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Effect of Heparan Sulfate on Vasculogenesis and Dentinogenesis of Dental Pulp Stem Cells

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ABSTRACT

Introduction: Heparan sulfate (HS) is a major component of dental pulp tissue. We previously reported that inhibiting HS biosynthesis impedes endothelial differentiation of dental pulp stem cells (DPSCs). However, the underlying mechanisms by which exogenous HS induces DPSC differentiation and pulp tissue regeneration remain unknown. This study explores the impact of exogenous HS on vasculogenesis and dentinogenesis of DPSCs both *in vitro* and *in vivo*. **Methods:** Human-derived DPSCs were cultured in endothelial and odontogenic differentiation media and treated with HS. Endothelial differentiation of DPSCs was investigated by real-time polymerase chain reaction and capillary sprouting assay. Odontogenic differentiation was assessed through real-time polymerase chain reaction and detection of mineralized dentin-like deposition. Additionally, the influence of HS on pulp tissue was assessed with a direct pulp capping model, in which HS was delivered to exposed pulp tissue in rats. Gelatin sponges were loaded with either phosphate-buffered saline or 10^1 – 10^2 $\mu\text{g/mL}$ HS and placed onto the pulp tissue. Following a 28-day period, tissues were investigated by histological analysis and micro-computed tomography imaging. **Results:** HS treatment markedly increased expression levels of key endothelial and odontogenic genes, enhanced the formation of capillary-like structures, and promoted the deposition of mineralized matrices. Treatment of exposed pulp tissue with HS in the *in vivo* pulp capping study induced formation of capillaries and reparative dentin. **Conclusions:** Exogenous HS effectively promoted vasculogenesis and dentinogenesis of DPSCs *in vitro* and induced reparative dentin formation *in vivo*, highlighting its therapeutic potential for pulp capping treatment. (*J Endod* 2024; ■:1–9.)

KEY WORDS

Dental pulp stem cells; dentinogenesis; heparan sulfate; pulp capping; vasculogenesis

Dental pulp is known for its capacity for tissue repair and regeneration in response to external stimuli, due to its rich diversity of stem and progenitor cells¹. Among these cell types, dental pulp stem cells (DPSCs) have a particular capacity for self-renewal and multipotent differentiation². When the dental pulp is inadvertently exposed because of injury or disease, DPSCs adjacent to the injury site differentiate into odontoblasts, which produce the reparative dentin that acts as a physical protective barrier^{3,4}. Moreover, DPSCs can differentiate into vascular endothelial lineages, forming blood capillaries that enable nutrient delivery to the injury site⁵. Therefore, an appealing strategy in the development of novel pulp capping therapies is the biomimetic approach, in which damaged pulp is treated with bioactive materials that induce DPSC differentiation and promote a natural healing cascade.

The extracellular matrix (ECM) of the dentin-pulp complex is an intricate composition of proteoglycans, growth factors, and cytokines^{6–8}. Studies have shown that the ECM is pivotal in regulating the dentinogenic process and the development of fibrous framework for newly developed dentin^{9,10}. Additionally, ECM remodeling during vasculogenesis is critical for triggering stem cell differentiation, leading to basement membrane formation, pericyte recruitment, and ultimately, the formation of nascent

SIGNIFICANCE

Exogenous heparan sulfate (HS) effectively promoted vasculogenesis and dentinogenesis of dental pulp stem cells *in vitro*, and facilitated capillary and reparative dentin formation *in vivo*. HS shows promise as a medical agent for pulp capping treatment.

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capillary^{11,12}. Thus, the bioactive and biocompatible components of pulp tissue ECM present promising opportunities and novel approaches for vital pulp therapy. Heparan sulfate (HS), a prevalent ECM component in mammalian tissues^{13,14}, is of particular interest in this context. The HS epitope 10E4, for example, is ubiquitous in the tooth germ during embryogenic development¹⁵. Although expression of HS in odontoblasts and ameloblasts diminishes after birth, it is highly abundant in the dental pulp region, underscoring its regulatory role in dental pulp dynamics¹⁵. Our prior research indicated that silencing of HS-specific glycosyltransferase *exostosin 1* hinders endothelial differentiation of DPSCs *in vitro*¹⁶. Furthermore, *exostosin 1* knockdown impaired vasculogenic functions of DPSCs¹⁶. Earlier studies using endosulfatases Sulf1 and Sulf2 mutant mice demonstrated that the sulfation and desulfation processes of HS are intricately involved in the process of dentinogenesis^{15,17}. Although reduced HS levels in dental pulp are known to hinder vasculogenesis and dentinogenesis in dental pulp tissues, the specific role of exogenous HS in DPSCs and pulp tissues remains unclear. Therefore, the purpose of this study was to evaluate the influence of exogenous HS on the vasculogenesis and dentinogenesis of DPSCs *in vitro*, as well as its impact on vascular and reparative dentin formation in dental pulp tissue using an *in vivo* direct pulp capping model.

MATERIALS AND METHODS

Cell Culture

Human-derived DPSCs isolated from adult third molars were purchased from Lonza Inc. (Basel, Switzerland). DPSCs at passages 3–6 were cultured in growth medium comprising Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 20% fetal bovine serum (Thermo Fisher Scientific, MA, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). To induce endothelial differentiation, DPSCs were cultured in an endothelial differentiation medium (EM) consisting of EGM2-MV (Lonza) supplemented with 50 ng/mL rhVEGF₁₆₅ (R&D Systems, MN, USA)¹⁸. For odontogenic differentiation, DPSCs were maintained in odontogenic differentiation medium comprising growth medium supplemented with dexamethasone (1×10^{-6} mol/L; Sigma-Aldrich), β -glycerophosphate disodium salt hydrate (1×10^{-2} mol/L; Sigma-Aldrich), ascorbic acid (50 μ g/mL; Sigma-Aldrich), and calcium chloride (1×10^{-2} mol/L; Wako)¹⁹.

DPSCs were incubated in a humidified incubator at 37°C with 5% CO₂.

Impact of HS on DPSC Proliferation

DPSCs were seeded at a density of 500 cells/well in a 96-well plate and cultured with EM containing the following HS concentrations: 0, 10⁻², 10⁻¹, 10⁰, 10¹, and 10² μ g/mL (MedChemExpress, NJ, USA). After 1, 3, and 5 days of culture, 10 μ L of WST-8 solution (Dojindo, Tokyo, Japan) was added to each well, and the plates were incubated for an additional 2 hours. The absorbance of each well at 450 nm was measured using the iMark microplate reader (Bio-Rad, CA). The cell morphology under different treatments was observed by light microscopy (CK40-F100; Olympus, Tokyo, Japan).

Impact of HS on the Endothelial Differentiation of DPSCs

To evaluate the expression of endothelial differentiation markers, DPSCs at a density of 500 cells/cm² were seeded and maintained in EM containing different concentrations of HS (0–10² μ g/mL). After 7 days of culture, mRNA expression of vascular endothelial growth factor A (VEGFA), C-X-C motif chemokine ligand 1 (CXCL1), Nanog and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was quantified with a StepOne Real-time Polymerase Chain Reaction System (Thermo Fisher Scientific). The expression levels of differentiation markers relative to the housekeeping gene *GAPDH* were calculated using the 2^{- $\Delta\Delta$ Ct} method.

The endothelial differentiation of DPSCs was also investigated with a capillary sprouting assay. After polymerization of 300 μ L Growth Factor-Reduced Matrigel (Corning, NY, USA) to 24-well plates, 1×10^4 DPSCs were seeded onto Matrigel-precoated plates and maintained in EM in the absence or presence of HS at 10¹ or 10² μ g/mL. Reticular-like structures formed by DPSCs were observed by light microscopy (CK40-F100) over a 14-day period. The numbers of sprouting branches and total lengths were measured by ImageJ software (National Institute of Health, MD).

Impact of HS on the Odontogenic Differentiation of DPSCs

Dentin-like mineralized depositions were visualized by von Kossa staining. Ten thousand DPSCs were seeded into a 24-well culture plate and maintained in odontogenic differentiation medium with or without HS at concentrations 0, 10⁰, 10¹, and 10² μ g/mL. After incubation for 21 and 28 days, plates were stained with a 5% silver nitrate solution

(Sigma-Aldrich) for 5 min under ultraviolet light irradiation. Stained specimens were observed by light microscopy (ECLIPSE Ci-L; Nikon, Tokyo, Japan) and a digital camera (D5500; Nikon). Mineralized nodule formation was semiquantified by ImageJ software.

Odontogenic differentiation was also investigated using real-time polymerase chain reaction. The mRNA expression levels of dentin matrix acidic phosphoprotein 1 (DMP1), dentin sialophosphoprotein (DSPP), and *GAPDH* were evaluated after 14 days of odontogenic induction.

Direct Pulp Capping *In Vivo* Model

All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry (Approval No. R-02-010-0).

Nine 8-week-old male Wistar rats (CLEA Japan, Tokyo, Japan) were anesthetized, and dental pulp was exposed from the mesial fossa of both maxillary first molars using a 0.5 mm diameter steel bur. The injury site was directly capped with a gelatin sponge (GC, Tokyo, Japan) loaded with the following aqueous solutions: phosphate-buffered saline (PBS) (Gibco, NY, USA) or HS (10¹ and 10² μ g/mL). Six teeth were used in each group. Subsequently, the cavity was sealed with glass ionomer cement (Fuji IX; GC). On postoperative day 28, maxillae were harvested and scanned by micro-computed tomography (R-mCT2; Rigaku, Tokyo, Japan) with a 5 \times 5 mm scan field of view and 10- μ m resolution.

Histological Evaluation

Harvested maxillae were fixed with 4% paraformaldehyde, followed by decalcification with Kalkitox solution (Wako) for 3 days at 4°C.

For hematoxylin and eosin staining, 5- μ m thick paraffin sections were rehydrated and stained with hematoxylin (Wako) for 5 min, followed by staining with 0.5% eosin Y (Wako) for 5 min. Stained sections were observed by light microscopy (ECLIPSE Ci-L).

Immunofluorescence staining was conducted to evaluate the localization of capillaries. Tissue sections were deparaffinized and incubated with mouse monoclonal antibody against rat CD31 (Thermo Fisher Scientific) and rabbit monoclonal antibody against rat von Willebrand Factor (vWF) (Abcam, Cambridge, UK) overnight at 4°C. These antibodies were visualized using Alexa Fluor 488 goat antimouse immunoglobulin G (Thermo Fisher Scientific) and Alexa Fluor 594 goat antirabbit IgG (Thermo Fisher Scientific), respectively. Cell nuclei were stained with

Hoechst 33342 (Thermo Fisher Scientific). The stained sections were observed by fluorescence microscopy (TE2000; Nikon).

Statistical Analysis

One-way analysis of variance with Tukey's honestly significant difference test was used for comparisons of more than two groups. Student's *t*-test was used for comparisons of two groups. $P < .05$ was considered significantly different.

RESULTS

Impact of HS on DPSC Proliferation

Microscopic examination of DPSCs treated with various concentrations of HS is illustrated in Figure 1A. No differences in cell morphology were observed throughout the culture period. The cell proliferation showed no significant difference among the experimental conditions at day 1, thereafter cell numbers gradually increased with the increasing dosage of HS, which reached to plateau at the concentration of $10^0 \mu\text{g/mL}$ (Fig. 1B). Notably, higher

concentrations (10^1 and $10^2 \mu\text{g/mL}$ of HS) suppressed cell proliferation rates, which was evident after 5 days of endothelial induction.

Impact of HS on Endothelial Differentiation of DPSCs

Expression of endothelial markers VEGFA and CXCL1 was found to increase progressively with higher HS concentrations (10^1 and $10^2 \mu\text{g/mL}$) compared with the control group without HS (Fig. 1C). However, VEGFA expression did not differ significantly up to

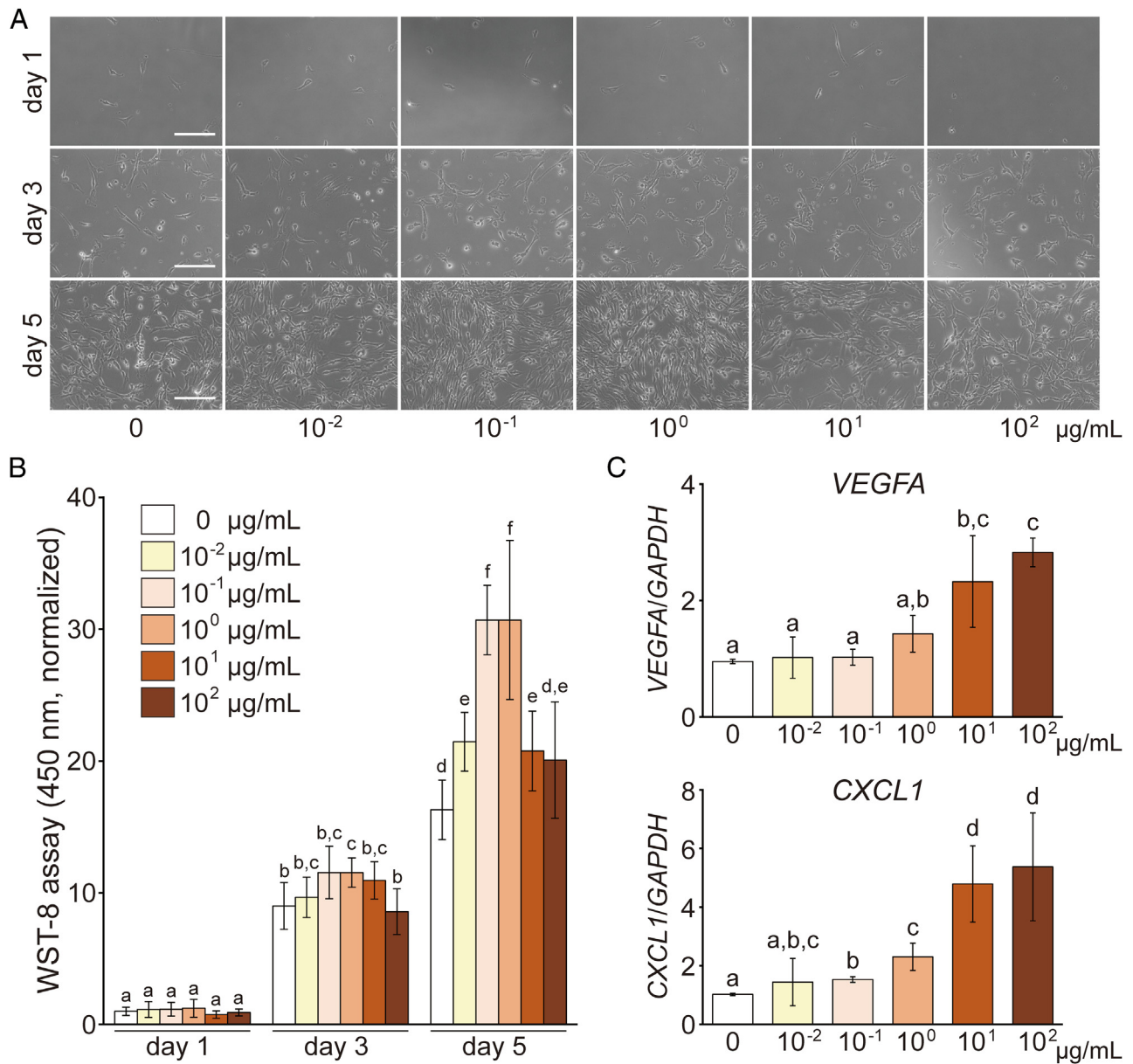


FIGURE 1 – The effect of HS on proliferation and endothelial differentiation of DPSCs. (A) Photomicrographs of DPSCs cultured in EM containing different concentrations of HS for up to 5 days. Scale bars: 200 μm (B) Quantification of relative cell numbers (WST-8 assay) at corresponding time points. (C) mRNA expression of VEGFA and CXCL1 after 7 days in EM containing various concentrations of HS. *GAPDH* served as the reference gene. Different letters indicate significant differences among groups ($P < .05$). Mean \pm SD; $n = 4$. HS, heparan sulfate; DPSC, dental pulp stem cell; EM, endothelial differentiation medium; VEGFA, vascular endothelial growth factor A; CXCL1, C-X-C motif chemokine ligand 1; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

10^0 $\mu\text{g/mL}$ of HS and CXCL1 expression remained consistent at 10^{-2} $\mu\text{g/mL}$ compared with the control.

In the capillary sprouting assay, DPSCs exhibited sprouting as early as 3 days into

endothelial induction, forming capillary-like structures over a period of 14 days, regardless of HS treatment (Fig. 2A). There was no significant divergence in the number of branches and total sprouting length during the

initial 3 days (Fig. 2B). However, by day 7, DPSCs treated with HS demonstrated a greater total length of sprouts compared with the untreated group. After 14 days, both the number and total length of branches in HS-stimulated DPSCs surpassed those of the control group, even though no difference in sprouting formation was detected between the groups treated with the low and higher concentrations of exogenous HS (Fig. 2B).

Impact of HS on Odontogenic Differentiation of DPSCs

An upward trend was observed in the expression of odontogenic markers correlated with increasing HS concentrations, although no significant changes in DMP1 expression were observed among the groups (Fig. 3A). DSPP expression, however, significantly increased with HS concentrations by day 7 (Fig. 3A).

The result of von Kossa staining revealed a few mineralized nodules formed in DPSCs without HS by 21 days of odontogenic induction. In contrast, exogenous HS treatment resulted in the precipitation of mineralized matrices (Fig. 3B). By day 28, mineral deposition had increased among all experimental groups (Fig. 3C). Semiquantitative analysis demonstrated that 10^1 and 10^2 $\mu\text{g/mL}$ of exogenous HS led to significant increase of mineralized area compared with that without HS at all experimental times (Fig. 3D).

Impact of HS on Dental Pulp Tissue *In Vivo*

Treatment with a PBS-loaded gelatin sponge resulted in incomplete tissue formation beneath the capping area (Fig. 4A1–A3). Notably, application of HS at both 10^1 and 10^2 $\mu\text{g/mL}$ led to the formation of a continuous reparative dentin bridge above the exposed dental pulp, with a densely packed odontoblast-like layer underneath the newly formed dentin bridge (Fig. 4B1–B3 and Fig. 4C1–C3).

Immunofluorescence staining indicated faint expression of CD31 and vWF in PBS-treated group (Fig. 4A4–A7). In contrast, blood capillaries beneath the reparative dentin layer were observed in groups treated with 10^1 and 10^2 $\mu\text{g/mL}$ HS (Fig. 4B4–B7 and Fig. 4C4–C7). In HS-treated specimens, the expression of these vascular markers was observed around the newly formed dentin bridge and odontoblast layer.

DISCUSSION

The dentin-pulp complex contains a wide variety of bioactive molecules and has been

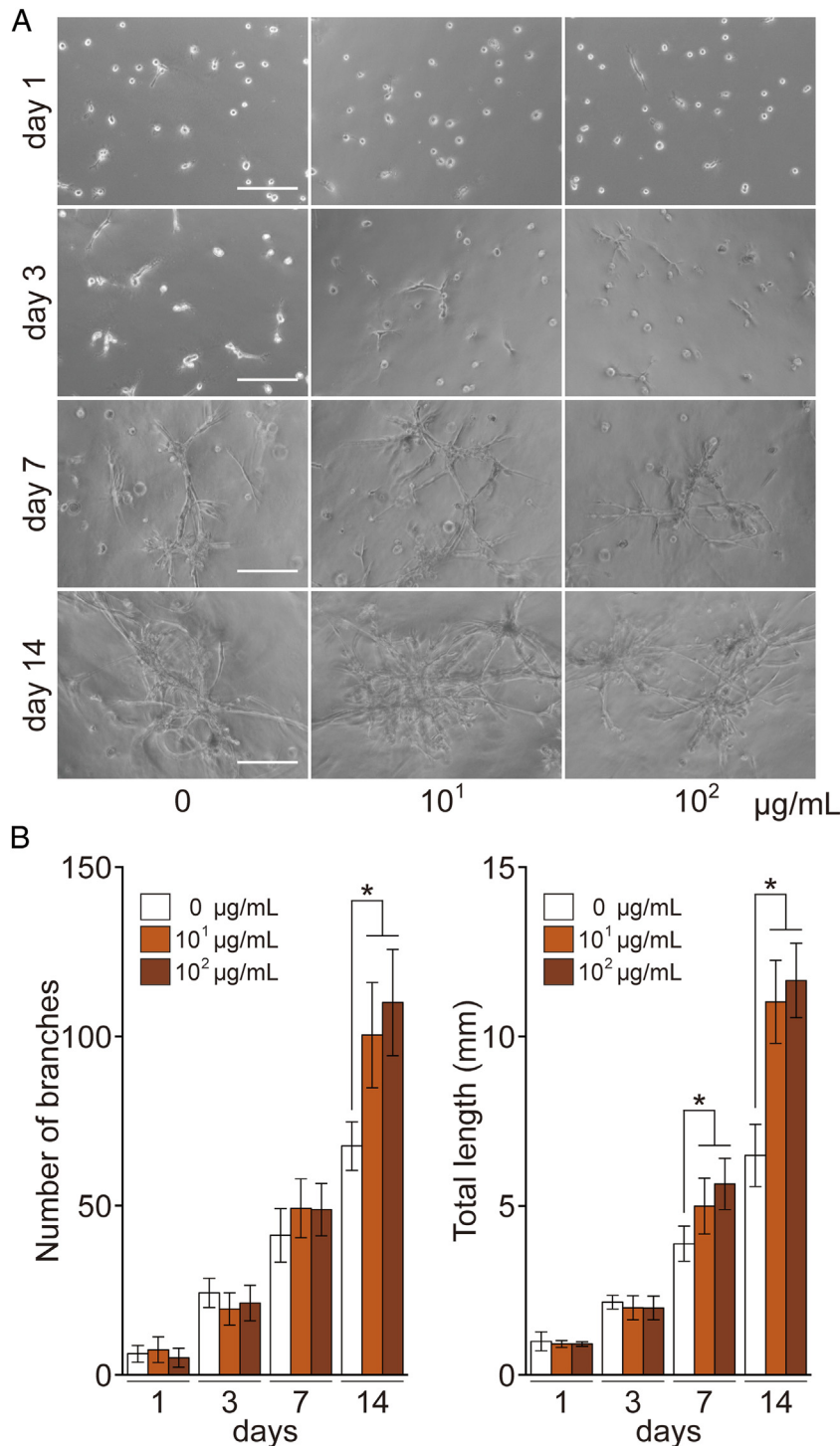


FIGURE 2 – The effect of HS on capillary sprouting in DPSCs. (A) Representative bright-field images showing sprouting of DPSCs on Matrigel for up to 14 days. Scale bars: 200 μm . (B) Analysis of sprout branch numbers and lengths at corresponding time points. Significance indicated with data shown as mean \pm SD for $n = 4$ ($*P < .05$). HS, heparan sulfate; DPSC, dental pulp stem cell; SD, standard deviation.

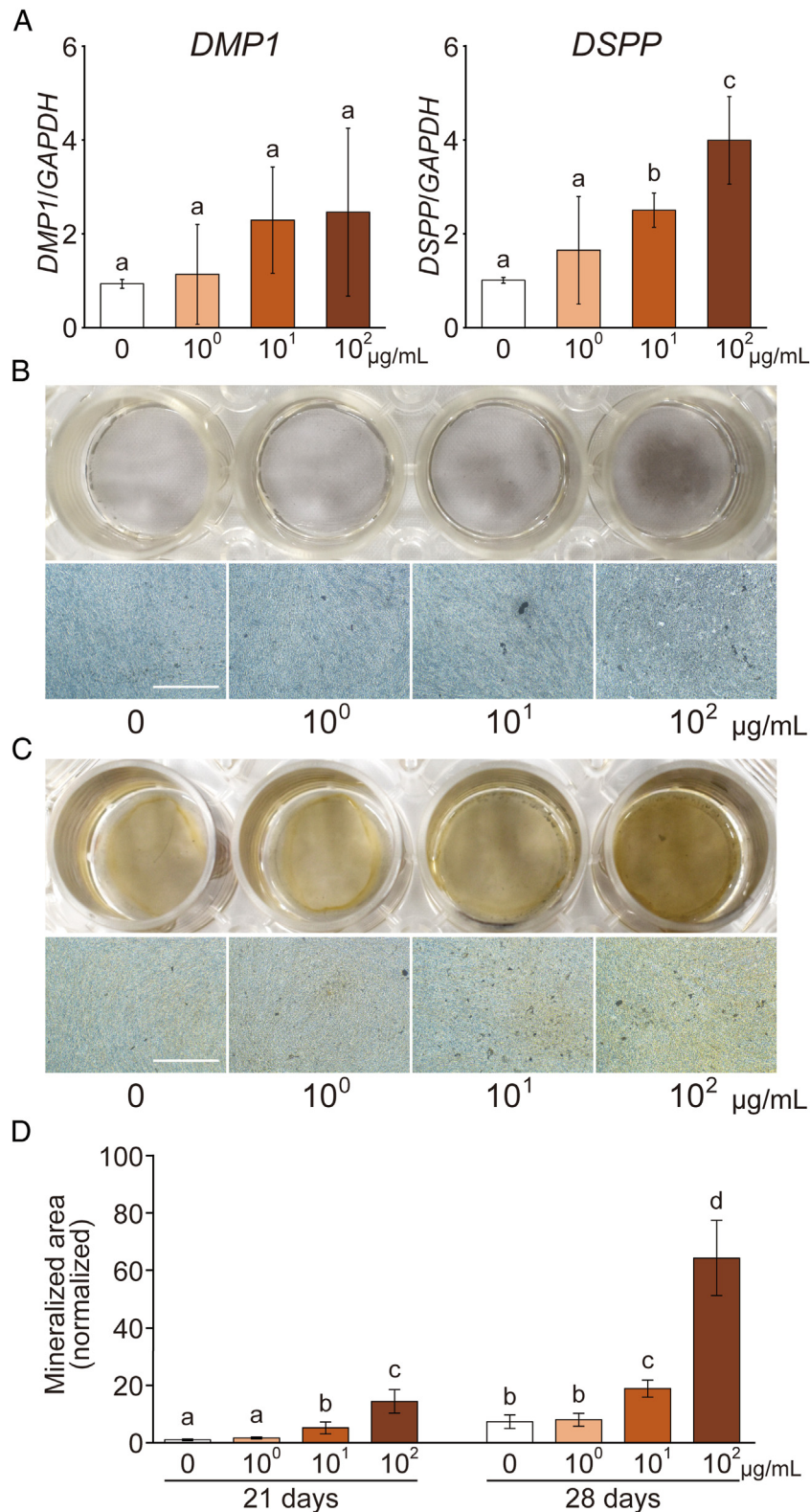


FIGURE 3 – The effect of HS on odontogenic differentiation of DPSCs. (A) mRNA expression of dentin matrix acidic phosphoprotein 1 (DMP1) and dentin sialophosphoprotein (DSPP) in DPSCs cultured for 7 days in OM supplemented with HS, using *GAPDH* as a reference gene. Different letters indicate significant differences among groups ($P < .05$). Mean \pm SD; $n = 4$. (B and C) von Kossa staining of DPSCs cultured with different concentrations of HS for 21 days (B) and 28 days (C). Scale bars: 500 μm . (D) Semiquantitative analysis of a mineralized area formed by DPSCs with different concentrations of HS at corresponding time points. Different letters indicate significant differences between groups ($P < .05$). Mean \pm SD; $n = 4$. HS, heparan sulfate; DPSC, dental pulp stem cell; OM, odontogenic differentiation medium; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

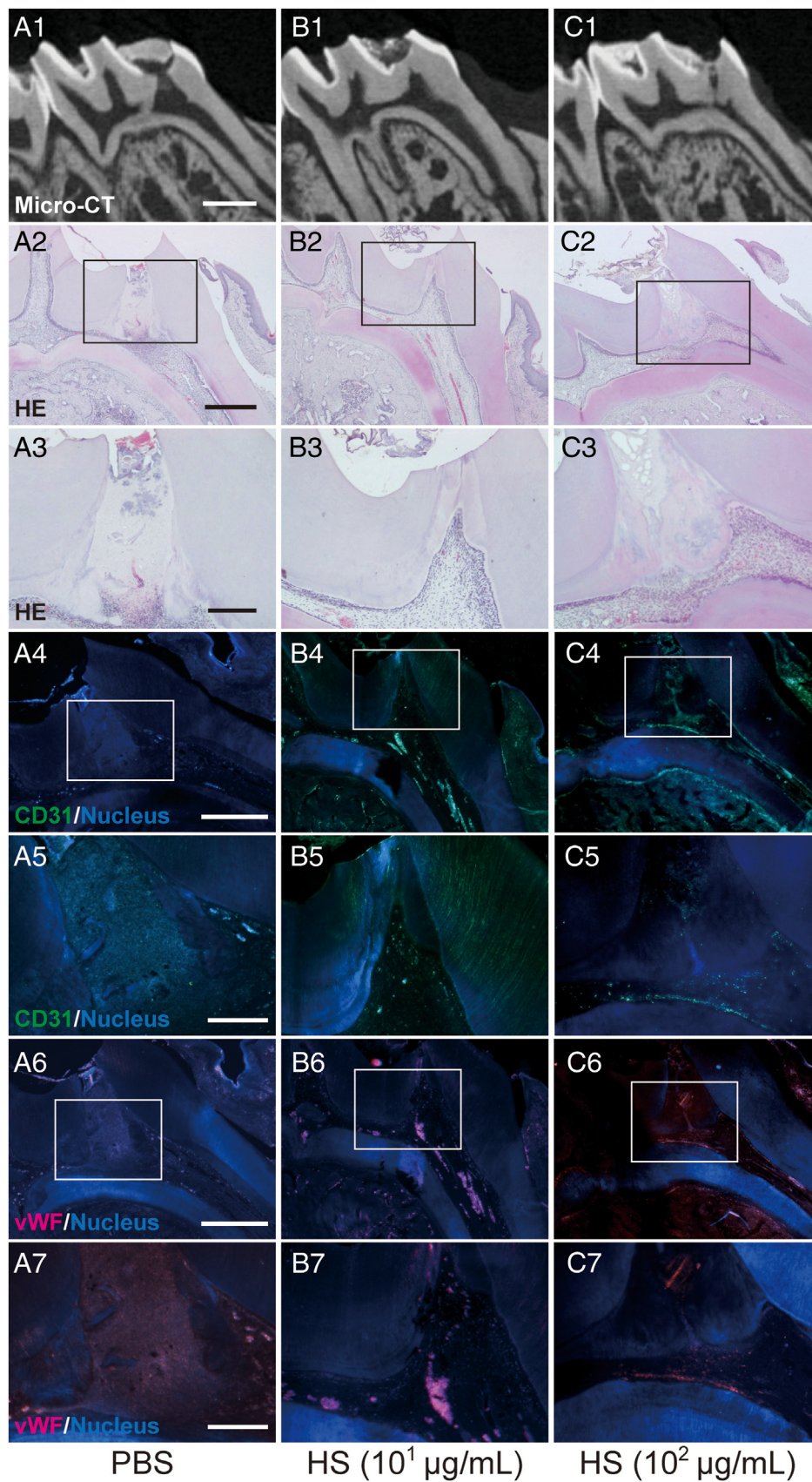


FIGURE 4 – The effect of HS on reparative dentin and capillary formation in a direct pulp capping model at day 28. (A1–C1) Micro-computed tomography (micro-CT) images of the experimental group of gelatin sponge with phosphate-buffered saline (PBS) (A1), HS-loaded sponges at 10¹ μg/mL (B1), and 10² μg/mL (C1). Scale bar: 1 mm. (A2–C2) HE staining of tissue

extensively studied for its therapeutic potential in dental tissue regeneration²⁰⁻²². The present study focused on the bioactive properties of HS to investigate its effects on the vasculogenesis and dentinogenesis of DPSCs.

We observed that DPSC proliferation was stimulated at a low HS concentration (10^{-2} – 10^0 $\mu\text{g/mL}$), however this effect was diminished at higher HS concentrations (10^1 – 10^2 $\mu\text{g/mL}$). Notably, at the two higher HS concentrations, the expression levels of both VEGFA and CXCL1 were upregulated compared with the control group, indicating that endothelial differentiation of DPSCs was enhanced by HS at 10^1 – 10^2 $\mu\text{g/mL}$. These results indicate that an increase in cell differentiation capabilities often coincides with a decrease in proliferative capacity^{23,24}. This study aimed to evaluate the influence of exogenous HS on DPSC differentiation and further availability of HS for pulp capping therapy; therefore HS concentrations 10^1 and 10^2 $\mu\text{g/mL}$ were selected for further experiments.

The capillary sprouting assays confirmed that DPSCs treated with HS can differentiate into vascular endothelial cells and contribute to the formation of vascularized networks^{18,25}. The results of this study indicate that exogenous HS at concentrations between 10^1 and 10^2 $\mu\text{g/mL}$ promoted the formation of reticular-like structures compared with the cells without HS. Our previous study showed that downregulation of HS diminished endothelial differentiation of DPSCs¹⁶; this study demonstrated that exogenous HS enhanced DPSC vasculogenesis *in vitro*.

DSPP and DMP1 are predominantly expressed in odontoblasts and play a crucial role in tooth formation and mineralization²⁶⁻²⁸. Here, significantly increased DSPP expression was observed in response to increasing

concentrations of HS, while no significant difference in DMP1 expression was found among all groups. This result might be attributed to the dentin formation stage with odontogenic induction. D'Souza et al²⁹ observed coexpression of DMP1 and DSPP in the young odontoblasts; however, DMP1 mRNA expression was notably decreased in secretory odontoblasts. They also observed sustained upregulation of DSPP during tooth mineralization. Thus, it is reasonable to assume that a portion of DPSCs in the present study were differentiated into mature odontoblasts with HS stimulation, followed by accelerating the production of mineralized matrices. Taken together, these results demonstrate that exogenous HS promoted dentinogenesis of DPSCs as well as vasculogenesis *in vitro*.

The establishment of a stable vascular network is a crucial factor in regulating tissue repair in dental pulp³⁰⁻³². To investigate the effects of HS on dental pulp healing *in vivo*, we employed a rat model of direct pulp capping. In this model, HS treatment resulted in a significant increase in the quantity of CD31 and vWF-positive cells compared with the PBS-treated group, indicating a marked increase in capillary formation. This finding suggests that HS not only promotes endothelial differentiation of DPSCs *in vitro*, but also accelerates vasculature development *in vivo*. Furthermore, application of HS induced the formation of reparative dentin, which might have been formed by DPSCs that had undergone differentiation within the dental pulp tissue. The presence of this dentin bridge implies that HS treatment not only enhances odontogenic differentiation in DPSCs but also promotes subsequent mineralization processes. Additionally, the enhanced vascular network observed in the HS-treated groups may accelerate dentinogenesis

in vivo^{33,34}. Future studies should determine the quality of the reparative dentin bridge formed by HS stimulation compared with that generated by hydraulic calcium silicate cement. Given these findings, it becomes evident that the interplay between vasculogenesis and dentinogenesis is complex and multifaceted. Therefore, further research is warranted to unravel the underlying signaling mechanisms by which HS influences these processes, which could provide deeper insight into potential applications of HS in dental tissue regeneration and improve pulp capping treatments.

CONCLUSION

This study demonstrated that exogenous HS significantly promotes both endothelial and odontogenic differentiation of DPSCs, leading to the formation of capillary structures and reparative dentin. These findings position HS, with its dual role in promoting both vascularization and dentin regeneration, as a promising agent for enhancing pulp capping treatment outcomes.

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

sections from different treatment groups. Scale bar: 500 μm . (A3–C3) High magnification views of HE-stained regions, as highlighted in A2–C2, with black boxes indicating areas of interest. Scale bar: 200 μm . (A4–C4) Immunofluorescence staining of CD31 (green) and nuclear staining (blue). Scale bar: 500 μm . (A5–C5) High magnification images of the corresponding A4–C4 images marked with white boxes. Scale bar: 200 μm . (A6–C6) Histological images of von Willebrand Factor (vWF)-positive capillary networks (red) and nuclear staining (blue). Scale bar: 500 μm . (A7–C7) High magnification views of the vWF-stained sections from A6–C6, guided by white boxes for detailed observation. Scale bar: 200 μm . HS, heparan sulfate; HE, hematoxylin and eosin.

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