The potential application of concentrated growth factor in pulp regeneration: an in vitro and in vivo study

Fangfang Xu 1†, Lu Qiao 2†, Yumei Zhao 2†, Weiting Chen 1, Shebing Hong 1, Jing Pan 1 and Beizhan Jiang 1*†

Abstract

Background: Concentrated growth factor (CGF), as a natural biomaterial, is known to contain platelets, cytokines, and growth factors to facilitate the healing process, but there has been little information acquired in regenerative endodontics. The purpose of this study was to investigate the effects of CGF on proliferation, migration, and differentiation in human dental stem pulp cells (hDPSCs) exposed to lipopolysaccharide (LPS) in vitro and its potential role in pulp regeneration of the immature teeth in vivo.

Methods: In vitro experiments: CGF-conditioned medium were extracted by freeze-dried method. hDPSCs were isolated and identified. The proliferative potential of hDPSCs with different concentration of CGF and LPS was evaluated by Cell Counting Kit-8. Migration capacity was analyzed by Transwell assays, odonto/osteoblastic differentiation was determined by measuring alkaline phosphatase (ALP) activity using ALP staining, and the extent of mineralization was evaluated by using Alizarin red S staining. The mRNA expression level of DMP-1, DSPP, OPN, Runx2, and OCN were determined by quantitative polymerase chain reaction (qPCR).

In vivo experiments: CGF were used as root canal filling agent of the immature single-rooted teeth in the beagle dogs. The teeth were then radiographed, extracted, fixed, demineralized, and subjected to histologic analyses at 8 weeks. The newly formed dentine-pulp complex and the development of apical foramen were evaluated by the hematoxylin-eosin (HE) and Masson trichrome technique. Soft tissues were analyzed by immunohistochemical staining of vascular endothelial growth factor (VEGF) and Nestin.

Results: In vitro experiments: The cultured cells exhibited the characteristics of mesenchymal stem cell. The treatment of LPS significantly increased the expression of TNF-α, IL-1β, IL-6, and IL-8 in hDPSCs, and CGF inhibited the mRNA expression of IL-8 in LPS-stimulated hDPSCs. The proliferation values of the CGF group in LPS-stimulated hDPSCs were significantly higher than that of the control group from day 3 to day 7 (P < 0.05). In addition, the number of migratory cells of the CGF group was greater than that of the control group at 24 h with or without LPS treatment. ALP activities increased gradually in both groups from day 4 to day 7. The mineralized nodules and the expression of odontogenesis-related genes DMP-1 and DSPP, osteogenesis-related genes OPN, Runx2, and OCN were dramatically enhanced by CGF in LPS-stimulated hDPSCs at days 21 and 28.

In vivo experiments: In CGF treated group, the results of radiograph, HE, and Masson trichrome staining showed a continuing developed tooth of the immature teeth in the beagle dogs (i.e., the ingrowth of soft tissues into the root). (Continued on next page)
Background

Pulpitis and periapical periodontitis in immature tooth are common diseases in dental clinical treatments that derived from bacterial infection. The emergence of pulp inflammation is caused by the response of dental pulp tissue to foreign bacterial component and its byproducts. Dental pulp tissue has the ability to repair and regenerate, minor inflammation can stimulate dental pulp stem cells reside in the pulp to migrate to the injured site, where they subsequently differentiate into odontoblasts and participate in repairing the dentine-pulp complex [1]. Although current treatment for pulpitis and periapical periodontitis in immature tooth are apecification and apical barrier technique, there still exist many undeniable drawbacks including postoperative fractures and reinfections result from the arrested root development [2]. Therefore, how to facilitate the repairing process of dental tissues under inflammatory micro-environment to achieve pulp regeneration has drawn more attention in current endodontic researches.

As an alternative approach, regenerative endodontic treatment (RET) aims to replace inflamed/necrotic pulp tissue with regenerated pulp-like tissue hereby to achieve the maximum conservation of tooth vitality and the continued development of immature tooth [3]. Three key elements which are essential for tissue regeneration are stem cells, bioactive molecules, and scaffolds. Concentrated growth factor (CGF), which is known as bioscaffolds and a reservoir of cytokines, has been used for bone regeneration in current clinical implantological application [4, 5]. As the latest generation of platelet concentrate products, the modified production process of CGF is simpler and requires repeatedly switching the centrifugation speed [6]. As a result, the relatively stiffer structure of CGF is more similar to a natural fibrin and contains abundant growth factors and proteins from autologous platelets and leucocytes [7]. CGF contains large amounts of growth factors including platelet-derived growth factor-BB (PDGF-BB), transforming growth factor β-1 (TGF-β1), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF). All these factors were intimately involved in the regulation of cell differentiation, proliferation, and angiogenesis which were vital for tissue regeneration [8]. However, it also contains several pro-inflammatory cytokines including IL-6 and IL-1β that may exert negative effects on tissue repair and regeneration [9].

Gram-negative bacteria species are the most common microorganisms that account for necrotic pulp. Lipo polysaccharides (LPS) are a major molecular component of the cell wall of these bacteria and the prime toxic factor that contribute to the bacterial-induced immune response [10, 11]. In several experiments, LPS has been used to form a model of inflammation [12–14]. Current studies about CGF in tissue regeneration have been mainly focused on bone regeneration [15, 16], and our previous studies have investigated the effects of CGF on human stem cells of the apical papilla (SCAPs) and proved its potentials in regenerative endodontics [17]. Therefore, the purpose of this study was to evaluate the effects of CGF on the proliferation, migration, and differentiation of hDPSCs under LPS-inflamed condition in vitro and its potential role in pulp regeneration of the immature teeth in vivo.

Materials and methods

The present work was developed according to the principles recommended for experimentation with human beings and the animals determined by the Institutional Review Board of Tongji University, and ethics committee approval was obtained (No. 2018-012). All subjects enrolled were informed about the procedures and objectives of the study and signed a consent form.

Isolation and characterization of hDPSCs

Normal impacted third mandibular molars were collected (N = 6, aged 14–20 years) from healthy patients with informed consent in a dental clinic at the Affiliated Stomatology Hospital of Tongji University. In brief, the DPSCs were isolated by enzyme digestion according to a previously described method [18]. The separated cells were cultured in Dulbecco’s modified Eagle's medium...
(HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Gibco BRL, USA) and 100 U/ml penicillin-G (Sigma, St Louis, MO, USA) and streptomycin in a humidified atmosphere of 5% CO$_2$ at 37 °C. Cells at passage 3 (P3) were used for the following study.

The characterization of DPSCs was analyzed by flow cytometry and three multilineage differentiation assays as previously described [19, 20]. In brief, CD105, CD90, and CD146 were used as surface markers of mesenchymal stem cell and CD34 and CD45 were used to confirm that the hDPSCs were not hematopoietic lineage cells (R&D Systems, Minneapolis, MN, USA). The isotype served as the negative control. Each experiment was performed with a BD FACsCalibur (BD Biosciences, San Jose, CA, USA). For the three multi-lineage differentiation assay, alizarin red S staining, alcian blue staining, and oil red staining were used to identify the mineralized nodule, glycosaminoglycans, and lipid droplet after the cells were incubated with a different inducing medium for 3 weeks.

**Conditioned medium preparation**
Venous blood (10 mL) was collected from each participant after providing informed consent; the tubes were immediately centrifuged in a special centrifuge device by 30 s acceleration, 2 min at 2700 rpm (600 g), 4 min at 2400 rpm (400 g), 4 min at 2700 rpm (600 g), 3 min at 3000 rpm, and 36 s deceleration (MEDIFUGE™, Silfradentsrl, S. Sofia, Italy). Conditioned medium (CM) was prepared as described previously with slight modifications [17]. In brief, the isolated CGF membranes were frozen overnight in a vacuum freeze dryer. To harvest the cytokines, the lyophilized membrane was pulverized and immersed in 50 mL DMEM. The medium was collected after incubation at 4 °C for 24 h and was centrifuged to remove the red blood cells. CM was completed after being supplemented with 10% fetal bovine serum and 1% antibiotic. Four concentrations of 1× CGF (CGF isolated from 10 mL venous blood dissolved in 50 mL DMEM), 1/2×, 1/4×, and 1/8× CGF were used.

**LPS treatment and the detection of inflammation-related genes**

hDPSCs were treated with 0, 0.1, 1, and 10 μg/mL LPS (Escherichia coli 0111:B4, Sigma) and/or 1× CGF for 24 h. The messenger RNA expression levels of IL-6, IL-8, IL-1β, and TNF-α were determined by quantitative polymerase chain reaction. The cells of the different group were lysed by Trizol reagent (Life Technologies, Carlsbad, CA, USA), and total RNA was isolated. The extracted RNA was reverse transcribed using a complementary DNA synthesis kit (Roche, Schlieren, Switzerland), and the relative messenger RNA expression of the target gene was analyzed using the FastStart Essential DNA Green Master (Roche, Schlieren, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control to normalize the RNA expression levels. The primer sequences for IL-6, IL-8, IL-1β, and TNF-α (Sango Biotech, Shanghai, China) are listed in Table 1.

**Cell proliferation assay**
The hDPSCs were seeded on a 96-well plate (Corning, NY, USA) at a density of 2 × 10$^3$ cells per well and were cultured with different concentrations of CGF and 0.1 μg/mL, 1 μg/mL, and 10 μg/mL LPS (E. coli 0111:B4, Sigma) for 1, 3, 5, and 7 days. The normal medium was used as the control group. The culture medium was replaced with fresh culture medium every 2 days. Cell Counting Kit-8 (Keygen, Nanjing, China) was used to analyze the cell numbers. The optical density values were measured using a microplate reader (BioTek, Winooski, VT) at 450 nm. The results from different groups were compared. After analyzing the results comprehensively and for the sake of research convenience, we selected the concentration of 1× CGF for this study.

| Table 1 Primers sequences used in the real-time PCR |
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| Gene | Forward primer | Reverse primer |
| IL-6 | GGTGTGCGCTGCTGCCTTC | GCTCTGAAGAGGTGAGTGGCTGTC |
| IL-8 | CAAGCTGGCCGCTGGCTCTC | GTTCTGAAGAGGTGAGTGGCTGTC |
| IL-1β | TGGCTTATTACAGTGCCCAATGAGGAGT | GTTCTGAAGAGGTGAGTGGCTGTC |
| TNF-α | CGTGGACCTGGCGCCAGGAG | AGGAAGGAAGAGGCTGAGGAAC |
| DSPP | GGAGCCACAAACAGAAAGCAACA | TGGACAAACAGGACATCCCAT |
| DMP1 | CAGGAAGAGGTGGTGAGTGGT | TGGATACGCTCTGCTGCTG |
| Runx2 | TCCAGACGGACAGCCTCCCTCAA | CTCAAGCTGCTGCTGCTG |
| OPN | TGCTACAGACGGGACCATCACC | TCTGGACGCTGCTGCTGCTG |
| OCN | CCGCAGCTCCTACAAAAAACAAAT | GCCAGCTCCAGCACTGCTT |
| GAPDH | CCAAGAACATCATCCCTGCTCTC | GAGCCTCTGTTCCACACCTT |
Cell migration assay
Twenty-four plates of Transwell filter inserts (Corning, NY, USA) were used to investigate the migratory capacity of hDPSCs after being treated with CGF with or without 1 μg/mL LPS presence at 24 h. Normal media with or without 5% serum served as the positive and negative control groups, respectively. The migrated cells were stained by crystal violet and were counted randomly in six microscope fields. The cell numbers per field were calculated using ImageJ software (version 10.2; National Institutes of Health, Bethesda, MD, USA), and the average was analyzed using GraphPad Prism v4.0 software (GraphPad Software, La Jolla, CA, USA).

Detection of ALP activity
hDPSCs were cultured with 1 μg/mL LPS, 1x CGF, or a combination of LPS and CGF for 4 and 7 days. The culture medium was replaced with fresh culture medium every 2 days. For ALP staining, the media were removed, and the cells were fixed in 70% ethanol for 1 h. After the cells were rinsed three times with deionized water, a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Beyotime, Shanghai, China) was added to each well. Then, the stained cells were photographed after several steps of washing. For quantitative analysis, the stain was extracted with 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich) for 15 min, and stain intensity was quantified by measuring the absorbance at 562 nm on an absorbance microplate reader (BioTek, Winooski, VT, USA).

Alizarin red S staining
Alizarin red S staining was performed to detect the mineralization nodules of the hDPSCs after culture in CGF and/or LPS for 21 and 28 days. In brief, the paraformaldehyde-fixed cells were stained with 0.5% alizarin red S solution (Sigma-Aldrich, St Louis, MO, USA) and were photographed after several steps of washing.

Real-time quantitative polymerase chain reaction
The messenger RNA expression levels of dentin matrix protein 1 (DMP-1), dentin sialophosphoprotein (DSPP), osteopontin (OPN), Runt-related transcription factor 2 (Runx2), and osteocalcin (OCN) were determined by quantitative polymerase chain reaction. The method was the same as the qPCR procedure mentioned above. The primer sequences for DSPP, DMP-1, OPN, Runx2, and OCN (Sango Biotech, Shanghai, China) are listed in Table 1.

Orthotopic transplantation assay of immature tooth with CGF in the beagle dogs
The beagle dogs (N = 3) approximately 5 months old were obtained from the Experimental Animal Center of Jiagan Biotechnological Limited Company (Shanghai, China). Thirty-six single-rooted anterior teeth were randomly divided into three groups including CGF group (N = 12), positive control group (normal teeth with no treatment, N = 12), and negative control group (root canal were prepared only, N = 12). Preoperative radiographs were obtained to confirm the presence of an open apex and the absence of preexisting pathosis in each canine tooth.

All experimental procedures were conducted under a clean protocol with the use of sterile materials and equipment according to a previously described method [21]. Under general anesthesia induced by Pentothal (Sinopharm Chemical, China) 13.5 mg/kg intravenously and intubation and maintenance with isoflurane (Sinopharm Chemical, China) supplemented with local anesthesia (Alticaine epinephrine; Sinopharm Chemical, China), venous blood (10 mL) was collected from the carotid arteries. The animals were killed under general anesthesia provided by Jiagan Biotech Company (pentobarbital; Sinopharm Chemical, China) at 30 mg/kg intravenously. The carotid arteries were exposed and cannulated. The animals were euthanized with additional pentobarbital (Sinopharm Chemical, China) at a dose of 90 mg/kg.
intravenously. The animals were perfused with 4% para-formaldehyde (Sangon Biotech, Shanghai, China). The involved teeth were extracted and fixed in 4% paraformaldehyde (Sangon Biotech, Shanghai, China) for 24 h at 4 °C. The samples were demineralized in 10% EDTA for 2 months at 37 °C then embedded in paraffin. The sections with a thickness of 5 μm were cut in a mesiodistal direction for HE staining and immunohistochemistry (IHC).

Histologic evaluation
For the HE and Masson trichrome staining, the slides were deparaffinized and rehydrated by gradient elution using xylene and ethanol, and then were stained by hematoxylin-eosin (Keygen, Nanjing, China) and Masson trichrome staining reagent (Keygen, Nanjing, China) according to the manufacturer's instructions.

IHC
For the IHC, the primary antibodies used for immunohistochemistry were as follows: VEGF (Bioss, bs-1665R) and Nestin (Abcam, ab7659). Secondary antibodies were all purchased from Boster in China. In brief, the slides were deparaffinized and rehydrated by gradient elution using xylene and ethanol, followed by incubation in 3% H₂O₂ to suppress endogenous peroxidase activity. For antigen retrieval, the slides were incubated in hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) for 1 h at 37 °C. After the sealing of 5% BSA, the specimens were incubated with primary antibody diluted 1:100 at 4 °C overnight. Then sections were then rinsed in PBS and incubated with biotinylated secondary antibody for 20 min at room temperature. SABC kit purchased from Boster (Wuhan, China) was used for the staining process. 3,3′-Diaminobenzidine (DAB) was used as a color developing agent, and then slides were counterstained with hematoxylin. Finally, slides were mounted with Permount TM Mounting Medium and observed by the stereomicroscope (Carl Zeiss, Stemi 508, Germany) and the microscope (Nikon Eclipse 80i, Japan). Pictures were modified by PhotoshopCS6 (Adobe, CA, USA). Negative controls were incubated with normal anti-rabbit or anti-mouse IgG instead of the primary antibodies.

Statistical analysis
All experiments were performed in triplicate, and statistical analysis was performed by using SPSS (IBM SPSS, Armonk, NY, USA) version 20.0. Mean values were calculated and presented with an error bar representing ±SD. The one-way analysis of variance (ANOVA) test was used for statistical analysis. Statistical significance was accepted at P < 0.05 and graphics software used was GraphPad Prism 6.0.

Results
Mesenchymal stem cell characteristics of hDPSCs
Cells successfully grew out from the extracted dental pulp tissue after cultured for 3–7 days. The primary cells showed plastic adherence and exhibited a spindle shape. The flow cytometry analysis indicated that hDPSCs expressed the mesenchymal stem cell-related antigen CD90, CD105, and CD146 but did not express the hematopoietic cell antigen CD45 and CD34 (Fig. 1A). hDPSCs also could be induced to three lineages of differentiation; after 3 weeks of osteogenic, adipogenic, and chondrogenic induction, extensive amounts of mineralized nodules, lipid droplets, and glycosaminoglycans were observed with alizarin red, oil red O, and alcian blue staining, respectively (Fig. 1B).

Consequence of LPS on hDPSCs
After 1, 3, 5, and 7 days of LPS (0.1, 1, 10 μg/mL) treatment, the expression of IL-6, IL-8, TNF-α, and IL-β were all significantly increased and LPS have increased the expression of IL-6 and IL-8 in a dose-dependent manner at days 3, 5, and 7 (Fig. 2).

Effect of LPS and CGF on hDPSC proliferation
After 5 days treatment with LPS (0.1, 1, 10 μg/mL), the proliferation ratio of hDPSCs was significantly higher than that of the control group (P < .05) (Fig. 3a). 1×, 1/2×, 1/4×, and 1/8× CGF showed no accelerated effect on hDPSC proliferation on day 1. However, 1× CGF increased proliferation ratio of hDPSCs on day 3, and on days 5 and 7, different concentrations of CGF could significantly increase cell proliferation in a dose-dependent manner, and the promoting effect on cell proliferation of 1/8× CGF was less obvious than that in 1×, 1/2×, and 1/4× CGF group (Fig. 3b). When hDPSCs were treated with 1× CGF and 1 μg/mL LPS at the same time, the proliferation ratio was also enhanced as compared with the control group from day 3 to day 7 (Fig. 3c).

Effects of CGF on the expression of inflammation-related genes in LPS-stimulated hDPSCs
To determine the effect of CGF on proinflammatory cytokines, hDPSCs were cultured with or without 1× CGF in the presence of 1 μg/mL LPS for 1, 3, 5, and 7 days and examined by qPCR to detect the release of cytokines including IL-6, IL-8, and TNF-α. The results showed that IL-8 was highly detected in CGF at initial 24 h, and the treatment with CGF can significantly attenuate the LPS-stimulated release of IL-8 in hDPSCs at 1, 3, 5, and 7 days (Fig. 4). Besides, the expression of IL-6 was significantly suppressed by CGF in LPS-stimulated hDPSCs at initial 24 h and then exhibited no significant decrease on that as compared with the LPS treatment group (Fig. 4). Moreover, the expression of TNF-α was
upregulated at day 1 after CGF treatment in LPS-stimulated hDPSCs (Fig. 4a), and then slightly inhibited by CGF in LPS-stimulated hDPSCs at days 3 and 7 (Fig. 4b). At day 5, qPCR results demonstrated that CGF significantly decreased the LPS-stimulated release of TNF-α in hDPSCs (Fig. 4c).

Effect of CGF on cell migration in LPS-stimulated hDPSCs

To investigate the effect of CGF on the migration capacity of LPS-stimulated hDPSCs, cells were treated with 1× CGF with or without the presence of 1 μg/mL LPS for 24 h for transwell assay (Fig. 5). The results showed that the migratory cells in 1× CGF group with or without LPS presence were significantly denser than those in the control group (P < .05), and migratory cells in the LPS group were more than those in the positive serum group (Fig. 5).

The effect of CGF on the differentiation in LPS-stimulated hDPSCs

To investigate the effect of CGF on the odonto/osteogenic capability of LPS-stimulated hDPSCs, cells were treated with or without 1× CGF in the presence of 1 μg/mL LPS for 4 and 7 days for ALP analysis and 21 and 28 days for alizarin red S staining and quantitative polymerase chain reaction analysis.

At day 4, osteogenic-induction medium (OM) with or without LPS could promote ALP activity of the hDPSCs
as compared with the negative control group; however, 1× CGF suppressed this process under osteogenic induction with or without LPS (Fig. 6a). At day 7, 1× CGF significantly promoted the ALP activity of LPS-stimulated hDPSCs as compared with OM and LPS group (P < 0.05), LPS could also enhance the ALP activity of hDPSCs (Fig. 6a). These results were inconsistent with the quantitative ALP results (Fig. 6b).

Alizarin red staining showed that the mineralized areas were significantly increased by the co-treatment of LPS and CGF at day 21, and the staining gets stronger at day 28 (Fig. 7a). The gene expression levels of DSPP, DMP-1, OPN, Runx2, and OCN were greatly upregulated after incubation in CGF for 21 and 28 days (P < 0.05) (Fig. 7b). As compared with the LPS group, the gene expression of DSPP, DMP-1, OPN, Runx2, and OCN were significantly increased in the co-treatment of LPS and CGF group at day 21. Under LPS-stimulated condition, CGF could also enhance the mRNA expression of DMP-1, OPN, and Runx2 as compared with that in the LPS group at day 28 (Fig. 7b).

The effect of CGF on the generation of dentine-pulp complex and the development of apical foramen of the immature teeth in the beagle dog

The radiographic images revealed the presence of an open apex and the absence of preexisting pathosis in the pre-operative canine teeth (Fig. 8a, c). The positive control group exhibited a normally thickened root canal wall and a closed apex (yellow arrowhead and dotted circle in Fig. 8d). Meanwhile, after 8 weeks of the operation, the root canal wall (green arrowhead in Fig. 8d) of the CGF group presented different levels of root thickening as compared with the images before the operation (green arrowhead in Fig. 8d). The contrast, the root canal walls of the negative control group (pulp removed

Fig. 2 The effects of LPS on the expression of proinflammatory cytokines in hDPSCs. The release of TNF-α, IL-1β, IL-6, and IL-8 of hDPSCs after 1 (a), 3 (b), 5 (c), and 7 (d) days incubation of different dose of LPS were determined by qPCR. The results are mean ± standard deviation of triplicate measurements from three independent experiments.
and left no filling material inside the root canal (red arrowhead in Fig. 8b) showed no significant changes as compared with the images before the operation (red arrowhead Fig. 8a); however, the apical foramen of the negative control group was almost closed as well (red dotted circle in Fig. 8b).

**Histologic results**

In the positive control group, we could see the thickened root canal wall and the closed apical foramen. Inside the root canal, there were normal pulp tissues which have numerous pulp cells, scattered blood vessels, and the surrounding odontoblasts (Fig. 9b, e). In the CGF group, there also exists a histological evidence of hard-tissue deposition in the internal root dentin wall (Fig. 9m, p) and an apical closure (Fig. 9o, r); the root canal was filled with the regenerated connective tissues with different degrees of similarity to normal dental pulp (Fig. 9n, q), which also has the palisading-arranged odontoblasts which were adjacent to the newly formed pre-dentin (asterisk in Fig. 9n, q). Besides, the scattered blood vessels and cerulean stained collagen fibers could also be seen in the central area of the regenerated pulp-like tissues (arrowhead in Fig. 9n, q). In the negative control group, the results exhibited empty lumina with no regenerated tissues and a closed apical foramen (i.e., no wall thickening or ingrowth of soft tissues into the root canal)(Fig. 9B).

IHC was performed for VEGF and Nestin in the newly formed pulp-like tissues in the CGF group and the positive control group (Fig. 10). The results revealed that the positive immunoreactivity for VEGF in the CGF group was obvious throughout the regenerated soft tissues (Fig. 10c), which was similar to the positive staining in the normal pulp tissues (Fig. 10a), but it was particularly intense in the perivascular areas (arrowhead in Fig. 10a, c). The overall moderate Nestin staining was also seen in the regenerated pulp-like tissues, and it was particularly intense in the nerve-like cells (arrowhead in Fig. 10b, d). Negative controls for IHC using normal rabbit or mouse
IgG never showed positive reactions in any sections examined (data not shown); we examined three to four different blocks for each sample and obtained the same results.

**Discussion**

Dental pulp is a highly specialized loose connective tissue that preserves the teeth. A healthy dental pulp is not only responsible for tooth vitality, but also for pain sensation, immune defense, and tissue repair/regeneration after tooth injury. The major clinical injury to the teeth is bacterial penetration through caries lesions [22]. Several most common bacteria associated with dental caries are gram-negative bacteria. And LPS, as a main component of this bacterial membrane, is the prime toxic factor that contribute to the bacterial-induced immune response [10, 11]. According to the previous reports, LPS in the infected root canals was detected approximately ranging from 0.001 to 2 μg/mL [23, 24]. The method they used was by directly collecting samples through a paper point in the root canals and then quantified by limulus amebocyte lysate assay. However, the sampling they collected may not obtain all LPS molecules in the root canals. Therefore, the virtual concentration of LPS in the infected root canals might be higher than that in these reports. In order to better simulate the in vivo inflamed microenvironment in our in vitro research, we selected the concentrations of 1 μg/mL LPS on the basis of it was well used in many studies and could induce the biological responses of many mesenchymal stem cells [25, 26].

LPS can induce the expression of proinflammatory cytokines and chemokines, such as TNF-α, IL-6, and...
IL-8, and elicit a variety of immune responses in the odontoblasts, fibroblasts, and monocytes of dental pulp tissue [27–29]. Different doses and times of these pro-inflammatory cytokines may yield different effects in dental pulp cells. Chronic exposure (>3 days) of dental pulp cells to TNF-α and IL-1β impairs its ability to differentiate into odontoblasts, suggesting that inflammatory cytokines may inhibit the repair and regeneration of dentin and pulp during inflammation [30]. Shorter and appropriate exposure of inflammatory molecules induced by TNF-α and LPS to dental pulp cells can up-regulate the odontoblastic-related gene expression of DSPP and DMP-1 [31, 32]. In accordance with the previous study [33], our results showed that different doses of LPS can stimulate the expression of several inflammatory cytokines including IL-6, IL-8, IL-1β, and TNF-α in hDPSCs at days 1, 3, 5, and 7, which are the representative proinflammatory molecules detected in the inflamed pulp tissue [34]. As a member of the CXC chemokine family of cytokines, IL-8, along with TNF-α, can not only mediate the migration of neutrophils but also promote the recruitment of tissue stem cells to the injury sites and contribute to tissue healing [35–37]. The high detection of IL-8 in CGF may suggest its ability to recruit DPSCs that resided in distant pulp to the injury site. The initial inflammatory period is important for recruiting leucocytes and surrounding connective tissue cells for tissue healing. On the other hand, feedback signaling from the cells surrounding the injury site modulates the activation of resident macrophages by secretion of anti-inflammatory factors. As one of the most important proinflammatory cytokines, TNF-α can not only participate in vasodilation and regulation of blood coagulation, but also contribute to increasing the synthesis of anti-inflammatory factors, such as IL-10 [38]. Therefore, CGF may increase the expression of TNF-α in LPS-stimulated hDPSCs at day 1 as the result of initial regulated inflammatory response. In addition, the expression of TNF-α was significantly decreased by CGF in LPS-stimulated hDPSCs at day 5, we speculated that these may result from the highly promoted cell proliferation by LPS. The enhanced cell proliferation can accelerate the process of tissue regeneration and wound healing and therefore inhibit the expression of TNF-α at day 5 [39]. According to Chiche et al.’s report at 2017 [40], IL-6 have a promoted effect on cellular reprogramming via senescent cells in the context.
of tissue injury, indicating us that IL-6 may have a positive role in tissue repairment. These may explain why the treatment of CGF did not inhibit the expression of IL-6 at days 3, 5 and 7. Meanwhile, CGF can attenuate the expression of IL-8 under LPS-stimulated condition from day 1 to day 7, indicating that CGF may have an inhibitory effect on the inflamed dental pulp cells and play a positive role in tissue repair.

In tissue regeneration strategies, an ideal scaffold is specifically designed to promote adhesion, proliferation, migration, and/or differentiation of the incorporated cells [41]. CGF, as an autologous biomaterial, has been used in oral, maxillofacial, plastic, and bone surgery as well as in gingival tissue engineering research [15, 16, 42]. As similar to our previous study that CGF can make the proliferation and migration of SCAPs elevated [17], in this study, CGF was revealed to promote the proliferation and migration of hDPSCs whether under LPS stimulated or not. Although LPS alone was found to promote the proliferation and migration of hDPSCs, the upregulation level of CGF in LPS-stimulated hDPSCs was much higher, indicating CGF can still accelerate the proliferation and migration of hDPSCs under LPS-stimulated condition. Previous studies have reported that proinflammatory cytokines can be secreted by a range of cells including odontoblasts in response to bacterial secreted LPS, and those proinflammatory cytokines were accounted for the migration of neutrophil and stem cells [43]. Meanwhile, CGF contains a variety of platelet cytokines and growth factors, including PDGF-BB, TGF-β1, and VEGF, which were critical growth factors participating the regulation of the proliferation of various cells [44, 45]. The previous study has reported that bFGF, chemotactic factors released from the CGF, had the
same effect of migration on DPSCs as compared with G-CSF in vitro [46]. And PDGF-BB can also facilitate the migration of hDPSCs besides from their enhanced proliferation and odontoblastic differentiation ability [47]. Therefore, these chemotactic factors released from the CGF may play a vital role in pulp regeneration through mediating the inflammation and enhancing the proliferation and migration of the LPS-stimulated hDPSCs.

The critical step of pulp regeneration is the differentiation into odontoblasts and the formation of new dentin and capillaries. The odonto/osteoblastic differentiation of hDPSCs may be identified by detection of ALP activity and the mineralized nodule formation and the expression of several odonto/osteogenic genes, such as DSPP, DMP-1, Runx2, OPN, and OCN. In the present study, we analyzed ALP activity, which is considered as early markers of hard tissue formation or osteoblastic/
odontoblastic differentiation. Co-treatment of CGF and LPS resulted in suppressed ALP activity and mineralization in hDPSCs when compared with the LPS and OM group on day 4. And at day 7, the co-treatment of CGF and LPS upregulated the ALP activity in hDPSCs. In order to examine the odonto/osteoblastic capacity of CGF in LPS-stimulated hDPSCs at late stages, the alizarin red staining was used to reveal the mineralized nodules in the LPS/CGF. LPS and CGF groups were denser and larger than those in the control group after incubation.

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**Fig. 8** The radiographic results of CGF on the generation of dentine-pulp complex and the development of apical foramen of the immature canine teeth. a, c The radiographic images of the pre-operative teeth. b, d The radiographic images of the postoperative teeth after 8 weeks. Red arrowhead and dotted circle indicated the root canal walls and the apical foramen of the negative control group (pulp removed and left with no filling material) respectively. Yellow arrowhead and dotted circle indicated the root canal walls and the apical foramen of the positive control group (normal teeth) respectively. Green arrowhead and dotted circle indicated the root canal walls and the apical foramen of the CGF filling group respectively.

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**Fig. 9** The histological results of CGF on the generation of dentine-pulp complex and the development of apical foramen of the immature canine teeth. The HE and Masson trichrome staining results of the positive control group (A), the negative control group (B) and the CGF filling group (C). b, c, e, f, h, i, k, l, n, o, q, r The amplified images of the square box in a, d, g, j, m, and p. Arrowhead indicates the vessels; the asterisk indicates the odontoblasts. DP, dental pulp; AF, apical foramen; V, vessel; Od, odontoblast; D, dentin. Scale bar = 100 μm.
for 21 days and 28 days. At the same time, the co-treatment of LPS and CGF also increased the mRNA expression of DSPP, DMP1, Runx2, and OCN as compared with the control and LPS group although it resulted in no significant increase in DSPP, DMP-1, and OPN when compared with the CGF group. These results were in accordance with earlier findings in which LPS promoted the odontoblastic differentiation of hDPCs by increased mineralized nodule formation and gene expression of odontoblastic markers [48]. These results indicated that the CGF could significantly promote the odonto/osteogenic differentiation of the LPS-stimulated hDPCs at the late stage.

The criteria for evaluating successful pulp regeneration was the regenerated pulp-like tissues should be connective tissues that (i) deposit new dentine, (ii) show similar cell density and architecture to the natural pulp, and (iii) have vascularization and (iv) innervation [49]. The results of in vivo study indicated that CGF could induce the thickened formation of dentin walls and the ingrowth of soft connective tissues which included the palisading arranged odontoblasts and the scattered blood vessels. As we know, CGF is a three-dimensional network that consists of the cross-linked fibrins, platelets, and various growth factors; those growth factors can not only recruit the stem cells residing in periapical areas such as stem cells from apical papilla (SCAPs), but also promote the proliferation and differentiation of those dental mesenchymal stem cells. Meanwhile, the natural-fibrin-like structure of CGF could also enable stem cells to grow better. VEGF plays an important role in angiogenesis by promoting endothelial cell proliferation, increasing vascular permeability, and altering the biological effects of extracellular matrix [50]. Nestin is associated with the pluripotency of neural stem cells and is expressed in both precursors of neurons and glial cells [51]. The IHC results of VEGF and Nestin indicated that CGF could regenerate the pulp-like tissues that resemble the natural one which has vascularization and innervation.

**Conclusion**

The results of this study revealed that CGF can not only inhibit proinflammatory cytokines release and promote proliferation, migration, and odonto/osteogenic differentiation in vitro, but also promote the regeneration of dentine-pulp complex and achieve the continued development of the immature teeth in the beagle dog in vivo. Therefore, as a good combination of biomaterial and abundant growth factors and chemotactic factors, CGF can serve as a promising biomaterial due to its excellent regulatory properties in inflammation, proliferation, migration, and odonto/osteogenic differentiation to promote pulp regeneration in clinical pulp injury applications.

**Abbreviations**

ALP: Alkaline phosphatase; bFGF: Basic fibroblast growth factor; CGF: Concentrated growth factor; DMP1: Dentin matrix protein 1; DSPP: Dentinsialophosphoprotein; hPDPCs: Human dental pulp stem cells; IGF-1: Insulin-like growth factor-1; IL: Interleukin; LPS: Lipopolysaccharide; OCN: Osteocalcin; OM: Osteogenic-induction; OPN: Osteopontin; PDGF-BB: Platelet-derived growth factor-BB; Runx2: Runt-related transcription factor 2; SCAPs: Stem cells from apical papilla; SCAPs: Stem cells of the apical papilla; TGF-β1: Transforming growth factor β-1; TNF-α: Tumor necrosis factor-alpha; VEGF: Vascular endothelial growth factor
Acknowledgements

We would like to also thank the Shanghai Engineering Research Center of Tooth Restoration and Regeneration, Tongji University, for providing all experimental equipment.

Funding

This work was supported in part by Shanghai Municipal Commission of Health and Family Planning Program (Nos.201740223) for the design of the study and, partially by Shanghai Science and Technology Commission Program (18411969500) for collection, analysis, and interpretation of data and writing of the manuscript.

Availability of data and materials
Please contact the author for data requests.

Authors’ contributions

This study was based on the whole work of each author. All authors approved the final version of the manuscript. Each author is expected to have made substantial contributions to the conception, including the execution of all cell experiment, analysis, and writing by FX, the in vivo experiments by LQ and YZ, experiment planning and writing by BJ, and organization of the pictures by WC/Sr/P.

Ethics approval and consent to participate

The present work was developed according to the principles recommended for experimentation with human beings determined by the Institutional Review Board of Tongji University, and ethics committee approval was obtained (No.2018-012). All subjects enrolled were informed about the procedures and objectives of the study and signed a consent form.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author details

1 Department of Operative Dentistry and Endodontics, School and Hospital of Stomatology, Tongji University, Shanghai Engineering Research Center of Tooth Restoration and Regeneration, Shanghai 200072, China. 2 Department of Pediatric Dentistry, School and Hospital of Stomatology, Tongji University, Shanghai Engineering Research Center of Tooth Restoration and Regeneration, Shanghai 200072, China.

Received: 7 January 2019 Revised: 15 April 2019 Accepted: 29 April 2019

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