

Concentrated growth factor exudate enhances the proliferation of human periodontal ligament cells in the presence of TNF- α

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Abstract. The purpose of this study was to evaluate the effects of concentrated growth factor exudate (CGFe) on human periodontal ligament cells (hPDLs) stimulated by tumor necrosis factor (TNF)- α . From the peripheral blood of healthy donors, CGFe was prepared according to the Sacco protocol after 7 days of incubation. The hPDLs were cultured by a tissue explant method and identified with anti-vimentin and anti-cytokeratin antibodies. Cells were subjected to four different treatments: i) Control; ii) TNF- α (10 ng/ml); iii) CGFe (concentration 50%); and iv) CGFe+TNF- α . The proliferation of hPDLs was measured with Cell Counting Kit-8 assays. Osteogenic differentiation and mineralization were determined by Alizarin Red S staining, alkaline phosphatase activity, western blotting and reverse transcription-quantitative polymerase chain reaction. CGFe enhanced cell proliferation and upregulated ALP activity, the mineralization level, and osteogenic-associated osteocalcin, runt-related transcription factor 2 and Osterix gene expression in hPDLs under inflammatory conditions induced by TNF- α . The present study demonstrated that CGFe enhanced hPDL proliferation and osteogenic differentiation in the presence of TNF- α -induced inflammation. As CGFe can be obtained from the venous blood of patients, it generates no immune reaction. Thus, it is more economical and beneficial to use CGFe in clinical periodontal regeneration practice than synthetic growth factors.

Introduction

Periodontitis and chronic apical periodontitis often cause periodontal tissue destruction and severe alveolar bone defects (1). In periodontally compromised teeth, when partial or full socket wall destruction is evident, connective tissue will grow into the extraction site and lead to a deficient ridge (1). Periodontitis is widely treated with instant implant technology in clinical practice (2). However, periodontal tissue destruction and alveolar bone defects significantly weaken the stability and shorten the service life of dental implants (2). To improve the success rate of dental implants, concentrated growth factor (CGF) can be used to promote periodontal tissue regeneration and osteogenic differentiation (3). CGF is a gel-like substance, obtained by centrifugation of venous blood, which is rich in growth factors and fibrin (4-8), and CGF combined with bone graft material promotes immediate periodontal tissue regeneration and osteogenic differentiation (8,9).

Previous studies have reported that platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in the first and second phases promote tissue regeneration, inhibit infection, regulate inflammation and reduce postoperative reactions (9-14). CGF is a third-phase plasma extract, consisting of multiple growth factors (4-8). Due to the unique structure of CGF fibrin, it can replace the diaphragm used in guiding bone regeneration to a certain extent (8,9,15). CGF has been widely studied in the fields of oral implantation, extraction site preservation, jaw cyst treatment, and fracture healing promotion (4-8,16,17).

CGF exudate (CGFe) is extracted from CGF and used in research labs to study the effects of CGF. Previous studies have shown that CGFe significantly shortens the time for osteogenesis in the operational area and distinctly improves bone formation quality (8,9). CGFe has been demonstrated to stimulate the proliferation of human periodontal ligament fibroblasts (PDLFs) (17). However, these previous studies were performed without the influence of inflammatory factors, and therefore did not sufficiently reflect the clinical environment. Particularly, the effect of CGFe on enhancing the proliferation of hPDLs in the presence of tumor necrosis factor (TNF)- α -induced inflammation has not yet been investigated, to the best of our knowledge.

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TNF- α is a pro-inflammatory cytokine that is a critical pathological factor for the development of apical inflammation, which significantly inhibits osteogenic differentiation (16,18-21). As TNF- α -induced inflammation is commonly encountered in clinical practice (16,20), it is of great clinical interest to evaluate the effects of CGFe in the presence of TNF- α .

In the present study, the effects of CGFe on hPDLc proliferation and osteogenic differentiation were investigated in an inflammatory environment, stimulated by TNF- α . Specifically, the effects of CGFe on alkaline phosphatase (ALP) activity, mineralization, and osteocalcin (*OCN*), runt-related transcription factor 2 (*RUNX2*), and Osterix (*OSX*) gene expression in hPDLcs under TNF- α -induced inflammatory conditions were examined.

Materials and methods

Preparation of CGFe. The present study was approved by the Ethics Committee of the Jilin University Health Science Center (Changchun, China). In accordance with this committee, CGFe was obtained from three healthy male donors who had visited the outpatient clinic at the Health Center of Jilin University (Changchun, China) from August 2017 to March 2018. They were non-smokers and non-drinkers aged from 22 to 30 years old, and their informed consent was obtained. The experiments described below were carried out under similar conditions and procedures as the previous study (11).

Venous blood samples from the donors were used to produce human CGF according to a previously described protocol (22). In brief, blood samples were centrifuged at 750 $\times$ g for 12 min at 4°C. Between the acellular plasma and red blood cells (RBCs), a white CGF clot formed. The CGF clot was held with sterile forceps, separated from the RBCs using scissors, placed on the grid of an endo box and compressed by the endo box cover. After 1 min of applied pressure, the CGF clot was converted into CGF membrane, and the CGF exudate (CGFe) was collected in the tray of the endo box.

The CGFe was centrifuged at 500 $\times$ g for 5 min at 4°C to remove RBCs. Then, the CGFe was precipitated and filtered using a 0.22 μ m sterile syringe filter unit (Merck KGaA, Darmstadt, Germany). The pooled CGFe samples were stored at -80°C prior to use. The original concentration of CGFe was defined as 100%, and a 50% concentration of CGFe was obtained by dilution with Minimal Essential Medium- α (α -MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and used in subsequent experiments.

Human periodontal ligament cell (hPDLc) culture. In total, 10 healthy and noncarious premolars from three male and two female donors aged from 13 to 18 years old who had received orthodontic treatment at the Oral and Maxillofacial Surgery Department of the Stomatology School of Jilin University (Changchun, China) were obtained with informed consent. Periodontal ligaments were gently scraped from the middle third of the tooth-root surface with a sharp scalpel, minced with ophthalmic scissors and rinsed with α -MEM. These explants were cultured in α -MEM supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Chicago, IL, USA), 1% streptomycin and penicillin (Gibco; Thermo Fisher

Scientific, Inc.) at 37°C in 5% CO₂. Examination by inverted light microscopy (IX73; Olympus Corporation, Tokyo, Japan) at magnifications of $\times$ 20 or $\times$ 40 was performed daily, and the medium was replaced every 3 days. Once cells reached 80% confluence, they were transferred to a 75 cm² flask, and this was defined as passage one. The same procedure was performed repeatedly to produce multiple passages. Cells prior to passage 6 were used in the present study.

Immunocytochemistry staining. hPDLcs at passage 3 were seeded into several 24-well plates at a density of 1 $\times$ 10⁴/well and covered in advance with circular coverslips (diameter, 14 mm) and incubated for 48 h at 37°C. Cells were then rinsed three times with 0.01 M PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Following a further wash with PBS, 0.25% Triton X-100 was added into the 24-well plates, which were incubated at 37°C for 15 min. Endogenous peroxidase activity was eliminated by incubation with 3% H₂O₂ for 10 min at room temperature. Then, hPDLcs were incubated with 1% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) and 22.52 mg/ml glycine in PBS + 0.1% Tween-20 for 30 min at room temperature. Next, cells were incubated with anti-vimentin (1:100; cat. no. ab24525; Abcam, Cambridge, MA, USA) and anti-cytokeratin (1:200; cat. no. AM06387SU-N; OriGene Technologies, Inc., Beijing, China) primary antibodies overnight at 4°C. The SP immunohistochemistry assay kit (cat. no. SP9001; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China) was used for immunocytochemical staining according to the manufacturer's instructions, and a 3,3'-diaminobenzidine was used to stain positive cells. An inverted microscope (IX73; Olympus Corporation, Tokyo, Japan) was used to view the cells at magnifications of $\times$ 20 or $\times$ 40.

Cell Counting Kit (CCK)-8 assay. The objective of this experiment was to examine the effect of CGFe on the proliferation of hPDLcs *in vitro*. CGFe was obtained according to a previous protocol (16). The CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used to determine the effects of CGFe on hPDLc proliferation.

hPDLcs (2 $\times$ 10³/well) were seeded into a 96-well plates containing complete medium (α -MEM supplemented with 10% FBS, 1% streptomycin and penicillin) with 10% FBS and incubated for 24 h at 37°C. Then, hPDLcs were exposed to CGFe (concentration 50%), TNF- α (10 ng/ml), or CGFe+TNF- α for 24, 48 or 72 h at 37°C, respectively. There was one additional control group with no CGFe or TNF- α treatment (complete medium with 10% FBS only). Cell proliferation was determined using the CCK-8 kit at the specified time-points. Kit reagent (10 μ l) was added to the culture medium in each well. After a 90 min incubation at 37°C, absorbance at 450 nm was detected using an automatic microplate reader (Infinite 200 PRO; Tecan Group Ltd., Mannedorf, Switzerland). A well containing complete medium and CCK-8 solution without seeding cells was used as a further control. The assay was performed in duplicate, and the experiments were repeated six times under the same conditions.

ALP activity assay. hPDLcs (500 μ l; 1 $\times$ 10⁴/well) were seeded into 24-well plates and incubated for 24 h at 37°C. Next,

the cells were exposed to CGFe, TNF- α or CGFe+TNF- α for 7 or 14 days. At the given time-points, cells were lysed with 0.1% Triton X-100, and the lysates were centrifuged at 8000 x g for 10 min at 4°C. The supernatant (50 μ l/well) was added to 96-well plates, and ALP activity was examined with a ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. The optical density (OD) values were read at 520 nm with an automatic microplate reader (Infinite 200 PRO; Tecan Group, Ltd.).

Osteogenic differentiation induction and Alizarin Red S staining. hPDLs (500 μ l; 1×10^4 /well) were seeded into 24-well plates in standard medium (α -MEM supplemented with 10% FBS, 1% streptomycin and penicillin) until 60-70% confluence was reached. The medium was then replaced with four different media: i) Mineral induction medium (MM; α -MEM medium containing 10% FBS, 50 mg/ml ascorbic acid, 10 mM β -glycerolphosphate and 10^{-8} M dexamethasone); ii) MM with CGFe (concentration 50%); iii) MM with TNF- α (10 ng/ml); or iv) MM with CGFe+TNF- α (10 ng/ml). The medium was replaced every 3 days.

Alizarin Red staining was used to detect and quantify the formation of mineralized nodules after 21 days. This assay was performed according to a previously described protocol, with minor modifications (9,23). Specifically, the hPDLs were fixed with 95% ethanol for 15 min at room temperature, washed twice with dH₂O and stained with 0.1% Alizarin Red S solution (pH 4.1) for 20 min at room temperature. hPDLs were observed under an inverted phase-contrast microscope (IX73; Olympus Corporation, Tokyo, Japan; magnification, x20 or x40). Next, the hPDLs were washed three times with dH₂O. To semi-quantify the content of mineralized matrix nodules generated from the hPDLs, 100 mM cetyl pyridinium chloride was added to the 24-well plates to dissolve and release the calcium-combined Alizarin Red S into solution. OD values were read at 570 nm, which represented the relative quantity of mineralization nodules.

Western blotting. Cell lysates were prepared in radioimmuno-precipitation assay buffer (150 mM NaCl, 0.1% SDS, 1 mM PMSF, 10 mM Tris-Cl, pH 7.4, 1% sodium deoxycholate, 1% Triton X-100). The cells were treated with four different media (control, CGFe, TNF- α , or CGFe+TNF- α) for 14 days. The cell lysates were incubated for 30 min on ice, then clarified by centrifugation at 6,000 x g for 10 min at 4°C. Protein concentration was determined with a bicinchoninic acid assay. Lysate samples (20 μ l) were denatured and resolved by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes and run at 300 mA for 2 h. Membranes were blocked in 5% non-fat milk at room temperature for 1 h, then incubated with primary antibodies against RUNX2 (1:1,000; cat. no. 12556; Cell Signaling Technology, Inc., Danvers, MA, USA) and Osterix (1:1,000; cat. no. ab229258; Abcam) and GAPDH overnight at 4°C. Membranes were subsequently washed three times in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500; cat. no. 10285-1-AP; ProteinTech Group, Inc., Chicago, IL, USA) for 1 h at room temperature. Following three washes with TBST, the protein bands were

visualized by using an Enhanced Chemiluminescence kit (GE Healthcare Life Sciences) and exposed to X-ray film. GAPDH was used as internal reference and the ImageJ (version 1.48; National Institutes of Health, Bethesda, MD, USA) was used for densitometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). hPDLs (2 ml; 1×10^5 /well) were seeded into 6-well plates in standard medium until 60-70% confluence was reached. The cells were treated with four different media (control, CGFe, TNF- α , or CGFe+TNF- α). The control group was incubated with osteogenic induction medium [α -MEM, 150 μ g/ml ascorbic acid and 10 mM β -sodium glycerophosphate (Sigma-Aldrich; Merck KGaA)]. The experimental groups were treated with osteogenic induction medium plus CGFe, osteogenic induction medium plus TNF- α (10 ng/ml), or osteogenic induction medium plus CGFe+TNF- α (10 ng/ml). RNA was extracted and reverse transcribed into cDNA using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) after 4, 7 and 14 days. cDNA synthesis was performed with 1 μ g of total RNA, random hexamer primers, 5X First-Strand Buffer, and dNTP Mix (10 mM each) using SuperScript[™] II RT (cat. no. 18064014; Invitrogen; Thermo Fisher Scientific, Inc.). The temperature protocol for RT was: Denaturing RNA at 65°C for 5 min, then incubation at 25°C for 10 min and 42°C for 60 min, followed by 70°C for 15 min.

A 10 μ l volume reaction system was adopted for qPCR determination of the expression of the osteogenic genes *OCN*, *RUNX2* and *OSX* using the SYBR[®] Premix Ex Taq[™] II kit (Takara Bio, Inc., Otsu, Japan). qPCR primers were designed to span an intron so that only RNA-specific amplification was possible. PCR was performed with the following thermocycling conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 3 sec and 60°C for 60 sec. Each sample was tested in triplicate, and fold differences in gene expression were calculated using the $2^{-\Delta\Delta C_q}$ method (24) with normalization to β -actin. The primer sequences are presented in Table I.

Statistical analysis. hPDL proliferation and ALP activity assays were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Western blotting and Alizarin Red staining assay data were analyzed using two-way ANOVA, followed by Bonferroni's post-hoc comparisons test for independent samples. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference. All data were expressed as the mean $\pm$ standard deviation, and experiments were performed in triplicate.

Results

Characterization of hPDLs. hPDLs grew out from the tissue explant after 7 and 10 days of culture (Fig. 1A). Spindle shapes were observed, and a number of cells were distributed in a circinate pattern with rapid proliferation (Fig. 1B). The CGFe obtained was a yellowish clear fluid, and each 50 ml blood sample produced 4 ml of CGFe. The cells were vimentin positive (Fig. 1C) and keratin negative (Fig. 1D) according to immunocytochemistry staining, indicating that these primary cells were of mesenchymal origin.

Table I. Primers used for quantitative polymerase chain reaction analysis.

Gene	Primers		PCR products (bp)
	Forward (5'-3')	Reverse (5'-3')	
β -actin	AGAAAATCTGGCACCACACC	GGGGTGTGTAAGGTCTAAA	139
Osteocalcin	GGCGCTACCTGTATCAATGG	TCAGCCAACTCGTCACAGTC	106
<i>RUNX2</i>	CACCATGTCAGCAAACTTCTT	TCACGTCGCTCATTTTGC	96
Osterix	TGCTTGAGGAGGAAGTTCAC	AGGTCACTGCCCCACAGAGTA	148

RUNX2, runt-related transcription factor 2; bp, base pairs.

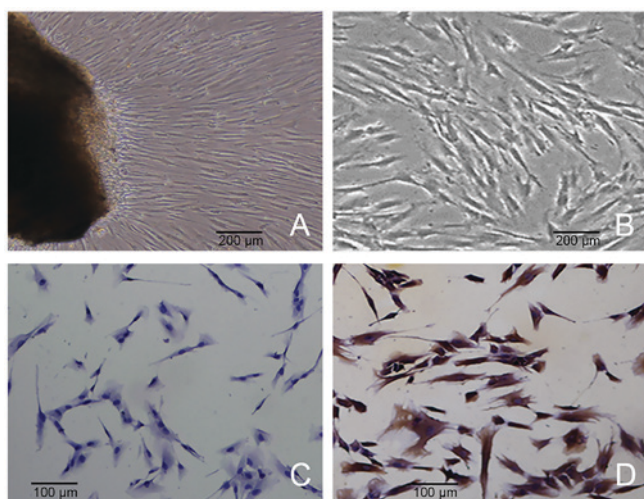


Figure 1. Identification and characterization of primary hPDLCs. (A) Primary cells grew out from the tissue explants and (B) were spindle shaped. Scale bar, 200 μ m. (C) Immunocytochemistry staining showed that cells were vimentin positive and (D) cytokeratin negative. Scale bar, 100 μ m. hPDLCs, human periodontal ligament cells.

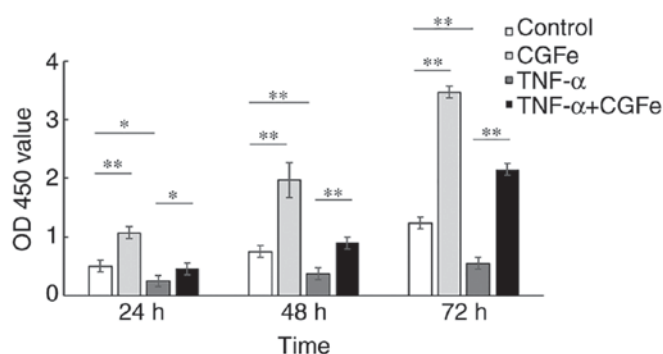


Figure 2. CGFe increases the proliferation of hPDLCs *in vitro*. The proliferation activity of hPDLCs was promoted by CGFe, but inhibited by TNF- α . CGFe mitigated the effect of TNF- α . * P <0.05, ** P <0.01. CGFe, concentrated growth factor exudate; hPDLCs, human periodontal ligament cells; TNF- α , tumor necrosis factor- α ; OD, optical density.

CGFe increases hPDLC proliferation. The proliferation of hPDLCs was measured with CCK-8 assays (Fig. 2). After 24, 48 and 72 h, CGFe significantly enhanced the proliferation of hPDLCs compared with the control group (P <0.01), while TNF- α markedly inhibited the proliferation of hPDLCs (for

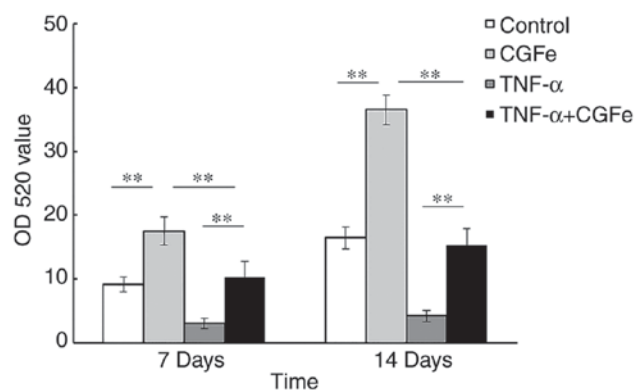


Figure 3. CGFe increases ALP activity in hPDLCs. After 7 or 14 days of culture, the ALP activity in the CGFe group was significantly higher than that of the control group. Compared with the control group, the ALP activity in the TNF- α group was decreased. ** P <0.01. CGFe, concentrated growth factor exudate; hPDLCs, human periodontal ligament cells; TNF- α , tumor necrosis factor- α ; ALP, alkaline phosphatase; OD, optical density.

TNF- α vs. control, P <0.05 at 24 and 48 h; P <0.01 at 72 h). In addition, the proliferation rate of the CGFe group was significantly higher than that of the CGFe+TNF- α group, and the proliferation of the CGFe group exceeded the TNF- α group substantially (for TNF- α vs. CGFe+TNF- α , P <0.05 at 24 h; P <0.01 at 48 and 72 h).

CGFe increases ALP activity. After 7 and 14 days of culture, the hPDLCs cultured in the CGFe group showed the highest levels of ALP activity compared with the other experimental groups (Fig. 3). Treatment with TNF- α significantly inhibited the ALP activity of hPDLCs, as shown by comparison of the TNF- α treatment group with the CGFe and TNF- α +CGFe groups, and the difference between these groups was significant (P <0.01) at both time-points. As expected, the ALP activity in all groups progressively increased over time.

Alizarin Red S staining and semi-quantification of mineralized matrix nodules. In order to detect the formation of mineralized matrix nodules, Alizarin Red S staining was performed (Fig. 4A-D). After 21 days of osteogenic induction, mineralized nodules were observed with an inverted phase-contrast microscope, and the number of nodules was notably higher in the CGFe group (Fig. 4C), compared with the control (Fig. 4A) or CGFe+TNF- α (Fig. 4D) groups. The TNF- α group (Fig. 4B) formed the fewest mineralized nodules.

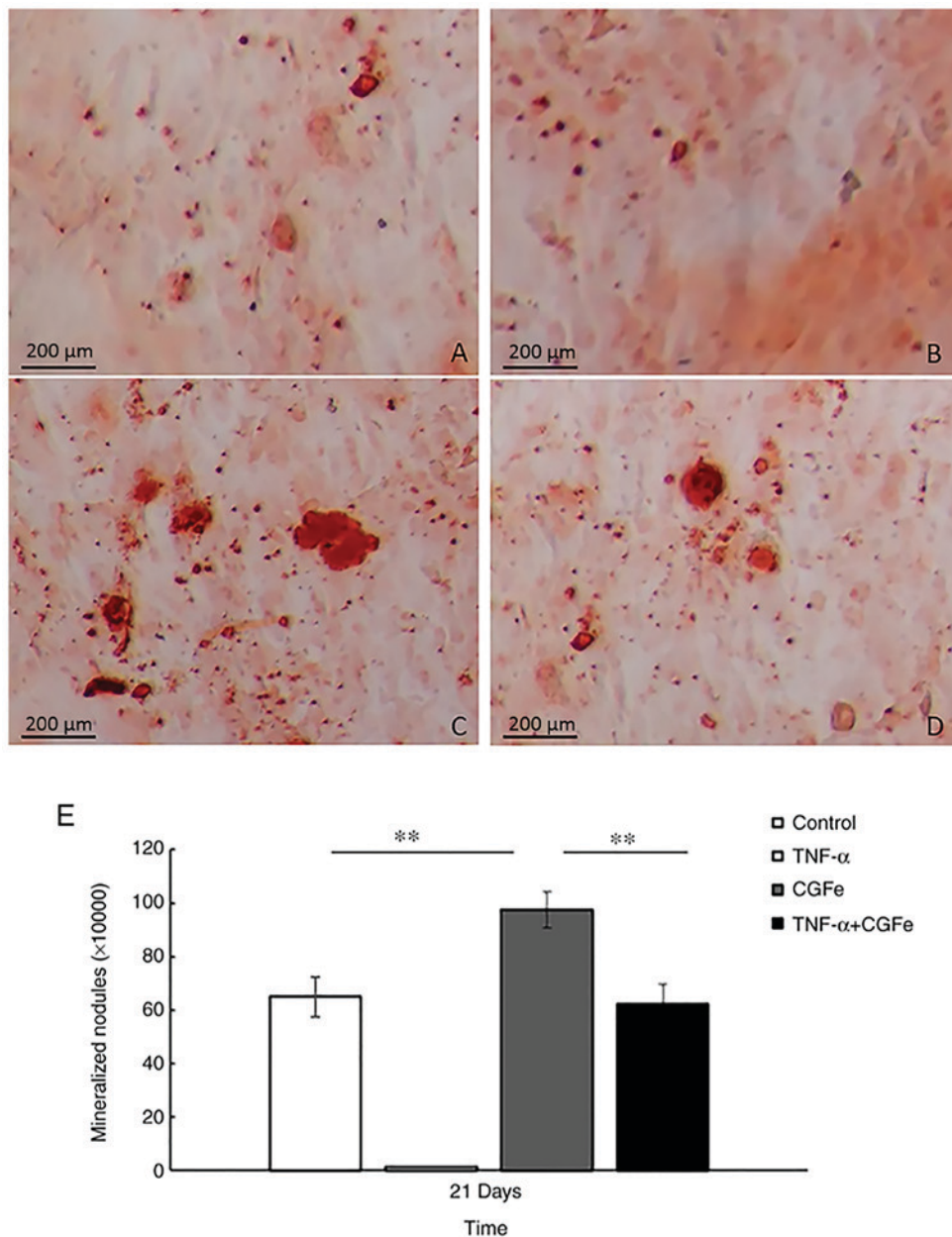


Figure 4. Alizarin Red S staining of hPDLCs. hPDLCs were cultured in (A) MM, (B) MM+TNF- α , (C) MM+CGFe or (D) MM+CGFe+TNF- α (scale bar=200 μ m). After 21 days of osteogenic induction, the number of mineralized nodules in the MM+CGFe group was much greater than those in the other three groups. (E) Mineralized nodule number was quantified after 14 days of osteogenic induction. The number of mineralized nodules in the MM+CGFe group was increased compared with the MM+CGFe+TNF- α and control groups, and the control (MM) group formed more mineralized nodules than the TNF- α group. ** $P < 0.01$. MM, mineralization-inducing medium; CGFe, concentrated growth factor exudate; hPDLCs, human periodontal ligament cells; TNF- α , tumor necrosis factor- α .

The mineralized matrix nodules were quantified after 21 days of induction (Fig. 4E). The absorbance values at 570 nm revealed that extracellular calcium deposition in the CGFe group was significantly higher than the control or CGFe+TNF- α groups ($P < 0.01$).

CGFe increases osteogenic-associated gene and protein expression. The gene expression of the *RUNX2*, *OSX* and *OCN* was determined after 4, 7 and 14 days of osteogenic induction (Fig. 5). It was observed that on days 4, 7 and 14, the expression of *RUNX2* and *OSX* in the TNF- α (10 ng/ml) groups was decreased compared with the control group ($P < 0.01$; excluding *OSX* expression on day 7, $P < 0.05$), and the expression of these

two genes in the CGFe+TNF- α group showed no significant increase compared with the control group. By contrast, on days 4, 7, and 14, expression of the *RUNX2* and *OSX* genes in the CGFe group was significantly increased, compared with the control group ($P < 0.01$; Fig. 5A and B). The expression of the downstream *OCN* gene was not significantly different between groups on day 4. By day 7, the CGFe group began to surpass the control group ($P < 0.01$). After 14 days of culture, *OCN* expression in the TNF- α group was lower than that of the control group ($P < 0.01$; Fig. 5C).

Western blotting was performed to examine the effects of CGFe on hPDLc differentiation. hPDLc were cultured in each medium for 14 days. As shown in Fig. 6A, compared

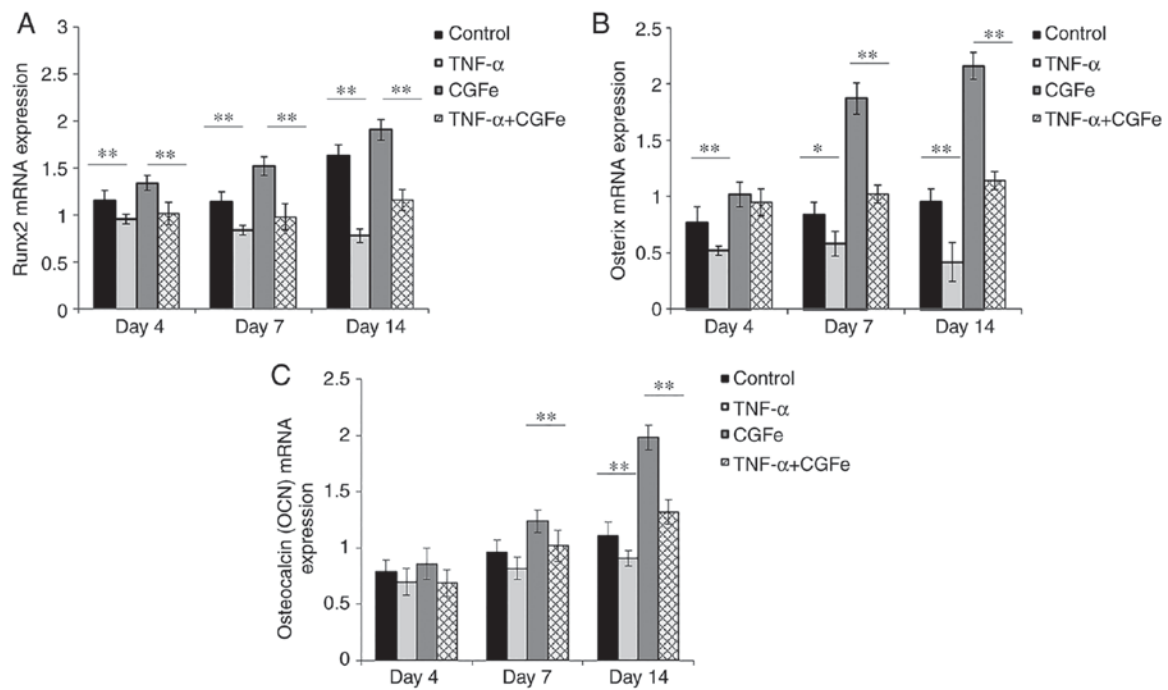


Figure 5. CGFe increases the expression of osteogenic-associated genes. Following osteogenic induction for 7 or 14 days, the expression of (A) *RUNX2*, (B) *OSX* and (C) *OCN* was determined by reverse transcription-quantitative polymerase chain reaction. The expression of *RUNX2* and *OSX* in the TNF- α groups was lower than that of the control group. On days 4, 7 and 14, the expression of *RUNX2* and *OSX* in the CGFe groups was higher than the control group. *OCN* gene expression was not significantly different between the experimental groups on day 4. By day 7, *OCN* expression in the CGFe group began to surpass the control group. After 14 days of culture, *OCN* expression in the TNF- α was lower than that of the control group. ** $P < 0.01$. CGFe, concentrated growth factor exudate; TNF- α , tumor necrosis factor- α ; *RUNX2*, runt-related transcription factor 2; *OCN*, osteocalcin; *OSX*, Osterix.

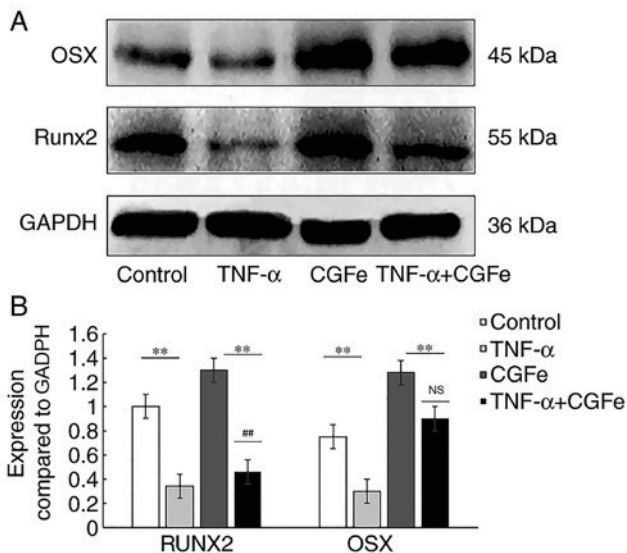


Figure 6. CGFe increases RUNX and OSX protein expression. (A) Western blot analysis of RUNX2 and OSX following treatment with various culture conditions for 14 days. GAPDH was used to monitor equal protein sample loading. (B) Quantitative analysis of RUNX2 and OSX protein expression. RUNX2 was upregulated in the CGFe group and downregulated in the CGFe+TNF- α and TNF- α groups. The protein level of OSX was upregulated in the CGFe+TNF- α group, but not significantly upregulated compared with the control group. ** $P < 0.01$; NS, CGFe+TNF- α group vs. control group; ## $P < 0.01$, CGFe+TNF- α group vs. control group. CGFe, concentrated growth factor exudate; TNF- α , tumor necrosis factor- α ; *RUNX2*, runt-related transcription factor 2; *OCN*, osteocalcin; *OSX*, Osterix.

with the control group, the protein expression of RUNX2 was upregulated in the CGFe group, and downregulated in the

CGFe+TNF- α and TNF- α groups ($P < 0.01$; Fig. 6B). A similar trend was observed for OSX expression. Although the protein level of OSX was upregulated in the CGFe+TNF- α group, there was no significant difference compared with the control group ($P > 0.05$; Fig. 6B).

Discussion

CGFe can be obtained from the venous blood of patients and thus is convenient and economical to prepare. CGFe contains high concentrations of growth factors and provokes no immune reaction (8-10,22,25). According to previous studies (4-8), the CGFe obtained from different patients in the present study contained different inventories of components, yet the major components were the same. These included epidermal growth factor, platelet-derived growth factor, transforming growth factor (TGF- β), vascular endothelium growth factor, insulin-like growth factor, bone morphogenetic protein (BMP), and fibroblast growth factor (4-8). In the last decade, CGFe has been widely used in the reconstruction of bone tissue in the surgical field (8,9). These growth factors have functions in accelerating the revascularization of injured tissues and inducing the migration, proliferation, and differentiation of fibroblasts and osteoblasts (8-10,25).

TNF- α is a common and crucial inflammatory factor in the development of periodontal inflammation and alveolar bone resorption (20). It has been established that TNF- α significantly inhibits the proliferation of hPDLs and mesenchymal stem cells (5,26,27). In addition, activation of the nuclear factor (NF)- κ B signaling pathway inhibits the expression of *RUNX2*, which is a downstream transcription factor induced

by the exogenous BMP2-mothers against decapentaplegic homolog (Smad) signaling pathway controlling osteoblast differentiation, thus inhibiting the formation of bone (28).

In the present study, TNF- α (10 ng/ml) was used to construct an inflammatory microenvironment. It was demonstrated TNF- α reduced ALP activity, mineralization ability and the expression of RUNX2 and OSX, compared with the control group. This result was concordant with the findings of Yang *et al* (18), which revealed that the bone formation efficiency of gingival mesenchymal stem cells and periodontal ligament stem cells decreased under TNF- α -induced inflammatory conditions.

Previous studies have shown that RUNX2 and OSX are two essential transcription factors in the osteogenic pathway (29,30). In particular, RUNX2 regulates cell osteogenic differentiation and serves a central role in multiple osteogenic signaling pathways (29). OSX is an osteogenic-specific transcription factor with a zinc finger structure. A previous study revealed that no bone formation occurs in mice lacking the *OSX* gene (30). The level of ALP activity reflects the tendency of cells to transform in the osteogenic direction midway during the osteoproliferative process, while OCN is a late marker of osteogenesis. OCN is the most abundant non-procollagen protein in bone tissue and promotes osteogenic differentiation by combining with minerals (31,32). Positive Alizarin Red S staining of the mineralized nodules formed indicates the expression of OCN and osteogenesis at a later stage (31,32).

In the present study, CGFe was added to cell cultures to observe the osteogenic efficiency of hPDLs, by detecting the expression of *RUNX2*, *OSX* and *OCN* genes, as well as ALP activity and mineralized nodule formation in each group of hPDLs. The formation of mineralized nodules was used to determine the osteogenic capacity of each group of hPDLs. It was observed that even in the presence of inflammatory cytokine stimulation, hPDLs cultured with CGFe still exhibited osteogenic differentiation and mineralization ability. By comparing the assay and imaging results of the CGFe group with the CGFe+TNF- α group, it was demonstrated that ALP activity in the CGFe group was stronger than that of the CGFe+TNF- α group, and the formation of mineralized nodules was notable in both groups, although the CGFe group was stained more prominently. In addition, ALP activity in the CGFe+TNF- α group was significantly higher than the TNF- α group, and the CGFe+TNF- α group had deep mineralized staining with a large area and high density, while no mineralized nodule formation was observed in the TNF- α group.

The western blotting results of the present study revealed that RUNX2 and OSX expression was higher in the CGFe group, compared with the CGFe+TNF- α group, indicating that TNF- α may play an inhibitory role upstream of protein translation. By contrast, growth factors contained in the CGFe may antagonize the inhibitory effect of TNF- α on cell proliferation. For example, TGF- β 1 induces the phosphorylation of Smad2/Smad3, which upregulates *RUNX2* transcription (29), thereby impeding TNF- α activation of the NF- κ B signaling pathway, which would result in RUNX2 inhibition.

In conclusion, CGFe not only has an osteogenic effect on hPDLs in a normal culture, but also promotes hPDL osteogenesis in a TNF- α -induced inflammatory microenvironment. In the present work, a single inflammatory factor, TNF- α , was

used to mimic the microenvironment of periodontal disease, and this imposed certain limitations. The enhancing effect of CGFe on osteogenic differentiation of hPDLs stimulated by other inflammatory factors will be examined in follow-up experiments. To study the effects of CGFe in the treatment of alveolar bone defects, *in vivo* should be performed. In addition, the exact mechanism of how CGFe enhanced the proliferation and osteogenic differentiation of hPDLs should be determined.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL, HY and BW conceived and designed the study. XL, YZ, and HL performed cell culture, immunostaining and proliferation analysis. XL and YZ performed the experimental procedures of osteogenic differentiation induction, RT-qPCR, and western blotting. HY, ZZ and ZY provided reagents and interpreted the data. XL, HY and BW performed data analysis and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Jilin University Health Science Center (Changchun, China). Written consent was obtained prior to experimentation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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