Wnt4 prevents apoptosis and inflammation of dental pulp cells induced by LPS by inhibiting the IKK/NF-κB pathway

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Abstract. Wnt4 has been shown to promote the recovery of odontogenic differentiation of dental pulp stem cells under inflammatory conditions, but its role in inflammation and apoptosis of pulpitis remains to be elucidated. Lipopolysaccharide (LPS) (10 μg/ml) was applied to treat the human dental pulp cells (HDPCs) for 24 h. Western blotting measured the expressions of inflammatory cytokines and apoptosis-related proteins. Cell apoptosis was measured by flow cytometry. The level of Wnt4 was evaluated by reverse transcription-quantitative PCR and western blotting. The results indicated that LPS could promote inflammatory response and apoptosis in HDPCs and downregulated Wnt4 expression was found in LPS-HDPCs. Overexpression of Wnt4 ameliorated cell inflammatory response and apoptosis, presented by reduced expressions of IL-8, IL-6, TNF-α, IL-1β, Bax, cleaved-caspase 3 and enhanced Bcl-2 expression as well as decreased apoptosis rate. Moreover, overexpression of Wnt4 reduced the phosphorylation levels of IKK2, IκBα and p65 proteins upregulated by LPS. Finally, overexpression of IKK2 reversed the effects of Wnt4 on inflammation and apoptosis of LPS-HDPCs and NF-κB inhibitor reversed the effect of IKK2 overexpression in LPS-HDPCs. Wnt4 inhibited LPS-triggered inflammation and apoptosis in HDPCs via regulating the IKK/NF-κB signaling pathway, which provided a new viewpoint for understanding the pathological mechanism of pulpitis.

Introduction

Pulpitis is one of the most common dental pulp diseases, which brings a great burden to the life of afflicted individuals (1). Pulpitis includes reversible pulpitis and irreversible pulpitis (IP). IP indicates a more severe degeneration process, which, if left untreated, leads to pulp necrosis followed by apical periodontitis (2). Dental pulp cells (DPCs), the main components of the dental pulp, serve a deterministic role in innate immune response and inflammatory reactions (3), which can secrete inflammatory factors and chemokines, thus initiating and regulating the inflammatory response (4). As potential critical targets, DPCs have therefore acted as the anti-inflammation target in pulpitis treatment.

Wnts belong to a highly conserved family of secreted growth factors, which couple to various receptors, thereby activating different downstream pathways which classify as either canonical or noncanonical signaling pathways (5). The Wnt pathway controls cellular processes, such as proliferation, differentiation and migration (5). Wnt4 is known to regulate noncanonical Wnt signaling (6). It has been proved to serve a role in pulpitis. Wnt4 promotes the recovery of differentiation of dental pulp stem cells into dentin cells in pulpitis through the JNK signaling pathway (7). However, the role of Wnt4 in pulpitis remains to be elucidated.

The NF-κB pathway mediates inflammation, immunity and cell survival (8,9). Extracellular stimulation is recognized by the receptor and transmitted to initiate a cascade and IKK is activated by a cascade signal. Activated IKK phosphorylates IκB at specific N-terminal serine residue and the phosphorylated IκB is ubiquitinated and degraded. Then, the NF-κB is released from the inhibitory complex and translocated into the nucleus, regulating the expression of multiple genes (10,11). Evidence has shown that NF-κB controls the development of pulpitis (12,13). Meanwhile, Wnt4 regulates the expression of NF-κB. For example, Wnt4 alleviates bone loss and inflammation by suppressing NF-κB *in vivo* (14). Melatonin inhibits NF-κB in a dependent manner with Wnt4 (15).

Based on the aforementioned studies, it was hypothesized that Wnt4 serves an anti-inflammatory and anti-apoptotic role in pulpitis through the NF-κB signaling pathway. The present study investigated the effect and underlying mechanisms of Wnt4 on the inflammatory levels and apoptosis of lipopolysaccharide (LPS)-induced human dental pulp cells (LPS-HDPCs), which might provide a novel therapy target for pulpitis. In addition, exploring effective treatment target therapeutic targets combined with advanced materials such as polyvinylidene fluoride/barium titanate composites (16) and antibacterial scaffold (17) applications could advance the treatment of pulpitis.

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Materials and methods

Isolation, culture and treatment of cells. Normal human impacted third molars free of carious lesions and oral infection were obtained from patients in Anhui Medical College after informed consent was collected from each patient. HDPCs were isolated from the dental pulp tissues of non-decayed third molars. In brief, human dental pulp tissue obtained from sectioned teeth was removed aseptically and minced into small pieces. The pulp was treated with 3 mg/ml collagenase type I solution for 0.5 h at 37˚C. The digested tissues were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 300 mg/ml L-glutamine (Thermo Fisher Scientific, Inc.) and 100 U/ml antibiotics (Thermo Fisher Scientific, Inc.) in an incubator at 37˚C with 5% CO_2; 3 to 5 passages were used for the experiments. For LPS stimulation, LPS (10 μg/ml) was administrated for 24 h as reported previously (18). NF-κB inhibitor (BAY11-7082; 10 Mm; Sigma-Aldrich; Merck KGaA) was used to treat HDPCs at 37˚C for 6 h and cells were acquired for follow-up experiments.

Immunofluorescence. HDPCs were cultured in an 8-well slide for 24 h. Then cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.5% Triton X-100 (Thermo Fisher Scientific, Inc.) and blocked with goat serum (cat. no. SL038; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 20 and 30 min, respectively. Then primary anti-keratin (cat. no. 13063; 1:200; CST), anti-vimentin (cat. no. 5741; 1:600; CST) antibodies and anti-p-p65 (cat. no. 3033; 1:800l; CST) were applied to incubate HDPCs at 4˚C overnight. Next, the cells were cultured in secondary antibody goat against rabbit IgG (cat. no. A32731; 1:200; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. DAPI (1:1,000) was applied for nuclei staining. The images were obtained using a confocal microscope (Leica Microsystems GmbH) (19).

Western blotting. Cells were harvested and lysed a lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentrations were measured using a BCA kit (Thermo Fisher Scientific, Inc.). Proteins (25 μg per lane) were separated using 10% SDS-PAGE and transferred onto a PVDF membrane (BD Biosciences). The membrane was blocked with 5% skimmed milk at room temperature for 60 min and incubated with following primary antibodies: IL-8 (cat. no. ab289967; 1:1,200; Abcam), IL-6 (cat. no. ab233706; 1:1,000; Abcam), TNF-α (cat. no. ab183218; 1:1,000; Abcam), IL-1β (cat. no. ab254360; 1:1,000; Abcam), cleaved-caspase-3 (cat. no. ab32042; 1:800; Abcam), caspase-3 (cat. no. ab32351; 1:3,000; Abcam), Bax (cat. no. 2774S; 1:1,000; CST), Bcl-2 (cat. no. 15071; 1:1,000; CST), Wnt4 (cat. no. ab277798; 1:800, Abcam), p-IKK2 (cat. no. 2694; 1:1,000; CST), IKK (cat. no. ab124957; 1:1,000; Abcam), p-p65 (cat. no. 3033; 1:1,000; CST), p65 (cat. no. 8242; 1:1,000; CST), p-IκBα (cat. no. 2859; 1:1,000; CST), IκBα (cat. no. 9242; 1:1,000; CST), β-actin (cat. no. 4970; 1:5,000; CST), GAPDH (cat. no. 5174; 1:5,000; CST) at 4˚C overnight. Then, the HRP-conjugated secondary antibody (goat against rabbit IgG (cat. no. 7074; 1:2,000; CST) and goat against mouse IgG (cat. no. 96714; 1:3,000; CST) was administrated to the cells at room temperature for 1 h. The results were detected using an ECL kit (Beyotime Institute of Biotechnology) and quantitated using ImageJ (National Institutes of Health) (18).

Flow cytometry. Treated HDPCs were collected, washed and resuspended with binding buffer (PBS with 5% FBS). Subsequently, HDPCs were incubated with FITC annexin V at room temperature for 0.5 h and washed using PBS. Propidium iodide (PI; 50 mg/ml) was used to culture HDPCs at room temperature for 5 min in the dark. Analysis was performed using flow cytometry by a Cytomics FC500 flow cytometer (Beckman Coulter, Inc.) and Cytomics CXP software version 2.2 (Beckman Coulter, Inc.). Untreated HDPCs were used as the control (20). Apoptosis rate=the percentage of early + late apoptotic cells.

Reverse transcription-quantitative (RT-q) PCR. HDPCs were seeded into six-well plate ($1x10^6$ cells/well) and total RNA was extracted using TRIzol® according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.) after cells were treated with LPS or transfected for 48 h. Reverse transcription was executed with PrimeScript RT reagent kit (Takara Bio, Inc.) at 42˚C for 40 min and 85˚C for 5 min and 4˚C for 60 min according to the manufacturer's protocol. RT-qPCR was conducted with SYBR-Green I Master Mix (Roche Diagnostics) via LightCycler 480 (Roche Diagnostics) according to the manufacturer's protocols. Three parallel spaces were conducted for each sample and experiment was performed three times. PCR amplification conditions were: 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing 60˚C for 1 min and extension 72˚C for 20 sec. The relative changes were calculated using the $2^{-\Delta\Delta Cq}$ method (18). The primer sequences were: Wnt4 forward 5'-ACCTGGAAGTCATGGACTCG-3', reverse 5'-TCAGAGCATCCTGACCACTG-3'. GAPDH Forward: 5'-CTGCCCAGAACATCATCC-3', Reverse: 5'-CTCAGATGCCTGCTTCAC-3'.

Cell transfection. The Wnt4 overexpression plasmids (pcDNA-Wnt4) were constructed using pcDNA3.1 vector by Shanghai GenePharma Co., Ltd. and empty plasmids were used as normal control (NC). The constitutive activator of IKK2 (pCMV-IKK2EE) constructed using pCMV-MCS vector (21) and empty plasmids (pCMV-NC) were obtained from OriGene Technologies, Inc. IKK2EE primer: Forward: 5'-GCTCTAGAGCCACCATGAGCTGGTCACCTT-3', and reverse: 5'-TATGCGGCCGCATCAAGCGTAGTCTGGGACGTCGTATGGGTATGAGGCCTGCTCCAGGCAGCTGTGCTCTTCTT-3'. HDPCs were inoculated into six-well plates ($2x10^5$ cells/well) and when confluence reached 70-80%, the cells were transfected with 2.5 μg pcDNA-NC/pcDNA-Wnt4 and pCMV-IKK2EE/pCMV-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. Subsequent experiments were performed 48 h after transfection (21).

Statistical analysis. All experiments were conducted three times and the data were presented as mean ± standard deviation. Analysis used GraphPad Prism 8.0 via one-way ANOVA followed by a Bonferroni test or an unpaired Student's t-test. $P<0.05$ was considered statistically significant.

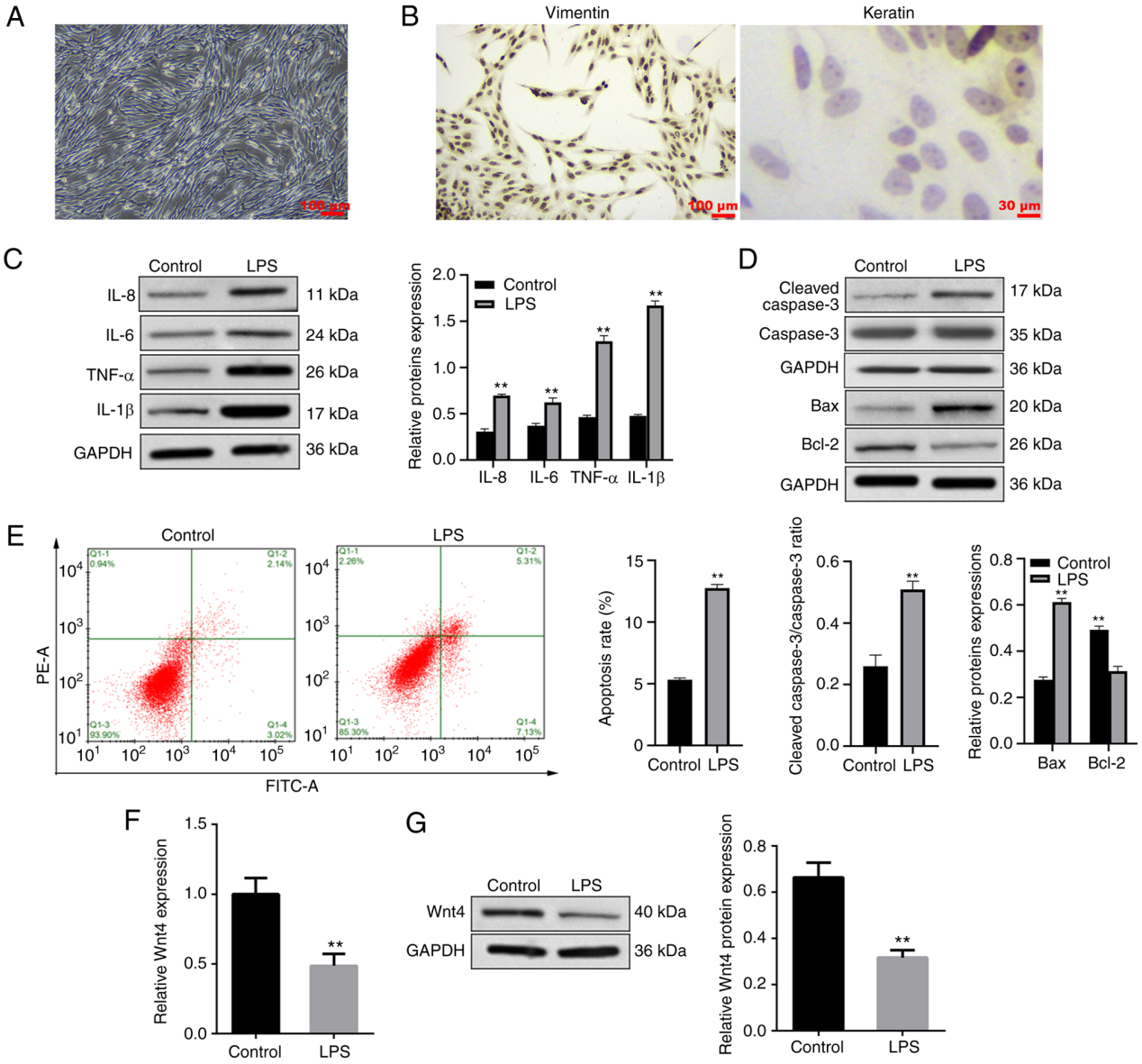

Figure 1. Effects of LPS on inflammation and apoptosis in HDPCs. The HDPCs were treated with LPS for 24 h. (A) The morphology of HDPCs was observed under an optical microscope. Scale bar, 100 μm. (B) Expressions of vimentin and keratin in HDPCs were measured by immunofluorescence. Scale bar, 100 μm/30 μm. (C) The expressions of inflammatory factors and (D) apoptosis-related proteins were detected by western blotting. (E) Flow cytometry was applied to measure cell apoptosis. (F) Reverse transcription-quantitative PCR and (G) western blotting was used to detect the expression of Wnt4. Data represent the mean values ± standard deviation of three experiments. **P<0.01. LPS, lipopolysaccharide; HDPCs, human dental pulp cells.

Results

LPS induces inflammatory response and apoptosis in HDPCs. Morphological observation identified that HDPCs of the third generation were fusiform or polygon (Fig. 1A). Immunofluorescence demonstrated that the HDPCs were positive for vimentin and negative for keratin (Fig. 1B), indicating that HDPCs were derived from mesenchymal tissue and conformed to the histological features of the pulp. HDPCs were treated with 10 μg/ml LPS for 24 h to construct a pulpitis cell model. Western blotting was applied to measure the expressions of proinflammatory cytokines and apoptosis-related proteins. It was found that the expressions of proinflammatory cytokines (IL-8, IL-6, TNF-α and IL-1β) were significantly increased in the LPS group (Fig. 1C). The expressions of Bax and cleaved-caspase 3 were upregulated while Bcl-2 was downregulated in the LPS group (Fig. 1D). Meanwhile, the results of flow cytometry indicated that LPS dramatically induced the apoptosis of HDPCs (Fig. 1E).

Overexpression of Wnt4 inhibits inflammation and apoptosis in HDPCs induced by LPS. The expression of Wnt4 in HDPCs was detected. In Fig. 1F and G, LPS significantly inhibited the expression of Wnt4 in HDPCs. To explore the effect of Wnt4 on LPS-induced inflammation and apoptosis, Wnt4 was overexpressed with plasmids in LPS-HDPCs. The results showed that the mRNA and protein levels of Wnt4 were significantly increased in the LPS + OE-Wnt4 group compared with LPS + OE-NC and LPS groups (Fig. 2A and B, P<0.01). Overexpression of Wnt4 significantly inhibited the upregulation of inflammatory factors induced by LPS in HDPCs

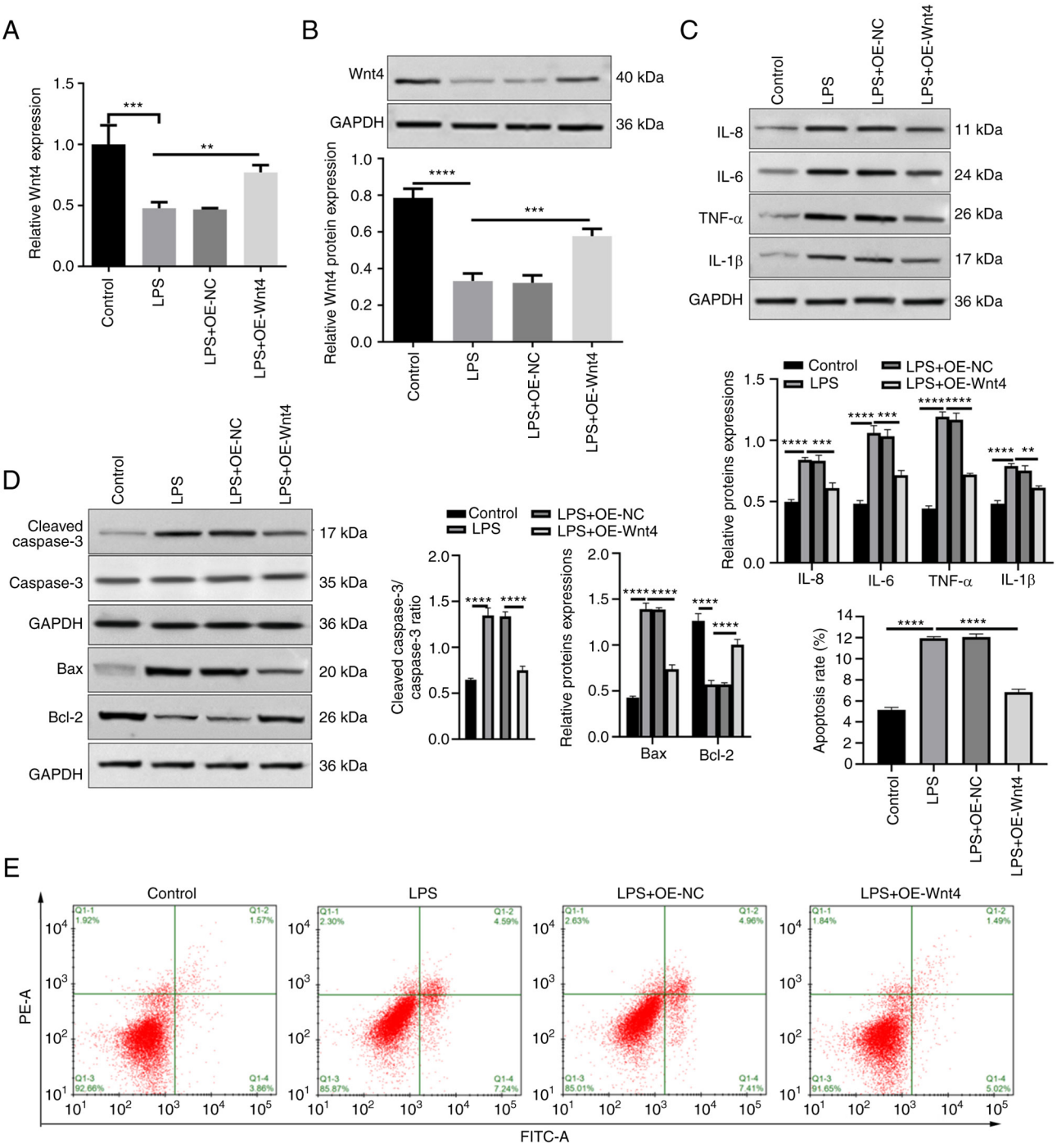

Figure 2. Overexpression of Wnt4 inhibits inflammation and apoptosis in LPS-induced HDPCs. pcDNA-Wnt4 or pcDNA-NC plasmid was transfected into LPS-HDPCs. (A) Reverse transcription-quantitative PCR and (B) western blotting were applied to detect the expression of Wnt4 after transfection with pcDNA-Wnt4 or pcDNA-NC in LPS-HDPCs. The expressions of (C) inflammatory factors and (D) apoptosis-related proteins were detected by western blotting. (E) Flow cytometry was applied to measure cell apoptosis. Data represent the mean values ± standard deviation of three experiments. **P<0.01, ***P<0.001, ****P<0.0001. LPS, lipopolysaccharide; HDPCs, human dental pulp cells; OE-Wnt4, overexpression of Wnt4; OE-NC, negative control; p-, phosphorylated.

(Fig. 2C). The results of western blotting showed that Bax and cleaved-caspase 3 were reduced, while Bcl-2 was increased in LPS + OE-Wnt4 group compared with the LPS + OE-NC group and LPS groups (Fig. 2D). Meanwhile, the result of flow cytometry showed that overexpression of Wnt4 significantly reduced LPS-induced apoptosis (Fig. 2E).

Wnt4 suppresses the activation of the IKK/NF-κB pathway by LPS. It has been found that Wnt4 inhibits the NF-kB pathway in the bone disease mouse model (14). To explore whether Wnt4 regulated the activation of the NF-kB pathway in LPS-HDPCs, the present study detected the expressions of NF-κB pathway-related proteins. The results of western blotting presented that LPS upregulated the phosphorylation levels of IKK2, IκBα and p65, while overexpression of Wnt4 downregulated the phosphorylation levels of IKK2, IκBα and p65 (Fig. 3A). In addition, the result of immunofluorescent assay demonstrated that p65 nuclear translocation was promoted in

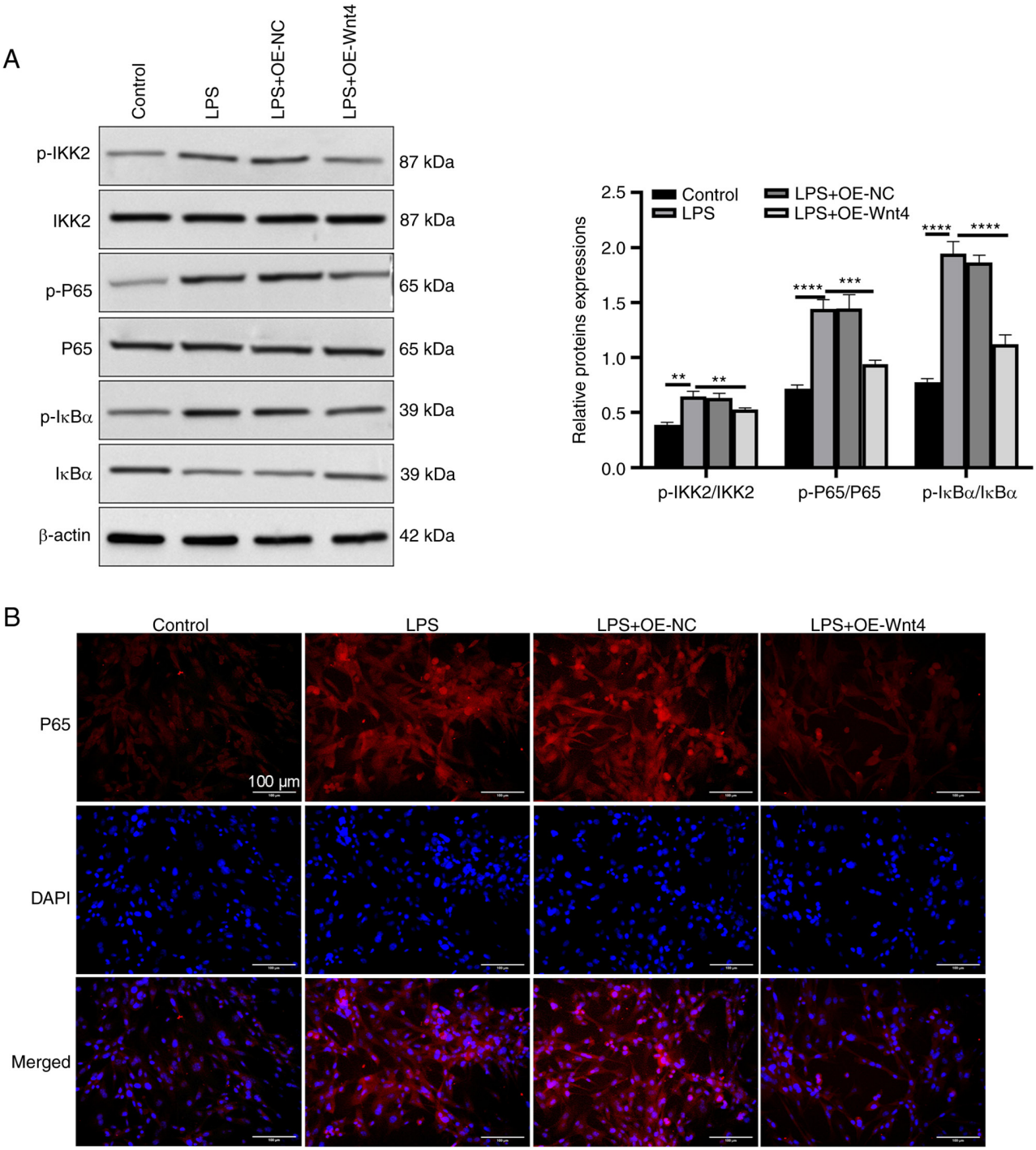

Figure 3. Wnt4 suppresses the activation of IKK/NF-κB pathway in LPS-induced HDPCs. (A) Western blotting and (B) immunofluorescence staining were used to analyze the activation of IKK/NF-κB pathway. Data represent the mean values ± standard deviation of three experiments. **P<0.01, ***P<0.001, ****P<0.0001. LPS, lipopolysaccharide; HDPCs, human dental pulp cells; p-, phosphorylated; OE-Wnt4, overexpression of Wnt4; OE-NC, negative control.

LPS-HDPCs compared with the control group, overexpression of Wnt4 effectively inhibited the nuclear translocation of p65 (Fig. 3B).

Wnt4 inhibits inflammation and apoptosis through IKK/NF-κB pathway in LPS-HDPCs. To investigate whether IKK/NF-κB pathway was involved in the regulation of Wnt4 on inflammation and apoptosis in LPS-HDPCs, pcDNA-Wnt4 or negative control and pCMV-IKK2EE which could mimic the IKK2 loop to activate the downstream NF-κB signaling pathway (21), or negative control were transfected into LPS-HDPCs. The results of western blotting revealed that overexpression of Wnt4 reduced the upregulated phosphorylation levels of IKK2, IκBα and p65 induced by LPS while overexpression of IKK2EE reversed the effect of Wnt4 (Fig. 4A). The result of immunofluorescence demonstrated that Wnt4 significantly suppressed p65 nuclear translocation induced by LPS, while overexpression of IKK2 significantly promoted p65 nuclear translocation in the LPS + OE-Wnt4 + OE-IKK2EE group (Fig. 4B). Moreover, the detection results

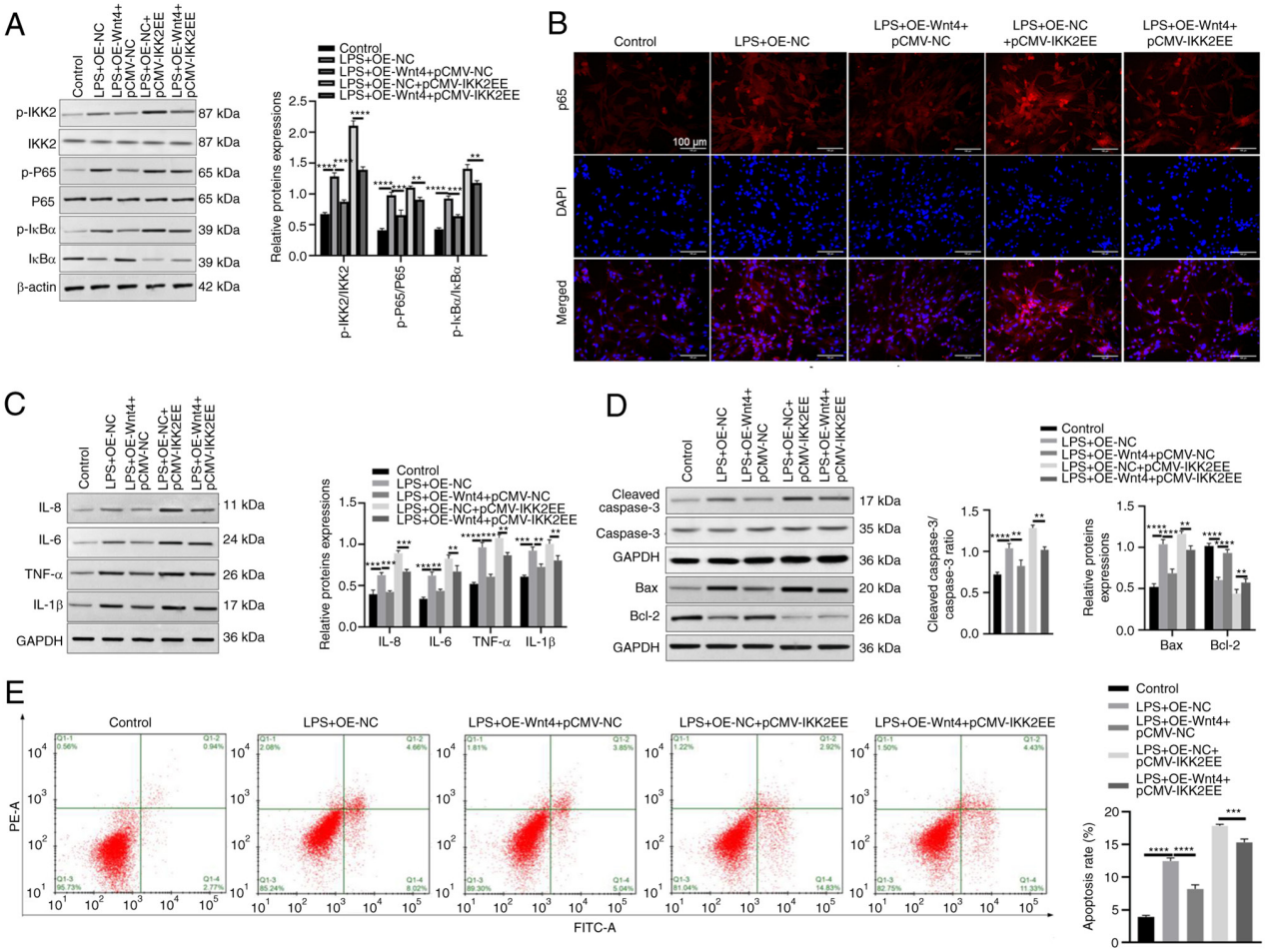

Figure 4. Wnt4 inhibits inflammation and apoptosis through the IKK/NF-κB pathway. pcDNA-Wnt4 or negative control and pCMV-IKK2EE or negative control plasmids were transfected in LPS-HDPCs. (A) Western blotting and (B) immunofluorescence staining were used to measure protein levels of the IKK/NF-κB pathway. The expressions of (C) inflammatory factors and (D) apoptosis-related proteins were detected by western blot. (E) Flow cytometry was applied to measure cell apoptosis. Data represent the mean values ± standard deviation of three experiments. **P<0.01, ***P<0.001, ****P<0.0001. LPS, lipopolysaccharide; HDPCs, human dental pulp cells; OE-Wnt4, overexpression of Wnt4, OE-NC, negative control.

of inflammatory factors showed that overexpression of IKK2EE increased the expressions of inflammatory factors (IL-8, IL-6, TNF-α and IL-1β) and reversed the inhibitory effect of Wnt4 on LPS induced upregulation of inflammatory factors IL-8, IL-6, TNF-α and IL-1β (Fig. 4C). Moreover, the results of western blotting showed that the expressions of Bax and cleaved-caspase 3 were significantly downregulated, Bcl-2 was upregulated in the LPS + OE-Wnt4 + pCMV-NC group as compared with the LPS + OE-NC + pCMV-NC group, while the expressions of Bax and cleaved-caspase 3 were strikingly increased, Bcl-2 was decreased in the LPS + OE-Wnt4 + pCMV-IKK2EE group as compared with LPS + OE-Wnt4 + pCMV-NC group (Fig. 4D). Meanwhile, the result of flow cytometry indicated that the apoptosis rate of HDPCs was significantly downregulated in the LPS + OE-Wnt4 + pCMV-NC group as compared with the LPS + OE-NC + pCMV-NC group, while the apoptosis rate was remarkably increased in the LPS + OE-Wnt4 + pCMV-IKK2EE group as compared with LPS + OE-Wnt4 + pCMV-NC group (Fig. 4E)

To further explore the effect of the IKK/NF-κB pathway on the inflammatory and apoptosis of HDPCs overexpressing of Wnt4. NF-κB inhibitor (BAY11-7082) was used to treat HDPCs co-transfected with OE-Wnt4 and pCMV-IKK2EE. The results showed that BAY11-7082 decreased the expression levels of IL-8, IL-6, TNF-α and IL-1β in the LPS + OE-Wnt4 + pCMV-IKK2EE + BAY group compared with the LPS + OE-Wnt4 + pCMV-IKK2EE + DMSO group (Fig. 5A). Similarly, The expression levels of Bax and cleaved-caspase 3 were markedly weakened and Bcl-2 expression was raised by in the LPS + OE-Wnt4 + pCMV-IKK2EE + BAY group compared with the LPS + OE-Wnt4 + pCMV-IKK2EE + DMSO group (Fig. 5B). Adding BAY11-7082 reduced the apoptosis of OE-Wnt4 and pCMV-IKK2EE co-transfected HDPCs (Fig. 5C). Collectively, these results suggested that Wnt4 alleviated inflammation and apoptosis via IKK/NF-κB pathway in LPS-HDPCs (Fig. 6).

Discussion

Pulpitis is a physiological response to bacterial infections, physical and chemical injuries (22). Bacterial invasion triggers immune reactions to initiate pulpitis (23). Pulpitis may continuously develop into pulp necrosis, periapical periodontitis and severe infections (24). Exploring the abnormal molecular changes in the pathological process of pulpitis can provide new ideas for the prevention and treatment of pulpitis.

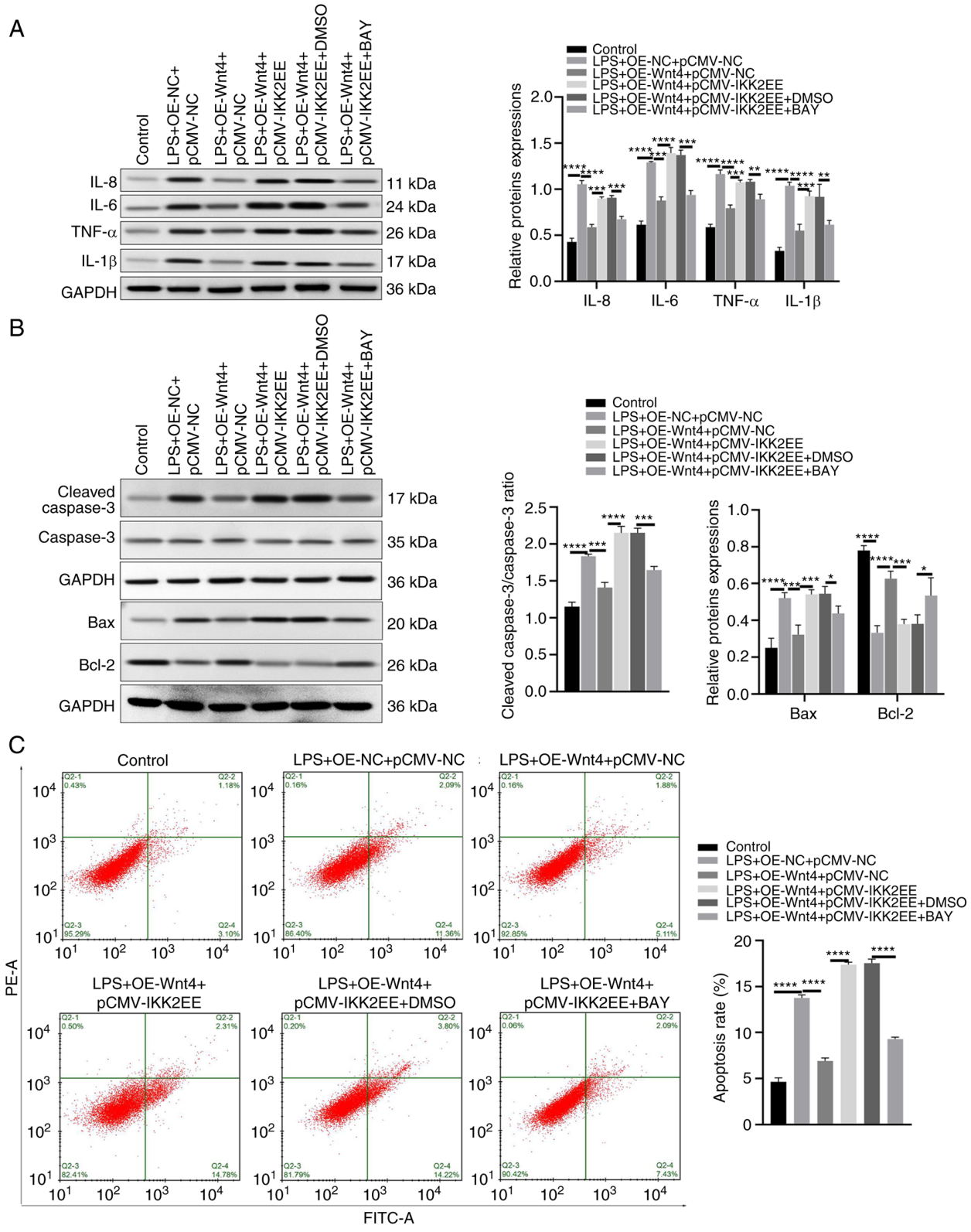

Figure 5. NF-κB participates in the effect of Wnt4 on LPS-induced HDPCs. The expressions of (A) inflammatory factors and (B) apoptosis-related proteins were detected by western blot. (C) Flow cytometry was applied to measure cell apoptosis. Data represent the mean values ± standard deviation of three experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. LPS, lipopolysaccharide; HDPCs, human dental pulp cells; OE-Wnt4, overexpression of Wnt4, OE-NC, negative control.

LPS is an important inducer of pulpitis. Administration of LPS to dentinal surfaces can trigger pulpal inflammatory responses in mice (25) and can cause upregulation of inflammatory factors and adhesion molecules in HDPCs (26). LPS also induces apoptosis of odontoblast-like cells (27). The present study also verified that LPS significantly induced the increased expressions of inflammatory factors IL-8, IL-6, TNF-α and IL-1β as well as the increase of apoptosis rate in

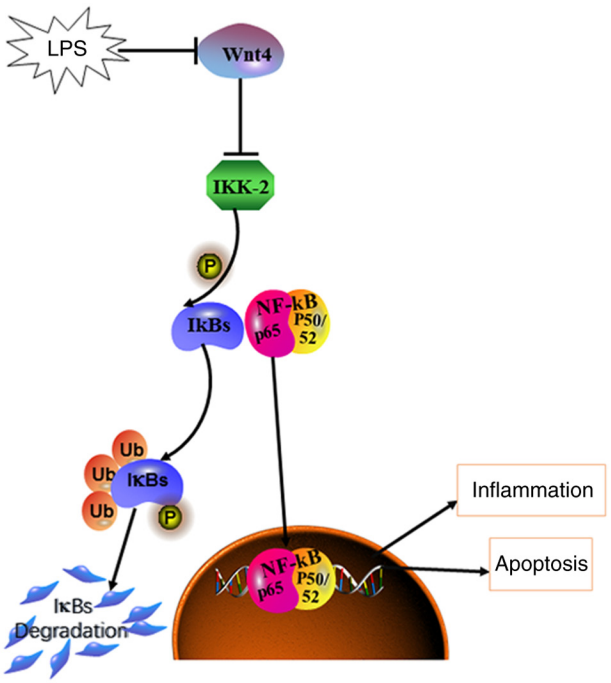

Figure 6. Schematic model for the molecular mechanism of Wnt4 in LPS-induced HDPCs. LPS, lipopolysaccharide; HDPCs, human dental pulp cells.

HDPCs, which were consistent with previous studies that LPS increased the expression of IL-1β and IL-6 (26,28).

Wnt4 is reported as a nonclassical Wnt signaling pathway component to exert crucial roles in physiological and pathological conditions (29). Wnt4 mediates the protective effect of mesenchymal stromal cells on vascular endothelial cell apoptosis (30). Wnt4 contributes to cisplatin-induced acute kidney injury (31). In addition, Wnt4 can promote bone repair by improving the osteogenic potential of inflammatory dental pulp stem cells (32). Wnt4 has also been shown to have a potential effect on pulpitis (7). The present study found that the expression level of Wnt4 was decreased in LPS-stimulated HDPCs, and that the upregulation of inflammatory factors induced by LPS was significantly decreased after overexpression of Wnt4. Overexpression of Wnt4 also significantly reduced the levels of apoptosis-related proteins and the apoptosis of HDPCs.

NF-κB is a transcription factor that controls the inflammatory response, cell cycle and apoptosis (33). In the canonical NF-κB pathway, IκB is phosphorylated, ubiquitinated and degraded, which triggers the nuclear translocation of the NF-κB complex and regulates the transcription of the downstream gene (34). LPS can significantly induce the activation of NF-κB (35). The present study also found that LPS treatment activated the IKK/NF-κB signaling pathway in HDPCs. Wnt signaling presents a critical effect in the regulation of cell proliferation and differentiation, while Yu *et al* (14), found that Wnt4 could reduce the inflammatory response by inhibiting NF-kB in osteoclasts. IKK2 (IKKβ) is the main IKK activated by proinflammatory stimuli and is the kinase primarily responsible for regulating NF-κB activation (36). The present study verified that the phosphorylation levels of IKK2, IκBα and p65 were decreased in Wnt4-overexpressed HDPCs, which indicated that Wnt4 inactivated IKK2/NF-κB pathway in HDPCs. Studies have reported that IKK2/NF-κB signaling mediates neuroinflammation in the cerebellum (37) and regulates the apoptosis of pulmonary arterial smooth muscle cells (38). In the present study, to verify the role of the IKK2/NF-κB pathway on Wnt4 mediation of inflammation and apoptosis, the IKK2/NF-κB pathway was activated by overexpressing constitutively active IKK2 and inhibiting the activation of NF-κB. The results indicated that IKK2/NF-κB pathway promoted the inflammation and apoptosis of HDPCs and Wnt4 inhibited LPS-induced activation of IKK2/NF-κB and thereby inhibiting inflammation and apoptosis induced by LPS in HDPCs.

In conclusion, Wnt4 could inhibit inflammation and apoptosis by hindering the activation of the IKK2/NF-κB pathway in LPS-HDPCs, which might provide a potential therapeutic target in pulpitis.

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Availability of data and materials

The datasets generated and/or used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors contributed to the conception and design of the study. GW, TM and JX performed the experiments, data collection and analysis and wrote the manuscript. CN conceived and designed the experiments and revised the manuscript. CN and GW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Anhui Medical College (approval no. 2022-LLBG-001) and obeyed the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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