

Fluocinolone Acetonide Promotes the Proliferation and Mineralization of Dental Pulp Cells

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Abstract

Introduction: The aim of this study was to investigate the role of the steroid fluocinolone acetonide on the proliferation and mineralization of human dental pulp cells (DPCs). The potential effect of fluocinolone acetonide on reparative dentin formation and the recovery of injured dental pulp were evaluated. **Methods:** The proliferative effect of fluocinolone acetonide on DPCs was analyzed by cholecystokinin octapeptide assay and flow cytometry. The mineralized effect of fluocinolone acetonide was investigated by the detection of mineralization-related biomarkers including alkaline phosphatase (ALP), bone sialoprotein, and osteocalcin by using ALP histochemical staining, ALP activity, immunostaining, alizarin red staining, and reverse-transcriptase polymerase chain reaction. The molecules, including dentin sialophosphoprotein and Wnt4, involved in the process of mineralization were detected by real-time polymerase chain reaction and Western blot analysis. **Results:** Low concentrations of fluocinolone acetonide (0.1–40 $\mu\text{mol/L}$) promoted the proliferation of DPCs. The flow cytometry results showed that the CD146-positive subpopulation of DPCs was significantly increased after treatment with fluocinolone acetonide at 1 and 10 $\mu\text{mol/L}$ for 48 hours, respectively. The messenger RNA expression and activity of the early-stage mineralization marker ALP were evidently increased in fluocinolone acetonide-treated DPCs compared with the untreated control group, so did the middle-stage mineralization marker bone sialoprotein and the late-stage mineralization marker osteocalcin. Meanwhile, Wnt4 and the dentin-specific marker dentin sialophosphoprotein were obviously up-regulated by fluocinolone acetonide compared with the untreated controls. **Conclusions:** Fluocinolone acetonide can promote the proliferation of DPCs, especially for the CD146+ subpopulation. Fluocinolone acetonide can initiate the mineralization of DPCs and has the potential role in repairing injured pulp tissues. (*J Endod* 2013;39:217–222)

Key Words

Dental pulp cells, fluocinolone acetonide, mineralization, proliferation

The recovery of injured dental pulp is still one of the unresolved clinical problems. When unexpected pulp exploration occurs, the survival of vital pulp will be more difficult. Because healthy and vital pulp can promise a better prognosis of the injured tooth, preserving the vitality of pulp tissue is of key importance for long-time tooth preservation (1). The ideal dental pulp capping agent should not only induce the formation of reparative dentin but also inhibit inflammatory processes. The widely used capping agents (ie, calcium hydroxide and mineral trioxide aggregate) are mainly focused on the closure of the exposed pulp; however, they are short of the anti-inflammation effect. So far, there is no ideal dental pulp capping material for the repair of slightly inflammatory pulp cases. Using anti-inflammatory medicine may be an alternative approach for the contaminated or mild infected pulp tissues to recover.

The commonly used anti-inflammatory agents include steroidal and nonsteroidal medicines. One representative nonsteroid medicine is aspirin. Recently, it has been shown that aspirin can improve the bone marrow mesenchymal stem cells–based calvarial repair by reducing the release of interferon γ or tumor necrosis factor α and therefore increases the survival of bone marrow mesenchymal stem cells (2). As the representation of steroid medicines, fluocinolone acetonide was primarily used in dermal and mucosal disorders and has the potential to inhibit the release of interleukin 1 α , tumor necrosis factor α , and interferon γ (3–6). The first reported case of using steroidal medicines in dental pulp capping was performed in 1958 (7), whereas there were only a few reports relating to steroidal medicines used in dental pulp treatment. In recent years, steroids were systemically applied to endodontic treatment (8–10). The literature showed that fluocinolone acetonide can promote the proliferation and extracellular matrix formation in human dental pulp cells (DPCs) (11). However, the role of fluocinolone acetonide in regulating pluripotent stem cell–mediated mineralization, especially for dental pulp stem cells, remains unknown. In this study, we investigated the effect of fluocinolone acetonide on the mineralization of DPCs and the underlying mechanism involved.

Materials and Methods

Isolation and Culture of Human DPCs

The study protocol was approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China. DPCs were isolated from healthy permanent premolars extracted for orthodontic reasons at the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology (18- to 28-year-old donors). Briefly, pulp tissues were digested in a mixture of 3 mg/mL

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collagenase type I (Sigma-Aldrich, St Louis, MO) and 4 mg/mL dispase (Roche, Indianapolis, IN) for 1 hour at 37°C. Then, the DPCs' suspensions were passed through a 70- μ m strainer to obtain separated dental pulp cells (12). The single-cell suspensions were inoculated in Dulbecco modified Eagle medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone, Logan, UT) (growth medium) and incubated at 37°C under 5% CO₂. The fourth to sixth passage of DPCs was used for further experiments.

Cell Proliferation Assay

Cell proliferation was measured by cholecystokinin octapeptide (CCK-8). This method was applied according to the manufacturer's instructions. Briefly, 5 × 10³ DPCs were plated in 100 μ L growth medium in a 96-well plate. After an overnight incubation, cells were treated with fluocinolone acetonide (Sigma-Aldrich) at series of concentrations (0, 0.1, 1, 10, 20, 40, 60, and 100 μ mol/L) for an additional 24 hours. Then, 10 μ L CCK-8 solution was added to each well, and the cultures were incubated for 4 hours at 37°C. Cell viability was measured by conversion of the Dojindo highly water-soluble tetrazolium salt WST-8 to a yellow-colored water-soluble formazan. The amount of the formazan dye generated by the activity of mitochondrial dehydrogenases in cells was directly proportional to the number of living cells. Color development was quantified photometrically at 450 nm using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winoski, VT). Viability is given in percent of the control value.

Flow Cytometry Assay

The DPCs were seeded into 6-well plates (2 × 10⁵ cells/well). On the following day, the cells were treated with fluocinolone acetonide at the final concentration of 1 and 10 μ mol/L and continued to culture for additional 7 days. Then, the cells were trypsinized, rinsed with phosphate-buffered saline (PBS), and labeled with fluorescein isothiocyanate (FITC)-conjugated CD146 antibody (Ansell Corp, Bayport, MN) or immunoglobulin G1-FITC isotype control (BD Biosciences, San Diego, CA) for 30 minutes, respectively. After being washed with PBS, cells were resuspended in PBS and subjected to flow cytometric analysis using an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA).

ALP Staining and Activity

The DPCs at a density of 2 × 10⁴ cells/well were seeded in 24-well plates for ALP staining or 6 × 10⁴ cells/well seeded in 12-well plates for ALP activity analysis. When 80% confluency was reached, the cells were treated with fluocinolone acetonide at the final concentration of 1 and 10 μ mol/L, respectively, and then incubated at 37°C in 5% CO₂ atmosphere. DPCs were treated with osteogenic media (100 nmol/L dexamethasone, 10 mmol/L sodium β -glycerophosphate, and 10 nmol/L L-ascorbic acid phosphate magnesium salt n-hydrate in growth medium) as the positive control. The cells continued to culture for an additional 7 days for ALP analyses by using an ALP histochemical staining kit (CWBioTech, Beijing, China) or an ALP activity assay kit

(Jiancheng, Nanjing, China), respectively, according to the manufacturers' protocols.

Immunostaining of Osteogenesis-related Biomarkers

The DPCs were seeded into 12-well plates (6 × 10⁴ cells/well). On the following day, the cells were treated with fluocinolone acetonide at the final concentration of 1 and 10 μ mol/L and continued to culture for an additional 7 days for immunostaining. DPCs were treated with osteogenic medium as the positive control. The DPCs were washed 3 times with PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes, and rinsed 3 times with PBS. Then, the procedure was performed according to the protocol for the SP immunohistochemical kit and the 3, 3'-diaminobenzidine (DAB) coloration kit (Zhongshan Bioengineering Co Ltd, Beijing, China). The primary antibodies were antihuman bone sialoprotein (BSP, Santa Cruz, CA) and antihuman osteocalcin (OCN), which was kindly provided by Dr Larry Fisher (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD), respectively. The immunostaining results were observed with a light microscope equipped with a camera (BX51 microscope and DP72 camera; Olympus, Tokyo, Japan) after samples dealt with DAB color development substrate and hematoxylin counterstain.

Alizarin Red Staining

The DPCs were seeded into 12-well plates (6 × 10⁴ cells/well). On the following day, the cells were treated with fluocinolone acetonide at the final concentration of 1 and 10 μ mol/L and continued to culture for additional 21 days for alizarin red staining. The cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes and then stained using alizarin red (Sigma-Aldrich).

Real-time Polymerase Chain Reaction

The DPCs were seeded into 6-well plates (2 × 10⁵ cells/well). On the following day, the cells were treated with fluocinolone acetonide at the final concentration of 1 and 10 μ mol/L and continued to culture for additional 7 days for RNA extraction. DPCs were treated with osteogenic media as the positive control. Media changes were performed every other day with a medium volume of 2 mL per well. The total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Complementary DNA was reverse transcribed using 2 μ g RNA as a template and Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI) in a total volume of 20 μ L. The real-time polymerase chain reaction (PCR) was performed in duplicate using 0.5 μ L complementary DNA template in a 20- μ L reaction volume for amplification with 200 nmol/L of the following primers (Table 1); the thermal cycling conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes and 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. Reactions were performed by using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) and FastStart Universal SYBR Green PCR master mix (Rox) from Roche Applied Science. The

TABLE 1. Quantitative PCR Primers

Gene	Forward primers	Reverse primers
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA
ALP	ATGGGATGGGTGTCTCCACA	CCACGAAGGGGAAGTCTGTC
BSP	GAATGGCCTGTGCTTTCTCAA	TGGATGAGTCACTACTGCCC
OCN	CACTCCTCGCCATTGGC	CCCTCCTGCTGGACACAAG
DSPP	TGGAGCCACAACAGAAGCAA	TCCAGTACTTGAGGTCCATC
Wnt4	GAGACGTGCGAGAACTCAAG	TGGTACTGGCACTCTCAATG

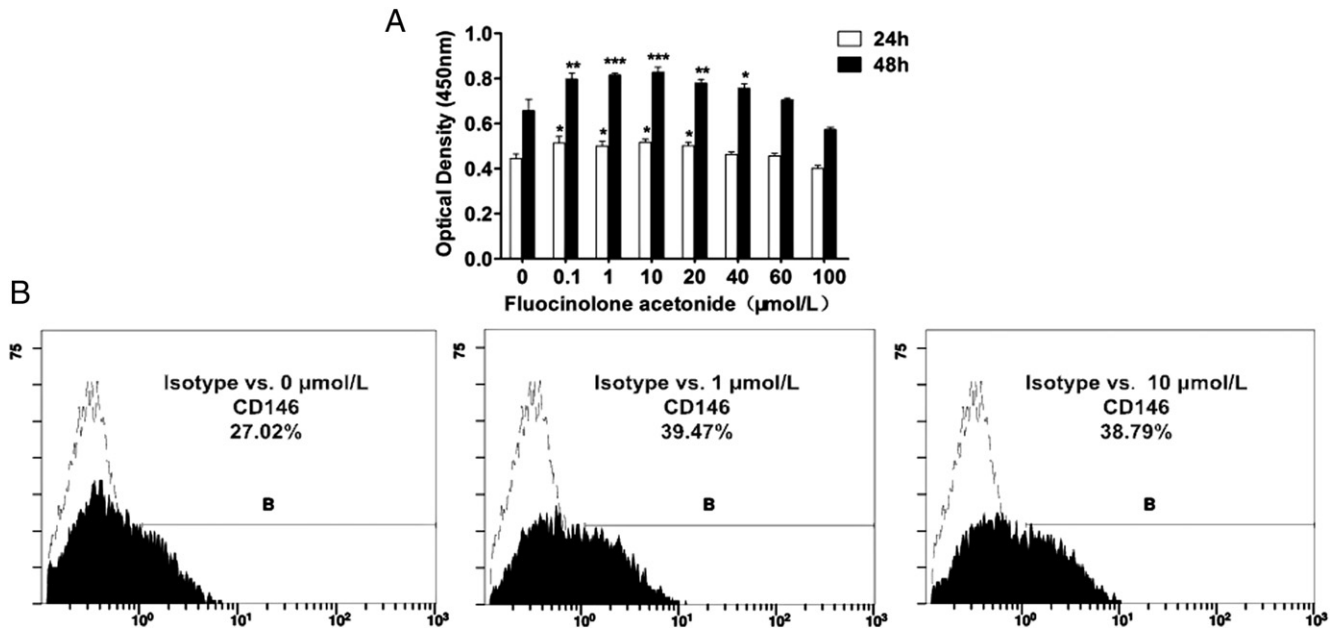


Figure 1. (A) Cell proliferation was enhanced by the different concentrations of fluocinolone acetonide (* $P < .05$, ** $P < .01$, *** $P < .001$). (B) The flow cytometry results for mesenchymal stem cell marker CD146 at 0, 1, and 10 $\mu\text{mol/L}$ fluocinolone acetonide.

housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Western Blot

The DPCs were treated with fluocinolone acetonide for 7 days for Western blot assay. The cells were harvested by using a protein lysis buffer (Applygen, Beijing, China). The suspensions were centrifuged at 12,000g for 30 minutes at 4°C. The protein concentration was deter-

mined using the BCA Protein Assay (Pierce, Rockford, IL), and equal aliquots of total protein (40 μg) were loaded in each lane. Sample lysates were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked in 5% nonfat dry milk for 1 hour, and probed with the following antibodies at 4°C overnight: dentin sialophosphoprotein (DSPP), Wnt4, and β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated antirabbit or mouse immunoglobulin

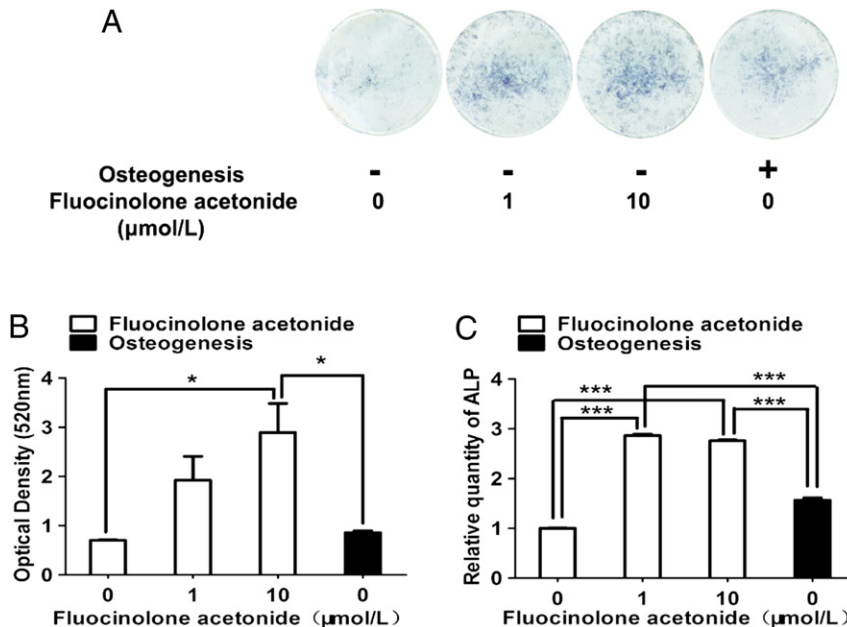


Figure 2. The ALP activity was evaluated on day 7 with the addition of 1 and 10 $\mu\text{mol/L}$ fluocinolone acetonide and osteogenic media as the positive control. (A) The ALP staining of DPCs showed that with 1 or 10 $\mu\text{mol/L}$ fluocinolone acetonide ALP staining was enhanced more evident than controls. (B) The ALP activities of DPCs cultured in 10 $\mu\text{mol/L}$ fluocinolone acetonide on day 7 was significantly higher than the 0 $\mu\text{mol/L}$ group and the positive control (* $P < .05$). (C) The induction of ALP messenger RNA expressions by 1 and 10 $\mu\text{mol/L}$ fluocinolone acetonide were significantly greater than the 0 $\mu\text{mol/L}$ group and the positive control (***) ($P < .001$).

G (Jackson ImmunoResearch, West Grove, PA) was used for 1 hour at room temperature based on the source of the corresponding primary antibody, and the immunoblots were detected by the Western-enhanced chemiluminescence blotting kit (ECL, Applygen).

Statistical Analysis

Data were expressed as the mean ± standard error of the mean. Between-group differences were compared using 1-way analysis of variance. Statistical difference was determined as $P < .05$.

Results

The Effects of Fluocinolone Acetonide on DPCs' Viability

Using a CCK-8 viability assay, we evaluated the effects of fluocinolone acetonide on DPCs. The CCK-8 assay showed that the growth rate of DPCs was significantly promoted with the addition of a low concentration of fluocinolone acetonide (from 0.1 to 20 μmol/L) at 24 hours ($P < .05$), and the difference was significantly enhanced at the concentration of 0.1 and 40 μmol/L ($P < .05$), 1 and 10 μmol/L ($P < .01$), and 40 μmol/L ($P < .001$) at 48 hours (Fig. 1A). Flow cytometry was shown in Figure 1B. The expression of CD146 was significantly high in the 1-μmol/L group ($39.47\% \pm 0.91\%$) and the 10-μmol/L group ($38.79\% \pm 2.72\%$) when compared with the 0-μmol/L group ($27.02\% \pm 1.29\%$).

Fluocinolone Acetonide Enhanced ALP Activity in the DPCs

As an early osteogenic marker, ALP was detected on day 7 after being treated with fluocinolone acetonide. Staining for ALP showed a higher ALP expression with 1 μmol/L and 10 μmol/L fluocinolone

acetonide compared with the 0-μmol/L group, which was even higher than the osteogenic media group (Fig. 2A).

The ALP activity assay showed the same tendency as ALP staining. The concentration of 10 μmol/L (2.89 ± 0.42) can significantly promote the ALP activity compared with that of the 0-μmol/L (0.70 ± 0.02) and osteogenic media groups (0.86 ± 0.06) ($P < 0.05$) (Fig. 2B). The messenger RNA levels of ALP were significantly increased at 1- and 10-μmol/L groups when compared with the 0-μmol/L group ($P < .001$) and the positive control ($P < .001$) (Fig. 2C).

The Effect of Fluocinolone Acetonide on BSP and OCN Expression via Immunostaining

To evaluate the effect of fluocinolone acetonide on osteogenesis in DPCs, osteogenic differentiations were investigated on day 7 by immunostaining and on day 21 by alizarin red staining. As osteogenic markers, BSP and OCN were immunostained on day 7 after being treated with fluocinolone acetonide. More positively stained cells of BSP (Fig. 3A–C) and OCN (Fig. 3D–F) were presented in the 10-μmol/L fluocinolone acetonide group when compared with the negative control (0 μmol/L).

Alizarin red staining results showed that there were no any mineralized node found in both 1 and 10 μmol/L fluocinolone acetonide treated DPCs in 21 days. Although cultured in osteogenic media for 21 days, alizarin red staining of the DPCs was positive (Fig. 3G).

Analysis of OCN, BSP, DSPP, and Wnt4 Expression at the Messenger RNA Level

Some osteogenesis-related genes were detected by quantitative PCR. Total RNA was isolated from DPCs that were treated with

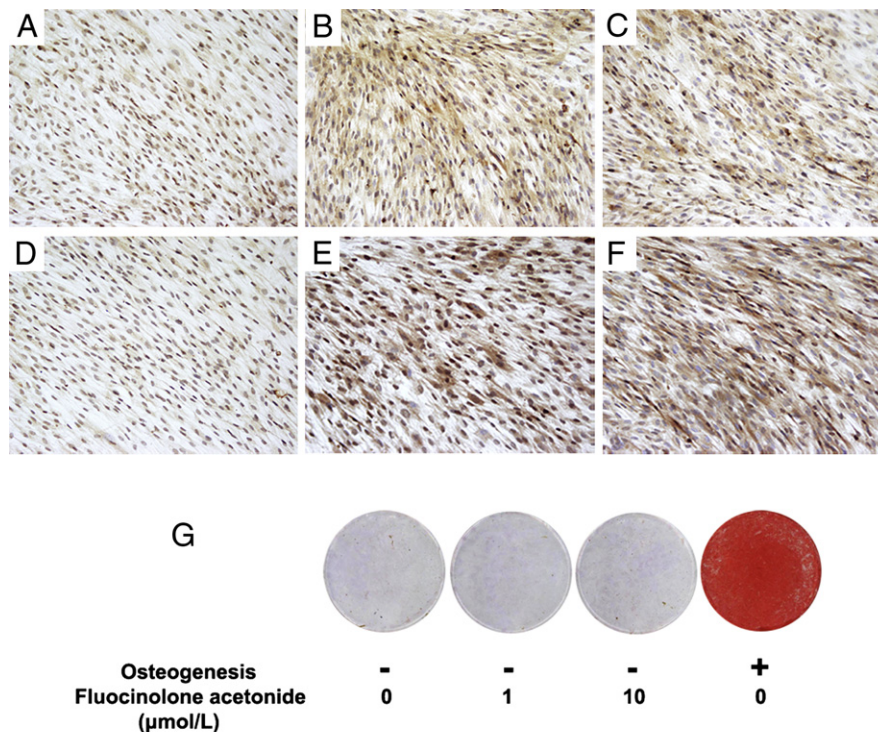


Figure 3. Immunostaining of BSP and OCN for cultured DPCs on day 7; alizarin red staining was detected on day 21. (A–C) BSP was positive in (B) the 10-μmol/L fluocinolone acetonide group and (C) the osteogenic media group, whereas they were negative in (A) the control group. (D–F) OCN was more positive in the (E) 10-μmol/L fluocinolone acetonide group and (F) the osteogenic media group than (D) the control group. (G) Alizarin red staining was positive in the osteogenic media group. No mineralized nodes were observed in the fluocinolone acetonide groups.

fluocinolone acetonide at 1 and 10 $\mu\text{mol/L}$ for 7 days, and then subjected to do quantitative PCR after reverse-transcription. The quantitative PCR results showed that fluocinolone acetonide significantly promoted the expressions of BSP, OCN, DSPP, and Wnt4, respectively (Fig. 4A, $*P < .05$, $**P < .01$, $***P < .001$).

Analysis of DSPP and Wnt4 Expression at Protein Level

The protein expressions of DSPP and Wnt4 in the 0-, 1-, and 10- $\mu\text{mol/L}$ fluocinolone acetonide groups were detected by Western blot. The expressions of DSPP and Wnt4 protein in the 1- and 10- $\mu\text{mol/L}$ fluocinolone acetonide groups were evidently higher than the negative control (0 $\mu\text{mol/L}$) (Fig. 4B).

Discussion

Evidence has shown that dental pulp cells have the characteristics of stem cells, which are capable of differentiating into odontoblast-like cells and forming a dentin bridge (12, 13). Theoretically, dental pulp could regenerate as well. However, the specific anatomic structure of tooth limits pulp tissues' repair capability. Inflammation-induced pressure is a main reason that contributes to the limited repair capability of

dental pulp tissues. Therefore, reducing the release of inflammatory mediators may give a chance to repair contaminated or mild infected pulp tissues. Anti-inflammatory medicine might be beneficial to inflammation control and inflammation subsiding. Fluocinolone acetonide is a potential treatment for this purpose because it has the anti-inflammation property as well as osteoinductive function.

Anti-inflammation and the proliferation of DPCs are required for the initial step of inflamed pulp healing. Previous studies showed that fluocinolone acetonide has the capacity to improve the proliferative rate in DPCs (11). Our results showed that fluocinolone acetonide not only had a stimulatory effect on cell proliferation in a range of concentrations but also distinctly increased the number of the CD146+ DPC subpopulation. CD146 is supposed to be a marker for mesenchymal stem cells, and its expression is linked to multipotency (14, 15). Thus, we deduced that fluocinolone acetonide could have the ability to promote the proliferation of CD146+ DPCs with greater differentiation potential. The increase in the number of stem cells would be beneficial to the healing process in the dental pulp.

The primary function of the dental pulp is to form dentin, which is involved in the process of biomineralization. The mineralized tissue-related biomarkers include ALP, BSP, OCN, and DSPP. They were

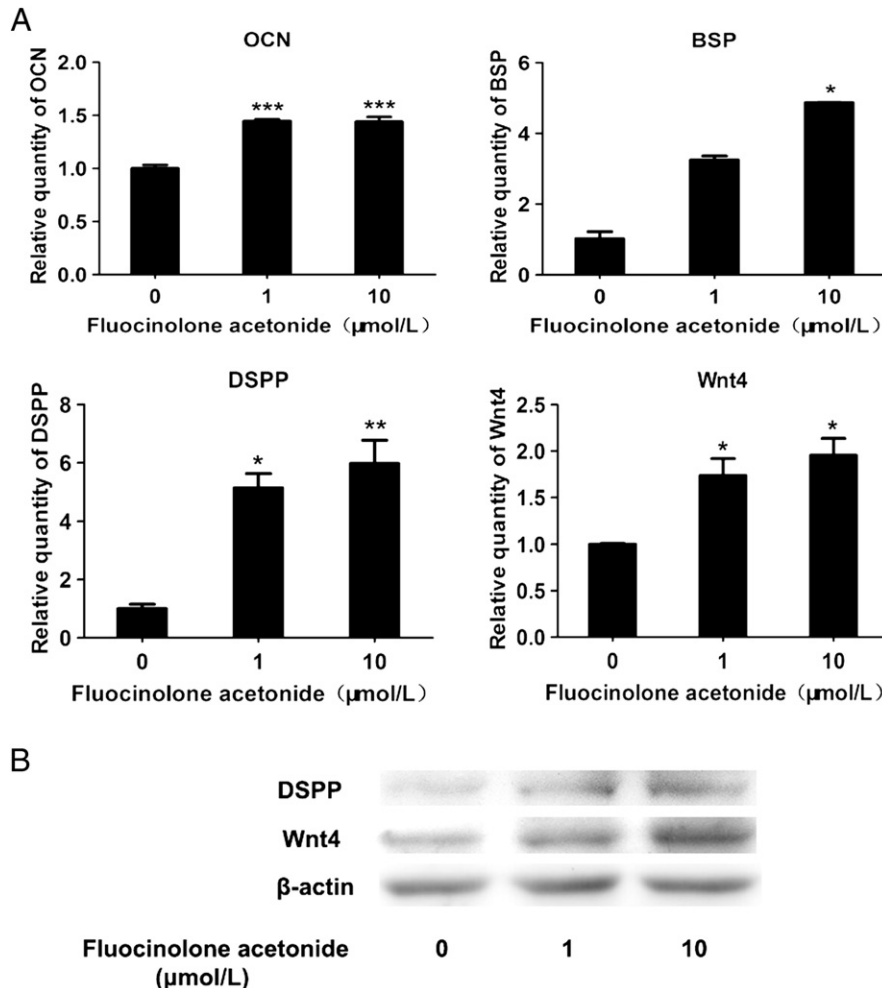


Figure 4. The expression of osteoblast-related markers at both the messenger RNA and protein levels. (A) The messenger RNA expressions of OCN, BSP, DSPP, and Wnt4 were detected by real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase messenger RNA served as an internal control ($*P < .05$, $**P < .01$, $***P < .001$). (B) The protein expressions of DSPP and Wnt4 in fluocinolone acetonide were detected by Western blot on day 7 with the addition of 0, 1, and 10 $\mu\text{mol/L}$ fluocinolone acetonide.

investigated to determine whether fluocinolone acetonide has the potential osteodifferentiation of DPCs. ALP is the early-stage biomarker of osteodifferentiation, which is highly expressed in mineralized-forming cells. BSP is the middle-stage biomarker of osteogenesis, which is highly expressed in mature mineralized tissues. OCN is the late-stage biomarker of osteogenesis, which is restricted to cells of mineralized tissues, including bone, dentin, and cementum. DSPP is a major non-collagenous dentin matrix protein expressed specifically by odontoblasts during dentinogenesis, which has been considered to be a dentin-specific protein (16).

Our results showed that the quantity and activity of ALP was significantly up-regulated in fluocinolone acetonide-treated DPCs, whereas the expression level of BSP and OCN was higher in fluocinolone acetonide-treated DPCs than untreated control. These indicated that fluocinolone acetonide had an ability to induce osteodifferentiation in DPCs, whereas alizarin red staining showed fluocinolone acetonide-induced DPCs could not form mineralized nodules *in vitro*. These results hinted that fluocinolone acetonide had only a limited capacity of osteodifferentiation in DPCs. In another experiment, bone marrow mesenchymal stem cells were treated with fluocinolone acetonide (10 $\mu\text{mol/L}$) and sodium β -glycerophosphate (10 mmol/L) for alizarin red staining. The staining showed that mineralized nodules were formed on 21 days *in vitro* (results not shown). Considering that the phosphate radical is essential for the formation of mineralized nodules, the absence of it may explain the blocked late mineralized process in fluocinolone acetonide groups.

DSPP was significantly up-regulated in fluocinolone acetonide-treated DPCs at both messenger RNA and protein levels. Of particular importance is that DSPP is found in high concentrations in mature dentin and is less expressed in the odontoblasts. The mutations in the DSPP gene are associated with dentinogenesis imperfecta in humans (17). DSPP knockout mice display a wide predentin zone and develop defective dentin mineralization (18). Evidence showed that DSPP was necessary for the dentin formation and important to control the mineralization (19, 20).

Numerous studies showed that the Wnt pathway was involved in the process of biomineralization (21). To investigate whether Wnt pathway is involved in the fluocinolone acetonide-induced mineralization of DPCs, Wnt4, one of the noncanonical Wnt pathway members, was detected by real-time PCR and Western blot. We found that Wnt4 was markedly up-regulated by fluocinolone acetonide at both the messenger RNA and protein levels. In another article, Chang et al (22) showed that Wnt4 could promote bone regeneration via the p38 MAPK pathway in mesenchymal stem cells (22). Our results revealed that Wnt4 was involved in fluocinolone acetonide-mediated biomineralization of DPCs, which was consistent with Chang et al's study.

In conclusion, fluocinolone acetonide has the potential to promote the proliferation of DPCs, especially for the CD146+ stem cell subpopulation. Fluocinolone acetonide can initiate the biomineralization in DPCs, but it has no capacity to form mineralized nodes in DPCs. Fluocinolone acetonide promotes the mineralization process of DPCs via the up-regulation of ALP, BSP, OCN, and DSPP. In addition, Wnt4 could be involved in this process. However, the concise underlying mechanisms remain unclear; further study is needed. In a word, fluocinolone acetonide has a potential value in the recovery of injured dental pulp tissues.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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