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ORIGINAL ARTICLE

Histological analysis for pulp mineralisation after severe intrusive luxation of immature molars in rats

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Abstract

Background/Aim: Pulp mineralisation is a survival process that may occur in the pulp of immature teeth following trauma. However, the mechanism of this process remains unclear. The aim of this study was to evaluate the histological manifestations of pulp mineralisation after intrusion in immature molars of rats.

Materials and Methods: Three-week-old male Sprague–Dawley rats were subjected to intrusive luxation of the right maxillary second molar by an impact force from a striking instrument through a metal force transfer rod. The left maxillary second molar of each rat was used as a control. The control and injured maxillae were collected at 3, 7, 10, 14, and 30 days after trauma ($n = 15$ per time group) and evaluated using haematoxylin and eosin staining and immunohistochemistry. Independent two-tailed Student's *t*-test was used for statistical comparison of the immunoreactive area.

Results: Pulp atrophy and mineralisation were observed in 30%–40% of the animals, and no pulp necrosis occurred. Ten days after trauma, pulp mineralisation, with osteoid tissue rather than reparative dentin, formed around the newly vascularised areas in the coronal pulp. CD90-immunoreactive cells were observed in the sub-odontoblastic multicellular layer in control molars, whereas the number of these cells was decreased in the traumatised teeth. CD105 localised in cells around the pulp osteoid tissue of the traumatised teeth, whereas in control teeth, it was only expressed in the vascular endothelial cells of capillaries in the odontoblastic or sub-odontoblastic layers. In specimens with pulp atrophy at 3–10 days after trauma, hypoxia inducible factor expression and CD11b-immunoreactive inflammatory cells increased.

Conclusions: Following intrusive luxation of immature teeth without crown fractures in rats, no pulp necrosis occurred. Instead, pulp atrophy and osteogenesis around neovascularisation with activated CD105-immunoreactive cells were observed in the coronal pulp microenvironment characterised by hypoxia and inflammation.

KEYWORDS
animal model, histological analysis, immature teeth, intrusive luxation, pulp osteogenesis

1 | INTRODUCTION

Intrusive luxation causes injury to the periodontal ligament, cementum, alveolar bone, and dental pulp. Complications of intruded permanent teeth include external replacement resorption, external inflammatory resorption, pulp necrosis with infection, and pulp mineralisation.[1] Pulp necrosis after intrusion is less frequent in immature permanent teeth than in mature teeth, but immature teeth are more prone to pulp mineralisation, which occurs in 35%–42% of immature teeth after intrusive luxation.[2,3]

The histological manifestations of normal and aging dental pulp mineralisation are pulp stones or diffuse calcifications exhibiting smooth and compact surface structures, respectively.[4,5] However, the morphology of mineralisation in the traumatised dental pulp of immature teeth has been rarely reported. Andreasen proposed that pulp mineralisation may present as reparative dentin, osteoid dentin, or even as bone (tissue metaplasia).[6] Using an electron microscope, Heling et al. showed bone-like tissue growing in the root canals of five young permanent teeth after trauma.[7] In vivo studies of avulsion in rat molars have shown both reparative dentin and osteoid mineralisation in the pulp.[8–10] No studies on the histology and development of pulp mineralisation in intrusive teeth were found in the literature.

Pulp mineralisation is an abnormal process of pulp survival in traumatised teeth that might be initiated by long-term, low-intensity stimuli. Severed or injured dental pulp produces angiogenesis-related growth factors that promote angiogenesis.[11–13] Various hypotheses for excessive mineralisation and dentin deposition in dental pulp have been proposed, and the most common cause is hypoxia or inflammatory stimulation caused by alterations in blood flow.[14–16] Whether intrusive luxation induces pulp hypoxia or inflammation needs to be determined.

Infection or trauma can cause cells in the dentin-pulp complex, including immune cells, fibroblasts, and dental pulp stem cells (DPSCs), to mount a defence response.[17] During tissue repair, low-level inflammation can promote stem cell migration.[18] The main pluripotent stem cells in dental pulp repair are DPSCs, which often express mesenchymal stem cell (MSC) surface markers, including CD29, CD44, CD59, CD73, CD90, CD105, and CD146, but rarely express haematopoietic stem cell markers (CD14, CD34, CD45, and CD11b).[19] DPSCs primarily differentiate into odontoblasts but they can differentiate into osteoblasts in vitro.[19] However, the specific differentiation mechanism is unknown. With the advent of single-cell sequencing technology, researchers have investigated different DPSC subpopulations.[20–24] Comparison of sequencing data from extracted and monolayer-cultured DPSCs has revealed that their cell compositions differ. The proportion of CD146-positive DPSCs was similar in vivo and in vitro, although these cells had stronger proliferation and osteogenesis abilities in vitro.[21] Whether the mineralised tissues in dental pulp and normal dentin originate from the same subpopulation of MSCs and the mechanism underlying mineralisation is unclear.

A rat model of intrusive luxation of immature molars has been established, and in this model, pulp mineralisation developed in the coronal pulp at 14 days after injury.[25] The aim of this study was to evaluate the histology and cell activities in traumatised pulps with mineralisation and to preliminarily explore their origin and development. The proposed hypothesis was that dental pulp mineralisation after intrusion is reparative dentin formed by activated DPSCs.

2 | MATERIALS AND METHODS

This manuscript of an animal study was written in accordance with the Preferred Reporting Items for Animal Studies in Endodontology (PRIASE) 2021 guidelines (Nagendrababu et al. 2021, doi: 10.1111/iej.13477) (Figure 1).

Male Sprague–Dawley rats (n = 75; age, 3 weeks) purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) were housed in individually ventilated cage (IVC)-supervised facilities (three rats per cage; ad libitum food and water) under climate-controlled conditions and a 12-h light–dark cycle. All animal procedures were approved by the Animal Research Ethics Committee of the Peking University Health Science Center, Beijing, China (LA2021160).

In the prior study, two or three of the five rats developed pulp mineralisation.[25] In the present study, to ensure at least five samples with pulp mineralisation for statistical analysis of the histology (in case of animal death or other loss), the final sample size was increased to three times that of the prior experiment (n = 15 per time point).

All experimental procedures were performed under anaesthesia with an intraperitoneal injection of 2% sodium pentobarbital (0.20 mL/100 g body weight). Traumatic intrusion was experimentally induced as previously described by applying a striking instrument to the occlusal surface of the right maxillary second molar.[25] The left maxillary second molar was used as a control. The animals were resuscitated on a thermostatic appliance. No intervention was administered after intrusion.

Animals were sacrificed at 3, 7, 10, 14, and 30 days (n = 15/time point) after injury. Fifteen control and injured maxillae were collected for each time point and fixed in 4% paraformaldehyde, followed by decalcification in 10% ethylenediaminetetraacetic acid (EDTA) (pH 7.0) at 37°C for 4–6 weeks. Tissues were then dehydrated, embedded in paraffin the mesiodistal direction, and sectioned into 4-μm thick sagittal sections using a microtome (Leica Instruments, Hubloch, Germany) for haematoxylin and eosin (HE) staining and immunohistochemistry.

For immunohistochemistry staining, sections were deparaffinised with xylene and graded ethanol (100%–70%). Heat-mediated antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) or citrate buffer (pH 6.0) for 20 min at 70°C. After cooling and washing in phosphate-buffered saline (PBS, pH 7.2), the sections were incubated in 3% hydrogen peroxide and 10% goat serum for 20 min

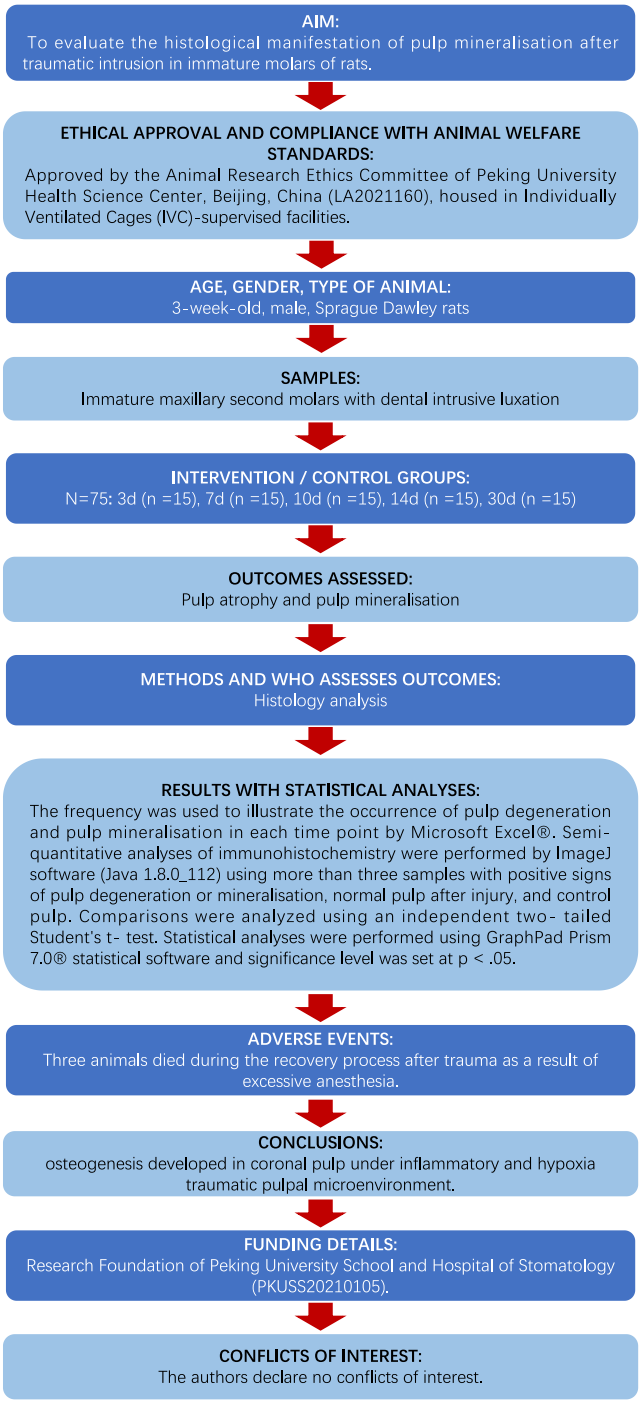

FIGURE 1 PRAISE 2021 flow chart.

at room temperature to block endogenous peroxidase activity and prevent non-specific binding, respectively. Thereafter, the sections were probed with rabbit or mouse anti-rat antibodies overnight at 4°C. Specific information regarding the host, concentration, and manufacturer of the antibodies is shown in Table 1. Negative controls were probed with PBS (pH 7.2). All sections were then incubated with biotin-conjugated goat anti-rabbit or anti-mouse IgG and horseradish peroxidase (HRP)-conjugated streptavidin (ZSGB-BIO, Beijing, China) at room temperature for 20 min. After washing in PBS, the slides were incubated with 3,3-diaminobenzidine-tetrahydrochloride (DAB) in 0.05 M Tris–HCl buffer (pH 7.6) for visualisation and with haematoxylin (HE) for 30 s for nuclear staining.

To differentiate mineralised pulp from dentin, expression of the osteoblast-related proteins (runt-related transcription factor 2 [Runx2], osterix [OSX], and osteocalcin [OCN]) and the odontoblast-related proteins (nestin, dentin sialophosphoprotein [DSPP], and dentin matrix protein 1 [DMP1]) was evaluated. To investigate the origin of the mineralised tissue and the activities of different MSC subtypes in the coronal pulp, the expression of CD90, CD105, and CD146, which are surface markers of MSCs, was evaluated via immunohistochemistry. In addition, to explore the mechanism of osteoid mineralisation in the traumatised coronal pulp, the presence of inflammatory-related myeloid cells ($CD11b^+$) and the expression of hypoxia inducible factor (HIF-1α) were evaluated.

All sections were observed under a light microscope (Olympus, Tokyo, Japan). In HE stained images, the coronal pulp was observed at 4× and 20× magnification. The frequency of pulp degeneration and mineralisation were determined. For immunohistochemical analyses, sections with intact tooth structure (including coronal pulp, root pulp and apical foramen) were involved. The coronal pulp segments in each specimen were examined at 20× magnification. Semi-quantitative immunohistochemical analyses were performed by measuring the percent immunoreactive area in the matrix and cytoplasm (area%) of the whole coronal pulp using ImageJ software (Java 1.8.0_112). For each time point, three specimens from teeth with signs of pulp degeneration or mineralisation, normal pulp after injury, and control pulp were chosen randomly for measurements. The data were analysed using an independent two-tailed Student's *t*-test in GraphPad Prism 7.0 statistical software, and the significance level was set at $p < .05$.

3 | RESULTS

Of the 75 rats (average weight 67.1 g), three died during recovery owing to excessive anaesthesia. The mean weight increases (g) of the animals at 3, 7, 10, 14, and 30 days after dental trauma were 21.5 ± 2.5, 59.4 ± 7.3, 71.2 ± 6.5, 94.3 ± 11.2, and 263.5 ± 17.9, respectively. The remaining animals were alive and had no systemic symptoms. No crown fractures or pulp necrosis occurred in any of the teeth. However, pulp atrophy and mineralisation initially developed in the coronal pulp but not in the root pulp during healing at 3–30 days after intrusion (Figure 2). The occurrence of coronal pulp atrophy and mineralisation are presented in Table 2.

After injury, multicellular layer disappearance and vacuolar degeneration of odontoblasts or fibroblasts were observed in the coronal pulp at 3 days, followed by vasodilatation at 7 days. From day 10, the number of cells around the vascularisation area increased. At 14–30 days after trauma, osteoid mineralisation appeared in the coronal pulp of some teeth and filled the coronal pulp chamber, replacing the connective tissues. There were no histological differences of pulp between the non-mineralised injured specimens (Figure 2U–Z,I–IV) and the control specimens (Figure 2A–J).

TABLE 1 Antibodies used in this study.

Antibody	Host	Dilution	Manufacturer	ID number
CD11b	Rabbit	1:4000	Abcam	ab133357
HIF-1α	Rabbit	1:500	GeneTex	GTX127309
Runx2	Rabbit	1:2000	Abcam	ab236639
OSX	Rabbit	1:1000	Abcam	ab209484
Nestin	Mouse	1:10,000	Abcam	ab6142
OCN	Rabbit	1:200	Bioss	bs-4917R
DMP1	Rabbit	1:200	Bioss	bs-12359R
DSPP	Mouse	1:50	Santa Cruz	sc-73,632
CD34	Rabbit	1:2500	Abcam	ab81289
CD90	Rabbit	1:200	Abcam	ab92574
CD105	Rabbit	1:1000	Abcam	ab252345
CD146	Rabbit	1:250	Abcam	ab75769

Runx2 and OSX are usually expressed in pre-osteoblasts. Nestin, a marker of neural stem cells, is expressed in pre-odontoblasts. In this study, Runx2, OSX, and OCN, but not Nestin, DSPP, or DMP1, were highly expressed in immature and mature mineralisation tissues (Figure 3G–R), indicating that the coronal mineralised matrix was bone and not dentin. Thus, the functional cells around the osteoid tissue were more likely to be osteoblasts than odontoblasts.

In control molars, CD90-immunoreactive cells were primarily observed in the sub-odontoblastic multicellular layer, and some were observed in the perivascular region of the coronal pulp. In intrusive molars with pulp atrophy and mineralisation, CD90-immunoreactive cells were decreased in the sub-odontoblastic layers and were not present around the osteoid tissue (Figures 4H–N,III and 5A).

More CD105 localised in cells outside the vascular endothelial cells around the pulp osteoid tissue at 3–14 days after dental trauma compared with that in normal teeth after injury and control teeth, where it was only expressed in the vascular endothelial cells of capillaries in the odontoblastic or sub-odontoblastic layers (Figures 4O–U,III and 5B). At 30 days after trauma, it disappeared, indicating that when osteoid tissue development was complete, the role of CD105-positive cells was complete.

CD146 was expressed in the odontoblastic layer of control molars and normal teeth after injury. In molars with pulp atrophy and mineralisation, CD146 was highly expressed in the vascular endothelial cells around the osteoid tissue of the coronal pulp and did not disappear until 30 days after the dental trauma, which was consistent with the expression of CD34, a marker of neovascularisation (Figures 4A–G,V–Z,I–III and 5C).

CD11b expression gradually increased in the coronal pulp from day 3 to day 10 after trauma and decreased in the mineralised region (Figure 6F–J). HIF-1α was primarily expressed in the odontoblastic layer of control molars where dentin formation was active (Figure 6K). After injury, it was highly expressed in the pulp tissue at 3–10 days (Figure 6L–N). At 14 days after trauma, cells enclosed in the osteoid mineralisation (osteocytes) were negative for HIF-1α, and the cells around the mineralised region (osteoblasts) had weak HIF-1α staining (Figure 6O), which was similar to the expression in the odontoblastic layer.

4 | DISCUSSION

This study used an animal model of dental trauma and described the pulp-healing processes following intrusive luxation in young permanent teeth of rats. In the model, osteoid mineralisation developed in the coronal pulp.

Osteoclasts and bone marrow-like structures were observed around the pulp osteoid tissue. The presence of OSX/OCN-positive and Nestin/DSPP/DMP1-negative cells during osteogenesis indicated that the osteoblastic, not odontoblastic, differentiation occurred in the coronal pulp. Those intra-pulp osteoblasts might originate from: (1) DPSCs or (2) stems cells recruited from Hertwig's epithelial root sheath, periodontal ligament and/or bone marrow migrating to the pulp through the open apical foramen.

Many studies have shown that DPSCs can differentiate into osteoblasts in vitro, which may support the first possibility.[26–29] The results of this study show that MSCs in the dental pulp space may form histologically different structures in the mineralised tissue (i.e., dentin or bone). However, the key factors and mechanisms that induce MSCs differentiation into odontoblasts or osteoblasts have yet to be confirmed.

Some studies have used models of tooth avulsion in immature rat molars.[8–10] During the process of pulp healing, reparative dentin and osteoid tissue were simultaneously observed, which was very similar to the findings in this study. In addition, 30%–40% of the specimens had osteogenic pulp mineralisation, while others had reparative dentin formation. Although avulsion and intrusion are different types of dental trauma, there are some similarities between them, since, both keep the crown intact (without bacterial infection) and the apical blood supply is severed. It is suggested that osteoid mineralisation in the pulp may be related to apical blood supply reconstruction. In an experiment involving the transplantation of a GFP-transgenic rat tooth into a wild-type rat tooth

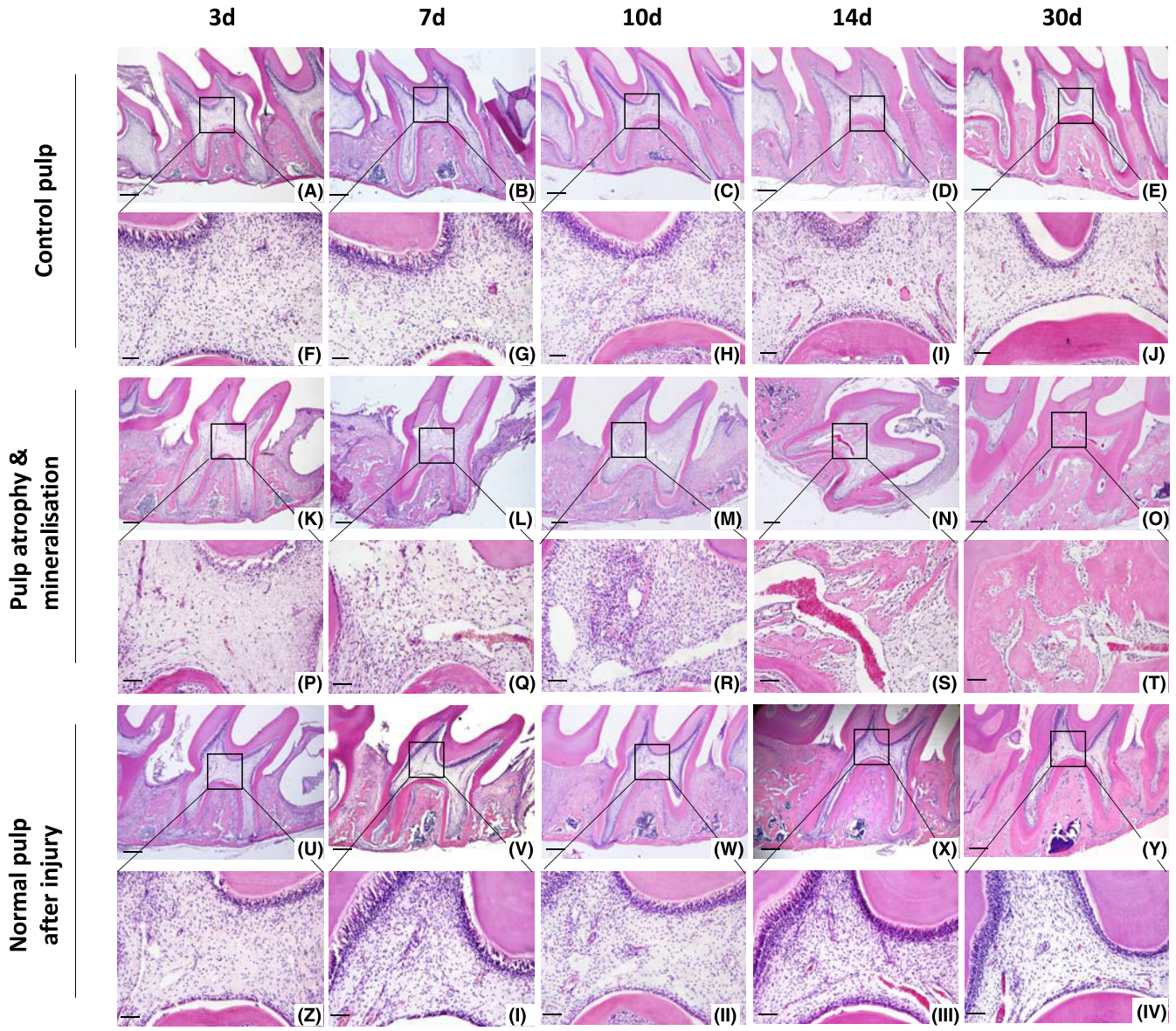

FIGURE 2 Healing of dental pulp in the intrusive immature molars of rats. (A–J) The coronal pulp of control teeth consisted of fibrous connective tissue. The dentin continued to develop as the distance between the top and bottom of the pulp cavity gradually decreased; (K–T) Pulp atrophy occurred in the early stage after trauma (K, L, P, Q), and repair began 10 days later (M, R). Osteoid mineralisation was observed in the coronal pulp around the vascularisation area, and the development of normal dentin was interfered (N, O, S, T); (U–Z, I–IV) Normal pulp after injury: no histological difference between non-mineralised injured pulp and control pulp. Scale bars: 200 μm (A–E, K–O, U–Y), 50 μm (F–J, P–T, Z, I–IV).

TABLE 2 Occurrence of atrophy and mineralisation in traumatic dental pulp in the immature molars of rats.

	n/N				
	Day 3	Day 7	Day 10	Day 14	Day 30
Pulp atrophy	6/14	5/14	4/14	1/15	0/15
Pulp mineralisation	0/14	0/14	5/14	5/15	5/15
Normal pulp	6/14	9/14	5/14	9/15	10/15

Note: *n*: Number of animals with pulp atrophy, pulp mineralisation, or normal pulp.
N: Total number of animals at each time point.

socket, GFP-positive cells were detected on the surface of dentin-like tissue, but not in osteoblast-like cells on the surface of newly formed intra-pulp osteoid tissue. This implies that osteoblast-like cells came from MSCs that migrated from the recipients.[9] Further studies are required to verify the origin of osteoid tissue in the dental pulp after trauma.

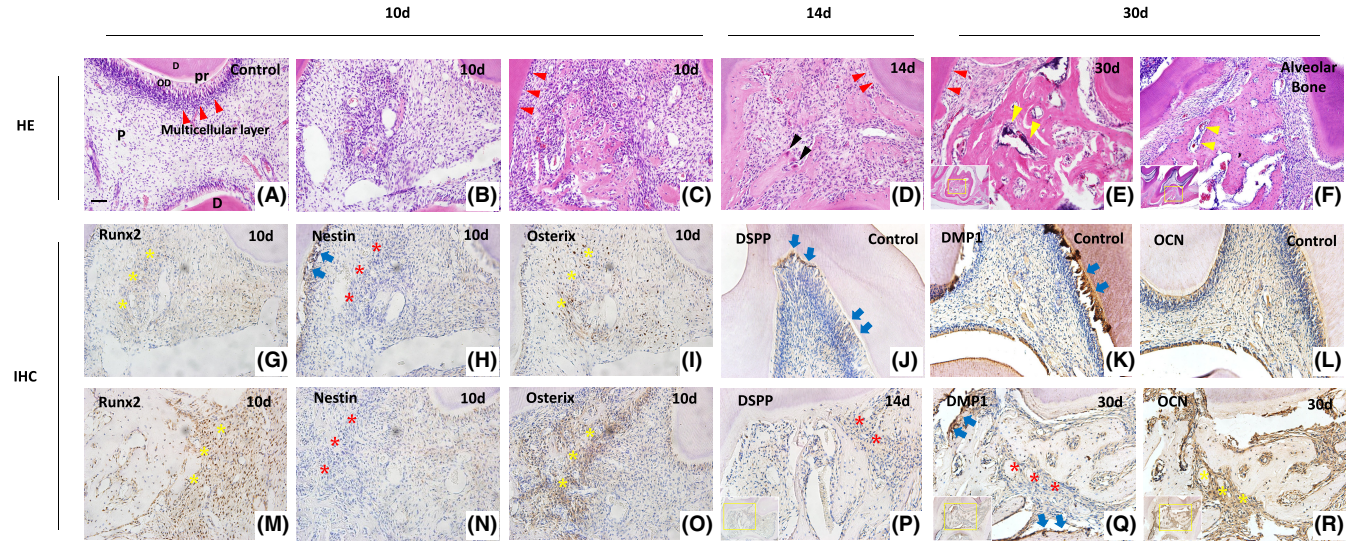

FIGURE 3 Coronal pulp mineralisation after dental trauma. The coronal pulp tissue of control rat molars consisted of the dentin, pre-dentin, odontoblast layer, multicellular layer (red arrow), and pulp core with fibrotic connective tissues and equally distributed cells (A). Beginning at 10 days after trauma, cell proliferation (B), followed by osteoid mineralisation (C–E) appeared around the vascularisation area, wherein osteoclasts were observed (black arrows). The osteoid tissue in the pulp cavity at 30 days after trauma (E) was similar to the normal alveolar bone at the furcation of teeth (F), which had bone marrow structures (yellow arrows). The multicellular layer and development of pre-dentin were interfered (C–E, red arrow) in teeth with central osteoid tissue formation. In the cell proliferation region (G–I) and premature osteogenic site (M–O), the osteoblast-related proteins Runx2 (G, M) and OSX (I, O) were expressed rather than the odontoblastic-related protein Nestin (H, N). In mature traumatic pulp mineralised sites, OCN was highly expressed in surrounding cells (R), while in control teeth it was weakly expressed in odontoblasts (L). Compared with the strong expression in odontoblasts closely related to dentin formation (J, K, Q, blue arrow), DSPP and DMP1 were not shown in cells around pulp osteoid mineralisation (P, Q). Scale bar: 50 μm. D, dentin; OD, odontoblast layer; pr, pre-dentin; P, pulp core.

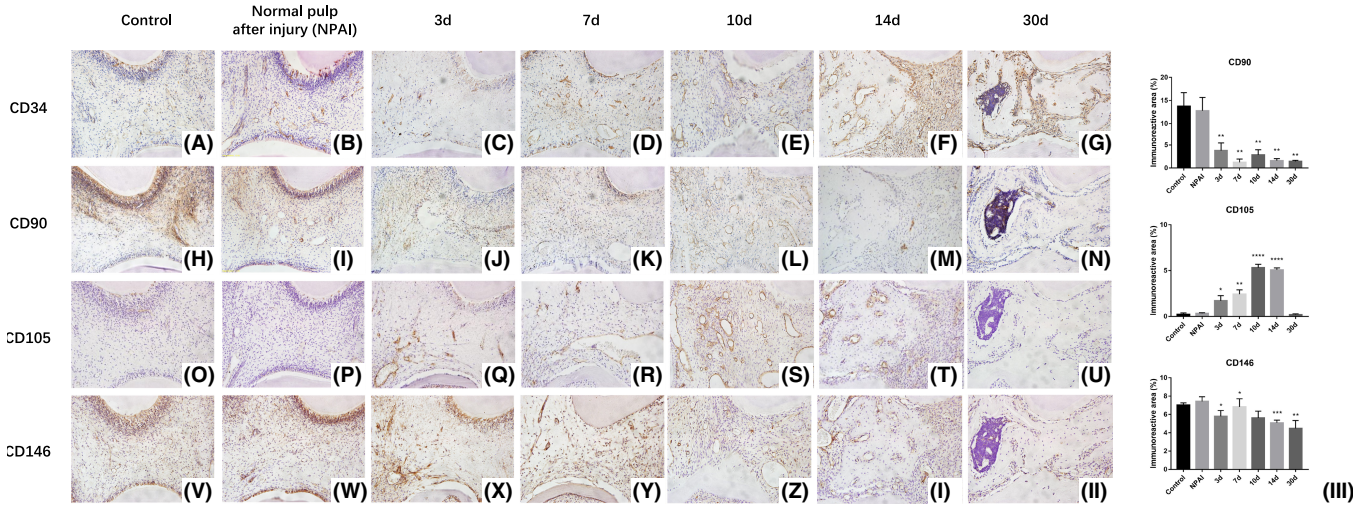

FIGURE 4 Localisation of mesenchymal stem cell subtypes in normal and traumatic dental pulp. (A–G) CD34 localised in neovascular endothelial cells around the neovascularisation area in traumatic dental pulp compared with that in the control molar. (H–N) CD90-immunoreactive cells were observed in the sub-odontoblastic multicellular layer and perivascular region of control coronal pulp (H) and in normal pulp after injury (I). In the abnormal pulp of intrusive molars at 3–30 days after dental trauma, CD90 expression was decreased in the sub-odontoblastic layers and disappeared around osteoid tissue (O–U), CD105 localised in cells of pulp with atrophy and osteoid mineralisation of injured teeth at 3–14 days after dental trauma (Q–T). In control teeth and in normal pulp after injury, CD105 was only expressed in the vascular endothelial cells of capillaries in the odontoblastic or sub-odontoblastic layers (O, P). (V–Z, I–II) CD146 located in the odontoblastic layer in control pulp and normal pulp after injury. In abnormal traumatic pulp, CD146 was expressed in the vascular endothelial cells and continued until 30 days after dental trauma. (III) Semi-quantitative analysis of CD90, CD105, and CD146 immunohistochemistry. Scale bar: 50 μm. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

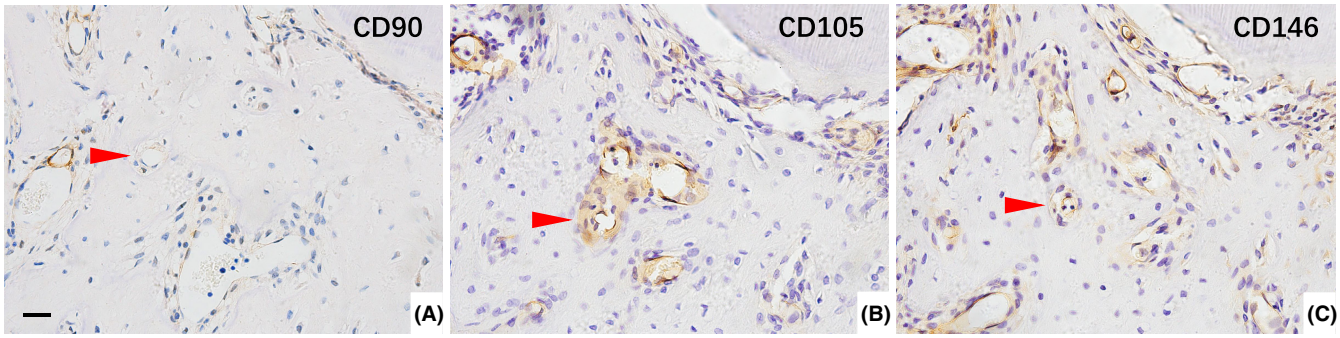

FIGURE 5 Cell morphology and localisation of CD90, CD105 and CD146 in mineralised pulp at higher magnification. (A) No signs of CD90 were detected around neovascularisation and osteoid tissue. (B) CD105 located in cells outside the vascular endothelial cells around neovascularisation at intra-pulp osteogenic region. (C) CD146 was expressed in the intra-pulp vascular endothelial cells. Scale bar: 20 μm.

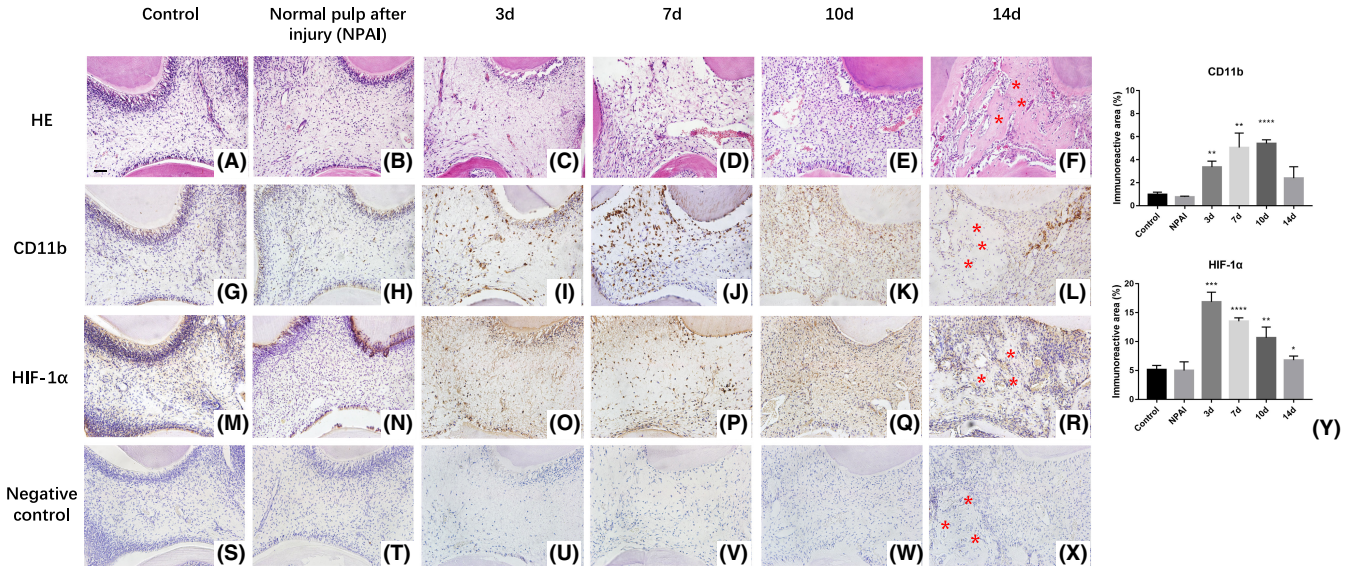

FIGURE 6 Evaluation of the coronal pulp microenvironment after intrusive luxation. Normal pulp consists of the dentin, pre-dentin, odontoblast layer, multicellular layer, and pulp core (A, B), and there was no expression of the inflammatory cell marker CD11b (G). HIF-1α was primarily expressed in the odontoblast layer rather than at the centre of control pulp (M). CD11b and HIF-1α expression in normal pulp after injury (H, N) were the same as that in the control. In contrast, in the coronal pulp of abnormal traumatic molars, the multicellular layer disappeared, and vacuolar degeneration of odontoblasts or fibroblasts occurred at 3–10 days after intrusion (C–E) along with increases in CD11b-positive inflammatory cells (I–K) and HIF-1α expression (O–Q). At 14 days after trauma, osteoid tissue (red asterisk) was observed in coronal pulp (F), and CD11b and HIF-1α expression were decreased in the mineralised region (L and R, red asterisk). (S–X) Negative control of specimens probed only with secondary antibody. (Y) Semi-quantitative analysis of CD11b and HIF-1α immunohistochemistry. Scale bar: 50 μm. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

This study preliminarily detected the changes in stem cell markers in dental pulp tissue and found that CD90-immunoreactive cells were distributed in the sub-odontoblastic layer, which was close to the odontoblasts and pre-dentin, but were not related to post-traumatic dental pulp osteogenesis, suggesting that they might be related to dentin development. Other studies have also shown that CD90-immunoreactive cells are related to reparative dentin formation in molars and continuous dentin development in mouse incisors.[30–32] Although CD146 immunoreacted with cells around the osteoid tissue, it was highly expressed in the vascular endothelial cells and kept stable until the process of osteogenesis finished. This finding indicated that CD146-positive cells might have differentiated into vascular endothelial cells in the traumatised pulps, but not into osteoblasts. CD105 was not found in the sub-odontoblastic layer close to the pre-dentin in the control pulps, while it was activated in cells outside the vascular endothelial cells around the neovascularisation after trauma and disappeared when osteogenesis stopped, suggesting that they might be related to perivascular pulp osteogenesis. However, whether CD105-positive cells are derived from pericytes, MSCs or vascular endothelial cells need further confirmation.

It has been reported that MSC subpopulations with different combinations of surface markers exhibit different tendencies towards osteo/odontogenic, adipogenic, and neurogenic commitment,[33,34] but the specific stem cell subtype most closely related to odontoblast differentiation is unknown. Distinguishing stem cell subsets and detecting their functions is another potential research

direction. Alvarez et al. found that CD271 was the most useful surface marker for identifying dental MSCs with high odontogenic potential.[33] Gli1$^+$ cells around the artery of the neurovascular bundle can proliferate and differentiate into dental pulp cells and odontoblasts in the incisors of mice, and their offspring cells nearly fill the dental pulp within 4 weeks.[35] These markers could be tracked in this rat model of dental intrusion to verify their impact on MSCs and explore the post-injury activities of MSCs. This would provide a theoretical basis for determining the mechanisms of dentin development and post-traumatic pulp repair.

CD11b-positive inflammatory cells infiltrated the traumatised dental pulp. CD11b is a marker of myeloid-derived cells, including monocytes, macrophages, dendritic cells, neutrophils, and lymphocytes. The specific inflammatory cells involved in dental pulp recovery require further investigation. Rungvechvuttivittaya et al. reported that dendritic cells concentrated in the normal pulp tissue and showed a marked accumulation along the pulp-dentine border.[10] While, in replanted avulsed teeth, the macrophages, TRAP-positive and CK-positive cells (typical markers of osteoclasts) were more active and contacted with MSCs around intra-pulp osteoid mineralisation.[8] This suggests that immune microenvironment may have effects on the differentiation of MSCs, providing a valuable research direction for pulp regeneration.

Trauma always induces ischaemia, leading to local hypoxia caused by blood deficiency. HIF-1α is a hypoxia-related protein. In experimental molars, pulp mineralisation developed in the coronal pulp with expansive vascularisation, and expression of CD34 and HIF-1α increased, indicating hypoxia. Hypoxia and HIF-1α can promote osteo/odontoblast differentiation of human DPSCs in vitro,[36] which is partially dependent on Wnt/β-catenin signalling, and B-cell CLL/lymphoma 9 (BCL9) is a key mediator.[16] It was suggested that enhanced angiogenic potential through VEGF-A and Ang-2 released from hDPSCs under hypoxia supported vascularised osteogenesis in the pulp.[14,15]

Differentiation of hDPSCs into osteo/odontoblasts in vitro under different hypoxic conditions (1%–5%) resulted in increased expression of osteogenesis- and odontogenesis-related proteins, such as BSP, OCN, DSPP, and DMP1.[36] In contrast, in this study, expression of only the osteogenesis-related proteins (Runx2, OSX, and OCN) was increased in the region of pulp mineralisation and the odontogenesis-related proteins (Nestin, DSPP, and DMP1) were not detected. This suggests that hypoxia promotes mineralisation, and MSC differentiation, that is, osteogenesis or odontogenesis, might be regulated by other factors. Pasiewicz et al. reported that the complement fragment C5a modulated the odontogenic differentiation of hDPSCs under normoxic conditions. However, a low oxygen tension microenvironment could reverse its differentiation effect.[37] Understanding microenvironmental control of stem cell differentiation is essential for establishing successful dentin repair strategies in injured pulps. C5a and hypoxia and the interaction between the immune system and oxygen levels might be key initial signals controlling odontogenic differentiation of DPSCs. Therefore, reversing the hypoxic environment in the pulp might be a solution to promote normal dentin development after trauma. For instance, oxygen-carrying biomaterials for pulp capping or hyperbaric oxygen therapy using a periodontal approach may be potential treatment options. However, before applying these treatment options, the hypoxic status of the dental pulp must be confirmed. This can be achieved via Laser Doppler Flowmetry to detect blood oxygen saturation.[38–40]

This study used a previously established rat model of intrusive luxation. The advantage of this model is that the force and direction of trauma are consistent and repeatable. No crown fracture occurred during the experiment, indicating no bacterial infection, thus providing a good model to investigate the host response to trauma. However, this model has some limitations. In clinical practice, trauma often occurs to the incisors. In this study, rat molars were used, as rat incisors continue to grow throughout their lives, thereby differing from human incisors. Whether pulp healing of molars is equivalent to that of incisors needs to be confirmed. Additionally, because of the thin bone plate in the molar area of rats, traumatic intrusion of molar is often accompanied by alveolar bone fracture. This does not always occur in incisor's intrusion and instead the tooth becomes wedged into the alveolar bone. Although pulp osteoid mineralisation has substantial research value, periodontal tissue complications in this animal model, such as external replacement resorption, do not occur frequently. Therefore, research on periodontal tissue healing cannot be conducted. Other models with controllable and standardised intrusive luxation using more accurate forces are needed.

5 | CONCLUSION

After intrusive luxation of immature teeth without crown fractures in rats, no pulp necrosis was observed. However, pulp atrophy and osteogenesis occurred in the coronal pulp. The number of CD90-immunoreactive cells in the sub-odontoblastic layer decreased, whereas CD105-immunoreactive cells appeared around the intra-pulp mineralisation during healing after trauma in the pulp microenvironment, which is characterised by hypoxia and inflammation.

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AUTHOR CONTRIBUTIONS

Nan Wang contributed to the animal model generation, design of the study, experiment conducting, collection and analysis of data, manuscript preparation, and editing. **Yike Gao and Huihui Ren** contributed to the experiment operation and data analysis. **Linhai He** contributed to the design of the study and data analysis. **Yuming Zhao** contributed to the design of the study and critical manuscript revision. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors deny any conflicts of interest related to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Andreasen J, Andreasen F, Tsilingaridis G. Intrusive luxation. In: Andreasen JO, Andreasen FM, Andreasen L, editors. Textbook and color atlas of traumatic injuries to the teeth. Volume 6. 5th ed. Copenhagen: Munksgaard; 2019. p. 469–85.
2. Wigen TI, Agnalt R, Jacobsen I. Intrusive luxation of permanent incisors in Norwegians aged 6–17 years: a retrospective study of treatment and outcome. Dent Traumatol. 2008;24:612–8.
3. Tsilingaridis G, Malmgren B, Andreasen JO, Wigen TI, Maseng Aas AL, Malmgren O. Scandinavian multicenter study on the treatment of 168 patients with 230 intruded permanent teeth-a retrospective cohort study. Dent Traumatol. 2016;32:353–60.
4. Carvalho TS, Lussi A. Age-related morphological, histological and functional changes in teeth. J Oral Rehabil. 2017;44:291–8.
5. Palatyńska-Ulatowska A, Fernandes MC, Pietrzycka K, Koprowicz A, Klimek L, Souza RA, et al. The pulp stones: morphological analysis in scanning electron microscopy and spectroscopic chemical quantification. Medicina (Kaunas). 2022;58:5.
6. Andreasen JO. Pulp and periodontal tissue repair-regeneration or tissue metaplasia after dental trauma. Dent Traumatol. 2012;28:19–24.
7. Heling I, Slutzky-Goldberg I, Lustmann J, Ehrlich Y, Becker A. Bone-like tissue growth in the root canal of immature permanent teeth after traumatic injuries. Endod Dent Traumatol. 2000;16:298–303.
8. Tsukamoto-Tanaka H, Ikegame M, Takagi R, Harada H, Ohshima H. Histochemical and immunocytochemical study of hard tissue formation in dental pulp during the healing process in rat molars after tooth replantation. Cell Tissue Res. 2006;325:219–29.
9. Zhao C, Hosoya A, Kurita H, Hu T, Hiraga T, Ninomiya T, et al. Immunohistochemical study of hard tissue formation in the rat pulp cavity after tooth replantation. Arch Oral Biol. 2007;52:945–53.
10. Rungvechvuttivittaya S, Okiji T, Suda H. Responses of macrophage-associated antigen-expressing cells in the dental pulp of rat molars to experimental tooth replantation. Arch Oral Biol. 1998;43:701–10.
11. Grando Mattuella L, Westphalen Bento L, de Figueiredo JA, Nör JE, de Araujo FB, Fossati AC. Vascular endothelial growth factor and its relationship with the dental pulp. J Endod. 2007;33:524–30.
12. Mullane EM, Dong Z, Sedgley CM, Hu JCC, Botero TM, Holland GR, et al. Effects of VEGF and FGF2 on the revascularization of severed dental pulps. J Dent Res. 2008;87:1144–8.
13. Zhang R, Cooper PR, Smith G, Nör JE, Smith AJ. Angiogenic activity of dentin matrix components. J Endod. 2011;37:26–30.
14. Aranha AM, Zhang Z, Neiva KG, Costa CA, Hebling J, Nör JE. Hypoxia enhances the angiogenic potential of human dental pulp cells. J Endod. 2010;36:1633–7.
15. Fujio M, Xing Z, Sharabi N, Xue Y, Yamamoto A, Hibi H, et al. Conditioned media from hypoxic-cultured human dental pulp cells promotes bone healing during distraction osteogenesis. J Tissue Eng Regen Med. 2017;11:2116–26.
16. Orikasa S, Kawashima N, Tazawa K, Hashimoto K, Sunada-Nara K, Noda S, et al. Hypoxia-inducible factor 1α induces osteo/odontoblast differentiation of human dental pulp stem cells via Wnt/β-catenin transcriptional cofactor BCL9. Sci Rep. 2022;12:682.
17. Cooper PR, Holder MJ, Smith AJ. Inflammation and regeneration in the dentin-pulp complex: a double-edged sword. J Endod. 2014;40:S46–51.
18. Cooper PR, Takahashi Y, Graham LW, Simon S, Imazato S, Smith AJ. Inflammation-regeneration interplay in the dentine-pulp complex. J Dent. 2010;38:687–97.
19. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Proc Natl Acad Sci USA. 2000;97:13625–30.
20. Krivanek J, Soldatov RA, Kastriti ME, Chontorotzea T, Herdina AN, Petersen J, et al. Dental cell type atlas reveals stem and differentiated cell types in mouse and human teeth. Nat Commun. 2020;11:4816.
21. Cui Y, Ji W, Gao Y, Xiao Y, Liu H, Chen Z. Single-cell characterization of monolayer cultured human dental pulp stem cells with enhanced differentiation capacity. Int J Oral Sci. 2021;13:44.
22. Pagella P, de Vargas RL, Stadlinger B, Moor AE, Mitsiadis TA. A single-cell atlas of human teeth. iScience. 2021;24:102405.
23. Yin W, Liu G, Li J, Bian Z. Landscape of cell communication in human dental pulp. Small Methods. 2021;5:2100747.
24. Ren H, Wen Q, Zhao Q, Wang N, Zhao Y. Atlas of human dental pulp cells at multiple spatial and temporal levels based on single-cell sequencing analysis. Front Physiol. 2022;13:993478.
25. Wang N, Lei S, Luo R, Chen J, Han J, Zhao Y. Radiographic and histologic evaluation of experimentally induced severe intrusive luxation of immature teeth in rats. J Endod. 2020;46:1631–8.
26. Goto N, Fujimoto K, Fujii S, Ida-Yonemochi H, Ohshima H, Kawamoto T, et al. Role of MSX1 in osteogenic differentiation of human dental pulp stem cells. Stem Cells Int. 2016;2016:8035759.
27. Tabatabaei FS, Torshabi M. *In vitro* proliferation and osteogenic differentiation of endometrial stem cells and dental pulp stem cells. Cell Tissue Bank. 2017;18:239–47.
28. Mortada I, Mortada R. Dental pulp stem cells and osteogenesis: an update. Cytotechnology. 2018;70:1479–86.
29. Yoshida S, Tomokiyo A, Hasegawa D, Hamano S, Sugii H, Maeda H. Insight into the role of dental pulp stem cells in regenerative therapy. Biology (Basel). 2020;9:160.
30. Hosoya A, Hiraga T, Ninomiya T, Yukita A, Yoshiba K, Yoshiba N, et al. Thy-1-positive cells in the subodontoblastic layer possess high potential to differentiate into hard tissue-forming cells. Histochem Cell Biol. 2012;137:733–42.
31. An Z, Sabalic M, Bloomquist RF, Fowler TE, Streelman T, Sharpe PT. A quiescent cell population replenishes mesenchymal stem cells to drive accelerated growth in mouse incisors. Nat Commun. 2018;9:378.
32. Sano Y, Sugiuchi A, Mitomo K, Yanagisawa A, Kambe R, Furusawa M, et al. Changes of CD90 expression and immunoreactive cell localization in rat dental pulp after cavity preparation. Aust Endod J. 2019;45:189–95.
33. Alvarez R, Lee HL, Hong C, Wang CY. Single CD271 marker isolates mesenchymal stem cells from human dental pulp. Int J Oral Sci. 2015;7:205–12.
34. Pisciotta A, Carnevale G, Meloni S, Riccio M, de Biasi S, Gibellini L, et al. Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations. BMC Dev Biol. 2015;15:14.
35. Zhao H, Feng J, Seidei K, Shi S, Klein O, Sharpe P, et al. Secretion of shh by a neurovascular bundle niche supports mesenchymal stem cell homeostasis in the adult mouse incisor. Cell Stem Cell. 2014;14:160–73.
36. Li L, Zhu YQ, Jiang L, Peng W, Ritchie HH. Hypoxia promotes mineralization of human dental pulp cells. J Endod. 2011;37:799–802.
37. Pasiewicz R, Valverde Y, Narayanan R, Kim JH, Irfan M, Lee NS, et al. C5a complement receptor modulates odontogenic dental

pulp stem cell differentiation under hypoxia. Connect Tissue Res. 2021;24:1–10.
38. Ma KF, Kleiss SF, Schuurmann RCL, Nijboer TS, el Moumni M, Bokkers RPH, et al. Laser doppler flowmetry combined with spectroscopy to determine peripheral tissue perfusion and oxygen saturation: a pilot study in healthy volunteers and patients with peripheral arterial disease. J Pers Med. 2022;12:853.
39. Komaki S, Ozaki H, Takahashi SS, Wada-Takahashi S, Fushima K. Gingival blood flow before, during, and after clenching, measured by laser Doppler blood flowmeter: a pilot study. Am J Orthod Dentofacial Orthop. 2022;161:46–52.
40. Igna A, Igna C, Miron MI, Schuszler L, Dascălu R, Moldovan M, et al. Assessment of pulpal status in primary teeth following direct pulp capping in an experimental canine model. Diagnostics (Basel). 2022;12:2022.

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