogenic differentiation, as evidenced by the remarkable increase in calcium deposits detected through Alizarin red S staining at 14 days. This observation emphasizes the importance of carefully selecting the appropriate concentration of Chrysin to achieve optimal outcomes in promoting mineralization and odontogenic differentiation of hDPCs.

To gain deeper insights into the molecular mechanisms underlying the observed effects, we examined the expression levels of odontogenic markers, including DSPP, DMP-1, and ALP, at the mRNA level. The Chrysin-induced group treated with 10 μM Chrysin exhibited a significant increase in the expression of DSPP, DMP-1, and ALP at 7 days. These findings further confirm the pro-odontogenic effects of Chrysin, supporting its role in enhancing the differentiation of hDPCs towards an odontogenic lineage.

Comparing our results with existing literature, our study aligns with the emerging consensus on the non-cytotoxic nature of Chrysin on dental pulp cells [12,13]. Moreover, our concentration-specific findings echo similar trends reported in related investigations, thereby reinforcing the significance of optimal Chrysin dosage in promoting odontogenic differentiation [12,14]. The upregulation of key odontogenic markers further solidifies Chrysin's potential as a beneficial adjunct in the field of dental regenerative medicine. Our study contributes to the growing body of evidence supporting the promising role of Chrysin in enhancing odontogenic differentiation, opening avenues for future research and clinical applications in dental tissue engineering.

Despite the promising outcomes of our study, certain limitations should be acknowledged. First, our investigation primarily focused on in vitro experiments using hDPCs, and the translation of these findings to an in vivo setting or clinical applications warrants cautious consideration. The complex microenvironment of the dental pulp in vivo, which involves interactions with surrounding tissues and physiological conditions, may influence the behavior of Chrysin-treated hDPCs differently than observed in our controlled in vitro conditions. Additionally, our study concentrated on a specific Chrysin concentration (10 μM) that exhibited optimal effects, and the potential dose-response relationship across a broader range of concentrations remains unexplored.

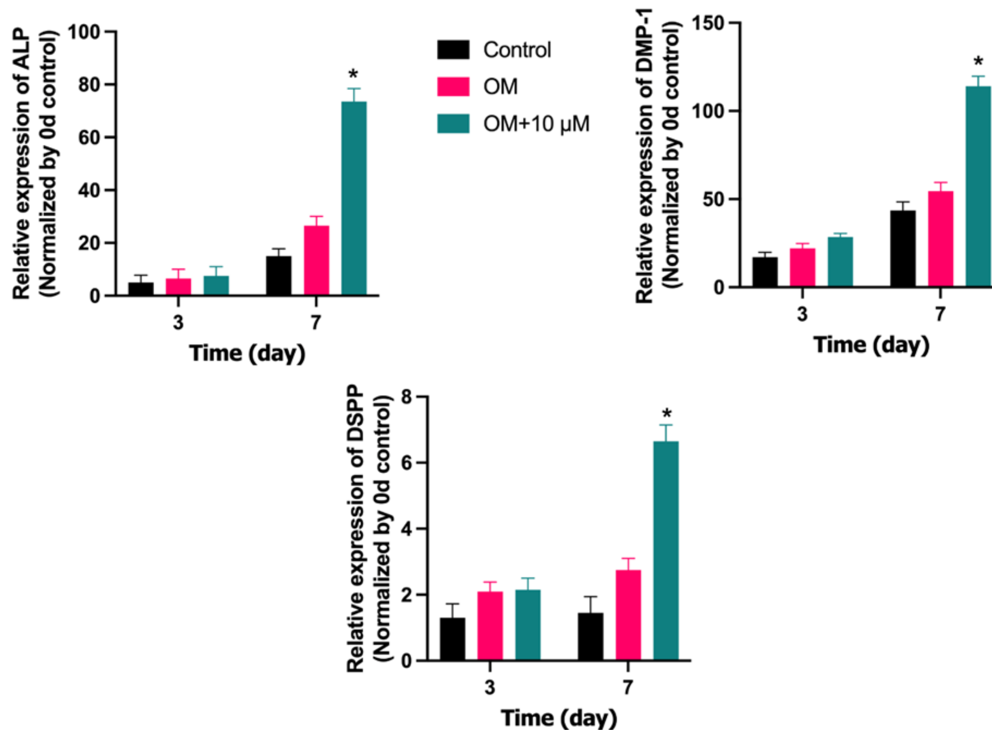

Fig. 2. DSPP, DMP-1, and ALP gene expression in hDPCs treated OM with or without 10 μM Chrysin was analyzed by quantitative real-time PCR analysis for 3 and 7 days. (*$P < .05$, n = 3) All data were normalized by 0-day control.

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Furthermore, the study primarily assessed short-term effects up to 14 days, and long-term investigations are essential to elucidate the sustainability and stability of the observed odontogenic differentiation. Finally, while our focus was on key odontogenic markers, a more comprehensive analysis of gene expression and protein levels could provide a more detailed understanding of the molecular mechanisms underlying Chrysin-induced odontogenic differentiation. Addressing these limitations will be crucial for the translational potential and broader applicability of Chrysin in the context of dental tissue regeneration.

5 Conclusion

In conclusion, our findings suggest that Chrysin, particularly at a concentration of 10 μM, promotes odontogenic differentiation of hDPCs without inducing cytotoxicity. The positive effects observed in terms of ALP activity, mineralization ability, and the expression of odontogenic markers highlight the potential therapeutic relevance of Chrysin in dental tissue engineering and regenerative medicine. Future studies could explore the in vivo effects of Chrysin and its potential application in clinical settings for dental tissue regeneration.

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Ethical consideration: The study was approved by Islamic Azad University.
Data Availability: No data was used for the research described in the article.

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