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Hakki, Sema S.; Bozkurt, Serife Buket; Sculean, Anton; Božić, Darko

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Hyaluronic acid enhances cell migration, viability, and mineralized tissue-specific genes in cementoblasts

Sema S. Hakki[1] | Serife Buket Bozkurt[2] | Anton Sculean[3] | Darko Božić[4]

[1]Department of Periodontology, Faculty of Dentistry, Selcuk University, Konya, Turkey

[2]Department of Biochemistry, Faculty of Medicine, Niğde Ömer Halisdemir University, Niğde, Turkey

[3]Department of Periodontology, School of Dental Medicine, University of Bern, Bern, Switzerland

[4]Department of Periodontology, School of Dental Medicine, University Clinical hospital, Zagreb, Croatia

Correspondence
Serife Buket Bozkurt, Department of Biochemistry, Faculty of Medicine, Niğde Ömer Halisdemir University, Niğde, Turkey.
Email: buketbozkurt@yahoo.com and buketbozkurt@ohu.edu.tr

Abstract

Background/Objectives: It has been repeatedly demonstrated that cementum formation is a crucial step in periodontal regeneration. Hyaluronic acid (HA) is an important component of the extracellular matrix which regulates cells functions and cell–cell communication. Hyaluronic acid/derivatives have been used in regenerative periodontal therapy, but the cellular effects of HA are still unknown. To investigate the effects of HA on cementoblast functions, cell viability, migration, mineralization, differentiation, and mineralized tissue-associated genes and cementoblast-specific markers of the cementoblasts were tested.

Materials and Methods: Cementoblasts (OCCM-30) were treated with various dilutions (0, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128) of HA and examined for cell viability, migration, mineralization, and gene expressions. The mRNA expressions of osteocalcin (OCN), runt-related transcription factor 2 (Runx2), bone sialoprotein (BSP), collagen type I (COL-I), alkaline phosphatase (ALP), cementum protein-1 (CEMP-1), cementum attachment protein (CAP), and small mothers against decapentaplegic (Smad) -1, 2, 3, 6, 7, β-catenin (Ctnnb1) were performed with real-time polymerase chain reaction (RT-PCR). Total RNA was isolated on days 3 and 8, and cell viability was determined using MTT assay on days 1 and 3. The cell mineralization was evaluated by von Kossa staining on day 8. Cell migration was assessed 2, 4, 6, and 24 hours following exposure to HA dilutions using an in vitro wound healing assay (0, 1:2, 1:4, 1:8).

Results: At dilution of 1:2 to 1:128, HA importantly increased cell viability ($p < .01$). HA at a dilution of 1/2 increased wound healing rates after 4 h compared to the other dilutions and the untreated control group. Increased numbers of mineralized nodules were determined at dilutions of 1:2, 1:4, and 1:8 compared with control group. mRNA expressions of mineralized tissue marker including COL-I, BSP, RunX2, ALP, and OCN significantly improved by HA treatments compared with control group both on 3 days and on 8 days ($p < .01$). Smad 2, Smad 3, Smad 7, and β-catenin (Ctnnb1) mRNAs were up-regulated, while Smad1 and Smad 6 were not affected by HA administration. Additionally, HA at dilutions of 1:2, 1:4, and 1:8 remarkably enhanced CEMP-1 and CAP expressions in a dilution- and time-dependent manner ($p < .01$).

Conclusions: The present results have demonstrated that HA affected the expression of both mineralized tissue markers and cementoblast-specific genes. Positive effects of HA on the cementoblast functions demonstrated that HA application may play a key role in cementum regeneration.

KEYWORDS
cell viability, cementoblast, hyaluronic acid, migration, mineralization, mRNA expression

1 | INTRODUCTION

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan that occurs naturally and its structure consists of polyanionic disaccharide units of glucuronic acid and N-acetyl glucosamine.[1] The periodontium, as well as the skin, joints, eyes, and the majority of other organs and tissues, contain HA, a significant natural carbohydrate component of the extracellular matrix. It is generated in the periodontium by HA synthase enzymes found in numerous cells such as cementoblasts, gingival fibroblasts, and osteoblasts.[2,3] Because HA is a crucial molecule in inflammation, epithelium formation, and tissue remodeling, it has been proposed that it also plays a role in periodontal wound healing.[4] It can regulate the inflammatory response because chronically inflamed tissue extensively degrades the high-molecular-weight HA produced by hyaluronan synthase enzymes in periodontal tissues, gingiva, periodontal ligaments, and alveolar bone. Additionally, HA prevents the breakdown of extracellular matrix by serine proteinases generated during the healing phase from inflammatory cells, thus reducing inflammation and stabilizing the granulation tissue.[5] Cementum has a similar structure to bone in that it is composed of a mineralized collagen matrix and contains non-collagenous proteins such as bone sialoprotein, osteocalcin (OCN), and osteopontin, but it lacks vasculature and innervation. Contrary to bone, cementum lacks bone marrow and has a minimal capacity for rebuilding.[6] Cementoblasts are the cellular elements of cementum, that produce hard tissue that connects to the alveolar bone via the periodontal ligament.[7] Under pathological conditions, cementum tissue has limited or no regeneration potential when compared to bone.[8] However, cementoblasts can be activated during wound healing and are very important for the development of cementum.[9] Cementum is anatomically a component of the tooth, functionally a component of the periodontium and its extracellular matrix shows similarities with other mineralized tissues.[10] However, cementum has a distinct physiology and contains molecules that have not been found in any other tissues. Periodontal cells respond differently to some types of periodontal cells than to others in response to cementum components, which affect their behavior.[9] Migration, adhesion, proliferation, and differentiation of several cell types occur during wound healing. When polypeptide mediators attach to their cell-surface receptors and when integrins bind to ECM elements, all of these processes are set into motion[11]. There is evidence to support each of these theories for how cementum constituents can control cellular functions. Cementum, for example, contains chemicals that stimulate chemotactic migration, adhesion, proliferation, and differentiation of certain periodontal cell types more than others, and these compounds are not found in other periodontal structures.[12–14] According to earlier research, HA serves a wide range of purposes. For example, it helps to maintain the integrity of tissues in terms of osmotic pressure and tissue lubrication as well as the viscosity of joint synovial fluid by acting as an anti-inflammatory during the healing of oral wounds.[15–18] Hyaluronic acid has become widely employed in dental treatments because of its biocompatible properties and involvement in cellular mechanisms associated with tissue repair.[1] In the periodontium, hyaluronic acid is expressed in various cells and is a major component of the extracellular matrix.[19] Its major receptor CD44, a cell surface molecule, is expressed both in cementoblasts[20] and periodontal ligament cells where it plays an important role in periodontal ligament mineralization[21] indicating that HA could have a potential role in periodontal regeneration by attaching to CD44 in these cells.[22] Further support in using HA in periodontal regeneration comes from recent animal histological studies that investigated recession, intrabony, and furcation defects which showed that the application of HA by itself or with a carrier induced a significant amount on new cementum and periodontal ligament regeneration compared to the control group.[23–25] Additionally, several recently published human clinical trials have shown that HA significantly improves pocket depth reduction and clinical attachment gains when applied in intrabony defects.[26–28] Therefore, current evidence shows that HA has a positive effect on periodontal regeneration; however, nothing is known about the molecular mechanisms of how HA affects cementoblast functions.

The hypothesis of the present study is that HA would have biological functions on cementoblasts. Therefore, in this study, we aimed to assess the impact of HA on cell viability, migration, mineralization, expression of mineralized tissue-associated genes (COL I, BSP, Runx2, ALP, OCN), cementoblast-specific markers (CEMP-1 and CAP genes), and Smad genes which are the main signal transducers of the transforming growth factor-beta superfamily member's receptors in cementoblasts. To the best of our knowledge, this is the first in vitro study examining the effects HA has on cementoblast functions in vitro.

2 | MATERIALS AND METHODS

2.1 | Cementoblast (OCCM-30) source

The immortalized mouse cementoblasts (OCCM-30) given by Dr. Martha J. Somerman were used in this study. The method for characterization of OCCM-30 was defined by D'Errico et al.[29]

Twenty-fourth passage cementoblasts cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA) 600 mg/mL L-glutamine*, 100 U/mL penicillin*, and 125 mg/mL streptomycin (Gibco, USA) in an incubator with 5% CO_2.

2.2 | Hyaluronic acid (HA) application

Hyaluronic acid used as hyaDENT BG (BioScience GmbH, Germany) and hyaDENT BG contains 2.0 mg/mL of hyaluronic acid and 16.0 mg/mL of hyaluronic acid cross-linked with sodium chloride. The eight groups were evaluated as follows: (1) control (only DMEM) (2) dilution of HA (1:2), (3) dilution of HA (1:4), (4) dilution of HA (1:8), (5) dilution of HA (1:16), (6) dilution of HA (1:32), (7) dilution of HA (1:64), and (8) dilution of HA (1:128). In short, HA was diluted in 5% FBS-DMEM to make experiment groups that contained (1:2), (1:4), (1:8), (1:16), (1:32), (1:64), and (1:128), and these dilutions were used to treat the cementoblasts. For this purpose, (1:2), (1:4), (1:8), (1:16), (1:32), (1:64), and (1:128) dilutions were applied for cell viability assay. Additionally, cementoblast cells were treated with (1:2), (1:4), and (1:8) dilutions of HA for cell migration assay, mineralization, and real-time polymerase chain reaction (RT-PCR).

2.3 | Cell viability assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) test was used to investigate the impact of HA on the cementoblasts on 1 and 3 days. A 200 μL cell solution containing 5×10^3 cells from the twenty-fourth passage was trypsinized for this experiment and then placed into each well. After 24 h, (1:2), (1:4), (1:8), (1:16), (1:32), (1:64), and (1:128) dilutions of HA were applied to cementoblasts. No HA was administered to the controls. 1 day following the HA treatment, MTT was applied to the wells, followed by a 2-h incubation period. Following incubation, 200 μL of dimethyl sulfoxide was added to each well to create blue formazan, the optical density of which was determined at 540 nm. Using the same methodology, cell viability on 3 day was also measured.

2.4 | Cell migration: wound healing template

The wound healing migration experiment was carried out in cell culture dishes of 60 mm in diameter. In each cell dish, 5×10^4 cementoblasts were cultured overnight in 4 mL of DMEM with 10% FBS. When the cells were >90% confluent, a vertical wound was created with 1000 μL of a sterile pipette tip. The first wound pictures were captured using an inverted microscope (0 h). Following that, the cementoblasts were treated with 4 mL of fresh DMEM containing 5% FBS and different HA dilutions (1:2, 1:4, 1:8). Cell migration into the wound region was imaged using an inverted microscope after 2, 4, 6, and 24 h of incubation in 60-mm cell culture dishes. The wound distances were measured, and cell mobility was calculated.

2.5 | Effects of hyaluronic acid on cementoblast mineralization

Von Kossa staining was used to assess mineralization of extracellular matrix in cementoblast cell culture on day 8. In 24-well plates with 5×10^4 cells/cm^2 of DMEM containing 5% FBS for 24 h, the following variables were introduced to the cells:

(a) 5% FBS DMEM (negative control),

(b) 5% FBS DMEM + Mineralization Media [MM = ascorbic acid (AA, 50 μg/mL) (Sigma Aldrich, St. Louis) and β-glycerophosphate (Sigma Aldrich, St. Louis, Missouri, USA) (BGP, 10 mM)] (positive control),

(c) 5% FBS DMEM + MM + 1:2 HA,

(d) 5% FBS DMEM + MM + 1:4 HA,

(e) 5% FBS DMEM + MM + 1:8 HA.

Cells were rinsed twice with PBS before being fixed in 100% ethanol for 1 h at 37°C and washed in a declining alcohol series (90% to 50%) to deionized water. After 15 min of dark incubation at 37°C with 5% $AgNO_3$ (Sigma Aldrich), cells were rinsed with deionized water. Photographs were taken after plates were exposed to fluorescent light for 20 min.

2.6 | Analysis of mRNA expression levels

For mRNA expression levels of BSP, OCN, COL-I, ALP, RunX2, Smad 1, 2, 3, 6, 7, β-catenin (Ctnnb1), CEMP-1, and CAP, cementoblasts were treated with 1:2, 1:4, and 1:8 dilutions of HA and total RNA was isolated from cells on days 3 and 8. The EZ-RNA Total RNA Isolation Kit's (Kibbutz Beik Haemek, Israel) recommended methodology for RNA extraction was followed. Utilizing a complementary DNA (cDNA) synthesis kit (Applied Biosystems High-Capacity RNA-to-cDNA kit, Foster City, USA) first-strand complementary DNA was produced from 1.0 μg of total RNA. Real-time PCR was carried out with 1.0 μL of cDNA in a total volume of 25 μL using the Brilliant SYBR Green Q-PCR Master Mix (Agilent, USA). Analysis of the melting curves of the PCR products was done to see whether any nonspecific PCR amplifications had occurred. The primer sequences are shown in Table 1. The amplification profiles of OCN, $RunX_2$, BSP, COL-I, ALP, and GAPDH used for the BIORAD-CFX Connect[§] were 94/180; 94/45, 55/45, 95/54, 55/30, and 95/30 [temperature (°C)/time (s)], respectively, for 35–40 cycles. The amplification profiles for Smad 1, 2, 3, and GAPDH performed with the BIORAD-CFX Connect were 94/180; 94/45, 55/45; 95/60, 55/30, and 95/30 [temperature (°C)/time (s)], respectively, for 35–40 cycles. The amplification profiles for Smad 6, 7, β-catenin (Ctnnb1), and GAPDH performed with the BIORAD-CFX Connect were 94/180; 94/45, 57/45; 95/60, 55/30, and 95/30 [temperature (°C)/time (s)], respectively, for 35–40 cycles. The amplification profiles for CEMP-1, CAP,

TABLE 1 Primer sequences for mineralized tissue-associated genes and cementoblast-specific genes for mouse.

Primer	Forward	Reverse
COL-I	GCAACATTGGATTCCCTGGACC	GTTCACCCTTTTCTCCCTTGCC
BSP	GAGACGGCGATAGTTCC	AGTGCCGCTAACTCAA
OCN	TGAACAGACTCCGGCG	GATACCGTAGATGCGTTTG
RunX2	CTTCATTCGCCTCACAAAC	GTCACTGCGCTGAAGA
ALP	ATTGCCCTGAAACTCCAAAACC	CCTCTGGTGGCATCTCGTTATC
CAP	TCTGACGACTCTGCTTCACG	TTCAGGGCATGTGTGATGCT
CEMP-1	ACCAAGAGTGCTTCCCCCACAC	CCCATGTGGCAAACACGGGC
Smad 1	ACCTGCTTACCTGCCTCCTGA	GCAACTGCCTGAACATCTCCTCT
Smad 2	TTCACAGACCCATCAAACTCGG	CTATCACTTAGGCACTCAGCAAACA
Smad 3	GACCACCAGATGAACCACAGCA	TAGGAGATGGAGCACCAGAAGG
Smad 6	AGGTGTTCGACTTTGAGCGC	CAGGAGGTGATGAACTGTCGC
Smad 7	GCTGTCCAGATGCTGTACCTTCC	GAGTCTTCTCCTCCCAGTATGCC
β-catenin (Ctnnb1)	GGGTCCTCTGTGAACTTGCTC	TGTAATCCTGTGGCTTGTCCTC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

and GAPDH used for the BIORAD-CFX Connect were 94/180; 94/45, 58/45; 95/60, 55/30, and 95/30 [temperature (°C)/time (s)], respectively, for 35–40 cycles.

2.7 | Statistical analysis

A one-way analysis of variance (ANOVA) with Dunnett's test and Tukey's HSD test was used to assess the cell viability and RT-PCR results. The data are displayed as mean and standard deviation. A p value of $< .05$ was regarded statistically significant, while a value of $< .01$ was regarded highly significant.

3 | RESULTS

3.1 | Hyaluronic acid positively affected the cell viability of cementoblasts

To investigate the effects of HA on cell viability, cementoblasts were cultured for 1 and 3 days with hyaluronic acid at dilutions ranging from 1:2 to 1:128. Cell numbers and the average of viability cells were analyzed at each time point for each dilution (Figure 1). HA dilutions of 1:2 or higher resulted in increased cell viability after 1 day of treatment to control group (Figure 1A). All dilutions of HA increased cementoblasts cell viability ($p < .001$) in a dilution- and time-dependent manner (Figure 1). From day 3, analysis of cementoblasts displayed a higher number of cell viability when stimulated with higher HA dilutions (1:2, 1:4, 1:8) (Figure 1B). Lower dilutions (1:16, 1:32, 1:64, 1:128) resulted in cementoblast cell viability to remain at the same level as the control group (Figure 1B). Thus, all dilutions of HA seem to promote the viability potential of cementoblasts in the short-term and this effect of HA on cell viability of cementoblasts was determined especially high dilutions in the long-term.

3.2 | The effect of HA on cell migration of cementoblasts

After the initiation of a vertical wound and addition of various HA dilutions (1:2, 1:4, 1:8), photographs were taken over several hours (0, 2, 4, and 6 h) and overnight (Figure 2A). A comparative analysis of the data demonstrated that all dilutions of HA had the same effect as compared with control group ($p > .05$) (Figure 2B). As shown by the analysis of cementoblasts cell migration, cell migration (wound healing) was significantly higher 4 and 6 h after the initiation of the wound in the cells exposed to 1:2 and 1:4 HA ($p < .01$ and $p < .01$, respectively) (Figure 2B). As compared with the control, at HA dilutions of 1:2, 1:4, and 1:8, cell migration was significantly higher 24 h after the initiation of the assay ($p < .05$). These results demonstrated that HA upregulated cementoblasts migration in a dilution- and time-dependent manner.

3.3 | Mineralization

Figure 3 shows a significant increase in nodule number as quantitated using image analysis in the cultures treated with 1:2, 1:4, and 1:8 HA with ascorbic acid plus bGP compared with control (MM) or cultures treated only with DMEM (no MM). A 1.7-fold increase in the number of nodules was observed in cultures treated with 1:2 HA compared with control (MM). Also, we determined a 1.8-fold increase and a 1.9-fold increase in the number of nodules in cultures treated with 1:4 and 1:8 HA compared with control (MM).

3.4 | Hyaluronic acid regulation of cementoblasts mineralized tissue-associated gene expression

Real-time PCR of genes coding for the mineralized tissue-associated markers osteocalcin (OCN), runt-related transcription factor

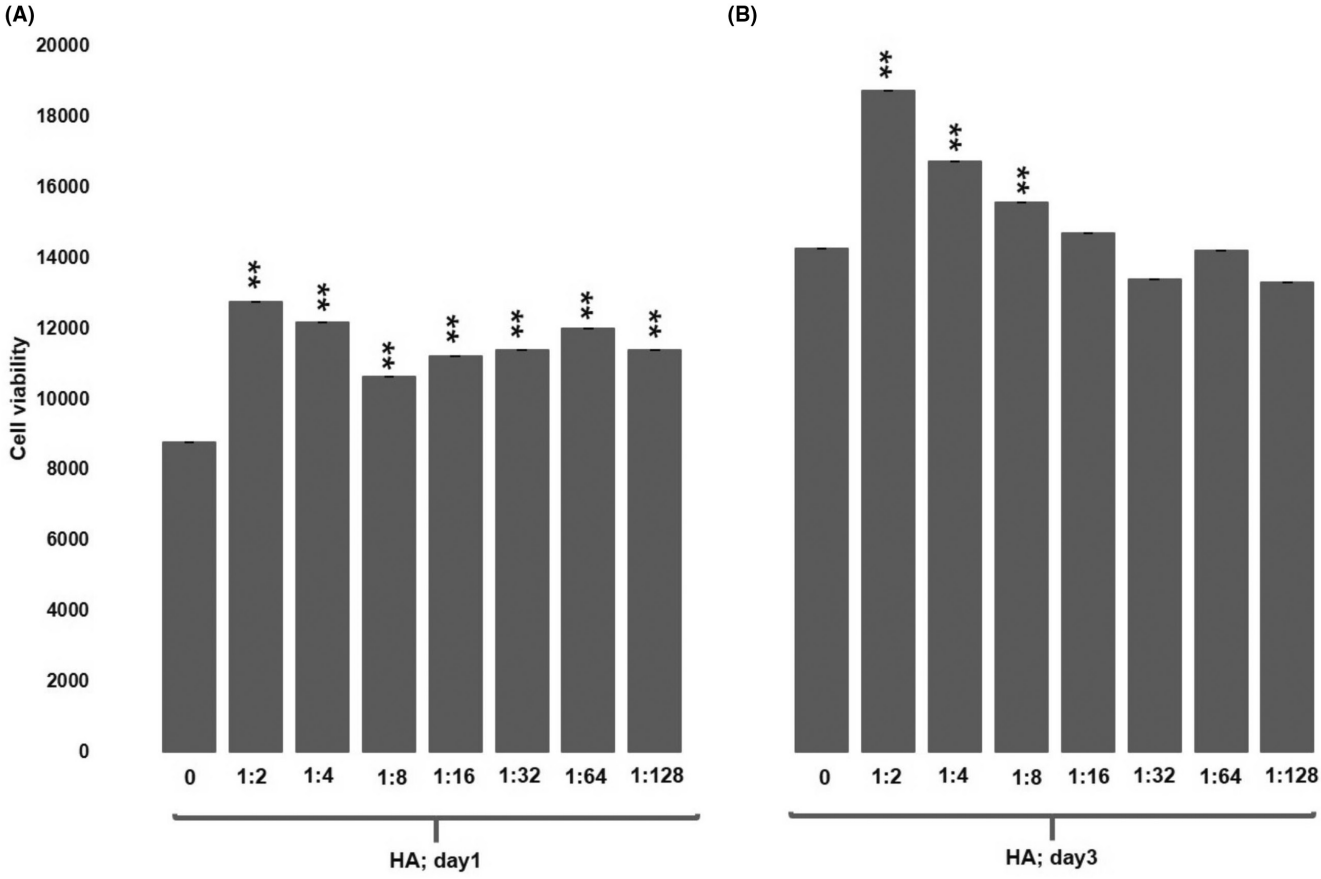

FIGURE 1 The impact of HA on cell viability of cementoblasts. The cementoblasts were treated with 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 dilutions of HA for 1 day (A) 3 days (B). $^{**}p < .01$, compared with the control.

2 (Runx2), bone sialoprotein (BSP), collagen type I (COL-I), alkaline phosphatase (ALP) as well as for cementoblast-specific markers cementum protein-1 (CEMP-1), cementum attachment protein (CAP) was performed in cementoblasts cultured for 3 and 8 days with 1:2, 1:4, and 1:8 diluted hyaluronic acid. After 3 days of treatment, COL-I was significantly increased under all applied dilutions (1:2, 1:4, 1:8) of HA ($p < .01$) (Figure 4A). Additionally, 1:2 dilution HA demonstrated higher COL-I mRNA expression than 1/4 and 1:8 HA dilutions compared to control group on day 8 ($p < .01$) (Figure 4B). While the BSP expression was still increased in cementoblasts stimulated by 1:2 and 1:4 HA for 3 days ($p < .01$) (Figure 4C), BSP levels less increased by 1:8 HA dilution compared to control for 8 days ($p < .01$) (Figure 4D). In line with this, RunX2, ALP, and OCCM mRNA expressions were upregulated by HA in a dilution- and time-dependent manner ($p < .01$) (Figure 4G,H,J–M). All HA treatments, when compared to the control, significantly increased the expressions of CEMP-1 and CAP expression on days 3 and 8 ($p < .01$) (Figure 4L–O).

3.5 | Effects of hyaluronic acid on cementoblast smad gene expressions

The study showed that Smad genes including Smad 1, 2, 3, 6, 7, and β-catenin (Ctnnb1) were differently affected by the three concentration levels of HA (1:2, 1:4, 1:8) for the 3 and 8 days of incubation (Figure 5). Significant upregulation in all groups was observed in Smad 2, 3, and 7 (Figure 5C–F,J,K), while Smad 1 and 6 transcripts were not affected by HA treatment (Figure 5A,B,G,H) ($p < .01$). 1:2 HA increased Smad2, 7 expression more than 1:4 and 1:8 HA compared with the control group on day 3 (Figure 5C,J), while 1:4 and 1:8 HA later upregulated Smad2, 7 levels by the same ratio as 1:2 HA on day 8 (Figure 5D,K). Smad 3 mRNA expression reached peaks compared with the control group (Figure 5E) within 3 days and continued on day 8 (Figure 5F). Compared with the control, a concentration of 1:2 HA was able to upregulate the expression of the β-catenin (Ctnnb1) more than 1:4 and 1:8 HA on day 3 (Figure 5L) and then 1:4 and 1:8 HA reached the same level as 1:2 HA on day 8 (Figure 5M).

4 | DISCUSSION

The present study has shown that HA promotes cell viability, mineralization, and upregulated, over a longer period of time, expression of mineralization-associated genes in cementoblasts. Hyaluronic acid, an innately occurring non-sulfated glycosaminoglycan with a high molecular weight polysaccharide formed, is a key element in the extracellular matrix of periodontium such as gingiva, periodontal ligament, alveolar bone, and cementum.[1] The treatment of three

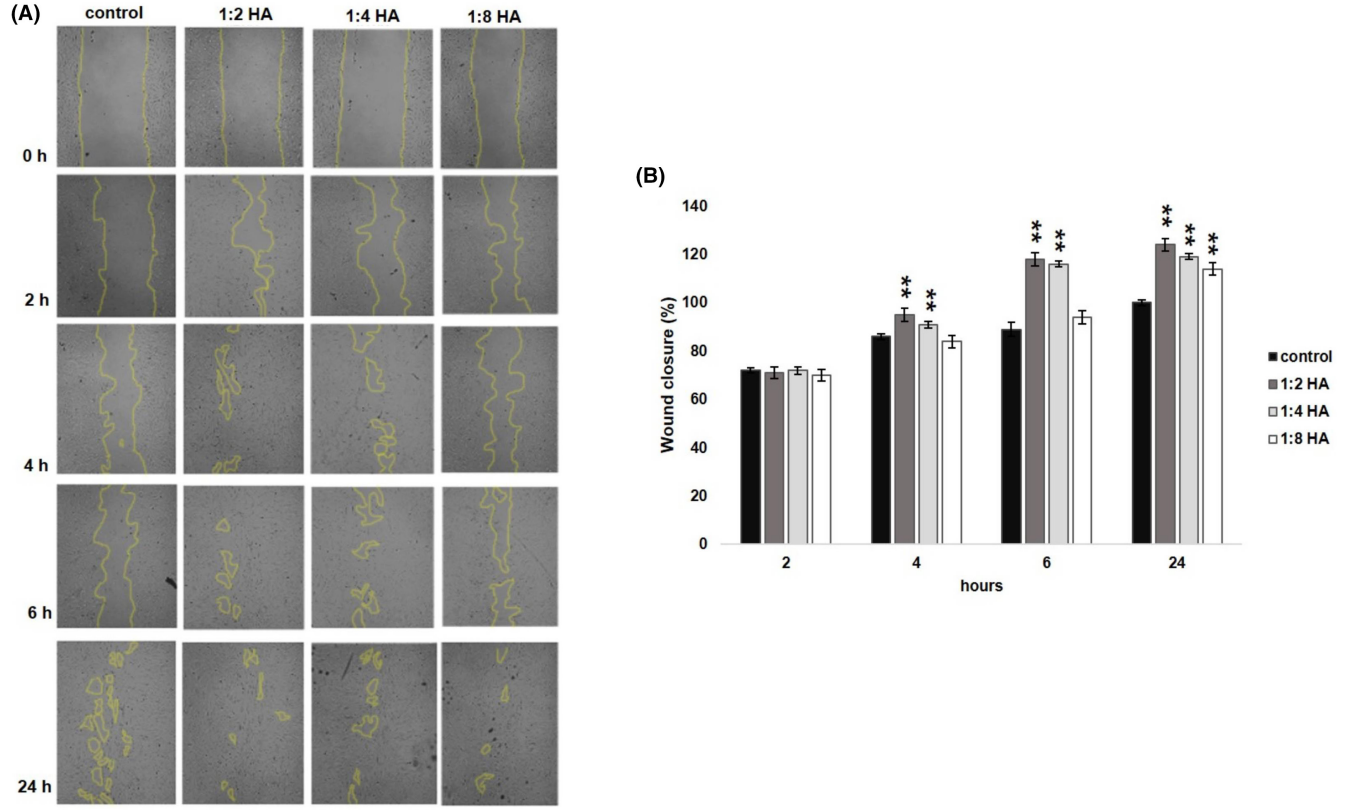

FIGURE 2 The effect of HA on the wound closure ability of cementoblast cells. (A) The cementoblast cells were treated with different HA dilutions (1:2, 1:4, 1:8). (B) Quantification of the wound closure rate. Statistical significance was evaluated at each HA dilution as compared with the control, $^{**}p < .01$.

distinct tissues, including the periodontium, the cementum, and the periodontal ligament, cooperatively results in the management of periodontal diseases. Several in vitro studies have shown that hyaluronic acid has an effect on oral tissue cells including periodontal ligament cells and gingival fibroblast,[30–32] however, there are no studies examining the effect hyaluronic acid has on cementoblasts. In the present study, the impact of high molecular weight hyaluronic acid was assessed on in vitro cementoblast cell activity. It was first found that hyaluronic acid was shown to be biocompatible on cementoblast cells compared with the control group. Fujioka-Kobayashi et al. reported that both non-cross-linked hyaluronic acid and cross-linked hyaluronic acid displayed high cell viability in human periodontal ligament cells.[30] Additionally, human periodontal ligament cells were implanted on control and hyaluronic acid-coated dentin discs, and all samples showed almost 100% survival rates, confirming great biocompatibility of hyaluronic acid at both dilutions of 1:10 and 1:100, respectively.[31] Hyaluronic acid is a vital part of the periodontal ligament matrix and is mediated by a number of hyaluronic acid-binding proteins and cell-surface receptors, including CD44, which play critical roles in cell adhesion, migration, and differentiation.[33] Findings obtained in the present study show that hyaluronic acid at the 1:2, 1:4, and 1:8 concentrations induced increased mineralized nodule formation and in vitro wound healing rates compared with the control group. In addition, Fujioka-Kobayashi et al. demonstrated that both non-cross-linked and cross-linked hyaluronic acid significantly decreased mineralization in human periodontal ligament cells.[30] The contrasting findings may have been the result of different sensitivity to hyaluronic acid because of functional and morphological differences between the murine-derived cementoblasts and human-derived periodontal ligament. However, is it interesting to note that HA was able to induce mineralization of cementoblasts in a manner similar to that seen in two other studies using the same cementoblast cell line treated with the highly cementogenic rhBMP-7 a potent growth factor, indicating a high potential of HA to induce cementoblasts differentiation and hence cementogenesis.[20,34] Further support for this observation comes from recent animal histological studies that clearly showed new cementum formation following application of the same HA preparation used in this study.[23–25] The basic glycosaminoglycan, hyaluronic acid, plays a key role in a number of crucial biological processes, such as mediating cellular signaling, controlling cell adhesion and proliferation, and influencing cell differentiation. Additionally, hyaluronic acid is appealing in tissue engineering and disease therapy due to its high viscosity, elasticity, strongly negative charge, low toxicity, biodegradability, and non-immunogenicity.[35] Cementoblasts are an important cell type for maintaining/restoring tissue attachment after periodontal disease because they synthesize the mineralized tissue, cementum, which is essential for attaching the soft periodontal tissue to the tooth.[36] Cementoblasts express similar gene expression patterns to that of osteoblasts. One of the essential key transcriptional factors in osteogenesis, Runx2[37] has

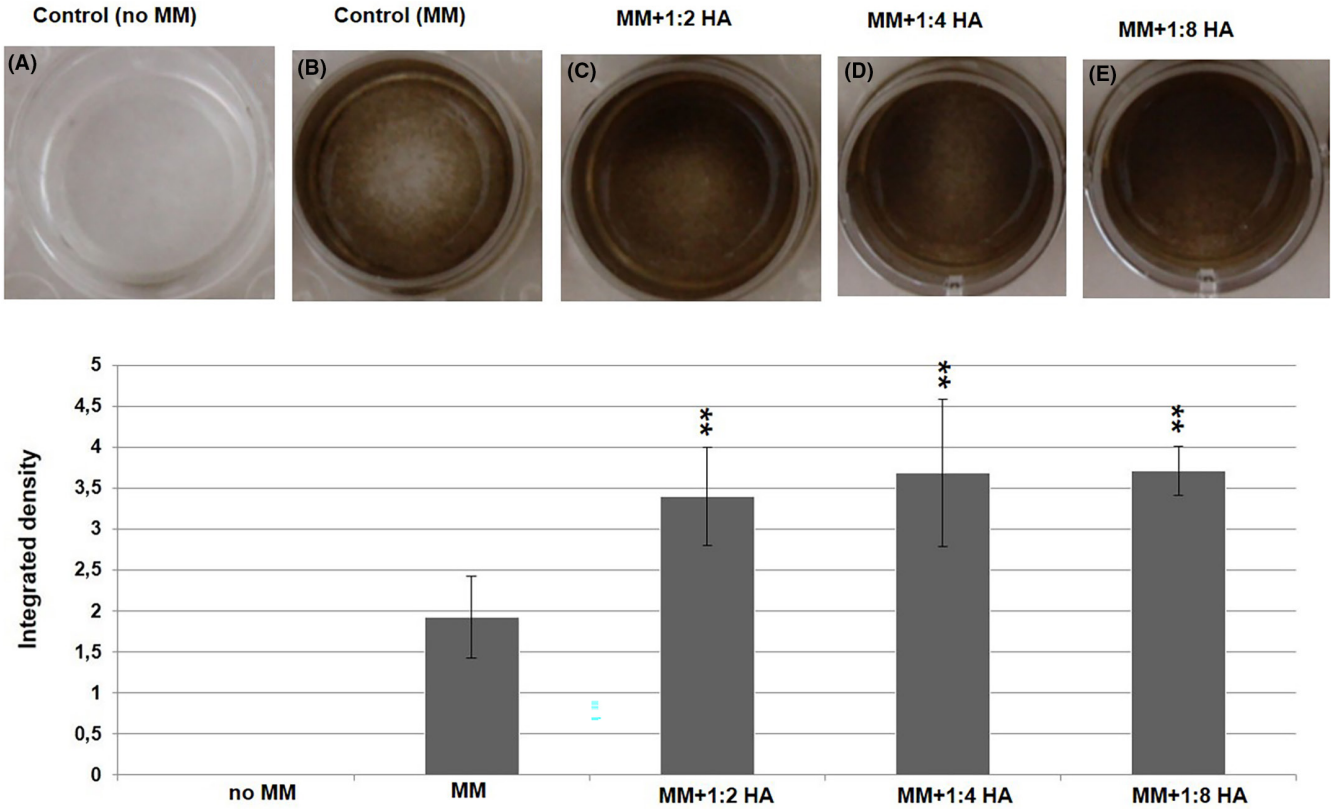

FIGURE 3 Biomineralization of cementoblasts. The development of calcified nodules stained with von Kossa was indicative of osteogenic differentiation on day 8. (A–E) von Kossa staining macroscopic images. (A) Negative control (without mineralization media), (B) positive control (with mineralization media), (C) 1:2 HA; (D) 1:4 HA; (E) 1:8 HA, $^{**}p < .01$, compared with the control.

also been found to be expressed in cementoblasts with co-localization with Osterix.[38] It has been shown that Runx2 promotes differentiation and mineralization of dental follicle cells by up-regulating genes such as BSP, osteopontin, collagen I, and two cementum-specific genes, cementum protein-1, and CAP.[39,40] In our study, all three hyaluronic acid concentrations increased the expression of Runx2 both on 3 and 8 days. All concentrations of hyaluronic acid up-regulated gene expression of mineralization markers COL-I, BSP, RunX2, ALP, and OCN, suggesting that hyaluronic treatment of cementoblast cells stimulated extracellular matrix mineralization. Also, cementoblast-specific markers such as CEMP-1 and CAP expressions increased with hyaluronic acid administration. In the literature, there are results that non-cross-linked hyaluronic acid and cross-linked hyaluronic acid significantly down-regulate early osteogenic differentiation markers such as Runx2, COL-I, and ALP in standard cell growth medium. However, the expression of early osteogenic markers was shown to importantly enhance COL-I and ALP levels with the addition of osteogenic differentiation medium.[30] Asparuhova et al. displayed that the expression of COL-I was increased by primary human palatal and gingival fibroblast.[32] Cementoblasts and osteoblasts, as well as fibroblasts and keratinocytes in the gingival and periodontal ligament, contain the hyaluronic acid synthase enzymes that are necessary for the periodontium to produce hyaluronic acid.[3] The present study indicates that hyaluronic acid may be an effective stimulator of the cementoblast action on gingival tissue. Hyaluronic acid apparently is a natural key component, and has been found to vital role in the functioning of periodontium extracellular matrices. In the present study, hyaluronic acid dilutions positively affect CAP and CEMP-1 expressions compared to controls on either 3 or 8 days. Both of these genes have been exclusively found in the cementoblasts. Since cementoblasts and their progenitors are known to express CEMP-1 in the paravascular zone of the periodontal ligament, this molecule can be used as a particular marker for cementoblasts and their precursor cells.[41] Further research revealed that CEMP-1 promoted the migration and proliferation of periodontal ligament cells. These findings imply that CEMP-1 mediates a number of biological processes, such as cementoblast development, wound healing, and periodontal tissue regeneration.[42]

Cementum attachment protein has been shown to be able to enhance the recruitment of putative cementoblastic cells onto the root surface and is capable of enhancing their differentiation by inducing a higher gene expression of ALP, BSP, and CAP which altogether indicates that CAP does play a role in the differentiation of putative cementoblasts progenitor cells. It is also interesting to note that CAP Is capable of binding to PDL progenitor cells that are able to form mineralized tissue.[43] Therefore, the HA-enhanced gene expression of CAP in cementoblasts over a period of 8 days could have a positive impact on new cementum and mineralized tissue formation which actually has been shown in the recently published animal histological studies.[23–25] Another cementoblast-specific gene that was found to

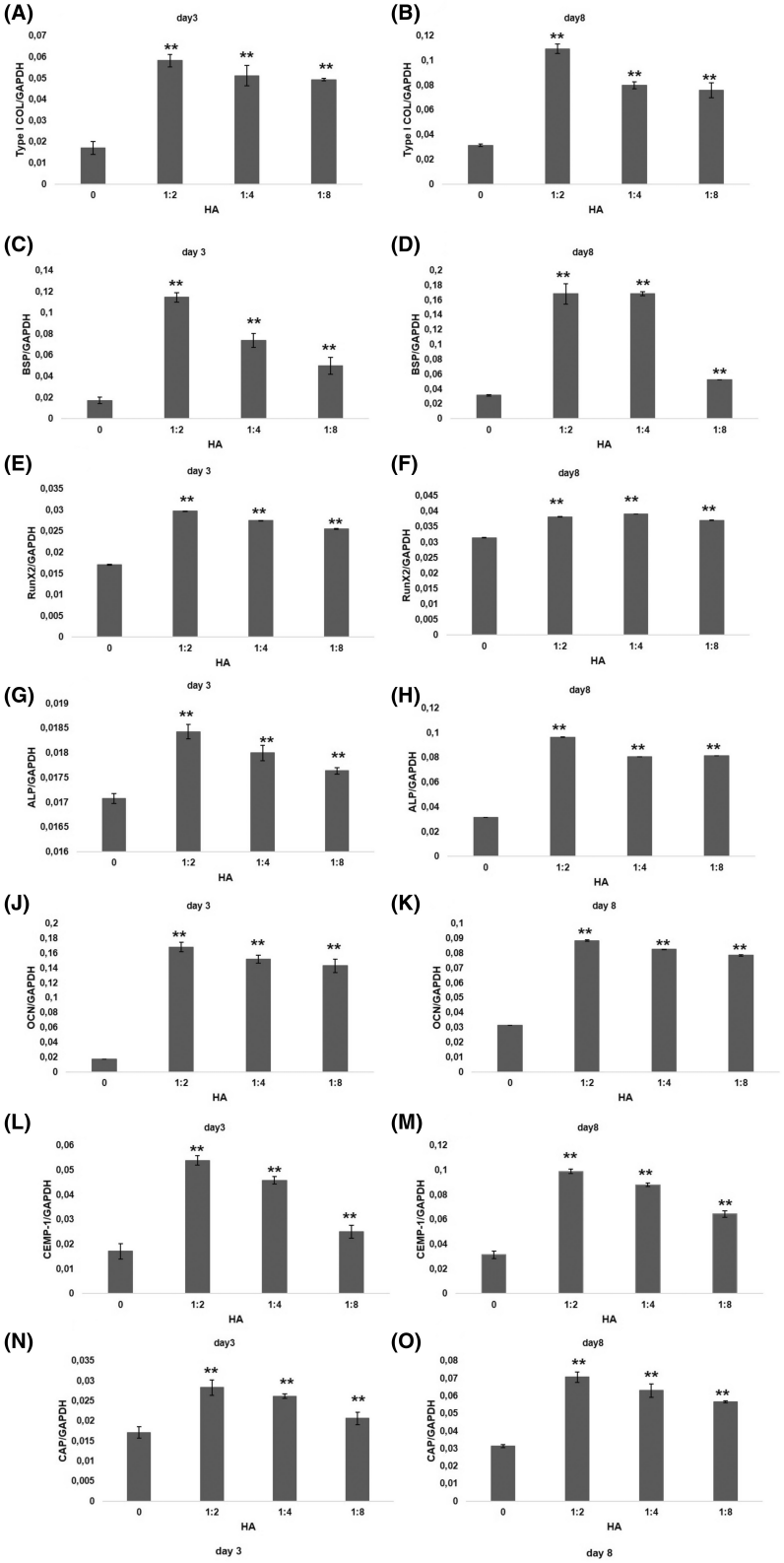

FIGURE 4 Hyaluronic acid (HA) affected the mRNA expressions of mineralized tissue-associated genes and cementoblast-specific markers HA-applied cementoblasts. The expression of target genes in cementoblasts from each group detected by quantitative RT-PCR (target genes were normalized to the housekeeping gene GAPDH). The cells were treated with 1:2, 1:4, and 1:8 HA on 3 and 8 days. (A) COL-I mRNA expression on day 3, (B) COL-I mRNA expression on day 8; (C) BSP mRNA expression on day 3, (D) BSP mRNA expression on day 8; (E) RunX2 mRNA expression on day 3, (F) RunX2 mRNA expression on day 8; (G) ALP mRNA expression on day 3, (H) ALP mRNA expression on day 8; (J) OCN mRNA expression on day 3, (K) OCN mRNA expression on day 8; (L) CEMP-1 mRNA expression on day 3, (M) CEMP-1 mRNA expression on day 8; (N) CAP mRNA expression on day 3, (O) CAP mRNA expression on day 8; $^{**}p < .01$, compared with the control.

be significantly increased in cementoblasts following HA application is CEMP-1. This gene has been shown to be a key regulator in the biomineralization process in cementoblasts[41,44] and furthermore, in a recent animal study, it was shown that CEMP-1 was able to induce periodontal regeneration by influencing cementoblasts progenitors' cells recruitment and inducing cementogenesis with newly embedded Sharpey's fibers.[45] Taken together, the ability of HA to increase the gene expression of these two cementoblast-specific genes could have an impact on the crucial event of promoting cementogenesis during periodontal regeneration in intrabony defects.

We also looked, for the first time, into the potential signaling pathway of HA in cementoblasts. We examined the BMP, TGF-β, and

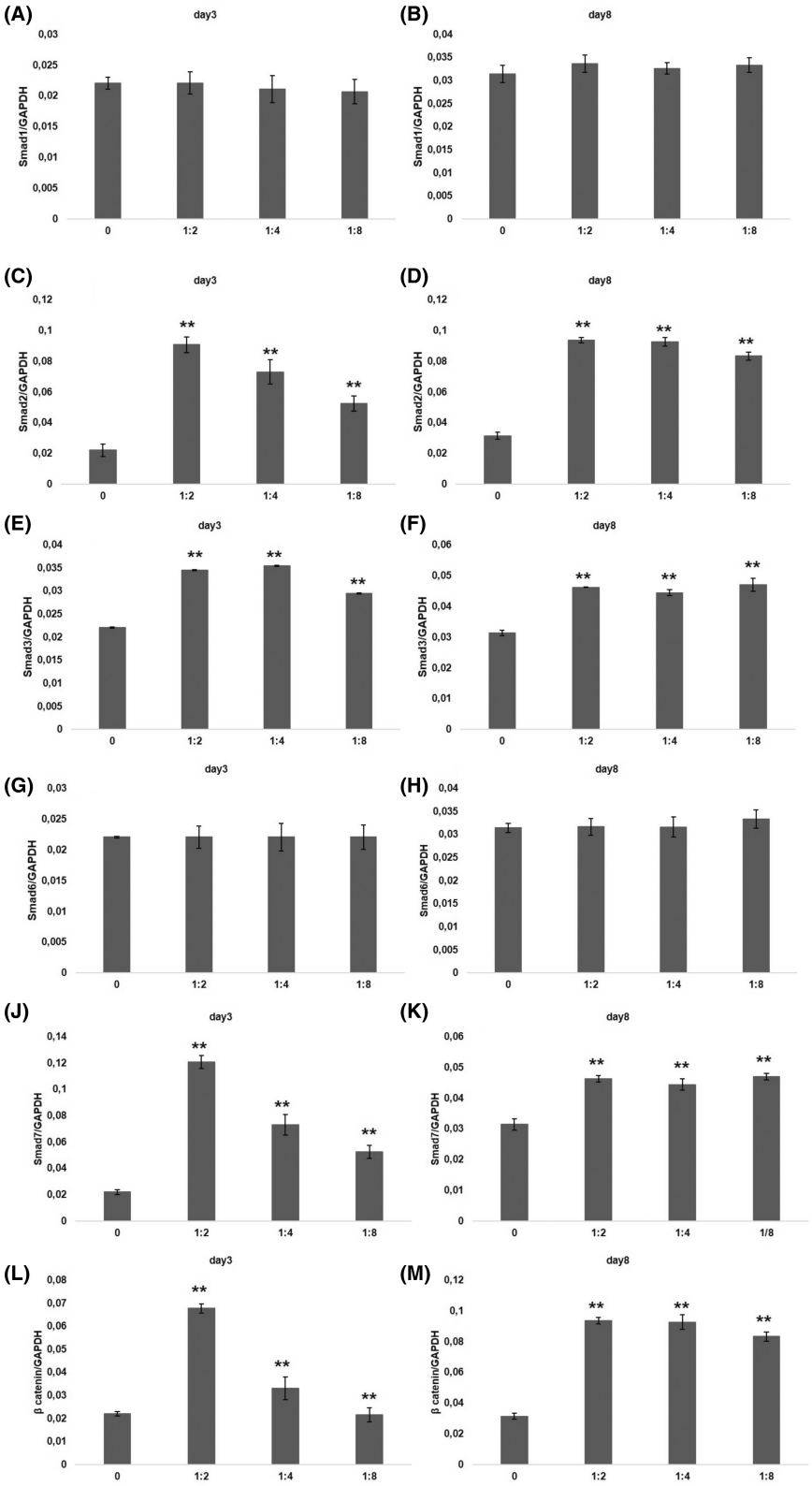

FIGURE 5 Effects of hyaluronic acid (HA) on Smad genes by RT-PCR. The cells were applied with 1:2, 1:4, and 1:8 HA on 3 and 8 days. (A) Smad-1 mRNA expression on day 3, (B) Smad-1 mRNA expression on day 8; (C) Smad-2 mRNA expression on day 3, (D) Smad-2 mRNA expression on day 8; (E) Smad-3 mRNA expression on day 3, (F) Smad-3 mRNA expression on day 8; (G) Smad-6 mRNA expression on day 3, (H) Smad-6 mRNA expression on day 8; (J) Smad-7 mRNA expression on day 3, (K) Smad-7 mRNA expression on day 8; (L) β-catenin (Ctnnb1) mRNA expression on day 3, (M) β-catenin (Ctnnb1) mRNA expression on day 8, $^{**}p < .01$, compared with the control.

Wnt/β-catenin signaling pathways. Periodontal tissue development is a dynamic process including Wnt/β-catenin signaling pathway and TGF-β/BMP signaling pathway.[46] Transforming growth factor beta (TGF-β) superfamily members, such as TGF-β1 and bone morphogenetic proteins (BMPs), play critical roles in numerous developmental processes. BMP signaling has been implicated in the proliferation and differentiation of osteoblasts, as well as bone production in mice.[47] Also, BMP-2 is a potent cytokine that promotes osteogenic differentiation and bone production in human mesenchymal stem cells and PDL cells.[48] In addition to BMP-2, it has been reported that BMP-7 induces the expression of cementogenic markers such as cementum attachment protein (CAP) and cementum protein 1

(CEMP1) in human PDL cells through a mechanism different from odontogenic differentiation.[49]

It is interesting to note that Smads-1, 5, and 8 are anchored at the plasma membrane by CD44, the receptor for HA, and they are activated by BMP type I receptors, while Smad2 and Smad3 are activated by activin and TGF-b type I receptors.[50] In our study, HA signaling pathway seems to go through the TGF-β type I receptors since Smad2 and Smad3 showed a significant increase in mRNA expression, compared to no effect of HA on Smad1 gene expression. Also, Smad-1 and Smad6 expressions were not affected by HA treatment, while Smad-7 was up-regulated by 1:2 HA compared with the control group on day 3. This increase reached the same level with 1:4 HA and 1:8 HA compared with the control group on day 8. Inhibitory Smads such as Smad-6 and -7 are their roles as inhibitors of Smad activation and regulators of receptor stability for Smad genes.[51] Another interesting finding is the increase in β-catenin expression in cementoblasts when treated with HA. The Wnt/β-catenin signaling pathway has been shown to be important in proper tooth development indicating its important role in cementogenesis[52] and more recently it was shown that β-catenin promotes cementum formation through Osterix activation in a reciprocal interaction during cementum formation.[53] Furthermore, the Wnt/β-catenin signaling pathway seems to be responsible for physiological cementum repair[54] and in human cementoblasts rh-BMP2 seems to induce differentiation also through the Wnt/β-catenin signaling pathway[55] and other proteins such as the brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein-1 (Bmal1) also seems to induce cementoblasts differentiation and mineralization through the Wnt/β-catenin signaling.[56] Taken together, there is growing evidence of the importance of the Wnt/β-catenin signaling pathway in cementoblasts differentiation and mineralization. The activation of this pathway by HA might be an important biological process during periodontal regeneration. A limitation of this study is that BMP, TGF-β, and Wnt ligands study results could not be included.

In summary, this study has for the first time demonstrated that hyaluronic acid is able to induce migration, proliferation, and mineralization of cementoblasts. Addition of hyaluronic acid increased mineralized tissue-associated genes and cementoblast-specific markers in a time-dependent manner. These findings suggest that hyaluronic acid might help in periodontal tissue regeneration by inducing cementogenesis.

CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Sema S. Hakki https://orcid.org/0000-0001-8665-6235
Serife Buket Bozkurt https://orcid.org/0000-0002-8641-2844
Darko Božić https://orcid.org/0000-0002-0391-0574

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