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ORIGINAL ARTICLE

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Effect of propolis on preserving human periodontal ligament cells and regulating pro-inflammatory cytokines

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Abstract

Background/Aim: Propolis has been suggested as a storage medium for avulsed teeth. The aim of this study was to compare the effectiveness of Brazilian propolis with Hank's balanced salt solution and milk in maintaining the viability of human periodontal ligament cells, their osteogenic differentiation potential, and pro-inflammatory cytokine expression.

Material and Methods: Cell Counting Kit 8 assays were performed to test human periodontal ligament cell viability in different storage media. The preservative effect on osteogenic differentiation was evaluated using alkaline phosphatase staining and activity assays, Alizarin Red S staining, and western blotting. Quantification of pro-inflammatory cytokines was performed using real-time PCR and enzyme-linked immunosorbent assays.

Results: Brazilian propolis at 10 μg/mL was not cytotoxic toward human periodontal ligament cells. The milk group showed the highest cell viability. Brazilian propolis and Hank's balanced salt solution groups showed similar cell viabilities. Alkaline phosphatase staining and activity were similar in all groups. Calcium deposition and mineralization nodule formation were similar in the Brazilian propolis and Hank's balanced salt solution groups but were higher in the milk group. Osteogenic marker gene and protein levels were similar in all groups. The genes and protein expression levels of IL1β, IL6, and IL8 decreased significantly after treatment with Brazilian propolis. *TNF*α mRNA expression showed no significant difference among the experimental groups. Pro-inflammatory cytokine levels in the milk group were higher than in the Brazilian propolis and Hank's balanced salt solution groups.

Conclusions: Brazilian propolis, Hank's balanced salt solution, and milk maintained the viability of human periodontal ligament cells and preserved their osteogenic differentiation ability similarly. However, Brazilian propolis showed a better anti-inflammatory effect.

KEYWORDS
human periodontal ligament cells, inflammation, osteogenic differentiation, propolis, tooth avulsion

1 | INTRODUCTION

Avulsion, the total displacement of a tooth from the alveolar socket, is one of the most severe forms of dental trauma. When avulsion occurs, it is better to replant the tooth immediately back into the alveolar socket. However, this is not always possible. In most cases, the primary goal is to preserve the viability of the human periodontal ligament cells (hPDLCs) until the tooth is replanted.[1] Selection of a suitable storage medium to maintain the viability of the hPDLCs is necessary for periodontal healing. An ideal storage medium should be capable of preserving the viability of the hPDLCs, presenting a compatible physiological pH and osmolality, clonogenic capacity, antioxidant properties, antimicrobial activity, have no or minimal microbial contamination, high availability, and should be inexpensive.[2]

A variety of media, including Hank's balanced salt solution (HBSS), milk, coconut water, egg albumen, and other solutions, have been advocated as storage media for avulsed teeth. The International Association of Dental Traumatology recommends using HBSS for avulsed teeth.[3] Milk is also a proven storage medium when avulsion occurs.[4]

Recently, natural products have been investigated as alternative sources of drugs, and interest in the area of complementary and alternative medicine has increased, resulting in many studies on the use of natural products for tissue repair.[5] Propolis, a natural product produced by honeybees, has been used successfully in medicine as an anti-inflammatory and antimicrobial agent. Studies have shown that propolis was effective in periodontal ligament cell conservation.[6,7] Furthermore, Roberta et al[8] demonstrated that propolis inhibited the late stages of osteoclast maturation, including fusion of osteoclasts precursors to form giant cells and formation of actin rings, which may prove useful as a medicament to reduce resorption associated with traumatic injuries to teeth.

External replacement resorption and external inflammatory resorption are the 2 main causes of loss of replanted teeth.[9] Tooth resorption is believed to be very similar to bone resorption,[10] involving an elaborate interaction among stromal and immune cells, mediated by soluble factors, such as cytokines and chemokines. Storage media should prevent the unpredictable sequelae of inflammatory root resorption or replacement resorption after tooth replantation. Although several studies have analyzed the viability of PDLs in different tooth storage media using in vitro techniques, little research has been carried out on the osteogenic potential and pro-inflammatory cytokine expression levels in hPDLCs after treatment with different storage media.[11–13] These two aspects are important for researchers to understand the role of storage media in the reparative processes in hPDLCs after replantation.

Thus, the aim of this study was to compare the effect of propolis with other storage media on the viability of hPDLCs. Furthermore, the study explored the changes in osteogenic differentiation potential and pro-inflammatory cytokines production in hPDLCs after treatment with different storage media.

2 | MATERIAL AND METHODS

All protocols used to obtain hPDLCs were approved by the Ethical Guidelines of Peking University (PKUSSIRB-201732003).

The hPDLCs were obtained from clinically healthy premolars and wisdom teeth extracted for orthodontic reasons (n = 4). The teeth were washed in sterile saline solution to remove residual blood. Each tooth was stored in a tube filled with HBSS. The coronal region of the teeth was held with forceps, and hPDLCs were obtained by scraping with #15 scalpel blades from the lower two-thirds of the root surfaces. The tissues were split into small pieces and cultured in Alpha Minimum Essential medium (α-MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ScienCell, USA) in a humidified atmosphere containing 5% CO_2 at 37°C (Thermofisher, USA). Subsequently, hPDLCs from passages 2-6 were used for the experiments.

Brazilian propolis (BP) was dissolved in dimethylsulfoxide (Sigma, USA). BP was then serially diluted in DMEM (Gibco, USA) without supplementation with FBS (DMEM(–)) from 20 mg/mL to final concentrations of 5, 10, 20, 40, and 80 μg/mL. The hPDLCs were seeded in 96-well plates (Corning, USA) at a density of 5×10^3 cells per well and cultured until they reached 70%-80% confluence. Subsequently, the cells were treated with different concentrations of BP and DMEM (–) (solvent control) for 1 hour at room temperature. A CCK8 assay was performed to assess the optimum concentration of BP for use in subsequent experiments. Furthermore, the viability of hPDLCs after treatment with different storage media, including BP, HBSS, milk, α-MEM, and tap water, was determined by conducting CCK8 assays. α-MEM was used as the positive control and tap water was used as the negative control. Plates without cell seeding were used as the blank control. The viability of hPDLCs stored in different storage media was calculated as the relative absorbance of a sample vs control wells, as follows: cell viability = (mean absorbance value of experimental group—blank control group)/(mean absorbance value of positive control group—blank control group).

hPDLCs were incubated with α-MEM (positive control, 37°C), BP, HBSS, and milk at room temperature for 1 hour and then induced in osteogenic medium (OM) containing 10 μmol/L dexamethasone, 100 μmol/L L-ascorbic acid phosphate, and 1.8 mmol/L potassium phosphate monobasic (Sigma, USA). Cells treated with proliferation medium (α-MEM) without OM treatment served as the negative control. After 7 days of osteogenic induction, ALP staining was performed using a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium Alkaline Phosphatase Color Development kit according to the manufacturer's instructions (CWbio, China). After 5 days of osteogenic induction, ALP activity was measured using an ALP kit (Jiancheng Bioengineering, Nanjing, China) according to the manufacturer's instructions. After 21 days of osteogenic differentiation induction, Alizarin Red S staining was conducted to evaluate calcium deposition and mineralized nodules formed by hPDLCs. Calcium deposition was determined quantitatively using the cetylpyridinium chloride (CPC; Sigma, USA) method. Absorbance was measured at 570 nm using a multifunctional microplate reader (Bio-Tek, USA)

TABLE 1 Real-time PCR primer sequences

Primer	Sequence	Product	GenBank Number
GAPDH	F:5′-ATGGGGAAGGTGAAGGTCG-3′ R:5′-GGGGTCATTGATGGCAACAATA-3′	108 bp	NM_002046.5
ALP	F:5′-ATGGGATGGGTGTCTCCACA-3′ R:5′-CCACGAAGGGGAACTTGTC-3′	108 bp	NM_000478.5
RUNX2	F:5′-TCCTATGACCAGTCTTACCCCT-3′ R:5′-GGCTCTTCTTACTGAGAGTGGAA-3′	190 bp	NM_001024630.3
OCN	F:5′-CACTCCTCGCCCTATTGGC-3′ R:5′-CCCTCCTGCTTGGACACAAAG-3′	112 bp	NM_199173.5
OSX	F:5′-CCTCCTCAGCTCACCTTCTC-3′ R:5′-GTTGGGAGCCCAAATAGAAA-3′	148 bp	NM_001300837.1
IL-1β	F:5′-AGCTCGCCAGTGAAATGATG-3′ R:5′-GCCCTTGCTGTAGTGGTGGT-3′	164 bp	NM_000576.2
IL-6	F:5′-GAAAGCAGCAAAGAGGCACT-3′ R:5′-TTTCACCAGGCAAGTCTCCT-3′	108 bp	NM_000600.4
IL-8	F:5′-TTTTGCCAAGGAGTGCTAAAGA-3′ R:5′-AACCCTCTGCACCCAGTTTTC-3′	194 bp	NM_000584.3
TNF-α	F:5′-CAGAGGGAAGAGTTCCCCAG-3′ R:5′-CCTCAGCTTGAGGGTTTGCTAC-3′	124 bp	NM_000594.3

ALP, Alkaline phosphatase; OCN, osteocalcin; OSX, osterix.

to compare the concentration of Alizarin red S staining between groups.

To analyze osteogenic marker gene expression, hPDLCs were treated in α-MEM (positive control, 37°C), BP, HBSS, and milk at room temperature for 1 hour. The liquid was removed after 1 hour of incubation, OM was added to each well, and the cells were cultured for 5 days and 21 days. Total RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the RNA was resuspended in diethyl pyrocarbonate-treated water (Solarbio, China). RNA (1 μg) was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit (Takara, Japan) according to the manufacturer's protocol. Primers for specific genes, including *ALP*, Runt-related transcription factor 2 (*RUNX2*), osteocalcin (*OCN*), osterix (*OSX*), and *GAPDH* (control), were designed and synthetized by the Sangon Company (Shanghai, China). Details for the primers used are shown in Table 1. Real-time PCR was performed using a Light-Cycler 480 SYBR Green I Master kit (Roche, USA). Expression data were reported as the ratio between each investigated mRNA and *GAPDH* mRNA according to the formula $2^{-\triangle\triangle CT}$.

To analyze pro-inflammatory gene expression, cells were seeded in 24-well plates (Corning, USA), grown until their density reached 70%-80% confluence, and then starved for 12 hour in α-MEM (without FBS supplementation). The optimum time for lipopolysaccharide (LPS) stimulation was determined by incubating cells with LPS for different times at 1, 3, 6, 12, and 24 hour. Subsequently, hPDLCs were stimulated with 1 μg/mL LPS for the optimum time and then exposed to BP, HBSS, and milk for 1 hour at room temperature. α-MEM without LPS stimulation was used as the negative control, whereas α-MEM with LPS stimulation was used as the positive control. Osteogenic marker gene expression in hPDLCs was detected using real-time PCR. Details of the primers are provided in Table 1.

To confirm the osteogenic marker gene expression result, the protein levels of ALP and OSX were detected by western blotting. hPDLCs treated with different storage media were induced in OM for 5 and 21 days, and cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors to extract the proteins. The concentration of total protein was measured using a BCA Protein Assay Kit (CWbio, China). Forty micrograms of total protein extract were separated by electrophoresis and electro-blotted onto polyvinylidene fluoride (PVDF) membranes and nitrocellulose filter membranes. The membranes were blocked in 5% non-fat milk for 1 hour and then incubated with the following primary antibodies: rabbit-polyclonal anti-ALPL (Proteintech, Wuhan, China) and mouse-monoclonal anti-OSX (Santa Cruz, CA, USA) overnight. An anti-beta-actin mouse-monoclonal antibody (Proteintech, Wuhan, China) served as the internal control. After incubation in the primary antibodies, the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies. Protein detection was performed using an enhanced chemiluminescence kit, and the immunoreactive protein bands were visualized using Fusion-Capt software (Vilber Lourmat, Paris, France).

hPDLCs were stimulated by LPS (1 μg/mL) for 3 hour and then treated with α-MEM, BP, HBSS, and milk for 1 hour. hPDLCs treated in α-MEM without LPS stimulation were used as the control. The supernatants from the cultures were obtained, and the concentrations of inflammatory cytokines IL-1β, IL-6, and IL-8 were measured using an ELISA assay according to the manufacturer's protocols.

Statistical analysis of the data was performed using one-way analysis of variance, complemented by Tukey's test. The level of significance was 5% ($P < .05$). Data were expressed as the mean ± standard deviation (SD). The statistical analysis was performed using the GRAPHPAD Prism software (GRAPHPAD Prism version 5.0, San Diego, CA, USA).

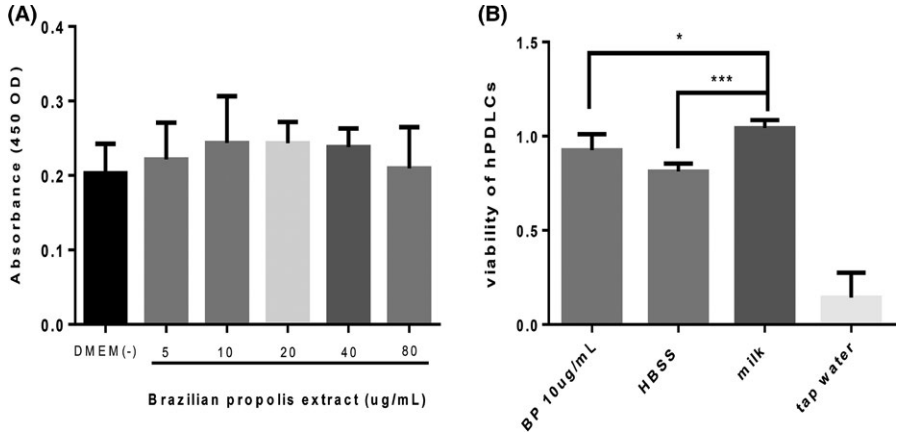

FIGURE 1 Preservative effect of different media on the viability of human periodontal ligament cells (hPDLCs). A, Selection of the optimum concentration of Brazilian propolis (BP) at room temperature for 1 h, as assessed by a Cell Counting Kit (CCK-8) assay. B, Comparison of the effects of different storage media on the viability of hPDLCs at room temperature for 1 h. *P < .05, ***P < .001. Hank's balanced salt solution (HBSS), Hank's balanced salt solution; Alpha Minimum Essential Medium (α-MEM, positive control); tap water (negative control)

3 | RESULTS

Different concentrations of BP, from 5 to 80 μg/mL, had no toxic effect on hPDLCs. The difference in viability was not statistically significant among the BP groups and the solvent control group ((P > .05; Figure 1A). BP at 10 μg/mL was used in subsequent experiments because of its relatively higher ability to preserve cell viability. Among the different storage media groups, cell viability was the highest in the milk group (P < .05), followed by the BP and HBSS groups. Most cells died when incubated in tap water (Figure 1B).

Cells stored in BP, HBSS, and milk showed no significant difference in ALP staining and ALP activity compared with those of the positive control group (Figure 2A). In addition, no statistically significant difference was found among the BP, HBSS, and milk groups (P > .05; Figure 2B).

Alizarin Red S staining and CPC qualitative analysis showed similar results in the BP, HBSS, and positive control groups (P > .05; Figure 2C and D). In the milk group, the cells produced significantly more calcium nodules than did the cells in the BP and HBSS groups (P < .05; Figure 2C and D).

The expression levels of *ALP* and *RUNX2* were not statistically significantly different among the BP, HBSS, milk, and positive control groups (P > .05; Figure 2E and F). The *OCN* expression level was slightly higher in the HBSS and milk groups compared with that in the BP and positive control groups; however, the difference was not statistically significant (P > .05, Figure 2G). The *OSX* expression level was slightly higher in the milk group compared with that in the BP, HBSS, and positive control groups, but the difference was not statistically significant (P > .05, Figure 2H). Western blotting revealed no differences in the protein levels of ALP and OSX among the experimental groups (Figure 2I).

The mRNA expression levels of pro-inflammatory cytokines in hPDLCs increased significantly after stimulation with LPS for 3 hour (P < .05; Figure 3A-D). Thus, hPDLCs were stimulated with LPS for 3 hour in the following experiments. The *IL1β* expression level decreased significantly in the 3 storage media groups compared with that in the positive control group. The expression level in the BP group decreased more than that in the other two groups, but the difference was not statistically significant (P > .05; Figure 3E). The *IL6* mRNA expression level in hPDLCs treated with BP was significantly lower than that in cells treated with milk (P < .05; Figure 3F). The *IL6* expression level in the HBSS group was slightly higher than that in the BP group, but without statistical significance (P > .05; Figure 3F). The *IL8* expression level decreased significantly in the BP group compared with that in the positive control and the other two experimental groups (P < .01; Figure 3G). *TNFα* mRNA expression was increased in the BP, HBSS, and milk groups, although no statistically significant differences were observed among the 3 groups (P > .05; Figure 3H). As shown in Figure 3I-K, the protein levels of IL-1β, IL-6, and IL-8 decreased significantly in the BP group.

4 | DISCUSSION

In cases of tooth avulsion, selection of a suitable storage medium to preserve the viability of hPDLCs is very important. In recent years, a number of studies have focused on the effectiveness of different solutions for use as storage media. Although the International Association of Dental Traumatology has recommended HBSS and milk as storage media for avulsed teeth, there is no consensus regarding their ability to preserve the viability of hPDLCs. The application of HBSS is restricted to laboratory or hospital environments and it is not available at the accident site, which makes it impractical as a storage medium.[14] Media such as milk should not be considered as "physiological" because ankylosis may occur when avulsed teeth are placed in milk, and concern has also been raised over its sterility and temperature.[15] Propolis has been applied medically because of its antimicrobial, anti-inflammatory, and antioxidant properties.[16,17]

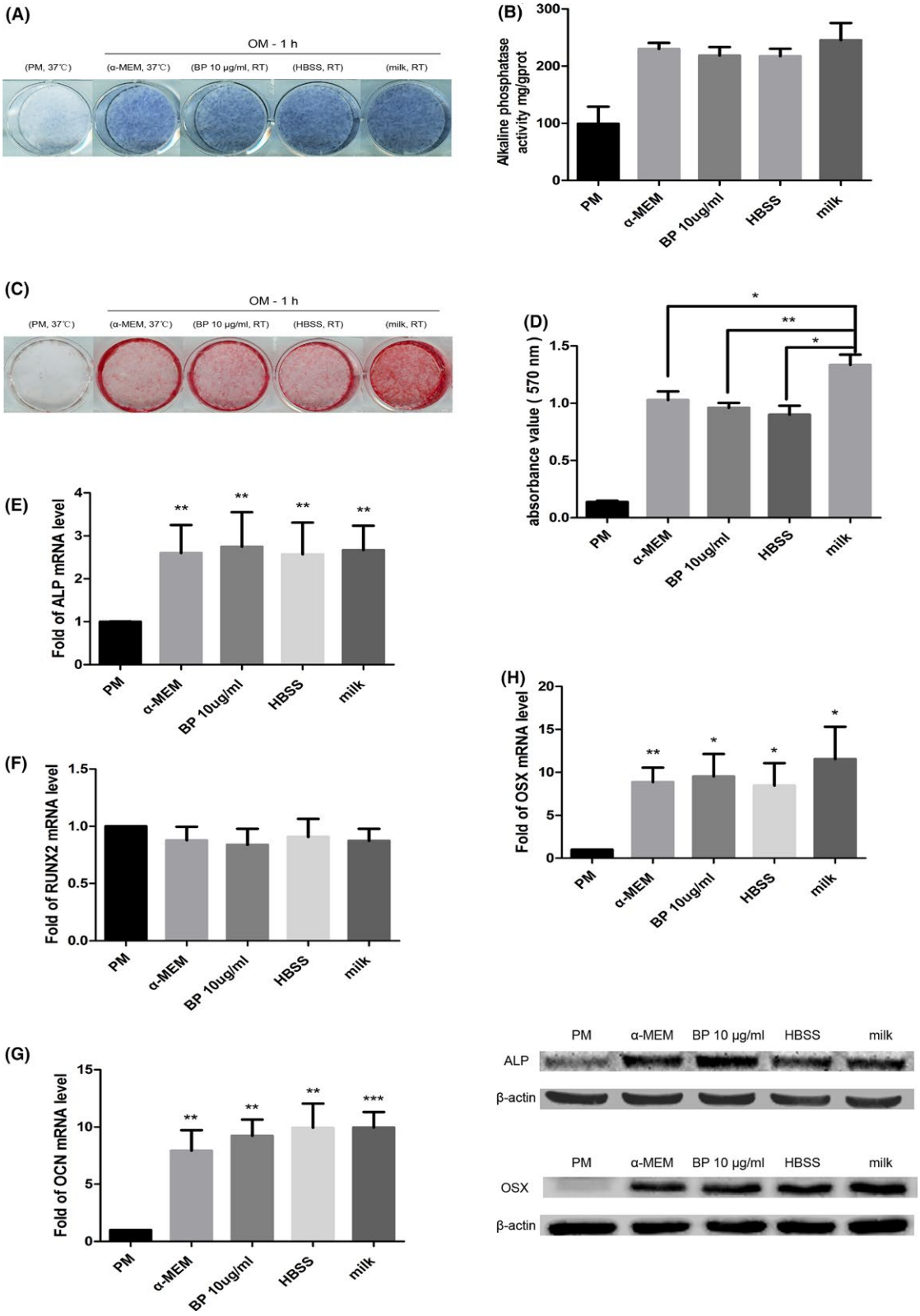

FIGURE 2 Effect of different storage media on preserving the osteogenic potential of human periodontal ligament cells (hPDLCs). hPDLCs were cultured in different storage media for 1 h at room temperature, then induced in osteogenic media for 5, 7, and 21 days. A, Alkaline phosphatase (ALP) staining, B, ALP activity assay, C, Alizarin Red S staining and D, calcium deposition. E, F, G, H, *ALP*, Runt-related transcription factor 2 (*RUNX2*) osteocalcin (*OCN*) and osterix(*OSX*) mRNA expression levels and (I) ALP and OSX protein production in hPDLCs. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Alpha Minimum Essential Medium (α-MEM) with osteogenic media was used as the positive control, whereas α-MEM without osteogenic media (PM) was used as the negative control

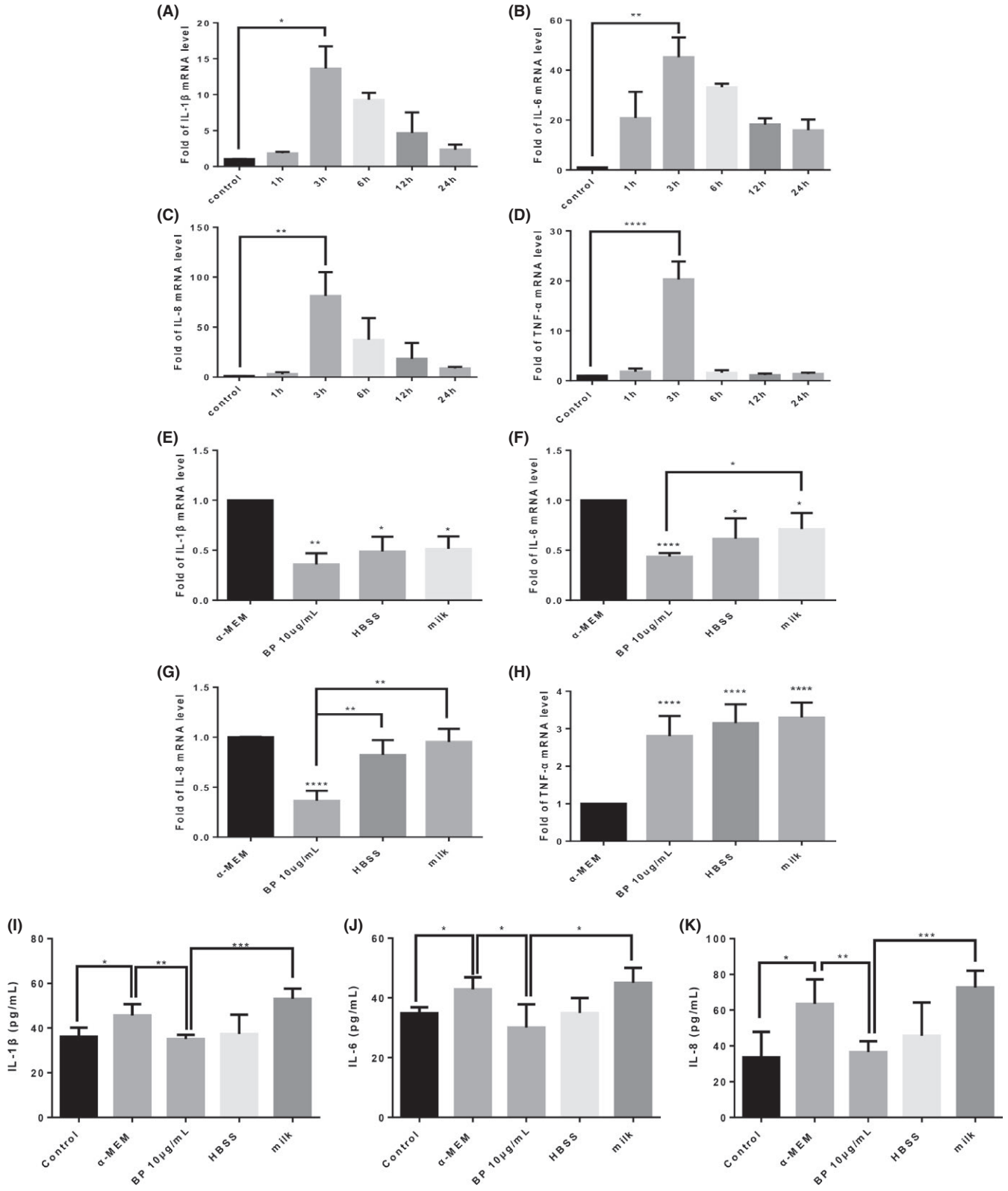

FIGURE 3 Effects of different storage media on pro-inflammatory gene expression and protein production in human periodontal ligament cells (hPDLCs). A, B, C, D, Optimum lipopolysaccharide (LPS) stimulating time selection. hPDLCs were stimulated with LPS for 3 h, and then treated with different storage media for 1 h at room temperature. E, F, G, H, *IL1β*, *IL6*, *IL8*, and *TNF*-α mRNA expression and I, J, K, IL1β, IL6, IL8 protein production in hPDLCs. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. Alpha Minimum Essential Medium (α-MEM) without LPS stimulation was used as the control

One study has shown that avulsed teeth stored in a medium containing antioxidants might improve the outcome of replantation.[18] Thus, propolis might be an alternative storage medium for avulsed teeth.

The present study evaluated the efficacy of BP, HBSS, and milk on the viability of hPDLCs. The results showed that the propolis yielded a slightly higher number of viable hPDLCs than HBSS, but the difference was not statistically significant. This result was in agreement with previous studies,[19–21] which found that propolis was similar to, or more effective than, HBSS in preserving the viability of hPDLCs. The current study also showed that both BP and HBSS were inferior to milk in maintaining the viability of hPDLCs, which is not consistent with the following studies. Hasan et al[12] compared the effect of HBSS, milk, and egg white, and found that that the number of hPDLCs was reduced, the extracellular matrix degenerated, and the occurrence of ankylosis increased in the milk group. Sousa et al[15] also reported reduced cellularity of hPDLCs after 1 hour of immersion in milk. The different results might reflect the different experimental methods, milk brands, and observation times.

An easily available tooth storage medium should not only maintain the viability of hPDLCs but also should prevent the occurrence of root resorption and ankylosis, which are essential factors to improve prognosis after avulsion. In the clinical situation, avulsed teeth are often kept in non-physiological ways, for example, kept dry, wrapped in plastic or paper sheets, or stored in improper storage media. These could lead to the development of ankylosis. Unfavorable storage conditions after extraction (ie, desiccation) also encourage the growth and differentiation of osteogenic cells from the root side of the periodontal ligament, and the selective loss of periodontal ligament fibroblasts.[22] Therefore, it is essential to explore the effect of different storage media in preserving the osteogenic differentiation potential of hPDLCs.

In this study, the osteogenic differentiation potential of hPDLCs in different media was compared using ALP staining, ALP activity, osteogenic marker gene expression and protein production. The results showed that cells stored in BP, HBSS, and milk presented no significant difference in ALP activity, *ALP* expression, and protein levels compared with those of the positive control group, which suggested that these three media maintained the original early osteogenic differentiation character of hPDLCs. The expression of *RUNX2* was similar among the different groups, which reflect that *RUNX2* was not affected by different storage media. The result may be partly due to the expression of *RUNX2* being earlier than *ALP*. To examine the late stage osteogenic differentiation potential, Alizarin Red S staining and real-time PCR of *OCN* and *OSX* showed that BP and HBSS exhibited similar effects to the positive control while in the milk group, both Alizarin red S staining and CPC qualitative analysis showed more nodules formation than in the positive control, BP, and HBSS groups. A previous study reported that cells kept in milk are more likely to develop ankylosis after replantation.[23] These results may partially explain the mechanisms of this phenomenon. However, western blotting analysis showed similar ALP and OSX protein levels among the experimental groups, which is inconsistent with the Alizarin Red S staining results. A possible explanation for this is that the large amount of calcium ions and other mineral substances in milk might induce mineralization within the culture system directly, rather than influencing the osteogenic ability of hPDLCs. Further experiments are needed to test this hypothesis. Taken together, in terms of preserving the osteogenic differentiation potential when used as a storage medium, BP maintained the original osteogenic ability of hPDLCs similarly to HBSS.

Storage media with anti-inflammatory properties might be useful to prevent root resorption. Clinical data have demonstrated that atopic patients, who predominately produce anti-inflammatory cytokines, had a more favorable outcome after replantation.[24] Target cytokine actions may be used to reduce the effects of inflammatory diseases on tooth resorption. Pro-inflammatory or osteoclastogenic cytokines, including IL-1β, IL-6, IL-8, and TNF-α have been identified in inflamed human pulp, periodontal disease, and periodontitis.[25–28] In addition, IL-1 has an important role during physiological root resorption in primary teeth[29] and during external apical root resorption during orthodontic treatment.[30] A previous clinical study[9] showed that the *IL-1β* level was higher in the inflammatory tooth resorption group, especially in moderate stages. Thus, in the present study, a comparison was made to investigate the influence of different storage media on pro-inflammatory gene expression and protein production. hPDLCs were stimulated with LPS to increase the expression of inflammatory cytokines.[31] After treatment with BP, the expression levels of inflammatory cytokines were significantly downregulated. Previous studies also showed that propolis attenuates the expression of pro-inflammatory cytokines in different cell types,[32,33] which is consistent with the current findings. Pro-inflammatory cytokine expression levels were higher in the HBSS group than in the BP group, although the difference was not statistically significant. In the milk group, the gene expression levels of *IL1β*, *IL6*, and *IL8*, but not *TNFα*, were higher than those in the BP group. This result was consistent with protein production results. Previous reports showed that pro-inflammatory cytokines were highly expressed when hPDLCs were kept in milk.[34] Another study[35] found that *IL-1β* expression was upregulated in the co-culture of mouse periodontal ligament cells and RAW macrophage-like cells kept in milk for 6 hour. Khademi et al[36] found that loss of teeth was presumably caused by a severe inflammatory reaction, which, because it occurred in the milk-treated teeth after periods of 6 and 10 hour, suggested that milk is not a suitable medium for long-term storage. In terms of its anti-inflammatory effect, BP is superior to HBSS and milk.

In conclusion, BP could be used as an alternative storage medium to preserve the viability and osteogenic potential of hPDLCs. It also has a superior effect in downregulating the expression of pro-inflammatory cytokines in hPDLCs when used as a storage medium.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

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