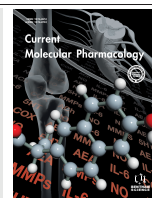


## RESEARCH ARTICLE

BENTHAM  
SCIENCE

# Nuezhenide Exerts Anti-Inflammatory Activity through the NF- $\kappa$ B Pathway



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**Abstract: Background:** Nuezhenide (NZD), an iridoid glycoside isolated from *Ilex pubescens* Hook. & Arn. var. *kwangsiensis* Hand.-Mazz., used as a traditional Chinese medicine for clearing away heat and toxic materials, displays a variety of biological activities such as anti-tumor, antioxidant, and other life-protecting activities. However, a few studies involving anti-inflammatory activity and the mechanism of NZD have also been reported. In the present study, the anti-inflammatory and antioxidative effects of NZD are illustrated.

**Objective:** This study aims to test the hypothesis that NZD suppresses LPS-induced inflammation by targeting the NF- $\kappa$ B pathway in RAW264.7 cells.

**Methods:** LPS-stimulated RAW264.7 cells were employed to detect the effect of NZD on the release of cytokines by ELISA. Protein expression levels of related molecular markers were quantitated by western blot analysis. The levels of ROS, NO, and Ca<sup>2+</sup> were detected by flow cytometry. The changes in mitochondrial reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were observed and verified by fluorescence microscopy. Using immunofluorescence assay, the translocation of NF- $\kappa$ B/p65 from the cytoplasm into the nucleus was determined by confocal microscopy.

**Results:** NZD exhibited anti-inflammatory activity and reduced the release of inflammatory cytokines such as nitrite, TNF- $\alpha$ , and IL-6. NZD suppressed the expression of the phosphorylated proteins like IKK $\alpha$ / $\beta$ , I $\kappa$ B $\alpha$ , and p65. Besides, the flow cytometry results indicated that NZD inhibited the levels of ROS, NO, and Ca<sup>2+</sup> in LPS-stimulated RAW264.7 cells. JC-1 assay data showed that NZD reversed LPS-induced MMP loss. Furthermore, NZD suppressed LPS-induced NF- $\kappa$ B/p65 translocation from the cytoplasm into the nucleus.

**Conclusion:** NZD exhibits anti-inflammatory effects through the NF- $\kappa$ B pathway on RAW264.7 cells.

**Keywords:** Nuezhenide, RAW264.7 cells, LPS, anti-inflammation, NF- $\kappa$ B pathway, cytotoxicity.

## 1. INTRODUCTION

The inflammatory response is a common pathological reaction, which occurs in all tissues and organs of the body [1-3]. It is the defensive response of the body to various external stimuli [4]. Most of the inflammatory reactions are transduced through the NF- $\kappa$ B signaling pathway [5, 6]. When human immune cells are affected by inflammatory factors, they secrete some proteins or peptides with specific immunoregulatory functions, thereby performing the auto-

immune regulation of the body, and thus further protecting the body [4, 7, 8]. However, an excessive inflammatory response can lead to a range of diseases [9, 10]. During the inflammatory process, the NF- $\kappa$ B pathway always plays an important role. NF- $\kappa$ B is composed of I $\kappa$ Bs, p65, and p50 and in the cytoplasm, it is inactive [11]. When I $\kappa$ Bs are degraded, the active heterodimers p65 and p50 translocate into the nucleus, resulting in pro-inflammatory cytokines release [12, 13]. Therefore, to seek out an anti-inflammatory agent targeting the NF- $\kappa$ B pathway is an effective strategy.

*Ilex pubescens* Hook. et Arn. var. *kwangsiensis* Hand.-Mazz. (Chinese name called *Guangxi Maodongqing*), used as a traditional Chinese medicine for clearing away heat and toxic materials, belongs to genus *Ilex*. The previous studies indicated that *Guangxi Maodongqing* contains many ingredi-

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ents such as flavonoids, iridoid glycosides, and triterpenoid saponins, which display a variety of biological activities [14-16]. Nuezhenide (NZD), an iridoid glycoside isolated from *Guangxi Maodongqing*, displays a variety of biological activities such as anti-tumor, antioxidant, and life-protecting, lipid-lowering, antiviral, and antibacterial activities [17]. However, there are a few studies about the anti-inflammatory activity and mechanism of exploration. The present study, using the LPS-stimulated RAW264.7 cells model, mainly illustrates the anti-inflammatory effects and mechanism of NZD, which provided the fundamental and scientific basis for the research and development of an anti-inflammatory agent.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

NZD was isolated and identified by our laboratory. The purity of NZD was determined by HPLC (over 98%). Griess reagent (modified-G4410), Lipopolysaccharides from *Escherichia coli* O111:B4. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Life Technologies/Gibco Laboratories (Grand Island, NY, USA). IL-6 and TNF- $\alpha$  ELISA kits were obtained from Neonbioscience (Shenzhen, China). Antibodies: the NF- $\kappa$ B pathway sampler kit (#8242T), iNOS (#13120), COX-2 (#4842), and GAPDH (#5174) were obtained from Cell Signaling Technologies (Beverly, MA, USA). Dulbecco's modified eagle medium (DMEM), Fluo-3/AM, NO detector DAF-FM, fetal bovine serum (FBS) were purchased from Life Technologies/Gibco Laboratories (Grand Island, NY, USA).

### 2.2. Cell Cultures

RAW264.7 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM with 10% FBS, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. Cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 2.3. Cell Viability Assay

RAW264.7 cells were cultured in 96-well plates at a density of  $5 \times 10^4$  cells/well overnight. Cells were treated with NZD (0, 10, 20, 40  $\mu$ M) for 24 h, and subsequently, MTT (5 mg/mL) was added to each well and cultured for 4 h. After that, the culture medium was removed and replaced by DM-SO (100  $\mu$ L/well) to dissolve the crystals. The absorbance was read at 570 nm with a microplate reader (BioTek, Winooski, VT, USA).

### 2.4. Griess Reagent Assay

RAW264.7 cells were cultured in 24-well plates at a density of  $1 \times 10^5$  cells/well overnight. The cells pretreated with NZD (0, 10, 20, 40  $\mu$ M) for 1 h were co-cultured with or without LPS (1  $\mu$ g/mL) for another 18 h. The medium was collected to determine the nitrite level by Griess reagent.

### 2.5. Determination of IL-6 and TNF- $\alpha$

RAW264.7 cells were cultured in 24-well plates at a density of  $1 \times 10^5$  cells/well overnight. The cells pretreated with NZD (0, 10, 20, 40  $\mu$ M) for 1 h were co-cultured with or without LPS (1  $\mu$ g/mL) for another 24 h. The medium was collected and IL-6 and TNF- $\alpha$  were determined by ELISA according to the manufacturers' instructions.

### 2.6. Flow Cytometry Assay

RAW264.7 cells were cultured in 12-well plates at a density of  $2 \times 10^5$  cells/well overnight. Subsequently, cells were pretreated with NZD (0, 10, 20, 40  $\mu$ M) for 1 h, and then treated with or without LPS (1  $\mu$ g/mL) for 8 h. DCFH<sub>2</sub>-DA (1  $\mu$ M), DAF-FM (1  $\mu$ M), and Fluo-3/AM (1  $\mu$ M) were employed to label cells for 30 min, 1 h, and 1 h, to detect ROS level, NO level, and Ca<sup>2+</sup> level, respectively. After probes incubation, cells were collected and tested by the flow cytometry at the FITC channel (Becton-Dickinson, Franklin Lakes, NJ, USA).

### 2.7. Western Blot Analysis

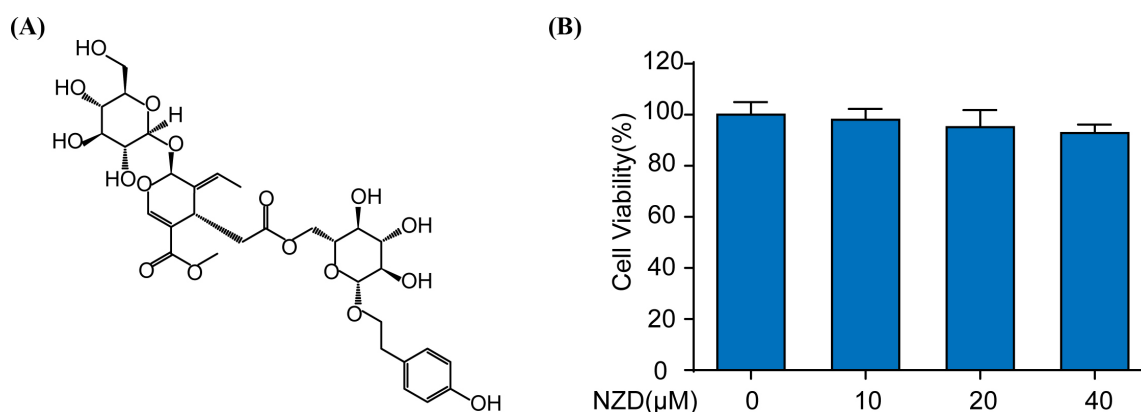
RAW264.7 cells were cultured in 6-well plates at a density of  $3 \times 10^5$  cells/well overnight. The cells were pretreated with NZD (0, 10, 20, 40  $\mu$ M) for 1 h, and subsequently, LPS induction was done for a certain time. Total cell proteins were extracted using RIPA (1% PMSF and 1% cocktail). BCA protein kit (Waltham, MA, USA) was employed to determine protein concentrations. The denatured proteins were separated by 8% or 10% SDS-PAGE gels and were transferred to the PVDF membrane (Millipore, Billerica, MA, USA). After blocking the PVDF membrane with 5% nonfat milk for 1 h, the PVDF membrane was incubated with primary antibodies (1:1000) for more than 12 h at 4 °C. After the membrane was washed with TBST and incubated with secondary antibody (1:5000) for 2 h at room temperature, the membrane was exposed to ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

### 2.8. Fluorescence Assay

RAW264.7 cells were seeded in 96-well plates with a density of  $5 \times 10^4$  cells/well overnight. Cells were pretreated with NZD (40  $\mu$ M) for 1 h and then incubated with or without LPS for another 8 h. Staining was performed JC-1 (10  $\mu$ g/mL) and DCFH<sub>2</sub>-DA (100  $\mu$ M) for 30 min in dark. Fluorescence images were captured by fluorescence microscopy (Leica, Wetzlar, Germany).

### 2.9. Immunofluorescence Assay

RAW264.7 cells were cultured on confocal dishes (SPL, Pocheon, Korea) at a density of  $2 \times 10^5$  cells/well overnight and then pretreated with NZD (40  $\mu$ M) for 1 h. After stimulation with LPS (1  $\mu$ g/mL) for another 1 h, cells were fixed, permeabilized, blocked, and incubated with rabbit anti-mouse NF- $\kappa$ B p65 antibody (1:200) for 1 h at room temperature. Cells were then incubated with Alexa Fluor 488-conjugated secondary antibody for 1 h. Hoechst 33342 was used



**Fig. (1).** NZD shows no significant cytotoxicity on RAW264.7 cells. (A) The chemical structure of NZD. (B) The cytotoxicity of NZD was detected by MTT assay after 24 h of treatment.

to stain the nuclei for 10 min. The image was captured by a confocal laser scanning microscopy (Leica, Wetzlar, Germany) at an excitation/emission wavelength of 588/615-690 nm.

### 2.10. Statistical Analysis

All results were independently repeated at least thrice, and presented as mean  $\pm$ SD. The significance of the intergroup differences was analyzed with the one-way analysis of variance (one-way-ANOVA) and Dunn's multiple comparison tests using GraphPad Prism 6.0 software. \* $p < 0.05$  was considered a significant difference.

## 3. RESULTS

### 3.1. NZD Shows no Significant Cytotoxicity on RAW264.7 Cells

Nuezhenide (Fig. 1A), a natural product, was isolated from Guangxi Maodongqing. The cytotoxicity of NZD (0, 10, 20, 40  $\mu$ M) was investigated by MTT assay in RAW264.7 cells. According to the results of this study, no significant cytotoxicity was observed (Fig. 1B).

### 3.2. NZD Suppresses LPS-stimulated Inflammatory Response in RAW264.7 Cells

The expression of COX-2, an enzyme whose degree of expression is related to the severity of inflammation, can be induced [18-20]. Reducing or inhibiting the activation of COX-2, the synthesis and release of inflammatory mediators iNOS and NO can be regulated, which can effectively control the occurrence of inflammation [21, 22]. In order to detect and evaluate whether NZD could reduce the nitrite level and NO generation in LPS-stimulated RAW264.7 cells, we adopted the Griess reagent and flow cytometer. The results showed that the levels of nitrite level and NO release were significantly increased after the stimulation of LPS in RAW264.7 cells, and this phenomenon was reversed after NZD pretreatment (Fig. 2A-C). Besides, we further investigated whether NZD could reduce the expressions of proteins, iNOS and COX-2 in LPS-stimulated RAW264.7 cells. The results of this study indicate that the expressions of pro-

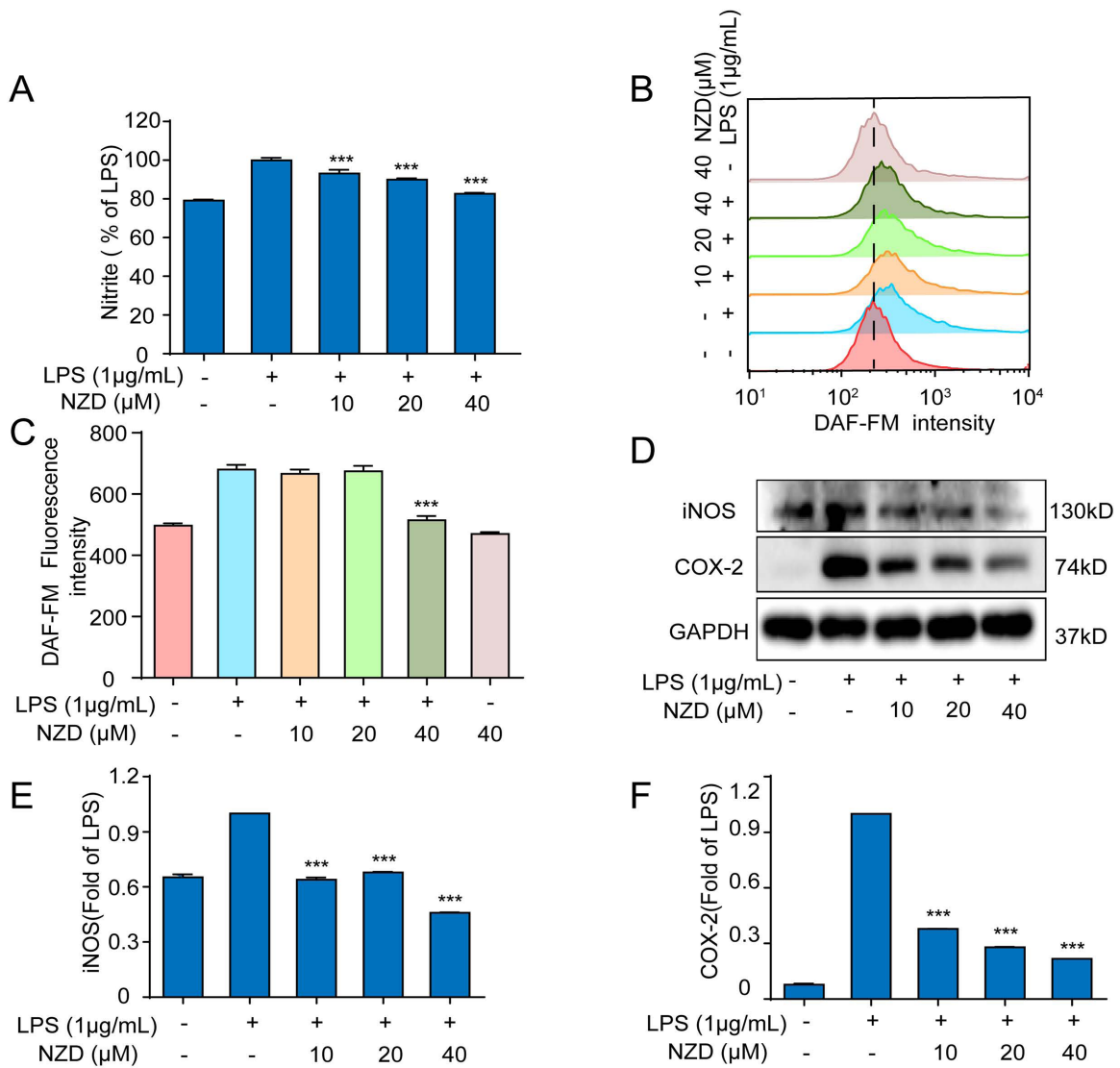
teins, iNOS and COX-2 were significantly increased in the LPS-treated group, and pretreatment with NZD for another 18 h inhibited the expressions of proteins (Fig. 2D-F). Collectively, these results suggested that NZD displayed anti-inflammatory activities in RAW264.7 cells.

### 3.3. NZD Inhibits LPS-induced Release of Pro-Inflammatory Cytokines in RAW264.7 Cells

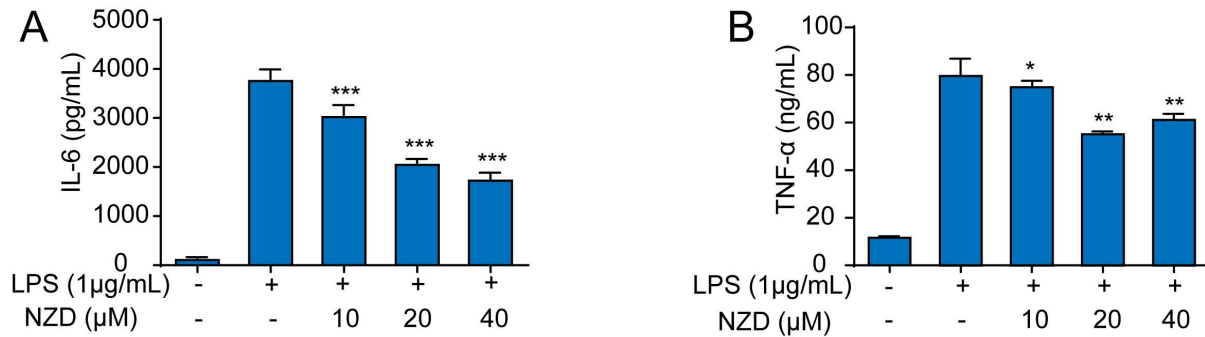
Inflammation is a complicated process, which is regulated by a series of inflammatory mediators and cytokines [23-25]. A great deal of evidence has confirmed that pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, are able to induce inflammatory responses and aggravate the development of inflammation [26-28]. To further investigate the effect of NZD on the release of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells, we detected the secretion levels of IL-6 and TNF- $\alpha$  by ELISA. The results suggested that NZD decreased the levels of TNF- $\alpha$  and IL-6 levels, in LPS-stimulated RAW264.7 cells (Fig. 3A-B). Taken together, the results of this study prove that NZD suppresses LPS-induced release of pro-inflammatory cytokines in RAW264.7 cells.

### 3.4. NZD Reverses LPS-Induced ROS Generation in RAW264.7 Cells

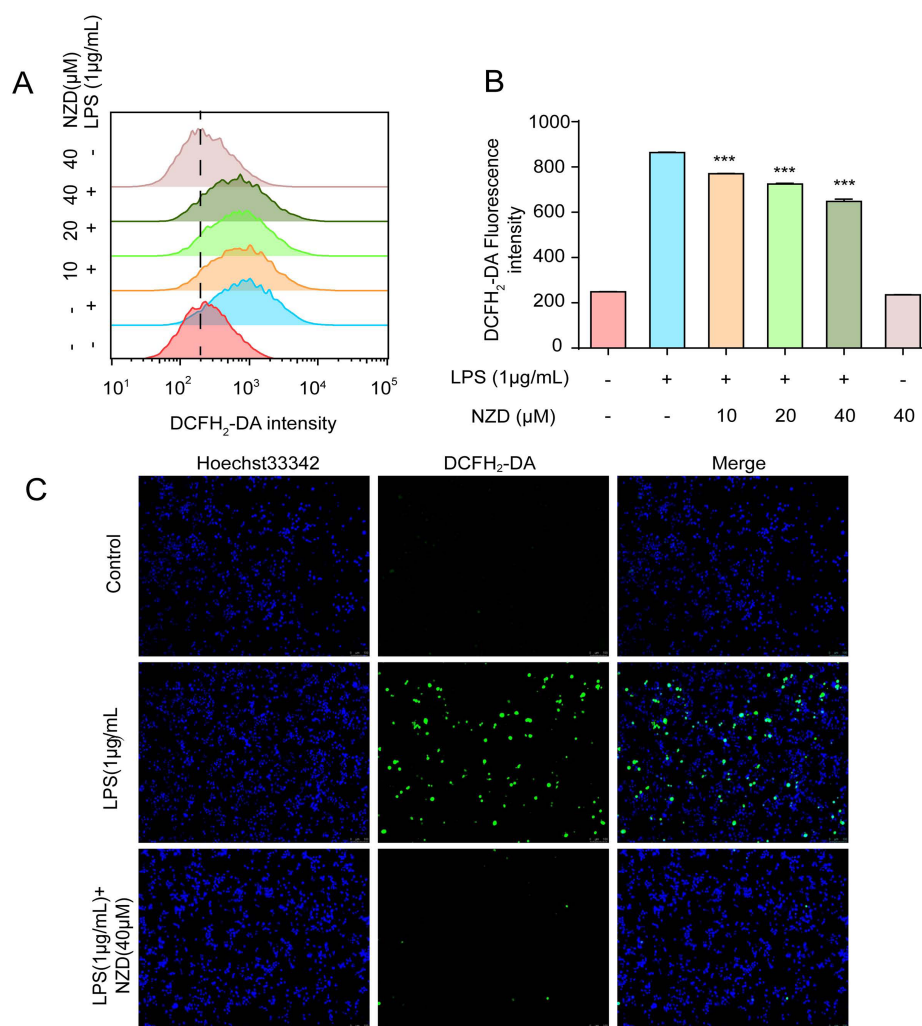
Generally, the term reactive oxygen species (ROS) refers to the oxygen in the body or natural environment, containing the properties of oxygen and active substances [29-31]. A large number of studies have shown that when the cell membranes of phagocytes in the body were stimulated, a deluge of ROS is produced through the respiratory outbreak mechanism [32-34]. Therefore, in an inflammatory response, ROS were greatly increased in cells. We used the flow cytometry to test the effects of NZD on the production of ROS. The results showed that LPS induced ROS production. NZD effectively inhibited LPS-induced ROS generation (Fig. 4A-B). Furthermore, we observed the production of ROS by fluorescence microscopy and the results were consistent with those obtained by flow cytometry (Fig. 4C). Thus, NZD decreased the LPS-induced ROS level in RAW264.7 cells.



**Fig. (2).** NZD suppresses LPS-stimulated inflammatory response in RAW264.7 Cells. RAW264.7 cells were pretreated with NZD for 1 h and then stimulated with LPS (1 µg/mL) for 18 h. (A) The level of nitrite was determined by Griess reagent. (B, C) RAW264.7 cells were pretreated with NZD for 1 h and then stimulated with LPS (1 µg/mL) for 8 h. The level of NO was measured by a flow cytometer with a fluorescence probe DAF-FM. (D, E, F) The expressions of iNOS and COX-2 were detected by Western blotting. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$  vs. LPS group.



**Fig. (3).** NZD inhibits LPS-induced release of pro-inflammatory cytokines in RAW264.7 cells. RAW264.7 cells were pretreated with NZD for 1 h and then stimulated with LPS (1 µg/mL) for 18 h. (A, B) IL-6 and TNF-α, in the culture medium, were determined by ELISA kits. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$  vs. LPS group.



**Fig. (4).** NZD decreases LPS-induced ROS generation in RAW264.7 Cells. RAW264.7 cells were stimulated with LPS (1 μg/mL) for 8 h with or without NZD pretreatment for 1 h. (A, B) The ROS levels were measured by a flow cytometer with a fluorescence probe DCFH<sub>2</sub>-DA. (C) The ROS were detected by fluorescence microscopy. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. LPS group.

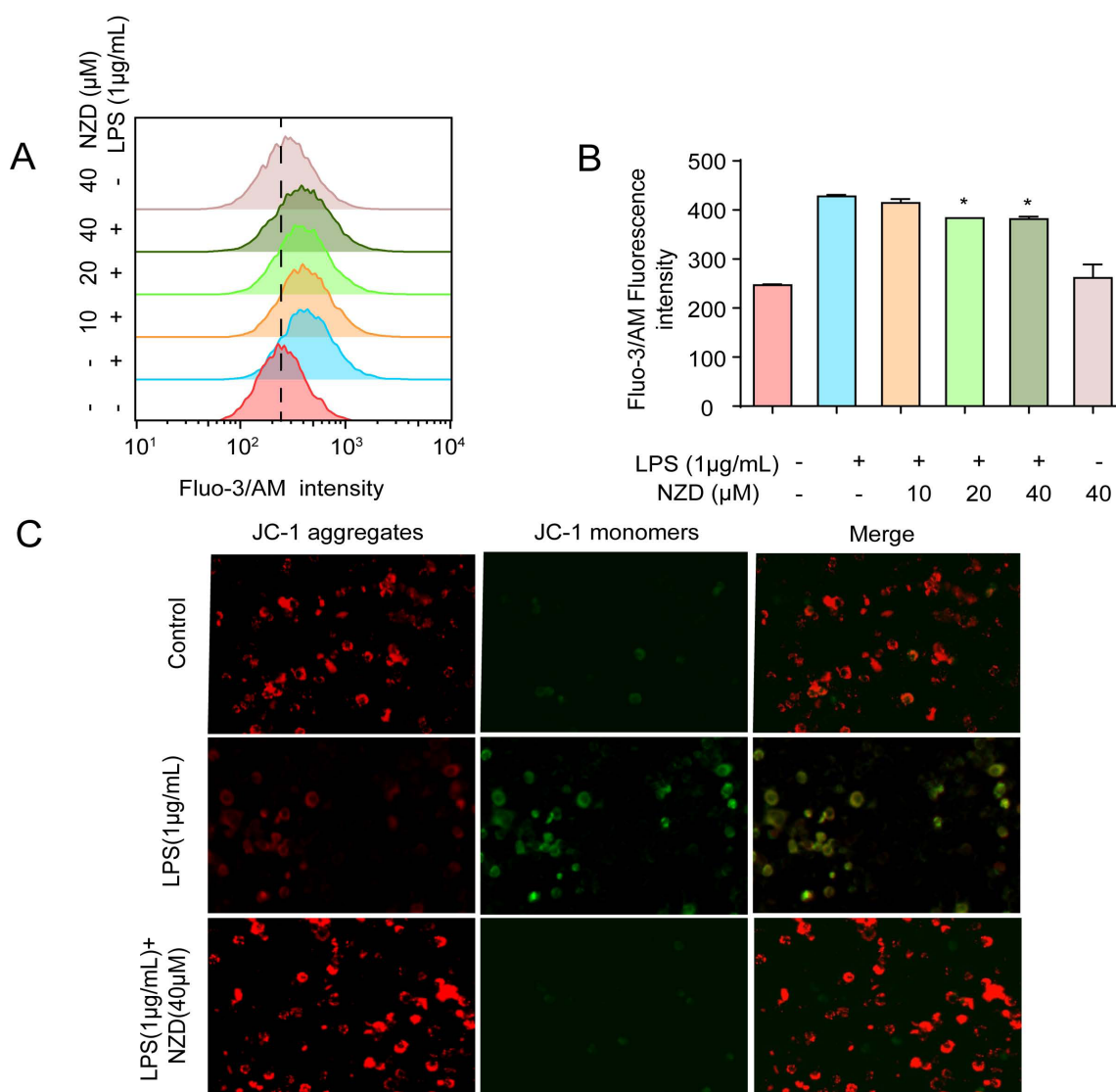
### 3.5. NZD Ameliorates LPS-Induced Calcium Level and MMP Loss in RAW264.7 Cells

The change in mitochondrial membrane potential and calcium concentration is closely related to inflammatory diseases [35, 36]. In this study, flow cytometry was employed to assess the effects of NZD on the level of calcium. The results showed that NZD inhibited LPS-induced calcium influx. (Fig. 5A-B). Mitochondrial membrane potential (MMP) is generated by the asymmetric distribution of protons and other ions on both sides of the inner membrane during respiratory oxidation, the stabilization of which is beneficial to maintain the normal physiological function of cells [37, 38]. LPS could disrupt the stability of MMP, which is not conducive to maintain normal physiological functions of the cell. Using the JC-1 assay and fluorescence microscopy, it was observed that NZD reversed MMP loss in LPS-stimulat-

ed RAW264.7 cells (Fig. 5C). Taken together, NZD reversed MMP loss and inhibited calcium influx.

### 3.6. The NF-κB Pathway Participates in the Anti-Inflammatory Effect of NZD in RAW264.7 Cells

LPS could activate the NF-κB signaling pathway, spawning a pathogen-specific innate immune response through the release of pro-inflammatory cytokines [39, 40]. To further study the mechanisms of the anti-inflammatory effect of NZD, the NF-κB signaling pathway was investigated. As shown in Fig. (6A), LPS led to the translocation of NF-κB/p65 from the cytoplasm into the nucleus, which was reversed by NZD pretreatment. Besides, the results of this study showed that NZD suppressed the phosphorylation expression of IKKα/β, p65, and IκBα without altering the total expression of IKKα/β, p65, and IκBα. (Fig. 6B-E). Thus, the activity of the NF-κB pathway was suppressed by NZD in LPS-stimulated RAW264.7 cells.

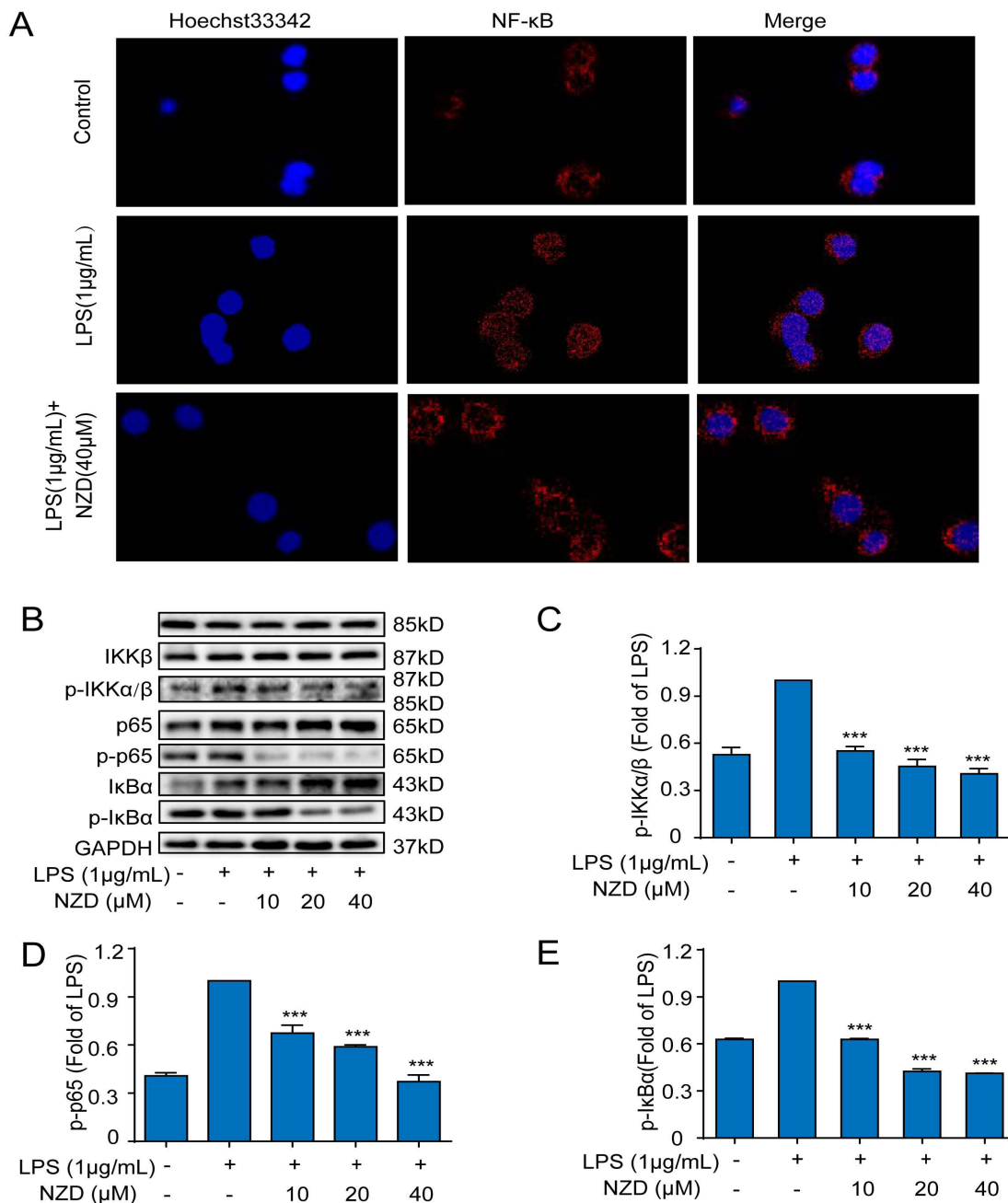


**Fig. (5).** NZD Ameliorates LPS-Induced Calcium Level and MMP Loss in RAW264.7 cells. (A, B) RAW264.7 cells were treated with LPS (1 μg/mL) and the calcium levels were measured by cytometer with a fluorescence probe Fluo-3/AM. (C) RAW264.7 cells were stimulated with LPS (1 μg/mL) for 6 h with or without NZD (40 μM) pretreatment for 1 h. The MMP was detected by fluorescence microscopy. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. LPS group.

#### 4. DISCUSSION

Inflammation is a defensive response of the body to various stimuli. However, long chronic inflammation damages the body. Thus, the homeostasis of inflammation in the body should be maintained [41, 42]. Macrophages, an important effector cells of human innate immune response, can secrete a variety of pro-inflammatory cytokines such as IL-6, IL-12, IL-23, TNF- $\alpha$ , iNOS, and COX-2 [43, 44]. TNF- $\alpha$ , a pro-inflammatory factor, is a marker of early inflammation, mediating immune response and tissue damage [45, 46]. Excessive TNF- $\alpha$  can induce the production of other cytokines, thus aggravating inflammatory damage, and various inflammation-related diseases that represent high levels of TNF- $\alpha$  [47, 48]. IL-6 is both a pro-inflammatory factor and an anti-inflammatory factor [49]. When the concentration of IL-6

in the body is low, it can enhance the immune defense response of the body [50]. When the concentration of IL-6 in the body is high, it will lead to a series of inflammatory reactions, causing certain damage to tissues and organs [27, 51, 52]. Furthermore, IL-6 is one of the most important mediators of various chronic and acute inflammatory reactions [53]. NO is an important inflammatory mediator, which can remove pathogens in the early stage of inflammation and provide protective measures for the body [54]. However, excessive NO reacts with superoxide anion to generate peroxide nitrite, leading to local tissue damage and promoting the occurrence of inflammatory diseases [55]. In this study, it was found that the secretion of NO, TNF- $\alpha$ , and IL-6 was significantly inhibited after the pretreatment of NZD, suggesting that NZD exerts its anti-inflammatory effect *via* inhibiting

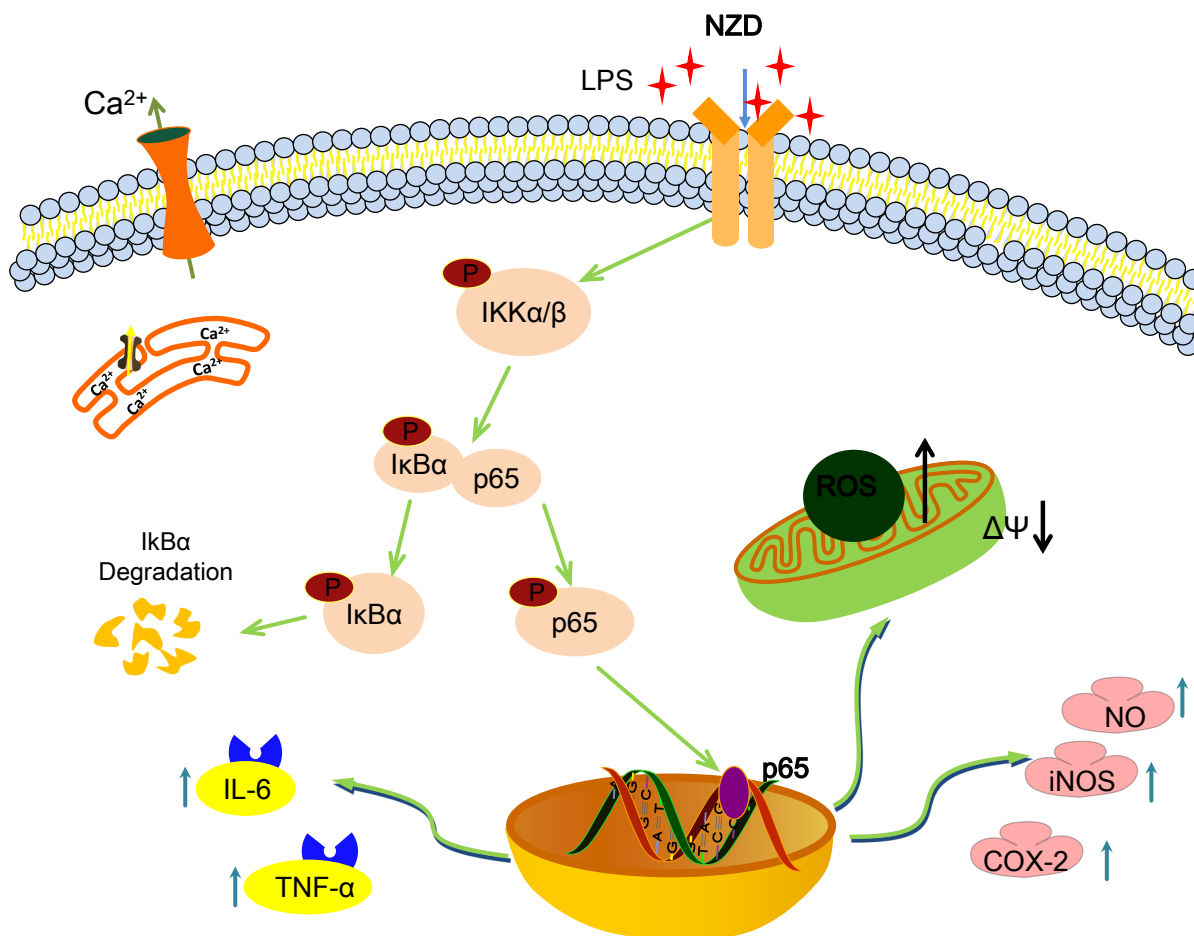


**Fig. (6). The NF-κB pathway participates in the anti-inflammatory effect of NZD in RAW264.7 cells.** RAW264.7 cells were stimulated with LPS (1 μg/mL) for 8 h after NZD pretreatment for 1 h. (A) The localization of p65 in the cytoplasm and nucleus was measured by immunofluorescence staining. The expressions of proteins involved in NF-κB (B) pathway, including p-IKKα/β (C), p-p65 (D), p-IκBα (E) proteins, were measured by Western blotting. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. LPS group.

the release of NO, TNF-α, and IL-6 in RAW264.7 cells. As already known, SARS-CoV-2-induced COVID-19 has outbreak in the world has lead to over one million pneumonia patients and 50,000 deaths, and no effective drugs to treat COVID-19 have been reported until now. Previous studies indicated that a significant inflammatory storm was discovered in COVID-19 patients. More specifically, IL-6 was found to be significantly increased in most COVID-19 patients [56]. Thus, a drug that inhibits IL-6 release may ame-

liorate lung injury induced by SARS-Cov-2. According to the results of the present study, it can be speculated that NZD can not directly kill the virus due to the absence of cytotoxicity. However, NZD can significantly suppress IL-6 release, suggesting that NZD can indirectly effectively treat COVID through anti-inflammatory effects.

COX has three isozymes: COX-1, COX-2, and COX-3 [57]. COX-2, an inducible expression enzyme, is closely



**Fig. (7). The schematic representation of the anti-inflammatory effect and mechanisms of NZD.** NZD showed an inhibitory effect on LPS-induced inflammation in RAW264.7 cells. The underlying mechanism is suppressing the NF-κB pathway.

related to inflammatory diseases [58, 59]. Its expression is related to the severity of inflammation [60]. The synthesis and release of inflammatory mediator COX-2 affected the occurrence of inflammation [61]. In this study, we found that NZD inhibited the expression of COX-2 in LPS-stimulated RAW264.7 cells, suggesting that NZD could be used as an inhibitor of COX-2 and considered for further study.

iNOS is closely related to inflammation, which can catalyze the continuous production of NO by L-arginine [62-64]. Excessive NO leads to cell damage and tissue necrosis, thus promoting the development of inflammatory diseases [65]. In this study, the data indicated that NZD suppresses NO release, nitrite level, and the expression of iNOS protein.

Reactive oxygen species (ROS), a by-product of aerobic metabolism, have strong chemical reactivity and play an important role in cell signaling transduction and body stability [66, 67]. Once stimulated, ROS increase sharply, and then induce inflammation through a series of signal transduction pathways influenced by oxidative stress of cells [68]. Moreover, excessive ROS cause damage to the mitochondria by decreasing MMP [69, 70]. Besides, calcium, as a second signal message, plays an important role in the activation of

macrophages, such as transcriptional control, and the activation of kinases, and phosphatases [71, 72]. The results of this study indicate that NZD recused MMP loss and reversed ROS generation and Calcium influx in LPS-stimulated RAW264.7 cells.

The NF-κB signaling pathway plays a vital role in the pathological process of inflammatory responses [73-75]. NF-κB is a key transcription factor in the inflammatory responses. It exists in the cytoplasm in the form of inactive NF-κB/IκBα complex at rest [76, 77]. Furthermore, the nucleus translocation of NF-κB/p65 into the nucleus can regulate the release of large amounts of inflammatory mediators such as TNF-α, IL-6, etc [78, 79]. Therefore, the changes in NF-κB/p65 can be a marker of inflammation. When cells are stimulated by LPS, IκBα is phosphorylated and degraded, and then NF-κB is translocated into the nucleus to regulate the release of cytokines like NO, ROS, IL-6, and TNF-α, chemokines and inflammatory response [75, 80]. As shown in Fig. (7), the results of this study suggest that NZD can inhibit the translocation of NF-κB/p65 from the cytoplasm into the nucleus and also inhibit the expression of the proteins of p-IKKα/β, p-p65, and p-IκBα proteins, indicating that



NZD exerts anti-inflammatory activity through the NF- $\kappa$ B pathway.

## CONCLUSION

In summary, this study shows that NZD displays anti-inflammatory effects through the NF- $\kappa$ B pathway. Thus, NZD can be considered a potential new drug for the clinical treatment of inflammation.

## LIST OF ABBREVIATIONS

COX-2	= Cyclooxygenase-2
DCFH <sub>2</sub> -DA 2'	= 7'-Dichlorodihydrofluorescein diacetate
DAF-FM 4-ami-no-5-methylamino-2'	= 7'-difluorofluorescein diacetate
NZD	= Nuezhenide
DMEM	= Dulbecco's Modified Eagle Medium
DMSO	= Dimethyl Sulfoxide
ELISA	= Enzyme-linked Immunosorbent Assay
Fluo-3AM	= Fluo-3-pentaacetoxymethyl Ester
FBS	= Fetal Bovine Serum
IL-6	= Interleukin-6
IKK $\alpha$	= Inhibitor of Nuclear Factor Kappa-B Kinase
IKK $\beta$	= Inhibitor of Nuclear Factor kappa-B Kinase
I $\kappa$ B $\alpha$	= Inhibitor of Kappa B $\alpha$
iNOS	= Inducible Nitric Oxide Synthase
LPS	= Lipopolysaccharide
MMP	= Mitochondrial Membrane Potential; NO Nitric Oxide
MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NO	= Nitric Oxide
NF- $\kappa$ B	= Nuclear Factor-kappa B
NO	= Nitric Oxide
PVDF	= Polyvinylidene Fluoride
ROS	= Reactive Oxygen Species
TNF- $\alpha$	= Tumor Necrosis Factor- $\alpha$
SD	= Standard Deviation

## ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

Not applicable.

## FUNDING

We would like to appreciate the support from the National Natural Science Foundation of China (NSFC, 81803807), Guangxi Natural Science Foundation (2018JJB140265), Guangxi Science and Technology Base and Talent Special Project (2018AD19034), the Project of China-ASEAN Traditional Chinese Medicine Development Research Center (05J1601602 and 0501801715), the Project of cultivating High-level Talent Teams in the Qi Huang Project of Guangxi University of Chinese Medicine (2018002), Study the effect and mechanism of YZZ4 405 in the treatment of nephritis based on the influence of P2X7/ROS signaling pathway 406 (YCSZ20190012) and Ph.D. Fund of Guangxi University of Chinese Medicine (B170023), and the project of Guangxi overseas "100 persons plan" high-level expert.

## CONFLICT OF INTEREST

The authors have no conflicts of interest, financial or otherwise.

## ACKNOWLEDGEMENTS

Declared none.

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