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Original Article

Alkali-extracted proteins from the tooth dentin matrix as a mixture of bioactive molecules for cartilage repair and regeneration

Sainan Wang [a, *, 1], Sicong Mao [b, 1], Guibin Huang [a], Peipei Jia [a], Yanmei Dong [a], Jinxuan Zheng [c, **]

[a] Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology & National Center for Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Beijing, 100081, China
[b] Department of General Dentistry, Peking University School and Hospital of Stomatology & National Center for Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Beijing, 100081, China
[c] Department of Orthodontics, Hospital of Stomatology, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Stomatology, Guangzhou, Guangdong Province, 510080, China

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ABSTRACT

Introduction: Dentin matrix extracted protein (DMEP) is a mixture of proteins extracted from the organic matrix of a natural demineralized dentin matrix that is rich in a variety of growth factors. However, the effect of DMEP on cartilage regeneration is unclear. The aim of this study was to investigate the efficacy of DMEP extracted via a novel alkali conditioning method in promoting cartilage regeneration.
Methods: Alkali-extracted DMEP (a-DMEP) was obtained from human dentin fragments using pH 10 bicarbonate buffer. The concentration of chondrogenesis-related growth factors in a-DMEP was measured via enzyme-linked immunosorbent assay (ELISA). Human bone marrow mesenchymal stem cells (hBMMSCs) in pellet form were induced with a-DMEP. Alcian blue and Safranin O staining were performed to detect cartilage matrix formation, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess chondrogenic-related gene expression in the pellets. Rabbit articular osteochondral defects were implanted with collagen and a-DMEP. Cartilage regeneration was assessed with histological staining 4 weeks after surgery.
Results: Compared with traditional neutral-extracted DMEP, a-DMEP significantly increased the levels of transforming growth factor beta 1(TGF-β1), insulin-like growth factor-1(IGF-1) and basic fibroblast growth factor (bFGF). After coculture with hBMMSC pellets, a-DMEP significantly promoted the expression of the collagen type II alpha 1(COL2A1) and aggrecan (ACAN) genes and the formation of cartilage extracellular matrix in cell pellets. Moreover, compared with equivalent amounts of exogenous human recombinant TGF-β1, a-DMEP had a stronger chondrogenic ability. *In vivo,* a-DMEP induced osteochondral regeneration with hyaline cartilage-like structures.
Conclusions: Our results showed that a-DMEP, a compound of various proteins derived from natural tissues, is a promising material for cartilage repair and regeneration.

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Abbreviations: ACAN, aggrecan; a-DMEP, alkali-extracted DMEP; bFGF, basic fibroblast growth factor; CIM, chondrogenic inductive medium; COL2A1, collagen type II alpha 1; DMEP, dentin matrix extracted protein; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GFs, growth factors; hBMMSCs, human bone marrow mesenchymal stem cells; IGF-1, insulin-like growth factor-1; LAP, latency-associated propeptide; MSCs, marrow stem cells; qRT-PCR, quantitative real-time polymerase chain reaction; TGF-β1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor.

* Corresponding author. Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, Beijing 100081, China.
** Corresponding author. Department of Orthodontics, Hospital of Stomatology, Sun Yat-sen University, Guangzhou, Guangdong Province, 510080, China.
E-mail addresses: bdkqwsn@bjmu.edu.cn (S. Wang), zhengjx@mail.sysu.edu.cn (J. Zheng).
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[1] These authors contributed equally to this work.

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1. Introduction

Tissue regeneration involves complex cascade events that require the participation of many different growth factors (GFs), cytokines and signaling molecules. Although the administration of a single exogenous GF or a couple of exogenous GFs has therapeutic effects on some tissues, the appropriate integration of multiple biological cues that mimic the natural restorative microenvironment may result in synergistic effects [1,2]. The natural extracellular matrix is rich in GFs and other cytokines that promote the migration and proliferation of numerous cell types [3,4]. The cocktail of biomolecules harvested from natural tissue sources provides promising therapeutic opportunities. For example, concentrated growth factors derived from peripheral blood can be used to promote the regeneration of various tissues [5,6]. Non-collagen proteins extracted from bone, cartilage and tooth tissues are also used to induce the regeneration of various soft and hard tissues [3], even though their functions and mechanisms of action are not yet completely understood.

Dentin, the main hard tissue of teeth, contains a variety of glycoproteins, proteoglycans, GFs, etc. [7,8]. Many biomolecules can be released from the dentin matrix when dentin is damaged and play important roles in regulating and promoting pulp repair [9–11]. Dentin matrix extracted protein (DMEP) is a mixture of biomolecules extracted from the organic matrix of demineralized dentin and can be obtained from extracted deciduous teeth, wisdom teeth, or discarded teeth due to trauma or fracture. DMEP reportedly promote the adhesion, proliferation and osteogenic differentiation of different types of cells, such as dental pulp cells, periodontal ligament cells and bone marrow mesenchymal cells [12–14]. Studies have also shown that demineralized dentin can induce embryonic muscle-derived mesenchymal stem cell differentiation into chondrocytes and promote new bone formation and significant cartilage repair [15,16]. However, the effect of DMEP on cartilage regeneration has not been determined.

Transforming growth factor beta 1 (TGF-β1) plays a central role in the entire process of chondrocyte differentiation, including cell aggregation, proliferation, and terminal chondrogenic differentiation [17,18]. *In vivo*, TGF-β1 can induce ectopic cartilage formation by marrow stem cells (MSCs) and repair full-thickness cartilage defects [19]. TGF-β1 ubiquitously exists in the extracellular matrix in mammals. However, TGF-β is secreted and restored in organisms as an inactive complex consisting of an active cytokine dimer and a latency-associated propeptide (LAP) [20]. The latent cytokine TGF-β1 cannot exert its biological effects without ligand activation. TGF-β1 is one of the major GFs in the dentin matrix and exists mainly in an inactive latent form [21]. Traditionally, a neutral (pH 7.2) 10% ethylenediaminetetraacetic acid (EDTA) buffer solution has been used to demineralize dentin and extract released DMEP, but this method has not been reported to activate latent TGF-β1. Acid/alkali treatment can effectively activate latent TGF-β1 [22]. Our previous study confirmed that alkali treatment can effectively activate latent TGF-β1 derived from various tissues, including dentin, cartilage, serum and bone marrow fluid, thereby promoting cell migration [14]. Therefore, alkali conditioning could be a valid extraction method for improving the content and concentration of chondrogenesis-related growth factors in DMEP.

In this study, we hypothesized that DMEP extracted using a novel alkali conditioning method, designated alkali-extracted DMEP (a-DMEP), would increase the content of chondrogenesis-related growth factors. a-DMEP can promote the chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMMSCs) *in vitro* and induce cartilage regeneration *in vivo*. a-DMEP may be a suitable natural mixture of bioactive molecules for cartilage regeneration.

2. Materials and methods

2.1. DMEP extraction

De-identified, surgically extracted healthy human teeth were obtained from the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology with the approval of the ethics committee (PKUSSIRB-202053006). Periodontal and peri-apical soft tissues of the teeth were removed by scalpel. Enamel and cementum were removed using high-speed diamond bur and dental pulp was extirpated. The remaining dentin was grinded into fragments and treated with 17% EDTA at pH 7.4 for 10 min, followed by washing with distilled water for three times. DMEP was extracted from dentin fragments in PBS (pH 7.4) at 1 g/mL for 24 h, while a-DMEP was extracted from dentin fragments in Na_2CO_3–$NaHCO_3$ buffer (0.15 M, pH 10) solutions at 1 g/mL for 24 h. The concentrations of active TGF-β1, insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in DMEP and a-DMEP were assayed with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, USA) strictly according to the manufacturer's instructions. The freeze-drying process was performed to obtain DMEP and a-DMEP powders and then stored in −20 °C before use.

2.2. Cell culture

hBMMSCs (ATCC, PCS-500-012, Gaithersburg, MD, USA) were cultured in MSCs human Basal Medium (ATCC, PCS-500-030) with 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, USA), 125 pg/mL bFGF, 15 ng/mL IGF-1, 7% fetal Bovine Serum and 2.4 mM L-Alanyl-L-Glutamine (ATCC, PCS-500-041). A total of 5 independent hBMMSCs samples were used (age range: 20–35 years old). Cells were incubated in a humidified incubator with 5% CO_2 at 37 °C. hBMMSCs were passaged at 80% confluence, with medium change every 2 days. Less than passage 3 cells were used for all experiments.

2.3. Chondrogenic induction

To induce chondrogenic differentiation, hBMMSCs were collected and resuspended chondrogenic inductive medium (CIM) consisting of high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco, USA), 10% fetal bovine serum (FBS, ScienCell, USA), 1% penicillin–streptomycin (ScienCell), 0.01% dexamethasone (Sigma–Aldrich), 50 mg/mL ascorbic acid (Sigma–Aldrich), and 1% ITS (Sigma–Aldrich). Cells (2×10^5) in 200 μL suspension was added to each well in a 96-well plate with round bottoms and centrifuged at 200 g for 5 min to form chondrogenic pellets. Pellets in control group were cultured in CIM, and pellets in the positive control group were incubated in CIM with 20 ng/mL recombinant human TGF-β1 (rhTGF-β1). Pellets in DMEP/a-DMEP group were cultured in CIM with 10% DMEP/a-DMEP extract. Pellets in 1.17 ng/mL rhTGF-β1 group were cultured in CIM with 1.17 ng/mL rhTGF-β1. Pellets in a-DMEP + SB431542 group were cultured in CIM with 10% a-DMEP extract and 10 μM SB431542. The medium was exchanged every second day. The pellets were collected at days 14 for quantitative real-time polymerase chain reaction (qRT-PCR) and days 21 for histology.

2.4. qRT-PCR

After 14 days of chondrogenic differentiation induction, pellets (at least four per group) were manually homogenized in Trizol solution (Thermo Fisher Scientific, USA). RNA was extracted with

Direct-zol RNA Purification Kit in accordance to the manufacturer's protocol (Zymo Research, Irvine, USA). cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT PCR was performed using a Qiagen miScript SYBR green PCR Kit on an ABI 7500 instrument (Applied Biosystems, Courtaboeuf, France) following the manufacturer's instructions. The following primers were used: GAPDH Primer F, 5′-ATGACTCTACCCACGGCAAG-3′; R, 5′-CTGGAAGATGGTGATGGGTT-3′; ACAN Primer F, 5′-CAGTGCGATGCAGGCTGGCT-3′; R, 5′-CCTCCGGCACTCGTTGGCTG-3′; COL2 Primer F, 5′-AAGGGACACCGAGGTTTCACTGG-3′; R, 5′-GGGCCTGTTTCTCCTGAGCGT-3′; COL10 Primer F, 5′-ATGCTGCCTCAAATACCCT-3′; R, 5′-TGCCTTGTTCTCCTCTTACT-3′; SOX9 Primer F, 5′-AGCGACAACTTTACCAG-3′; and R, 5′-GGAAAACAGAGAACGAAAC-3′. GAPDH was used as a housekeeping gene. Relative mRNA expressions of target genes were calculated based on $2^{-\Delta\Delta Ct}$ method. There were three replicate samples for each group.

2.5. Histological staining of pellets

After 21 days of chondrogenic differentiation induction, the pellets were fixed in 4% paraformaldehyde for 2 h at room temperature. One layer of gauze was placed onto a cassette and pellets were transferred onto the gauze. Dehydration was performed with sequential ethanol solutions. Dehydration solutions were cleared with graded ethanol and zylene mixtures and paraffin was infiltrated overnight. The next day, pellets were fixed to a paraffin block and 4 μm sections were obtained using a microtome. Slides were dried for 10 min at 60 °C. Sections were deparaffinized by two cycles of zylene. Sections were rehydrated with decreasing sequential ethanol series and sections were rinsed under running tap water for 5 min. Hematoxylin (Mayer's Hematoxylin, catalogue number: S3309, DakoCytomation, Carpinteria, USA) and Eosin (catalogue number: 230251, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) staining was performed according to the manufacturer's protocol (DakoCytomation). Chondrogenic staining was performed with Alcian Blue and Safranin O staining. Sections were incubated in 1% alcian blue solution (catalogue number: A3157, Sigma-Aldrich Chemie GmbH) for 30 min. Safranin O staining was performed by incubating the slides in Weigert's iron hematoxylin for 10 min. Slides were washed and incubated in 0.1% safranin O solution (Solarbio, Beijing, China) for 5 min. After the staining process, sections were washed and passed through increasing sequential ethanol series. Slides were cleared with xylene, mounted with xylene, and covered with glass. Cartilage glycosaminoglycans appeared blue. Pictures were taken with a light microscope (DM-1000 Microscope, Leica Microsystems, Wetzlar, Germany) and processed using the Leica Manager software (Leica Microsystems).

2.6. In vivo experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center (LA2019279). New Zealand white rabbits (weight: 2.0–2.5 kg) were used in the articular cartilage defects experiment. Rabbits were anesthetized by intravenous injection of a 3% pentobarbital sodium solution at 35 mg/kg 2% lidocaine hydrochloride was used to provide local anesthesia in the surgical region. A medial parapatellar incision was made to the knee, and the patella was dislocated to access the patellar groove of the femur. A 4-mm surgical drill was used to create an osteochondral defect (diameter = 4 mm; depth = 3 mm). Slight bleeding from the subchondral bone was confirmed, and physiological saline was used to clean the defect and prevent thermal damage. The rabbits were randomly divided into three transplantation groups: 1. Empty group (nothing was implanted into the defects), (2) Collagen group (60 μL of Collagen I was implanted into the defects), (3) a-DMEP group (60 μL of Collagen I with a-DMEP was implanted into the defects, the dose of TGF-β1 in a-DMEP was 5 ng). The rabbits (n = 4) were euthanized at 4 weeks. Femoral condyles were subsequently retrieved. Macroscopic images of the condyles were initially observed; the samples were then fixed in 20% formalin for 3–5 days. The sample was decalcified in 10% EDTA for 3–4 months and embedded in paraffin wax, and 4-μm sections were cut near the central of the defect area, parallel to the long axis of the femur. Standard protocols were used for histological staining. Deparaffinized sections were stained with HE, Alcian Blue with Safranin O-Fast Green staining. The percentage of neocartilage area was quantified from the Safranin O-Fast Green staining histology images (n = 4) using ImageJ software by dividing the red-staining area of the defect by the total area of defect.

2.7. Statistics

Data were expressed as mean ± SD and were analyzed with statistical software SPSS 26.0 software. The normality and homogeneity test of variance was performed and passed by all quantitative data before the statistical test. All quantitative data were analyzed by using the ANOVA test combined with the Tukey-Kramer multiple comparison posttest. For all statistical tests, P values < 0.05 were considered significant.

3. Results

3.1. Alkaline extraction increased chondrogenesis-related growth factor levels in DMEP

DMEP extracted at pH 7.4 contained 1.79 ± 0.33 ng/mL TGF-β1, 4.43 ± 2.16 ng/mL IGF-1, 0.89 ± 0.35 bFGF and 33.07 ± 2.77 pg/mL VEGF (Fig. 1). DMEP extracted at pH 10 (a-DMEP) contained significantly greater levels of TGF-β1 (11.34 ± 1.41 ng/mL), IGF-1 (8.84 ± 0.87 ng/mL) and bFGF (3.40 ± 0.42 ng/mL). Moreover, there was no significant difference in the VEGF level between the a-DMEP group (36.31 ± 10.02 pg/mL) and the DMEP group.

3.2. a-DMEP promoted the chondrogenic differentiation of hBMMSCs

After 3 weeks of induction, all the harvested pellets exhibited a smooth spherical morphology (Fig. 2A). Pellets cultured with 20 ng/mL TGF-β1 were used as a positive control and were larger in size than were those in the negative control groups. The pellets cultured in the presence of DMEP were similar in size to those in the control groups. In contrast, the pellets cultured with a-DMEP were the largest in all the groups.

Safranin O and alcian blue staining were used to detect cartilage matrix proteoglycans (GAGs) formation. In the negative control group, GAG was negatively stained, whereas in the positive control group, chondrocytes in distinct lacunae were embedded in strongly positively stained GAG, and almost the entire cross-section was positively stained. Pellets in the DMEP group had a small area of positive GAG staining. Pellets in the a-DMEP group had stronger positive staining than those in the DMEP group, but weaker than those in the positive control group. A lacuna structure was visible in both DMEP and a-DMEP groups.

To further determine the chondrogenic-inducing potential of a-DMEP, the expression of chondrogenic marker genes in the pellets was analyzed after 2 weeks (Fig. 2B–D). The expression of COL2A1 and ACAN in the a-DMEP-treated chondrogenic pellets was significantly higher than that in the control pellets but lower than

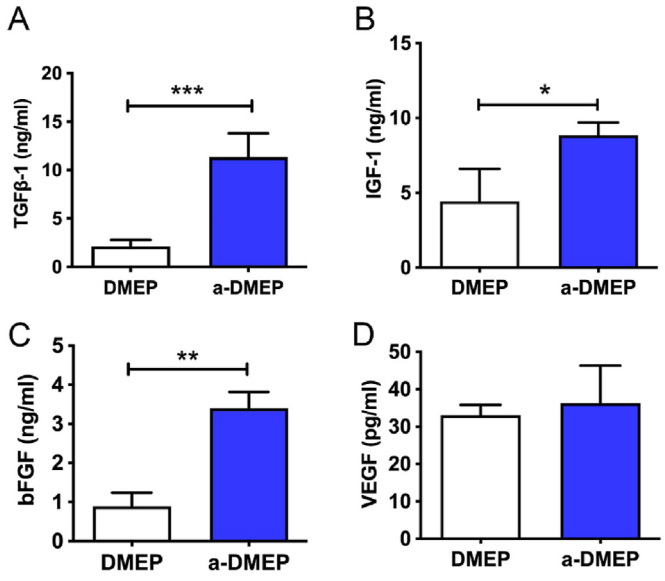

Fig. 1. Concentrations of chondrogenesis-related growth factors: TGF-β1 (A), IGF-1 (B), bFGF (C) and VEGF (D) in DMEP and a-DMEP groups determined via ELISA, showing the concentration of TGF-β1, IGF-1, bFGF in a-DMEP is significantly higher. *$P \leq 005$, **$P \leq 001$, ***$P \leq 001$.

that in the positive control group. Furthermore, the expression of these two genes was significantly greater in a-DMEP-treated pellets than in the DMEP group. Similarly, compared with that in the control pellets, the expression of SOX9 in the cultured DMEP and a-DMEP pellets was slightly higher, but the difference was not significant.

3.3. a-DMEP had a stronger chondrogenic ability than did an equivalent amount of rhTGF-β1

The effect of the TGF-β1 component of a-DMEP on the ability of a-DMEP to induce chondrogenesis was analyzed. The concentration of TGF-β1 in a-DMEP we used was 1.17 ng/mL; therefore, 1.17 ng/mL rhTGF-β1 was added to the control media to induce cell chondrogenic differentiation. In addition, the TGF-β1 inhibitor SB431542 was added to a-DMEP-treated pellets to observe whether the chondrogenic ability of a-DMEP could be inhibited by SB431542.

Histological examination (Fig. 3A) revealed that the 1.17 ng/mL rhTGF-β1-treated pellets were slightly larger in size and showed slightly more positive staining for GAG than did the control pellets, but the area of positive staining was much less than that of the a-DMEP-treated pellets. Furthermore, SB431542 strongly prevented GAG-positive staining in a-DMEP-treated pellets, and no lacunae structures were observed in a-DMEP + SB431542-induced pellets.

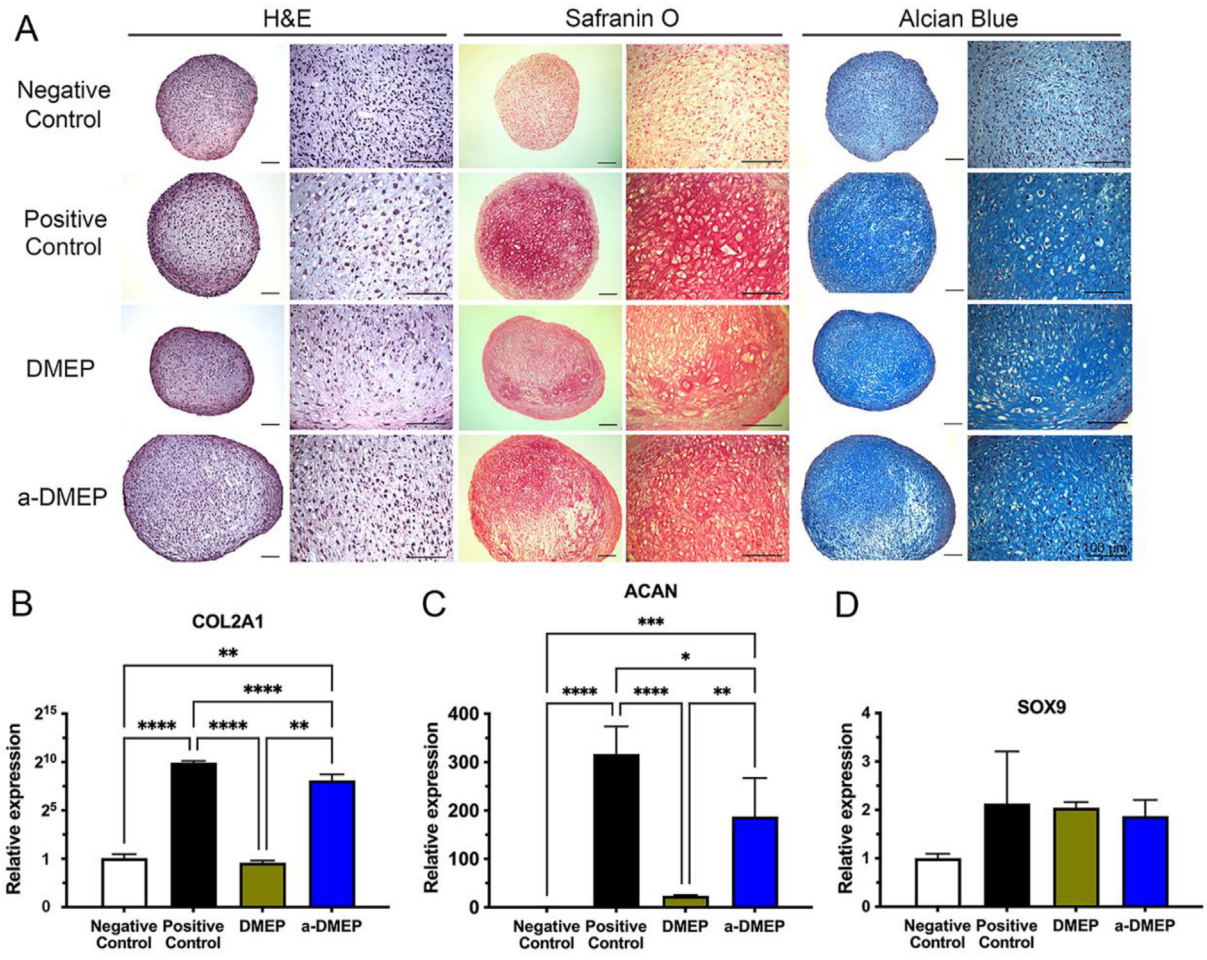

Fig. 2. Histological and chondrogenesis-related gene expression analyses of pellets exposed to CIM (control group), CIM supplemented with 20 ng/mL rhTGF-β1 (positive group), DMEP and a-DMEP. (A) Images of HE-, safranin O- and alcian blue-stained pellets on day 21. All scale bars represent 100 μm. Pellets cultured with a-DMEP are the largest in size and have the strongest positive staining. The gene expression of COL2A1 (B), ACAN (C) and SOX9 (D) determined by qRT–PCR on day 14. *$P \leq 005$, **$P \leq 001$, ***$P \leq 001$. The expression of COL2A1 and ACAN in the pellets of a-DMEP group is significantly higher than that in the control and DMEP group.

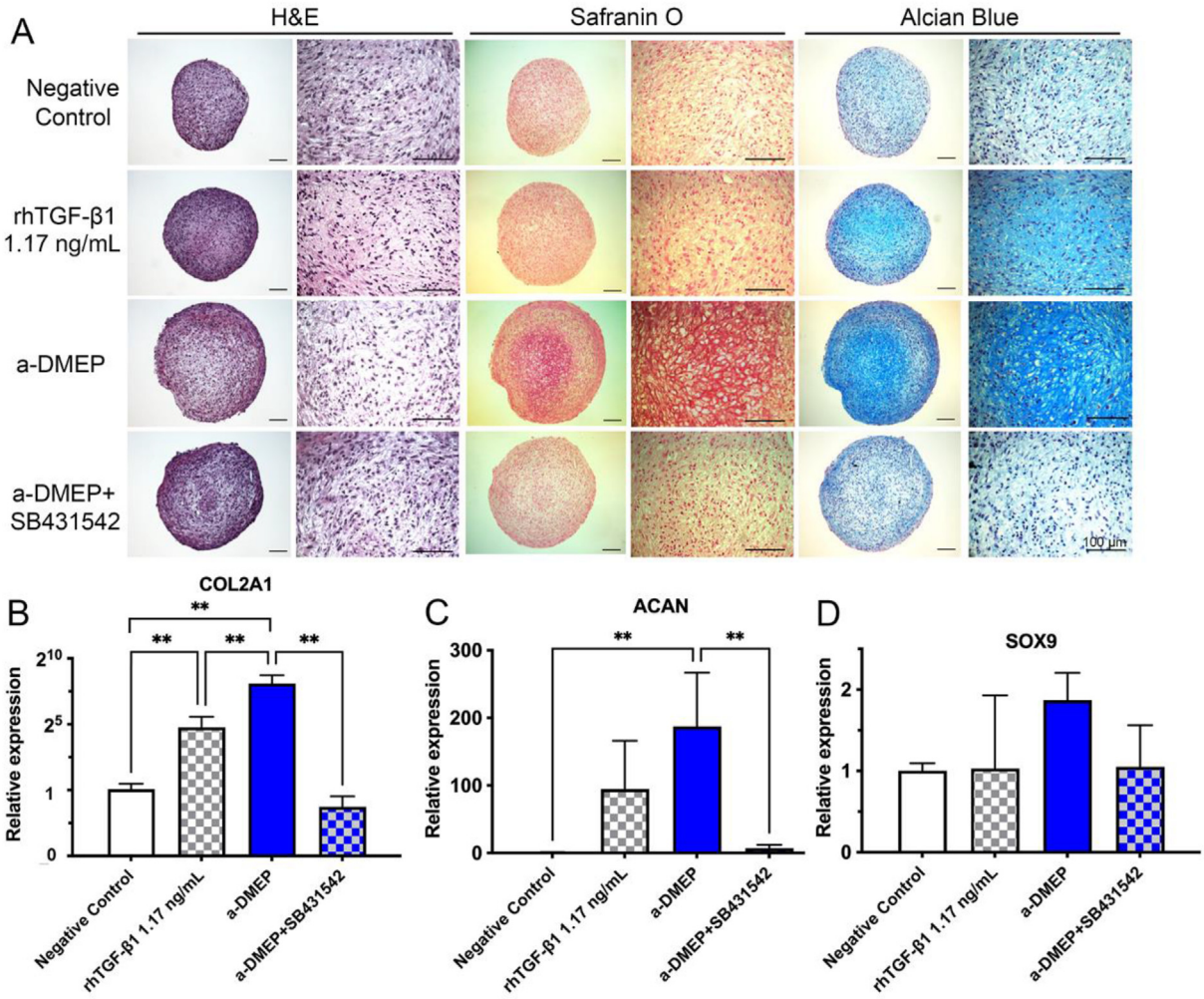

Fig. 3. Histological and chondrogenesis-related gene expression analyses of pellets exposed to CIM (control group), CIM supplemented with 1.17 ng/mL rhTGF-β1, a-DMEP or a-DMEP plus the TGF-β1 inhibitor SB431542. (A) Images of HE-, safranin O- and alcian blue-stained pellets on day 21. All scale bars represent 100 μm. The area of positive staining in rhTGF-β1-treated pellets is less than that of a-DMEP-treated pellets and a-DMEP + SB431542-induced pellets show less GAG-positive staining than a-DMEP-treated pellets. The gene expression of COL2A1 (B), ACAN (C) and SOX9 (D) determined by qRT–PCR on day 14. *P ≤ 005, **P ≤ 001, ***P ≤ 001. The expression of COL2A1 and ACAN in a-DMEP-induced pellets is higher than that in other groups.

The expression of COL2A1 and ACAN in the 1.17 ng/mL TGF-β1-induced pellets was markedly lower than that in the a-DMEP-induced pellets (Fig. 3B and C) but higher than that in the control group. The upregulation of COL2A1 and ACAN expression in a-DMEP-induced pellets was strongly prevented by SB431542. The above results suggest that TGF-β1 plays an important role in chondrogenic induction by a-DMEP. However, a-DMEP, as a mixed growth factor, has a stronger chondrogenic effect than does TGF-β1 alone.

3.4. a-DMEP promoted cartilage regeneration in a rabbit model

Representative macroscopic images of each group of defect regions are shown in Fig. 4. General morphological observation revealed that the defects in the empty group (Fig. 4A1) and the collagen group (Fig. 4B1) were filled with heterogeneous new tissue and that the central part of the defect remained unfilled. In contrast, defects in the a-DMEP group were filled with smooth white tissue (Fig. 4C1).

In the empty group (Fig. 4A2-A5) and the collagen group (Fig. 4B2-B5), HE staining revealed that only disorganized fibrous tissues with blood vessels (yellow arrows) filled the cartilage defect area. Both groups exhibited unhealed subchondral bone defects with significantly less trabecular bone structure than the surrounding bone tissue. In the a-DMEP group, the cartilage defect was filled with stratified hyaline cartilage-like tissue with surface perichondrium (Fig. 4C2-C5). Numerous hyaline-like chondrocytes located within the lacuna (yellow arrowhead) were detected. Additionally, no blood vessels were observed in the new cartilage layer, similar to natural avascular cartilage. New subchondral bone and trabecular bone appeared to be integrated with nearby bone tissue.

Alcian blue staining and Safranin O-fast green staining showed that the defects in the blank group (Fig. 5A1-A4) and collagen group (Fig. 5B1-B4) had small patches of blue or orange–red staining separated from nearby natural tissue, with no cartilage matrix staining observed in the central part of the defect area. However, the entire layer of newly generated tissues in the a-DMEP group exhibited strong blue staining by alcian blue staining and intense orange–red staining by safranin O-fast green staining. Additionally, the new cartilage was thicker than the nearby host cartilage. The histological quantification revealed that the percentage of neocartilage area of a-DMEP group was significantly higher than that in blank and collagen group (Fig. 6).

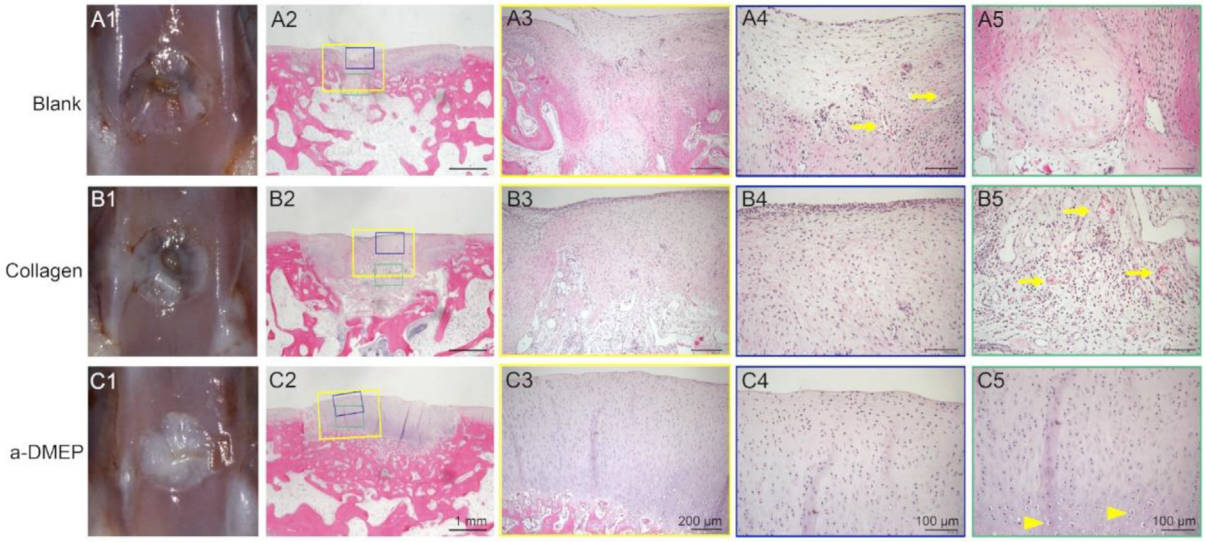

Fig. 4. Macroscopic views and histological evaluation of rabbit cartilage repair 4 weeks after surgery. (A) Cartilage defects (blank group) show disorganized fibrous tissues with blood vessels. (B) Cartilage defects repaired with collagen (collagen group) also show plenty fibrous tissues ingrowth and the osteochondral defects remain unhealed. (C) Cartilage defects repaired with collagen and a-DMEP (a-DMEP group) show hyaline cartilage-like tissue with chondrocytes locate within the lacuna. The yellow, blue and green boxed areas in A2-C2 are magnified in A3-C3, A4-C4 and A5-C5, respectively. The yellow arrows indicate blood vessels. The yellow arrowheads indicate hyaline-like chondrocytes located within the lacuna. Scale bars = 1 mm in A2-C2, scale bars = 200 μm in A3-C3, and scale bars = 100 μm in A4-C4 and A5-C5.

4. Discussion

The results of the present study showed that DMEP could induce chondrogenic differentiation *in vitro* and cartilage regeneration *in vivo*, suggesting that the tooth dentin matrix may be a suitable natural source of bioactive molecule mixtures for cartilage repair and regeneration. In contrast to bone and cartilage tissue, dentin has no embedded cell bodies and little tissue remodeling [23]; therefore, the composition of dentin extracts may be more consistent and immunologically safer than other tissue sources. In addition, dentin tissue is easy to obtain from extracted deciduous teeth, wisdom teeth, or teeth discarded after extraction due to trauma or fracture. Teeth from animals can also be a source of bioactive molecules for potential clinical applications. For example, in the past 20 years, enamel matrix proteins from porcine tooth germ have been used clinically to repair periodontal bone defects

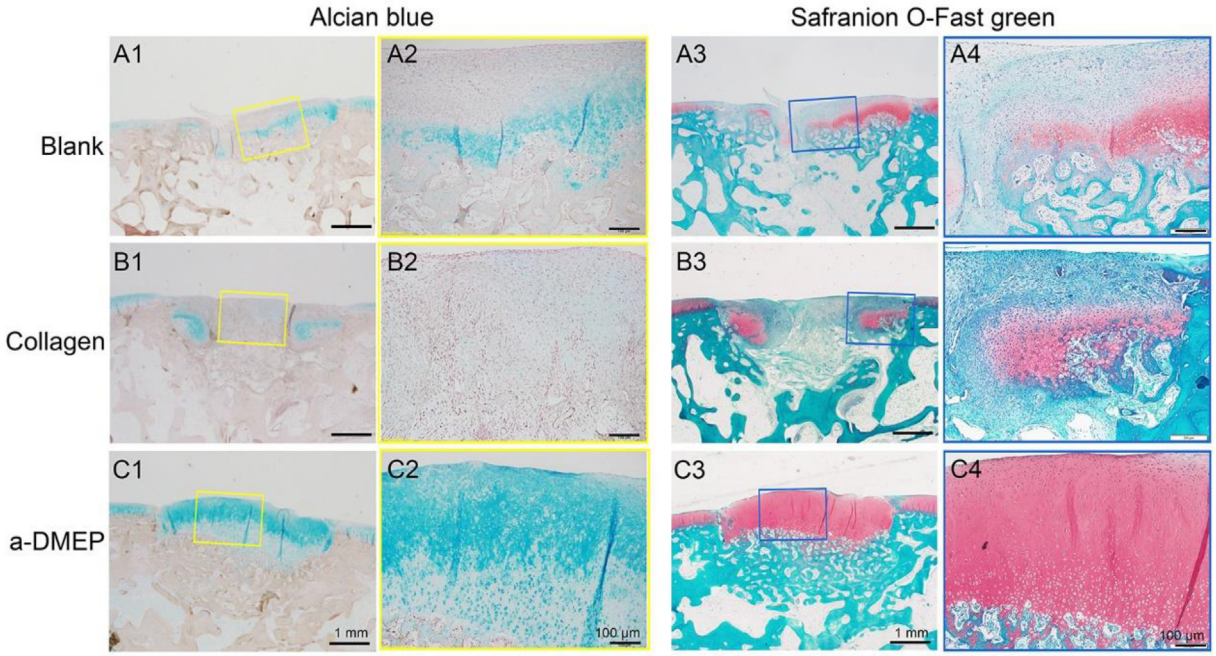

Fig. 5. Alcian blue and Safranin O-fast green staining of rabbit cartilage repair 4 weeks after surgery. (A) Cartilage defects (blank group) present scarce positive staining. (B) Cartilage defects repaired with collagen (collagen group) show only few positively stained regions. (C) Cartilage defects repaired with collagen and a-DMEP (a-DMEP group) exhibit abundant strongly positive-staining area. The yellow boxed areas in A1-C1 are magnified in A2-C2. The blue boxed areas in A3-C3 are magnified in A4-C4. Scale bars = 1 mm in A1-C1 and A3-C3, and scale bars = 100 μm in A2-C2 and A4-C4.

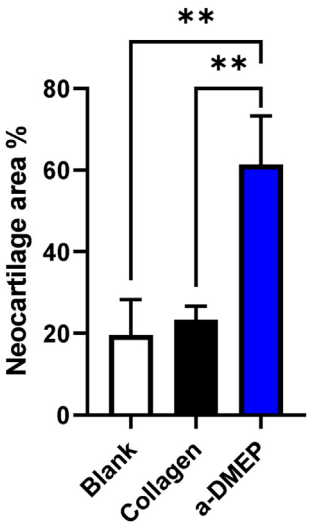

Fig. 6. Quantification of Safranin O-fast green staining, showing a significant increase in the percentage of neocartilage area of a-DMEP group. *P ≤ 005, **P ≤ 001, ***P ≤ 001.

[24–26]. Therefore, tooth derivatives have good clinical application prospects.

DMEPs contain many GFs, including TGF-β1, bFGF, BMP-2, PDGF, IGF-1, and VEGF [27,28], which may play important roles in chondro-induction [29]. The TGF-β family can enhance the expression of proteoglycans and type II collagen and plays a central role in the entire process of chondrocyte differentiation [18]. Remarkably, our previous study showed that the TGF-β1 content in dentin was dozens of times greater than that in cartilage and bone marrow tissue [14]. The FGF and IGF families also have important regulatory effects on cartilage repair. bFGFs are present in the extracellular matrix structure of hyaline cartilage and can prevent cartilage damage and the development of osteoarthritis [30]. IGF-1 regulates MSC chondrogenesis by stimulating proliferation, regulating apoptosis, and inducing chondrocyte phenotypic expression [31].

Compared with neutral extraction, alkali extraction of dentin can effectively increase the contents of bFGF, IGF-1 and TGF-β1 in DMEPs. High alkaline conditions can disrupt the intermolecular bonds in dentin collagen fibrils, thereby releasing proteins bound to the collagen [32]. The chondrogenesis-inducing effect of TGFβ1 is reportedly dose related. After the addition of 10 ng/mL TGF-β1 to the cartilage microsphere culture model for 21 days, the cartilage matrix was completely and uniformly formed inside the microsphere, whereas in the cartilage induction cultures containing 1 ng/mL and 0.5 ng/mL TGF-β1 in the medium, only the outer part of the cartilage extracellular matrix was formed [33]. An IGF-1 concentration less than 1 ng/mL had no obvious effect on promoting chondrocyte proliferation, but this effect increased at concentrations greater than or equal to 10 ng/mL [34]. Thus, the upregulation of these chondrogenesis-related GFs in a-DMEP may increase the chondrogenesis-inducing ability of the fungus.

DMEP, as a cocktail of concentrated compound GFs, may have synergistic chondrogenic effects superior to those of a single GF. Our results showed that a-DMEP had a stronger chondrogenic ability than did the equivalent amount of exogenous rhTGFβ1. Furthermore, the TGF-β1 concentration in the a-DMEP group was only 1.17 ng/mL, but the a-DMEP-induced chondrogenic pellets were larger than those in the positive control group treated with 20 ng/mL TGF-β1. Cartilage repair and regeneration are the result of the combined action of a variety of biological factors. When IGF-1 is combined with TGF-β superfamily GFs (TGF-β1, BMP2, BMP7, etc.), cartilage matrix synthesis significantly increases [34,35]. The combined use of IGF-1 and TGF-β1 can regulate the proliferation and differentiation of periosteal mesenchymal cells during chondrogenesis and maintain type II collagen mRNA expression for 2–6 weeks [36]. FGF-18 and TGF-β3 have synergistic effects on chondrogenesis and differentiation, and their effects are better than those of TGF-β3 alone [37]. Treatment with 10 ng/mL bFGF and 10 ng/mL TGF-β3 can effectively induce the chondrogenic differentiation of adipose stem cells, while bFGF itself does not promote differentiation at this concentration [38]. Therefore, the synergistic effect of cocktail GFs in DMEP makes them attractive for inducing cartilage tissue repair.

The results of our animal studies demonstrated that hyaline-like cartilage with abundant GAG was regenerated in a-DMEP-transplanted defects. The osteochondral defects we used offered an open environment for recruiting cells from the subchondral bone marrow, joint cavity, or synovium. Articular cartilage repair based on the recruitment of endogenous stem cells, such as synovium stem cells (SSCs) and bone marrow mesenchymal stem cells (BMSCs), and the generation of cartilage tissue under the action of specific cues is a promising strategy for treating cartilage diseases [39,40]. Several chemokines and GFs in DMEP are known to recruit a great variety of progenitor and stem cells [41]. Our previous study showed that DMEP could promote the chemotaxis of hBMMSCs [14]. Because of the potential of a-DMEP to recruit host endogenous stem cells, this approach does not require additional cell transplantation.

This study yielded promising results regarding the potential of dentin matrix proteins for application in cartilage repair and regeneration. However, importantly, DEMP also contains several ingredients that may have potentially adverse effects, such as MMP13, which may lead to chondrocyte hypertrophy. It is important to elucidate the molecular and biological mechanisms underlying the chondroinductivity of DMEP, with the aim of designing innovative strategies for cartilage tissue engineering applications.

5. Conclusion

We demonstrated that a-DEMP improved the chondrogenic phenotype of hBMMSCs and induced hyaline-like cartilage regeneration *in vivo*. These findings suggest that dentin is a promising source of natural GFs for cartilage repair and that alkali treatment is an effective method for extracting chondrogenesis-inducing mixtures from dentin.

Author's contributions

Sainan Wang: Conceptualization, Funding acquisition, Investigation, Formal analysis, Writing-original draft.

Sicong Mao: Investigation, Formal analysis, Writing - original draft.

Guibin Huang: Investigation.

Peipei Jia: Investigation.

Yanmei Dong: Supervision, Writing-review & editing, Project administration.

Jinxuan Zheng: Conceptualization, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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