# Regeneration of Dental Pulp Tissue by Autologous Grafting Stem Cells Derived from Inflammatory Dental Pulp Tissue in Immature Premolars in a Beagle Dog

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**Objective:** *To compare the biological characteristics of dental pulp stem cells (DPSCs) and inflamed dental pulp derived stem cells (I-DPSCs) in vitro and their regeneration potential in Beagle immature premolars.*

**Methods:** *Pulpitis was induced in the premolars of one beagle dog by opening the pulp chamber for 2 weeks, and inflammation was histologically confirmed. DPSCs and I-DPSCs were isolated from normal and inflamed dental pulp, and cell morphology, expression of mesenchymal stem cell markers, clone formation ability, cell proliferation and osteogenic/odontogenic differentiation potential were compared. The dental pulp of 20 roots from 10 immature premolars was extracted and divided into two groups. DPSCs or I-DPSCs with scaffolds were transplanted into the root canals. The roots were extracted after 3 months, and pulp regeneration was evaluated by histological analysis. The data were statistically analysed using one-way ANOVA and a Student t test.*

**Results:** *Histological analyses showed lymphocyte infiltration and elevated TNF-α expression, which confirmed the diagnosis of pulpitis. I-DPSCs showed similar morphology, marker gene expression and clone formation ability but greater proliferation ability and osteogenic/odontogenic differentiation potential. Pulp-like tissue formation and bone- and dentine-like tissue deposition were observed in both DPSC- and I-DPSC–transplanted roots.*

**Conclusion:** *DPSCs derived from inflammatory dental pulp tissue have similar biological characteristics to those from normal dental pulp and could mediate pulp and dentine regeneration in immature premolars.*

**Key words:** *dental pulp stem cells, inflamed dental pulp, tissue regeneration*

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Treatment of pulpitis due to pulp necrosis in immature teeth is a challenge for dentists worldwide. Traditional methods include calcium hydroxide–based apexification and mineral trioxide aggregate apexification. However, neither can solve the issue of cessation of root development and fragile root canal walls[1].

Regenerative medicine and tissue engineering are emerging fields focused on developments in organ repair, and have achieved substantial progress in recent years[2,3]. The key factors in tissue engineering and regenerative medicine are stem cells, scaffolds and growth factors[4].

Stem cells are first found in embryo and cord blood, and can differentiate into multiple lineages. Numerous adult tissues have been found to harbour stem cells. Animal studies have been particularly useful in observing that in vivo adult stem cells usually reside in specific areas called "niches"[5,6]. Mesenchymal stem cells (MSCs) are considered a good cell source for tissue regeneration. MSCs have been isolated from dental tissues, including the dental pulp, periodontal ligament and dental follicle[7-9]. Since dental pulp stem cells (DPSCs) were isolated in 2000, numerous studies

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have proven that pulp regeneration can be achieved by using DPSCs[7,10]. However, its clinical application may be limited due to the lack of availability of autologous DPSCs.

Alongi et al[11] reported that DPSCs also existed in inflamed pulps and retained tissue regeneration potential. Moreover, Pereira et al[12] isolated stem cells from inflamed dental pulps and found that DPSCs derived from inflamed and normal tissues were similar in morphology, proliferation rate and differentiation potential. Similarly, Liao et al[13] showed that cells isolated from inflamed periapical tissue expressed MSC markers and were highly osteogenic. In addition, several other studies have revealed that DPSCs with retained potential for tissue regeneration could be isolated from inflamed dental pulp[14-16]. Thus, DPSCs from inflamed dental pulp could be the new source of autologous stem cells for dental pulp tissue regeneration.

However, all previous studies about inflamed dental pulp stem cells (I-DPSCs) have focused on their characteristics and multiple differentiation potential in vitro. In the present study, we wished to investigate whether I-DPSCs could achieve the same behaviour as DPSCs in pulp regeneration, and systematically characterised DPSCs and I-DPSCs and investigated their regenerative potential.

## Materials and methods

### *Animal preparation*

One 5-month-old male beagle dog was selected from Marshall Biotechnology (Beijing, China). Care and handling of the animal were performed according to the guidelines set out by the Institutional Authority for Laboratory Animal Care at Peking University. This animal study was reviewed and approved by the animal care and use committee of Peking University Health Science Centre (No. LA2011-045).

With the animal under general anaesthesia, radiographs were taken of the premolars to ensure the apex was not closed. Subsequently, the pulp chambers of the 5 right premolars were opened using a high-speed turbine drill at the buccal side with a 2-mm diameter hole. The pulp chambers of the right premolars were exposed for 2 weeks while the 5 left premolars were intact. Thereafter, the pulp tissues of all 10 premolars from both sides were extracted, and the root canals were filled with calcium hydroxide paste. Pulps extracted from the right premolars were referred to as "inflamed dental pulps" and the others were referred to as "normal pulps".

### *Histological evaluation of pulps*

Two normal pulps and two inflamed pulps were fixed using 4% paraformaldehyde. Four-millimetre sections were deparaffinised and stained using haematoxylin-eosin. Immunohistochemical analysis was also performed on all samples to determine TNF-α expression levels.

### *Cell isolation and culture*

After extraction, the pulp tissues were soaked in Hanks' balanced salt solution. In a biological safety cabinet, the tissues were minced and digested in a solution of 3 mg/ml collagenase type I (Sigma-Aldrich, St Louis, MO, USA) and 4 mg/ml dispase (Sigma-Aldrich) for 1 hour at 37ºC. Single-cell suspensions were obtained by passing the cells through a Falcon 70-μm strainer (Corning Life Sciences, Tewksbury, MA, USA). These suspensions were seeded in 75-$cm^2$ culture plates containing α-minimum essential medium supplemented with 15% foetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA), 2 mmol/l glutamine (Sigma-Aldrich), 100 U/mL penicillin and 100 mg/ml streptomycin (Sigma-Aldrich), and the plates were cultured under 5% $CO_2$ at 37ºC. The medium was replaced every 3 days, and the cells were subcultured at 70% confluence. After having been cultured and passed 3 or 4 times, the cells were used in the experiment.

### *Characterisation of DPSCs and I-DPSCs*

DPSCs and I-DPSCs were labelled using fluorescein isothiocyanate–conjugated or phycoerythrin-conjugated antibodies and analysed using flow cytometry. Cells were harvested with 0.25% trypsin, and cell aliquots ($1.0 \times 10^6$ cells) were incubated for 1 hour at room temperature with monoclonal antibodies specific for Stro-1, CD105, CD90 and CD34 (BD Biosciences, Franklin Lakes, NJ, USA). The expression profiles were analysed using a BD FACSCalibur flow cytometer (BD Biosciences).

### *Cell proliferation*

DPSCs and I-DPSCs were seeded at a density of $1.0 \times 10^3$ cells/well into 96-well plates. Cells were counted at 1, 3, 5, 7, 9 and 10 days by adding a Cell Counting Kit-8 (CCK-8) solution (Solarbio, Dojindo, Kumamoto, Japan) to each well of the plate, and absorbance was measured at 450 nm after culturing for 3 hours according to the manufacturer's protocol.

*Osteo/odontogenic differentiation*

DPSCs and I-DPSCs were seeded at a density of $1.0 \times 10^5$ cells/well into 6-well plates, grown to 70% confluence and incubated for 2 weeks with a differentiation medium containing 10 nmol/l dexamethasone, 10 mmol/l β-glycerophosphate, 50 mg/ml ascorbate phosphate, 10 nmol/l 1,25-dihydroxyvitamin D3 and 10% foetal bovine serum (GE Healthcare Life Sciences). To examine mineral nodule formation, the cultured cells were fixed in 4% paraformaldehyde and washed in water, and mineralisation of the extracellular matrix was assayed using 1% alizarin red S staining. For real-time reverse transcriptase polymerase chain reaction (PCR), RNA of cultured cells was extracted and 2 μg aliquots of RNA were synthesised with random hexamers and transcriptase according to the manufacturer's protocol (Invitrogen, Waltham, MA, USA). Real-time PCR was performed using an SYBR Green PCR kit (Qiagen, Dusseldorf, Germany) and an Icycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The gene-specific primer sequences are listed in Table 1.

*Pulp regeneration using DPSCs and I-DPSCs*

With the animal under general anaesthesia, 20 roots of 10 premolars of both sides were irrigated with 10 ml of 1.25% NaOCl and sterile saline. Subsequently, $1.0ene^6$ cells were transplanted into each root of the right premolars, mixed with Puramatrix™ (BD Biosciences) as a scaffold. For the experimental group, I-DPSCs were transplanted into the 10 roots of the right premolars, and for the control group, DPSCs were transplanted into the 10 roots of the left premolars. After 3 months, all roots of the experimental and control groups were extracted, and histological analyses were performed. The anterior teeth were extracted as normal controls.

*Statistical analysis*

Cell proliferation and real-time RT-PCR were analysed using one-way ANOVA, and the histological results were analysed using a Student *t* test. SPSS (version 13.0; SPSS, Chicago, IL, USA) was used for all statistical analysis. The mean difference was considered significant at 0.05 and a 95% confidence interval.

## Results

*Histological analyses showed pulp inflammation*

After pulp exposure for 2 weeks, there were no signs of swelling or mobility in the teeth. Intact dental pulp tissue could be harvested from normal teeth as well as teeth with pulp exposure. The histological images showed clear collagen fibres and a large number of spindle fibre cells with little lymphocyte infiltration in the normal dental pulps (Fig 1a). For teeth with dental pulp exposure for 2 weeks, the histological images showed numerous lymphocytes with smaller and dyed dark nuclei as well as a nodule with lymphocyte infiltration (Fig 1d). However, there were some residual spindle fibre cells. Immunohistochemical staining showed high expression levels of TNF-α in the lymphocyte infiltration nodule of the inflamed pulp (Figs 1e and 1f) but no such expression in the normal pulp (Figs 1b and 1c).

*I-DPSCs had similar biological characteristics to DPSCs*

Like with normal dental pulp, stem cells could be isolated from inflamed dental pulps, and these were named I-DPSCs. I-DPSCs showed typical fibroblast-like morphology. I-DPSCs notably exhibited similar morphology and clone formation ability to DPSCs.

Cell proliferation was monitored over a period of 10 days after seeding. Cell growth curves showed that cell growth rates were similar for DPSCs and I-DPSCs at 1, 3 and 5 days but significantly higher for I-DPSCs after 7 days (Fig 2a). DPSCs and I-DPSCs were both positive for STRO-1, CD105 and CD90 but negative for CD34 (Fig 2b).

**Table 1** Gene-specific primer sequences.

| Gene name | Primer (5'-3') | |
|---|---|---|
| | Forward | Reverse |
| BSP | ACCCTGCCAAAAGAATGCAG | TGCCACTAACATGAGGACGT |
| ALP | TCTCACTACGTCTGGAACCG | CTCGGGGTTCTTGCTCAGA |
| DSPP | GTGGTGTCCTGCGTGAAAT | ACCCTCACTATTCCCCTCCT |
| GAPDH | CAAGGCTGAGAACGGGAAGC | AGGGGGCAGAGATGATGACC |

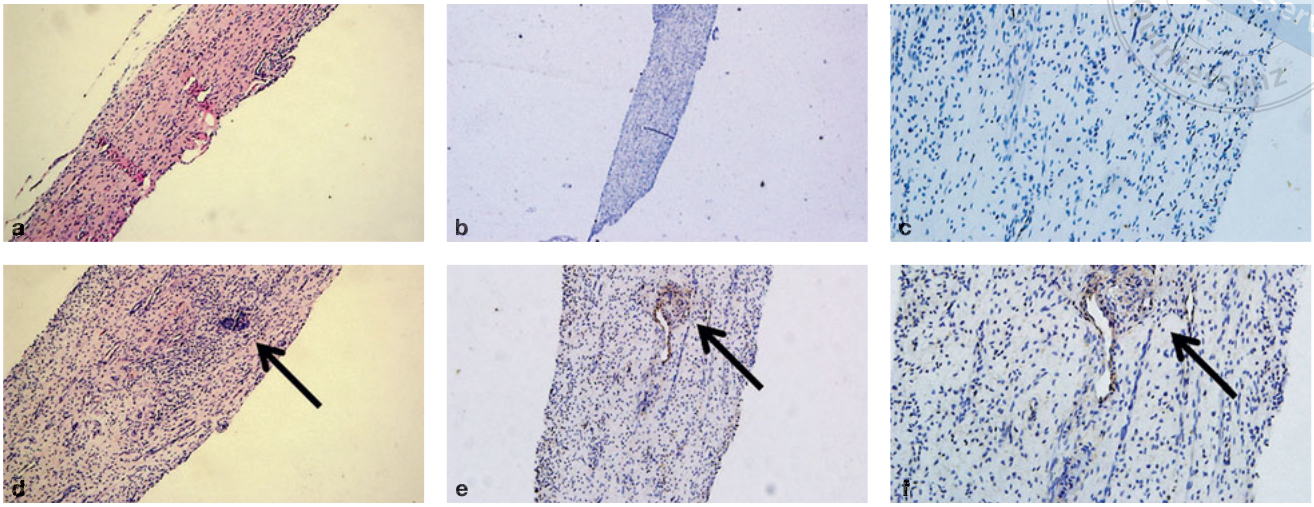


**Fig 1** Histological images of premolar tissue samples of normal dental pulp (a, b and c) and inflamed dental pulp (d, e and f). **(a)** Haematoxylin and eosin (HE) staining of normal pulp (4×). **(b)** and **(c)** Immunohistochemical staining showed no expression of TNF-α (b: 4×; c: 20×). **(d)** HE staining of inflamed pulp showed infiltrated lymphocytes (arrow) (4×). **(e)** and **(f)** Immunohistochemical staining showed expression of TNF-α in the lymphocyte infiltration area (e: 4×; f: 20×).

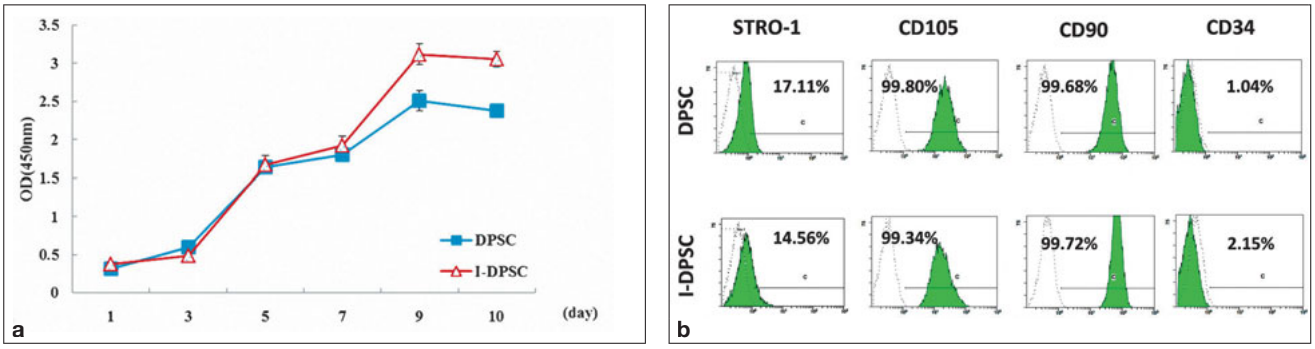


**Fig 2** Proliferation rate and surface marker expression of DPSCs and I-DPSCs. **(a)** I-DPSCs have a better proliferation rate than DPSCs. **(b)** Immunophenotype analysis by flow cytometry; I-DPSCs have similar MSC surface marker expression to DPSCs.

Osteogenic/odontogenic differentiation potential was investigated by culturing DPSCs and I-DPSCs in an osteogenic induction medium. Two weeks later, total RNA was extracted and real-time RT-PCR results showed that the expression levels of ALP, BSP and DSPP were higher in I-DPSCs than in DPSCs (Fig 3b). After induction with the osteogenic medium for 2 weeks, alizarin red staining of the two types of cells revealed similar mineralised nodules (Fig 3a).

### *DPSC- and I-DPSC-induced tissue regeneration*

After 3 months, all restorations were intact, and there were no signs of swelling or mobility in the teeth from either group. Radiographs showed no periradicular lesions along with the development of the roots of all the teeth in both the experiment and control groups.

In the experiment group, histological evaluation revealed that regenerated vital tissue could be observed in all roots: 4 roots exhibited tissues reaching the coronal third, 2 exhibited tissues reaching the apical third, while others were nearly empty in the roots with tissues just around the apex.

In the control group, vital tissue could also be observed in all roots: 3 roots exhibited tissues reaching the coronal third, 2 exhibited tissues reaching the apical third, while others exhibited tissues at the apex. No significant differences were noted in tissue regeneration between the groups. The results are summarised in Table 2.

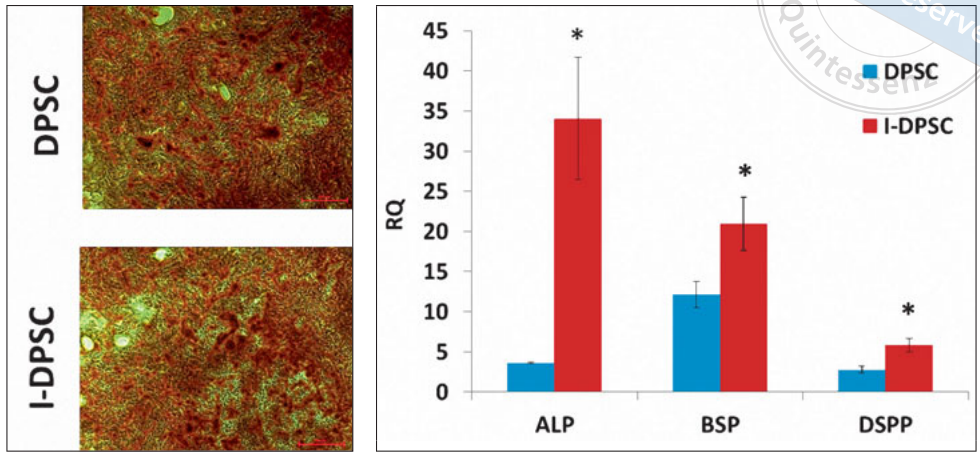


**Fig 3** Osteogenic differentiation of DPSCs from healthy and inflamed premolar dental pulp tissues. **(a)** Mineralised nodules were detected after alizarin red staining following osteogenic induction for 4 weeks. **(b)** Real-time PCR showed that expression of osteogenic genes (ALP, BSP and DSPP) was much higher in I-DPSCs than in DPSCs after osteogenic induction for 2 weeks.

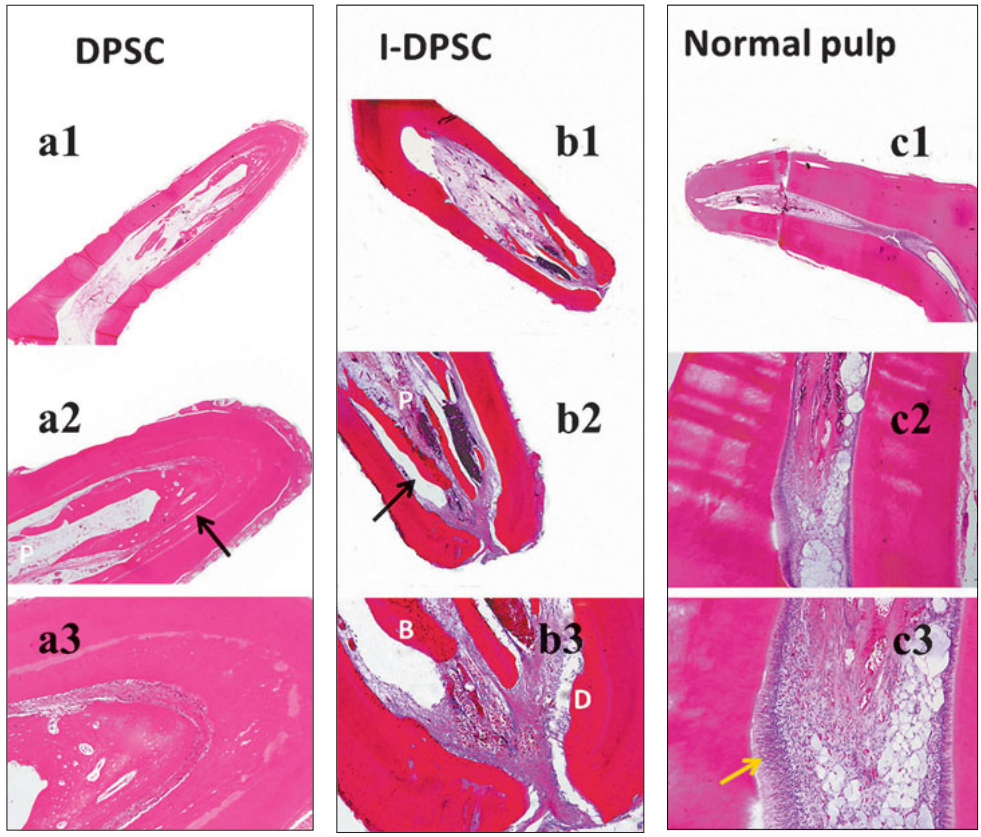


**Fig 4** Histological images of tooth roots in the DPSC group (a1–3), I-DPSC group (b1–3) and control group (c1–3). P, pulp-like tissue; B, bone-like tissue; D, secondary dentine; C, cementum. Black arrows indicate bone-like tissue and the red arrow indicates odontoblasts.

**Table 2** Volume of regenerated pulp tissue in two groups.

| Vital tissue | To coronal third | To middle third | To apical third |
|---|---|---|---|
| Experimental group (n = 10) | 4 | 2 | 4 |
| Control group (n = 10) | 3 | 2 | 5 |
| Statistical analysis | P > 0.05 | P > 0.05 | P > 0.05 |

Histological analysis showed a large amount of regenerated tissue in the roots, among which regenerated dentine could be observed in both groups. Irregular fibrous connective tissue and blood vessels could be observed in the roots with a few cells (Fig 4). Compared with normal dental roots, the regenerated tissue in the canal space consisted of pulp-like, bone-like and dentine-like tissue but with odontoblasts observed (Fig 5).

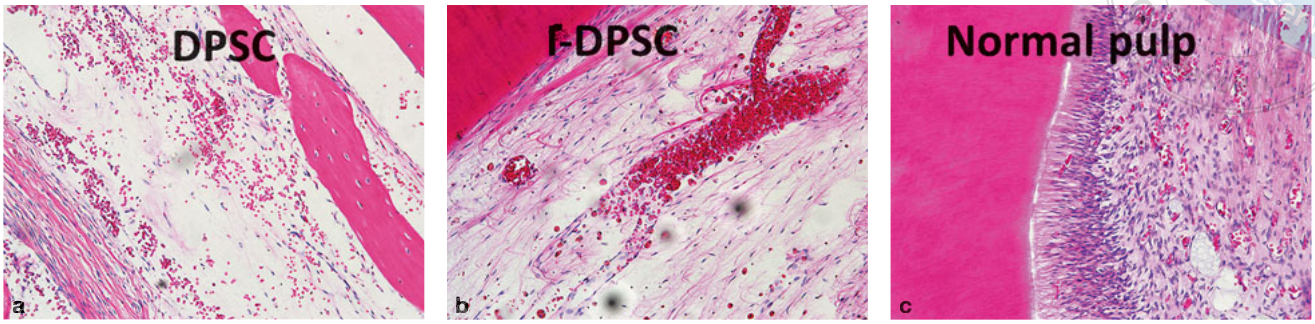


**Fig 5** Histological images of roots in the middle in the DPSC group, I-DPSC group and control group (20×). Arranged odontoblasts could be observed in the normal pulp but there were few odontoblasts in the DPSC and I-DPSC groups, where only irregular fibrous connective tissue and blood vessels could be observed.

## Discussion

In the present study, I-DPSCs were successfully isolated from inflamed dental pulp, and had similar cellular properties to DPSCs from normal pulp. Both DPSCs and I-DPSCs had the potential to induce pulp tissue regeneration in immature premolars.

Several studies have demonstrated successful pulp tissue regeneration with DPSCs, with characteristics such as root wall development and the presence of bone-like tissue[17-19]. This could therefore be a more promising strategy for the treatment of irreversible pulpitis or pulp necrosis of immature permanent teeth.

In this study, we extracted and obtained inflamed dental pulp tissue samples after pulp exposure for two weeks. The histological findings revealed a nodule with lymphocyte infiltration with high expression levels of TNF-α, which proved the diagnosis of pulpitis. Previous studies have elaborated on the relationship between inflammatory cytokines and inflamed pulps[20-22], which provided evidence for diagnosis in our study.

Our results revealed no differences between I-DPSCs and DPSCs in terms of morphology, cell markers and clone formation ability, but I-DPSCs revealed greater proliferation ability and osteogenic/odontogenic differentiation potential. This is consistent with previous experimental results[11-13,15]. Thus, the results from these studies indicate that inflammation could enhance the migratory capacity of human periodontal ligament stem cells[23], and hypoxia could enhance the angiogenic potential and proliferation of DPSCs in the inflammation environment[24,25]. In addition, the expression of TNF-α could promote odontoblastic differentiation[26]. Because of the increased osteogenic/odontogenic differentiation potential, I-DPSCs have a potential application in bone formation. Studies have found that stem cells derived from inflamed dental pulp and gingival tissue could be used for the repair of human periodontal bone defects[27,28].

Based on the histological results of pulp tissue regeneration in roots noted in the present study, pulp-like tissue was observed in both groups with no significant differences, indicating that I-DPSCs have similar regeneration potential to DPSCs. The regenerated tissues in the canal space consisted of pulp-like, bone-like and dentine-like tissue, but no odontoblasts, and this was consistent with other studies[29-32]. However, similar to our results, regeneration of odontoblasts and dental pulp with a complete structure could not be achieved in all related research.

Stem cells possess anti-inflammatory and immunomodulating properties[33,34]. Previous studies have demonstrated that DPSCs can attenuate inflammatory processes and modulate macrophage function via TNF-α signalling; this could be helpful for the treatment of heart failure, as well as wound healing[35-37]. Moreover, DPSCs could activate macrophages and suppress TNF-α secretion, thereby downregulating the inflammatory response[34]. It is well known that TNF-α can promote an odontoblastic phenotype in dental pulp cells, which, in turn, could be helpful for pulp regeneration using I-DPSCs[26]. Furthermore, matrix metalloproteinase-3 in tissues has anti-inflammatory effects, and the cytokine interferon-γ can improve impaired dentinogenic and immunosuppressive functions of I-DPSCs[38,39]. All these factors may help to preserve the regenerative potential of I-DPSCs isolated from inflamed dental pulp.

In this study, vital tissue regeneration was observed in all roots, but not all tissues reached the coronal third, which may be attributed to the varying blood supply from the apex as well as other specific surgical

details. In fact, rubber dam and more careful aseptic surgical procedures would be needed for the next study. Compared with DPSCs, I-DPSCs showed similar biological characteristics but a better proliferation rate, osteogenic/odontogenic differentiation potential and tissue regeneration in immature canine teeth. However, actual dental pulp regeneration with complete structure warrants further research. Studies have shown that growth factors such as combined granulocyte colony stimulating factor (G-CSF) or drugs such as simvastatin have anti-inflammatory effects and could promote the regenerative potential of stem cells[40,41]. Simultaneously, betamethasone could decrease inflammatory response and increase the regenerative potential of reserved cells in pulpitis[42]. These findings may be helpful for future studies on I-DPSCs.

In conclusion, our study verified that there were no significant differences between DPSCs and I-DPSCs in terms of their proliferation and osteogenic/odontogenic differentiation potential. After implantation in immature canine teeth for 3 months, vital tissues could be observed in both DPSC and I-DPSC groups, with no significant difference. Our results suggested that I-DPSCs might be a novel, viable source of cells for tissue regeneration, and might provide a new treatment option for pulpitis in immature permanent teeth.

## Conflicts of interest

The authors declare no conflicts of interest related to this study.

## Author contribution

Dr Long LING contributed to the project design, in vitro experiments, data analysis and manuscript drafting; Drs Xiao Tong WANG and Quan WEN performed the animal experiments; Prof Yu Ming ZHAO reviewed the manuscript; Prof Li Hong GE supervised the overall progress of the project. All authors read and approved the final manuscript.

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