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The Enhancing Effects of Amelogenin Exon 5-Encoded Peptide from Enamel Matrix Derivative on Odontoblast-Like KN-3 Cells

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Featured Application: Amelogenin exon 5 could have potential for application in dental pulp capping.

Abstract: Enamel matrix derivative (EMD) is applied for periodontal therapy. We created a synthetic amelogenin peptide (SP) derived from EMD, and have previously investigated the biological function of SP. However, it is unknown whether SP affects odontoblastic differentiation. In this study, we tested the effects of SP in the odontoblast-like cells, KN-3 cells. KN-3 cells were cultured with SP (0 to 1000 ng/mL) and then cultured for 3, 8, 24, or 48 h in order to determine the effects of SP on cell proliferation and detect its optimum concentration. KN-3 cells were treated with SP in odontogenic differentiation medium cultured for 3 or 7 days. Odontogenic markers were measured by the detection of alkaline phosphatase (ALP) activity and dentin sialo phosphoprotein (DSPP) expression, the calcified nodule formation, and calcium deposition. The addition of SP significantly promoted cell proliferation at 100 ng/mL, generating the greatest change in cell proliferation. SP also showed increased odontogenic expression markers and mineralization. These results suggest that SP, derived from EMD, could have potential for application in dental pulp capping.

Keywords: emdogain; amelogenin; odontoblast; differentiation; mineralization

1. Introduction

Enamel matrix derivative (EMD) can induce the formation of hard tissue, such as alveolar bone and cementum tissue [1,2]. EMD is applied for periodontal therapy and bone regeneration. We previously showed that subcutaneous injections of EMD can induce the growth of cartilage tissue and eosinophilic round bodies (ERBs) [3]. We further analyzed these ERBs by using MALDI-TOF, and found fragments of exon 5 of amelogenin.

We synthesized a 7-amino acid (WYQNMIR) peptide based on these fragments and tested whether the synthetic peptide (SP) would behave similarly to EMD [4]. We found that the SP could induce bone-like tissue formation in artificial periodontal defects in rats [5,6]. Moreover, we found that SP could enhance the cell proliferation of periodontal ligament (PDL) cells [7] and enhance osteoblastic

differentiation in mesenchymal stem cells (MSCs) [8–10]. These findings also could help to clarify the biological functions of amelogenin exon 5.

EMD induces anti-EMD antibodies [11]. Only peptides of greater than approximately ten residues (or >5 kDa) can function as antigens [12,13]. SP is 7 amino acids long, with a mass of 1118 Da. Therefore, SP has no effect with respect to inducing an immunological response.

Dental caries, tooth fractures, and other types of dental trauma induce tooth loss. Direct pulp treatment requires materials that protect the pulp tissue but induce hard tissue formation, in order to repair and maintain dental pulp tissues [14–19]. This requirement has led to the design and introduction of new, bioactive agents for dental pulp tissue engineering materials.

To investigate dental pulp tissue regeneration, a rat odontoblast-like cell line, KN-3, was created by Prof. Kitamura and Prof. Nishihara [20]. KN-3 cells showed high levels of odontogenic expression and the ability to form calcium nodules [21]. KN-3 cells have also been used as an authentic control to study the differentiation of induced pluripotent stem (iPS) cells and embryonic stem (ES) cells into odontoblast-like cells [22,23].

The biological response to SP regarding odontogenic differentiation has not yet been investigated. In the present study, we evaluated the odontogenic effects of SP in KN-3 cells.

2. Materials and Methods

2.1. Cell Culture

The rat odontoblast-like cell, KN-3, was provided by Prof. Chiaki Kitamura and Prof. Tatsuji Nishimura (Kyushu Dental College). KN-3 cells were maintained as described previously. Twenty KN-3 cells were cultured in normal medium comprising Eagle's minimal essential medium (α-MEM) with 10% FBS (Gibco BRL, Life Technologies, Grand Island, NY, USA), 500 U/mL penicillin, 500 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque, Kyoto, Japan). For differentiation assays, KN-3 cells were incubated in medium containing 50 μM L-ascorbic acid 2-phosphate (Nacalai) and 10 mM β-glycerophosphate (Wako Pure Chemical Industries Ltd., Tokyo, Japan).

2.2. Cell Proliferation Assay

KN-3 cells were cultured in normal medium. After 24 h, the medium was changed to normal culture medium containing varying concentrations of SP (0 to 1000 ng/mL); KN-3 cells were incubated for 3, 8, 24, or 48 h. Cell proliferation was measured via the amount of formazan. We measured the absorbance and analyzed results using the SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA).

2.3. Morphological Analysis

KN-3 cells were incubated in normal culture medium containing SP (0 to 1000 ng/mL) for 48 h. The images were analyzed using the all-in-one fluorescence microscope (BZ-II, Keyence Corporation, Osaka, Japan).

2.4. Alkaline Phosphatase (ALP) Activity Assay

KN-3 cells were cultured with osteogenic medium for 7 days, and measurements were made at days 3 and 7. After cells were washed using PBS and lysed using 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), the activity of ALP was investigated by one-step p-nitro phenyl phosphate (pNPP) (Pierce Biotechnology Inc., Rockford, IL, USA). ALP activity was normalized to the quantity of DNA. DNA content was investigated by DNA assay kit (Invitrogen, Paisley, UK). Data were investigated using the SoftMax Pro software.

2.5. Extracellular Matrix Mineralization

For measurements of calcium production, KN-3 cells were melted using 10% formic acid, and then calcium deposition (Ca) was investigated by calcium detection kit (Wako). For qualitative

histology, cells were then stained using 1% alizarin red. Calcified nodules were captured using a BZ-II microscope (Keyence).

2.6. Quantitative Real-Time Polymerase Chain Reaction (PCR)

RNA was obtained using a kit (RNeasy Mini Kit; Qiagen, Venlo, The Netherlands). and then the extracted RNA was transcribed into cDNA using a kit (Prime Script Reagent kit, Takara, Kyoto, Japan). mRNA expression was examined by real-time polymerase chain reaction (PCR) assay. The expression of dentin sialoprotein (DSPP; Rn02132391_s1) was investigated in accordance with standard protocols.

2.7. Immunofluorescence Staining

KN-3 cells were fixed by 70% ethanol. The cells were treated using 0.2% Triton X-100, after which they were blocked using 3% bovine serum albumin (BSA) and then incubated using mouse anti-rat DSPP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After cells were washed with phosphate-buffered saline (PBS), they were incubated using a fluorescence-labeled secondary anti-mouse antibody (Santa Cruz). The samples were stained using DAPI solution (Dojindo Laboratory, Kumamoto, Japan). Images were captured using fluorescence microscope.

2.8. Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test using IBM SPSS. Significant differences ($p < 0.05$) were determined.

3. Results

3.1. Cell Proliferation

We first tested varying concentrations of SP on KN-3 cells to determine an effective concentration. We found that 100 ng/mL of SP significantly promoted KN-3 proliferation at 8, 24, 48, and 72 h (Figure 1A, $p < 0.05$). Based on these results, we chose 100 ng/mL SP as the optimal concentration for subsequent experiments.

3.2. Cell Morphology

In the SP control group (0 ng/mL SP), the cell morphology exhibited round-shaped morphology. On the other hand, the cell morphology exhibited spindle-shaped morphology in the SP 100 ng/mL group. Figure 2 indicates that the effect of SP on cell morphology.

3.3. ALP Activity

ALP activity in the SP-treated group was significantly increased after 7 days, compared with cells solely treated with differentiation media (Figure 3; $p < 0.05$).

3.4. Extracellular Matrix Mineralization

Calcified nodules were increased in the SP-treated group. Calcium deposition in the SP-treated group significantly increased after 7 days ($p < 0.05$). Figure 4 indicates that the effect of SP on extracellular matrix mineralization in KN-3 cells. Figure 4A shows the effect of SP on calcified nodule formation by Alizarin red staining. Figure 4B shows the effect of SP on extracellular matrix mineralization.

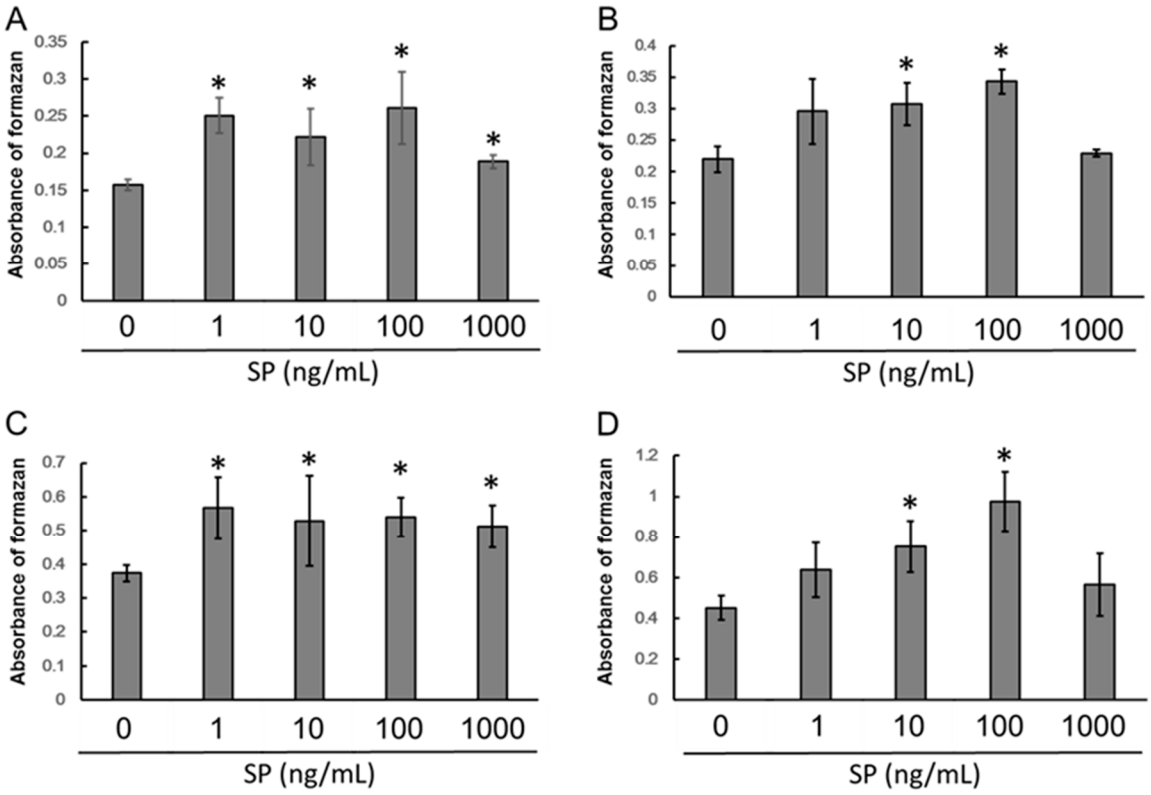

Figure 1. Effect of synthetic peptide (SP) on KN-3 cell proliferation. KN-3 cells were treated with 0 to 1000 ng/mL SP diluted in 100 μL culture medium. Cell proliferation was measured at 3, 8, 24, and 48 h. Significant differences (*, $p > 0.05$) were determined in comparison with the control (0 ng/mL SP). (**A**) 3 h, (**B**) 8 h, (**C**) 24 h, (**D**) 48.

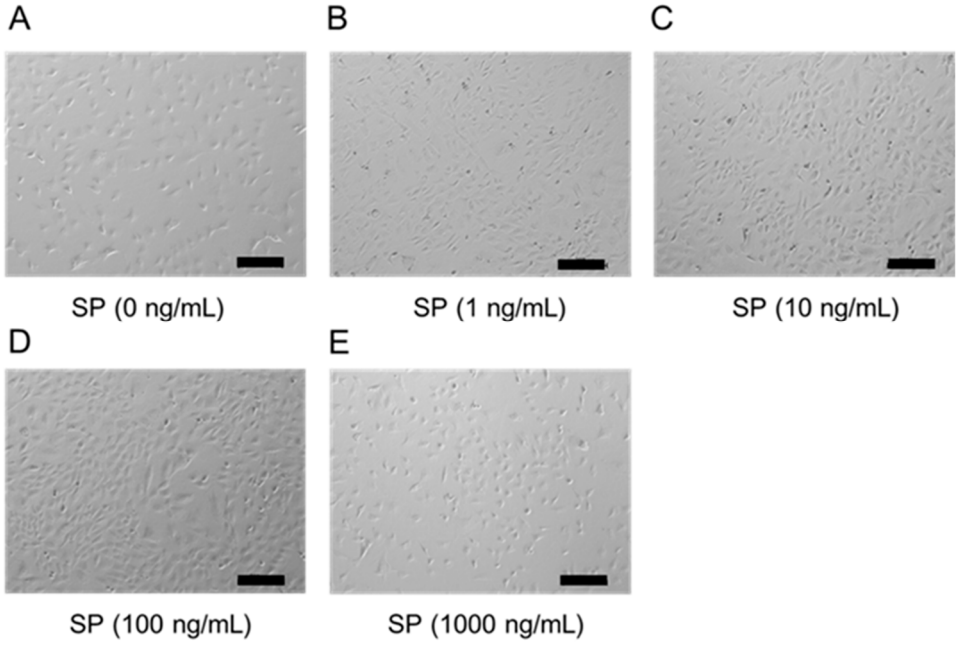

Figure 2. Effect of SP on cell morphology in KN-3 cells. Cell morphology in KN-3 cells treated with SP. Cells were cultured with SP (0 to 1000 ng/mL) for 48 h. Scale bar indicates 200 μm. (**A**) SP (0 ng/mL), (**B**) SP (1 ng/mL), (**C**) SP (10 ng/mL), (**D**) SP (100 ng/mL), (**E**) SP (1000 ng/mL).

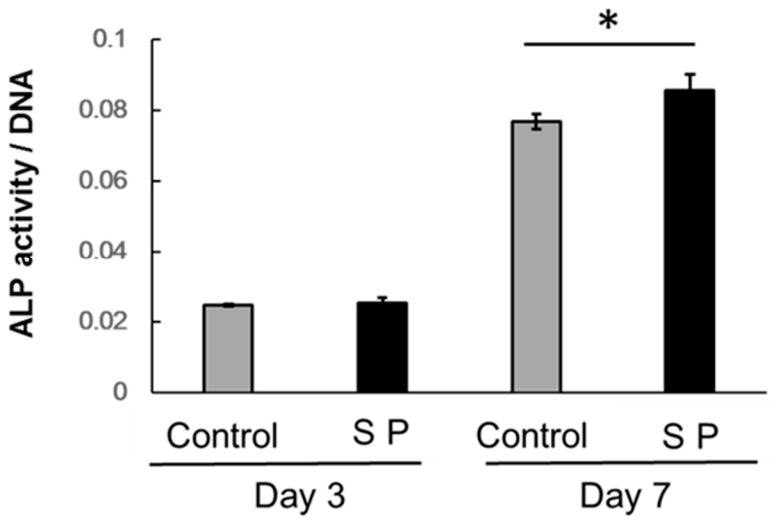

Figure 3. Effect of SP on alkaline phosphatase (ALP) activity. Confluent cells were treated with differentiation medium for 7 days (*, $p > 0.05$), and changes in ALP activity were investigated at days 3 and 7.

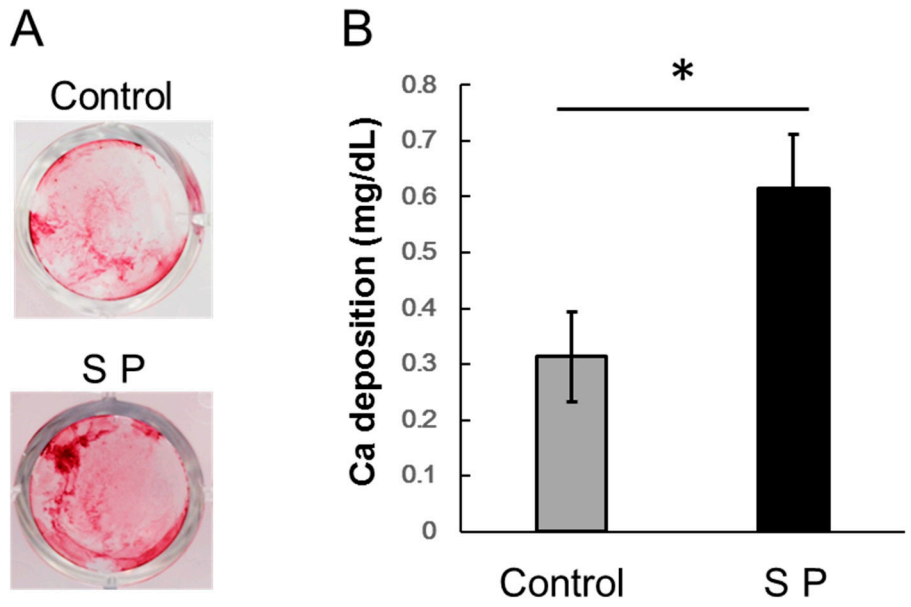

Figure 4. Effect of SP on mineralization in KN-3 cells. (**A**) Alizarin Red staining for detection of calcified nodule formation, (**B**) Measurement of extracellular matrix calcium deposition.

3.5. mRNA Expression of Dentin Sialoprotein (DSPP)

DSPP was significantly enhanced in the SP group at 1, 3, and 5 h ($p < 0.05$). Figure 5 indicates that the effect of SP on mRNA expression of DSPP.

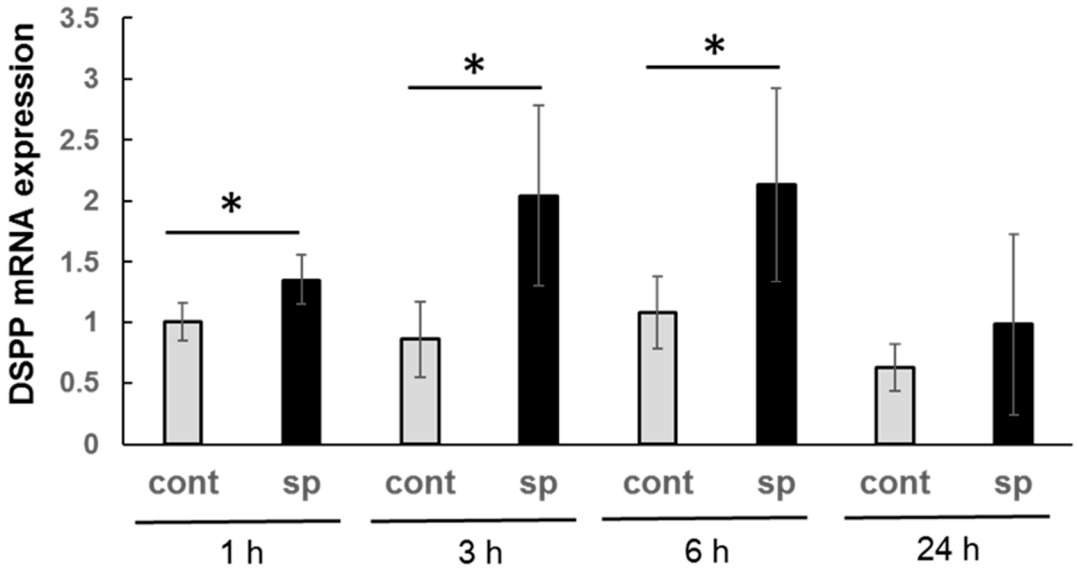

Figure 5. Effect of SP on odontoblastic differentiation in KN-3 cells. Quantitative real-time PCR analysis of dentin sialoprotein (DSPP) in KN-3 cells at 1, 3, 6, and 24 h.

3.6. Immunofluorescence Expression of DSPP

Sigillary of mRNA expression, the fluorescent intensity of DSPP by immunofluorescence staining was enhanced in the SP group. Figure 6 indicates that the effect of SP on immunofluorescence expression of DSPP in KN-3 cells.

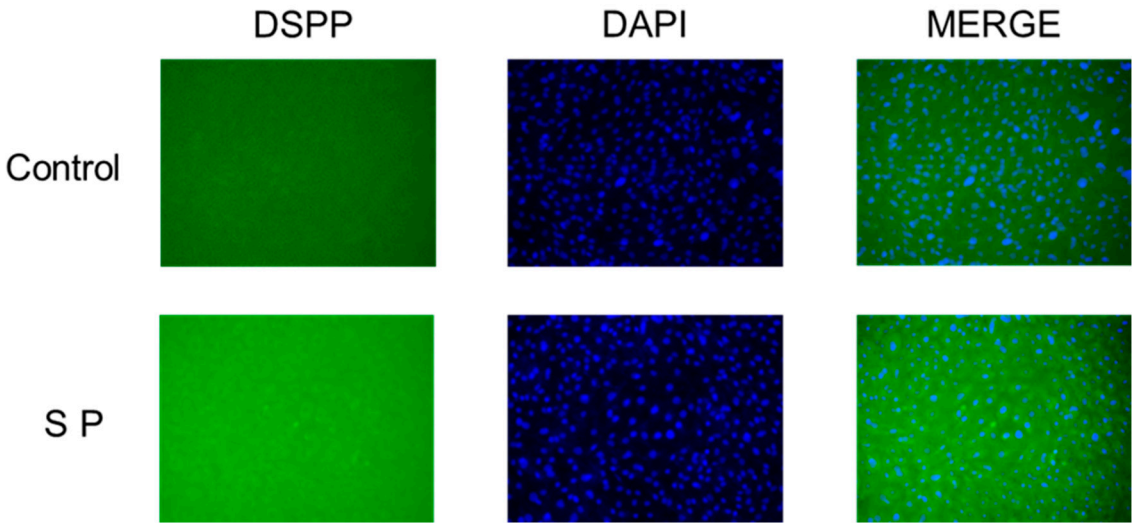

Figure 6. KN-3 cells treated with or without SP were incubated with anti-DSPP monoclonal antibody. Cells were incubated with a fluorescence-labeled secondary antibody. The samples were stained with DAPI solution. Scale bar = 100 μm.

4. Discussion

In this study, we found that SP enhances the proliferation and odontogenic differentiation of KN-3 cells.

EMD and amelogenin peptide promote the proliferation of dental tissue cells, such as PDL fibroblasts and bone marrow stromal cells (BMSCs) [24,25]. In a previous study, we showed that SP, derived from EMD, can also promote cell proliferation of human BMSCs [8]. However, the effects of SP on proliferation in odontoblasts had not been previously investigated.

We showed that SP promotes KN-3 proliferation at 100 ng/mL, similar to the optimal concentration for PDL stem cells [10]. Therefore, we further examined the odontogenic differentiation at this concentration of SP (100 ng/mL).

ALP is considered to be an odontogenic marker [26]. We found that SP enhanced ALP activity, which is similar to the effect of EMD in hard tissues and the effect of SP in KN-3 cells. Therefore, SP can also induce odontogenic differentiation in KN-3 cells.

We quantified calcium levels in cultures of KN-3 cells by using alizarin red staining and investigation of extracellular matrix calcium deposition. Alizarin red staining is performed for the detection of calcified nodules formed by osteoblasts or odontoblasts [27]. We qualitatively and quantitatively determined changes in mineralization in response to treatment with SP. We found that SP enhanced mineralized nodule formation stained with Alizarin red and calcium deposition in cultures treated for 7 days, compared with untreated cultures. Our previous study showed that SP can also promote mineralization of PDL stem cells [16]. Our results suggest that SP can be used for the dentin formation for earlier protection of dental pulp tissue.

We also investigated the effect of SP on odontogenic differentiation. We found that it impacted the levels of DSPP, which is a noncollagenous dentin matrix protein that is known as an early-stage marker of odontoblastic differentiation [28,29]. KN-3 cells cultured for 1–12 h expressed high levels of DSPP [30]. This exhibited that KN-3 cells have the ability to differentiate into odontoblasts. In this study, we showed that DSPP expression was upregulated by SP in KN-3 cells. These results indicate that SP promotes odontoblastic differentiation.

5. Conclusions

We found that SP, an amelogenin peptide derived from EMD, can enhance the proliferation, odontogenic differentiation, and formation of calcified nodules of KN-3 cells in vitro. Our findings suggest that SP could be a new biomaterial in dental pulp therapy. Moreover, the present study partially clarified the function of amelogenin exon 5 in odontogenesis.

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Conflicts of Interest: The authors declare no conflict of interest.

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