EFFECTS OF LPS AND HYDROGEN PEROXIDE ON CELL VIABILITY AND EXPRESSION OF INFLAMMATORY CYTOKINES IN HUMAN DENTAL PULP CELLS: A PRELIMINARY STUDY

Suphachart CHAYAWATTO[1], Chatvadee KORNSUTHISOPON[2] and Pairoj LINSUWANONT[3]

1 Master of Science Program in Endodontics, Department of Restorative Dentistry, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand; suphachart.dent@gmail.com
2 Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand; Chatvadee.K@chula.ac.th
3 Department of Restorative Dentistry, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand; linspairoj@gmail.com

ARTICLE HISTORY
Received: 7 April 2025 **Revised:** 21 April 2025 **Published:** 6 May 2025

ABSTRACT

This study aimed to evaluate the effects of lipopolysaccharide (LPS) and hydrogen peroxide (H_2O_2) on cell viability and the expression of inflammatory cytokines in human dental pulp cells (DPCs). DPCs were treated with various concentrations of LPS (20, 50, and 100 μg/mL), 400 μM H_2O_2, or their combination. Cell viability was assessed using the MTT assay at days 1 and 3, while the expression of IL-1β, IL-6, TNF-α, and IFN-γ was analyzed by RT-qPCR after 24 hours. LPS alone did not significantly affect cell viability, even at higher concentrations, whereas co-treatment with H_2O_2 markedly reduced viability at both time points. The combination of LPS and H_2O_2 significantly upregulated the expression of all measured inflammatory cytokines compared to the control group, though differences between LPS alone and the combined treatment were not statistically significant. These findings suggest that H_2O_2 enhances the inflammatory response initiated by LPS and effectively reduces DPC proliferation, thereby better simulating the *in vivo* inflammatory microenvironment. Further study is recommended to investigate the expression of inflammatory genes at multiple time points to optimize the use of LPS and H_2O_2 as inducers in an inflammation model.

Keywords: Human Dental Pulp Cells, Lipopolysaccharide, Hydrogen Peroxide

CITATION INFORMATION: Chayawatto, S., Kornsuthisopon, C., & Linsuwanont, P. (2025). Effects of LPS and Hydrogen Peroxide on Cell Viability and Expression of Inflammatory Cytokines in Human Dental Pulp Cells: A Preliminary Study. *Procedia of Multidisciplinary Research, 3*(5), 44.

INTRODUCTION

Inflammation is a fundamental biological response that plays a crucial role in dental pulp tissue following injury. The dentin-pulp complex reacts to bacterial invasion through a cascade of inflammatory events, which, when properly regulated, facilitate tissue repair and regeneration. While excessive or chronic inflammation can hinder healing, evidence indicates that a controlled inflammatory response is crucial for initiating the healing process (Goldberg et al., 2015). This process involves inflammation, proliferation and migration of mesenchymal stem cells and differentiation of stem cells to generate hard tissue (El karim et al., 2021)

Given the importance of preserving pulp vitality, vital pulp therapy (VPT) has emerged as a promising approach for treating inflamed pulp tissue affected by caries. Clinical studies have demonstrated high success rates, which exceed 90% regardless of the type of VPT (direct pulp capping, partial pulpotomy or full pulpotomy) (Asgary et al., 2024). However, despite its clinical potential, a deeper understanding of the cellular and molecular mechanisms underlying pulpitis is necessary to optimize treatment strategies (Richert et al., 2022). To bridge this knowledge gap, various in vitro and in vivo models have been developed to study pulp inflammation and healing. In particular, lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, is widely used in vitro to induce inflammation. LPS activates Toll-like receptor 4 (TLR4), leading to the upregulation of key pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), all of which contribute to pulp tissue inflammation (Brodzikowska et al., 2022). Due to its ability to mimic bacterial-induced inflammation, LPS is frequently employed as an inducer in pulpitis studies (Richert et al., 2022).

Nevertheless, a single inflammation inducer, like LPS may not fully replicate the complex microenvironment of clinical pulpitis (Richert et al., 2022). To better simulate the inflammatory profile observed *in vivo*, studies have explored the use of more complex inducers, such as cytokine cocktails and bacterial co-culture models (Akamp et al., 2024). Hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), is a byproduct of phagocytosis by immune cells. An accumulation of ROS contributes to oxidative stress, which can lead to cellular apoptosis (Hong et al., 2024). A recent study has reported that the combination of LPS and H_2O_2 generates a more pronounced inflammatory response, leading to decreased cell proliferation, impaired differentiation, mitochondrial imbalance and increased apoptosis. This combined model has been proposed as a more clinically relevant approach to replicating pathological conditions in inflamed pulp tissues (Vaseenon et al., 2023).

However, the effects of LPS and H_2O_2 on the expression of key inflammatory genes involved in the inflammation are still not known. Understanding how these two agents interact at different concentrations is essential for optimizing *in vitro* model and improving therapeutic intervention for pulpitis. Therefore, this study aims to investigate the effects of LPS and H_2O_2 on cell viability and the expression of inflammatory genes in human dental pulp cells.

LITERATURE REVIEWS

The dental pulp initiates a complex cascade of immune responses upon exposure to microbial by-products. In the early stages of carious lesion development, the innate immune response is activated through increased outward fluid movement in the dentinal tubules, secretion of immunoglobulins, and the release of neuropeptides from sensory nerve endings. As the lesion advances and microorganisms penetrate deeper into the pulp chamber, phagocytic responses intensify, contributing to a heightened inflammatory environment (Hahn & Liewehr, 2007). LPS, a key component of the outer membrane of gram-negative bacteria, is widely used in vitro to simulate pulpal inflammation, particularly in studies involving DPCs. Upon interaction with TLR4, LPS triggers *Myeloid differentiation primary response*88-dependent signaling, leading to the activation of the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase

(MAPK) signaling pathways. These pathways orchestrate the transcription of pro-inflammatory mediators that closely resemble those observed in clinical pulpitis (Brodzikowska et al., 2022). An in vitro study demonstrated that stimulation of DPCs with *Escherichia coli*-derived LPS significantly reduced cell proliferation while promoting cellular migration. This was associated with the upregulation of TLR4 expression, which in turn modulated cellular behavior and cytokine production (Liu et al., 2014). Another study found that exposure of DPCs to both *Porphyromonas gingivalis* and *E. coli* LPS resulted in increased expression of inflammatory cytokines, including IL-6, IL-10, and IL-18, with *P. gingivalis* LPS eliciting a more pronounced early-phase response (Mojtahedi et al., 2022). In contrast, a separate investigation revealed that *E. coli* LPS significantly enhanced the expression of IL-1β, IL-6, IL-8, and TNF-α compared to *P. gingivalis* LPS (Lan et al., 2022). This variation may be attributed to differences in the molecular structure of LPS from different bacterial species, their affinity for TLR subtypes, or experimental variables such as passage number, LPS concentration, and exposure duration. A study evaluating the effects of LPS in the presence of dentin matrix proteins found that although LPS alone had a limited impact on cell viability. LPS altered gene expression related to odontogenic differentiation and increased IL-6 production over time (Widbiller et al., 2018). Additionally, more recent evidence indicates that LPS stimulation reduces DPC viability and simultaneously upregulates key pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, highlighting its suitability as an inflammation inducer in in vitro pulpitis models (Azaryan et al., 2023).

H_2O_2 plays a concentration-dependent dual role in biological systems—as a signaling molecule at physiological levels and a pro-oxidant causing cytotoxicity under stress conditions. Under inflammation condition of DPCs, H_2O_2 readily diffuses across cell membranes and generates hydroxyl radicals that disturb redox homeostasis. This oxidative stress activates cellular signaling cascades, including NF-κB and MAPK, thereby influencing the expression of inflammatory genes and stress-response elements (Hong et al., 2024). An in vitro study reported that H_2O_2 exposure at concentrations ranging from 0.1-0.3 mg/mL significantly impaired DPC viability and odontogenic differentiation, as reflected by reduced alkaline phosphatase (ALP) activity and the absence of mineralized nodule formation (Soares et al., 2015).

Although single-agent models are useful for mechanistic insights, they often fail to replicate the complex interplay of factors present in clinical pulpitis. A recent critical analysis of pulpitis models emphasized the limitations of single-molecule stimulation and advocated for dual-agent systems to better mimic the chronic and multifactorial nature of dental pulp inflammation (Richert et al., 2022). Supporting this approach, a study by Vaseenon et al. (Vaseenon et al., 2023) examined the effects of LPS, H_2O_2, and their combination on human DPCs. Their findings revealed that while LPS alone induced inflammatory responses and disrupted mitochondrial dynamics without affecting cell proliferation, H_2O_2 alone, and more so in combination with LPS, significantly reduced cell proliferation, impaired mineralization, and elevated TNF-α levels. Notably, the combined treatment exacerbated mitochondrial imbalance and induced apoptosis, highlighting its potential as a more representative in vitro model of pulpitis. These insights reinforce the value of using combined inflammatory and oxidative stress inducers to simulate complex pulpal pathologies and evaluate therapeutic interventions in a controlled experimental setting.

MATERIAL AND METHODS

Culture of Human Dental Pulp Cells

The DPCs isolation protocol for this study was approved by the Human Research Ethics Committee, Faculty of Dentistry, Chulalongkorn University (No: 058/2023). Permanent third molars with no carious lesion were extracted from the Department of Oral Surgery. The tooth

was stored in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific) and transferred to the laboratory. The tooth was rinsed three times with phosphate-buffered saline (PBS) before being sectioned into two halves. The pulp tissue was then harvested, minced into 2×2 mm^2 fragments, and cultured in growth medium containing DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), 1% L-glutamine (Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphothericin B (Antibiotic-Antimycotic, Gibco). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was refreshed every two days. Upon reaching confluence, the cells were detached using 0.25% trypsin-EDTA (Gibco). DPCs at passages 3-5 were used in this study.

Material preparation

E. coli LPS (Sigma-Aldrich, MO, USA) was stored at 4°C according to the manufacturer's instructions. A 200 µM stock solution was prepared by dissolving LPS in 5 mL of serum free medium (SFM, Cytiva) and aliquoted into 1 mL tubes, which were stored at -20°C. On the day of the experiment, the stock was thawed and diluted in SFM to final concentrations of 20, 50, and 100 µg/mL for inflammation induction. Likewise, 30% H_2O_2 (Sigma-Aldrich, MO, USA) was diluted in SFM to a concentration of 400 µM.

Cell viability assay

DPCs were seeded in 24-well plates at a density of 20,000 cells per well in growth medium and incubated for 24 hours. The medium was then replaced, and the cells were divided into eight groups as follow:

- Group 1: DPCs cultured in SFM
- Group 2: DPCs cultured in SFM with 20 µg/mL LPS
- Group 3: DPCs cultured in SFM with 50 µg/mL LPS
- Group 4: DPCs cultured in SFM with 100 µg/mL LPS
- Group 5: DPCs cultured in SFM with 400 µM H_2O_2
- Group 6: DPCs cultured in SFM with 20 µg/mL LPS and 400 µM H_2O_2
- Group 7: DPCs cultured in SFM with 50 µg/mL LPS and 400 µM H_2O_2
- Group 8: DPCs cultured in SFM with 100 µg/mL LPS and 400 µM H_2O_2

DPCs were collected after 1 and 3 days and subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT test. To measure cell viability, the growth medium was removed and washed with PBS. 300 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for 30 minutes at 37°C in a humidified environment with 5% CO2. After incubation, the MTT solution was removed, and 300 µL of DMSO was added to each well to dissolve the formed formazan crystals and stop the reaction. The optical density (OD) at 570 nm was measured using a microplate reader (ELx800, BIO-TEK®). DPCs cultured in SFM served as the control group. The data were expressed as the percentage of cell viability, with the control group set at 100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

DPCs were seeded in 12-well plates with growth medium at a density of 100,000 cells per well and incubated for 24 hours. After 24 hours, the growth medium was replaced, and DPCs were exposed to SFM containing 20 µg/mL LPS, either alone or with 400 µM H_2O_2. DPCs cultured in SFM served as the control. DPCs were collected after 24 hours and subjected to RT-qPCR. Total RNA from each well was extracted using 1 mL of TRIzol reagent (GeneAll, Seoul, Korea). The RNA was then reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The expression of inflammatory-related genes was analyzed by RT-qPCR using LightCycler 96 machine (Roche Diagnostics). The thermal cycling condition were as follows: 95ºc for 20s, then 60ºc for 20s, followed by 72ºc for 20s for 45 cycles. RT-qPCR was carries out in a total volume of 10 µL of FastStart Essential DNA Green Master (Roche Diagnostics), cDNA, and the following primer (Biobasic, CA): IL-1β (5'-

GCAGAAGTACCTGAGCTCGC-3' and 5'-CTTGCTGTAGTGGTGGTCGG-3'), IL-6 (5'-CCTGAACCTTCCAAAGATGGC-3' and 5'-CTGACCAGAAGAAGGAATGCC-3'), IFN-γ (5'-CCAACTAGGCAGCCAACCTAA-3' and 5'-AGCACTGGCTCAGATTGCAG-3'), TNF-α (5'-CACAGTGAAGTGCTGGCAAC-3' and 5'- ACATTGGGTCCCCCAGGATA-3'). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference (5'-CACTGCCAACGTGTCAGTGGTG-3' and 5'-GTAGCCCAGGATGCCCTTGAG-3'). The expression each target gene were normalized to GAPDH and quantified. Briefly, The average threshold cycle (CT) values of the target gene and GAPDH were used to calculate the relative expression using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Statistical analysis

This study utilized individual cultures of DPCs from four patients. Statistical analyses were conducted using SPSS Statistical Software version 25 (IBM Corp.). Data are presented as mean ± standard error of the mean (SEM). Shapiro-Wilk test was used to assess the normality of the data. If the data passed the normality test, one-way ANOVA followed by Tukey's post-hoc test was used; otherwise, the Kruskal-Wallis test followed by Dunn's test was used. Statistical significance was set at $p<0.05$.

RESULTS

Cell viability assay

Cell viability was assessed using the MTT assay. DPCs were treated with LPS, H_2O_2 or their combination at varying concentrations for 1 and 3 days. There was no statistically significant difference in cell viability between the LPS-treated groups and the control group on both day 1 and day 3, regardless of the LPS concentration. When comparing groups with the same LPS concentration, the addition of 400 µM H_2O_2 significantly reduced cell viability on both day 1 and day 3 ($p <.05$). Cell viability in the groups treated with H_2O_2 was comparable on both day 1 and day 3, regardless of the LPS concentration.

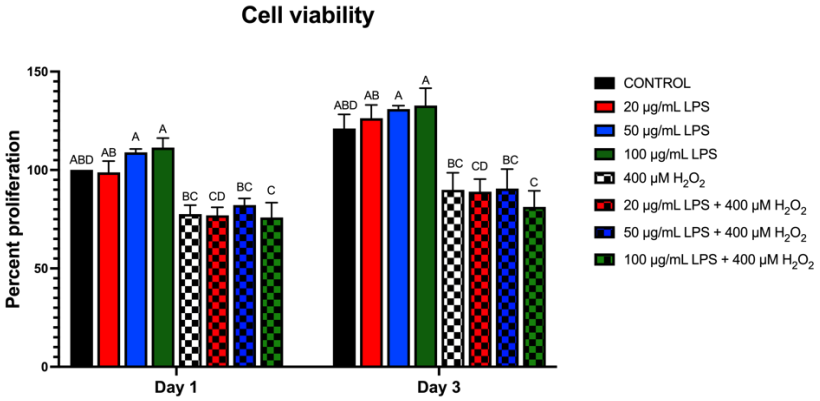

Figure 1 Cell viability of DPCs following exposure to various concentrations of LPS and H_2O_2 was evaluated using the MTT assay on days 1 and 3. DPCs cultured in SFM were used as the control group. Statistical analysis was performed using one-way ANOVA, and significant differences between groups on the same day are indicated by different letters ($p < 0.05$).

Reverse transcription-quantitative polymerase chain reaction

The expression of inflammation-related genes was analyzed using RT-qPCR. DPCs were treated with 20 µg/mL LPS, either alone or in combination with 400 µM H_2O_2, for 24 hours. The treatment with LPS combined with H_2O_2 significantly increased the expression of IL-1β, IL-6, IFN-γ, and TNF-α compared to the control group ($P <.05$). However, no significant difference was found between the LPS-only group and LPS combined with H_2O_2 group.

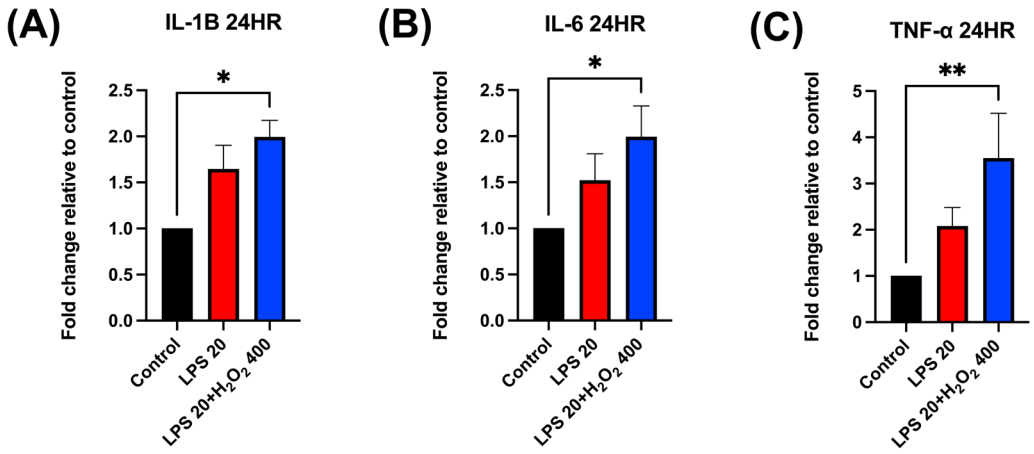

Figure 2 LPS and H_2O_2 upregulated the expression of inflammatory-related genes. DPCs cultured in SFM served as the control. Statistical significance was determined using the Kruskal-Wallis test, with * $P < 0.05$ and ** $P < 0.005$ indicating significant differences.

DISCUSSION

LPS primarily exerts its effects through TLR4, which is highly expressed in odontoblasts, fibroblasts, and dental pulp stem cells. The binding of LPS to TLR4 activates NF-κB and MAPK) signaling pathways. These pathways play a crucial role in upregulating pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, which mediate the inflammatory response in dental pulp cells (Brodzikowska et al., 2022). Additionally, activation of the NF-κB and MAPK pathways produces ROS, which serve as secondary messengers in the inflammatory process. ROS further amplifies the activation of these pathways, intensifying the expression of pro-inflammatory cytokines and exacerbating the inflammatory response (Hong et al., 2024).

The inflammatory cytokines analyzed in this study play a key role in modulating the inflammatory response. The expression of these cytokines reflects the pathological condition of dental pulp tissue. IL-1β contributes to the recruitment of immune cells to the site of injury and stimulates the production of other inflammatory cytokines (Kaur et al., 2021). IL-6 regulates immune cell activity by activating T cells, promoting B-cell differentiation, and enhancing antibody production (Hirsch et al., 2017). TNF-α promotes leukocyte infiltration into inflamed tissues, and its elevated levels have been detected in inflamed pulpal tissues, particularly in cases of irreversible pulpitis (Zanini et al., 2017).

In vitro studies have used LPS to induce inflammation in dental pulp cells with various concentrations and stimulation duration (Richert et al., 2022). This study used 20 μg/mL LPS to stimulate DPCs for 24 hours, which aligns with previous studies (Vaseenon et al., 2023; Weekate et al., 2021). Previous studies have demonstrated a significant upregulation of IL-1β, IL-6, and TNF-α in LPS-stimulated DPCs [12-14]. In our study, LPS alone also increased these inflammatory cytokines, though the changes were not statistically significant. Interestingly, compared to the control group, significant upregulation was observed only in the group treated with both LPS and H_2O_2. The role of H_2O_2 has been highlighted in a recent study, which reported that TNF-α protein levels were significantly higher in DPCs stimulated with LPS and H_2O_2 than those treated with LPS alone (Vaseenon et al., 2023). This suggests that H_2O_2 can amplify the inflammatory response by enhancing cytokine expression. One study found that the expression levels of these cytokines varied depending on the duration of DPC stimulation (Lan et al., 2022). Therefore, future studies assessing inflammatory cytokine expression at

multiple time points would be beneficial in determining the optimal conditions for using LPS and H_2O_2 as an effective inducer of inflammation.

Cell proliferation is essential for healing as it facilitates the regeneration of tissue lost during the inflammatory process. This phase involves the production of new cells that replace the damaged ones. Proliferation begins once the inflammatory response starts to subside. Therefore, reduced proliferation is a characteristic of DPCs in the laboratory that could mimic the clinical situation of inflamed pulp tissue, as one study found lower proliferation in DPCs from inflamed pulp tissue compared to those with healthy teeth (Wang et al., 2010). Our findings were consistent with previous studies, which showed that LPS at a concentration of 20 µg/mL did not affect the viability of DPCs (Vaseenon et al., 2023; Weekate et al., 2021). Additionally, no inhibitory effect on cell survival was observed when the concentration was increased up to 100 µg/mL. In contrast, a concentration of 400 µM H_2O_2 effectively inhibits the proliferation of DPCs (Min et al., 2008). Our findings align with the previous study (Vaseenon et al., 2023), which reported that supplementation of H_2O_2 to LPS significantly reduces cell viability in DPCs.

Mild inflammation is known to promote odontoblastic differentiation and hard tissue formation. The previous study has shown that pulp tissue harvested from inflamed teeth exhibits greater mineralization than those from healthy teeth (Wang, 2005). However, 20 µg/mL LPS and 400 µM H_2O_2 significantly reduce mineralization in an *in vitro* model (Vaseenon et al., 2023). Thus, further studies using various concentrations of H_2O_2 could help develop a more specific model to induce inflammation in DPCs. Furthermore, exploring other aspects of inflammation will provide insight into the effectiveness of LPS and H_2O_2 as an inflammation inducer.

CONCLUSION

The combination of LPS and H_2O_2 significantly reduced cell viability and upregulated inflammatory cytokine expression. These findings support the use of LPS and H_2O_2 as an inflammation-induced model for DPCs.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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