Original Article

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Effect of mineral trioxide aggregate mixed with Thai propolis extract on matrix metalloproteinase-2 expression in inflamed human dental pulp cells

Nutnicha Tiyapitsanupaisan[1], Nutthapong Kantrong[2], Subin Puasiri[3], Pattama Nathapakti[1]

[1] *Department of Restorative Dentistry, Faculty of Dentistry, Khon Kaen University, Thailand*
[2] *Faculty of Dentistry, Mae Fah Luang University, Thailand*
[3] *Department of Community Dentistry, Faculty of Dentistry, Khon Kaen University, Thailand*

Objective: To investigate the inhibition activity of mineral trioxide aggregate (ProRoot®MTA) mixed with Thai propolis extract on the expression of Matrix Metalloproteinase-2 (MMP-2) in IL-1β-stimulated human dental pulp cells (HDPCs), compared to calcium hydroxide (Dycal®), a commonly used dental pulp capping material.

Materials and Methods: HDPCs cultured from three freshly extracted intact third molars were incubated with 10 ng/ml of IL-1β to induce MMP-2 activity. Concentrations of Thai propolis extract (0.25-1.50 mg/ml) were evaluated with PrestoBlue cytotoxic assay. ProRoot®MTA mixed with distilled water, ProRoot®MTA mixed with non-toxic Thai propolis extract, non-toxic Thai propolis extract, and DyCal® were used to treat IL-1β-stimulated HDPCs *in vitro*. After 24 h of incubation, culture supernatants were collected and evaluated for MMP-2 expression using gelatin zymography.

Results: Thai propolis extract 0.25, 0.50, and 0.75 mg/ml were not toxic to inflamed pulp cells at 24, 48, and 72 h. MMP-2 expression was upregulated in IL-1β–challenged HDPCs. Pro-form of MMP-2 was significantly reduced when IL-1β–stimulated HDPCs were incubated with 0.75 mg/ml Thai propolis extract and Dycal®, lower than MTA mixed with propolis and untreated control. In addition, Thai propolis extract significantly decreased the levels of active MMP-2 than Dycal®. However, the MMP-2 levels were not statistically different among MTA mixed with distilled water and MTA mixed with the propolis extract.

Conclusion: Thai propolis extract reduced an active form of MMP-2, greater than Dycal®. Although Thai propolis extract and Dycal® provide better MMP-2 reduction than MTA groups, Thai propolis extract mixed with MTA showed an insignificant effect on MMP-2 reduction in IL-1β – stimulated HDPCs.

Keywords: inflammation, matrix metalloproteinase, mineral trioxide aggregate, propolis

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Introduction

Extracellular matrix (ECM) is an integral part of development, morphogenesis, and tissue remodeling of many organs. The integrity of ECM can be affected by matrix metalloproteinases (MMPs) which MMPs play a role in matrix remodeling [1]. Gelatinases including MMP-2, and MMP-9 have a crucial role in soft tissue and dentin collagen breakdown. Both are synthesized and released by pulp cells and osteoblasts [1, 2]. However, MMP-2 expression was more predominant than MMP-9 in sound dentin and carious lesions [2]. MMP-2 was up-regulated in acute pulpitis in comparison with healthy pulp [3].

Corresponding Author: Nutnicha Tiyapitsanupaisan
Department of Restorative Dentistry, Faculty of Dentistry, Khon Kaen University
123 Mittraphap Rd., Muang, Khon Kaen 40002, Thailand
Tel: +668 1862 4922
E-mail: nutnicha.ti@kkumail.com
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In addition, an inflammatory cytokine, interleukin (IL)-1β induced an elevated level of MMP-2, and only minimal amounts of MMP-9 in human pulp cells [4]. MMP-2 might play a role in pulpal inflammation. Interestingly, the expression of cyclooxygenase-2 (COX-2) derived prostaglandin E 2 associated with the expression of MMP-2, a specific inhibitor of COX-2 inhibited MMP-2 production in human pulp cells [5].

In case of deep caries, physical or mechanical trauma that exposed pulp in reversible pulpitis, inhibition of early pulp inflammation and maintaining pulp vitality is important to promote dentinogenic potential of pulpal cells for the reparative process. The pulp capping procedure recruits pulp cells to produce the ECM that turns to be a scaffold for mineralized tissue barrier across the exposed surface, commonly referred to as dentinal bridge. Pulp capping material is one of the elements that contribute to the success of dental pulp viability [6]. Calcium hydroxide has been widely used as pulp capping material that can stimulate the formation of dentin bridge but it can be gradually degraded and creates the tunnel defect in the newly formed dentin. Moreover, it has a high solubility in oral fluids, affecting the sealing ability and microleakage prevention. Mineral trioxide aggregate (MTA) is a pulp-seal, bioactive material agent able to promote pulp cell proliferation, cytokine release, and dentinogenic process. In a prospective histologic study of pulp capping, MTA-treated teeth presented a lower inflammatory response and improved dentin bridge formation (thicker and less porous dentin) than calcium hydroxide [7]. A systematic review and meta-analysis has demonstrated that MTA-treated teeth have a higher clinical success rate than calcium hydroxide-treated teeth [7]. Despite many advantages of MTA, unfavorable properties include delayed setting time, tooth staining over time, poor handling, and high costs. MTA has toxic elements in its freshly mixed state due to a high alkalinity during setting. During an early MTA implantation, an inflammatory molecule IL-1β was increased between days 1 and 3, and COX-2 expression peaked at 12 h and slightly decreased in 3 days after MTA implantation [8, 9]. A recent study found that MTA-treated macrophages did not increase MMP-2 and MMP-9 concentrations [10]. In contrast, MTA increased the production of MMP-9 in neutrophils [11].

Propolis or bee glue has been reported antimicrobial and anti-inflammatory properties. Flavonoid, an active component of propolis inhibited nuclear factor-kappa B (NF-kB) by decreasing the expression of IL-1 in dental pulp. [12]. Thai propolis extract suppressed inflammatory COX-2 expression in pulp cells resulting in an increase of pro-inflammatory cytokines [13]. The effect on pulpal wound healing and pulp exposure in rabbits' teeth treated with the propolis extract showed less inflammation and well-organized dentinal tubules in the dentin bridge, compared with that treated with calcium hydroxide paste [14]. Thai propolis extract had preservative effects on dental pulp cells and PDL, as a storage solution for avulsion tooth [15]. Propolis also has demonstrated excellent anti-inflammatory activity against MMP-2 and MMP-9, better than Biodentine in tooth stem cells. Indeed, the dentin matrix depends on MMPs up-regulated in pulp cell response. Direct pulp capping agents consisting of MMP-digested dentine matrix components have shown similar regenerative properties [16]. It is of interest to know how MTA mixed with propolis modifies the initial inflammatory state of the dental pulp during the regenerative or reparative process by investigating MMP-2 activity in HDPCs using MTA mixed with Thai propolis extract.

Materials and Methods

Preparation of Thai propolis extract

Propolis was collected from an apiary in Nong Khai provinces, Thailand. These were ground into fine particles and dissolved in 70% analytical ethanol. The samples were vortexed and followed by ultrasonic-assisted extraction at 60 Hertz for 20 minutes at ambient temperature. The tubes were centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected in a new tube. The extraction process was repeated three times per tube. The combined supernatant was evaporated to dryness using a rotary evaporator overnight and redissolved in 1 ml ethanol. The extract was kept in a tube that was wrapped with aluminum foil and stored at -20°C until further use [17].

Culture of primary human dental pulp cells

Normal pulp tissues were obtained from three freshly extracted upper or lower nonfunctional third molars, from three healthy volunteers (18–25 years old) with their informed consent. These teeth exhibited no evidence of carious lesions, cracks, restorations, or involvement with periodontal disease. The research protocol was approved by the Khon Kaen University Ethics Committee in Human Research (#HE660252). The extracted tooth was immediately stored in the growth medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco®, by Life Technologies Corporation, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml amphotericin B. Each tooth was separated using a carborundum disc and extraction forceps. Pulp tissue was removed and immediately submerged in a 35-mm culture dish containing DMEM. The tissue explant was cut into several pieces using a scalpel blade #15 and cultured in the growth medium at 37°C under 5% CO2 atmosphere with 95% relative humidity. The medium was replenished every other day. Pulp cells were subcultured until they reached 80% cell and separated independently at the third to the eighth passages were used for further experiments [13].

Cytotoxic analysis of Thai propolis extract

The PrestoBlue cytotoxic assay was used to determine non-toxic concentrations of Thai propolis extract on cultured dental pulp cells. $1x10^4$ pulp cells were seeded into a 96-well plate (Corning Incorporated, NY, USA) and cultured in the growth medium (1%FBS/DMEM) at 37°C for 24 h. On the following day, human recombinant IL-1β (R&D Systems, Inc., Minneapolis, MN, USA) at 10 ng/ml was added to induce inflammation. IL-1β-induced cells were treated with 0.25-1.5 mg/ml of the Thai propolis extract for 24, 48, and 48 h. 1%FBS/DMEM and 10%DMSO are set as negative and positive control, respectively. After 24, 48, and 72 h, cultured supernatant was removed, and cells were incubated with a 50 µl of the PrestoBlue reagent (Gibco®, by Life Technologies Corporation) per well for 60 min. The fluorescence activity was measured at 560/590 nm using a microplate reader (Varioskan Flash, Thermo Fisher Science, Vanntaa, Finland). The percentage of cell viability was calculated using (z-x/y-x)x100, x, y, and z are the fluorescence values of negative control, positive control, and experimental samples, respectively. The percentage of cell viability was calculated from three independent experiments, each of which was performed in triplicate. The highest of non-toxic concentrations of the Thai propolis extract was used to mixed with ProRoot®MTA for the further test [13].

Material preparation and extract

White ProRoot®MTA and DyCal® (Dentsply International Inc., DE, USA) were prepared following the manufacturers' instructions into a vinylpolysiloxane addition-silicones mold (1 mm high and 10 mm radius). Distilled water and non-toxic concentration propolis extract were mixed with ProRoot®MTA in a ratio of 1:3 and left for 24 h at 37°C in 100% humidity before removing the mold [18]. All samples were sterilized by ultraviolet irradiation for 30 min and implanted in 5 ml DMEM for 24 h (ISO 10993-5:2012 part 12) [19]. The extraction was filtered through 0.22 µm pore size and kept at -20 °C until used [18].

Gelatin zymography

Induced dental pulp cells were divided into 4 experimental groups: ProRoot®MTA mixed with distilled water, ProRoot®MTA mixed with non-toxic Thai propolis extract, non-toxic propolis extract, DyCal®, a positive control (0.2% chlorhexidine) and, a negative control (1%FBS/DMEM). All groups were incubated at 37°C for 24h then supernatant was collected and kept at -20°C. Zymography was used to assay the gelatinolytic activity of all samples. Supernatant media from the treated cells were collected and mixed with 5X nonreducing sample buffer in a ratio of 6:1 for preparing the sample. Twenty µl of the samples were electrophoresed on 10% polyacrylamide gels copolymerized with 0.1%, 1%, and 3% gelatin. After electrophoresis, sodium dodecyl sulfate (SDS) was removed from the gel, rinsed twice with 2.5% Triton X-100 for 30 min at room temperature, and incubated at 0.05 M Tris (pH 7.5), 5 mM $CaCl_2$, and 1 M $ZnCl_2$ at 37°C overnight. The gels were stained with 0.25% Coomassie brilliant blue solution and de-stained with 5% methanol and 8% acetic acid. Gels were scanned using an Epson scanner and converted into digital images. Clear zones of gelatin digestion indicated MMPs activity. The band intensities and areas of the relevant bands were measured through Image J software (version 1.41; National Institutes of Health, Bethesda, MD, USA) in triplicate [20, 21].

Statistical analysis

All data were presented as mean±standard deviation. Mean percentages of cell viability and MMP-2 levels were analyzed using One-way ANOVA and Bonferroni's post hoc comparison (SPSS® version 27, IBM, Chicago, IL, USA). The differences were considered statistically significant at the p-value < 0.05.

Results

Effect Thai propolis extract on cell viability

In observed time, 0.25, 0.50, and 0.75 mg/ml of Thai propolis showed no significant difference of cell viability between 24, 48, and 72 h. Higher concentrations of Thai propolis (1.00, 1.25, 1.50 mg/mL) exhibited cytotoxicity in HDPCs Therefore, the highest non-toxic dose of Thai propolis extract was conducted using 0.75 mg/ml propolis to mixed with ProRoot®MTA (Table 1 and Figure 1).

Table 1 Mean and standard deviation of percentage of cell viability at different time points

Time	Cell viability (%) n=3					
	Concentrations of Thai Propolis extract (mg/ml)					
	0.25	0.50	0.75	1.00	1.25	1.50
24 h	96.67±1.46	91.68±2.21	87.20±1.49	42.15±0.36	25.67±2.28	20.34±3.70
48 h	96.19±3.00	95.76±2.13	85.86±2.63	38.43±0.80	16.39±3.22	7.84±1.05
72 h	98.70±0.50	97.24±1.45	92.56±1.95	49.13±7.18	15.71±2.68	6.11±0.16

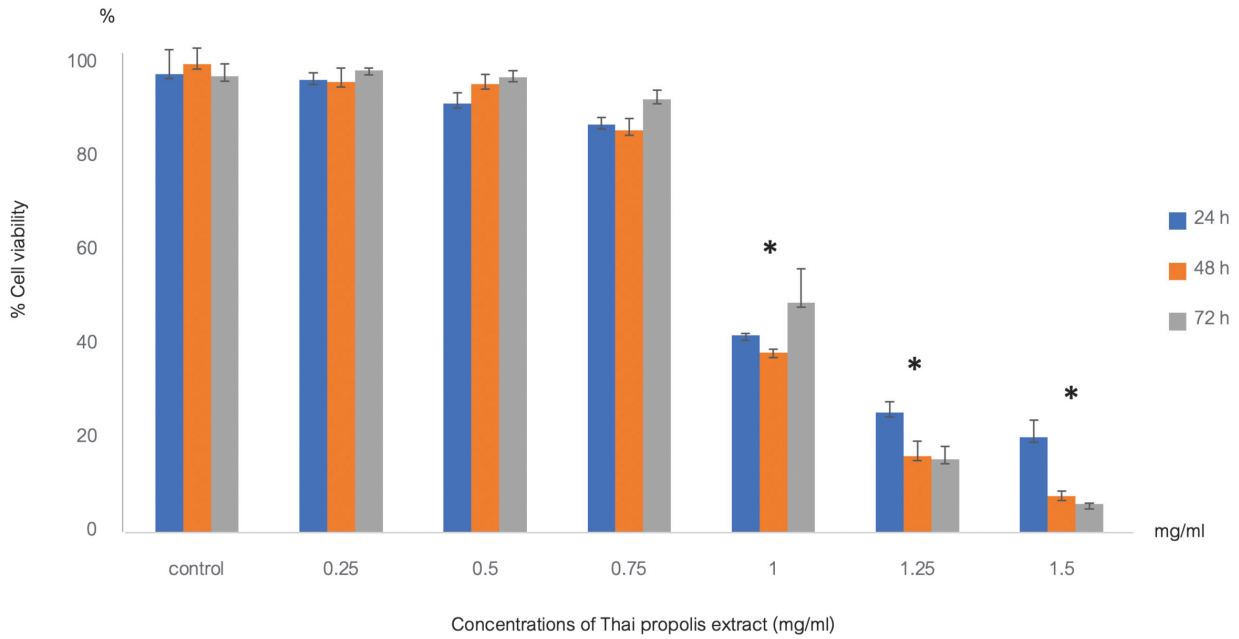

Figure 1 Determination of the cytotoxicity of Thai propolis extract by the PrestoBlue assay. 10 ng/mL IL-1β stimulated human dental pulp cells were treated with 0.25-1.5 mg/mL of the extract at 24, 48, and 72 h. Error bars = standard deviation; *$p<0.05$, as compared with the percentage of cell viability in control.

Effect ProRoot®MTA mixed with Thai propolis extract on MMP-2 production

In gelatin zymography, 10% polyacrylamide gels copolymerized 3% gelatin is a suitable gel condition due to the presence of clearer and sharper bands. Expression profiles of detected MMPs are shown in Fig.2a, the main gelatinase secreted by HDPCs migrated at 72 kDa, and 63 kDa which represent the pro MMP-2 and active MMP-2, respectively. Minor gelatinolytic bands were observed at 92 kDa, which represents pro MMP-9. Densitometric analysis of the total expression of MMP-2 of each band was converted to areas and compared with positive control (0.2% chlorhexidine) to show the fold change of MMP-2 expression (Table 2) Pro and active MMP-2 expression in inflamed (IL-1β stimulated) HDPCs were significantly higher than healthy (no IL-1β stimulated) HDPCs. In inflamed HDPCs, four experiments significantly reduced MMP-2 expression (pro MMP-2 and active MMP2). Thai propolis extract (0.75 mg/ml) and Dycal® potentially reduced pro MMP-2 production than MTA groups as no IL-1β stimulated HDPCs ($p<0.05$). As with the inhibitory effect, the propolis extract significantly decreased active MMP-2 than Dycal®. No different MMP-2 levels between ProRoot® MTA mixed with distilled water and ProRoot® MTA mixed with the propolis extract (Table 2, Figure 2).

Table 2 Mean and standard deviation of fold of MMP-2 for each experiment to negative control

	Mean ± SD (fold of negative control) n=1					
	Standard control	Stimulated with IL-1β				
		MTA+W	MTA+PL	PL	Dycal	Positive control
Pro MMP-2	0.81±0.05	0.73±0.08	0.75±0.07	0.40±0.10	0.29±0.05	0.17±0.01
Active MMP-2	0.43±0.01	0.58±0.01	0.55±0.08	0.47±0.04	0.72±0.05	0.28 ±0.01

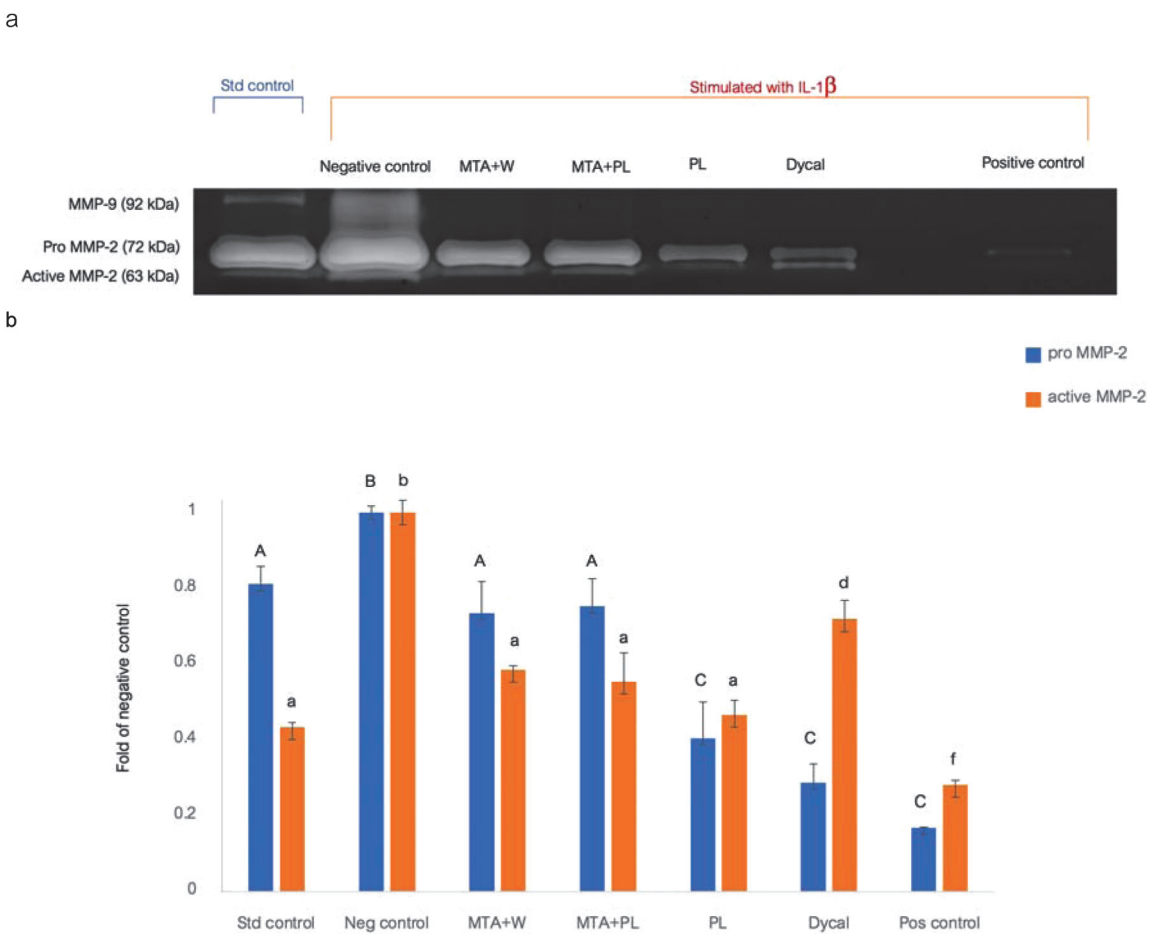

Figure 2 **Effect of treatments on MMP-2 expression.** (a) MMPs expression was determined by zymogram. (b) Densitometric analysis of total expression of MMP-2. 10 ng/ml IL-1β stimulated human dental pulp cells were treated with media extract from ProRoot®MTA mixed with distilled water (MTA+W), ProRoot®MTA mixed with non-toxic propolis extract (MTA+PL), non-toxic propolis extract (PL) and DyCal® at 24 h. The positive control group contains 0.2% chlorhexidine and the negative control contains 1%FBS/DMEM. Std control group is no IL-1β stimulated pulps. The fold expression was compared with negative control. Error bars = standard deviation; groups with similar upper-case letters are not significantly different between pro MMP-2. Similar lower-case letters indicate a non-statistically significant difference of active MMP-2 levels. A significance difference was determined at values of $p<0.05$.

Discussion

Dental pulp exposure may occur during deep carious dentin removal or cavity preparation. Inflammation in the superficial layer of pulp should be restricted. To mimic the clinical condition of pulp inflammation, HDPCs were treated with 10 ng/mL of IL-1 which is the concentration increasing the six-fold of COX-2 expression [13]. and upregulating levels of MMP-2 [4]. A direct link between COX-2 and MMP-2 had been shown in many studies [5, 22]. The MMP-2 expression was examined at 24 h because a previous study has shown that MTA produced a significant increase in the COX-2 expression when compared with calcium hydroxide on day 1 [9]. Corresponding to the rat dental pulps capped with MTA, a few scattered inflammatory cells were found beneath the capping material after 1 day [23]. Likitpongpipat *et al*., 2019 revealed that the histological analysis of rabbit teeth with propolis pulp capping showed no pulp inflammation and more orderly arranged dentinal tubules of the dentin compared with that of calcium hydroxide pulp capping [14]. This could be related to flavonoids, caffeic acid, and phenethyl ester (CAPE) in propolis that inhibit the lipoxygenase (LOX) and cyclooxygenase (COX) pathway of arachidonic acid [24]. In this study, the highest non-toxic concentration of the extract was 0.75 mg/ml, close to the highest Thai propolis extract concentration (0.63 mg/ml) that inhibits COX-2 mRNA expression in HDPCs [13]. Accordingly, the reduction of MMP-2 and MMP-9 expressions was observed in a dose-dependent manner [25, 26]. Thus, 0.75 mg/ml propolis extract mixed with MTA before medium extraction was used to treat HDPCs. A current study demonstrated that Thai propolis extract 0.75 mg/ml could suppress the MMP-2 expression significantly in inflamed HDPCs. However, the mechanism of propolis in MMP-2 regulation remains unclear, it could be explained by the flavonoid from propolis that decreased MMP-2 and MMP-9 expression [26], and/or CAPE reduced the protein level MMP-2 and MMP-9 [27, 28].

MMP-2 and MMP-9 play roles in matrix remodeling by regulating ECM digestion. In carious lesion, MMP-2 contributes to the breakdown of collagenous matrices [2]. Interestingly, cancer studies have reported the increased MMP-2 level by an acidic extracellular pH. Corresponding to acidic pH during carious progression, MMP-2 expression was up-regulated [29]. In this study, the extracted medium of MTA mixed with propolis extract resulted in lower alkalinity than MTA-mixed distilled water. However, comparing the treatments between groups after MMP-2 induction, we did not find any different levels between these groups. Markedly reduced MMP-2 expression was shown in the acidic pH of propolis extract-treated inflamed pulp cells. Chlorhexidine digluconate (CHX) has MMP inhibitor effects. The 0.2% CHX was able to inhibit MMP-2 activity in dental pulp cells [30].

Regulations of MMPs by dentin-derived growth factors may affect reparative dentin formation. MMP-2 has been proposed to release active TGF-β2 in the carious lesion, which can then stimulate the repair process [31]. Moreover, MMP-2 was shown to cleave the dentin matrix protein-1 (DMP-1) and dentin sialophosphoprotein (DSPP), which are key dentin matrix signaling proteins, and are able to enhance pulp healing capacity. (31,32) Notably, the combination of MTA with propolis upregulated DSPP and DMP-1 expression in dental pulp stem cells [33].

The present study used the ISO 10993-5 protocol to fabricate material used in the cell culture [19]. Pulpal fibroblasts were selected because of their contact with the pulp capping material in clinical conditions. ProRoot®MTA is rich in calcium and hydroxide ions, resulting in increased pH and calcium ion release. Initially,

the high surface pH of ProRoot®MTA can cause the denaturation of proteins in the adjacent cells. The high toxicity of fresh ProRoot®MTA affected cell viability lower than 50% [34]. To evaluate MMP-2 activity from active inflamed pulp cells, we extracted a 24-h set ProRoot® MTA to avoid apoptosis of HDPCs.

Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9. Other MMPs, such as MMP-1, MMP-8, and MMP-13 can also lyse gelatin but are very weak. MMPs will be detected as clear bands against a blue background [35]. In the present study, 10% polyacrylamide gel copolymerized with 0.1% gelatin does not appear band clearly in each sample and does not correspond to the gel condition in the previous study [20]. Testing substrates from various sources should determine the optimal substrate conditioned medium, and the optimal running and developing conditions. Gel copolymerized with 0.1% gelatin may be not enough for MMPs to digest. In 1% and 3% gelatin substrate gels show clear and sharper bands, respectively. Thus, the zymography can detect pro MMP-2, the activated form of MMP-2 and pro MMP-9 by molecular weight (72, 63, and 92 kDa, respectively) [35]. In the present study, the main gelatinolytic proteinase secreted by the inflamed pulp cells was MMP-2, and only minimal amounts of MMP-9 were detected similar to the study by Kim *et al*., 2014. [30] In contrast, Chang *et al*., 2001 and Gusman *et al*., 2002, determined the MMPs activity using zymography. They found that inflamed HDPCs with chronic inflammation exhibited significantly strong MMP-9 expression but weak MMP-2 expression [4, 20]. Although the overexpression of MMP-2 or MMP-9 in inflamed dental pulp cells was positively correlated with the gelatinolytic activity, over-destaining zymogram gel can lead to the loss of suitable bands for quantification. Using Western blot would be another examination nevertheless, this approach must performed with specific antibodies [35].

Our study investigated the effects of experiments on collagen matrix degradation by focusing on the MMP-2 expression in pulp cells. Based on tissue resorption, MMP activity is mainly regulated by tissue inhibitors of matrix metalloproteinases (TIMPs), and many different MMPs are involved [35]. These can be investigated in future study.

Conclusion

In inflamed pulp, Thai propolis extract reduces active MMP-2 greater than Dycal®. Thai propolis extract and Dycal® provide better MMP-2 reduction than MTA groups. MMP-2 expression between MTA mixed with or without propolis extract is similar.

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