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Original Article

Effects of different methods of demineralized dentin matrix preservation on the proliferation and differentiation of human periodontal ligament stem cells

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KEYWORDS

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Scaffold material

Abstract *Background/purpose:* Demineralized dentin matrix (DDM) is used as a tissue regeneration scaffold. Effective preservation of DDM benefits clinical applications. Cryopreservation and freeze-drying may be effective methods to retain DDM mechanical properties and biological activity.

Materials and methods: Human periodontal ligament stem cells (hPDLSCs) isolated using enzymatic dissociation were identified by multidirectional differentiation and flow cytometry. DDM was prepared with EDTA and divided into four groups: fresh DDM (fDDM), room temperature-preserved DDM (rtDDM), cryopreserved DDM (cDDM) and freeze-dried DDM (fdDDM). The DDM surface morphology was observed, and microhardness was detected. Transforming growth factor- β 1 (TGF- β 1), fibroblast growth factor (FGF) and collagen-I (COL-I) concentrations in DDM liquid extracts were detected by enzyme-linked immunosorbent assay (ELISA). The hPDLSCs were cultured with DDM liquid extracts. The effect of DDM on cells proliferation was examined by CCK-8 assay. The effect of DDM on hPDLSC secreted phosphoprotein-1 (SPP1), periostin (POSTN) and COL-I gene expression was examined by real-time qPCR.

Results: cDDM dentinal tubules were larger than those of the other groups. The three storage conditions had no significant effect on DDM microhardness and COL-I concentration. However, TGF- β 1 and FGF concentrations decreased after storage, with the greatest change in rtDDM, followed by fdDDM and cDDM. The liquid extracts of fDDM, cDDM and fdDDM slightly inhibited hPDLSCs proliferation, but those of rtDDM had no significant effect. The hPDLSCs cultured with fDDM, cDDM and fdDDM liquid extracts showed increased SPP1, POSTN and COL-I gene

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expression.

Conclusion: Cryopreservation and freeze-drying better maintain the mechanical properties and biological activity of DDM.

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Introduction

Periodontal disease is one of the most common diseases that endangers human oral health. The development of tissue engineering provides a new method for periodontal tissue regeneration. Periodontal tissue regeneration requires multipotent stem cells, scaffolds, and signaling molecules.¹ In recent years, demineralized dentin matrix (DDM) has been increasingly used in the dental field for applications such as alveolar bone regeneration, vital pulp preservation and tooth regeneration.^{2–10} Dentin is the extracellular matrix secreted by odontoblasts. During dentin mineralization, its bioactive molecules, including various growth factors such as TGF- β , FGF, vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP), which are related to periodontal regeneration, are embedded in matrix, but can be released after demineralization.¹¹

DDM is mainly derived from the third molar and teeth that need to be extracted for orthodontic or other reasons. However, DDM is not usually needed at the time of tooth extraction. Therefore, preservation of the activity of DDM within a certain period is a challenge in the clinic. At present, the temperatures commonly used to store biological samples are room temperature, 4 °C, –20 °C, –80 °C and –196 °C. In general, proteins, RNA and cells are usually stored at –80 °C or –196 °C because low-temperature damage is more likely to occur between 0 °C and –60 °C. Freeze-drying, also known as lyophilization, is a low-temperature dehydration process that can basically maintain the original chemical composition, physical properties and biological activity of the sample. Bone and teeth have similar compositions. Research has shown that demineralized bone matrix after freeze-drying can be transported and stored at room temperature for a long time.¹² Therefore, we speculate that cryopreservation (–196 °C) and freeze-drying better maintain the mechanical properties and biological activities of DDM than storage at room temperature.

In this study, we chose three storage methods, room-temperature preservation, cryopreservation and freeze-drying, with which to store fresh DDM and compared changes in surface morphology, microhardness, and protein concentration and the effects of preservation on the proliferation and differentiation of human periodontal ligament stem cells (hPDLSCs). Our purpose was to determine whether DDM can promote the proliferation and differentiation of hPDLSCs and to explore the effects of different storage methods on DDM activity to provide evidence for the further use of DDM for periodontal tissue regeneration.

Materials and methods

hPDLSC isolation and culture

Premolars and third molars without any disease were collected from the Department of Oral and Maxillofacial Surgery, Xiangya Stomatological Hospital, Central South University with patient consent and approval of the Ethics Committee. The patients were 18–25 years old with good health, no systemic diseases, normal tooth development and apical foramen closure. Immediately after each tooth was removed, it was placed in a phosphate-buffered saline (PBS, BI, Kibbutz, Israel) solution containing 100 U/ml penicillin and 100 μ g/ml streptomycin (BI). The periodontal ligament was scraped from the middle part of the root, rinsed with PBS at least three times, cut, placed into a solution containing type I collagenase (3 mg/ml, Sigma–Aldrich, Saint Louis, MO, USA) and Dispase II (4 mg/ml, Sigma–Aldrich), and digested at 37 °C for 40 min. The suspension was centrifuged at 1100 rpm \times 7 min, and the supernatant was removed. The cells were resuspended in α MEM (BI) containing 15% fetal bovine serum (FBS, BI), 2 mmol/L glutamine (BI), 100 U/ml penicillin and 100 μ g/ml streptomycin and stored in an incubator at 37 °C and 5% CO₂. The culture medium was changed every 3 days, and cells were subcultured when the cell confluence reached 80%. Cells from passages 3 to 4 were used for our experiments.

Osteogenic differentiation

The cells were seeded at 1×10^5 cells/well into 6-well plates. At 80% cell confluence, the medium was changed to osteogenic medium containing 15% FBS, 1% antibiotics, 100 μ mol/L ascorbic acid (Sigma–Aldrich), 10 mmol/L β -glycerophosphate (Sigma–Aldrich), 10 nmol/L dexamethasone (Sigma–Aldrich) and 10 μ mol/L glutamine. The control group was cultured in the previously described medium. After culture at 37 °C and 5% CO₂ for 14 days, the cells were washed twice with PBS, fixed with 4% paraformaldehyde and stained with alizarin red S for 30 min at room temperature. Excess dye was washed away with ddH₂O. The cells were observed and photographed under a microscope (Olympus, Tokyo, Japan).

Adipogenic differentiation

The cells were seeded at 1×10^5 cells/well into 6-well plates. At 80% cell confluence, the medium was changed

to MSCgo™ Adipogenic complete medium (BI). The control group was cultured in the previously described medium. After culture at 37 °C and 5% CO₂ for 21 days, the cells were washed twice with PBS, fixed with 4% paraformaldehyde and stained with oil red O for 30 min at room temperature. Excess dye was washed away with ddH₂O. The cells were observed and photographed under a microscope.

Flowcytometric analysis

The cells were detached and resuspended at a concentration of 1×10^6 cells/ml with PBS. The cell suspension (100 µl/tube) was added to 7 EP tubes, to which 5 µl of each of the following antibodies (Biolegend, San Diego, CA, USA) was added: PE-conjugated anti-human CD73, CD90, CD14, CD 105, and CD34 and PE-conjugated anti-mouse IgG2a and IgG1. The cells were incubated in the dark for 30 min, washed twice, resuspended in 300 µl of PBS and immediately analyzed on a flow cytometer (Beckman Coulter, Brea, CA, USA).

Preparation of DDM

DDM was prepared as described by Li et al.¹³ Premolars and third molars without any disease were collected from Xiangya Stomatological Hospital, Central South University. The tooth crowns, periodontal tissues, cementum, pulp and pre-dentin were mechanically removed. The dentin was made into 1 cm × 0.4 cm pieces and ultrasonically washed with ddH₂O for 30 min. Then, the dentin was treated with 17% EDTA for 5 min, 10% EDTA for 5 min, and 5% EDTA for 10 min and washed with ddH₂O for 30 min.

DDM storage

DDM after the above treatment was divided into the following four groups. (1) fresh DDM (fDDM) (no further processing required), (2) DDM preserved at room temperature for 6 months (rtDDM), (3) DDM cryopreserved in liquid nitrogen for 6 months (cDDM), and (4) DDM that was freeze-dried (FD5-6B, SIM, Newark, DE, USA) and preserved at room temperature for 6 months (fdDDM).

Analysis of the surface morphology of DDM

After the DDM (3 per group, 12 in total) was dried at the critical point and sprayed with gold, the surface morphology of the DDM was observed by scanning electron microscopy

(SEM, Quanta 250 FEG, FEI, Hillsboro, OR, USA). Dentinal tubule diameters were measured based on three images at 5000 × magnification, and the test was repeated three times.

Test of the microhardness of the DDM

The samples (3 per group, 12 in total) were embedded in denture base resin and polished with a polishing machine (UNIPOL-802, KEJING, Shenyang, China). A load of 0.025 kg was applied to the specimens for 10 s using the diamond pyramid indenter of a microhardness tester (HMV-2T, Shimadzu, Kyoto, Japan).

COL-I and growth factor detection

Liquid extract was prepared according to the International Standard ISO 10993 and Li et al.¹³ Briefly, fDDM, rtDDM, cDDM and fdDDM were ground into powder and sterilized by irradiation. In addition, the fdDDM was rehydrated for 30 min before subsequent use. Two grams of each powder were individually added to 10 ml of αMEM and incubated at 37 °C for 7 days. The filtered solutions were collected for growth factor detection. ELISA was used to determine the concentrations of TGF-β1, FGF-2 and COL-I in the liquid extracts with kits purchased from EXCELL (Taicang, China) and Dogesce (Beijing, China) according to the instructions. OD measurements were performed using a microplate reader (Epoch, BioTek, Winooski, VT, USA). Four-parameter logistic curve fitting was used to obtain the standard curve equation, and the concentrations of the samples were calculated by their OD. Two replicates were set for each group of samples, and the experiment was repeated three times.

Cell proliferation

The cells were seeded at 2×10^3 cells/well into 96-well plates (three parallel control wells per group). After cell attachment, the medium was changed to one of the following media (100µl/well) and cultured for 1, 3, 5 and 7 days: (1) αMEM + 15% FBS, (2) liquid extract of fDDM + 15% FBS, (3) liquid extract of rtDDM + 15% FBS, (4) liquid extract of cDDM + 15% FBS, and (5) liquid extract of fdDDM + 15% FBS. Then, 100 µl of culture medium and 10 µl of CCK-8 reagent were added to each well and incubated in the dark at 37 °C and 5% CO₂ for 2 h. The absorbance at 450 nm was determined by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1 Primer sequences.

Gene name	Primer sequence (5' -3')	Product size (bp)	Transcript ID
SPP1	F CTCCATTGACTCGAACGACTC R CAGGTCTGCGAAACTTCTTAGAT	230	NM_001,251,830
POSTN	F CTCATAGTCGTATCAGGGGTCG R ACACAGTCGTTTTCTGTCCAC	138	NM_001,135,935
COL-I	F GAGGGCCAAGACGAAGACATC R CAGATCACGTCATCGACAAC	140	NM_000088

Real-time qPCR to assess cell differentiation

The cells were seeded at 1×10^5 cells/well into 6-well plates, and the medium was changed as described above. After 7 days, RNA was extracted by using TRIzol (Ambion, Austin, TX, USA) and a small total RNA extraction kit (Tianmobio, Beijing, China). HiScript II Q RT SuperMix (Vazyme, Nanjing, China) and ChamQ Universal SYBR qPCR Master Mix (Vazyme) were used for reverse transcription PCR and real-time qPCR. All steps were performed according to the manufacturer's instructions. The sequences of the primers used are listed in Table 1. Endogenous reference gene (GAPDH) primers were purchased from Sangon Biotech (Shanghai, China), which also synthesized the other primers. Reverse transcription PCR was performed using Biometra TOne (Analytik Jena, Jena, Germany). Real-time qPCR was performed using an Applied Biosystems QuantStudio instrument (Thermo Fisher Scientific). Relative quantification was calculated using $2^{-\Delta\Delta C_t}$.

Statistical analysis

SPSS 21.0 was used for statistical analysis. One-way or 2-way ANOVA followed by LSD t-test were used for multiple comparisons among groups. A *P*-value below 0.05 indicated statistical significance. Error bar represents standard error (SE).

Results

hPDLSC culture and identification

The hPDLSCs at passage three had a typical long and fusiform shape (Fig. 1A). After 14 days of osteogenic induction, mineralized nodules were observed after alizarin red S staining (Fig. 1B). After 21 days of adipogenic induction, a large number of red lipid droplets were visible after oil red O staining (Fig. 1C).

The flow cytometry results (Fig. 2) showed that the cells were positive for the mesenchymal stem cell markers CD73, CD90, and CD105. The cells were negative for the monocyte marker CD14 and the hematopoietic stem cell marker CD34.

Combined with the results of multidifferentiation experiments, the above flow cytometry results confirmed that the cells were mesenchymal stem cells with multidirectional differentiation capability and thus met the needs of subsequent experiments.

Surface morphology and microhardness of DDM

The surface morphologies are shown in Fig. 3A–D. The dentinal tubule diameters were measured by ImageJ, and the results are shown in Fig. 3E. The diameters of dentinal tubules in the fDDM, rtDDM and fdDDM were smaller than that in the cDDM ($P < 0.001$).

The microhardness test results are shown in Fig. 3F; there was no significant difference in microhardness between any group ($P > 0.05$).

COL-1 and growth factor detection

The concentrations of COL-I and growth factors in the liquid extracts are shown in Fig. 4. The concentrations of TGF- β 1 and FGF-2 decreased in order from fDDM to cDDM, fdDDM and rtDDM. There were significant differences in their expression levels among the groups ($P < 0.05$). However, there were no significant differences in COL-I expression between any group ($P > 0.05$).

Cell proliferation

The cell proliferation results are shown in Fig. 5 and showed no significant differences in cell proliferation between the groups on the first and third days ($P > 0.05$). On the fifth day, cell proliferation in the fDDM group was lower than that in the other groups ($P < 0.05$). On the seventh day, cell proliferation in the fDDM, cDDM and fdDDM groups was lower than that in the control and rtDDM groups ($P < 0.05$).

Cell differentiation

The real-time qPCR results are shown in Fig. 5. After hPDLSCs were cultured with the liquid extracts for 7 days, cells in the fDDM, cDDM and fdDDM groups highly expressed

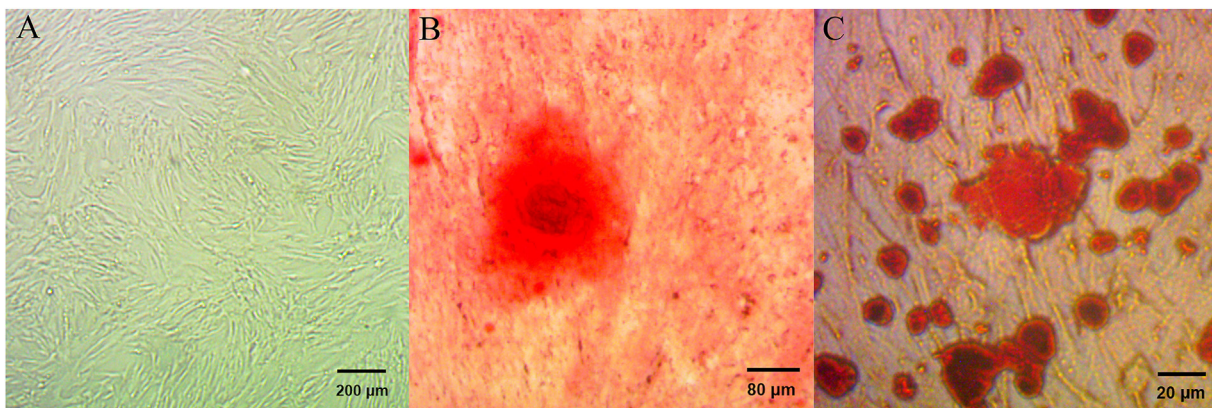


Figure 1 hPDLSCs were cultured and identified (A) Third-passage hPDLSCs (B) In osteogenic differentiation culture, mineralized nodules were stained with alizarin red S (C) In adipogenic differentiation culture, lipid droplets were stained with oil red O.

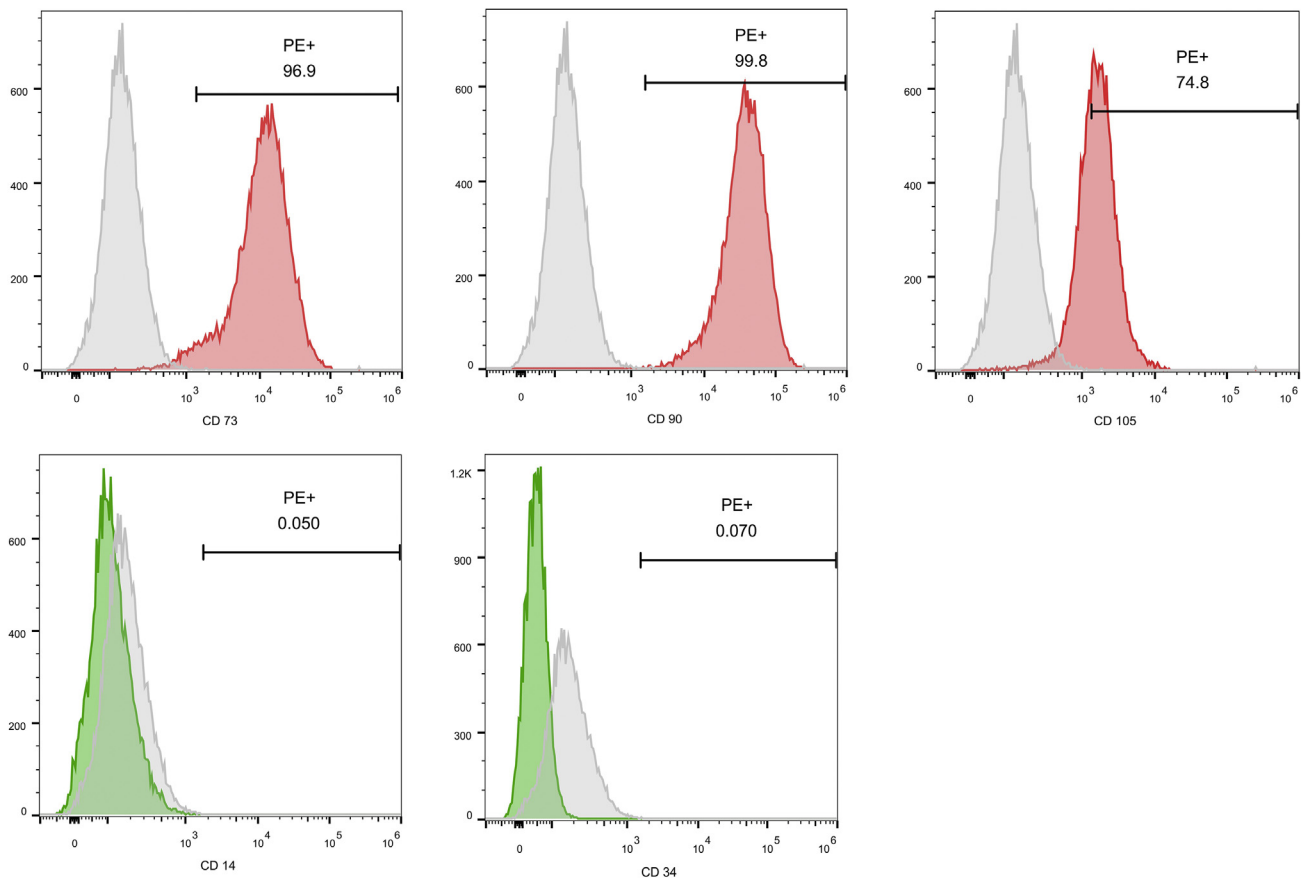


Figure 2 Flow cytometry results showed that the cells were positive for the mesenchymal stem cell markers CD73, CD90 and CD105 and negative for the monocyte marker CD14 and the hematopoietic stem cell marker CD34.

the genes SPP1, POSTN and COL-1 ($P < 0.05$), while the gene expression of SPP1, POSTN and COL-1 did not increase in the rtDDM group ($P > 0.05$). SPP1 gene expression was highest in the fDDM and cDDM groups, followed by the fdDDM group ($P < 0.05$). The expression of POSTN and COL-1 was highest in the fdDDM group, followed by the fDDM and cDDM group ($P < 0.05$).

Discussion

The mechanical properties and biological activity of a scaffold material may change after its long-term storage. We first tested the microhardness of each group of DDM. The average Knoop hardness value for dentin is 68.¹⁴ EDTA can chelate the Ca^{2+} in dentin and may reduce the hardness of dentin. Our experiment showed that the hardness of DDM in the fdDDM group was lower than that in the other groups, but this difference was not statistically significant. Then, we observed the surface morphology of each group by SEM and found that dentinal tubules in the cDDM group had larger pore sizes than those in the other groups. This may be because freeze-drying removes the water from a frozen sample by sublimation, so the material maintains the original skeleton structure during the drying process, and the dried material has a loose structure and larger pores.¹⁵ These two experiments showed

that the storage method had little effect on the mechanical properties of DDM.

The advantage of DDM over other scaffold materials is that it is rich in growth factors. However, proteins may denature and become inactivated under long-term storage. We chose DDM subjected to two feasible storage methods, cryopreservation and freeze-drying, as the experimental groups, fresh DDM as the positive control and room-temperature DDM as the negative control groups. We detected the contents of several proteins in the liquid extract of each group by ELISA. Growth factor levels were reduced after 6 months of storage, with the highest preservation rate observed in the cryopreservation group, followed by the freeze-dried group, while DDM in the room-temperature group exhibited low growth factor levels. This finding was somewhat different from the results of a study by Jiao et al.¹⁶ The authors found that the growth factor levels in the sample cryopreserved for 3 and 6 months were not significantly different from those in the fresh sample. This might be due to the addition of a protein-stabilizing cocktail, which can slow down the loss of proteins. In addition, both freeze-drying and storage may cause protein inactivation. Freeze-drying is more complicated than cryopreservation because it requires two drying steps in addition to freezing. The pressure during freeze-drying process can cause changes in protein structure. The drying process can increase the α -helical and random structure

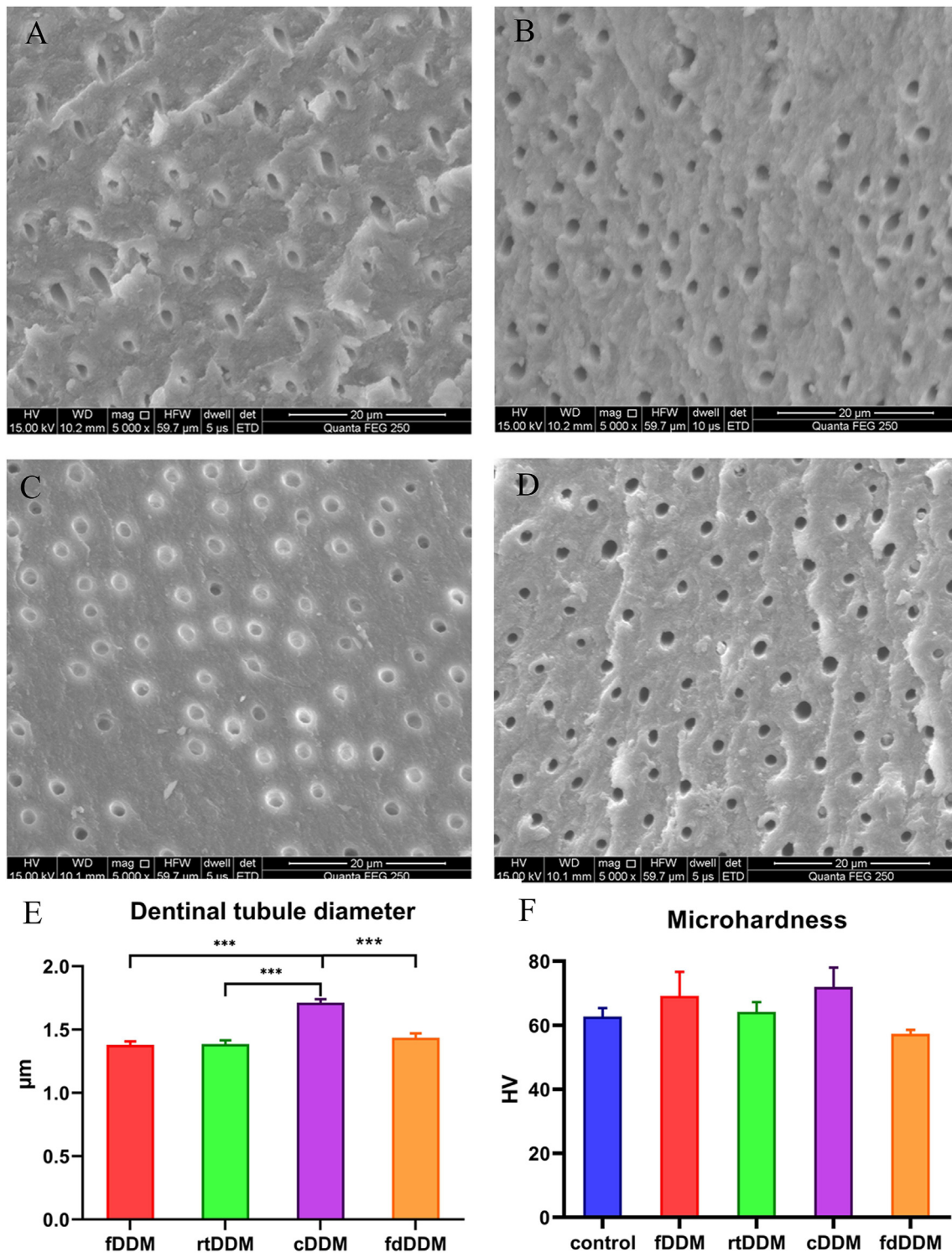


Figure 3 The surface morphology of fDDM (A), rtDDM (B), cDDM (C) and fdDDM (D) was observed by SEM (5000 ×) (E) Dentinal tubule diameter data were measured using ImageJ, the diameters of dentinal tubules in the fDDM, rtDDM and fdDDM were smaller than that in the cDDM ($***P < 0.001$) (F) The microhardness of DDM was measured with a microhardness tester, there was no significant difference in microhardness between any group ($P > 0.05$).

and decrease the β -sheet structure of proteins.¹⁷ Prestrelski et al.¹⁸ used enhanced Fourier transform infrared spectroscopy to find that FGF, γ -interferon and α -lactalbumin exhibited large conformational changes and

aggregation during freeze-drying. In the presence of moisture, freeze-dried proteins may undergo disulfide interchange reactions, resulting in protein inactivation.¹⁷ We also found that there were no differences in the

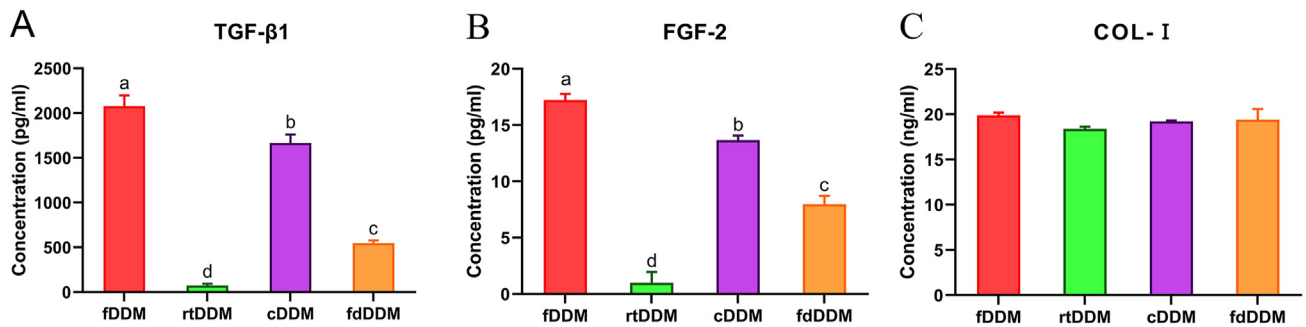


Figure 4 Concentrations of TGF- β 1, FGF-2 and COL-I in liquid extracts detected by ELISA. The concentrations of TGF- β 1 (A) and FGF-2 (B) decreased in order from fDDM to cDDM, fdDDM and rtDDM (groups labeled with different letters indicate significant differences with $P < 0.05$). There were no significant differences in COL-I (C) expression between any group ($P > 0.05$).

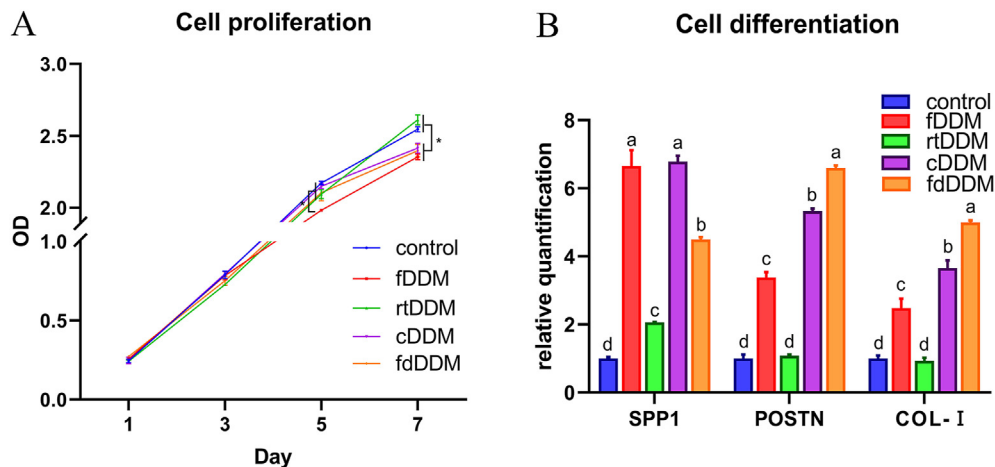


Figure 5 (A) Cell proliferation was assessed with the CCK-8 assay, cell proliferation in the fDDM, cDDM and fdDDM groups was lower than that in the control and rtDDM groups ($*P < 0.05$) (B) RT-qPCR was used to detect SPP1, POSTN and COL-I gene expression, after hPDLSCs were cultured with the liquid extracts for 7 days, cells in the fDDM, cDDM and fdDDM groups highly expressed the genes SPP1, POSTN and COL-I (groups labeled with different letters indicate significant differences with $P < 0.05$).

concentration of COL-I among liquid extracts of the different groups. This may be because COL-I is more stable than the other proteins examined or because its content was so high that no significant change was observed after 6 months.¹⁹ Notably, while different methods used to treat DDM altered the concentrations of the measured growth factors, previous work and our research results showed that TGF- β 1 was the most abundant growth factor in DDM.²⁰

By detecting cell proliferation through the CCK-8 assay, we found that except for rtDDM, the liquid extracts of other groups slightly inhibited the proliferation of hPDLSCs. This may be due to the levels of growth factors in the liquid extracts. The effect of TGF- β 1 on cell activities depends on the experiment conditions, the type of the cellular population as well as the presence of other growth factors.²¹ Studies have suggested that TGF- β 1 has dual effects on the proliferation of human periodontal ligament cells (PLCs) depending on their differentiation state. TGF- β 1 was found to stimulate the proliferation of PLCs but reduce the proliferation of a human periodontal ligament stem/progenitor cell line.²² The effect of TGF- β 1 on cell differentiation is also ambiguous. Depending on the culture condition and cell types, TGF- β 1 may show dual effects on bone sialoprotein expression.²³ Some researches showed that

TGF- β 1 upregulated COL-I mRNA expression of PLCs in the early phase of culture with TGF- β 1 in a dose-dependent manner, whereas the prolonged exposure to TGF- β 1 attenuated the ability of PLCs to express COL-I mRNA.^{24,25} In addition, TGF- β 1 can induce periostin expression in PLCs.²³ FGF-2 displays broad mitogenic and angiogenic properties.²⁶ It can promote hPDLSCs proliferation, while modulating the balance between their osteoblastic and fibroblastic phenotypes by affecting the gene expression of Runx2 and COL-I.^{27,28} Because the concentration of TGF- β 1 in the DDM was much higher than that of FGF, TGF- β 1 might play a major role in hPDLSCs proliferation. SPP1, also known as osteopontin or bone sialoprotein 1 (BSP-1), is related to the mineralization of bone, dentin and cementum.^{29,30} Collagen plays an important role in anchoring the tooth into the alveolar bone socket. The main collagen in periodontal ligament tissue is COL-I.³¹ The increased gene expression of SPP1 and COL-I is beneficial to the formation of extracellular matrix and the process of biomineralization. POSTN plays an important role in periodontium maintenance and regeneration and has been used as a successful periodontal regeneration marker.³² The results of our study showed that the liquid extracts of fDDM, cDDM and fdDDM could significantly increase the gene

expression of POSTN in hPDLSCs. This result indicated that under these stimulating conditions, hPDLSCs could differentiate into the periodontal ligament, which would be beneficial for periodontal tissue regeneration. Except for a slight increase in the expression of SPP1, the rtDDM group showed no significant change in the gene expression of POSTN and COL-1, indicating that DDM essentially lost its biological activity under room-temperature storage and could not promote periodontal tissue regeneration. Each group showed slight differences in its ability to promote gene expression, which might be related to the concentration of various bioactive components in DDM. Further studies are required to clarify the effects and mechanisms of the interactions between these components.

In our experiment, DDM was well preserved after freeze-drying or cryopreservation, but DDM stored at room temperature could not be used. In addition, the properties of cDDM were close to those of fDDM. Nevertheless, further *in vivo* experiments are needed to verify our conclusions. If fdDDM has the ability to induce periodontal tissue regeneration similar to that of cDDM in *in vivo* experiments, then fdDDM may be more clinically useful because it can reduce storage space and cost. In addition, DDM preparation methods need to be standardized, like demineralization and sterilization methods, as well as the shape and size of the DDM. These factors may cause deviations between research results.

In conclusion, our study indicates that cryopreservation, freeze-drying and storage at room temperature did not substantially change the mechanical properties of DDM. Storage at room temperature did not maintain the biological properties of DDM. Growth factor levels were reduced after cryopreservation and freeze-drying, which slightly inhibited cell proliferation, but these DDM could still promote the expression of osteogenic and periodontal genes in hPDLSCs.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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References

- Han J, Menicanin D, Gronthos S, Bartold PM. Stem cells, tissue engineering and periodontal regeneration. *Aust Dent J* 2014; 59:117–30.
- Kim YK, Lee J, Yun JY, Yun PY, Um IW. Comparison of autogenous tooth bone graft and synthetic bone graft materials used for bone resorption around implants after crestal approach sinus lifting: a retrospective study. *J Periodontal Implant Sci* 2014;44:216–21.
- Minamizato T, Koga T, Takashi I, et al. Clinical application of autogenous partially demineralized dentin matrix prepared immediately after extraction for alveolar bone regeneration in implant dentistry: a pilot study. *Int J Oral Maxillofac Surg* 2018;47:125–32.
- Bakhtiar H, Mirzaei H, Bagheri MR, et al. Histologic tissue response to furcation perforation repair using mineral trioxide aggregate or dental pulp stem cells loaded onto treated dentin matrix or tricalcium phosphate. *Clin Oral Invest* 2017;21:1579–88.
- Chen J, Cui C, Qiao X, et al. Treated dentin matrix paste as a novel pulp capping agent for dentin regeneration. *J Tissue Eng Regen Med* 2017;11:3428–36.
- Mehrvarzfar P, Abbott PV, Mashhadiabbas F, Vatanpour M, Tour Savadkouhi S. Clinical and histological responses of human dental pulp to MTA and combined MTA/treated dentin matrix in partial pulpotomy. *Aust Endod J* 2018;44:46–53.
- Luo S, Pei F, Zhang W, et al. Bone marrow mesenchymal stem cells combine with treated dentin matrix to build biological root. *Sci Rep* 2017;7:44635.
- Qin X, Zou F, Chen W, et al. Demineralized dentin as a semi-rigid barrier for guiding periodontal tissue regeneration. *J Periodontol* 2015;86:1370–9.
- Wang ZS, Feng ZH, Wu GF, et al. The use of platelet-rich fibrin combined with periodontal ligament and jaw bone mesenchymal stem cell sheets for periodontal tissue engineering. *Sci Rep* 2016;6:28126.
- Yang B, Chen G, Li J, et al. Tooth root regeneration using dental follicle cell sheets in combination with a dentin matrix-based scaffold. *Biomaterials* 2012;33:2449–61.
- Smith AJ, Duncan HF, Diogenes A, Simon S, Cooper PR. Exploiting the bioactive properties of the dentin-pulp complex in regenerative endodontics. *J Endod* 2016;42:47–56.
- Nather A, Tay LM. Processing of bone and musculoskeletal soft tissue allografts. In: Nather A, Yusof N, Hilmy N, eds. *Allograft procurement, processing and transplantation*. Singapore: World Scientific, 2010:335–60.
- Li R, Guo W, Yang B, et al. Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration. *Biomaterials* 2011;32:4525–38.
- Craig RG, Peyton FA. The micro-hardness of enamel and dentin. *J Dent Res* 1958;37:661–8.
- Krokida MK, Maroulis ZB. Effect of drying method on shrinkage and porosity. *Dry Technol* 1997;15:2441–58.
- Jiao L, Xie L, Yang B, et al. Cryopreserved dentin matrix as a scaffold material for dentin-pulp tissue regeneration. *Biomaterials* 2014;35:4929–39.
- Roy I, Gupta MN. Freeze-drying of proteins: some emerging concerns. *Biotechnol Appl Biochem* 2004;39:165–77.
- Prestrelski SJ, Tedeschi N, Arakawa T, Carpenter JF. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys J* 1993;65:661–71.
- Shoulders MD, Raines RT. Collagen structure and stability. *Annu Rev Biochem* 2009;78:929–58.
- Avery SJ, Sadaghiani L, Sloan AJ, Waddington RJ. Analysing the bioactive makeup of demineralised dentine matrix on bone marrow mesenchymal stem cells for enhanced bone repair. *Eur Cell Mater* 2017;34:1–14.
- Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. *Growth Factors* 2006;24:260–7.
- Fujii S, Maeda H, Tomokiyo A, et al. Effects of TGF- β 1 on the proliferation and differentiation of human periodontal ligament cells and a human periodontal ligament stem/progenitor cell line. *Cell Tissue Res* 2010;342:233–42.
- Maeda H, Wada N, Tomokiyo A, Monnouchi S, Akamine A. Prospective potency of TGF- β 1 on maintenance and regeneration of periodontal tissue. *Int Rev cell Mol Biol* 2013;304:283–367.
- Brady TA, Piesco NP, Buckley MJ, Langkamp HH, Bowen LL, Agarwal S. Autoregulation of periodontal ligament cell phenotype and functions by transforming growth factor- β 1. *J Dent Res* 1998;77:1779–90.

25. Silverio-Ruiz KG, Martinez AE, Garlet GP, et al. Opposite effects of bFGF and TGF-beta on collagen metabolism by human periodontal ligament fibroblasts. *Cytokine* 2007;39:130–7.
26. Kao RT. The use of biologic mediators and tissue engineering in dentistry. *Periodontol 2000* 2009;50:127–53.
27. An S, Huang X, Gao Y, Ling J, Huang Y, Xiao Y. FGF-2 induces the proliferation of human periodontal ligament cells and modulates their osteoblastic phenotype by affecting Runx2 expression in the presence and absence of osteogenic inducers. *Int J Mol Med* 2015;36:705–11.
28. Lee JH, Um S, Jang JH, Seo BM. Effects of VEGF and FGF-2 on proliferation and differentiation of human periodontal ligament stem cells. *Cell Tissue Res* 2012;348:475–84.
29. Foster BL, Ao M, Willoughby C, et al. Mineralization defects in cementum and craniofacial bone from loss of bone sialoprotein. *Bone* 2015;78:150–64.
30. Harahashi H, Odajima T, Yamamoto T, Kawanami M. Immunohistochemical analysis of periodontal reattachment on denuded root dentin after periodontal surgery. *Biomed Res* 2010;31:319–28.
31. Narayanan AS, Page RC. Connective tissues of the periodontium: a summary of current work. *Collagen Relat Res* 1983; 3:33–64.
32. Du J, Li M. Functions of periostin in dental tissues and its role in periodontal tissues' regeneration. *Cell Mol Life Sci* 2017;74: 4279–86.