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ORIGINAL ARTICLE

Cloning, purification and characterization of human dentine matrix protein 1(DMP1)

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KEYWORDS

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Abstract *Introduction:* Human teeth are composed mainly of dentin, formed by the odontoblasts. Dentin matrix protein 1 (DMP1) is one of odontoblast differentiation's most important growth factors. Human DMP1 has yet to be completely identified or studied. This study aimed to clone and characterize human DMP1.

Materials and methods: The DMP1 gene sequence was prepared and cloned by transfection of human 293 cells.

Results: The recombinant DMP1 was purified and characterized.

Conclusion: The results suggested its future use in dental tissue regeneration and tissue engineering.

1. Introduction

Dentin matrix protein one (DMP1) is one of five non-collagenous proteins found in the extracellular matrix of dentin and bone (Ahmad et al., 2019; Ravindran & George, 2014; Ten Cate, 1992; D'Souza et al., 1997; Kulkarni et al.,2000; Moses et al, 2006; Chaussain et al.2009). DMP1 is a small integrin-binding ligand N-linked acidic glycoproteins (Moses et al, 2006). DMP1 regulates intra and extracellular biochemical cascades (Kulkarn et al., 2005)[5]. DMP1 regulates osteoblast differentiation (Chaussain et al.2009)[7] and

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hydroxyapatite crystal growth during bone, enamel, and dentine formation (George et al, 1993; He et al, 2003))'. DMP1 is important during mineralized tissue formation and its loss causes rickets and osteomalacia (Hao et al, 2004; Feng et al, 2006).DMP1 is synthesized as a full-length 105 Kilo Dalton (KDa) proprotein and is divided into a 37 KDa N terminal fragment and a 57 KDa C terminal fragment (Feng et al, 2006; Ling, et al., 2005; Lu, e al., 2008; Qin, 2006). DMP1 is important in initiating dentin mineralization (He et al, 2003). DMP1 is important in alveolar bone and periodontal cell differentiation (Zhang, et al. 2008). A previous study has reported cloning and purification of recombinant DMP1; however, it was unclear if that produced recombinant DMP1 was for humans or animals (Srinivasan, et al, 1999). Therefore, this study aimed to clone and characterize human DMP1.

2. Materials and methods

2.1. The summary of the procedure is depicted in the schematic Fig. 1

This work was performed at the University of Alberta, Canada in collaboration with Rambam Health Center, Israel.

2.2. Transformation of competent cells and culturing of the single colonies

The plasmid carrying the human DMP1 gene (Trueclone, Origene, Rockville, MD, USA) was prepared according to the manufacturer's recommendation. An empty vector (Trueclone pCMV6-Neo vector, Origine, Rockville, MD, USA) was used as a control.

2.3. Plasmid DNA purification using miniprep

According to the manufacturer's recommendation (Purelink^{TM} quick plasmid Miniprep kit, Invitrogen, Burlington, ON., Canada), cell lysate preparation was followed by the binding of the DNA and finally eluting the DNA.

2.4. Quality assessment of the DNA

Not1enzyme (Invitrogen, Burlington, ON., Canada) was used to prepare the DNA insert. The DNA ladder (Qiagen, Venlo, The Netherlands) was used as a standard. The gel was stained with Ethidium Bromide and visualized by UV light. The results were compared to those of the manufacturing company (Origen, Rockville, MD, USA), the *BLAST* gene bank (Basic local alignment search tool) and *ExPASy* (Expert Protein Analysing System), Proteomics server (the Swiss Institute of Bioinformatics). The entire process was repeated using Maxiprep (Prelink TM pure plasmid DNA purification kit, Invitrogen, Burlington, ON., Canada) to obtain more DNA. Using the spectrophotometer (Beckman Coulter), readings were recorded. The amount of the recovered DNA was quantified on agarose gel. No1 digestion confined that the insert is present.

2.5. FS293 cells transient transfection for protein expression

FS293 cells (Freestyle TM 293 Expression System, Invitrogen Burlington, ON., Canada) were suspended and grown. Once the required numbers of cells (90% viable) were obtained, transfection began. First, 30 μg of the plasmid DNA was diluted in Opti-MEM 1 to a total volume of 1 ml. Then 40 μl of 293 fectin in Opti-MEM 1 was diluted to a total volume of 1 ml (add to 960 μl in a sterile tube). Finally, the diluted DNA was added to the diluted 293 fectin to obtain a total volume of 2 ml. The complex was then incubated, and the suspension was centrifuged. SDS-PAGE blot was done on the supernatant to evaluate the expression of DMP1, and then a large-scale (600 ml) transfection was performed.

2.6. Partial purification of DMP1

This was performed using DEAE-Sephacel (Sigma-Aldrich, Burlington, Massachusetts, USA) column equilibrated in 0.05 M Tris-HCl, 0.01 M NaCl, pH 7.4 (Tris buffer) at 10°C. The column was eluted using a linear gradient of 0.01–0.8 M NaCl in Tris buffer. The absorbance of eluted proteins was monitored at 230 nm (Holochrome monitor). Fractions (LKB fraction collector) were collected (2.4 ml), and those containing DMP1 were identified using Dot blotting.

2.7. Dot blotting

The control sample (FSEM containing DMP1) was added. Antibody staining was done using the primary antibody (LF 148 or Takkarra Inc., San Jose, CA, USA). The secondary antibody (Anti-rabbit IgG, Sigma-Aldrich, Burlington, Massachusetts, USA) was used. According to the manufacturer's manual, the antibody-positive fractions containing DMP1 were pooled and concentrated using Amicon Ultra-15 Filter Devices (Millipore 10,000NMWL). BCA protein Assay Kit (Pierce, Rockford, Illinois, USA) was used to quantify the amount of protein present in the pooled fractions by measuring the absorbance at or near 562 nm on a plate reader.

2.8. Protein visualization using SDS-PAGE and Western Blotting

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis was performed using NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen, Burlington, ON., Canada). Two staining techniques were used; the Coomassie blue was used to visualize the protein bands and estimate their molecular weight. The second technique, Stains All (Sigma-Aldrich, Burlington, Massachusetts, USA), was used to differentiate between acidic and glycoproteins which stain blue and other classes of proteins which stain red. Following SDS-PAGE, a gel was prepared for electroblotting.

3. Results

3.1. Assessment of DNA

The DNA was isolated from the cell using miniprep and maxiprep. The purified DNA was cleaved by the Not1 restriction enzyme to produce a full-length insert coding for the DMP1 gene. The cleaved DNA was applied on an agarose gel which following electrophoresis, illustrated the Not1-cleaved and uncleaved DNA bands (Fig. 2). The size of the DMP1 gene

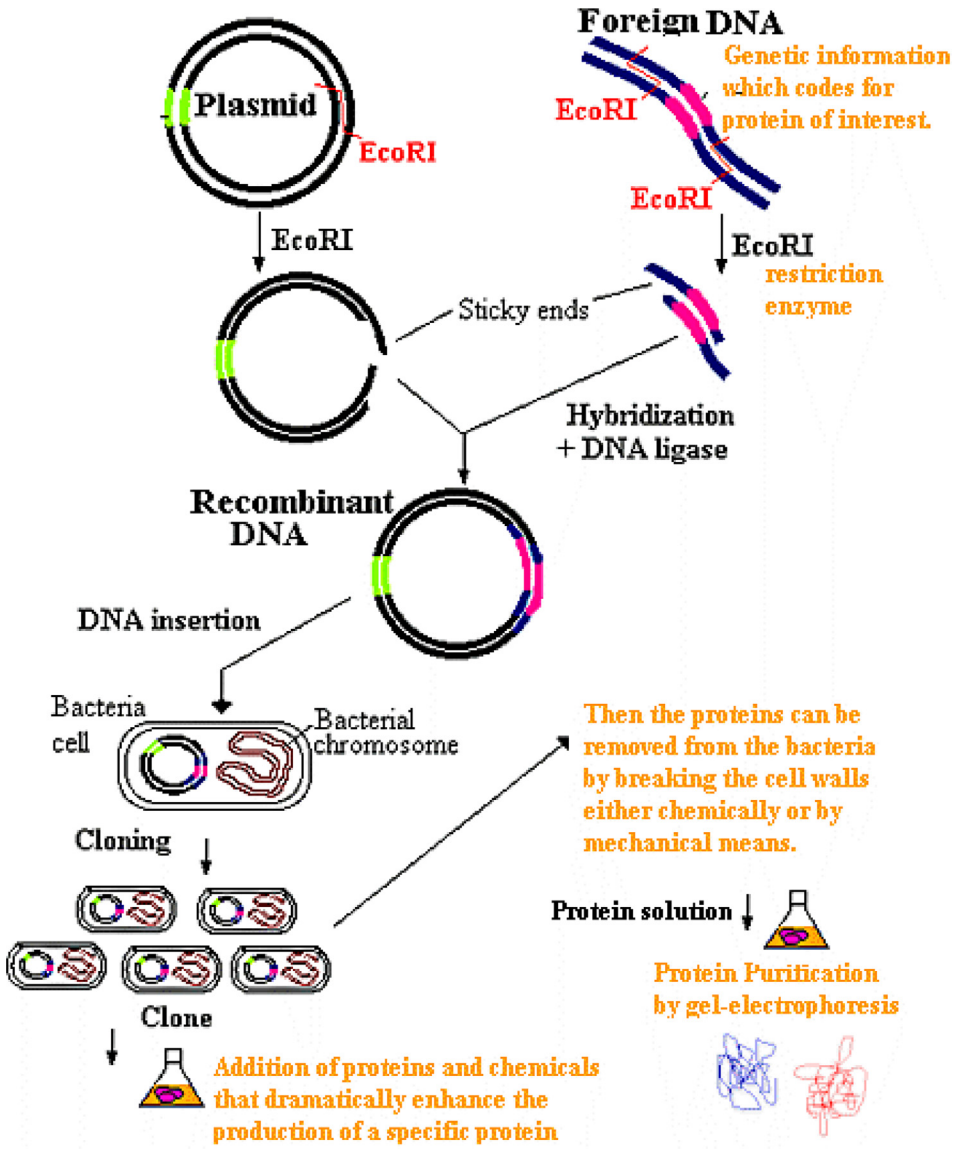

Fig. 1 Schematic representation of the methods. First, the plasmid and the DMP1 gene were transfected into the bacteria to perform recombinant DNA. Then the DMP1 DNA was purified, and its quality was assessed. Then the DMP1 DNA was transfected into bacterial cells for cloning and production of the DMP1 in large quantities. Once the protein has been produced, it has been purified and characterized.

was confirmed by the DNA ladder (Qiagen, Venlo, The Netherlands) as 1.5 kb. DNA quantification was estimated by spectrophotometer for miniprep and 1.78 µg/µl for maxiprep.

3.2. Characterization of DMP1

DMP1 protein was characterized using SDS-PAGE and Western Blot. The coomassie-stained SDS-PAGE gel demonstrated a band of 57kD in both lane 3 (empty vector) and lane 4 (DMP1 vector) corresponding to the size of the C-terminal portion of DMP1 but showed no band in the lane 1 (medium) and lane 2 (only cells). The proteins were then transferred to a PVDF membrane and exposed to anti DMP1 antibody (LF 148). The only band corresponding to the size of DMP1 protein appeared in lane 4, indicating that the band seen in lane 3 of SDS-PAGE was not DMP1 protein (Fig. 3A, B).

Two SDS-PAGE gels were loaded with the samples and stained with Commassie blue or All Stains to differentiate between acidic proteins and phosphoprotein (blue) and other proteins that stain purple. The results indicated that the 57kD band belongs to the C-terminal part of the DMP1 protein (Fig. 3 C, D).

3.3. DMP1 characterization by phosphoserine and phosphoteronine

SDS-PAGE was set up for DMP1 and the gel was stained using Stains All kit. DMP1 appeared as a double band on the gel of the C-terminal portion of the protein (57kD). Another band located at 37kD corresponds to the

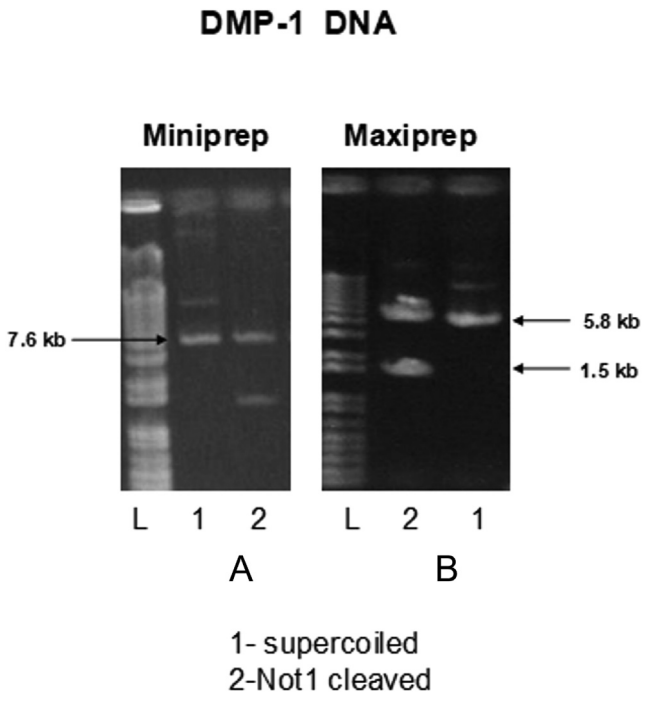

Fig. 2 A) Miniprep and B) Maxiprep is shown on the 1% agarose gel.

N-terminal portion of the DMP1 (Fig. 3 C, D). The gel was transferred to a nitrocellulose membrane for Western blot that was reacted with anti DMP1, anti phosphoserine, and anti phosphotheronine antibodies.

On the other hand, there are bands of ~ 100kD in sizes in lanes 2 and 3 corresponding to the full size of DMP1 protein. The reactivity of anti-phosphoserine and anti-phosphotheronine with DMP1 was primarily present for the whole DMP1. The comparison between the whole protein in lane 1 with lane 2 or 3 reveals that phosphorylation is not abundant, and the 52KD C-terminal part of DMP1 is not recognized by these anti-phosphor antibodies (Fig. 4 A, B).

3.4. Purification of DMP1 using DEAE column

DEAE column was connected to the holochrome monitor (230 nm, 0–2 absorbance). Eighty-five fractions were produced from 600 ml of the conditioned medium. Dot blotting of the fractions using anti DMP1 antibody (LF148) was performed to identify and exclude all non-DMP1-containing fractions (Fig. 4 C). A high-intensity dots corresponded to the fraction numbers 32–45.

3.5. BCA protein assay

The assay was performed to measure the protein concentrations of the sample fractions. First four fractions were pooled and concentrated using a filtration tube 10000NMWL (Amicon ultra-15, Millipore). Next, the volume and weight of the pooled fractions of each group were measured (Table 1). Then, four samples representing different fractions were applied to SDS-PAGE and transferred to the PVDF membrane. Samples number 1,2, and 3 illustrate a band at a size equal to the

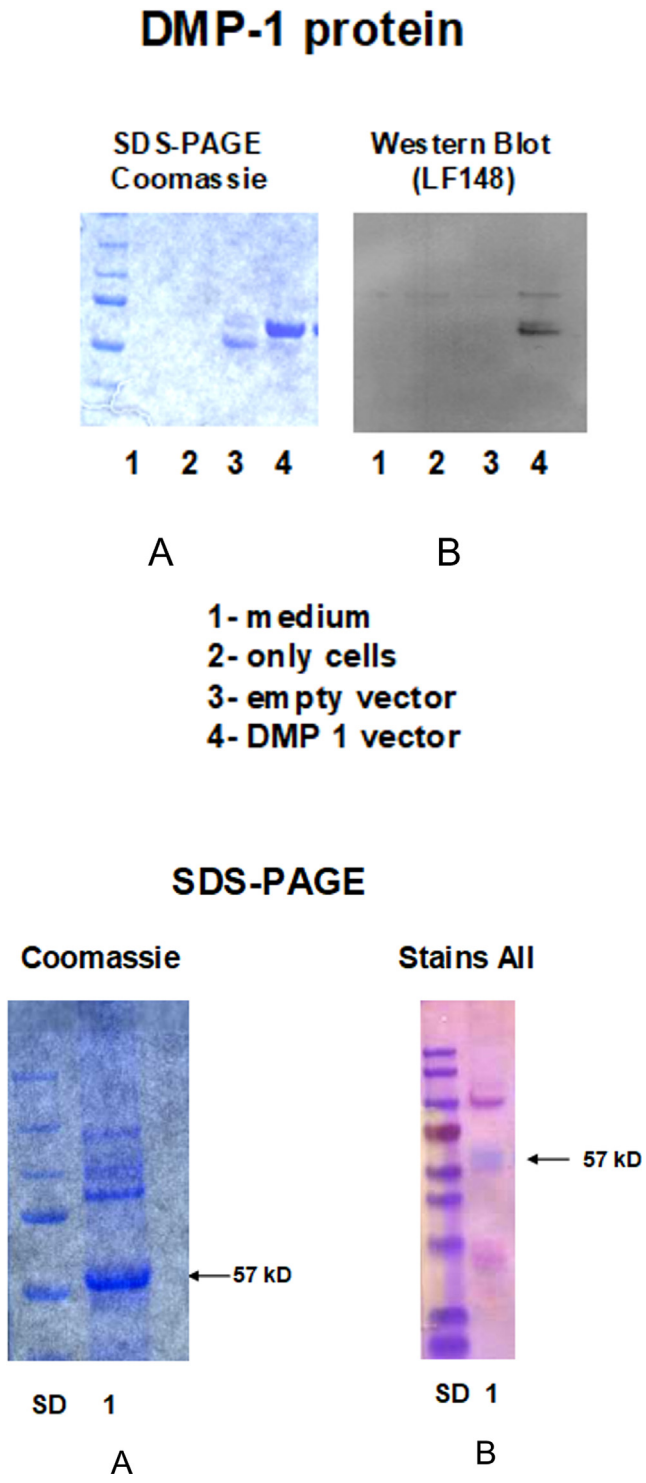

Fig. 3 *The 4–12% SDS-PAGE gels were loaded with preheated samples plus sample buffer. The prestained protein standard (SD) shown for the SDS-PAGE gel also corresponds to the band on the Western blot membrane. The membrane was subjected to anti DMP1 antibody (LF148) in a sealed bag and then similarly treated with the second antibody (anti-rabbit) before being developed by an Alkaline phosphatase detection kit (BCIP/NBT).* C) SDS-PAGE gels were stained by Commassie blue, and D) Stains All. In the Commassie, the C-terminal DMP1 protein is shown. In addition, the weak bluish DMP1 band stain (acidic) is shown on all gel.

C-terminal part of DMP1 (57kD), although this band is denser for sample number 2 (Fig. 4 D) (see Table 2.).

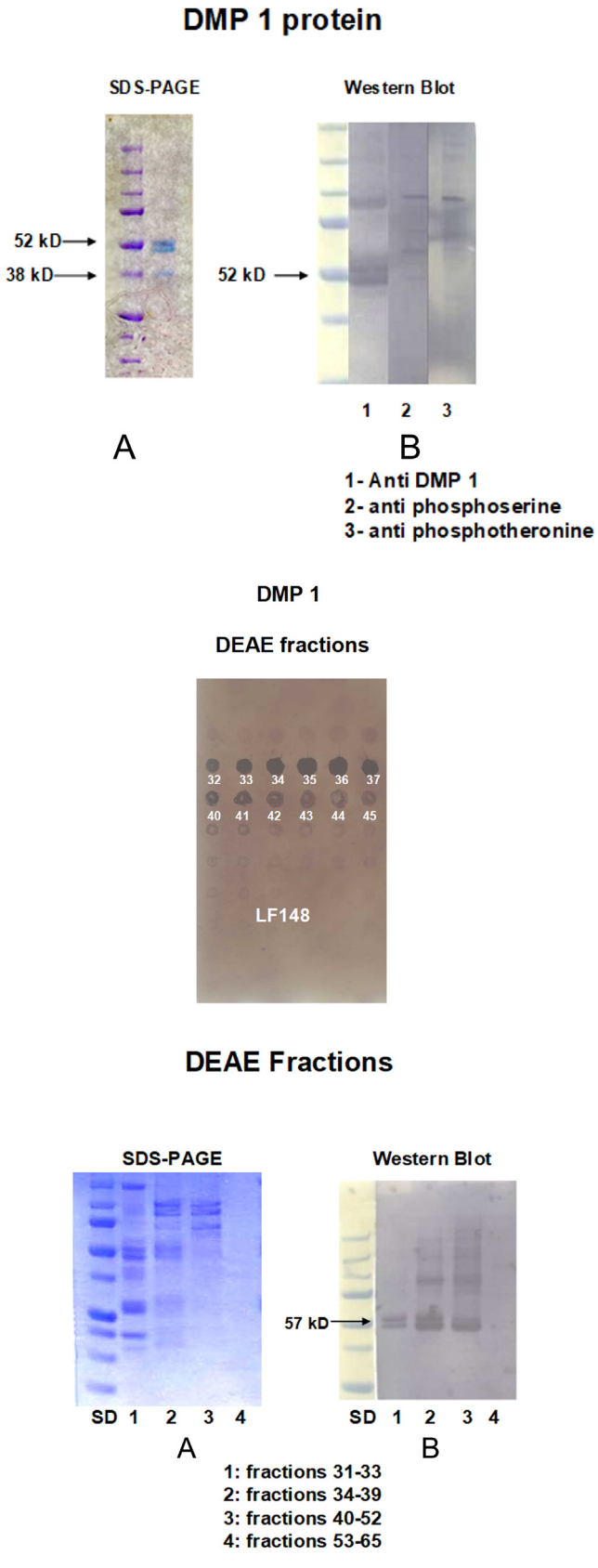

Table 1 Weight and volume of the pooled fractions.

Sample	Weight (gm)	Volume (µl)
1	0.932	930
2	1.04858	1048
3	1.27389	1270
4	2.280	2276

Table 2 Calculated concentrations sheet (raw data of BCA protein assay).

Sample number	1	2	3	4
A	12.073	8.010	2.635	0.413
B	9.298	6.328	3.204	0.401
C	11.255	7.168	4.567	0.50
D	2.238	6.375	11.255	
E	1.991	8.258	11.379	

4. Discussion

Although previous studies have reported on identifying, identifying, and sequencing of DMP1 in mice and rats, to our best knowledge, human DMP1 has not been cloned, purified or characterized yet. In the present study, human DMP1 has been cloned and characterized. The methodology used in this report is very similar to prior studies by George et al, 1993 and Srinivasan et al,1999. Our study results agree with the previous reports regarding the purified DMP1 molecular weight. Our study showed both C and N terminals expressed in the Coomassie blue and stained all stains, while the previous study by Srinivasan et al,1999 showed only DMP1 protein. Although our study is confirmatory to the previous studies, our study showed both C and N terminals expressions, indicating their possible future use in cell differentiation. Also, our study confirmed the production of DMP1 as indicated by its reaction to LF 148 or Takkarra DMP1 antibodies. DMP1 may provide a future solution to dental and alveolar tissue regeneration.

Limitations: Although this study is the first to report on the possibility of cloning and purification of human DMP1, we did not evaluate the potential differentiation of mesenchymal stem cells by the produced DMP1.Future studies may aim to evaluate similar products and optimize the produced DMP1 in differentiating undifferentiated cells into odontoblast-like cells.

Fig. 4 A) The SDS-PAGE gel stained with Stains All kit demonstrates the acidic portion of DMP1 protein as a bluish 57KD band. B) Western Blot showing protein expression against LF148 antibodies. C) DEAE fractioned sample dot blotted on a nitrocellulose paper. D) As indicated in the figure, the SDS-PAGE is loaded with the four samples corresponding to the pooled DEAE fractions. E) The Western blot anti-DMP1 antibody was used and showed a DMP1 fraction expressed at 57 KDa.

5. Conclusion

Human DMP1 has been successfully produced, purified and characterized. The produced DMP1 may provide a solution to dental and alveolar tissue regeneration.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sdentj.2023.05.006.

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