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Betamethasone suppresses the inflammatory response in LPS-stimulated dental pulp cells through inhibition of NF-κB

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ABSTRACT

Objective: This study aimed to investigate the anti-inflammatory effect of betamethasone on LPS-stimulated human dental pulp stem cells (DPSCs) and its associated mechanism. The osteo-/odontogenic differentiation and osteoclast effect of betamethasone on DPSCs and stem cells from human exfoliated deciduous teeth (SHED) were evaluated.

Design: The proliferative effect of betamethasone on DPSCs was analyzed using a cholecystokinin octapeptide assay. The anti-inflammatory effect of betamethasone was investigated using quantitative polymerase chain reaction (qPCR) and ELISA. The anti-inflammatory mechanism was explored using qPCR, Western blot, and immunofluorescence staining. The osteo-/odontogenic differentiation and osteoclast effect of betamethasone on DPSCs and SHED were detected by qPCR.

Results: 1 μg L^{-1} betamethasone was found to have the strongest effect on DPSCs proliferation. The expression of pro-inflammatory cytokines and mediators, as well as prostaglandin E_2 (PGE_2) were significantly decreased following treatment with betamethasone in LPS- stimulated DPSCs. They were also decreased in response to an NF-κB inhibitor, Bay 11-7082. Betamethasone and Bay 11-7082 significantly inhibited the expression of p-p65 and promoted the nuclear exclusion of p65. Gene expression associated with osteo-/odontogenic differentiation was significantly up-regulated in betamethasone and osteogenic media (OM) treated groups. The ratio of the receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) at the mRNA level was suppressed in DPSCs and elevated in SHED.

Conclusions: Betamethasone has an anti-inflammatory effect on LPS- stimulated DPSCs through a blockade of NF-κB activation and exhibits an osteo-/odonto-inductive effect on DPSCs and SHED. Although betamethasone displays an osteoclast effect on SHED.

1. Introduction

Irreversible pulpitis refers to inflammation involving the dental pulp tissue, for which the subjective symptoms include, lingering thermal pain, spontaneous pain, and referred pain (J. J. Lin et al., 2015). The inflammatory response always follows the infection, leading to the release of inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) and chemical mediators (e.g., prostaglandins and bradykinin) (Coil, Tam, & Waterfield, 2004), which are known to activate and sensitize nociceptors and lead to peripheral sensitization(Nakanishi, Shimizu, Hosokawa, & Matsuo, 2001; Rittner, Brack, & Stein, 2003). Moreover, such inflammation can also induce edema of the pulp tissue (Girolami, 1999; Trowbridge & Emling, 1997), causing strongly stressful pain. Clinically, vital pulp conservation is critically important for irreversible pulpitis. Thus, a drug that meets the requirement of controlling the pain, eliminating the infection of pulp tissue, and promoting the formation of a hard tissue barrier to protect the pulp tissue from the oral environment is essential for vital pulp conservation.

Steroids are the most commonly used anti-inflammatory drugs. Indeed, glucocorticoids have been used to treat endodontic diseases to diminish dentinal hypersensitivity and pulpal hyperemia since the 1960s. (Fry, Watkins, & Phatak, 1960). Moreover, dexamethasone and prednisolone have been used to reduce the incidence of postoperative pain in endodontic patients in some clinical trials (Baume & Fiore-Donno, 1970; Chance, Lin, Shovlin, & Skribner, 1987; Krasner & Jackson, 1986). Recently, it has been reported that glucocorticoids promote fibronectin synthesis and suppress nerve growth factor secretion, suggesting that they could be used clinically to reduce pain and promote healing of dental pulp tissue (Srisawasdi & Pavasant, 2007). In a study by Liu et al., it was found that glucocorticoids can initiate the

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mineralization of DPSCs and have a potential role in repairing injured pulp tissues (Liu, Jiang, Wang, & Wang, 2013). Betamethasone, one of the most commonly studied glucocorticoids, has been confirmed to have a greater anti-inflammatory potential compared to other glucocorticoids (Corbel et al., 1999; Fiéveza et al., 2009). It has been reported that the topical application of betamethasone as an intra-canal medicament could reduce inflammatory reactions in rat incisor pulp (Fachin & Zaki, 1991). Moreover, Fachin et al. showed that betamethasone applied on the dentin could reduce the vascular phase of pulp inflammation in vivo (Fachin, Scarparo, Pezzi, Luisi, & Manoel Sant'Ana, 2009). However, the mechanism by which betamethasone mediates such anti-inflammatory effects in inflamed pulp tissue remains unknown. Lipopolysaccharide (LPS) is a Gram-negative bacterial cell wall component and is commonly detected in pulpitis. Moreover, LPS can activate NF-κB and induce the expression of inflammatory cytokines in dental pulp (Chang, Zhang, Taniishii, Shi, & Wang, 2005; J. C. Lee et al., 2008).

In this study, we investigated the effect of betamethasone on LPS-stimulated DPSCs and the underlying mechanism involved. Then, the osteo-/odontogenic differentiation and osteoclast effect of betamethasone on DPSCs and SHED were studied separately to explore potential clinical applications. Our results demonstrate that betamethasone has an anti-inflammatory effect on LPS-stimulated DPSCs through inhibiting the activation of NF-κB and exhibits an osteo-/odonto-inductive effect on both DPSCs and SHED. While an osteoclast effect was observed on SHED, no impact was observed on the DPSCs.

2. Materials and Methods

2.1. Isolation and Culture of DPSCs and SHED

All experimental protocols were approved by the Ethics Committee of the Peking University School and Hospital of Stomatology, Beijing, China (Approval Number: PKUSSIRB-201732003). Briefly, DPSCs were obtained from healthy permanent premolars (14 to 15-year-old males and females) extracted from the donors during orthodontic treatment at the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology. SHED was obtained from normal exfoliated human deciduous teeth of five to seven-year-old children. Dental pulp tissue was isolated and digested in a mixture of 3 mg mL^{-1} type I collagenase (Sigma-Aldrich, St Louis, MO, USA) and 4 mg mL^{-1} dispase (Sigma-Aldrich, St Louis, MO, USA) for 1 h at 37 °C. The cell suspensions were passed through a 70-μm strainer (Falcon) to separate the cells. The single-cell suspensions were cultured in α-MEM (GIBCO/BRL), supplemented with 10% FBS (GIBCO), 100 U·mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, and incubated at 37 °C with 95% air and 5% CO_2. DPSCs and SHED between P4-P6 were used for the following experiments.

2.2. Cell Proliferation Assay

A cell proliferation assay was performed to evaluate the influence of betamethasone on the proliferation of DPSCs under different concentrations. Briefly, 5 × 10^3 DPSCs were seeded into a 96-well plate. After 24 h, the cells were treated with various concentrations of betamethasone (0, 1, 10, and 100 μg L^{-1}) and incubated for one, three, five, or seven days. Then, 10 μL CCK-8 solution was added to each well and the cells were incubated for 1 h at 37 °C with 95% air and 5% CO_2.The absorbance of each well was quantified photometrically at 450 nm using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT). A cholecystokinin octapeptide (CCK-8, Dojindo, Beijing) assay was carried out with five replications to evaluate the number of cells according to the manufacturer's instructions.

2.3. Quantitative polymerase chain reaction (qPCR)

The DPSCs and SHED were separately seeded into six-well plates (1.5 × 10^5 cells/well). To induce an inflammatory reaction, the DPSCs were treated with 1 μg mL^{-1} *Escherichia coli* LPS (Sigma-Aldrich, St Louis, MO, USA). After exposure to LPS for 1, 3, or 6 h, the DPSCs were treated with or without 1 μg L^{-1} betamethasone or the NF-κB inhibitor, Bay 11-7082 (Sigma-Aldrich, St Louis, MO, USA), respectively to measure the expression of inflammatory cytokines. DPSCs and SHED were exposed to osteogenic media (OM) (10 mM sodium β-glycerophosphate, and 50 μg mL^{-1} l-ascorbic acid 2-phosphate sesquimagnesium salt hydrate [Sigma-Aldrich, St Louis, MO, USA]) with or without 1 μg L^{-1} betamethasone to measure the expression of osteogenesis and osteoclastogenesis-related markers. At the indicated time points, RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Then 1 μg total RNA was converted to cDNA using a PrimeScript™RT reagent Kit (TAKARA, Shiga, Japan). qPCR was performed using the Power SYBR Green PCR Master Mix (Rox) from Roche Applied Science (Indianapolis, IN, USA). The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Reactions were performed using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a total volume of 20 μL, containing 1 μL diluted cDNA, 0.5 μL forward primer, 0.5 μL reverse primer, and 10 μL SYBR

Table 1
The set of primers used in qPCR.

Gene name	5' to 3'	Size(bp)	Gene bank number	NM_001115114.1
	R	GGATGAACGGCAATCCCCAT		
TNF-α	F	CTGGGGCCTACAGCTTTGAT	126	NM_000594.3
	R	GGCCTAAGGTCCACTTGTGT		
IL-1β	F	CTTCGAGGCACAAGGCACAA	108	NM_000576.2
	R	TTCACTGGCGAGCTCAGGTA		
IL-6	F	TACAGGGAGAGGGAGCGATAA	283	XM_011515390.2
	R	GGGCGGCTACATCTTTGGAA		
COX-2	F	CCCATAACCCCGCCAAAAGG	148	M90100.1
	R	CTGAGTACCAGGTCTGCAGTG		
ALP	F	CTCCATACCTGGGATTTCCGC	299	NM_031313.2
	R	GGCCCCAGTTTGTCCTTCTT		
DSPP	F	GGAATGGCTCTAAGTGGGCA	284	NM_014208.3
	R	CTCATTGTGACCTGCATCGC		
OCN	F	ATTGTGGCTCACCCTCCATCA	119	NM_199173.5
	R	AGGGCTATTTGGGGGTCATC		
RANKL	F	GAACACGCGTATTTACCTGC	120	AF019047.1
	R	GGTCAACCCGTAATTGCTCC		
OPG	F	GTGTGCGAATGCAAGGAAGG	89	NM_002546.3
	R	GCTTGCACCACTCCAAATCC		

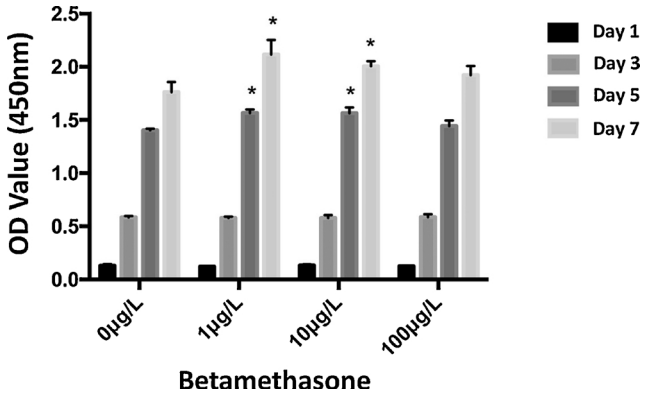

Fig. 1. Cell proliferation was enhanced by a low concentration of betamethasone (1 μg L^{-1},10 μg L^{-1}) at day 5, and the difference was significantly enhanced at day 7. A high concentration of betamethasone (100 μg L^{-1}) also promoted the proliferation but there is no significant difference ($p > 0.05$). (*$p < 0.05$)

Green Master Mix. Each gene analysis was performed in triplicate. The set of primers used are listed in Table 1.

2.4. Enzyme-linked immunosorbent assay (ELISA) for PGE_2

The DPSCs were seeded into six-well plates (1.5×10^5 cells/well). On the following day, the cells were exposed to 1 μg mL^{-1} LPS for 6 h with or without 1 μg L^{-1} betamethasone or the NF- κB inhibitor, Bay 11-7082. The supernatants from the cell cultures were harvested and kept at −80 °C until use. The level of PGE_2 in the supernatants was measured using a commercial PGE_2 ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Concentrations of PGE_2 were determined using the luminometer plate reader (Molecular Devices, Menlo Park, CA). Each sample was assayed in triplicate.

2.5. Western Blot

The DPSCs were exposed to 1 μg mL^{-1} LPS for 3 h with or without betamethasone and the NF- κB inhibitor, Bay 11-7082, for a Western blot assay. The cells were harvested using a protein lysis buffer with phosphatase inhibitor (Applygen Technologies Inc., Beijing, China). The suspensions were centrifuged at 12,000 × *g* for 30 min at 4 °C. The protein concentration was determined using a BCA Protein Assay (CWBIO, Beijing, China) and equal aliquots of total protein (25 mg) were loaded into each lane. The sample lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked in blocking sodium (Beyotime, Shanghai, China) for 1 h, and probed with the following antibodies at 4 °C overnight: p65 (NF-κB; 1:1000), p-p65 (1:1000), and β-actin (1:10000) (Cell Signaling Technology, Beverly, MA, USA). The membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin based on the source of the corresponding primary antibody, and the immunoblots were detected using a Western enhanced chemiluminescence blotting kit (ECL, SOLIBRO, Beijing, China).

2.6. Immunofluorescence staining

The DPSCs were seeded into 12-well plates and exposed to 1 μg mL^{-1} LPS with or without 1 μg L^{-1} betamethasone or the NF-κB inhibitor, Bay 11-7082, for 3 h, then fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min and blocked with 5% BSA for 2 h. Thereafter, the cells were incubated with a primary antibody (1:400) against p65 overnight at 4 °C then incubated with an anti-rabbit secondary antibody (1:500; Abcam, Cambridge, UK) for 1 h at room temperature. Nuclei were counterstained with 4',6-diami- dino-2-phenylindole (DAPI, SOLIBRO, Beijing, China) and the coverslips were mounted on a glass slide. Images were captured using an LSM 5 EXCITER confocal imaging system (Carl Zeiss, Oberkochen, Germany).

2.7. Statistical analysis

All statistical calculations were performed using SPSS21.0 statistical software. Comparisons between two groups were analyzed using independent two-tailed Student's *t*-tests, and comparisons between more than two groups were analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. All data are expressed as the mean ± standard deviation (SD) of three experiments per group. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of betamethasone on DPSCs Proliferation

The proliferation of DPSCs in response to different concentrations of betamethasone was evaluated using a CCK-8 assay. The results of the CCK-8 assay showed that the DPSCs growth rate was significantly enhanced with the addition of a low concentration of betamethasone (1 μg L^{-1},10 μg L^{-1}) on Day 5 ($P < 0.05$), and the difference was significantly enhanced on Day 7 ($P < 0.05$) (Fig. 1). A high concentration of betamethasone (100 μg L^{-1}) also promoted the proliferation of DPSCs, but the difference was not significant ($P > 0.05$). A betamethasone concentration of 1 μg L^{-1} played the strongest role in the proliferation of DPSCs on Day 7.

3.2. Effects of betamethasone on the level of pro-inflammatory cytokines and mediators mRNA expression in LPS-stimulated DPSCs

To explore the anti-inflammatory properties of betamethasone on LPS-stimulated DPSCs, the cells were treated with 1 μg mL^{-1} LPS for 1, 3, or 6 h with or without 1 μg L^{-1} betamethasone. Since TNF-α, IL-1β, IL-6, and COX-2 are the major pro-inflammatory cytokines associated with pulpitis, the inhibitory effect of betamethasone on their mRNA expression was measured using RT-PCR. Treatment with betamethasone significantly decreased the LPS-induced expression of TNF-α, IL-1 β, IL-6, and COX-2 at 1 h, 3 h, and 6 h ($P < 0.05$). Furthermore, the expression of pro-inflammatory cytokines at 3 h was significantly higher than that at 1 h and 6 h ($P < 0.05$) (Fig. 2A).

3.3. Effects of betamethasone and the NF-κB inhibitor, Bay 11-7082, on the expression of pro-inflammatory cytokines and mediators in LPS-stimulated DPSCs

To investigate the molecular mechanism by which betamethasone exerts an anti-inflammatory effect on LPS-stimulated DPSCs, the cells were treated with 1 μg mL^{-1} LPS for 3 h with or without 1 μg L^{-1} betamethasone and Bay 11-7082, an NF-κB inhibitor, which has been confirmed to have the ability to depress NF-κB activation in other articles (Pei, Wang, Chen, & Zhang, 2016; Zhao et al., 2014). The results revealed that treatment with both betamethasone or Bay 11–7082 could effectively inhibit the mRNA expression of pro-inflammatory cytokines and mediators (i.e., TNF-α, IL-1β, IL-6, and COX-2) (Fig. 2B). We performed a relationship analysis to determine the correlation between the effect of betamethasone and Bay 11-7082 on the level of mRNA expression, and obtained a positive correlation ($r = 0.98$) (Fig. 2D). The results of correlation analysis suggested the anti-inflammatory effect of betamethasone and the NF-κB pathway inhibitor Bay 11-7082 had a same tendency. Combined with the qPCR results, it was indicated that betamethasone may exert its anti-inflammatory effects via inhibiting NF-κB activation. The related results of betamethasone on the SHED

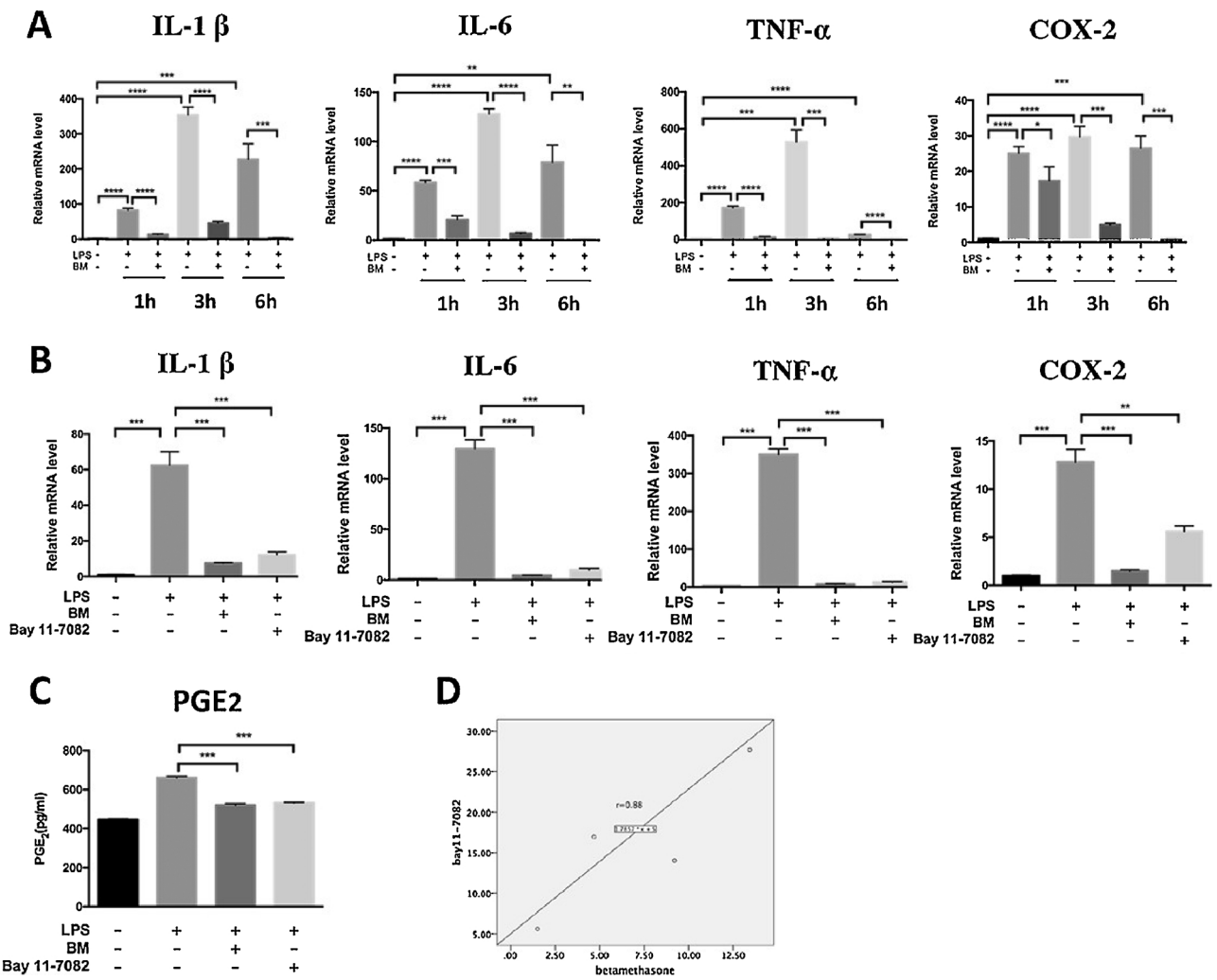

Fig. 2. (A) Effects of betamethasone on inflammatory mediators in LPS-stimulated DPSCs. LPS-stimulated DPSCs were incubated with 1 μmol L^{-1} betamethasone for 1, 3 or 6 h. The mRNA levels of inflammatory genes, TNF-α, IL-1 β, IL-6, and COX-2 were detected by qPCR. (B) Effects of betamethasone on the expression of inflammatory genes in DPSCs in response to LPS for 3 h. DPSCs were exposed to LPS for 3 h with either 1 μmol L^{-1} betamethasone or 1 μmol L^{-1} of NF-κB inhibitor, Bay11-7082. The expression of inflammatory genes TNF-α, IL-1 β, IL-6, and COX-2 was detected by qPCR. (C) The expression of PGE_2 after using 1 μmol L^{-1} betamethasone or 1 μmol L^{-1} of the NF-κB inhibitor, Bay11-7082 for 3 h in LPS- stimulated DPSCs was detected by ELISA. (D) Relationship analysis was carried out using the parameters mentioned above and a positive correlation (r = 0.88) was found between the effects of betamethasone and those of the NF-κB inhibitor. (Untreated DPSCs served as the normal control for comparison of the relative expression of all samples. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.)

were almost the same with DPSCs, and data was not shown.

3.4. Effects of betamethasone on the expression of PGE2 in LPS-stimulated DPSCs

DPSCs were exposed to 1 μg mL^{-1} LPS for 6 h with or without 1 μg L^{-1} betamethasone or Bay 11-7082. A commercial ELISA kit was used to measure the level of PGE_2 in the supernatants. It was found that the expression of PGE_2 increased in response to LPS stimulation, and both betamethasone and Bay11-7082 could significantly inhibits the expression of PGE_2 ($p < 0.001$) (Fig. 2C).

3.5. Betamethasone inhibits NF-κB activation in LPS-stimulated DPSCs

To investigate whether betamethasone suppressed inflammation by inhibiting NF-κB activation, DPSCs were exposed to 1 μg mL^{-1} LPS for 3 h with or without 1 μg L^{-1} betamethasone or Bay 11-7082. A Western blot analysis and immunofluorescence staining were performed to further confirm the inhibitory effect of betamethasone on NF-κB activation. Based on the Western blot analysis, it was found that the expression of p-p65 in DPSCs was increased in response to LPS, and both betamethasone and Bay11-7082 were able to significantly inhibit this effect (Fig. 3A-C). Consistently, confocal microscopy observations showed that p65 nuclear translocation was increased in LPS-stimulated DPSCs. Moreover, both betamethasone and Bay 11-7082 effectively promoted the nuclear exclusion of p65 (Fig. 3D). The ratio of nuclear exclusion of p65 was about 62%, and it was calculated by counting from four different fields. Taken together, these results demonstrate that betamethasone inhibits NF-κB activation by reducing p65 phosphorylation and promoting the nuclear exclusion of p65.

3.6. Glucocorticoid receptor antagonist mifepristone inverts the effect of betamethasone on NF-κB signaling

To further verify the anti-inflammatory mechanism of betamethasone, DPSCs were treated with 1 μg mL^{-1} LPS and 1 μg L^{-1} betamethasone for 3 h with or without 1 μmol L^{-1} mifepristone, a glucocorticoid receptor antagonist. Western blot analysis results showed that betamethasone significantly suppressed the expressions of p-p65 in LPS-stimulated DPSCs. This anti-inflammatory effect was clearly reversed by adding mifepristone: the expressions of p-p65 were significantly up-regulated in DPSCs treated with a combination of mifepristone,

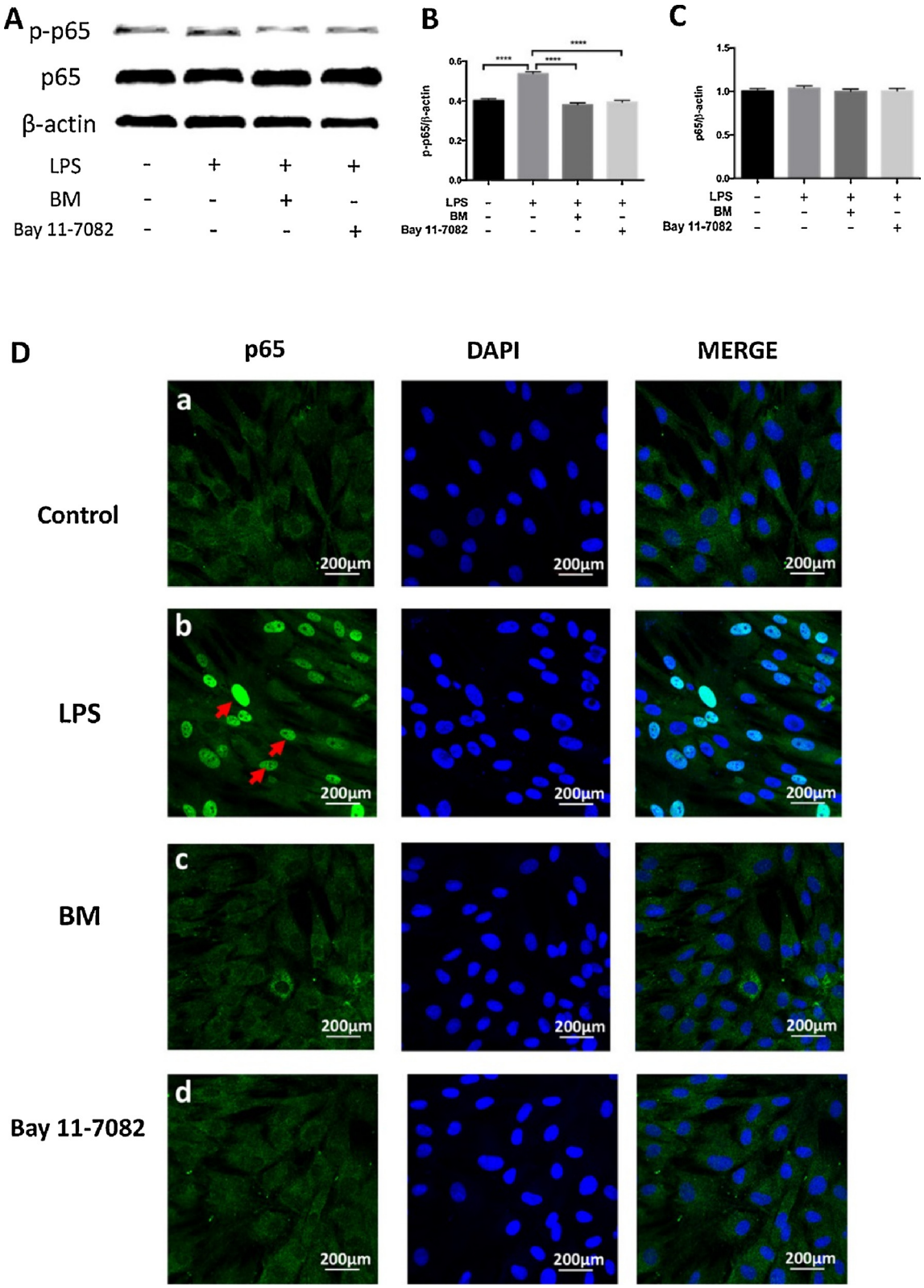

Fig. 3. Effects of betamethasone on NF-κB activity in DPSCs in response to LPS for 3 h were detected by western blots and immunofluorescence staining. DPSCs were exposed to LPS for 3 h with either 1 μmol L^{-1} betamethasone or 1 μmol L^{-1} NF-κB inhibitor Bay11-7082. (A) p-p65, p65 expression were examined by Western blot, and (B-C) the quantification of the western blots was determined by densitometry using Image J software. (D) Cellular localization of endogenous p65 observed by immunofluorescence staining under confocal microscopy. The green fluorescence represents endogenous p65 and the blue fluorescence represents the nucleus. (D-a) Green fluorescence represented endogenous p65 was weakly observed in the nucleus of DPSCs. (D-b) Green fluorescence represented that endogenous p65 was strongly expressed in the nucleus of LPS-induced DPSCs (stressed by the red arrowheads). (D-c, d) Green fluorescence represented endogenous p65 were weakly observed in the nucleus after using betamethasone or NF-κB inhibitor Bay11-7082. The ratio of nuclear exclusion of p65 was 62%. (****$p < 0.0001$)

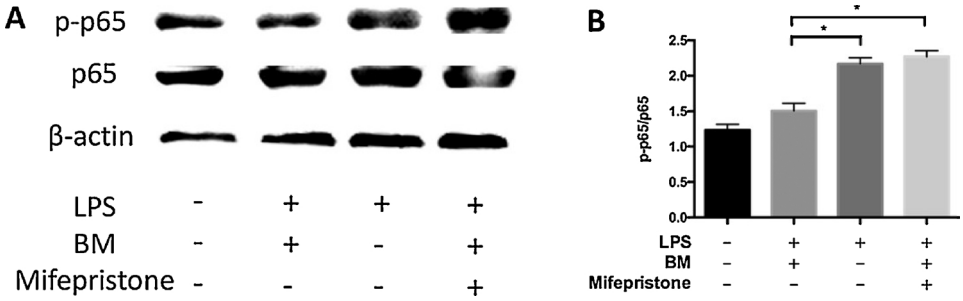

Fig. 4. The inverse effect of glucocorticoid receptor antagonist mifepristone on NF-κB signaling were detected by western blots. DPSCs were treated with 1 μg mL^{-1} LPS and 1 μg L^{-1} betamethasone for 3 h with or without 1 μmol L^{-1} mifepristone. (A) p-p65, p65 expression were examined by Western blot, and (B) the quantification of the ratio of p-p65 and p65 was determined by densitometry using Image J software. (*$p < 0.05$)

betamethasone and LPS (Fig. 4).

3.7. Effects of betamethasone on the odonto/osteogenic differentiation of DPSCs and SHED

The degree of DPSCs and SHED odonto/osteogenic differentiation following treatment with betamethasone was measured by qPCR, respectively. The qPCR findings on Day 7 revealed that the gene expression of alkaline phosphatase (ALP) was significantly up-regulated in both the betamethasone and OM-treated groups compared with control group. The expression of dentin sialophosphoprotein (DSPP), osteocalcin (OCN) on Day 14 exhibited responses similar to that of ALP. (Fig. 5A B).

3.8. Effects of betamethasone on DPSCs and SHED osteoclast induction

The osteoclast effect of betamethasone on DPSCs and SHED was also measured by qPCR. The qPCR findings on Days 1, 3, and 7 revealed that the gene expression of RANKL and OPG were differed between DPSCs and SHED. The results indicated that following treatment with betamethasone, the expression of RANKL decreased on Days 1, 3, and 7 in the DPSCs whereas the expression of OPG was not significantly different, which indicates that the RANKL and OPG ratio (RANKL/OPG) also decreased (Fig. 5C). Conversely, opposing results were found with SHED; the RANKL/OPG ratio increased on Day 7 and there was no significant difference between Days 1 and 3. (Fig. 5D).

4. Discussion

Previous reports have shown that the application of betamethasone as an intra-canal medicament could relieve the inflammatory reactions in rat incisor pulp and reduce the vascular phase of pulp inflammation (Corbel et al., 1999; Fachin, Scarparo, Pezzi, Luisi, & Manoel Sant'Ana, 2009). However, the mechanism of such anti-inflammatory effects in the pulp remains unknown. In this study, we investigated the effect of betamethasone on LPS-stimulated DPSCs and its potential anti-inflammatory mechanism.

DPSCs, as the main constitution of dental pulp tissue, have been used in lots of vitro tests to mimic the dental pulp (Tamaoki et al., 2010; Tziafas, Smith, & Lesot, 2000). SHED, which are the cells from human exfoliated primary teeth (Koyama, Okubo, Nakao, & Bessho, 2009; Miura et al., 2003; Wang et al., 2012), as a main component of dental pulp tissue from primary teeth, it was reported the corticoid has a similar anti-inflammatory effect to both of these two kinds pulp cells (Kojima, Yamaguchi, & Kasai, 2006). LPS is one of the major elements associated with the outer membrane of Gram-negative bacteria and can induce the expression of inflammatory cytokines and cellular apoptosis in DPSCs (Coil et al., 2004; Nakanishi et al., 2001; Rupf et al., 2000). In the present study, we demonstrated that LPS could create an inflammatory environment in DPSCs by up-regulating the expression of TNF-α, IL-1 β, IL-6, COX-2, and PGE_2. We also found that the level of inflammatory cytokines was increased in response to LPS stimulation and decreased following treatment with betamethasone. Interestingly, we observed changes in COX-2 expression following stimulation with LPS and betamethasone treatment. In addition, the secretion of its downstream molecule, PGE_2, also evoked attention. PGE_2 is produced from arachidonic acid (AA) stored in membrane phospholipids and has been reported to be involved in the pathogenesis of pulpal inflammation (Isett, Reader, Gallatin, Beck, & Padgett, 2003; S. K. Lin et al., 2002; Sundqvist & Lerner, 1996). Moreover, PGE_2 may activate and sensitize nociceptors, subsequently causing pain in irreversible pulpitis (Nakanishi et al., 2001; Rittner et al., 2003). Cyclooxygenase 2 (COX-2) is the rate-limiting enzyme involved in the synthesis of PGE_2. In our study, we detected the release of PGE_2 which responded in a similar manner to COX-2 in DPSCs (Kojima et al., 2006; Salvemini et al., 1993). Betamethasone treatment was observed to suppress the synthesis of PGE_2 through decreasing the expression of COX-2. These findings indicate that betamethasone may control the pain generated by the inflammation of dental pulp to some degree. Thus, betamethasone may have the potential to be used as an intracanal medication to suppress the local inflammatory response and control pain in irreversible pulpitis in order to save the vital pulp tissue.

The presence of LPS during infection is known to activate the classical NF-κB signaling pathway, which plays an important role in regulating inflammation (S. I. Lee et al., 2011). Thus, blocking such pathway may explain the potential mechanism by which betamethasone suppresses to production of various pro-inflammatory mediators and cytokines. It has been well-established that NF-κB is a transcription factor critically involved in the regulation of multiple genes, particularly in the inflammatory response (Baeuerle & Baltimore, 1996). There are five members of the NF-κB family: RelA (p65); c-Rel; RelB; NF-κB 1; and NF-κB 2. The NF-κB p65 subunit contains transactivation domains that are critical for NF-κB transcription. Following p65 phosphorylation, NF-κB will translocate into the nucleus and activate the transcription of appropriate genes (Chen, Parent, & Maniatis, 1996; Traenckner et al., 1995). In this study, LPS induced the expression of p-p65 at the protein level and promoted nuclear translocation, suggesting activation of NF-κB signaling in DPSCs. Following treatment with betamethasone, the expression of p-p65 decreased and the level of nuclear translocation was also inhibited. Moreover, this effect of betamethasone was found to be similar to that of Bay 11-7082. From the current results, it was hypothesized that betamethasone exerted a suppressive effect on the inflammatory response to LPS by inhibiting NF-κB signaling pathway. To verify this hypothesis, a pathway blockade experiment was performed by using mifepristone, a glucocorticoid receptor antagonist, to block the effect of betamethasone. It was found that mifepristone up-regulated the expression of p-p65 after treating with betamethasone which meant mifepristone reduced the anti-inflammatory effect of betamethasone. The results indicated that betamethasone may act on NF-κB signaling through stimulating the glucocorticoid receptor.

In a clinical setting, while previous studies do not support the use of betamethasone as a pulp capping agent since it is a glucocorticoid (Alshwaimi, Majeed, & Ali, 2015), it could be used as an intracanal medication to decrease inflammatory effect. According to the figures presented in this study, betamethasone exerts an anti-inflammatory

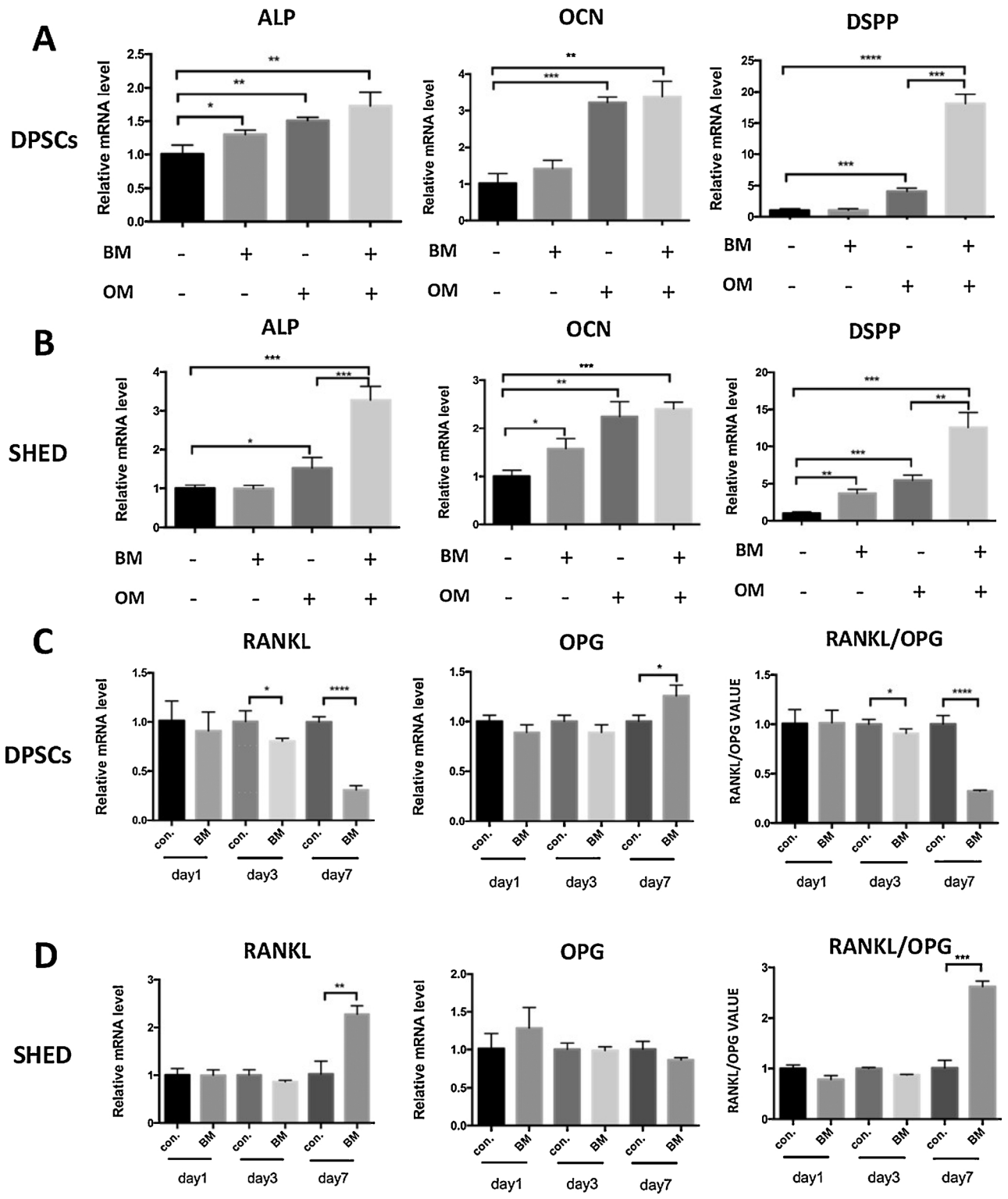

Fig. 5. Effects of betamethasone on the osteo-/odontogenic differentiation and osteoclast in DPSCs and SHED. (A-B) The mRNA expressions of ALP, DSPP and OCN were detected by qPCR. (C-D) The mRNA expressions of RANKL and OPG were detected by qPCR. The ratio of RANKL and OPG were also showed above. (Untreated DPSCs and SHED served as the normal control for comparison of the relative expression of all samples. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.)

effect on LPS-stimulated DPSCs. We also aimed to elucidate the effect of betamethasone on osteogenic/osteoclast induction in both permanent teeth and primary teeth. However, the osteogenic /osteoclast differentiation effects may be different in permanent teeth and primary teeth according to the previous studies. SHED were used to simulate the primary dental pulp tissue in order to explore the osteogenic /osteoclast induction effects (Koyama et al., 2009; Miura et al., 2003; Wang et al., 2012). First, we detected the osteogenic induction effect by the expression of genes related to osteogenesis (i.e., ALP, DSPP, and OCN) at the mRNA level. We found that the expressions of these genes were increased in both DPSCs and SHED following treatment with betamethasone and OM. Thus, betamethasone appears to exhibit an

osteogenic induction effect in both permanent and deciduous teeth, similar to that of OM. To determine the osteoclast effect, we detected the expression of RANKL and OPG at the mRNA level. Recently, the OPG and RANKL system has been recognized to play a crucial role in the signaling pathways associated with osteoclast differentiation. Since it is related to the osteoclastic effect, the RANKL receptor is expressed on the cell membrane of osteoclasts, whereas OPG acts primarily to inhibit osteoclast differentiation (Jiao et al., 2015). Moreover, OPG protects bone from excessive resorption by binding to RANKL (Zhan, Liu, & Wang, 2017). In this study, the "osteoclast-induced effect" referred to the ability to promote osteoclastogenesis indirectly dependent on the RANKL/OPG secreted by DPCSs and SHED. We found that the RANKL/OPG ratio was decreased in DPSCs on Day 7 following treatment with betamethasone; however, this ratio was increased at the same time point in SHED. This finding indicates that betamethasone should not be used as a long-term intracanal medicament in SHED.

In conclusion, our study demonstrates that betamethasone has an anti-inflammatory effect on LPS- stimulated DPSCs through a blockade of NF-κB activation and exhibits an osteo-/odonto-inductive effect on DPSCs and SHED. Although betamethasone displays an osteoclast effect on SHED, such effects were not observed on the DPSCs.

Conflict of interest

The authors declare that there are no potential conflicts of interest.

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