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Ornidazole Regulates Inflammatory Response and Odontogenic Differentiation of Human Dental Pulp Cells

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ABSTRACT

Aim: This study aimed to explore the potential of ornidazole as an alternative treatment for pulpitis, focusing on its effects on human dental pulp cells (hDPCs) and macrophages. We assessed the cytotoxicity of various concentrations of ornidazole, its safety and efficacy in treating inflamed hDPCs, and its regulatory impact on inflammatory markers during inflammation.

Materials and Methods: Inflammation in hDPCs was induced using lipopolysaccharides (LPS), and varying doses of ornidazole were introduced. Cell proliferation, migration, inflammation regulation, and dentinogenesis under inflammatory conditions were evaluated. Additionally, macrophages were cultured with different doses of ornidazole to analyse the expression of inflammatory genes. If statistically significant differences were observed between the control and treatment groups, this was considered evidence of ornidazole's effects on hDPCs. Statistical analysis was performed using SPSS 26.0, with one-way analysis of variance and Tukey's test for comparisons. A P-value of < 0.05 was considered statistically significant.

Results: Ornidazole influenced cell proliferation, inflammation regulation, and dentinogenesis. Concentrations below 10 μg/mL did not exhibit significant cytotoxic effects on hDPCs over a 7-day period, and the cytotoxicity of ornidazole was both concentration- and time-dependent. Ornidazole decreased the expression of proinflammatory markers (IL-6 and TNF-α) while enhancing the expression of anti-inflammatory markers (IL-1Ra and IL-8). It also suppressed alkaline phosphatase (ALP) activity but increased the expression of odontogenic differentiation markers at both mRNA and protein levels in the presence of inflammatory stimuli. Furthermore, ornidazole demonstrated immunomodulatory effects on macrophages.

Conclusions: Low concentrations of ornidazole were found to be safe for hDPCs. Ornidazole modulated the expression of inflammatory markers (IL-6, TNF-α, IL-8, IL-1Ra) in inflamed hDPCs and regulated odontogenesis-related markers. Furthermore, low concentrations of ornidazole enhanced the immune regulation in macrophages, highlighting its potential as a therapeutic agent for pulpitis.

Clinical Relevance: This study aimed to understand the interactions of ornidazole with hDPCs, its anti-inflammatory properties, and its regulatory effects on odontogenic processes. By examining the impact of different concentrations of ornidazole on cells associ-

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ated with pulp inflammation, this study provides valuable insights into its therapeutic potential for pulpitis and tends to support its clinical application.

Introduction

The dental pulp, a loose connective tissue encased by dentin, plays a crucial role in the vitality and health of teeth. It is essential for sensory perception, immune defense, tissue repair, and regeneration following tooth damage, maintaining the balance of the pulp chamber environment.[1,2] Pulp necrosis disrupts the supply channels of dental tissue, increasing the fragility of dentin and enamel. The leading cause of pulp necrosis is pulpitis, a condition in which chemotactic leukocytes increase the activity of lysosomal enzymes, resulting in tissue damage.[3] High levels of endotoxins and a more complex microbiota are commonly found in teeth with pulp symptoms. All teeth with irreversible pulpitis exhibit severe acute inflammation, necrosis, microabscesses, and bacterial infection within the pulp chamber. Bacteria were also observed in the vascular lumen around the necrotic lesion.[4,5]

Microorganisms, especially bacteria, are the primary cause of periapical and pulpal diseases.[6] *Streptococcus mutans* contributes to early carious lesions, whereas anaerobic and facultative anaerobic bacteria, such as *lactobacilli*, promote pulpal inflammation and necrosis progression.[7] Most anaerobic bacteria reaching the pulp lead to periapical disease.[6] When bacteria invade the body, the immune system triggers an inflammatory response to combat the infections. If left untreated, this inflammation can develop into autoimmune or autoinflammatory conditions, such as pulpitis, ultimately resulting in pulp death.[8,9] Research has shown that antibiotics effectively inhibit bacterial infections, mitigate the harmful effects of endotoxins, and reduce the body's inflammatory response.[10] Calcium hydroxide, due to its strong alkalinity and biocompatibility, is widely used in endodontic therapy for its ability to inhibit bacterial growth. However, it is susceptible to apical tissue fluids, is soluble outside the root canal, and its prolonged use can negatively impact tooth fracture resistance.[11] Furthermore, its antibacterial efficacy in severely infected pulp diseases is limited and difficult to remove.[12] Antibiotics are often employed in endodontic therapy to treat severe apical infections due to their potent anti-infective properties. Triple antibiotic paste (TAP), a combination of quinolones, tetracyclines, and nitroimidazoles, is frequently used in endodontic therapy and has a wide range of clinical applications. However, research has confirmed that tetracycline drugs in TAP can cause tooth discoloration, and combining multiple antibiotics enhances the drug's cytotoxicity.

Nitroimidazoles, a class of synthetically produced antibacterial agents, specifically target anaerobic bacterial infections.[13] Unlike tetracyclines and doxycycline, nitroimidazoles do not cause tooth discoloration.[14,15] The emergence of resistance to clindamycin, which also targets anaerobic bacteria, has become a significant clinical issue. Although nitroimidazoles are less effective than clindamycin against Gram-positive anaerobic bacteria, they are effective against Gram-negative anaerobes and are often preferred in treating infections caused by *Pseudomonas*.[16] Recent investigations have demonstrated that pulp capping materials containing nitroimidazoles significantly reduce inflammation, stimulate dental pulp cells (DPCs), and promote tissue repair following pulp injuries.[17]

Nitroimidazoles, including metronidazole, tinidazole, and ornidazole, differ in potency and toxicity. Ornidazole, a third-generation nitroimidazole derivative, has stronger disinfection properties and lower toxicity compared to metronidazole and tinidazole. Recent studies have shown that a mixture of ciprofloxacin, minocycline, and ornidazole was more effective than combinations using metronidazole or tinidazole.[18] Ornidazole has thus become a key component in TAP therapy and is widely used to treat anaerobic bacterial infections.[19] Although it is commonly administered orally, localised antibiotic delivery can enhance its efficacy and reduce side effects.[20] Studies have also shown that when incorporated into pulp capping systems or scaffolds for pulp regeneration, ornidazole exhibits excellent anti-inflammatory, antibacterial, and anti-infective properties, surpassing the performance of ornidazole alone.[21] This highlights ornidazole as a promising drug for controlling infections in pulpitis. However, the impacts of ornidazole on pulpitis cells, particularly DPCs and macrophages, remain underexplored. Hence, this study aimed to investigate ornidazole's potential application for dental pulp therapy.

Pulp tissue comprises various cell types, including odontoblasts, fibroblasts, stem cells, immune cells, and endothelial cells.[1] DPCs, especially stem cells, are essential for pulp regeneration, capable of differentiating into odontoblasts and nerve cells for tissue repair.[22] Their exosomes can modulate cytokine activity by interacting with immune cells, thereby exerting immunoregulatory effects.[23] Pharmaceuticals that positively regulate the immune response play a crucial role in preserving pulp vitality. Macrophages are also key players in vascular reconstruction and inflammation regulation, as they phagocytize bacteria and release cytokines.[24] These cells are pivotal in tooth development, infection control, and self-repair processes.[25] Understanding how ornidazole affects these cells is essential for assessing its potential in treating pulpitis.

Hence, this study investigated the effects of different concentrations of ornidazole on DPC proliferation, inflammation regulation, and odontogenic differentiation under inflammatory conditions, as well as its combined effect with macrophages in modulating inflammation.

Materials and methods

Cell isolation and culture

This experiment was conducted in compliance with the Basic Code of Medical Ethics and was approved by the Ethics

Committee of Jinan University (approval number: JNUKY-2022-070). The human dental pulp cells (hDPCs) were isolated from young permanent teeth obtained during orthodontic surgery in patients aged 18 to 24 years after patient-informed waiver. The teeth, isolated within 4 hours of extraction, were stored in 4°C phosphate buffer saline (PBS; pH 7.4). Pulp tissue was extracted and digested using 0.3 vol.% COL-1 collagenase (Cat. #C-0130, Sigma-Aldrich). The digested pulp was cut into pieces in a sterile petri dish, dispersed, and spread flatly on the culture flasks. The culture medium was changed every 3 days after observing cell growth. When the cell growth reached 80% to 90% confluence, the cells were digested, counted, and then cultured for 4 to 7 passages. The extracted cells were confirmed as hDPCs through flow cytometry analysis (NovoCyte2060R, ACEA, USA) of the cell surface marker CD90, along with alkaline phosphatase (ALP) staining and alizarin red staining using BCIP/NBT kit (Cat. #C3206, Beyotime, China) and ARS reagent (Cat. #A506786-0025, Sangon Biotech), respectively, as presented in Figure S1. RAW264.7 (Cat. #CL-0190, Procell) was used as a macrophage model. Cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (H-DMEM; Cat. #C11995500BT, Gibco) supplemented with 10% fetal bovine serum (FBS; Cat. #04-001-1ACS, Biological Industries).

Drug concentration selection

Preliminary experiments revealed that when ornidazole (Cat. #O824179, Macklin) was added to the hDPCs culture medium at concentrations exceeding 500 μg/mL, a significant number of hDPCs underwent apoptosis by day 21. To ensure that the experimental concentrations remained within a safe range for the cells, this study established 2 groups: a low-dose group (1, 2, 5, 10 μg/mL) and a high-dose group (50, 100, 200, 500 μg/mL).

Cell viability and migration

Cells were treated with various concentrations of ornidazole. Live/dead cell staining was performed with Calcein-AM (Cat. #C2012, Beyotime) and propidium iodide (Cat. #ST1569, Beyotime) observed by an inverted fluorescence microscope (TCS SP8, Leica8), and cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Cat. #CK04, Dojindo) assay. Cell migration was evaluated by scratch assays with images processed using ImageJ software (v1.54h, NIH).

ALP assay and alizarin red staining

The hDPCs were pretreated with 1 μg/mL *Escherichia coli* lipopolysaccharide (LPS; Cat. #L2880, Sigma-Aldrich) for 24 hours to establish an inflammatory model. Subsequently, the LPS-containing medium was replaced with fresh odontogenic induction medium supplemented with various concentrations of ornidazole, 50 mg/L vitamin C, 10 mM β-sodium glycerophosphate, and 100 nM dexamethasone. ALP staining, alizarin red staining, and a colourimetric ALP activity assay were conducted according to the manufacturer's instructions.

For macrophage-conditioned culture, RAW264.7 was first induced with 1 μg/mL LPS for 24 hours before being treated with ornidazole for an additional 1 or 3 days. The culture media from ornidazole-treated macrophages were collected on the first and third days of coculture for inflammatory gene expression analysis. These media were then mixed with fresh odontogenic induction medium at a 1:1 ratio to prepare conditioned media containing ornidazole, 50 mg/L vitamin C, 10 mM β-sodium glycerophosphate, and 100 nM dexamethasone, which were subsequently applied to hDPCs culture. ALP activity in hDPCs was then measured.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

RAW264.7 and hDPCs were seeded at densities of 4.0×10^5 and 2.0×10^5 cells/well in 6-well plates, respectively. Cells were treated with different concentrations of ornidazole (Cat. #ST1470, Beyotime) post-LPS induction. Total RNA was isolated using RNAex Pro Reagent (Cat. #AG21101, Accurate Biology), and cDNA was synthesised with the HiScript III RT SuperMix (Cat. #R333, Vazyme). The qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Cat. #Q711-02, Vazyme) on a CFX Connect Real-Time PCR System (Bio-Rad). Gene-specific primers (Table 1) quantified expression of interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-1Rα (IL-1Ra), interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), inducible nitric oxide synthase (iNOS), stromal cell-derived factor-1 (SDF1), collagen type I (COL-1), bone sialoprotein (BSP), runt-related transcription factor 2 (RUNX2), dentin matrix protein-1 (DMP-1), and dentin sialophosphoprotein (DSPP), normalised to GAPDH.

Western blot analysis

Following cell lysis with RIPA buffer (Cat. #P0013B, Beyotime), total protein concentrations were quantified using a BCA assay (Pierce BCA protein Assay Kit, Cat. #23225, Thermo Scientific). Proteins were separated by gel electrophoresis, transferred to PVDF membranes (0.45 μm, Cat. #IPVH00010, Millipore), and blocked with 5% skimmed milk in TBST for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies: anti-COL-1 (1:1000; Cat. #AF6524, Beyotime, China), anti-RUNX2 (1:1000; Cat. #AF2593, Beyotime), anti-DSPP (1:1000; Cat. #AF6741, Beyotime), and anti-GAPDH (1:2000; Cat. #AF1186, Beyotime; loading control). HRP-conjugated goat anti-rabbit IgG(H+L) secondary antibody (1:2000; Cat. #A0208, Beyotime) was used for detection. Protein bands were visualised using enhanced chemiluminescence (ECL) substrate (BeyoECL Star, Cat. #P0018AS, Beyotime) on an imaging system (KETA ML, Wealtec). Band density was quantitated using ImageJ software (v1.54h, NIH) with COL-1, RUNX2, and DSPP expression normalised to GAPDH.

Statistical analysis

All experiments utilised hDPCs from a single donor, with technical replicates performed at least 4 times under standardised conditions. Data were analysed using SPSS 26.0. (International Business Machines Corporation) and expressed as mean ± standard deviation. Statistical significance ($P < 0.05$)

Table 1 – Validated primer sequences for real-time PCR.

Gene	Direction	Sequence (5′-3′)
IL-6	Forward	GACAGCCACTCACCTCTTCAGAAC
(*human*)	Reverse	GCCTCTTTGCTGCTTTCACACATG
TNF-α	Forward	TGGCGTGGAGCTGAGAGATA
(*human*)	Reverse	TGATGGCAGAGAGGAGGTTG
IL-8	Forward	AACTTTCAGAGACAGCAGAGCACAC
(*human*)	Reverse	CACACAGTGAGATGGTTCCTTCCG
IL-1Ra	Forward	GTGCCTGTCCTGTGTCAAGTCTG
(*human*)	Reverse	GCCACTGTCTGAGCGGATGAAG
DSPP	Forward	AAGCAATAACAGTACAGACAATG
(*human*)	Reverse	TCTTGCTGTTGATCTGAGGTG
DMP-1	Forward	TCATAGCAGAAGACATTGGAG
(*human*)	Reverse	TCTTATCCAAAACAACTCACTG
BSP	Forward	CAGGGCAGTAGTGACTCATCC
(*human*)	Reverse	TAGCCCAGTGTTGTAGCAGA
COL1	Forward	TCTGCGACAACGGCAAGGTGT
(*human*)	Reverse	CGACGCCGGTGGTTTCTTGGT
RUNX2	Forward	GTCACTACCAGCCACCGAGA
(*human*)	Reverse	GCTGTTTGATGCCATAGTCCC
GADPH	Forward	TGACATCAAGAAGGTGGTGAAGCAG
(*human*)	Reverse	GTGTCGCTGTTGAAGTCAGAGGAG
CD86	Forward	CTGCTCATCATTGTATGTCAC
(*mouse*)	Reverse	ACTGCCTTCACTCTGCATTTG
iNOS	Forward	CAGAAGTGCAAAGTCTCAGACAT
(*mouse*)	Reverse	GTCATCTTGTATTGTTGGGCT
CD206	Forward	AGACGAAATCCCTGCTACTG
(*mouse*)	Reverse	CACCCATTCGAAGGCATTC
ARG-1	Forward	GGAATCTGCATGGGCAACCTGTGT
(*mouse*)	Reverse	AGGGTCTACGTCTCGCAAGCCA
IL-10	Forward	GAGAAGCATGGCCCAGAAATC
(*mouse*)	Reverse	GAGAAATCGATGACAGCGCC
IL-1Ra	Forward	GTGGCCTCGGGATGGAAAT
(*mouse*)	Reverse	CTTGCAGGGTCTTTTCCCAGA
TGF-β	Forward	TTGCTTCAGCTCCACAGAGA
(*mouse*)	Reverse	TGGTTGTAGAGGGCAAGGAC
SDF-1	Forward	TGCATCAGTGACGGTAAACCA
(*mouse*)	Reverse	CACAGTTTGGAGTGTTGAGGAT
TNF-α	Forward	CTGAACTTCGGGGTGATCGG
(*mouse*)	Reverse	GGCTTGTCACTCGAATTTTGAGA
IL-1β	Forward	AGTTGACGGACCCCAAAAGA
(*mouse*)	Reverse	GGACAGCCCAGGTCAAAGG
GADPH	Forward	TGTGTCCGTCGTGGATCTGA
(*mouse*)	Reverse	TTGCTGTTGAAGTCGCAGGAG

was determined by one-way analysis of variance with Tukey's test.

Results

Effects of ornidazole on the affinity of dental pulp-related cells

The hDPCs were cultured with varying concentrations of ornidazole added to the culture medium to explore the bioactivity of this drug. Comprehensive analysis of the CCK-8, cell migration, and live/dead cell staining showed that concentrations below 10 μg/mL exhibited a favourable affinity for hDPCs before day 7 of incubation. In contrast, concentrations higher than 50 μg/mL demonstrated concentration-dependent inhibition of hDPCs with increasing incubation time. At concentrations as high as 500 μg/mL, the drug exhibited significant cytotoxicity and minimal effect on migration, suggesting that high doses of ornidazole are significantly toxic to cells in the pulp chamber (Figure 1).

Effects of ornidazole on inflammation regulation of dental pulp-related cells

To evaluate the inflammation regulation capability of ornidazole, hDPCs induced by LPS for 24 hours were exposed to ornidazole (1-10 μg/mL). Results detected on day 3 by RT-qPCR revealed that ornidazole reduced the expression of IL-6 and TNF-α in LPS-induced hDPCs, while promoting the expression of IL-8 and IL-1Ra, in comparison to the control group (Figure 2A). Ornidazole demonstrated inflammatory modulation effects on LPS-stimulated hDPCs, primarily by reducing the pro-inflammatory effects of IL-6 and TNF-α. Additionally, ornidazole favoured IL-8 and IL-1Ra, regulating potential inflammation at concentrations below 10 μg/mL.[26-28]

Effects of ornidazole on odontogenic markers of dental pulp-related cells under inflammation conditions

Ornidazole's impact on odontogenesis-associated markers under inflammation conditions induced by LPS stimulation was investigated. The results revealed inhibited ALP activity and ARS staining but increased mRNA expression of markers such as DSPP, DMP-1, BSP, COL1, and RUNX2 at 1 to 10 μg/mL (Figures 2B-I). Western blot analysis of 3 selected odontogenic representative proteins showed that, at concentrations of 1 to 10 μg/mL, ornidazole up-regulated the expression of DSPP, RUNX2, and COL-1 proteins to varying degrees at 14 days, with continued up-regulation at 21 days except for COL-1 (Figures 2J-M).

Effects of ornidazole on regulating the immune microenvironment of dental pulp-related cells

RAW264.7 was used as a model to culture with varying concentrations of ornidazole under LPS-induced inflammatory conditions. Ornidazole treatment did not significantly alter RAW264.7 viability compared to untreated controls with LPS pre-exposure (Figure 3A). In response to prolonged inflammation stimuli, the expression of specific markers in RAW264.7 exposed to ornidazole (2 μg/mL) was assessed. RAW264.7 treated with ornidazole showed elevated expression levels of IL-10, IL-1Ra, TGF-β1, and SDF-1, while the expression of TNF-α and IL-1β was down-regulated compared to the control. Ornidazole appeared to be involved in regulating the immune microenvironment by modulating macrophage polarisation, thereby enhancing the later-stage odontogenic differentiation of hDPCs (Figures 3B-N).

Discussion

When the dental pulp is invaded by bacteria and becomes inflamed, the functionality of DPCs is compromised, with Gram-negative anaerobic bacteria being the primary cause.[29] These bacteria produce toxic LPS during replication or death. In vitro, LPS induces inflammation in pulp cells primarily by activating toll-like receptor (TLR) and stimulating NF-κB,

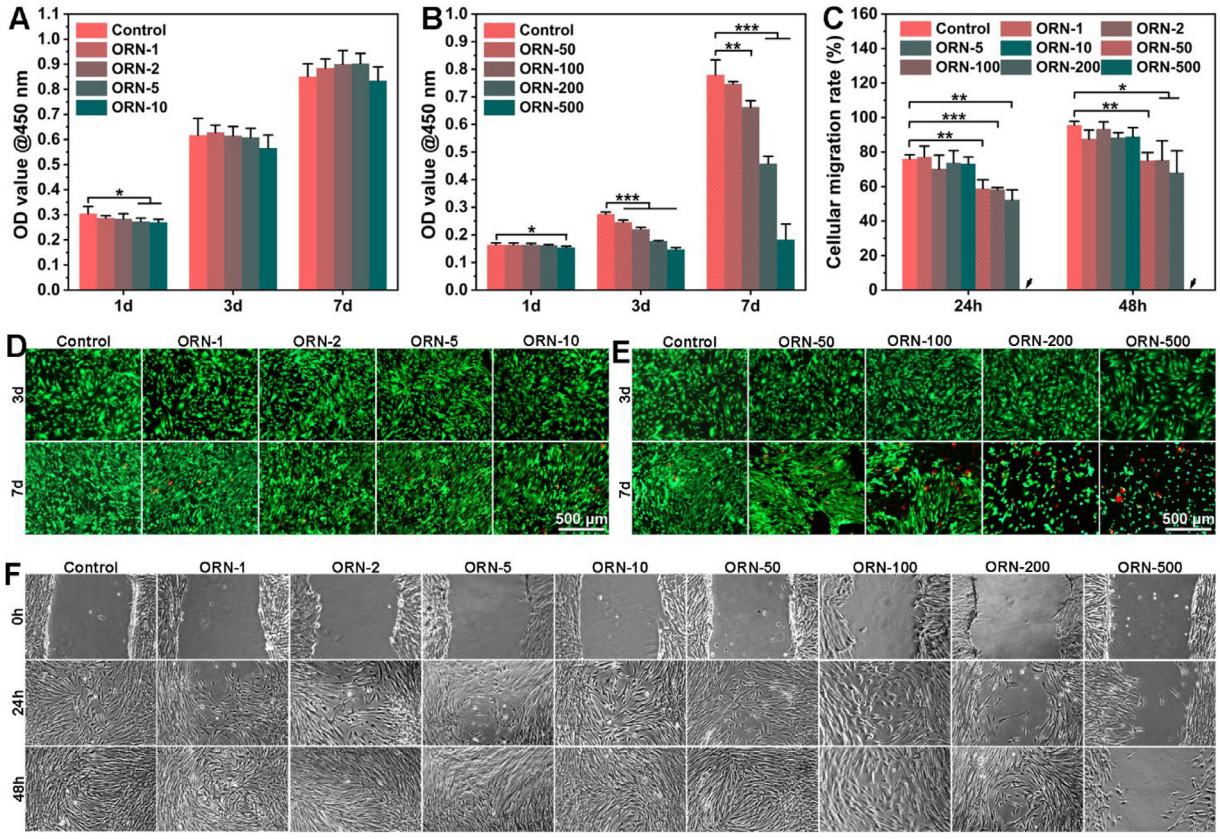

Fig. 1 – Proliferation ability (A, B), live/dead staining images (D, E), migration rate (C), and corresponding optical images (F) of hDPCs cultured with varying concentrations of ornidazole. Data were presented as mean ± S.D. *Ornidazole groups compared with control group. *P < 0.05, **P < 0.01, ***P < 0.001.

alongside other important molecules such as NLR and ROS.[30] LPS promotes DPCs to release various inflammatory factors, including TNF-α, IL-1Ra, IL-6, and IL-8, which ultimately lead to acute inflammatory responses.[31] While *Porphyromonas gingivalis* is a predominant pathogen in pulpitis, the disease pathogenesis involves polymicrobial synergy, with elevated TLR4 expression observed in both peripheral blood monocytes and pulp tissues from pulpitis patients.[32] Studies have shown that *Escherichia coli*-derived LPS effectively promoted inflammatory cytokines expression in pulp cells by increasing TLR4 expression in the dental pulp.[33]

Controlling infection reduces the release of inflammatory factors, thereby aiding in the recovery of dental pulp cell vitality. Infection control depends on inhibiting bacterial growth. The use of hydrogels as carriers for antimicrobial agents in treating infectious diseases is becoming increasingly common. Nitroimidazoles exert bactericidal effects by generating toxic metabolites that cause DNA strand breakage, with resistance being rare. Ornidazole, a nitroimidazole that inhibits anaerobic bacteria, is advantageous in the enclosed environment of the oral cavity and is widely used for treating oral infections. However, the effects of ornidazole on dental pulp activity remain unclear. This study found that the reasonable use of low concentrations of ornidazole did not inhibit dental pulp tissue activity. To verify the affinity between ornidazole and hDPCs, different concentrations of ornidazole were introduced to hDPCs culture, followed by CCK-8 assays, live/dead fluorescence staining, and cell migration experiments. This research concluded that the toxicity of ornidazole to hDPCs depended on both concentration and exposure duration. At concentrations below 10 μg/mL and treatment durations of less than 7 days, ornidazole was biocompatible with hDPCs. However, concentrations exceeding 500 μg/mL or exposure times beyond 7 days led to inhibited hDPCs proliferation and increased cell death. This suggested that high concentrations of ornidazole may inhibit cell migration and proliferation and promote apoptosis, likely due to the toxic effects of its degradation products.[34] Previous studies have shown that nitroimidazole drugs are neurotoxic in a concentration-dependent manner, supporting this hypothesis.[35]

In clinical practice, it is essential to strictly control the concentration of ornidazole and the duration of contact with hDPCs. As a drug for pulp therapy, ornidazole should be safe for pulp cells while retaining its antibacterial efficacy. Studies have shown that the minimum inhibitory concentration (MIC) of ornidazole against common oral bacteria such as *Porphyromonas gingivalis* and *Prevotella intermedia* range from 0.062 to 1 μg/mL.[36] Thus, ornidazole concentrations between 1 and 10 μg/mL are both effective against bacteria and safe for hDPCs, making it a suitable candidate for dental pulp therapy.

Qualified endodontic treatment drugs should prioritise both safety and the regulation of inflammation. Besides evaluating the safety of ornidazole for pulp cells, this study also examined its ability to regulate inflammation in inflamed

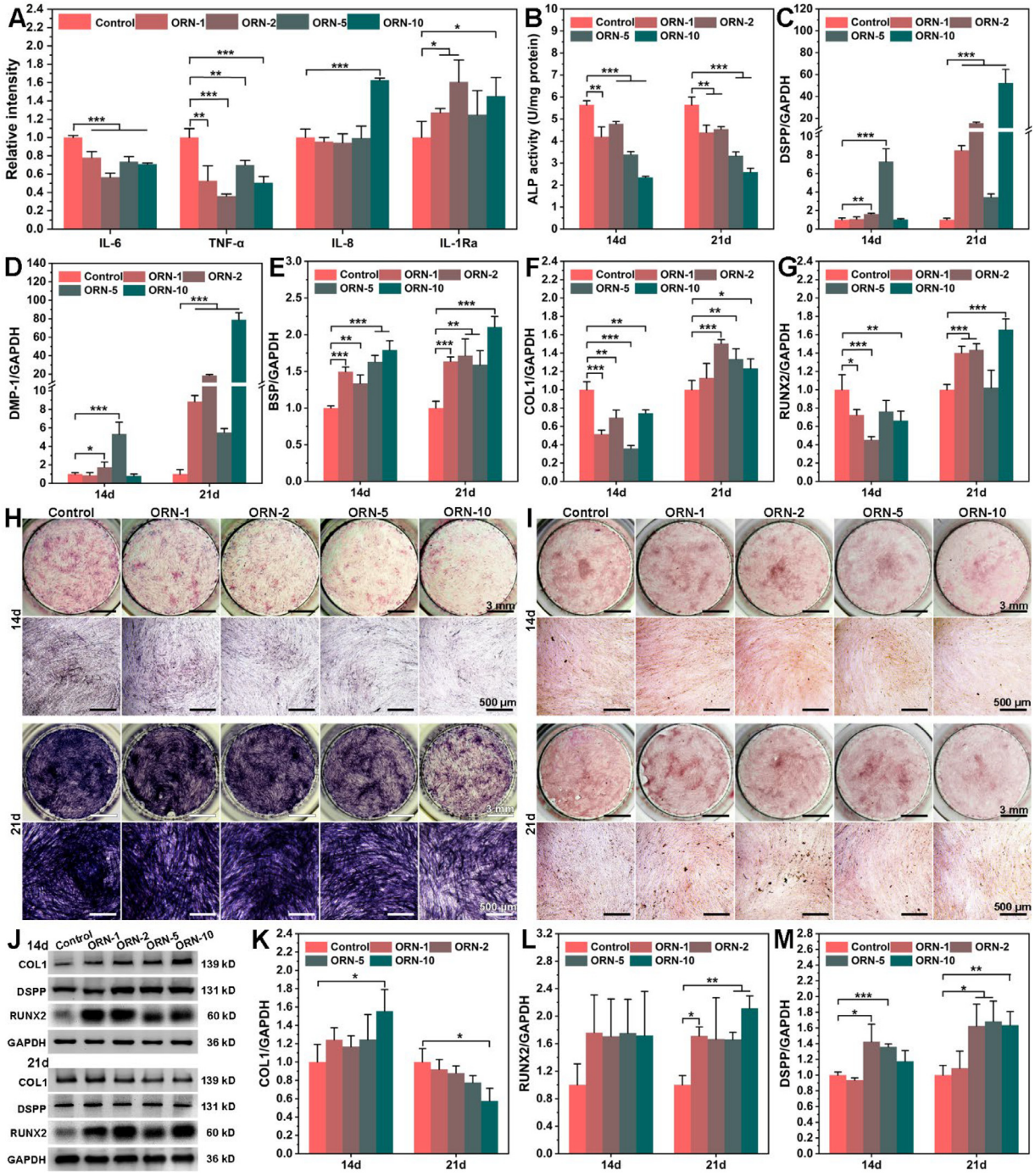

Fig. 2 – Inflammatory factors expression (A) and odontoblast differentiation (B-M) of hDPCs cultured with varying concentrations of ornidazole under LPS-induced inflammatory conditions. (A) mRNA expression of inflammatory factors; (B) ALP activity; (C-G) mRNA expression of dentinogenesis-associated markers; (H) ALP staining images; (I) ARS staining images; (J-M) Western analyses of dentinogenesis-associated markers. Data were presented as mean ± S.D. *Ornidazole groups compared with control group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

pulp cells. Recent studies showed that IL-6 and TNF-α played crucial roles in regulating pulpitis progression and influencing DPCs differentiation.[37-40] IL-6 levels are significantly elevated in pulpitis compared to normal dental pulp, while TNF-α, an early inflammation marker, affects pulp pain production and inhibits DPCs survival, migration, and odontogenic differentiation.[41-43] IL-1Ra acts as an anti-inflammatory agent by regulating inflammation.[44] IL-8, with its anti-inflammatory and strong angiogenic properties, is essential for DPCs survival and immune regulation.[45] Ornidazole exhibited a regulatory effect on the inflammatory genes in LPS-stimulated hDPCs on day 3, helping to control inflammation.

The continuous development of dental roots is driven by the ongoing differentiation of cells into dental pulp, with the

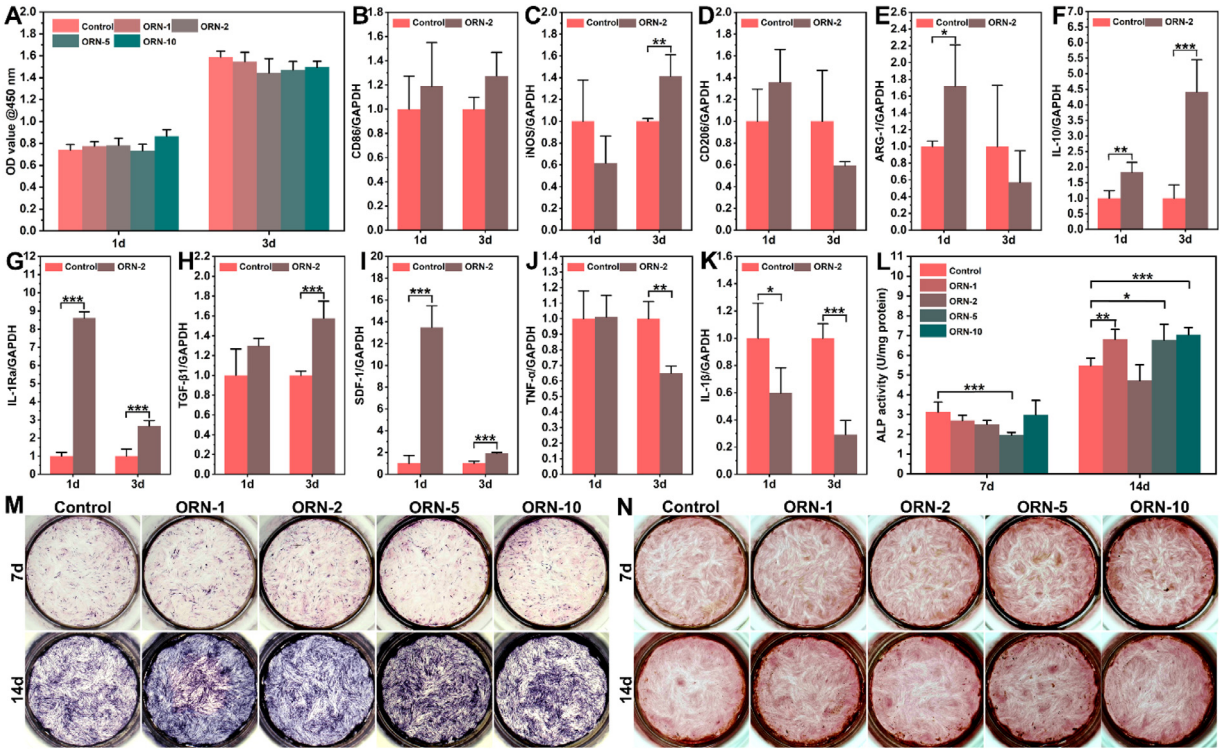

Fig. 3 – Proliferation ability (A) and mRNA expression of inflammatory factors (B-K) of RAW264.7 cultured with varying concentrations of ornidazole under LPS-induced inflammatory conditions. Odontoblast differentiation (L-N) of hDPCs cultured with the RAW264.7-conditioned media. L: ALP activity; M: ALP staining images; N: ARS staining images; Data were presented as mean ± S.D. *Ornidazole groups compared with control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

primary function of dental pulp being the formation of dentin. The proliferation and differentiation of hDPCs during tooth development are regulated by complex signalling molecules and pathways.[46] This study found that ornidazole may regulate the differentiation of hDPCs into dentin. Odontogenic genes such as DSPP, COL-1, and RUNX2 are crucial biomarkers affecting hDPCs differentiation into dentin.[47,48] COL-1 is recognised as an early differentiation marker that affects hDPCs differentiation into dentin.[49] RUNX2 inhibits odontoblast terminal differentiation and induces transdifferentiation of odontoblasts to osteoblasts at later stages.[50] The results showed that COL-1 expressions were negatively correlated with drug concentration, while RUNX2 expression was positively correlated. Additionally, expression of adult dentin-associated genes was reduced in the early phase and elevated in the late phase, which may be a negative feedback regulation by the suppression of protein expression by ornidazole.[51] These findings suggested that ornidazole may inhibit later-stage dentin formation under inflammation. ALP, a marker of mineralised tissue formation, was significantly inhibited after 14 days of exposure to ornidazole, with the inhibitory effect being time- and concentration-dependent, confirming previous results on odontogenic genes and proteins.[52] Previous studies have found that antibiotic treatment can compromise the mineralisation of dental tissues in both clinical and animal models.[53] This is because gut microbiota aids in maintaining continuous growth of incisors in injured rodents and differentiation abilities of hDPCs, while antibiotics suppress the microbial stimulation for tooth differentiation, weakening the differentiation capacity of hDPCs.[53] Our experimental results support this viewpoint.

Hemodynamic reconstitution and immune regulation are crucial for DPCs survival.[54,55] Although ornidazole-loaded nanofiber materials have demonstrated good biocompatibility and antibacterial activity, it remains unknown whether ornidazole exerts anti-inflammatory effects through modulation of immune cells.[56] Macrophages are pivotal in regulating inflammation and affecting the differentiation of hDPCs.[57] Regulating macrophage polarisation can regulate the inflammatory status of both reversible and irreversible pulpitis.[58,59] Macrophages are also crucial in the early stages of the wound-healing in reversible pulpitis.[60] When ornidazole enters the body, it controls inflammation by inhibiting bacterial growth and may also directly promote the secretion of anti-inflammatory factors; however, the specific mechanisms involved remain to be elucidated.[61] To understand the mechanism by which ornidazole inhibits inflammation in dental pulp-related cells, its inflammatory regulation of macrophages was investigated. RAW264.7 treated with ornidazole exhibited heightened expression of genes associated with anti-inflammatory responses and reduced expression of genes linked to pro-inflammatory processes. Furthermore, ornidazole-treated RAW264.7 exhibited significantly higher expression of SDF-1 compared to the control, suggesting a potential enhancement of pulp-associated cell migration.[62,63] The results from staining of conditionally cultured cells and

the determination of ALP enzyme activity suggested that ornidazole could promote hDPCs differentiation through immune cell stimulation. This contrasts with previous experimental results. It was hypothesised that low concentrations of ornidazole could enhance the anti-inflammatory response of macrophages in the inflammatory microenvironment, indirectly promoting differentiation into odontoblasts through macrophage secretion. By modulating metabolic pathways, ornidazole has the potential to optimise the macrophage secretome, thereby facilitating cellular differentiation. These results underscore the context-specific actions of ornidazole: while its direct application may disrupt early differentiation under inflammatory conditions, its indirect effects, mediated through macrophages, appear to support early differentiation by ameliorating the microenvironmental conditions. This experiment has certain limitations, particularly due to the lack of animal studies to confirm the regulatory effect of ornidazole on pulp differentiation. To validate this hypothesis, further experiments will be considered in future research.

Conclusions

Concentrations of ornidazole ranging from 1 to 10 μg/mL were not significantly cytotoxic to hDPCs over a 7-day period and can be safely used for therapeutic purposes. In the inflammatory immune environment, ornidazole exerted a regulatory effect on inflammatory genes by upregulating IL-8 and IL-1Ra expression while downregulating IL-6 and TNF-α expression. Additionally, ornidazole affected the expression of odontogenic genes such as DSPP, DMP-1, BSP, COL1, and RUNX2, as well as proteins including DSPP, RUNX2, and COL-1 in hDPCs under inflammatory conditions. However, ornidazole inhibited ALP activity and mineralisation in hDPCs, with the inhibitory effect being positively correlated with both concentration and duration of exposure. In a low-concentration ornidazole microenvironment, macrophage immune regulation and induction of odontogenic cell differentiation were promoted within the first 3 days.

Conflict of interest

None disclosed.

Author contributions

Jing Yang and **Zikai Li** contributed equally to this work: Methodology, investigation, validation, formal analysis, writing – original draft, and writing – review and editing; **Chengcheng Zhang** & **Jiaying Xiong**: Methodology, visualisation, writing – original draft, and writing – review and editing; **Xirui Yang**: Investigation and visualisation; **Dandan Zheng** & **Siming Xie**: Resources, supervision, and validation; **Haishan Shi**: Conceptualisation, methodology, validation, writing – review and editing, supervision, project administration and funding acquisition.

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Supplementary materials

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