Original Articles

Biodentine™ and MAC28 Inhibit Lipopolysaccharides-Induced Pulpal Inflammation in Human Dental Pulp Cells

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Abstract

Our previous results demonstrated that pretreatment with monocarbonyl analogue of curcumin compound 28 (MAC28) in lipopolysaccharides (LPS)-treated human dental pulp cells (HDPCs) could suppress inflammation. However, the pharmacological action of MAC28 co-incubated with LPS in HDPCs remained unclear. Furthermore, the impact of MAC28 in combination with Biodentine™ in LPS-treated HDPCs was still unclear. This study aimed to examine the cell viability and anti-inflammatory effects of Biodentine™ and MAC28 in LPS-treated HDPCs. HDPCs were assigned to five groups: (1) control, (2) LPS, (3) LPS + MAC28, (4) LPS + Biodentine™, and (5) LPS + MAC28 + Biodentine™. The concentration of LPS and MAC28 used in this study were 20 µg/mL and 10 µM, respectively. The Biodentine™ extract was mixed with Alpha modification of Minimum Essential Medium Eagle (α-MEM) at a 1:16 dilution ratio for cell treatments. Cell viability was assessed using the Alamar Blue assay. The expressions of toll-like receptor-4 (TLR-4), myeloid differentiation factor-2 (MD-2), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) mRNA were analyzed by qRT-PCR. Data was analyzed using one-way ANOVA with LSD post hoc tests at a 95% confidence interval. The results demonstrated that LPS treatment did not significantly affect HDPC viability compared with the control ($p > 0.05$). Similarly, co-treatment with MAC28, Biodentine™, or both combined did not affect cell viability ($p > 0.05$ vs. control), indicating no cytotoxicity under the experimental conditions. LPS significantly upregulated TLR-4, MD-2, TNF-α, and IL-6 mRNAs when compared with the control ($p < 0.05$). However, LPS-induced HDPCs co-incubated with MAC28, Biodentine™, or their combination significantly reduced the expressions of TLR-4, MD-2, and TNF-α mRNAs ($p < 0.05$), while the expression of IL-6 mRNA levels remained unchanged when compared with the LPS group ($p > 0.05$). MAC28 and Biodentine™ exhibited anti-inflammatory effects without cytotoxicity in LPS-treated HDPCs, supporting their potential as adjunctive agents for the treatment of pulpitis.

Keywords: Biodentine™, Dental pulp, Inflammation, Lipopolysaccharides, Pulpitis

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Introduction

Dental caries-induced pulpitis is among the most common oral diseases worldwide.[1] Toll-like receptor-4 (TLR-4) has received significant attention as it is involved in the inflammatory process of the dental pulp at the cellular level.[2] Myeloid differentiation factor-2 (MD-2) is a protein essential for the activation of TLR-4 signaling, which plays a critical role in inflammation across various tissues.[3,4] Several studies have demonstrated that the TLR-4/MD-2 complex is present on cell surfaces, including the membranes of dental pulp cells.[5,6] Lipopolysaccharide (LPS) is one of the most important molecules initiating TLR-4/MD-2 signaling. It binds to hydrophobic MD-2, thereby triggering inflammation.[4,7] Therefore, blocking TLR-4 signaling via MD-2 inhibitors represents a potential therapeutic strategy for the treatment of pulpitis (Fig. 1).[8]

The monocarbonyl analogue of curcumin compound 28 (MAC28), a curcumin-derived compound that functions as an MD-2 inhibitor, has been shown to block TLR-4 signaling in LPS-treated macrophages by directly binding to MD-2.[9] Our previous results demonstrated that pretreatment with MAC28 in LPS-treated human dental pulp cells (HDPCs) effectively inhibited inflammation (Fig. 1).[8] However, the pharmacological effects of MAC28 when co-incubated with LPS in HDPCs remain unclear. In addition, evidence regarding the pharmacodynamic effects of MAC28 in combination with Biodentine™ in LPS-stimulated HDPCs is still lacking.

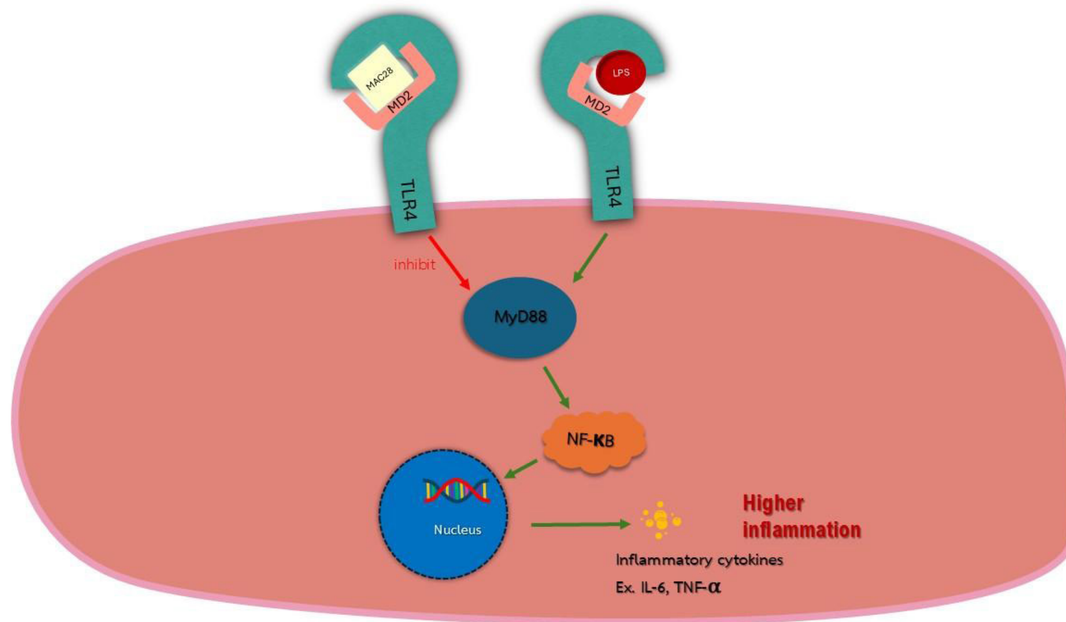

Figure 1 *The relationship between LPS, MD-2, TLR-4, and MAC28. IL-6, interleukin-6; LPS, lipopolysaccharides; MAC28, monocarbonyl analogue of curcumin compound 28; MD-2, myeloid differentiation factor-2; MyD88, Myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-alpha*

This study aimed to investigate the cell viability and anti-inflammatory effects of Biodentine™ and MAC28 in LPS-stimulated HDPCs. It was hypothesized that both materials would reduce inflammation in LPS-stimulated HDPCs and that their combination would synergistically inhibit the inflammatory response.

Materials and methods

Dental pulp tissue collection and dental pulp cell culture

This study was performed following approval from the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University, Thailand (ethical approval number: 53/2022). After informed consent was obtained

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from all participants, healthy dental pulp tissues were collected from individuals aged 18-21 years who were scheduled for the surgical removal or extraction of impacted teeth. Following tooth extraction, dental pulp tissues were aseptically removed from teeth and collected for cell culture as previously described.10 The HDPCs were cultured in Alpha modification of Minimum Essential Medium Eagle (α-MEM) (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/mL of penicillin, 100 µg/mL streptomycin (Gibco, USA), and 100 µmol/L of L-ascorbic acid at 37°C in an atmosphere of 5% CO_2. The medium was renewed every other day. HDPCs from passages two to five were used in the experiment.

The study design

HDPCs were divided into five groups, n = 3/group, as follows: 1) control group: cells were maintained in culture medium containing 0.0001% dimethyl sulfoxide (DMSO); 2) LPS group: cells were maintained in culture medium containing 20 µg/mL LPS; 3) LPS + MAC28 group: cells were maintained in culture medium containing 20 µg/mL LPS and 10 µM MAC28; 4) LPS + Biodentine™ group: cells were maintained in culture medium containing 20 µg/mL LPS and Biodentine™ extract; and 5) LPS + MAC28 + Biodentine™ group: cells were maintained in culture medium containing 20 µg/mL LPS, 10 µM MAC28, and Biodentine™ extract. After 24 hours of treatment, cell viability and inflammation were analyzed.

Preparation of LPS, MAC28, and Biodentine™

A concentration of 20 µg/mL LPS, 10 µM MAC28, and Biodentine™ extract was used in this study, in accordance with previous studies.[8-10] LPS from Escherichia coli (O111:B4; #0000110081) was purchased from Sigma-Aldrich. MAC28 was provided by Wenzhou Medical University, Zhejiang, China. A 1 M stock solution of MAC28 (molecular weight = 469.53) was prepared in 100% DMSO according to previous reports.[8] The absolute concentration of 10 µM MAC28 in culture medium was used for cell treatment.[8,9] Since the absolute concentration of DMSO diluted in culture medium consisting of MAC28 was 0.0001%, 0.0001% DMSO was also included in the control group, LPS group, and Biodentine™ group. Biodentine™ extract was prepared following a previously described method.[10-12] The Biodentine™ extract was diluted in culture medium at a ratio of 1:16 for cell treatments.

Determination of cell viability

To determine the viability of HDPCs in culture medium containing LPS, MAC28, and Biodentine™, HDPCs were divided into eight groups (n = 3/group), as follows: 1) control group; 2) LPS group; 3) MAC28 group; 4) Biodentine™ group; 5) MAC28 + Biodentine™ group; 6) LPS + MAC28 group; 7) LPS + Biodentine™ group; and 8) LPS + MAC28 + Biodentine™ group. After seeding 10,000 HDPCs per well in 96-well plates for 24 hours, cell treatments were performed as previously mentioned. In each assigned group, the cell treatment was performed in triplicate. At the end of the 24-hour experiment, cell viability was examined by an Alamar Blue assay as described by Weekate *et al.*[10] In brief, 10% AlamarBlue® was added to each well and incubated for four hours at 37°C. The absorbance reading was set at 570-600 nm using a multi-well scanning spectrophotometer (Tecan Group Ltd., Männedorf, canton of Zürich, Switzerland). HDPCs in the control group were interpreted as 100% viable.

Determination of inflammation by quantitative real-time polymerase chain reaction (qRT-PCR)

To examine the inflammatory markers, HDPCs were seeded in 6-well plates at a density of 2.5 × 105 cells/well. The HDPCs were assigned to five groups as previously stated in the study design. In each assigned group, the cell treatment was performed in triplicate. After 24 hours of treatment, qRT-PCR was conducted using the RNeasy Mini Kit (QIAGEN). Subsequently, cDNA was synthesized using PCRBIOSYSTEMS cDNA UltraScript kit according to the manufacturer's instructions. qRT-PCR was conducted using the SYBR Green-based method. The primer pairs for TLR-4, MD-2, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and GAPDH used in this study are presented in Table 1. The relative changes in gene expression were normalized with GAPDH and quantified using the 2– ∆∆CT method.[13]

Table 1 *List of primer pairs used for qRT-PCR analysis*[8]

mRNA	*Primer sequences (5′ to 3′)*
TLR-4	*Forward: CAA CAA AGG TGG GAA TGC TT* *Reverse: TGC CAT TGA AAG CAA CTC TG*
MD-2	*Forward: TTC CAC CCT GTT TTC TTC CA* *Reverse: AAT CGT CAT CAG ATC CTC GG*
TNF-α	*Forward: GCT GCA CTT TGG AGT GAT CG* *Reverse: CTT ACC TAC AAC ATG GGC TAC AG*
IL-6	*Forward: ATG AAC TCC TTC TCC ACA AGC GC* *Reverse: GAA GAG CCC TCA GGC TGG ACT G*
GAPDH	*Forward: ACC ACA GTC CAT GCC ATC AC* *Reverse: TCC ACC ACC CTG TTG CTG TA*

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin 6; MD-2, myeloid differentiation factor-2; qRT-PCR, quantitative real-time polymerase chain reaction; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-alpha.

Statistical analysis

The statistical analysis was performed based on n = 3/group for all experiments. The results were expressed as mean ± standard deviation (SD). One-way ANOVA followed by post hoc LSD tests were conducted for group comparison. All statistical analyses were carried out using GraphPad Prism 10 (version 10.2.1) software for macOS. A 95% confidence level for statistical significance was applied in this study ($p < 0.05$).

Results

Effects of LPS, MAC28, and Biodentine™ on HDPC viability

At the 24-hour incubation period, 20 µg/mL LPS did not influence HDPC viability when compared with the control group (Fig. 2, $p > 0.05$). In addition, HDPCs co-incubated with 10 µM MAC28, Biodentine™ extract, and the combination of MAC28 and Biodentine™ for 24 hours also showed comparable cell viability compared with the control group (Fig. 2, $p > 0.05$). Furthermore, LPS-induced HDPCs co-incubated with either MAC28, Biodentine™, or both combined for 24 hours showed no reduction in viability, as the percentages of the viable HDPCs were similar to those in the control (Fig. 2, $p > 0.05$).

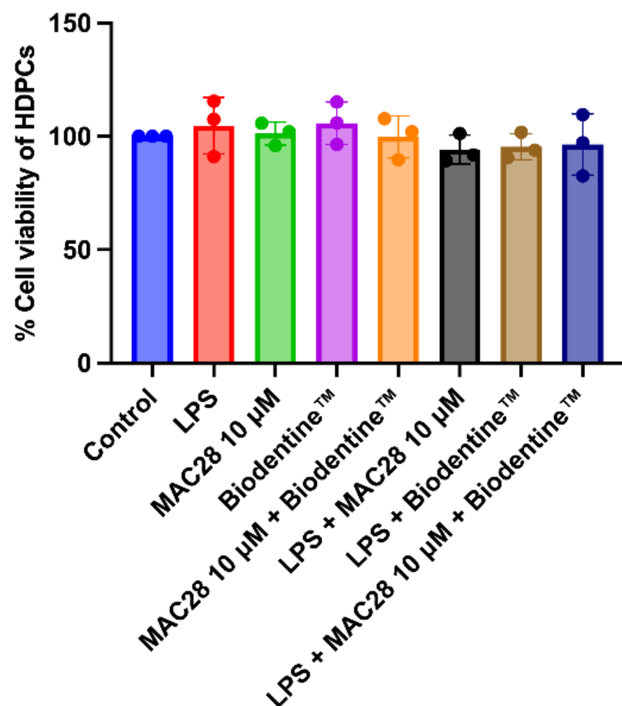

Figure 2 *Cell viability (% control) in LPS-treated HDPCs exposed to MAC28, Biodentine™, and their combination for 24 h (n = 3/group). * p < 0.05. Statistical analyses were performed by One-way ANOVA. HDPCs, human dental pulp cells; LPS, lipopolysaccharides; MAC28, monocarbonyl analogue of curcumin compound 28*

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Effects of MAC28, Biodentine™, and both combined on the LPS-induced inflammation in HDPCs

Treatment with 20 µg/mL LPS for 24 hours significantly induced inflammation as the levels of TLR-4, MD-2, TNF-α, and IL-6 mRNAs were upregulated when compared with the controls (Fig. 3A-D, $p < 0.05$). Co-incubation of either 10 µM MAC28, Biodentine™ extract, or the combination of MAC28 and Biodentine™ significantly reduced the levels of TLR-4, MD-2, and TNF-α mRNAs compared with the LPS group (Fig. 3A-C, $p < 0.05$). However, the expression of IL-6 mRNA remained unchanged in LPS-induced HDPCs treated with the MAC28, Biodentine™, or combined conditions (Fig. 3D, $p > 0.05$).

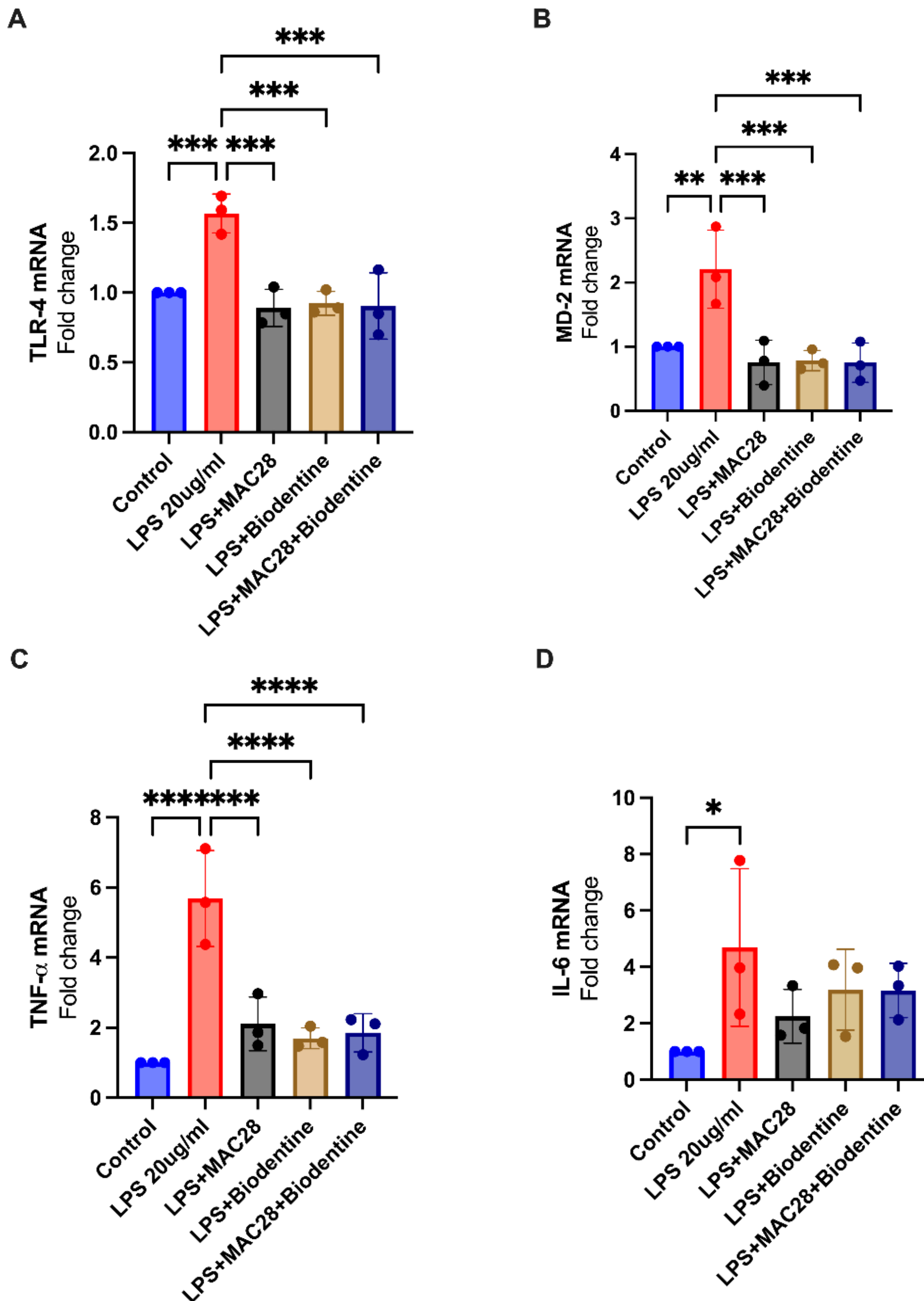

*Figure 3 Inflammatory profiles of LPS-treated HDPCs: A) Expression of TLR-4 mRNA (n = 3/group), B) Expression of MD-2 mRNA (n = 3/group), C) Expression of TNF-α mRNA (n = 3/group), and D) Expression of IL-6 mRNA (n = 3/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. IL-6, interleukin-6; LPS, lipopolysaccharides; MAC28, monocarbonyl analogue of curcumin compound 28; MD-2, myeloid differentiation factor-2; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-alpha*

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Discussion

The major findings from this study were: 1) 20 µg/mL LPS, 10 µM MAC28, Biodentine™ extract, and their combinations had no effect on HDPC viability at 24 hours; 2) LPS induced inflammation in HDPCs by upregulating TLR-4, MD-2, TNF-α, and IL-6 mRNAs; and 3) MAC28, Biodentine™, and their combination suppressed inflammation in LPS-treated HDPCs, but no synergistic anti-inflammatory effect was observed between MAC28 and Biodentine™. Therefore, the hypothesis that these materials reduce inflammation in LPS-treated HDPCs were partially accepted; however, their combination did not synergistically inhibit the inflammatory response.

A previous study demonstrated that 0.1 – 100 µg/mL LPS did not alter cell viability at 24 hours and induced cell proliferation in the dental pulp stem cells of young and aged rats.[14] Furthermore, our previous findings showed that 20 µg/mL LPS did not reduce HDPC viability at 24 hours.[8,10,15] In this study, it was confirmed that 24-hour exposure to 20 µg/mL LPS did not significantly affect the viability of HDPCs. In addition, these current results were in accordance with our previous study that 10 µM MAC28 did not compromise HDPC viability.[8] It was also reported that Biodentine™ extract applied in this study did not compromise HDPC viability, which agreed with a previous report that tested Biodentine™ extract at 10-25% dilution for 24-72 hours and discovered no impairment of HDPC viability.[16,17] Combining 20 µg/mL LPS with either 10 µM MAC28, Biodentine™ extract, or both combined did not impair HDPC viability either. These findings suggest that all the treatment conditions did not adversely affect HDPC viability.

LPS derived from Escherichia coli is one of the most commonly studied endotoxins and is widely used to model dental pulp cell inflammation due to its ability to induce proinflammatory cytokine production.[18] According to previous studies, LPS activates the TLR-4/myeloid differentiation primary response 88 (MyD88)/nuclear factor-κB (NF-κB) signaling pathway, leading to the secretion of pro-inflammatory cytokines such as TNF-α and IL-6.4,16-19 TLR-4, which recognizes LPS, requires MD-2 as a cofactor for effective signal transduction (Fig. 1).[4,22] Results from our previous study and findings from this study confirm that LPS upregulates TNF-α and IL-6 mRNAs with concomitant increases in TLR-4 and MD-2 mRNAs.[8] Therefore, the application of 20 µg/mL LPS for 24 h is suitable for inflammation induction in HDPCs without compromising cell viability.

Following the LPS treatment, our results revealed that MAC28, Biodentine™, and their combination reversed the LPS-induced inflammation in HDPCs as TLR-4, MD-2, and TNF-α mRNAs decreased. However, no synergistic effect was observed between MAC28 and Biodentine™, as the lowered TLR-4, MD-2, and TNF-α mRNA levels were not significantly lower than either MAC28 or Biodentine™ alone in LPS-induced HDPCs. The absence of an additional benefit with co-application can be explained by several biological factors. First, both MAC28 and Biodentine™ likely converge on the same signaling complex (TLR-4/MD-2/NF-κB), therefore, further suppression by combining these materials provides no additional effect. Second, a concentration mismatch is possible, as only one concentration of each material was tested. Third, the two materials may exhibit potential antagonism, which could hinder any additive effect when co-incubated. Finally, some mediators, such as IL-6, can be regulated through TLR-4-independent pathways, which may sustain their expression despite upstream suppression.[23] Further studies are needed to confirm these speculations. In line with this, our previous study reported that a 2-h pretreatment of MAC28 prior to LPS stimulation reduced TNF-α and IL-6 mRNA and protein expression through downregulation of TLR-4/MD-2.8 The present findings extend this by confirming that MAC28 co-incubated with LPS also suppressed inflammation via the TLR-4/MD-2 signaling pathway. Although none of the treatments tested reduced IL-6 mRNA compared with the LPS group, which is consistent with our earlier observation that, although MAC28 pretreatment inhibited inflammatory cytokines, IL-6 mRNA and protein levels did not return to control levels.[8] Taken together, these findings suggest that persistent IL-6 expression during co-treatment may be maintained

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through TLR-4-independent pathways,[23] thereby explaining the lack of additional anti-inflammatory benefit when MAC28 and Biodentine™ were combined.

Biodentine™ has been shown to possess anti-inflammatory and immunomodulatory properties in cells harvested from periapical lesions. It inhibited the production of TNF-α and IL-6, while enhancing the secretion of anti-inflammatory cytokines in granulocytes and other inflammatory cells from periapical lesions. Interestingly, the non-cytotoxic concentration of conditioned Biodentine™ medium that lowered IL-6 lay between 12.5%-25%.[17] In our study, the Biodentine™ extract was diluted at a ratio of 1:16, which corresponds to approximately 6.25% of the original extract. A 1:16 extract was intentionally selected to align with established HDPC protocols and to ensure cytocompatibility as confirmed in previous studies,[10-12] thereby providing a reliable concentration for assessing biological effects without confounding artifacts from higher extract concentrations. However, as discussed earlier, further studies are needed to verify the time course and determine the optimal concentration of Biodentine™ extract in the *in vitro* pulpitis model.

Both MAC28 and Biodentine™ reduced selected LPS-responsive genes (TLR-4, MD-2, TNF-α) at 24 hours without compromising HDPC viability, supporting the biological plausibility of attenuating early innate immune signals that are relevant to vital pulp therapy (VPT), hemostasis, and postoperative comfort. For regenerative or reparative procedures, early immunomodulation could help establish a more permissive microenvironment; however, these in vitro gene-level findings do not yet demonstrate clinical benefit, sealing performance, or effects on osteo/odontogenic differentiation. Co-application was biologically compatible but did not exceed the effects of either material alone at 24 hours. More studies are therefore required before concluding that the combination of MAC28 and Biodentine™ offers no advantage over Biodentine™ alone in clinical practice. Future VPT-related studies should confirm cytokine protein levels, assess the dose-response relationship of MAC28 and Biodentine™, evaluate extended time courses to determine cytokine kinetics, and investigate osteo/odontogenic differentiation and mineralization in both *in vitro* and *in vivo* models before any clinical inferences can be made.

Conclusion

Within the limitations of this study, MAC28 and Biodentine™ reduced inflammation without affecting HDPC viability; however, their combination did not demonstrate a synergistic effect. These findings suggest that MAC28 could serve as an alternative to Biodentine™ by targeting the modulation of selected LPS-responsive genes in HDPCs.

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Conflicts of interest

The authors declare no conflicts of interest.

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