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Concentrated Growth Factor Promotes Dental Pulp Cells Proliferation and Mineralization and Facilitates Recovery of Dental Pulp Tissue

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Background: Dental pulp cells (DPCs) play vital roles in the recovery of dental pulp tissue. Concentrated growth factor (CGF) can promote proliferation and mineralization of various cells. However, the functions of CGF on DPCs and dental pulp tissue are unclear. The object of our study was to identify the roles of CGF in DPCs proliferation and mineralization *in vitro* and to assess the effects of CGF on direct pulp capping *in vivo*.





Material/Methods: We performed CCK-8 and Transwell assay to detect proliferation and migration activity of DPCs. Alizarin Red staining was performed to examine mineralized nodules. Alkaline phosphatase activity test was used to measure the mineralization capacity of DPCs. We assessed the odontogenic differentiation gene expression level by Western blot and qPCR. The effect of CGF on direct pulp capping *in vivo* were evaluated by radiography and histopathology.

Results: CGF increased the number of proliferative and migratory DPCs. CGF enhanced DPCs mineralized nodules and improved the gene expression levels of DSPP, DMP-1, BSP, and ALP. CGF upregulated the protein levels of ALP, BMP2, SMAD5, Runx2, and p-Smad, and the effect could be partially reversed by Noggin. CGF promoted pulp recovery and kept its vitality in directly pulp capping.

Conclusions: CGF promotes DPCs proliferation and mineralization. It regulates the mineralization of DPCs via the BMP2/SMAD5/Runx2 signaling pathway. CGF can be used as the effective graft for direct pulp capping.

MeSH Keywords: **Cell Proliferation • Dental Pulp • Dental Pulp Capping • Platelet-Derived Growth Factor • Tooth Remineralization**

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Background

Dental pulp plays critical roles in the homeostasis of teeth, which includes the transit of oxygen and nutrients for metabolism of the tooth, producing dentin and maintaining the vitality of the pulp-dentin complex [1–3]. Dental pulp cells (DPCs) are oral-derived stem cells that have anti-inflammatory effects, form scaffolding, and play immunomodulatory roles, which contributes to the recovery of damaged tissue [4]. DPCs are isolated and cultured from dental pulp tissue. DPCs can differentiate into multiple cell types and possess high growth potential [5]. Due to their easy availability and better ethics profile, DPCs can be used as a new therapeutic option in tissue engineering [4]. When teeth suffered from trauma or caries, DPCs can differentiate into odontoblasts or other functional cells to repair the injured pulp tissue [5,6]. Dental pulp tissue healing is a complex, synergistic process that includes cell proliferation, migration, angiogenesis, differentiation, inflammation, and tissue remodeling [7]. Morphologically, pulp tissue recovery includes connective tissue formation, vascularization, innervation, and dentin-like tissue deposition [8]. DPCs can differentiate into various cell types and engage in cross-talk with other cells around them [7]. DPCs usually reside around the dental pulp blood vessels, called stem cell niches. The cross-talk between the dental pulp and endothelial cells (ECs) has mutual benefits. First, DPCs can release beneficial factors promoting vessel formation at the damaged tissue. Second, ECs can promote DPCs proliferation, and osteogenic/odontogenic differentiation by secreting molecules via signaling [9]. During dental pulp tissue repair and healing, dental pulp cells have high proliferative and migratory capacity, and reparative responses can involve direct differentiation or indirect stimulation [10]. Thus, the identification of effective material for pulp tissue recovery is urgently needed to improve clinical outcomes.

Generally, tissue engineering consists of 3 elements: seed cells, scaffolds, and signaling molecules [1]. Platelet concentrates contain numerous growth factors and have been used to promote the healing of hard and soft tissue in clinical treatments [11]. Concentrated growth factor (CGF) is a recently discovered platelet concentrate; it is generally obtained from autologous venous blood and generates much more, greater, denser fibrin matrix than any previously known platelet concentrates [12,13]. CGF usually acts as a scaffolding material and also delivers many growth factors at the site of application [13]. Three-dimensional (3D) scaffolds can provide space and support for cell growth and formation of tissue-specific shapes, but the remnants of scaffolds hamper tissue regeneration. CGF is a biological material that meets various criteria. It can adapt the tooth's morphology and minimize scaffold-related variability [14]. There are various growth factors in CGF, including fibroblast growth factor (FGF), transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF),

insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF). These growth factors can regulate cell proliferation, migration, matrix remodeling, differentiation, and angiogenesis [11]. Previous studies have demonstrated that CGF can regulate many stem cell biological functions, such as mesenchymal stem cells, bone marrow stromal cells [15], and periodontal ligament cells [13]. A previous study reported that CGF promotes DPSCs proliferation and odontoblastic and endothelial differentiation *in vitro*, in which DPSCs were treated with CGF extracts [16]. However, the effects and mechanism of CGF membrane on mineralization of DPCs have not been clarified.

Pulp exposure can cause bacterial contamination of pulpal tissue, even leading to pulp necrosis. Direct pulp capping is the preferred conservative treatment for preservation of vital pulp [17]. Calcium hydroxide (Ca(OH)₂) and mineral trioxide aggregate (MTA) are conventional and efficient pulp capping materials, but they also have disadvantages in clinical application, such as the presence of pulp chamber obliteration, tooth discoloration, and pulp degeneration [18]. Although there were many researches of CGF applied to clinical practice, the application of CGF on directly pulp capping have not been explored.

The goal of the present study was to elucidate the functions of CGF on proliferation and mineralization of DPCs and to explore the reparative role of CGF in dental pulp injuries. We found that CGF promoted proliferation and mineralization of DPCs and enhanced dental pulp tissue recovery in direct pulp capping. Understanding these effects will have important implications for the development of novel therapeutic strategies for dental pulp injury.

Material and Methods

The Ethics Committee of the Second Hospital of Hebei Medical University approved the experimental protocol. The animal experiments were executed in accordance with the guidelines and principles of the Institutional Animal Care and Use Committee of Hebei Medical University. In clinical treatment, the protocol was explained to the patients, and written consent was obtained from them prior to enrollment in the study.

Isolation, culture, and identification of human dental pulp cells

We obtained healthy human third molars or premolars from healthy human subjects with a mean age of 22 years (range 16–26 years old, 6 donors, 3 males and 3 females). In freshly extracted teeth, we cut a groove 0.5–1.0 mm deep around the cervical area using a sterile high-speed drill. The dental pulp was extracted with a broach, then cut into small pieces (1 mm³) using microsurgical scissors and digested

with type I collagenase (3.0 mg/ml; Sigma, St Louis, MO, USA) and dispase (4.0 mg/ml; Sigma-Aldrich) for 45 min at 37°C. The digested mixture was resuspended in PBS, and centrifuged (10 min, 1200 rpm). Cell suspensions were obtained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA), and we added 10% fetal bovine serum (FBS; Bioind, Bl, Israel), 2.0 mmol/L glutamine (Invitrogen, Carlsbad, CA, USA), and 100 IU/mL penicillin and streptomycin. Cells (1×10^5 /ml) were seeded into 35-mm dishes and cultured at 37°C in a humidified environment of 5% CO₂. Every 3 days, the medium was replaced. Cells between the 3rd and 5th passages were used in the following experiments.

CGF preparation

CGF was obtained from fresh venous blood of healthy volunteers. We collected about 9 mL intravenous blood into sterile Vacuette tubes (Silfradent Srl, Sofia, Italy) without anticoagulant solutions. The operator immediately put the tubes into a Medifuge MF200 (Silfradent Srl) performing CGF centrifugal program according to the following procedure: 30" acceleration, 2' 2700 r.p.m., 4' 2400 r.p.m., 4' 2700 r.p.m., 3' 3000 r.p.m., and 36" deceleration and stop. After the centrifugation, there were 3 layers in the tube: (1) the upper-layer serum, (2) the intermediate-layer CGF gel, and (3) the lower-layer red blood cells (RBCs). We took out the middle CGF gel, then pressed it onto the membrane. CGF membrane was cut into small piece (3×3 mm²) and immersed into saline for the next experiment. For cell culture, a piece of CGF membrane acted as 1 CGF. The cells were divided into 4 groups, each treated with a different dose of CGF: group 1 (0 CGF), group 2 (1 piece of CGF), group 3 (2 pieces of CGF), and group 4 (4 pieces of CGF).

Histomorphometric evaluation with scanning electron microscopy

The CGF membrane was fixed in 2% glutaraldehyde solution dehydrated serially with ethanol solutions. The ultrastructure was observed with a scanning electron microscope (SEM) (Hitachi, Tokyo, Japan) at 6000× magnification.

Cell proliferation assay

To examine DPCs proliferation, we performed cell counting kit-8 (CCK-8) assay to assess the number of viable cells. We seeded DPCs into 12-well plates (1×10^4 cells/well) in DMEM and incubated them in a humidified environment with 5% CO₂ for 24 h at 37°C. Subsequently, a different number of CGF membranes were immersed in the culture medium. At 1, 2, 3, 4, 5, 6, and 7 days following treatment, the cells were removed to 96-well plates, and 10 µl CCK-8 reagent (Dojindo, Kumamoto, Japan) was supplemented to each well. The cells were incubated for 2 h at 37°C. We measured the optical density (OD)

using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

Cell migration assay

Cell migration assays were performed using Transwell chambers. DPCs following culture with CGF at 24 h were seeded into the upper chamber at about 5×10^4 cells in 200 µl of serum-free medium. Culture solution was added to the lower chambers with 10% FBS. After incubation for 24 h at 37°C, we used a cotton swab to remove the DPCs remaining on the upper chamber, and the DPCs that migrated through the membrane to the lower surface of the membrane were fixed with 4% paraformaldehyde about 10 min, then we added crystal violet (Sigma) to the medium and counted the cells using a fluorescence microscope (Olympus, Tokyo, Japan).

Alizarin Red staining

For odontogenesis, DPCs were cultured in 6-well plates at ~70% confluence and incubated in DMEM supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/ml ascorbic acid, and 10% FBS for 21 days. After incubation for 21 days, 4% paraformaldehyde was added into DPCs, kept for 30 min, and mineral deposits were dyed with 0.1% Alizarin Red for 15 min. The mineralized nodules were observed under an inverted microscope to test the mineralization capacities of DPCs. For further quantitative analysis, calcified nodules were treated with 10% cetylpyridinium chloride (in 10 mM sodium phosphate). The absorbance of the supernatant was measured at OD=562 nm with a spectrophotometer.

Alkaline phosphatase (ALP) enzyme activity test

After mineralization induction, protein of DPCs was extracted and measured with a protein quantification kit (Jiancheng, Nanjing, China) at each experimental checkpoint. OD values at 520 nm were analyzed for the detection of ALP activity using the Enzyme Activity Reader (Labsystems Dragon, Wellscan MK3, Finland).

Reverse transcription-quantitative polymerase chain reaction

After culture, total RNA extraction was prepared using TRIzol reagent (Invitrogen), based on the manufacturer's protocol. The extracted RNA (2 µg) was reverse-transcribed to cDNA at 42°C for 1 h. The produced cDNA was acted as a template for qPCR reaction using the All-in-One™ qPCR mix containing 1 U Taq DNA Polymerase (Invitrogen) in a 20 µl final volume and PCR processes with the following program in a real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The following

primers were obtained from Invitrogen (Thermo Scientific, Waltham, MA, USA).

DSPP (130 bp): Forward: CCAGGGACAAGTAAGCATCA,
Reverse: GACAACAGCGACATCCTCAT;
DMP1 (121 bp): Forward: CAGGAAGAGGTGGTGAGTGAG,
Reverse: TTGAGTGGGAGAGTGTGTGC;
BSP (120 bp): Forward: GAAGAGGAGGAGGAAGAAGAGG,
Reverse: TCCATAGCCCAGTGTTGTAGC;
ALP (196 bp): Forward: CCACGTCTTCACATTGGTG,
Reverse: AGACTGCGCTGGTAGTTGT;
GAPDH (258 bp): Forward: AGAAGGCTGGGGCTCATTTG,
Reverse: AGGGGCCATCCACAGTCTTC

Amplification was performed as follows: Initial incubation at 95°C for 10 min, followed by 45 cycles of 95°C for 20sec, 63°C for 30sec, and 72°C for 20 s. The relative mRNA levels of each gene were standardized to the expression of GAPDH with $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

DPCs were lysed and total protein was extracted. Protein concentrations were quantified by the Bicinchoninic Acid Protein Assay (Thermo Fisher Scientific). About 50 µg of extracted protein samples were loaded into each well, separated with 10% SDS-PAGE, then the protein was transferred onto a PVDF membrane. We used 5% non-fat milk in TBST containing Tween-20 to block the membranes at 37°C for 2 h and then incubated them overnight at 4°C supplemented with primary antibodies against BMP2 (1: 200, sc-137087, Santa Cruz Biotechnology, CA, USA), SMAD5 (1: 200, sc-101151, Santa Cruz Biotechnology), p-Smad (1: 1000, mAb #13820, Cell Signaling Technology, Danvers, MA, USA), RUNX2 (1: 200, sc-390715, Santa Cruz Biotechnology), DMP-1 (1: 200, sc-73633, Santa Cruz Biotechnology), DSPP (1: 200, sc-73632, Santa Cruz Biotechnology), and ALP (1: 200, sc-373737, Santa Cruz Biotechnology). Membranes were washed with TBST, incubated with IRDye800 conjugated secondary antibody at 37°C for 60 min, and scanned with the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA). Data were normalized to β -actin (1: 1000, mAb #3700, Cell Signaling Technology) levels and analyzed with Image-Pro Plus software, version 7.0 (Rockville, MD, USA).

Pulp capping procedure

Six male beagle dogs about 12-months old were used in the present study. Each dog weighed about 11 kg. There were 48 second and third premolars in this study. We divided the teeth into 3 groups (n=16 per group), including CGF group, MTA group, Ca(OH)₂ group. During the experiment, the dogs were put under general anesthesia by intramuscular injection with 0.07–0.08 ml/kg of sumianxin (Shengda, Jilin, China).

Then, 0.3 ml/kg of pentobarbital sodium intramuscular injection was used for anesthesia maintenance, and 1% lidocaine with 1: 100 000 epinephrine was injected for local anesthesia.

The operative area was disinfected with 0.2% chlorhexidine. Rubber dam isolation was placed for each experimental tooth. The access cavity was prepared from the occlusal surface and a pulp chamber was mechanically exposed. The access cavity was flushed with a sterile saline solution, and a small moist cotton pellet was placed into the access cavity to stop bleeding. After hemostasis had been achieved, various pulp capping materials were placed directly over the exposed surface of pulp. The tooth was then sealed with glass ionomer cement (3M ESPE, Seefeld, Germany). Radiographs were taken for all experimental teeth after the treatment. The dogs were sacrificed 3 months after surgery. The block sections containing teeth were dissected out and placed in 10% buffered formalin for fixation.

Histologic analysis

After immersion in 10% buffered formalin for 7 days, the specimens were decalcified with 10% EDTA for 6 months. Then, the teeth were embedded in paraffin wax and cut along the long axis into 5-µm slices. Sections were dyed by hematoxylin and eosin (HE) stain. Each specimen was evaluated under a light microscope.

Statistical analysis

We used SPSS software (version 19.0; SPSS, Chicago, IL, USA) to analyze the data. The equal variance between individual groups was identified by a two-tailed *t* test or one-way analysis of variance (ANOVA). Multigroup comparisons of the means were carried out by one-way ANOVA test with post hoc contrasts by Student-Newman-Keuls test. Data are presented as mean value \pm standard deviation (SD). Experiments were conducted 3 times independently. A statistically significant difference was determined by $P < 0.05$.

Results

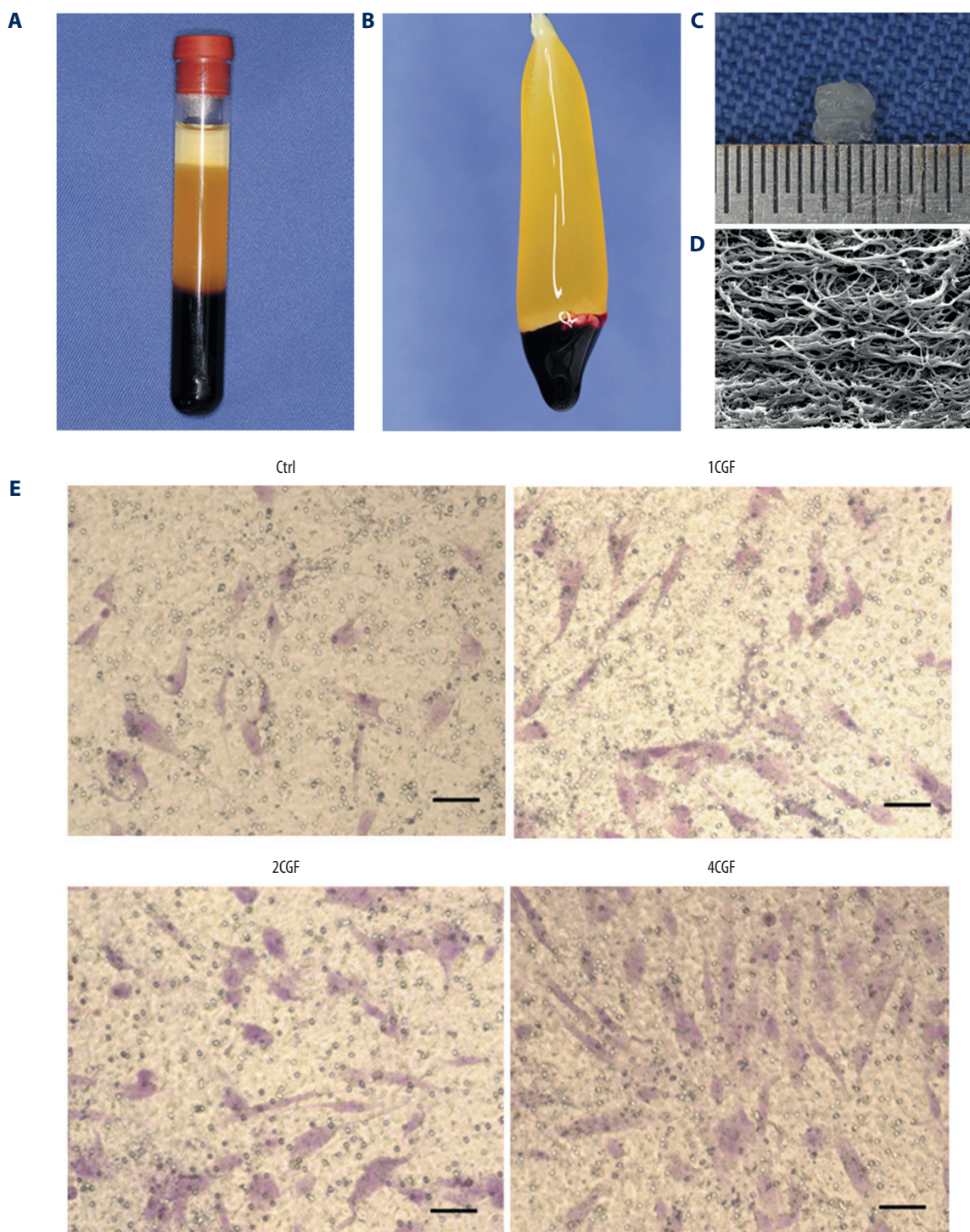
Characteristics of CGF

After centrifugation, the whole blood was divided into 3 layers. The upper layer was the blood serum. The lower layer was red blood cells. CGF gel was in the middle layer (Figure 1A). Then, the gel was removed (Figure 1B), pressed onto membranes, and cut into pieces (3×3 mm²) (Figure 1C). The ultrastructure of CGF samples were examined by SEM (Figure 1D). The image showed CGF contained numerous fibrin network constituted by extensive amounts of thin and thick fibrinogen fibers and exhibited porous structures.

The effects of CGF on DPCs migration and proliferation

Transwell assay was used to validate the effect of CGF on DPCs migration (Figure 1E). The results showed that CGF significantly increased the numbers of migrating DPCs compared to the

control group, and the effect was dose-dependent (Figure 1F). The proliferation of DPCs was investigated by CCK-8, showing that the numbers of proliferative DPCs in the CGF groups were remarkably higher compare to those in the control group after 3 days, although all CGF groups had no significant difference.



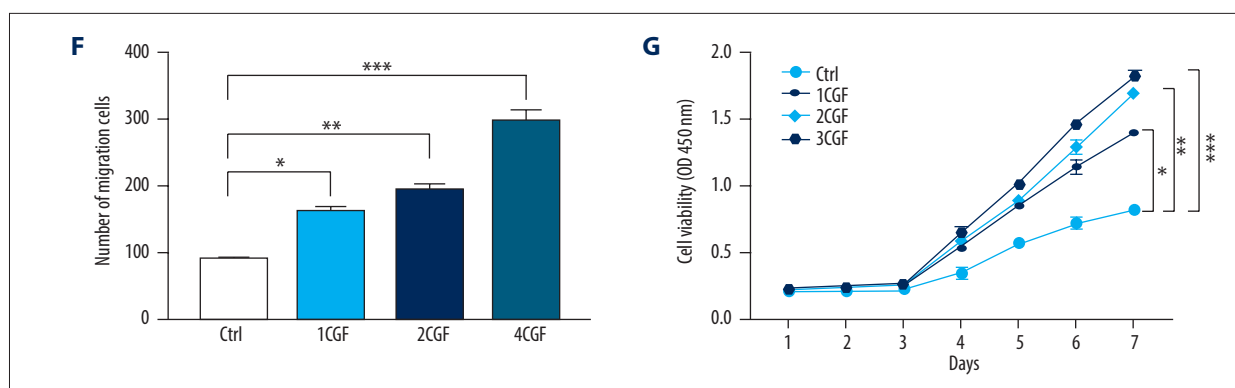


Figure 1. Concentrated growth factor (CGF) promotes DPCs proliferation and migration. (A) CGF gel in the middle of tube after centrifugation. (B) Macroscopic features of CGF gel. (C) The CGF membrane was cut into piece about 3×3 mm², and acts as 1 CGF. (D) Microstructure view of CGF membrane under scanning electron microscope (SEM, ×8000). (E) Crystal violet staining showed the cells traversed the Transwell membrane in different groups co-cultured with CGF at 24 h. Scale bar, 50 μm. (F) CGF remarkably improved the number of DPCs migrating. (G) The results of CCK-8 indicated that CGF groups had significantly facilitated DPCs proliferation compared with the control group after 3 days. * P<0.05; ** P<0.01; *** P<0.001. Ctrl – normal culture medium; 1 CGF – 1 piece of CGF membrane; 2 CGF – 2 pieces of CGF membrane; 4 CGFs – 4 pieces of CGF membrane.

The increase in cell numbers was most significant at day 7 (Figure 1G).

The effect of CGF on DPCs mineralization

To identify the ability of CGF to induce odontoblastic differentiation of DPCs, Alizarin Red staining was performed on day 21 after CGF treatment. There was more mineralization nodules formation in CGF groups compared to those in the control group (Figure 2A). The CGF groups had significantly increased calcium contents compared to the controls (Figure 2B).

To further clarify the effect of CGF on odontogenic differentiation, we used Western blot analysis to assess the protein expression of DSPP, DMP-1, and ALP, which were associated with mineralization (Figure 2C). The result showed the protein levels of DSPP, DMP-1, and ALP were significantly improved in the CGF group compare to the control group at day 7 and day 14, while 4 pieces of CGF had decreased expression of DSPP, DMP-1, and ALP at day 14 compared to other CGF groups (Figure 2D).

The ALP activities were detected to further evaluate the ability of CGF to modulate odontoblastic differentiation of DPCs. With increasing doses of CGF, ALP activity was gradually up-regulated on day 7 and day 14 (Figure 2E).

To confirm the effect of CGF on odontoblastic differentiation, we also evaluated mRNA levels of ALP, DSPP, DMP-1, and BSP in DPCs by real-time PCR at day 7 and day 14. The results demonstrated that CGF significantly increased mRNA levels of ALP, DSPP, DMP-1, and BSP in DPCs at day 7 (Figure 2F) and day 14 (Figure 2G) compared with the control group. Meanwhile,

4 pieces of CGF conversely repressed the ALP, DSPP, and BSP mRNA levels at day 14 compared to other CGF groups, which was consistent with the protein expression levels. The results showed CGF promoted the mineralization of DPCs.

CGF upregulates mineralization gene expression

To explore the possible mechanism by which CGF accelerates the mineralization of DPCs, we detected the protein expressions of BMP2, SMAD5, and Runx2 at day 7 by Western blot analysis (Figure 3A). The results showed the expression of BMP2 (Figure 3B), SMAD5 (Figure 3C), and Runx2 (Figure 3D) were significantly upregulated in a dose-dependent manner in DPCs mineralization co-cultured with CGF.

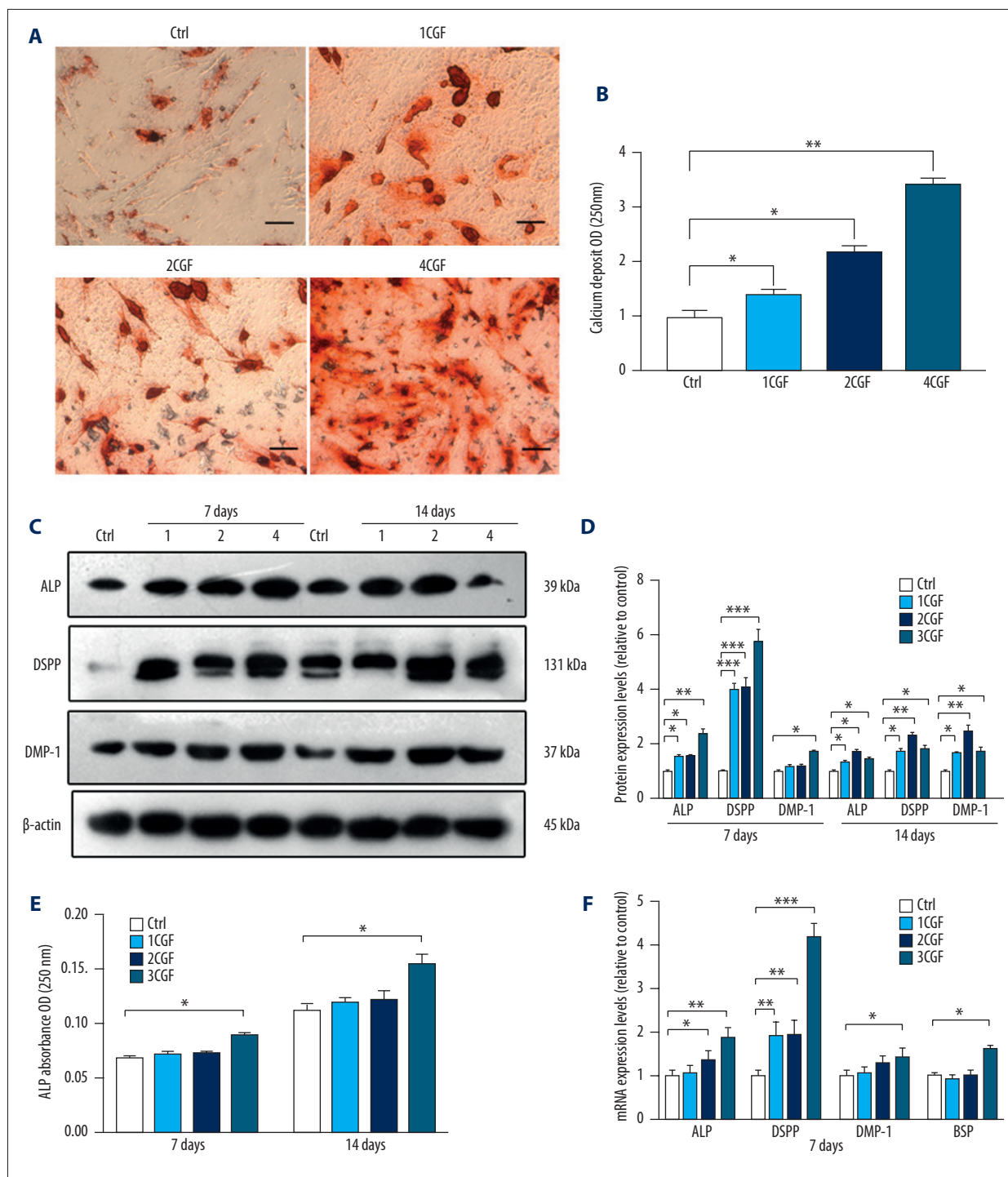
CGF promotes DPCs mineralization via BMP2/SMAD5/Runx2 signaling pathway

According to the results, we predicted that the BMP2/SMAD5/Runx2 signaling pathway is involved in mineralization of DPCs. Then, we co-cultured DPCs with 4 pieces of CGF and the inhibitor of BMP2 signaling pathway (Noggin) for 7 days. The Western blot results (Figure 4A) showed that CGF up-regulated the protein expression of ALP, BMP2, SMAD5, p-Smad1/5/8, and Runx2, while treatment with Noggin partially decreased protein expression levels of ALP (Figure 4B), BMP2 (Figure 4C), SMAD5 (Figure 4D), p-Smad1/5/8 (Figure 4E), and Runx2 (Figure 4F) compared to the CGF groups. The ALP activities and Alizarin Red staining were used to further evaluate the effect of the BMP2/SMAD5/Runx2 signaling pathway in mineralization of DPCs. The results showed CGF increased the ALP activity (Figure 4G) and mineralization nodules formation (Figure 4H), while Noggin partly abrogated the promoting

role of CGF. These results suggest that the BMP2/SMAD5/Runx2 pathway participates in the concentrated growth factor-mediated mineralization of DPCs.

Effects of CGF on pulp capping applied to canine teeth

After directly pulp capping for 3 months in beagle dogs, radiograph evaluation revealed that dentin bridging with $\text{Ca}(\text{OH})_2$ and MTA was thick, the pulp chamber was narrowed, and dentin of the root canal was thickened. CGF specimens had less dentin bridging, narrowed pulp chamber, and root canal.



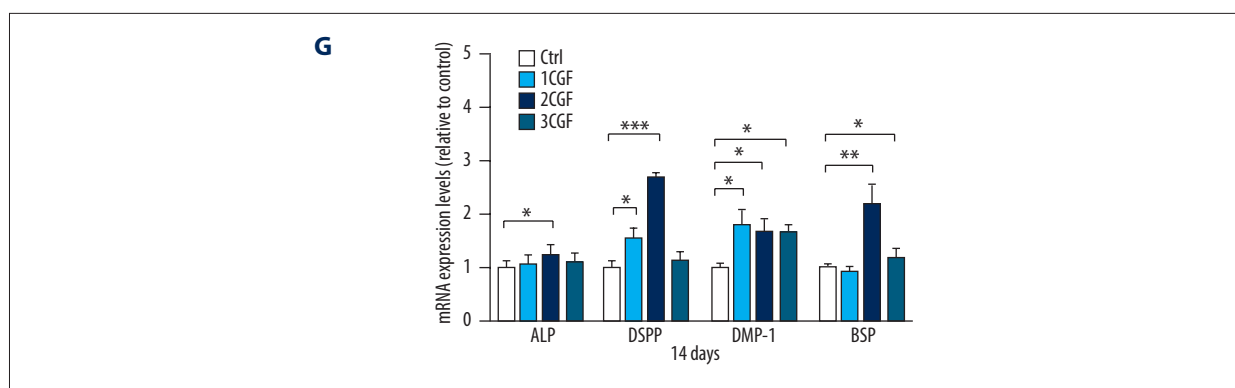


Figure 2. CGF regulates the mineralization of DPCs. **(A)** Alizarin Red staining shown mineralized nodules were formed in different groups after 21 days of osteogenic induction. Scale bar, 50 μ m. **(B)** Quantification measurement of calcium deposit with spectrophotometer after 21 days. **(C)** Western blot analysis of ALP, DSPP, and DMP-1 expression in DPCs at day 7 and 14. **(D)** Relative quantity of ALP, DSPP, and DMP-1 protein expression levels. **(E)** ALP activity was analyzed by the integrated optical density (IOD) of representative images at days 7 and 14. **(F)** mRNA expression of DMP-1, ALP, DSPP, and BSP in DPCs after being co-cultured with CGF at day 7. **(G)** mRNA expression of DMP-1, ALP, DSPP, and BSP in DPCs after being co-cultured with CGF at day 14. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Ctrl – differentiation medium; 1 CGF – 1 piece of CGF membrane; 2 CGF – 2 pieces of CGF membrane; 4 CGFs – 4 pieces of CGF membrane.

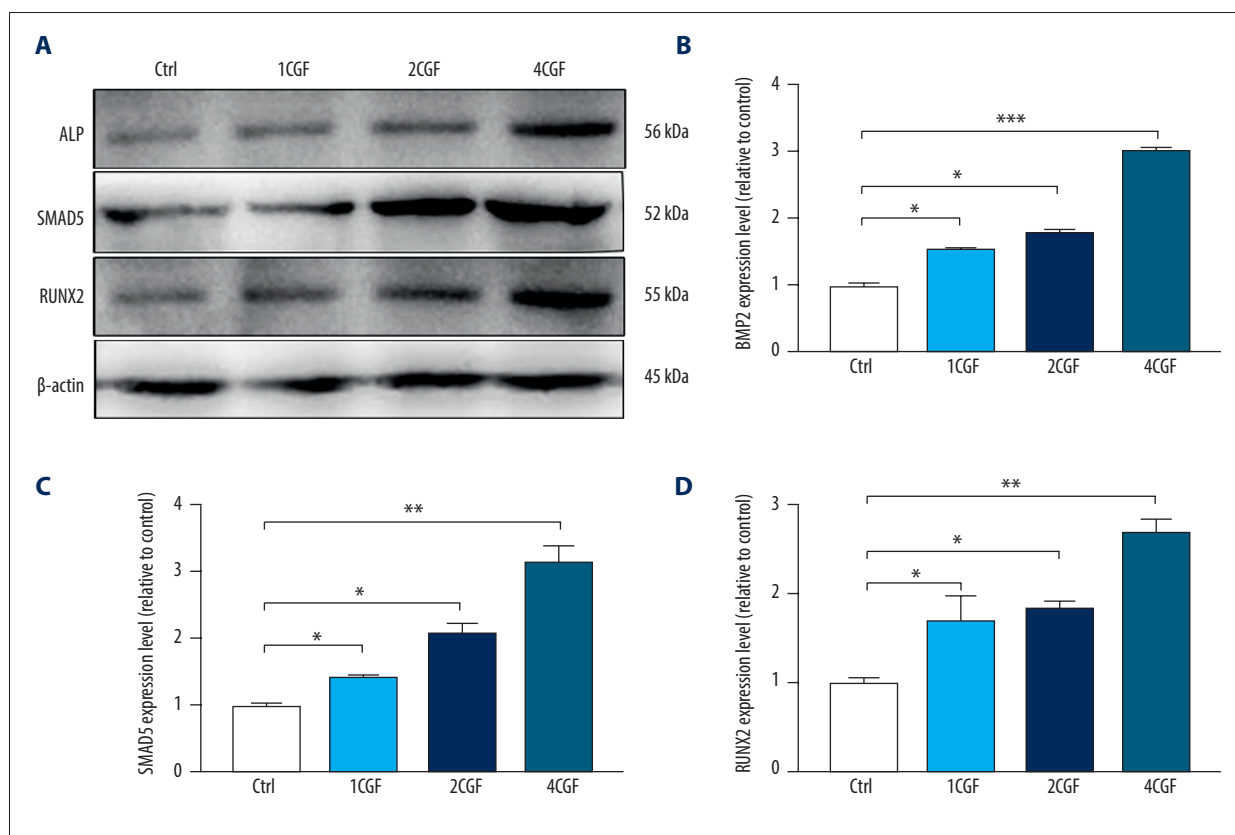


Figure 3. CGF mediated odontogenic differentiation of DPCs via BMP2 signaling pathway. **(A)** Western blot results of BMP2, SMAD5, and RUNX2 protein expression in DPCs treated with CGF after 7 days. **(B-D)** Relative quantity of BMP2, SMAD5, and Runx2 protein expression levels in DPCs treated with CGF. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Ctrl – differentiation medium; 1 CGF – 1 piece of CGF membrane; 2 CGF – 2 pieces of CGF membrane; 4 CGFs – 4 pieces of CGF membrane.

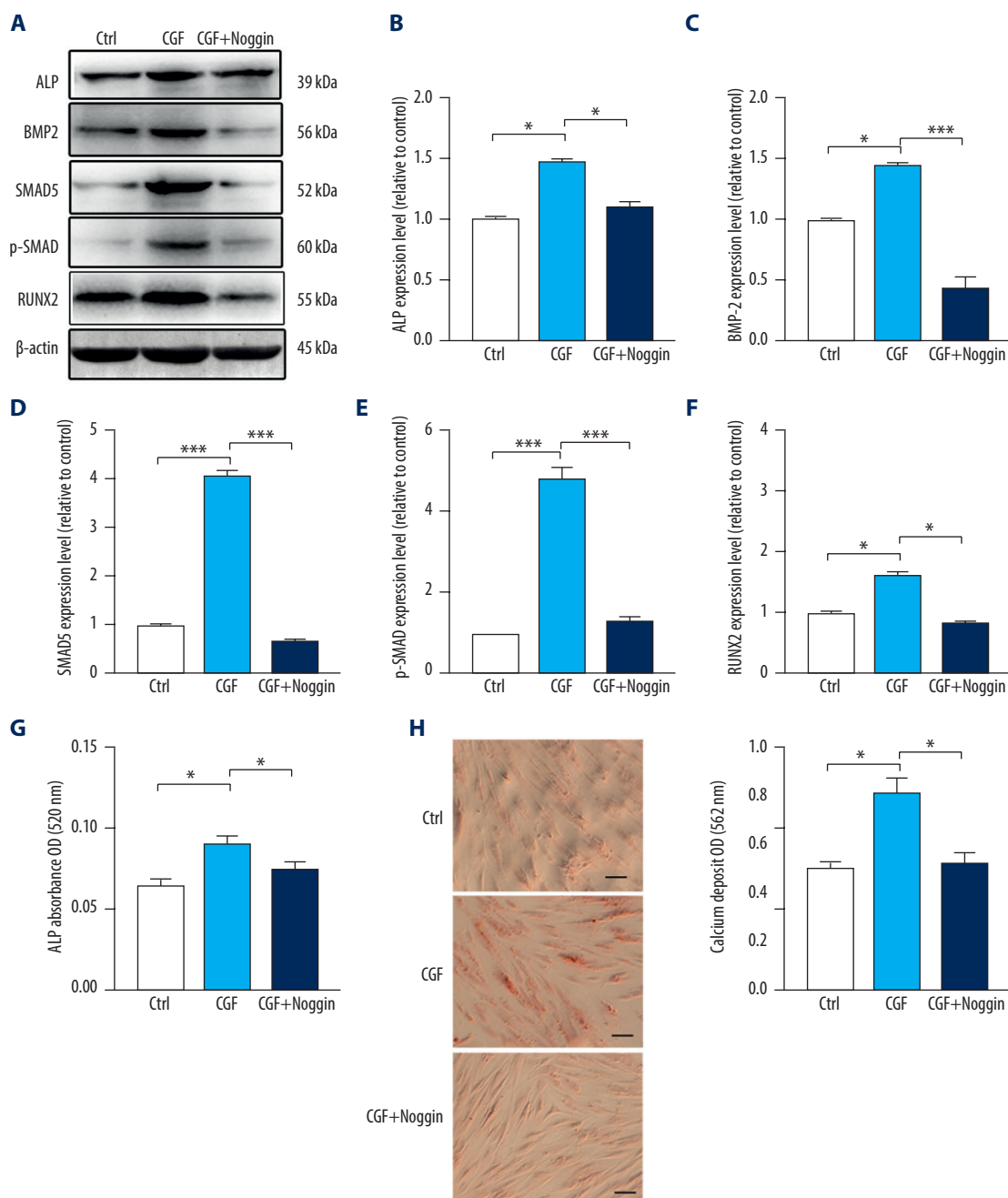


Figure 4. The rescue experiments for BMP2 signaling pathway in DPCs treated with CGF and Noggin. **(A)** Western blotting analysis of ALP, BMP2, SMAD5, Runx2, and phosphorylated SMAD (p-Smad) in DPCs after 7 days. **(B–F)** Relative quantity of ALP, BMP2, SMAD5, p-Smad, and Runx2 protein expression levels in DPCs treated with CGF and CGF+Noggin. **(G)** ALP activity was analyzed by spectrophotometer. **(H)** Alizarin Red stained shown mineralized condition of DPCs induced with CGF and CGF+Noggin for 7 days. Scale bar, 50 μm. * P<0.05; ** P<0.01; *** P<0.001. Ctrl – differentiation medium; CGF – 4 pieces of CGF membrane; CGF+Noggin – 4 pieces of CGF membrane+Noggin.

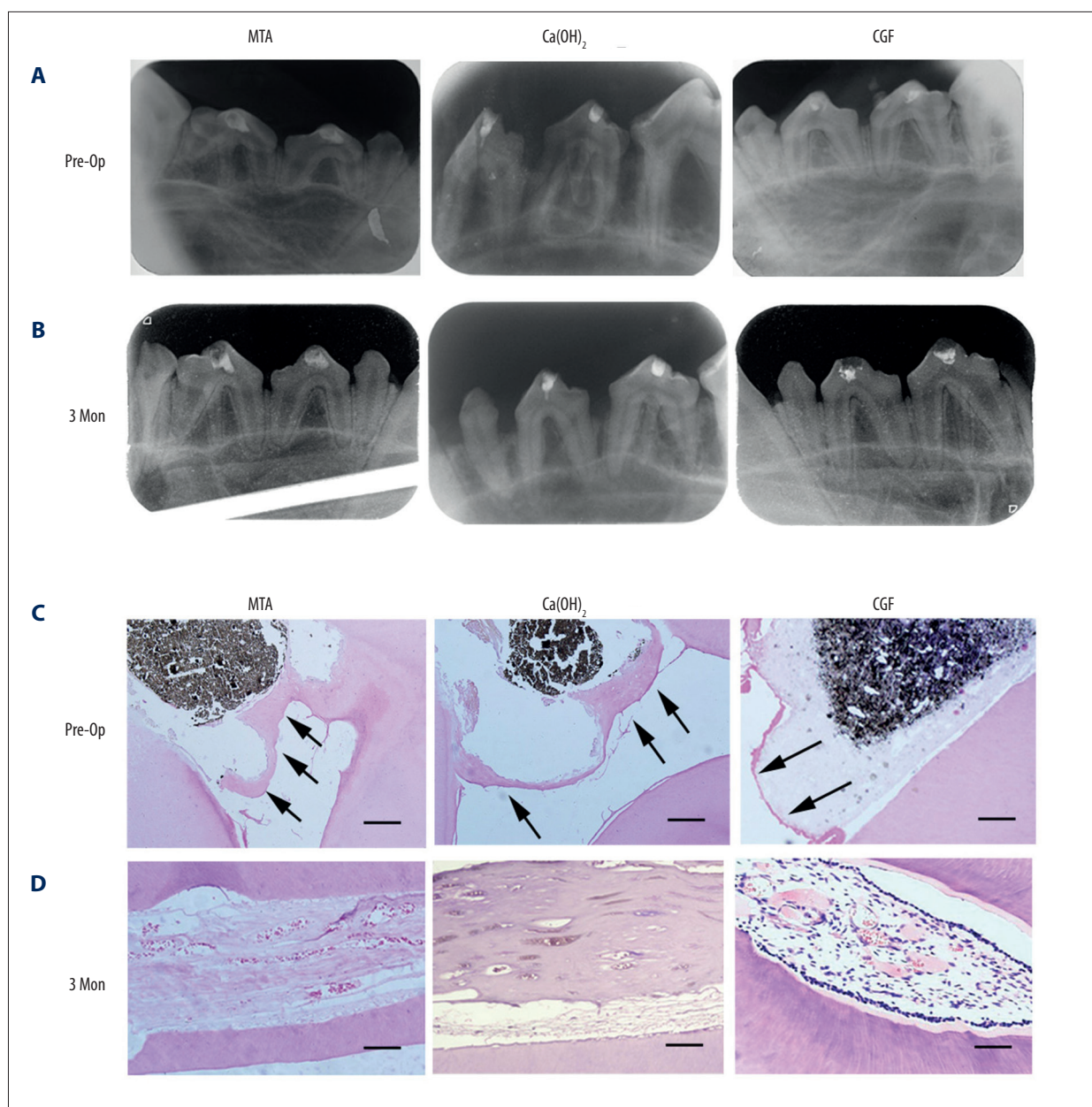


Figure 5. Effects of CGF on pulp capping applied to canine tooth. **(A)** Immediate postoperative radiographs. **(B)** Postoperative radiographs after 3 months showing thick dentin bridging formation in Ca(OH)₂ and MTA groups, narrowed pulp chamber, and thickened dentin of root canal. The CGF group had thin dentin bridging and wide pulp chamber. **(C)** Hematoxylin-eosin staining (HE) showing Ca(OH)₂ and MTA groups had complete calcific barrier formation and the CGF group had a thin calcific barrier (black arrows). Scale bar, 100 µm. **(D)** HE staining showing fiber hyperplasia, congestion, and odontoblasts disappeared in Ca(OH)₂ and MTA groups. The CGF group had odontoblasts regularly arranged and predentin formed. Scale bar, 100 µm.

There were no periapical lesions in any groups (Figure 5A, 5B). Hematoxylin-eosin-stained histopathologic evaluation showing complete calcific barrier formation in Ca(OH)₂ and MTA specimens (Figure 5C), and the pulp showed fiber partially hyperplasia, congestion, and disappearance of odontoblasts (Figure 5D). CGF specimens had thin calcific barrier formation,

and the pulp showed odontoblasts were regularly arranged and predentin formed after pulp capping with CGF for 3 months (Figure 5C, 5D).

Discussion

In this study, we showed CGF promoted proliferation and mineralization of DPCs *in vitro*, and the regulation role of CGF in mineralization was by activation of the BMP-2/SMAD5/Runx2 signaling pathway. Further studies demonstrated CGF facilitated the recovery of dental pulp tissue in a dog model of direct pulp capping.

Dental pulp is susceptible to injury, inflammation, microbial irritants, and other stimulations. With persistence of stimulus, the inflammation will cause pulp necrosis and degeneration. These processes can be terminated by appropriate treatment, and the biological structure and function of dental pulp can be partly or entirely regenerated [19]. Pulp tissue injury can be repaired by DPCs, which reside in normal pulp [3]. DPCs are derived from dental pulp, with strong proliferation and potent differentiation capacity [20]. Under the appropriate conditions, DPCs can be induced to form fibroblast, odontoblasts, osteoblasts, chondrocytes, neurons, and adipocytes *in vitro* [21]. DPCs appear to be promising seed cells and are widely applied in tissue engineering studies [22].

CGF is the third-generation platelet concentrate first achieved by Sacco in 2006 [23]. Generally, platelet concentrate contains quantities of growth factors, which can significantly promote healing of soft tissue and hard tissue [11]. Created with a special centrifuge program, CGF contains more growth factors and a harder fibrinogen structure than previous platelet concentrates [16,24]. In the present study, we found the ultrastructural of CGF was composed of multiple fibrin fibers, which interlaced together and formed a three-dimensional porous network structure. Thus, CGF may act as a biological scaffold in which DPCs can be embedded to differentiate into functional odontoblasts.

The aim of tissue engineering is to preserve the original tissue, replace injured tissues, and regenerate fresh tissue [25]. Cellular proliferation and migration are crucial elements for tissue repair and regeneration [8,26]. Growth factor has the important biology function of promoting cell proliferation activity and migration activity by modulating cellular recruitment [1]. In response to tooth injury, DPCs are induced by growth factors via cell homing to proliferate, migrate, and adhere to the damage sites to replace lost odontoblasts [13,27]. Most relevant studies detected the effects of a single growth factor, but growth factors generally do not act in isolation, and some of them work synergistically. Thus, various growth factors of CGF may be of greater importance than a single bioactive protein or agent [28]. In this study, DPCs were cultured with different doses of CGF membranes, which is more similar to clinical application and is more convenient. Moreover, our study investigated the mechanism of CGF membrane on

mineralization and the recovery effects of CGF on pulp tissue *in vivo*. We concluded that CGF progressively promoted DPCs proliferation and migration. This may be caused by the special structure of CGF, and growth factors can be sustained and slowly released [12,29,30]. CGF prolonged the effect of growth factors and promoted cell proliferation.

The mechanism of pulp injury and repair includes odontoblast differentiation of DPCs and secretion of tertiary dentine matrix [2]. ALP is considered the typical osteogenic marker playing a critical role in bone formation, and is associated with osteogenic differentiation of cells at early stage [31]. According to previous reports, day 7 and 14 were 2 important time points when cells were induced for odontogenic/osteogenic differentiation [5,15,16]. In the present study, we observed CGF significantly increased ALP activity and nodule mineralization. Furthermore, we confirmed that CGF significantly upregulated the gene expression levels of odontogenic differentiation biomarkers of DPCs, including DSPP, DMP1, and BSP. Interestingly, we found the gene levels of ALP, DMP-1, and DSPP were down-regulated in DPCs induced with 4 CGF at day 14, probably mainly due to the decrease of DPCs viability at the later stage of mineralization. These findings provided evidence that CGF promotes the mineralization of DPCs.

The BMP2/SMAD5/Runx2 signaling pathway is a key signaling pathway correlated with bone reconstruction and formation [32]. Bone morphogenetic protein 2 (BMP2) plays vital roles in tooth development and improves the terminal differentiation of odontoblasts [33]. BMP-2 can activate downstream cytokines, including Smad5, by inducing phosphorylation of SMAD5 signaling protein [34,35]. Runt-related transcription factor 2 (Runx2) is an essential osteogenic transcriptional factor that modulates the transcription of downstream cytokines in early bone- and tooth-related differentiation [36]. In the present study, we demonstrated that CGF promoted DPCs mineralization by upregulating the protein expressions of BMP-2, SMAD5, and Runx2 during odontogenic differentiation of DPCs. In addition, the effect of CGF-induced odontogenic was partially reverse by Noggin (an inhibitor of the BMP signaling pathway). These findings indicated that CGF promotes odontoblast differentiation via the BMP-2/SMAD5/Runx2 signaling pathway in DPCs.

After traumatic and carious exposures of dental pulp tissue, direct pulp capping is an efficient option to maintain pulp viability and function [17]. Ca(OH)₂ and MTA are widely used in pulp capping due to their high PH values [37]. They exert excellent antibacterial properties and dentin bridge formation capacity [18]. Although Ca(OH)₂ has been used as the criterion standard for clinical treatment, MTA is considered to have superior biocompatibility [38]. MTA can form a hard tissue barrier at the exposed site of pulp, and effectively prevent bacterial

leakage [17]. Despite its safety and efficacy, MTA has certain drawbacks, such as poor operability, tooth staining, long setting time, and high cost [39], possibly leading to pulp diffuse calcific deposition and finally resulting in pulp chamber and canal obliteration [1]. In this study, CGF provided evidences that the application of CGF for pulp capping is more similar to the physiological state than are Ca(OH)₂ and MTA. It appears to be effective for maintaining pulp normal functional structure and vitality. However, further research with longer observation time is needed before clinical application.

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Conclusions

The results of the present study suggest that CGF increases the proliferation and migration ability of DPCs in a dosage- and time-dependent manner, and facilitates the mineralization of DPCs by upregulating mineralization genes. CGF appears to be a promising pulp capping agent for pulp injury treatment.

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Conflict of interests

None.

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