

Effects of MTA and Brazilian propolis on the biological properties of dental pulp cells

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Abstract: The aim of this study was to evaluate the effect of mineral trioxide aggregate (MTA) and Brazilian propolis on the cell viability, mineralization, anti-inflammatory ability, and migration of human dental pulp cells (hDPCs). The cell viability was evaluated with CCK-8 kit after 1, 5, 7, and 9 days. The deposition of calcified matrix and the expression of osteogenesis-related genes were evaluated by Alizarin Red staining and real-time PCR after incubation in osteogenic medium for 21 days. The expression of inflammation-related genes in cells was determined after exposure to 1 µg/mL LPS for 3 h. Finally, the numbers of cells that migrated through the permeable membranes were compared during 15 h. Propolis and MTA significantly increased the viability of hDPCs compared to the control group on days 7 and 9. In the propolis group, significant enhancement of osteogenic potential and suppressed expression of IL-1β and IL-6 was observed after LPS exposure compared to the MTA and control groups. The number of migration cells in the propolis group was similar to that of the control group, while MTA significantly promoted cell migration. Propolis showed comparable cell viability to that of MTA and exhibited significantly higher anti-inflammatory and mineralization promotion effects on hDPCs.

Keywords: Propolis; Pulpotomy; Inflammation.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Pulpotomy is the most common method for preservation of the vital pulp of primary teeth or immature permanent teeth and is the preferred procedure when only the coronal pulp is inflamed due to bacterial penetration following carious, traumatic, or iatrogenic causes, and the radicular pulp is free from inflammation. Therefore, selection of pulpotomy medicaments largely affects the long-term outcomes.

Mineral trioxide aggregate (MTA) was introduced in the early 1990s¹ and has been successfully used in the endodontic field for perforation repair, root-end filling, one-visit apexification, and pulp capping in recent years. The clinical success of MTA is based on its superior sealing ability,² biocompatibility, and potential to induce odontoblast differentiation.³ MTA – which has been the gold standard⁴ – was used as conventional pulp capping material. Despite these advantages, MTA still has some drawbacks

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such as long setting time, weak anti-inflammatory effect, and tooth discoloration.

Propolis is characterized as a complex and resinous mixture produced by bees collected from variable vegetable sources. The properties and constituents of propolis change with its geographical origin, different species, and seasons. The putative therapeutic properties of propolis could be related to its antibacterial, anti-inflammatory, antioxidative, and/or tumoricidal activities.^{5,6,7,8,9,10,11,12} Animal experiment results indicate that the alcoholic extraction of propolis induces the production of a tubular and better quality dentin than calcium hydroxide groups.^{13,14}

The present study was designed to compare the effect of propolis and MTA on the biological properties of human dental pulp cells (hDPCs), and lay the experimental foundation for propolis application in pulp therapy in the future.

Methodology

Material preparation

The ethanolic extract of Brazilian propolis (EEP) was prepared using the same method as Al-Haj Ali¹⁵ and dissolved in dimethylsulfoxide (DMSO; Sigma, USA) as 10 mg/mL, and then stored at 4°C. MTA (ProRoot MTA; Dentsply Tulsa Dental, Johnson City, TN) was the other test material used in this study. This study is in accordance with ISO 10993-5:2012.¹⁶ MTA was mixed according to the manufacturer's instructions. Each sample disc (20 mm in diameter and 1 mm in thickness) was allowed to set for 24 h at 37°C in 100% humidity. Then all samples were sterilized by ultraviolet irradiation for 30 min and then exposed to 5 mL DMEM (Gibco, USA) for 24 h. The extraction was filtered through 0.22 µm pore size and stored at 4°C.

EEP was diluted with fresh culture medium to 10, 20, 40, 80, and 160 µg/mL; extraction of MTA was similarly diluted to 1:1, 1:2, 1:4, and 1:8.

Primary cell culture

Permanent teeth were collected from a 22-year-old individual for orthodontic reasons. The freshly extracted teeth were split and immediately placed in α-MEM (Gibco, USA). The teeth were cracked vertically

to remove the pulp. The pulp tissue was minced and cultured as explants in α-MEM supplemented with 15% fetal bovine serum (FBS; ScienCell, USA) and antibiotics. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. After achieving confluence, the cells were detached with 0.25% trypsin and 0.2% EDTA and subcultured at a ratio of 1:3. hDPCs between the third and sixth passages were used in the present study.

Cell viability assay

The cell viability of hDPCs was assessed using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, 5×10³ hDPCs/well were plated in a 96-well plate, preincubated for 24 h in normal media, and then incubated at different concentrations of EEP or MTA extracts for 24 h. Approximately 10 µL CCK-8 solution with 90 µL α-MEM was added to each well, and the mixture was incubated for another 1 h. The optical density was measured with a spectrophotometer at 450 nm. Experiments were performed in triplicate.

In the same way, hDPCs were treated with the minimum cytotoxic concentration of different medicaments for 1, 5, 7, and 9 days, and absorbance was then measured at 450 nm. Experiments were performed in triplicate.

Real-time PCR

The messenger RNA levels were assayed by quantitative reverse-transcriptase chain reaction using fluorescent markers.

Cells were collected after stimulation with EEP and MTA extracts included in osteogenic medium for 21 days.

The cells were activated with 10 µL of lipopolysaccharide (LPS) from *E. coli* serotype O111:B4 at 1 µg/mL. Materials, as mentioned before, were added to the cells at same time as LPS, and the plates were incubated for 3 h at 37°C in 5% CO₂.

Cells were harvested for the extraction of total RNA by the column-based method using thiocyanate guanidine (RNEasy; Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Total RNA was estimated using 2 µL of each sample with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Reverse transcription was then performed for complementary DNA synthesis followed by polymerase chain reaction. The primer sequences for dentin sialophosphoprotein (DSPP), alkaline phosphatase (ALP), osteocalcin (OCN), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) are shown in Table. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. Quantitative reverse-transcriptase polymerase chain reactions were performed in duplicate using a StepOne Plus real-time PCR system (Applied Biosystems). The results were analyzed based on cycle threshold values. Relative expression was calculated based on the equation $2^{-\Delta\Delta C_t}$. Data were obtained by averaging the results from three independent experiments.

Alizarin Red S staining

Briefly, hDPCs were seeded in 24-well plates and incubated in osteogenic medium with EEP and MTA extractions. The medium was changed every 3 days. After 21 days of incubation, the cell layer was washed twice with PBS, fixed with 4% paraformaldehyde, stained with 1% Alizarin Red S, and then photographed. The Alizarin Red-stained wells were destained with 100 mmol/L cetylpyridinium chloride for 2 h. The 1:10 dilution concentration of Alizarin Red S staining in the samples was determined by the absorbance of the eluted stain at 562 nm using a spectrophotometer. Experiments were performed in triplicate.

Cell migration assay

Cells (2×10^4 cells/well) were seeded on the bottoms of 6.5-mm diameter Transwell® inserts (Corning,

USA) containing 200 μ L medium without FBS, and the test materials were placed on the bottoms of 24-well plates. The Transwells contained permeable membranes (0.4 μ m pore size) and were used to prevent direct physical interaction between cells and the specimens while allowing for soluble compounds from the specimens to reach the cells. The Transwells containing the cells were treated for 3 h and then washed with PBS, fixed with 4% paraformaldehyde (Sigma, USA) and then stained with 0.1% crystal violet. The stained cells were counted under the microscope.

Statistical analysis

Statistical analysis was performed with data obtained from four independent experiments. Comparisons of real-time PCR were made by nonparametric tests. Other data were expressed as mean \pm standard deviation and were analyzed using one-way analysis of variance followed by Tukey's test. Statistical significance was established at $p < 0.05$.

Results

Toxicity of materials

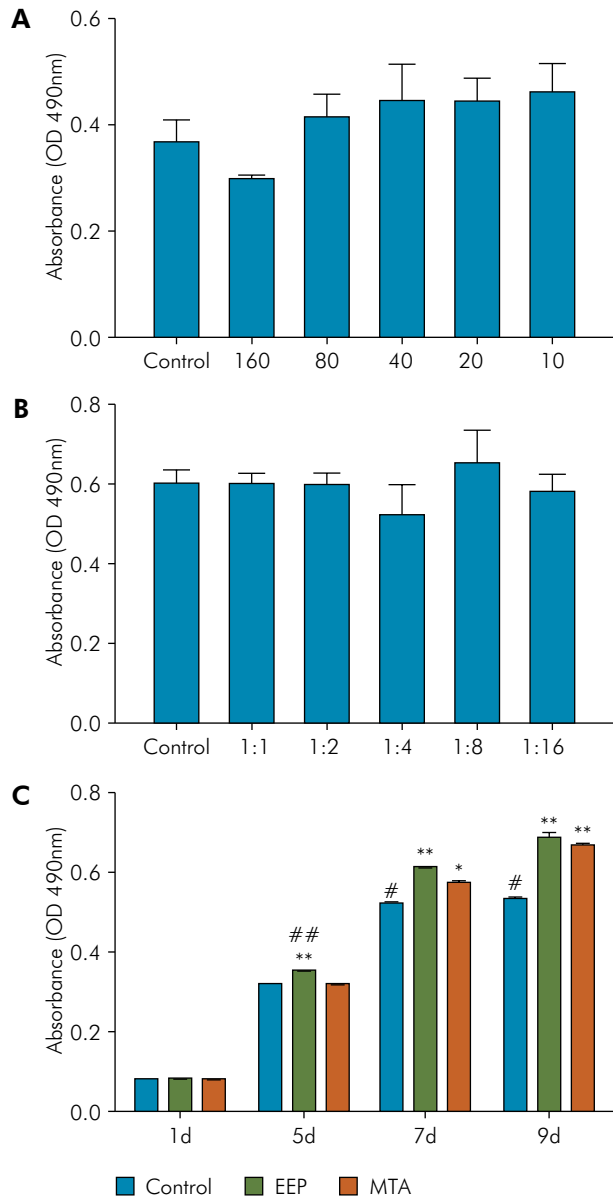
hDPC viability was slightly higher in 10 μ g/mL of EEP and 1:8 dilution of MTA extraction, and no significant differences were found among different dilutions of EEP or MTA extraction after 24 h. Both materials showed comparable cell viability to that of the negative control group ($p > 0.05$, Figures 1A and B). 10 μ g/mL of EEP and 1:8 dilution of MTA extraction were used in the subsequent experiments.

The cell proliferative ability of hDPCs was assessed after exposure to EEP and MTA for 1, 5, 7, and 9 days.

Table. Primer sequences of real-time PCR control: α MEM group; EEP: EEP group; MTA: MTA extraction group.

Gene	Primer sequences	
ALP	F:ATGGGATGGGTGTCTCCACA	R:CCACGAAGGGGAAGTCTGTC
DSPP	F:GCTGGCCTGGATAATTCCGA	R:CTCCTGGCCCTTGCTGTTAT
OCN	F:CACTCCTCGCCCTATTGGC	R:CCCTCCTGCTTGACACAAAG
TNF- α	F:CGAGTGACAAGCCTGTAGCC	R:TGAAGAGGACCTGGGAGTAGAT
IL-1 β	F:AGCTCGCCAGTGAAATGATG	R:GCCCTTGCTGTAGTGGTGGT
IL-6	F:GAAAGCAGCAAAGAGGCACT	R:TTTCAACCAGGCAAGTCTCCT
GAPDH	F:ATGGGGAAGGTGAAGGTCTG	R:GGGGTCATTGATGGCAACAATA

All materials exhibited no cytotoxicity for up to 9 days after culture. EEP groups showed a significant increase in cell numbers compared to the control group at 5, 7, and 9 days ($p < 0.05$, Figure 1C).

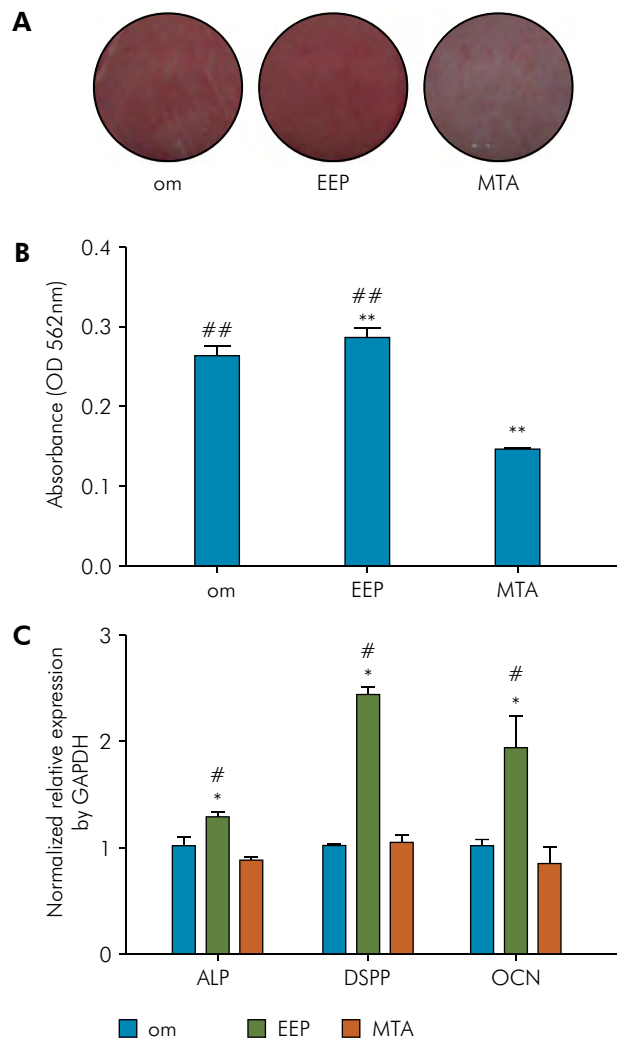


* $p < 0.05$ compared with control, ** $p < 0.01$ compared with control; # $p < 0.05$ compared with MTA, ## $p < 0.01$ compared with MTA; om: osteogenic medium group; EEP: EEP+ osteogenic medium group; MTA: MTA extraction + osteogenic medium group.

Figure 1. Effect of various concentrations of EEP and MTA extraction on cell viability in primary culture of dental pulp cells by CCK-8 after 1 day (A, B); effect of different materials on cell viability in primary culture of dental pulp cells by CCK-8 after 1, 5, 7, and 9 days (C).

Effects on odontoblast differentiation

A significantly higher level of Alizarin Red staining was exhibited in the EEP group than in the MTA and osteogenic medium groups ($p < 0.01$, Figures 2A, B). The expression levels of the odontogenic marker genes ALP, DSPP-1, and OCN were significantly increased in the EEP group compared with the MTA and osteogenic medium group at 21 days ($p < 0.05$, Figure 2C).



* $p < 0.05$ compared with control, ** $p < 0.01$ compared with control; # $p < 0.05$ compared with MTA, ## $p < 0.01$ compared with MTA; control: LPS group; EEP: EEP + LPS group; MTA: MTA extraction + LPS group.

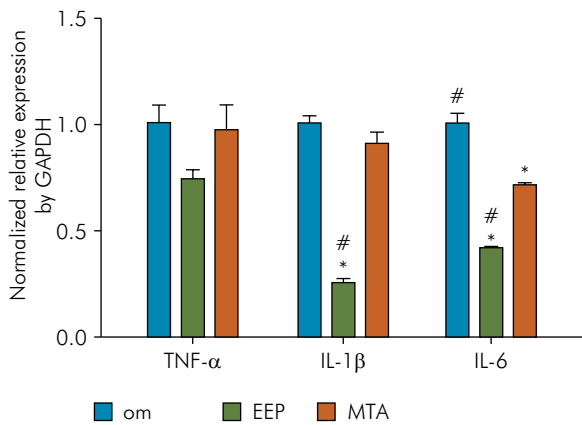
Figure 2. Cells cultured with different materials for 21 days. Alizarin Red staining of hDPCs (A); Quantification of Alizarin Red staining (1:10 dilution) (B). Expression of ALP, DSPP, and OCN in different groups for 21 days (C).

Pro-inflammatory cytokine expression

RT-PCR showed no significant difference between the groups for TNF- α expression at the 3-h time point ($p > 0.05$, Figure 3). A more significant decrease in IL-1 β and IL-6 mRNA expression was observed in the EEP group compared with the MTA and control groups ($p < 0.05$, Figure 3).

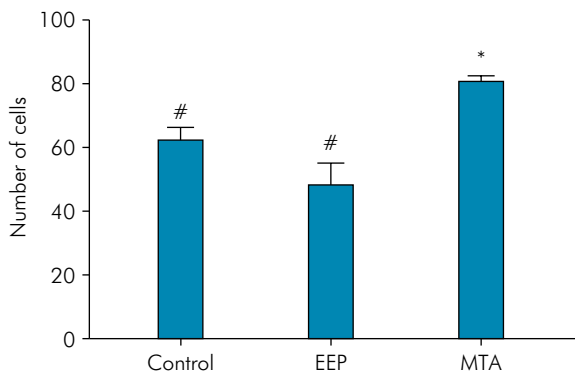
Cell migration

As shown in Figure 4, the MTA group (78.33 ± 1.53) significantly enhanced the migration ability of hDPCs compared with the control group (53.00 ± 4.08)



* $p < 0.05$ compared with control; ** $p < 0.05$ compared with MTA.

Figure 3. Expression of TNF- α , IL-1 β , and IL-6 in different groups after LPS stimulation for 3 h.



* $p < 0.05$ compared with control, ** $p < 0.01$ compared with control; # $p < 0.05$ compared with MTA, ## $p < 0.01$ compared with MTA; om: osteogenic medium group; EEP: EEP+ osteogenic medium group; MTA: MTA extraction + osteogenic medium group.

Figure 4. Number of cells migrated through permeable membranes in different groups after seeding at 15 h.

($p < 0.01$). However, results showed no significant difference in the number of migrated cells between the EEP (47.67 ± 7.23) and control groups ($p > 0.05$).

Discussion

The ideal capping medicaments should have the following properties: good biocompatibility, non-irritability, and non-toxicity, promoting pulp tissue regeneration, strong bactericidal and antibacterial ability, excellent sealing ability, stable and lasting efficacy, easy handling, among other factors. The existing clinical pulp capping materials are not perfect, so it is necessary to improve their properties. Propolis contains a wide variety of ingredients and several ingredients have been used in other clinical areas. In the field of stomatology, propolis is more widely used to prevent dental caries and to save traumatized teeth. Our experiments compared the effects of EEP and MTA on the proliferation, odontogenic differentiation, anti-inflammation effect, and cell migration of hDPCs to clarify the possibility of applying propolis in endodontic treatment.

The CCK-8 results showed that both propolis at $10 \mu\text{g/mL}$ and MTA at 1:8 dilutions were not cytotoxic to hDPCs after 1, 5, 7, and 9 days. Similarly, other researchers have found cell proliferation exposed to MTA extracts at 1:8 dilution was significantly higher, compared with the control group, 1:2 and 1:4 dilutions at 24 h.¹⁷ Previous studies have also suggested that treatment of dental pulp fibroblasts with 1 mg/mL of Brazilian propolis for 20 h was not toxic to pulp cells.¹⁸ Not only Brazilian propolis, but also Jordanian propolis (0.05 , 0.5 , and $5 \mu\text{g/mL}$) had comparable cell viability to the negative control and MTA groups.¹⁵

Alizarin Red S staining is a conventional method for testing calcium deposition in the extracellular matrix. In our study, more calcium deposition in the EEP group implied that EEP may have the ability to promote reparative dentin formation. ALP is one of the major enzymes expressed during the early maturation of osteoblasts and plays an important role in mineral deposition. In this study, there was increased ALP expression after 21 days in EEP culture, compared with the MTA and control groups. DSPP is a member of the small integrin-binding ligand

N-linked glycoprotein (SIBLING) family. DSPP was originally considered to be dentin-specific. Although several studies have recently shown its expression in bones, DSPP remains a major marker of odontoblast differentiation.¹⁹ Furthermore, OCN can be expressed by odontoblasts and is present in the dentin matrix, and it is also thought to be a reparative molecule within the dental pulp.^{20,21} In the present study, DSPP and OCN mRNA levels were significantly upregulated in the EEP-treated group. These results suggest that when propolis is applied to pulp capping, it could hopefully form the dentin bridges rapidly, thereby being able to block external stimuli. However, the strong ability to promote mineralization may, on the other hand, lead to the increasing risk of post-treatment pulp calcification and therefore cause increasing difficulties in future endodontic retreatment. Further *in vivo* animal studies are required to verify the exact long-term effects of propolis on pulp tissue mineralization.

Antibacterial and anti-inflammatory effects of pulp capping materials are important for the success of vital pulp preservation with mild inflammation. Previous studies have proven that propolis could break down the bacterial cell wall and prevent bacterial cell adhesion.^{12,22} Flavonoids and caffeic acid present in propolis are known to play an important role in reducing the inflammatory response by inhibiting the lipoxygenase pathway of arachidonic acid metabolism. Flavonoids and caffeic acid also aid the immune system by promoting phagocytic activities and stimulating cellular immunity.^{7,23,24,25} During the inflammatory reaction, activated macrophages will produce TNF- α , IL-1, IL-6, IL-10, chemokines, and short-lived lipid mediators to orchestrate a local inflammation.²⁶ Studies have shown significant increase in TNF- α and IL-1 in irreversible pulpitis. In this research, propolis reduced LPS-induced expression of IL-1 β and IL-6 in hDPCs 3 h after stimulation, and was superior to the MTA group. Neiva K G (2014) demonstrated the excellent anti-inflammatory effects of Brazilian propolis. In their research, mouse odontoblast-like cells were exposed to 20 ng/mL concentration of LPS for 1 h, and the expressions of MIP-1 α , G-CSF, TNF- α , and IL-6 were significantly inhibited by

treatment with 1:100 dilution of propolis for 24 h.²⁷ Unlike their results, we found the expression of TNF- α did not reduce significantly. Other groups found similar phenomena. i.e, that Brazilian red propolis decreased IL-1 β , IL-10, IL-6, and other cytokine levels in peritoneal macrophages; however, no difference was observed for TNF levels. This was due to the complex interactions between the various cytokines, including IL-10, IL-4, IL-13, and TNF.²⁸

The regulation of capping medicaments in hDPC migration may affect the healing of the remaining dental pulp and the formation of dentin bridges. In this experiment, propolis was found to inhibit cell migration to a certain degree, while MTA promoted cell migration significantly. Besides hDPCs, Kabala-Dzik et al.²⁹ confirmed that caffeic acid and caffeic acid phenethyl ester, the active component in propolis, interrupted the migration of breast cancer cells. Brazilian red propolis was also effective in decreasing the influx of neutrophils to the inflammatory site, acting through reduced TNF- α , IL-1 β , CXCL1/KC, and CXCL2/MIP-2 release and also reduced neutrophil chemotaxis by blocking calcium influx.³⁰ Some authors have claimed that Brazilian red propolis at the 10 μ g/mL concentration promotes visually faster wound closures, compared to control groups.³¹ More in-depth research studies are needed to confirm the effect of Brazilian propolis on cell migration. Most researchers agree that MTA extracts significantly promote dental pulp cell migration and wound closure after several hours of treatment.³²

The present study shows excellent osteogenesis and anti-inflammatory effects of Brazilian propolis, with no cytotoxicity. But as propolis contains some ingredients that trigger allergies to a certain degree, propolis extracts should be considered in the future for re-purifying and removing possible toxic components. Its active ingredients could be used as an independent inflammatory pulp capping material, as well as a supplement to other bioactive materials or used as an irrigant or intracanal medicament due to its excellent anti-inflammatory effect and good biocompatibility. All in all, Brazilian propolis is a promising material for vital pulp treatment in young permanent teeth with pulp inflammation and for the promotion of further root development.

In the literature, several sources of propolis have been used in animal and even clinical experiments, with good results. The use of Thai propolis in New Zealand white rabbits' incisors could promote wound healing responses in dental pulp comparable to those of calcium hydroxide paste.¹⁴ Indonesian propolis could suppress the expression of IL-6 in inflamed rat dental pulp tissue.³³ Some studies have used 33% Korean green propolis extract for pulpotomy, and at 6 months, propolis showed comparable clinical and radiographic success rate to that of formocresol in primary dentition.³⁴ Kusum et al.³⁵ showed similar results in spite of a significant decrease in success rate of Indian propolis at 9 months. Although the composition of propolis varies according to the source³⁶ and the effects on dental pulp cells may also differ accordingly, studies have provided evidence that suggest the potential application of Brazilian propolis in pulpotomy.

The limitations of this study include the lack of investigation into the antibacterial properties of propolis and failure to consider different propolis extraction techniques. Elgendy and Fayyad³⁷ showed

that nanoparticles of propolis extracts were less cytotoxic and induced less DNA apoptotic changes than their original particles counterpart. In the future, we could analyze propolis nanoparticles to check whether they are more biocompatible.

Conclusions

In summary, our *in vitro* study showed that Brazilian propolis exhibited similar cell viability to that of MTA. It had significantly higher anti-inflammatory and mineralizing effects on hDPCs than did MTA. Further animal studies are required to verify its possible application in the treatment of pulp inflammation in young permanent teeth.

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