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Effects of epigallocatechin gallate (EGCG) on the biological properties of human dental pulp stem cells and inflammatory pulp tissue

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ABSTRACT

Objective: This study aimed to investigate the effect of epigallocatechin gallate (EGCG) on the proliferation, mineralization, inflammation and hypoxia responses of human dental pulp stem cells (hDPSCs) *in vitro* and its effect on inflammatory pulp tissue in rats *in vivo*.

Design: The optimum concentration of EGCG was selected by creating a dose response curve. Expression of odontogenic/osteogenic-related genes and inflammatory cytokines after stimulation with Lipopolysaccharide (LPS) was detected by real-time PCR. Under hypoxic conditions, cell proliferation and expression of reactive oxygen species (ROS) and superoxide dismutase (SOD) were detected.*In vivo*, the maxillary first molars of SD rats were pulpotomized and stimulated with 5 mg/mL LPS for 30 min. Normal saline and EGCG were used to flush the pulp chamber. After 2 months, samples were removed for micro-CT scanning and HE staining.

Results: CCK-8 assay revealed that 10 μg/mL EGCG had no significant effect on the proliferation of hDPSCs. EGCG inhibited expression of IL-1β, IL-6, and TNF-α. Furthermore, EGCG rescued cell proliferation ability, increased SOD activity and reduced ROS expression under hypoxia.*In vivo*, reduced inflammatory cell accumulation was observed in the coronal pulp in the EGCG group, while in the control group, diffuse inflammatory cells were observed in the radicular pulp.

Conclusion: EGCG had no obvious effects on calcified nodule formation but significantly inhibited the inflammatory response of hDPSCs and inhibited apoptosis of hDPSCs caused by hypoxia injury. *In vivo*, EGCG exerts inhibitory effects on pulp tissue inflammation.

1. Introduction

Dental pulp is a type of loose connective tissue that functions in repair, defence, induction, absorption and nutrition. Inflammation of dental pulp caused by caries or dental trauma often involves infiltration of inflammatory cells and secretion of inflammatory cytokines by various cells (Anderson, Dumsha, McDonald, & Spitznagel, 2002; Lin et al., 2018). Dental pulp is very important for the root development of young permanent teeth. To preserve the vitality of dental pulp, indirect pulp therapy, direct pulp capping and pulpotomy can be performed, depending on the degree of inflammation. Clinically, pulpotomy is commonly used to treat young permanent teeth with carious pulp exposure, which is usually successful when inflammation is within the coronal pulp but fails when radicular pulp is involved (Cohenca, Paranjpe, & Berg, 2013). Studies have shown that the release of inflammatory cytokines in dental pulp affects odontogenic differentiation of dental pulp cells and induces pulp necrosis (Anthony, 2003; Ricucci, Loghin, Lin, Spangberg, & Tay, 2014). To preserve the inflammatory pulp as much as possible, it is important to control inflammation of the remaining pulp tissue during pulpotomy. Therefore, ideal preservation materials should be both biocompatible and anti-inflammatory. Current commonly used pulp capping materials, such as MTA, iRoot, and Biodentin, exhibit weak anti-inflammatory properties (Alqaderi, Al-Mutawa, & Qudeimat, 2014; Azimi et al., 2014; Qudeimat, Alyahya, & Hasan, 2017). On the other hand, flushing fluids commonly used in pulpotomy include normal saline and sodium hypochlorite (Tunc, Saroglu, Sari, & Gunhan, 2006). Normal saline has no anti-inflammatory effects, while a high concentration of sodium hypochlorite damages

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vital pulp tissue (Okino, Siqueira, Santos, Bombana, & Figueiredo, 2004). To date, researchers are still looking for ideal pulp preservation materials.

Previous studies on pulpotomy are primarily based on healthy pulp and have focused on biocompatibility (Azimi et al., 2014; Leites et al., 2011). However, the antibacterial and anti-inflammatory effects of pulp-preserving materials are often neglected, which is not conducive to the recovery of inflammatory pulp tissue. Therefore, we consider whether anti-inflammatory materials can be used in pulpotomy to control pulp inflammation and achieve pulp preservation.

Studies on the chemical constituents of tea polyphenols have shown that the primary components of tea polyphenols are catechins. Catechins primarily contain epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). Among them, EGCG accounts for 50–60 % of catechin content (Steinmann, Buer, Pietschmann, & Steinmann, 2013). Studies on EGCG have shown that it has a wide range of biological activities (Nagle, Ferreira, & Zhou, 2006), including antioxidant, antimicrobial, immune regulatory, anti-tumour and so on (Butt & Sultan, 2009; Jiao et al., 2015; Shin, Jeon, Park, & Chang, 2016; Trompezinski, Denis, Schmitt, & Viac, 2003; Xu, Zhou, & Wu, 2011). Some studies have reported that EGCG has an effect on the treatment of periodontitis due to its bactericidal effects against periodontal pathogens (Asahi et al., 2014; Jung et al., 2012; Lee et al., 2009). Moreover, Nakanishi et al. (2010) found that when dental pulp cells were exposed to LPS and prostaglandin (PG), expression of IL-6 and interleukin-8 (IL-8) was inhibited by addition of ECG and EGCG. Other studies also reported that after stimulation with LPS, EGCG inhibits expression of several proinflammatory factors (VEGF, COX-2 and PGE_2) in hDPSCs (Hirao et al., 2010; Nakanishi, Mukai, Hosokawa, Takegawa, & Matsuo, 2015). Therefore, we proposed that EGCG may have utility as an anti-inflammatory material in pulpitis. Although previous studies have indicated that EGCG may inhibit periodontal and pulp inflammation, it is necessary to explore the effects of EGCG on other biological properties of hDPSCs in addition to anti-inflammatory properties before its use in vital pulp therapy.

The objectives of this study were to investigate the effect of EGCG on the cell proliferation, mineralization, and inflammatory and hypoxic responses of hDPSCs *in vitro* and the effect of EGCG on the inflammatory pulp tissue of rats *in vivo*. Our hypothesis was that EGCG could inhibit the inflammatory response of hDPSCs without adverse effects on their biological properties.

2. Materials and methods

Epigallocatechin gallate (EGCG) powder was purchased from LuKang, Nanchang, Jiangxi, China. It was extracted from green tea, and the purity was 94 %. Molecular formula: $C_{22}H_{18}O_{11}$, molecular weight: 458.38.

2.1. Sample collection and cell culture

In the Department of Maxillofacial Surgery, Peking University Stomatology Hospital, healthy, complete and caries-free teeth (third molar) extracted for impacted reasons were collected (n = 5). Sample collection was approved by the Ethics Committee of the Health Science Center of Peking University (Beijing, China; PKUSSIRB-201732003). Collected teeth were removed from pulp tissue in a Steril CARDIII Clean Bench (Beckman Coulter, Brea, CA, USA). Extracted pulp tissue was minced into pieces and digested in 3 mg/mL type I collagenase (Sigma, St. Louis, MO, USA) and 4 mg/mL dispase enzyme (Roche, Cleveland, OH, USA) for 45 min. The reaction was terminated by adding α-MEM medium (Gibco; Grand Island, NY, USA) with 10 % foetal bovine serum (FBS; HyClone, Logan, Utah, USA). Then, pulp tissue was seeded into a 3 cm culture dishes (Corning, Corning, NY, USA) and cultured in α-MEM medium with 15 % FBS and 100 U/mL penicillin/100 μg/mL streptomycin (Gibco, Grand Island, NY, USA) and cultured in a SERIES II incubator (Thermo Forma, Waltham, MA, USA) at 37 °C and 5% CO_2. Three to five passages of cells were used in our experiments.

2.2. Identification of hDPSCs

The second generation of well-grown cells was digested and centrifuged routinely. Cells were resuspended in precooled stain buffer (HyClone, Logan, Utah, USA). Then, cells were counted and made into a single cell suspension (1×10^6 cells/mL). The suspension was separated into 1.5 mL EP tubes (Corning, NY, USA) with 1 mL in each tube. Cells were centrifuged on a 5415R cryogenic centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 4℃ and 1000 rpm. The supernatant was discarded, and 1 mL precooled Stain Buffer was added to wash gently. After centrifugation, the supernatant was discarded to obtain the cell precipitate. Then, cells were stained with CD45, CD73 and CD146 antibodies (BD Biosciences, USA) and analysed by Gallios flow cytometry (Beckman, Pasadena, CA, USA).

2.3. Cell proliferation experiments

The second generation of well-grown cells was seeded into 96-well plates (Corning, Corning, NY, USA) at a density of 5×10^3 cells per well. They were divided into 5 groups with 5 wells per group. The culture medium was changed every other day. After culturing for 1 d, 3 d, 5 d, 7 d or 9 d, 10 μL CCK-8 solution (Kumamoto, Kyushu, Japan) was added to each well. After 1 h of incubation, the optical density (OD) was determined by a Thermo3001 enzyme-labelled instrument (Thermo Forma, Waltham, MA, USA) at a wavelength of 490 nm.

Differentiation ability was assessed by osteogenic and adipogenic induction experiments. For preparation of osteogenic induction, 1.8 mM KH_2PO_4 (Sigma), 0.1 mM L-vitamin C phosphate (Sigma), and 10^{-8}M dexamethasone (Sigma) was added to the α-MEM medium with 15 % (volume fraction) FBS. For preparation of adipogenic induction, 200 μM indomethacin (Sigma), 0.5 mM IBMX (Sigma), 10 μg/mL insulin (Sigma), and 1 μM dexamethasone (Sigma) were added to α-MEM medium with 10 % (volume fraction) FBS. The well-grown second generation of cells were digested when cells reached 80–90 % confluence. Then, cells were seeded into 12-well plates (Corning, Corning, NY, USA) at approximately 1×10^5 cells per well, and 2 mL common culture medium was added to each well. After 24 h, the old culture medium was removed, and 2 mL osteogenic/adipogenic induction solution was added to each well. The control group was cultured with common medium. The culture medium was changed every three days, and cells were induced for 3 weeks. After 3 weeks, alizarin red (Sigma) staining was performed in the osteogenic induction group, and oil red O (Sigma) staining was performed in the adipogenic induction group.

2.4. EGCG concentration gradient experiment

EGCG (LuKang, Nanchang, Jiangxi, China) was added to the common medium at concentrations of 0, 10, 25, 50, or 75 μg/mL. The third generation of well-grown hDPSCs was seeded into 96-well plates (Corning, NY, USA) with medium containing EGCG at different concentrations. Cells were cultured in an incubator at 37℃ and 5% CO_2 (Thermo Forma). After culturing for 1 d, 3 d and 5 d, 10 μL CCK-8 solution was added to each well and incubated for 1 h. Optical density was determined by a Thermo3001 enzyme-labelled instrument (Thermo Forma) at a wavelength of 490 nm.

2.5. Alkaline phosphatase activity

After 7 d of culture in normal (blank group), osteogenic (control group), or EGCG-containing osteogenic medium (EGCG group), cells were lysed for 10 min in 100 mM Tris base with 1% Triton-X100. Alkaline phosphatase (AKP) activity was determined in the lysate by measuring the release of p-nitrophenol using SIGMAFASTTM p-

nitrophenyl phosphate (Sigma) as a substrate after 45 min at 37 °C. Absorbance was measured at 420 nm using a microplate reader (ELx808IU, BioTek, Winooski, VT, USA).

2.6. Alizarin red staining

After 21 d of culture in normal (blank group), osteogenic (control group), or EGCG-containing osteogenic medium (EGCG group), cells were rinsed three times in phosphate-buffered saline (PBS) (Sigma), and mineralization of the extracellular matrix was detected by staining with 2% alizarin red (Sigma-Aldrich). Pictures of alizarin red staining were captured using a scanner. Then, hDPSCs were divided into four groups: control, EGCG, LPS and LPS + EGCG. Osteogenic induction culture medium was added to the control group, osteogenic induction medium containing 10 μg/mL EGCG was added to the EGCG group, osteogenic induction medium containing 1 μg/mL LPS was added to the LPS group, and osteogenic induction medium containing 10 μg/mL EGCG and 1 μg/mL LPS was added to the EGCG + LPS group. After 21 d, cells were stained with 2% alizarin red (Sigma-Aldrich).

2.7. Real-time quantitative reverse transcription polymerase chain reaction (real-time PCR) analysis

After 21 d of culture in normal and osteogenic medium, total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized from oligo (dT) primers using a reverse transcriptase kit (Promega). Expression of ALP, osteocalcin (OCN) and dentin sialophosphoprotein (DSPP) genes was detected by a SYBR Green PCR Master Mix kit (Thermo Fisher, Waltham, MA, USA) using 7500 real-time PCR (Rockwell Allen-Bradley, Milwaukee, Wis, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. Primer sequences are shown in Table 1. The comparative cycle threshold ($2^{-\Delta\Delta CT}$) method was used to calculate relative expression levels of target genes.

E. coli lipopolysaccharide (LPS; InvivoGen, San Diego, CA, USA) was added at a concentration of 1 μg/mL to stimulate cells for 4 h. Expression of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) was detected by real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. Primer sequences are shown in Table 1.

2.8. Western blotting analysis

Thirty micrograms of protein were separated by 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane, which was blocked with 5% (w/v) non-fat dried milk, incubated overnight at 4 °C with primary antibodies (glyceraldehyde-3-phosphate dehydrogenase, GAP, Bioss, China; alkaline phosphatase, ALP, Bioss, China; osteocalcin, OCN, Bioss, China; DSPP, Bioss, China), and reacted with horseradish peroxidase-conjugated secondary antibodies (Origene, China). Immunoreactive bands were visualized by enhanced chemiluminescence (Cwbiotech, China) at room temperature and were digitized using the Fusion FX image analyser (Viber Loumat, Germany). Finally, band densitometry was measured using ImageJ 1.52v (NIH Image, USA).

Table 1
Specific primers of real-time PCR.

Gene	Forward primer	Reverse primer
GAPDH	CGGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC
ALP	CTATCCTGGCTCCGTGCTC	GCTGGCAGTGGTCAGATGTT
OCN	CTCACACTCCTCGCCCTATT	TTGGACACAAAGGCTGCAC
DSPP	AACTCTGTGGCTGTGCCTCT	TATTGACTCGGAGCCATTCC
IL-1β	AGCTCGCCAGTGAAATGATG	GCCCTTGCTGTAGTGGTGGT
IL-6	GAAAGCAGCAAAGAGGCACT	TTTCACCAGGCAAGTCTCCT
TNF-α	CAGAGGGAAGAGTTCCCCAG	CCTCAGCTTGAGGGTTTGCTAC

2.9. The effect of EGCG on hDPSCs under hypoxic conditions

hDPSCs cultured under normal conditions were digested in trypsin and seeded into 6-well plates (1×10^6 cells/well). The control group was cultured in common medium, and the experimental group was cultured in common medium containing 10 μg/mL EGCG. Both were cultured in a C21&C274 tri-gas incubator (Crystalclear Biotech CO., Hong Kong, China), which contained a 1% oxygen mixture. After culturing for 1 d, 3 d, 5 d or 7 d, cell proliferation was detected by CCK-8 assay.

Next, cells were divided into positive control, control and EGCG groups. Reactive oxygen species (ROS) production was monitored using a fluorescence microscope (Olympus, Tokyo, Japan) and fluorescence spectrometer (Hitachi F-4500, Tokyo, Japan) using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, Shanghai, China) after culture for 3 d. DCFH-DA (20 μM) was added to the medium for 20 min at 37 °C. Excitation was measured at 488 nm, and emission was measured at 525 nm. Superoxide dismutase (SOD) activity was monitored by a spectrophotometer (Hitachi F-4500) at a wavelength of 560 nm using an SOD kit (Beyotime, Shanghai, China).

2.10. Experimental model of rat molar pulpitis

In this study, 6-week-old male SD rats weighing 220−260 g (Charles River, Beijing, production licence: scxk (Beijing) 2016−0011) were used. Rats were housed in a specific pathway-free (SPF) animal laboratory at room temperature (18−25℃), 50 % humidity, and light/dark cycle (12 h/d) (ethical code: LA2019325). Twelve rats were randomly divided into 3 groups: (A) pulpitis model (Model, n = 4); (B) saline control (Control, n = 4); and (C) EGCG experimental (EGCG, n = 4). The bilateral maxillary first molars of each rat were used.

In the model group, rats were anaesthetized with 7% chloral hydrate (Yuanye Bio-Technology, Shanghai, China) at a rate of 0.5 mL/100 g. A sterile 0.8 mm high-speed round diamond bur was used to prepare a cavity at the mid-mesial surface. Dental pulp was then exposed using a sterile K-file (MANI, Tokyo, Japan). Then, the coronal pulp was removed with a slow-speed round diamond bur. LPS (5 mg/mL) was placed into the opening pulp chamber for 30 min.

2.11. Pulpotomy in the pulpitis model

In the control and EGCG groups, pulpotomy was performed in the bilateral maxillary first molars of SD rats. After LPS stimulation, the pulp cavities of the control and EGCG groups were flushed with 5 mL normal saline and 5 mL EGCG (10 μg/mL), respectively, for 2 min. Then, mineral trioxide aggregate (MTA) (ProRoot MTA, Dentsply Tulsa Dental, Tusal, OK, USA) was used to cover the pulp, and glass ionomer (GC Fuji IX, GC, Tokyo, Japan) was used for crown sealing. Rats were sacrificed after 2 months.

2.12. Micro-computed tomography (micro-CT) analysis

Specimens were scanned using a micro-CT scanner (GANTRY-STD CT 3121, Simens, Knoxville, TN, USA) at 80 kV and 500 μA, a camera exposure time of 1200 ms and 360° rotation around the vertical axis with a 1° rotation step. The aluminium filter was set to 0 mm during the scans. Raw data obtained in the scanning stage were reconstructed using Inveon Research Workplace 4.2 software 2 (Simens Medical Solutions, Knoxville, TN, USA).

2.13. Histological analysis

After 2 months, the entire maxilla was dissected out and separated into 2 halves. Tissues were placed in fixative solution, demineralized in 10 % EDTA, dehydrated in graded ethanol concentrations, and embedded in paraffin. Serial sections of 4 μm thickness were cut through the entire pulp and placed on glass slides. Staining of sections with

haematoxylin-eosin was used to evaluate cell and tissue morphology microscopically (BX51, Olympus, Japan).

2.14. Data analysis

One-way ANOVA was used for overall comparisons, and Tukey's post hoc test was used for paired data. The threshold of significant difference was set to $p = 0.05$ (bilateral). IBM SPSS 20.0 (Version 20.0, Released 2011; IBM, Armonk, NY, USA) was used for data analysis.

3. Results

3.1. Identification of hDPSCs

The results of flow cytometry showed that the cells we isolated were CD73 (99.5 %) and CD146 (88.6 %) positive and CD45 negative (1.2 %) (Fig. 1A), which was in accordance with the expression of mesenchymal stem cell surface markers.

The CCK-8 assay showed that cells grew well for the first 7 days and entered the plateau stage on days 7 and 9 (Fig. 1B). Osteogenic and adipogenic differentiation of cells was confirmed under certain inductive conditions (Fig. 1C).

3.2. The effect of EGCG on the proliferation and mineralization of hDPSCs

The effects of 0, 10, 25, 50, or 75 μg/mL EGCG solution on the viability of hDPSCs were compared by CCK-8 assay. Results showed that there was no significant difference among the 10 μg/mL/25 μg/mL EGCG solution groups and the control group on the first day. On days 3 and 5, only 10 μg/mL EGCG did not show a significant difference compared to the control group, while inhibitory effects were observed in the other EGCG groups ($p < 0.001$) (Fig. 2A). Therefore, the optimal concentration of EGCG for hDPSCs was 10 μg/mL, which was used in subsequent experiments.

In the study of the effect of EGCG on hDPSC mineralization, we found that there were obvious calcified nodules in both the control and EGCG groups compared to the blank group (Fig. 2B). On day 7, no significant difference in ALP activity was observed between control and EGCG groups ($p > 0.05$) (Fig. 2C). The results of real-time PCR revealed that expression of OCN was significantly increased in the EGCG group ($p < 0.001$), while expression of DSPP was significantly decreased ($p < 0.01$) (Fig. 2D). Results demonstrated that EGCG had no obvious effect on calcified nodule formation of hDPSCs but did affect expression of several odontogenic/osteogenic-related genes.

3.3. EGCG inhibits expression of proinflammatory cytokines and mineralization in hDPSCs after LPS stimulation

After treatment with 1 μg/mL LPS for 4 h, expression of IL-1β, IL-6 and TNF-α in the control group was significantly increased, indicating successful establishment of the inflammation model. After addition of 10 μg/mL EGCG, expression of proinflammatory cytokines decreased significantly ($p < 0.01$) (Fig. 3A).

Next, we examined the effect of 10 μg/mL EGCG on the mineralization of LPS-stimulated hDPSCs. After induction for 21 d, obvious mineralization nodules were observed in all four groups (Fig. 3B). Further semi-quantitative results showed that 1 μg/mL LPS promoted mineralization ($p < 0.01$), while EGCG slightly inhibited mineralization. When LPS and EGCG were added simultaneously, the results of alizarin red semi-quantitative analysis showed no significant difference between the LPS + EGCG and the control groups (Fig. 3C). Real-time PCR results revealed that expression of OCN and DSPP genes was upregulated by LPS stimulation ($p < 0.001$), but ALP expression was significantly

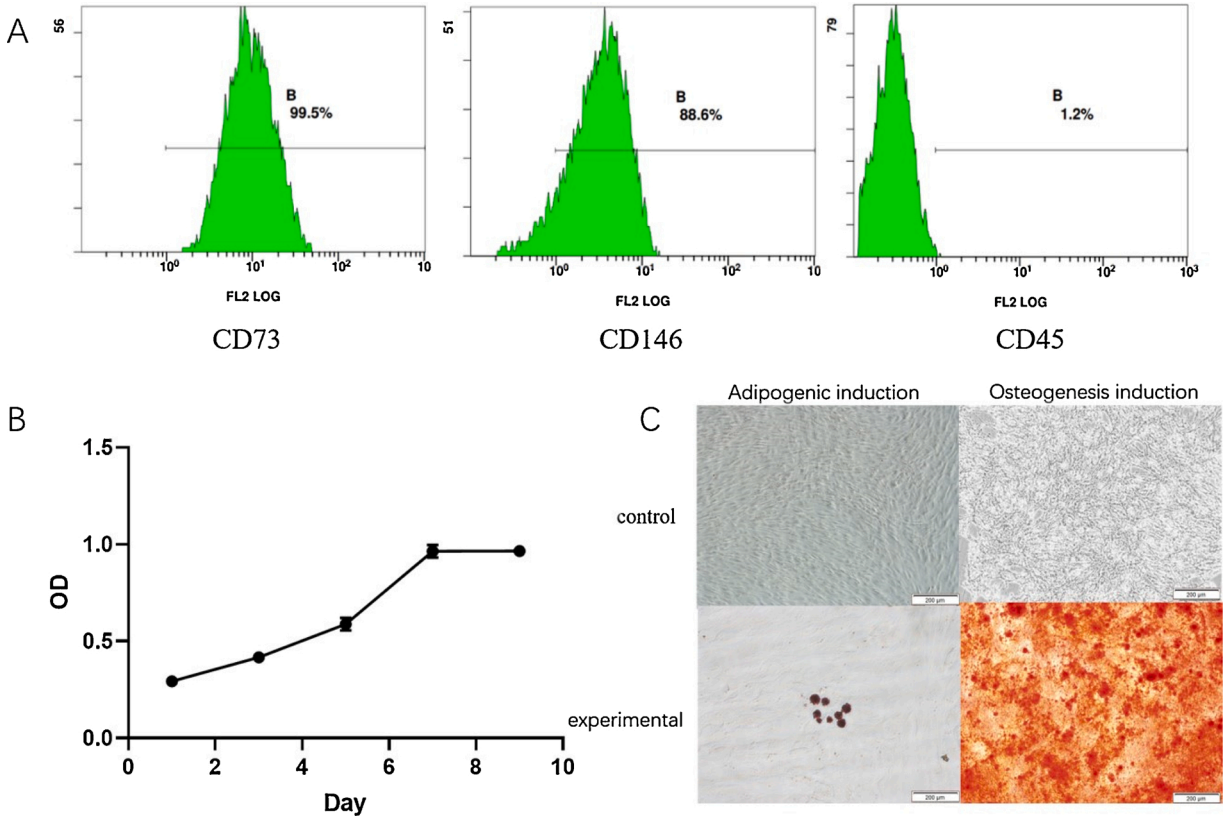

Fig. 1. Dental pulp stem cell identification. (A) Flow cytometry showed that cells were CD73 (99.5 %) and CD146 (88.6 %) were positive and CD45 negative (1.2 %). (B) Proliferation levels in cells at 1, 3, 5, 7 and 9 days was detected by CCK-8 assay. (C) After adipogenesis and osteogenesis induction for 21 d, lipid droplets and calcified nodules had formed.

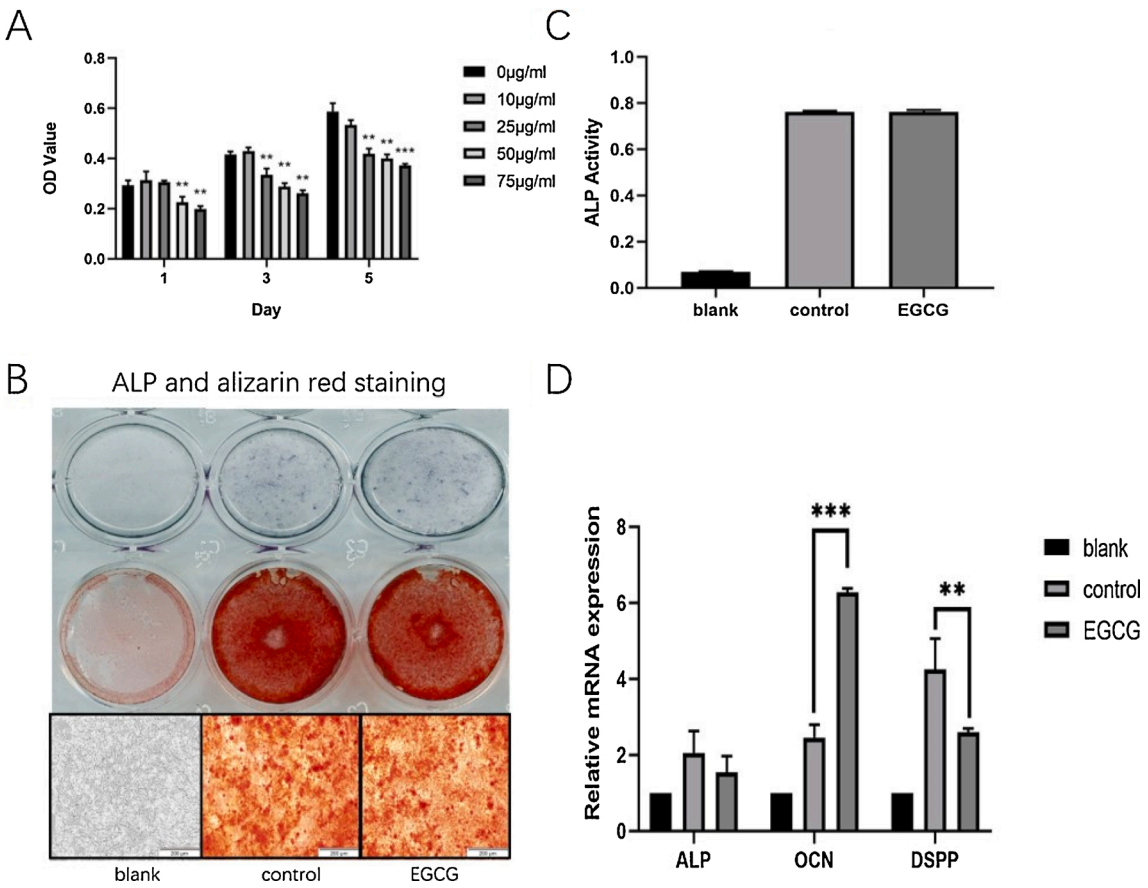

Fig. 2. The effect of EGCG on proliferation and mineralization of hDPSCs. (A) The effect of different concentrations of EGCG on cell viability detected by CCK-8 assay. (B) After osteogenesis induction for 21 d, calcified nodules were observed in both groups. (C) After osteogenesis induction for 7 d, there was no significant difference in ALP activity between the two groups ($p > 0.05$). (D) Expression of ALP, OCN and DSPP genes detected by real-time PCR. Data are expressed as the mean ± SD. **P < 0.01; ***P < 0.001.

downregulated ($p < 0.001$). When EGCG and LPS were added at the same time, compared to the LPS group, expression of DSPP significantly decreased but was still higher than the control, while expression of ALP increased but was still lower than the control (Fig. 3D). Western blot analysis confirmed these results (Fig. 3E, F).

3.4. EGCG inhibits apoptosis of hDPSCs under hypoxic conditions

When cultured under hypoxic conditions, proliferation of hDPSCs cultured in common medium entered the plateau stage on days 3–5, and apoptosis began on the days 5–7. After addition of EGCG, hDPSCs entered the plateau stage on the days 5–7 (Fig. 4A), suggesting that the proliferation potential of hDPSCs was partially recovered by EGCG.

Subsequently, expression of ROS in hDPSCs was detected by a ROS kit. DCFH-DA was used to measure levels of ROS production. A large amount of fluorescence was observed in the positive control group under a fluorescence microscope. There was some fluorescence observed in the control and EGCG groups, both of which were cultured under hypoxic conditions for 3 d. However, the fluorescence expression of the two groups was less than in the positive control group. Then, fluorescence was quantitatively analysed by a fluorescence spectrophotometer. Results showed that the production of ROS was significantly increased under hypoxic conditions compared to normal conditions. Between the two groups cultured under hypoxic conditions with a 1% oxygen mixture, the production of ROS in the EGCG group was reduced compared to the control group ($p < 0.01$) (Fig. 4B). The activity of SOD in hDPSCs was detected using a SOD kit. Results showed that the SOD activity in hDPSCs was significantly increased in the EGCG group at 1% oxygen ($p < 0.0001$) (Fig. 4C).

3.5. The effect of EGCG on inflammatory pulp tissue

HE staining showed that after 5 mg/mL LPS treatment for 30 min, a large number of inflammatory cells had infiltrated the coronal pulp, while the radicular pulp was basically normal. Odontoblasts were closely arranged on the surface of the pulp near the dentin surface (Fig. 5A–C). HE staining confirmed that the pulpitis model was established successfully.

Two months after pulpotomy, no loosening or gingival swelling was observed in any of the experimental teeth. MTA remained in the pulp cavity in all teeth. In each group of 6 samples, GICs were lost in both the control (n = 3) and EGCG (n = 2) groups. Micro-CT showed that hard tissues were formed under MTA in both groups. The bone mineral density (BMD) of the control group was 1232.28 ± 6.09 mg/cm^3, and that of the EGCG group was 1154.86 ± 29.35 mg/cm3 with no significant difference between the two groups ($p > 0.05$) (Fig. 5J–M). HE staining showed that there were no periapical lesions around the roots of teeth in either group. In the control group, a large number of inflammatory cells had infiltrated the coronal pulp and radicular pulp (Fig. 5D–F). In contrast, in the EGCG group, only a few inflammatory cells had infiltrated the coronal pulp, and no diffuse inflammatory cell infiltration was observed in the radicular pulp (Fig. 5G–I).

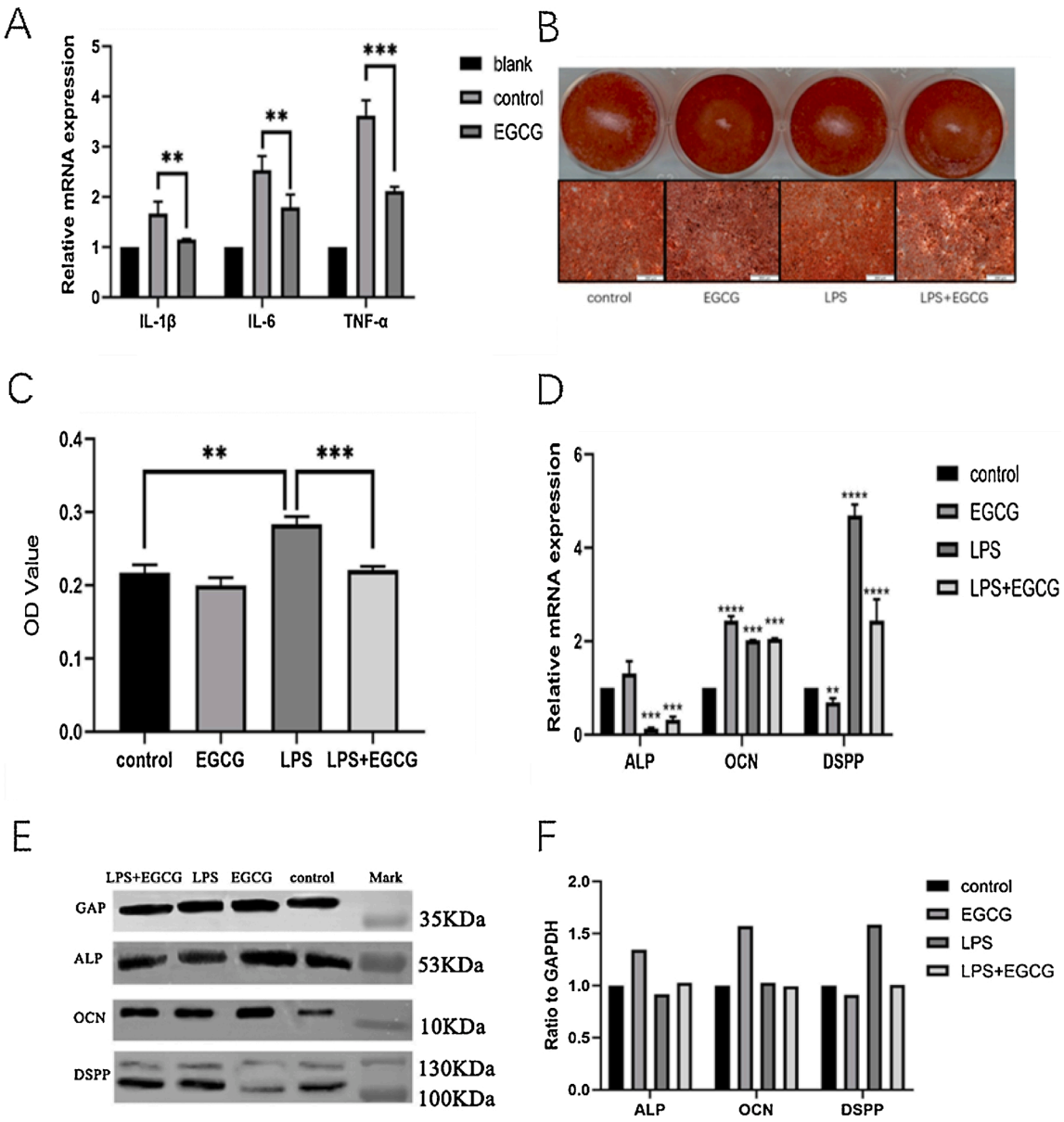

Fig. 3. Effects of EGCG on hDPSCs stimulated by LPS. (A) After 1 μg/mL LPS stimulation for 4 h, expression of IL-1β, IL-6 and TNF-α in the control group was significantly increased. Expression of proinflammatory cytokines decreased significantly after addition of EGCG ($p < 0.01$). (B) After induction for 21 d, obvious mineralization nodules were observed in all four groups. (C) Alizarin red semi-quantitative analysis was performed to detect the formation of mineralized nodules in each group. Expression of ALP, OCN and DSPP in the four groups was detected by real-time PCR (D) and Western blotting (E). Indicated protein quantitative data refer to the ratio to GAPDH (F). Data are expressed as the mean±SD. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

4. Discussion

Previous studies of EGCG on dental pulp cells have primarily focused on EGCG's inhibition of proinflammatory factors (Nakanishi et al., 2010, 2015). However, the cytotoxicity of EGCG and its effect on cell differentiation have not been examined. In vital pulp treatment, in addition to anti-inflammatory properties, materials play an important role in the proliferation, differentiation and mineralization of dental pulp stem cells (Giraud et al., 2019). We found that EGCG inhibited proliferation of hDPSCs in a dose-dependent manner, and 10 μg/mL EGCG exerted the least inhibitory effect. It has been reported that EGCG has differential effects on the proliferation of cells from different sources. Liu, Lu, Wang, Du, and Pei (2016) investigated the effects of different concentrations of EGCG on the proliferation of human periodontal ligament cells. They found that 3.7 μg/mL and 4.6 μg/mL EGCG exerted cytotoxicity and inhibited the proliferation of human periodontal ligament cells, while less than 2.8 μg/mL EGCG had no significant inhibitory effect on the proliferation of human periodontal ligament cells. Their results are consistent with those of Jin, Wu, Xu, Zheng, and Zhao (2014) in bone marrow stem cells. However, there are some different results as well. Jung et al. (2012) studied the effects of EGCG on human periodontal ligament cells and found that when the concentration of EGCG was 0–9.2 μg/mL, cell proliferation was unaffected. However, when the concentration of EGCG increased to 23 μg/mL or even 46 μg/mL, it significantly inhibited cell proliferation. The mechanism by which EGCG affects cell proliferation is still unclear. It has been speculated that low concentrations of EGCG enhance DNA antioxidant capacity and promote cell proliferation (Tian, Sun, Xu, & Hua, 2007). In our study, we found

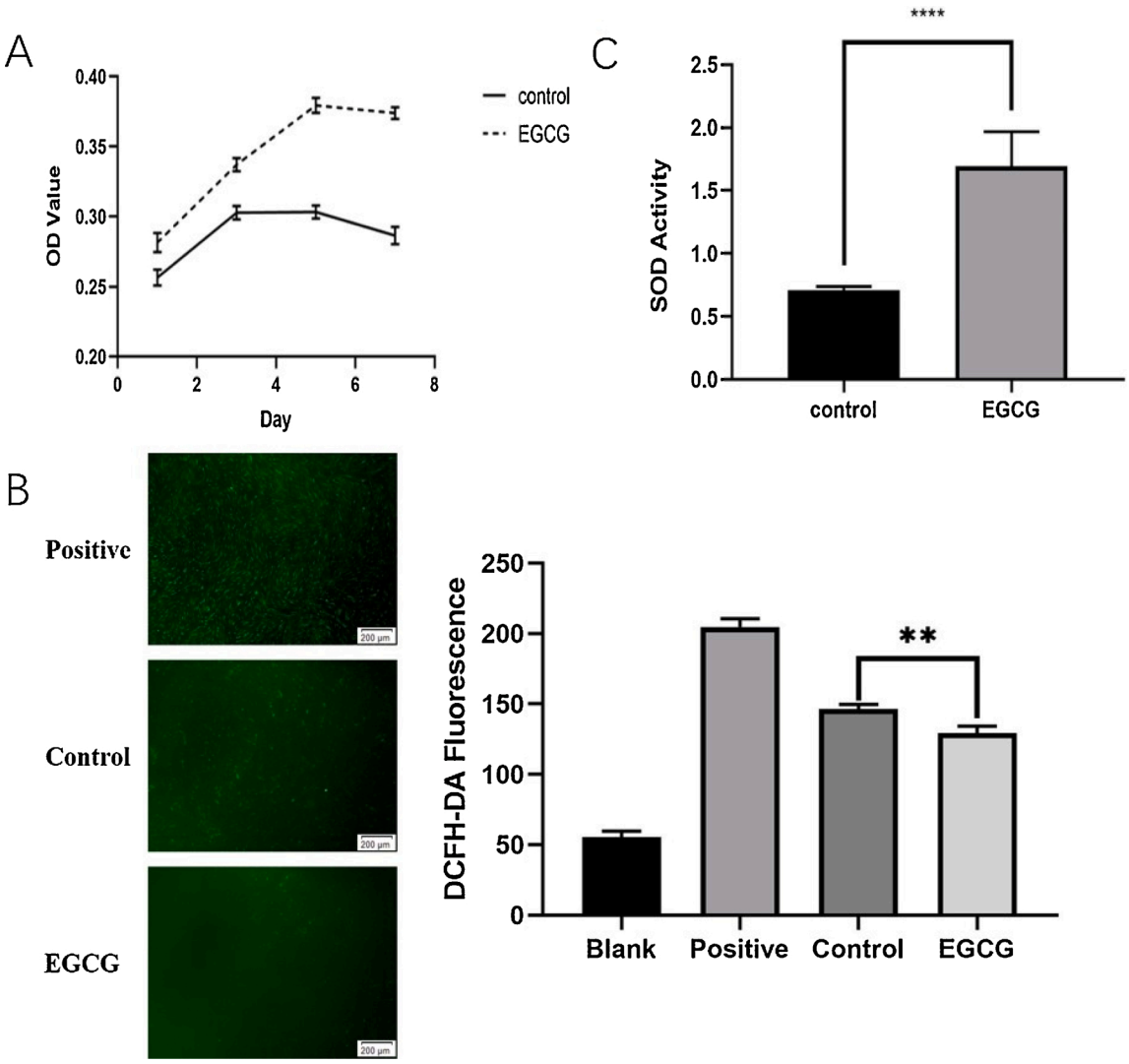

Fig. 4. Effect of EGCG on hDPSCs under hypoxic conditions. (A) After culture under hypoxic conditions, the proliferation abilities of hDPSCs at 1, 3, 5 and 7 days were detected by CCK-8 assay. (B) ROS production in hDPSCs was detected by a ROS kit. (C) SOD activity in hDPSCs. Data are expressed as the mean ± SD. $^{**}P < 0.01$; $^{****}P < 0.0001$.

that EGCG significantly inhibited cell proliferation when concentrations were greater than 10 μg/mL. The difference in results may be due to distinct cell types and experimental conditions.

Bacterial infection is the primary cause of pulp inflammation, and LPS is one of the most important pathogenic factors (Wang et al., 2019). In this study, hDPSCs were stimulated with *E. coli* LPS to simulate inflammation in the dental pulp and to explore the effect of EGCG on inflammation in hDPSCs. Results demonstrated that 10 μg/mL EGCG significantly inhibited expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in hDPSCs stimulated by LPS, consistent with previous studies. Nakanishi et al. (2010) found that when dental pulp cells were exposed to LPS and prostaglandin (PG), expression of IL-6 and interleukin-8 (IL-8) was inhibited by addition of ECG and EGCG. Subsequent studies showed that EGCG and ECG inhibited expression of vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) in inflammatory dental pulp cells (Nakanishi et al., 2015), further confirming the anti-inflammatory effects of EGCG. Hirao et al. (2010) reported that ECG and EGCG inhibited positive regulation of IL-8 and prostaglandin E_2 (PGE_2) in inflammatory dental pulp fibroblasts. Our results were consistent with previous studies. We found that 10 μmol/L EGCG inhibited inflammation while having no significant adverse effects on cell proliferation, which is important for clinical application.

In pulpotomy, capping materials should induce mineralization of the residual pulp tissue to form calcified bridges. However, to preserve the activities of pulp tissues, diffuse calcification of pulp tissue should be avoided. Diffuse pulp calcification leads to pulp inflammation and loss of pulp vitality, eventually resulting in treatment failure (Mello-Moura et al., 2017). Therefore, we next explored the effects of EGCG on osteogenic differentiation of LPS-stimulated hDPSCs. Based on previous studies (He et al., 2015; Huang, Jiang, Gong, Li, & Ling, 2015; Jun-qi, 2008), we chose 1 μg/mL LPS to stimulate hDPSCs *in vitro*. Results indicated that the formation of calcified nodules in hDPSCs was increased by LPS, suggesting enhanced mineralization ability of hDPSCs. Next, we further explored whether EGCG could alleviate the LPS-induced mineralization ability of hDPSCs. Results showed that 10 μg/mL EGCG inhibited excessive calcification of hDPSCs caused by LPS, suggesting that EGCG may prevent diffuse calcification of dental pulp under inflammatory stimulation.

The pulp is surrounded by hard tissue and lacks effective collateral

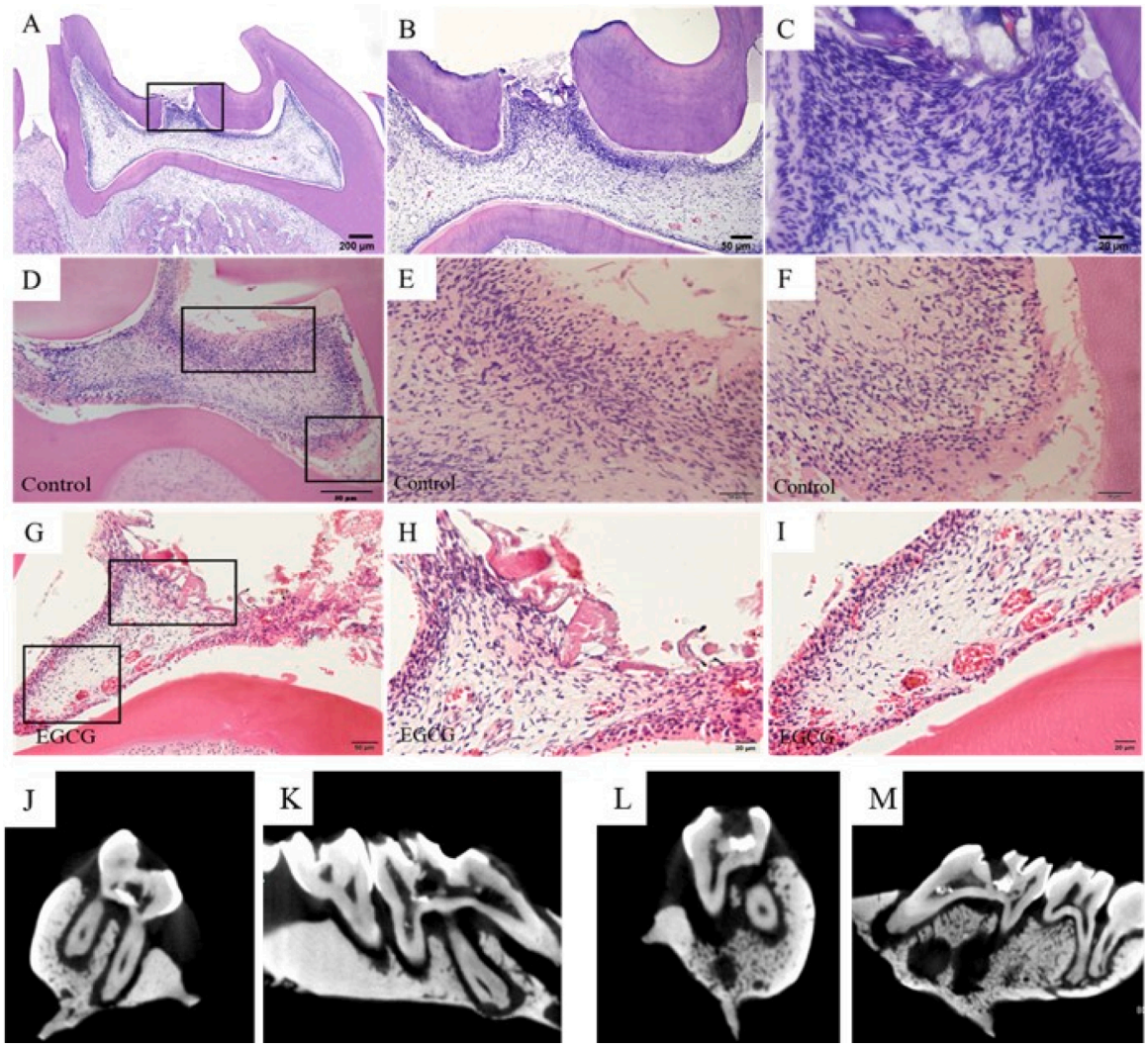

Fig. 5. HE staining of pulpotomied teeth. (A, B, C) After LPS stimulation for 30 min, a large number of inflammatory cells had infiltrated the coronal pulp (A, B), while the radicular pulp was mostly normal (C). (D, E, F) In the group with normal saline + MTA pulp capping, a large number of inflammatory cells infiltrated both the coronal pulp (D, E) and radicular pulp (F). (G, H, I) In the group with EGCG + MTA pulp capping, limited inflammation was observed in the coronal pulp (G, H), and no obvious inflammatory cell infiltration was found in the radicular pulp (I). A calcified bridge was observed in the pulp cavity on the sagittal plane in the control group (J, K) and EGCG group (L, M). (A 4×; B, D, G 10 ×; C, E, F, H, I 40 ×).

circulation. Once inflammation occurs, it leads to pulp ischaemia, hypoxia and increased pulp pressure (Amemiya, Kaneko, Muramatsu, Shimono, & Inoue, 2003). However, the specific oxygen concentration of pulp tissue undergoing inflammation has not yet been determined. Previous studies have shown that hypoxia affects both proliferation and differentiation of hDPSCs (Ahmed, Murakami, Kaneko, & Nakashima, 2016; Aranha et al., 2010; Iida et al., 2010; Sakdee, White, Pagonis, & Hauschka, 2009). Hypoxia/ischaemia has been widely recognized an important mediator and regulator of apoptosis (Shi et al., 2019). Low levels of ROS are essential for cell proliferation and differentiation, while excessive ROS can directly damage cell membranes, DNA and proteins, leading to loss of cell function, inhibition of proliferation and induction of apoptosis (Zorov, Juhaszova, & Sollott, 2014). Antioxidants, such as SOD, have been proven to inhibit apoptosis induced by stimulating factors (In, Hong, Choi, Jang, & Kim, 2016). Regarding the repair effects of EGCG on cell hypoxia, previous studies have focused on myocardial cells and tumours. Young Park, Jeong, Kim, Jung, and Kim (2013) showed that EGCG inhibits apoptosis by clearing ROS from cells. Wang et al. (2018) found that EGCG reduced the proportion of H9c2 myocardial cell apoptosis in response to hypoxia/reoxygenation (4/20 h), which may exert a myocardial protective effect by inhibiting the mitochondrial-dependent caspase pathway. Furthermore, Wei et al. (2018) found that EGCG decreased expression of hypoxia-inducible factor-1α (HIF-1α) and glucose transporter-1 (GLUT-1), critical players in regulating glycolysis. There are few studies on the effect of EGCG on hDPSCs under hypoxic conditions. In our study, a 1% oxygen concentration was selected as the hypoxia model. Results showed that EGCG inhibited hypoxia-induced apoptosis by downregulating ROS expression and upregulating SOD activity. The relevant mechanisms underlying these effects require additional investigation.

To further study the anti-inflammatory effects of EGCG *in vivo*, a high concentration of LPS (5 mg/mL) was selected to induce pulpitis in rats for 30 min. Histological analysis showed that a large number of inflammatory cells infiltrated the coronal pulp immediately after LPS treatment, while the radicular pulp was mostly spared, which met the requirements of the experiment.

Common flushing fluids used in pulpotomy include normal saline, sodium hypochlorite, hydrogen peroxide, *etc.* (Tunc et al., 2006). Some studies have shown that normal saline flushing removes debris and does not damage healthy pulp tissue (Asgary, Eghbal, Ghoddusi, & Yazdani,

2013). However, normal saline has no anti-inflammatory effects, which is essential for inflammatory pulp tissues. Abeer (2002) used sodium hypochlorite as a flushing fluid in pulpotomy and found that sodium hypochlorite has a good haemostatic effect. However, Okino et al. (2004) found that a high concentration of sodium hypochlorite destroys vital pulp tissue. In this study, EGCG was used in pulpotomy for the first time. Results revealed that EGCG inhibited the spread of inflammation in the pulp tissue. Compared to other flushing materials, EGCG has anti-inflammatory properties and good biocompatibility, evidencing its clinical applicability in the future. However, there are also some limitations in this study. *In vitro*, the anti-inflammatory effects of EGCG and its anti-apoptosis mechanisms under hypoxic circumstances need to be further studied. Furthermore, *in vivo*, the sample size needs to be expanded. Moreover, the results of *in vivo* experiments are lack of quantitative analysis. We plan to carry out immunohistochemical experiments to quantify the results, so as to make the research more clinical significance.

In conclusion, 10 μg/mL EGCG had a significant anti-inflammatory effect on hDPSCs without affecting cell proliferation or differentiation. In addition, EGCG inhibits apoptosis induced by hypoxia. *In vivo* studies demonstrated that EGCG inhibited the spread of inflammation in pulp tissues. These results provide support for the future application of EGCG in pulpotomy to preserve inflammatory pulp tissue.

Declaration of Competing Interest

The authors reported no declarations of interest.

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