Original Article

The effect of lipopolysaccharide contamination on gutta percha induced TNF-alpha and IL-1 beta gene expression by human monocyte cells

Hadi Assadian [1], Nassim Hashemi [2], Abdollah Ghorbanzadeh [3], Maryam Nourizadeh [4], Jamshid Hadjati [5]

[1] Assistant Professor, Department of Endodontics, School of Dentistry, Tehran University of Medical Sciences (TUMS), Tehran, IR Iran
[2] Postgraduate Student, Department of Endodontics, School of Dentistry, Tehran University of Medical Sciences (TUMS), Tehran, IR Iran
[3] Department of Endodontics, School of Dentistry, Tehran University of Medical Sciences (TUMS), Tehran, IR Iran
[4] Assistant Professor of Medical Immunology, Immunology, Asthma and Allergy Research Institute, Tehran, IR Iran
[5] Professor of Medical Immunology, Department of Immunology, School of Medicine, Autoimmune Bullous Diseases Research Center, Tehran University of Medical Sciences (TUMS), Tehran, IR Iran

Abstract

Background and Aim: The aim of this study was to assess gutta percha-induced TNF-alpha and IL-1 beta gene expression in the presence or absence of bacterial lipopolysaccharide (LPS) on cultured human monocyte cells *in vitro*.

Materials and Methods: Human monocytes from THP-1 cell line were cultured. Standardized gutta percha segments were prepared and directly placed at the bottom of the culture wells. Cultured cells were exposed to the materials in G1 and G2 groups and then LPS was added only to G1. Positive control included the bacterial LPS without the gutta percha segments and the negative control contained the cells in culture medium only. TNF-alpha and IL1-beta gene expression was evaluated using RT-PCR technique. Data were statistically analyzed using Kruskal-Wallis and Bonferroni-adjusted post-hoc tests.

Results: A statistically significant difference was noted between LPS-treated and untreated groups regarding IL-1-beta and TNF-alpha gene expression ($p < 0.05$). In G2 (the gutta-percha without LPS), IL-1-beta, and TNF-alpha gene expression was significantly higher compared to the negative control group ($p < 0.05$). In G1 (gutta-percha with LPS), TNF-alpha and IL-1-beta gene expression was significantly higher than in the positive control group ($p<0.05$).

Conclusion: It was indicated that gutta-percha could induce the expression of pro-inflammatory cytokines TNF-alpha and IL-1-beta, with this effect being significantly magnified by LPS contamination.

Key Words: Gutta-Percha, Lipopolysaccharides, Tumor Necrosis Factor-alpha, Interleukin-1-beta, Monocytes Cell Line, Gene Expression, Polymerase Chain Reaction, Inflammation Mediators, Endodontics

Corresponding author: Nassim Hashemi, Postgraduate Student, Department of Endodontics, School of Dentistry, Tehran University of Medical Sciences (TUMS), Tehran, IR Iran

nsm.hashemi@gmail.com

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Introduction

Successful endodontic treatment largely depends on the proper obturation of the root canal space, which prevents re-infection by sealing off the canal from microorganisms in the oral environment and periapical tissue fluids (1). Additionally, obturation helps entomb any remaining irritants within the root canal system (2). Among the various materials introduced for this purpose, gutta percha remains the most commonly used and one of the oldest dental materials still in practice (3). The apical extent of root filling materials is crucial in determining the success of treatment, making the biocompatibility of these materials especially important, particularly in cases where the material comes into direct contact with periapical tissue cells (4). Some studies suggest that extrusion of root canal filling material may lead to a foreign body reaction (5-7), although other research indicates that bacteria, rather than the material itself, play a more significant role in such reactions(8, 9). Gutta percha has been shown to cause minimal to no toxic reactions in various cell cultures, depending on the assays used (10). Interestingly, the tissue response to gutta percha is influenced by particle size: smaller particles (50–100 μm) implanted subcutaneously in guinea pigs triggered macrophage and foreign body giant cell activity, which could potentially interfere with the healing process. However, larger particles did not provoke any adverse biological reactions (11, 12).

A substantial number of teeth requiring root canal treatment have necrotic pulps in which contamination with bacterial toxins is a common finding. It has been well established that gram-negative anaerobic species are the predominant etiologic bacteria in endodontic infections(13). These bacteria have lipopolysaccharide (LPS) in their outer cell membrane as their virulence factor. LPS, also called endotoxin, stimulates the inflammatory mediators, activates the complement system and macrophages and causes host cell toxicity and bone resorption after being released during bacterial cell proliferation or death (14). Tumor necrosis factor-alpha (TNF-alpha) is the principal mediator of the acute inflammatory response to gram-negative bacteria and other infectious microbes and is responsible for many of the systemic complications of severe infections (15). The major cellular source of TNF-alpha is activated mononuclear phagocytes, although antigen-stimulated T cells, NK cells, and mast cells can also secrete this protein. TNF- alpha also acts on mononuclear phagocytes to stimulate secretion of interleukin-1 (lL-1). The function of lL-l, similar to that of TNF, is as a mediator of the host inflammatory response to infections and other stimuli. IL-1 works together with TNF in innate immunity and inflammation. The major cellular source of IL-1, like that of TNF, is activated mononuclear phagocytes. IL-1 production by mononuclear phagocytes is induced by bacterial products such as LPS and by other cytokines such as TNF (16, 17).

To the best of the authors' knowledge, evidence is lacking concerning biological effects of gutta-percha particles in contact with human defense cells when LPS is present. Therefore, the aim of this study was to assess the effect of *Escherichia coli* LPS contamination on gutta percha induced TNF-alpha and IL-1 beta gene expression by human monocyte cells.

Materials and Methods

a.Sample preparation

The root canal filling materials used in this study were #20, 0.02 gutta percha (VDW, München, Germany) points. The points were disinfected by being immersed into 5.25% sodium hypochlorite solution (Paxan Co., Tehran, Iran) for 1 minute and subsequently rinsed by sterile normal saline and distilled water at least three times to eliminate the effects of the disinfecting solution(18). Human monocyte cell line THP-1 was obtained from Pasteur Institution Cell Bank, Tehran, Iran and cultured under biologically safe and aseptic conditions in Department of Immunology, School of Medicine, Tehran University of Medical Sciences. Cells were cultured in 25 mm 2 culture flasks containing RPMI 1640 (Gibco BRL, Grand Island, NY), supplemented with penicillin (100 U/ml) (Gibco BRL, Grand Island,

NY), streptomycin (100 µg/ml) (Gibco BRL, Grand Island, NY), L-glutamine (2mM) (Sigma Chemical Co., St. Louis, MO), and 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) at 37 ° C in a 5% CO2 atmosphere. The cells were stored in their best growth state (i.e. logarithmic growth) in nitrogen tanks in a way that 3-5 million cells in 90% FBS and 10% DMSO were stored at -20◦C for 1 hour and then at -70◦C for another 24 hours and eventually preserved in liquid nitrogen at -137◦C. The viability of cells was assessed by trypan blue dye staining method through which bluish color change indicated non-viable cells.

According to Rezania et al.(19) study, LPS was extracted by hot phenol-water method from *Escherichia coli* suspensions (10^8 colony-forming *units/mL*).Three millimeters from the tip of each point was cut and directly placed at the bottom of the culture wells. Cultured cells were exposed to the following groups:

• Group 1 (G1), containing gutta percha with LPS (*Escherichia coli*; Sigma, St. Louis, MO) treatment

• Group 2 (G2), containing gutta percha without LPS treatment

• Group 3 (Ctrl+), positive control including no endodontic material with LPS treatment only

• Group 4 (Ctrl-), negative control including no endodontic material without LPS treatment

b. Evaluation of Cytokines Gene Expression

b.1: RNA Extraction

One ml of RNX solution (Cinnagen, Tehran, Iran) was added to an Eppendorf containing 3-5 million cells and then homogenized. The mixture was incubated at room temperature for 5 minutes. Two-hundred microliters of chloroform was added to the mixture and mixed well for 15 seconds. The mixture was incubated on ice at 4◦C for 5 minutes and then centrifugated at 12000 RPM and 4◦C for 15 minutes. The upper phase was transferred to an RNase/DNase-free tube in a way that the mid-phase was not disturbed. Then, equal volume of isopropanol was added, gently mixed and incubated on ice for 15 minutes. The supernatant was discarded and 1 ml of 75% ethanol was added, vortexed to dissolve the pellet and centrifugated at 4◦C for 8 minutes at 7500 RPM. The soup was discarded and the pellet dried completely at room temperature for a few minutes. Eventually, the pellet was dissolved in 50λ distilled water and the Optical Density (OD) was read spectrophotometric ally (NanoDrop Technologies, Wilmington, DE).

b.2: DNase Treatment

When optical absorbance ratio was determined at the wavelength of 260 to 280 nm, RNA volume was corrected as 1µgr/8µlit DEPC water, then the process went on through the following steps: RNA (8 µlit), RNase free DNase 10X Reaction Buffer (1 µlit), RNase Free DNase [2U/µg RNA (2µlit)], and RNAsin (0.5µlit) were added and incubated at 37◦C for 45 min. One microliter DNase stop solution was added to terminate reaction and incubated at 65◦C for 10 minutes to inactivate DNase. After the temperature reached 4◦C, 2µlit Random Primer was added to each sample.

b.3: Complementary DNA (cDNA) Synthesis

In order to synthesize cDNA, MasterMix was created per sample with the following components:

• Reverse Transcriptase(200u/µlit) (0.5µlit)
• dNTP's(5mM each) (2.5µlit)
• 5X RT First Strand Buffer(5.0µlit)
• DTT(0.1 m Dithiotritiol) (2 µlit)
• RNAsin(40U/ µlit) (0.5 µlit)
• Random Primer(2 µlit)

DNase treated RNA and 2.0 µlit random primer were pre-incubated at 70◦C for 5 minutes. The MasterMix was added when the temperature reached to 4◦C and incubated at 37◦C for 90 minutes. The temperature was then increased to 70◦C and remained constant for 15 minutes and then decreased to 4◦C and fixed for at least 5 minutes. cDNA was subsequently diluted to at least 100 µlit and stored at -20◦C.

b.4: Real-time Reverse Transcriptase Polymerase Chain Reaction

Polymerase Chain Reaction MasterMix (TAKARA-Bio Inc, Otsu, Japan) was utilized for this evaluation which consisted of the enzyme reverse transcriptase, the enzyme buffer, deoxynucleotide triphosphate (dNTP), $MgCl_2$, and SYBR Green (a dye to detect DNA amplification). The housekeeping gene selected for this evaluation was considered

glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The test was performed on template (the housekeeping gene as well as test and control) groups. Any of the aforesaid groups contained 4 microtubes (i.e. 2 for housekeeping genes and 2 for cytokines of interest). Besides, there were three additional microtubes serving as negative control for GAPDH, IL-1-beta and TNF-alpha. Eventually, each microtube contained 10µl MasterMix, and the remaining 10µl was composed of 10 pM forward primer (1λ), 1pM reverse primer (1λ), template (1λ) and water(7λ). The microtubes were subjected to PCR test (RotorGene 3000, Corbet Research, Australia). Primer sequences of pro-inflammatory cytokines used in this study are shown in table 1.

c.Statistical analysis

Given the distribution of the variables, the data were analyzed using Kruskal-Wallis test, followed by Bonferroni-adjusted post-hoc tests to ensure precise comparisons. The statistical analysis was conducted using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL).

Table 1. Primer sequences of pro-inflammatory cytokines used in this study

Gene	Direction	Sequence
IL-1-beta	Reverse	AAGCCCTTGCTGTAGTGGTG
	Forward	GAAGCTGATGGCCCTAAACA
TNF-alpha	Reverse	AGATGATCTGACTGCCTGGG
	Forward	CTGCTGCACTTTGGAGTGAT
GAPDH	Reverse	AATGAAGGGGTCATTGATGG
	Forward	AAGGTGAAGGTCGGAGTCAA

Results

Gene expression levels of IL-1-beta and TNF-alpha were quantitatively assessed in THP-1 cells following a 5-hour exposure to standardized gutta-percha segments, both in the presence and absence of bacterial LPS (10µg/ml). The measurements were conducted using real-time polymerase chain reaction (RT-PCR) to determine the relative expression levels of these inflammatory cytokines under the specified conditions.

a.Interleukin 1-beta (IL-1-beta)

In G2 (the group treated with gutta-percha without LPS), IL-1-beta gene expression was higher than in the negative control group. Statistical analysis (three-way ANOVA and *post hoc* statistical tests) revealed a significant difference between the G2 (gutta-percha without LPS group) and Ctrl- (the negative control group) ($p < 0.05$).

In G1 (the group treated with gutta Percha with LPS), IL 1 beta gene expression was significantly higher than that in Ctrl+ (the positive control group) ($p < 0.05$).

Additionally, a significant difference was noted between the all LPS-treated (G1 and Ctrl+) and untreated (G2 and Ctrl-) groups in terms of IL-1-beta gene expression ($p < 0.05$). (See fig.1.)

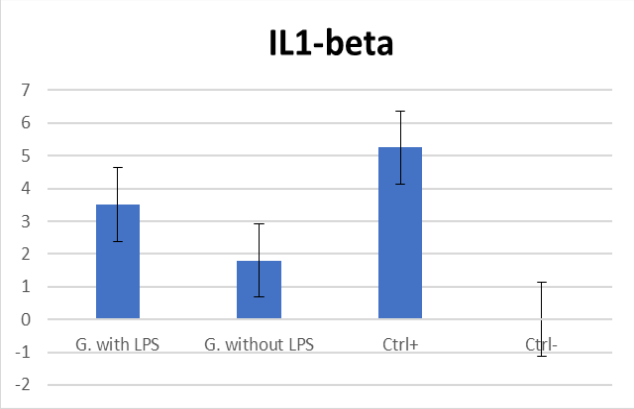

Figure 1. Interleukin-1- beta gene expression measured by real-time reverse transcriptase polymerase chain reaction in a 5-hour interval in both LPS treated and -untreated groups

b.Tumor Necrosis Factor-alpha (TNF-alpha)

In G2 (gutta-percha without LPS), TNF-alpha gene expression was higher than in Ctrl- (the negative control) group ($p < 0.05$). In G1(gutta-percha with LPS), TNF-alpha gene expression was significantly higher than in Ctrl+ (the positive control) group ($p < 0.05$). Furthermore, a significant difference was observed between the LPS-treated and untreated groups regarding TNF-alpha gene expression ($p < 0.05$). (See fig.2)

All pairwise comparisons (G1 vs Ctrl+, G1 vs G2, G1 vs Ctrl-, Ctrl+ vs G2, and Ctrl+ vs Ctrl-) showed p-values much less than 0.05 even after

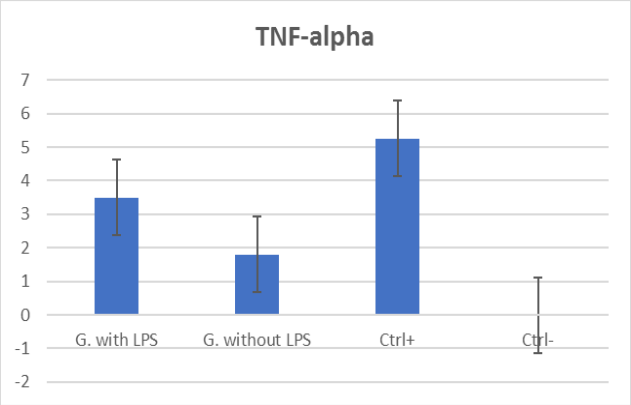

Figure 2. Interleukin-1- beta and TNF-alpha gene expression measured by real-time reverse transcriptase polymerase chain reaction in a 5-hour interval in both LPS treated and -untreated groups

adjustment with the Bonferroni procedure. The comparison conducted across all groups was determined to be statistically significant, signifying that the differences observed among the groups are not attributable to random variation but instead represent genuine and meaningful distinctions. Consequently, the results provide robust evidence that the differences are unlikely to have arisen by chance alone.

Discussion

The biocompatibility of endodontic materials is a topic of special concern. Consequently, the biological effects of root canal filling materials are worth of more meticulous investigations. This is characterized by various aspects including cytotoxicity, genotoxicity, immunogenicity, mutagenicity, histocompatibility, general toxicity, allergic reactions, systemic effects and microbial effects (20). The immunogenicity of dental Materials can be evaluated through *in vitro* (test tube or Culture dish) or *in vivo* (within a living organism) methods. The current investigation employed an *in vitro* approach to rigorously investigate the immunological effects and examine how the materials might provoke an immune response when introduced to biological systems. By using *in vitro* methods, variables can be controlled more precisely to gather detailed data on the immunotoxicity of the materials tested. Root filling materials are designed to be contained within the canal spaces, these materials might be inadvertently pushed into the periradicular tissues as a result of procedural errors, triggering apical tissue reactions that might delay healing and influence the outcome of treatment (21). As stated previously the exposure risk of periradicular tissues to root canal filling materials is not so low due mainly to inability of the clinicians to determine the exact termination point of endodontic therapy (22, 23). Gutta-percha as a widely utilized root filling material in contemporary dentistry is also prone to be extruded into periradicular tissues (7). Various toxicity assays conducted on different cell cultures have demonstrated that gutta-percha typically induces minimal to no toxic reactions (9). Moreover, previous studies also showed the acceptable biocompatibility of gutta-percha, which caused moderate to slight inflammatory reactions (24).

In this study, the effect of LPS contamination on gutta-percha-induced TNF-alpha and IL-1 beta gene expression in human monocyte cells was investigated *in vitro*. The results demonstrated that gutta-percha alone can induce an inflammatory response in THP-1 cells, which aligns with the findings of Belladonna et al. (21). Conversely, Mozayeni et al.(25) indicated that the inflammatory reaction caused by gutta-percha is temporary and decreases over time, suggesting that gutta-percha is biocompatible and could be a suitable endodontic obturation material. The current investigation suggests that LPS contamination further enhances the inflammatory response induced by gutta-percha. This finding supports the results of Martinho et al. (26), who demonstrated that endotoxin activates inflammatory signaling pathways. These findings suggest that LPS contamination can amplify the inflammatory reaction.

Immunogenicity refers to the ability of a particular substance, such as an antigen or a foreign body, to provoke an immune response in the body. This involves various components of the immune system, including antibodies, T

cells, and cytokines. TNF-alpha and IL-1 beta are critical cytokines that play significant roles in the immune response and inflammation (27). They are particularly crucial in the acute inflammatory response to gram-negative bacteria and other infectious agents, contributing to the systemic complications associated with severe infections (14).

The aim of this study was to compare elicitation of inflammatory responses by gutta percha particles with and without LPS contamination via gene expression of proinflammatory cytokines (IL-1-beta and TNF-alpha) from human monocyte cell line THP-1.

Monocytes and macrophages which are considered mononuclear phagocytes play an integral part in innate immunity and inflammatory responses against foreign objects (28). On the other hand, utilization of cell lines is a prevalent approach in assessing the cytotoxicity and immunogenicity of dental materials due to its simplicity and ability to produce consistent and manageable outcomes. *In vitro* testing further enables the comparison of multiple materials using identical cell lines in uniform conditions (29). THP-1 is a well-established human leukemia monocytic cell line that has been widely utilized in scientific research due to its ability to differentiate into macrophage-like cells under specific conditions. It has proven to be a valuable model for studying a broad range of monocyte and macrophage-related functions, including immune responses, inflammation, and host-pathogen interactions (30). Researchers have employed THP-1 cells extensively to investigate cellular mechanisms and signaling pathways involved in monocyte-to-macrophage differentiation, as well as in immune system regulation (31). Moreover, this cell line has been instrumental in exploring the molecular processes governing nutrient uptake, drug transport, and the immune system's reaction to pharmacological agents (32-34). Due to its versatility and physiological relevance, THP-1 has become a commonly used model system to evaluate how monocyte and macrophage activities can be modulated in various biological contexts, including studies of immunomodulation, disease pathogenesis, and therapeutic interventions (30). In the current study, RT-PCR technique was used to assess gene expression of pro-inflammatory cytokines. The purpose of RT-PCR technique is to amplify ribonucleic acid (RNA) targets. In RT-PCR an RNA template is the initial target, and the enzyme reverse transcriptase creates a complementary DNA (cDNA) copy of the RNA. When the cDNA is formed, it can be utilized as a template for amplification. This technique has the advantage of detecting messenger RNA (mRNA) encoding specific proteins, such as IL-1-beta and TNF-alpha (28, 35).

One important component of gram-negative bacteria is endotoxin, commonly used synonymously with the term lipopolysaccharide (LPS), which is a major constituent of the bacterial outer cell wall (36). It is well established that the classical stimulus for production of cytokines is through bacterial LPS (37). LPS in this study could have a stimulatory effect on THP-1 cells to produce pro-inflammatory cytokines and also was capable of simulating the condition present in infected periradicular tissues. The observed increase in TNF-alpha and IL-1 beta gene expression upon LPS contamination might be attributed to the activation of the NF-kB signaling pathway. LPS is known to bind to Toll-like receptor 4 (TLR4) on the surface of monocytes, leading to the activation of NF-kB and subsequent transcription of pro-inflammatory cytokines (13). This mechanistic insight underscores the role of LPS as a potent enhancer of inflammatory responses in the context of gutta percha-induced gene expression.

Our study represents a pioneering effort to evaluate the potential of gutta-percha, both with and without lipopolysaccharide (LPS), in inducing the expression of IL-1-beta and TNF-alpha in a human monocyte cell line. Consequently, it is not feasible to make detailed comparisons between our findings and those reported in the existing literature, as such comparative analyses are not applicable in this context. However, our findings revealed that all experimental groups were capable of inducing

the gene expression of both IL-1-beta and TNF-alpha. Notably, the presence of LPS further enhanced this effect, indicating a synergistic role of LPS in amplifying the inflammatory response. This observation underscores the inherent potential of gutta-percha, particularly when combined with LPS, to stimulate these pro-inflammatory cytokines in a human monocyte cell line. Such results highlight the significance of gutta-percha's role in modulating immune responses, which could have profound implications for its application in endodontic therapies.

The assessment of the initiation of inflammatory and immune responses to dental biomaterials and their constituents through *in vitro* methods has the potential to anticipate the biological responses that may occur *in vivo*. Gutta-percha particles are able to release factors which have a bone resorbing activity that is primarily due to enhanced production of IL-1alpha. Similar interactions might exist to enhance IL-1 beta production under the influence of gutta percha segments(6). The findings of this study have important clinical implications for endodontic treatments. The presence of LPS contamination in root canal materials, such as gutta-percha, could exacerbate inflammatory responses and potentially lead to post-treatment complications. Gutta-percha is not suitable for sterilization by wet or dry heat, which raises concerns since sterilizing endodontic instruments and materials is essential for maintaining the aseptic chain and preventing the introduction of pathogenic bacteria into the root canal system during non-surgical root canal treatment. Contamination of gutta-percha points can occur due to improper storage, exposure to aerosols, or incorrect handling during or after manufacture. Therefore, adopting a rapid Chairside Disinfection Protocol (CDP) for gutta-percha points before their use as a filling material is necessary (38). Additionally, our study demonstrated that gutta-percha exhibits a heightened capability to induce inflammatory reactions upon contact with monocytes. This finding suggests that caution is warranted when utilizing gutta-percha for root canal treatment in cases of acute apical periodontitis. In such scenarios, the periradicular tissues are densely populated with acute inflammatory mediators, including TNF-alpha and IL-1 beta. Consequently, the persistence of apical periodontitis following the extrusion of gutta-percha is a probable outcome. This underscores the importance of meticulous technique and consideration of the inflammatory status of the periradicular tissues during endodontic procedures.

One notable limitation of this study lies in its reliance on an *in vitro* model, which may not entirely capture the intricate and multifaceted environment of the human body. *In vitro* models, while invaluable for controlled experimentation, often fall short in replicating the complex interactions and dynamic conditions present *in vivo*. These models lack the systemic influences, cellular diversity, and physiological responses that are inherent to living organisms, thereby limiting the extrapolation of results to clinical situations. To truly validate these findings, future research endeavors should strive to conduct *in vivo* studies, thereby providing a more accurate reflection of the biological complexities at play. Moreover, it is imperative to explore the long-term effects of LPS contamination on inflammatory responses, as this could unveil critical insights into the chronic aspects of inflammation. Additionally, delving into the effects of varying concentrations and different types of LPS could yield a more nuanced and comprehensive understanding of its role in the inflammatory cascade.

Conclusion

The study demonstrated that gutta-percha alone upregulated IL-1 and TNF gene expression in THP-1 cells, indicating an induced inflammatory response. Additionally, LPS contamination further enhanced this inflammatory response. The investigation highlighted the necessity for stringent control of LPS contamination in endodontic materials to prevent exacerbated inflammatory responses. Further research was warranted to explore the clinical relevance of these findings and to develop effective strategies to mitigate the risks associated with LPS contamination.

Data Availability

The data supporting the findings of the present study are available from the corresponding author upon request.

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Conflict of Interest Statement:
The authors declare that they have no conflicts of interest related to this study. This research was conducted independently, without any financial or personal relationships that could have influenced the work reported in this paper.

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