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The pistil of nelumbo nucifera has anti-inflammatory effect in LPS-activated Raw 264.7 cells

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ABSTRACT

The pistil of nelumbo nucifera (PNN) is used in the treatment of nocturnal pollution, hematemesis, epistaxis, metrorrhagia and diarrhoea in traditional medicine.

The present study was examined to evaluate the effects of PNN on the production of pro-inflammatory mediators in vitro. After the treatment of PNN, cell viability was measured by MTT assay, nitric oxide (NO) production was monitored by measuring the nitrite content in culture medium. The protein bands were determined by immunoblot analysis and levels of cytokines were analyzed by sandwich immunoassays.

In the MTT assay, the doses of PNN extract (0.03, 0.10 mg/ml) had no significant cytotoxicity. The increases of NO production and inducible nitric oxide synthase expression were detected in lipopolysaccharide(LPS)-activated Raw 264.7 cells compared with control, in contrast, these increases were significantly attenuated by pre-treatment with PNN. In cytokine assay, the massive pro-inflammatory cytokines such as tumour necrosis factor-α, interleukin (IL)-1β and IL-6 were induced in LPS-activated Raw 264.7 cells, but pre-treatment of Raw 264.7 cells with PNN caused inhibition (TNF-α=14.17%, IL-1β=107.43%, IL-6=46.27%) the production of cytokines by LPS. In addition, PNN reduced prostaglandin E2 productions in a dose-dependent manner (0.03mg/ml=37.52%, 0.10 mg/ml=83.77%) as a consequence of the inhibition of

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cyclooxygenase-2 expression.

Taken together, our data indicates that PNN can regulate the inflammatory response in macrophage cells activated by Gram-negative infection.

Key word : nelumbo nucifera, iNOS, COX-2, cytokine

Ⅰ. Introduction

The pistil of nelumbo nucifera(PNN) is used in the treatment of nocturnal pollution, hematemesis, epistaxis, metrorrhagia and diarrhoea in East Asian traditional medicine. Recently, it has been shown that the biological properties of nelumbo nucifera, which include antidiarrheic[1], hypolipemic[2], antipyretic[3] and antioxidant effects[4]. However, the effect of PNN on the inflammatory gene expression and cytokine production has not been determined.

The pathology of inflammation includes the initiation of complex reactions stimulated by the microbial pathogens. Gram-negative bacteria are the most common pathogen. Lipopolysaccharide (LPS)-activated macrophages have usually been used for evaluating the anti-inflammatory effects about various materials. LPS is a typical endotoxin, which is derived from gram-negative bacteria membrane and leads to releasing of cytokines[5]. LPS can directly activate the macrophages, endothelial cells and complement initiating production of inflammatory mediators such as nitric oxide(NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukins (ILs) and leukotriens[6,7].

Inflammatory responses are advantageous for eliminating bacteria, in the responses are under control. However, when out of the control, deregulated inflammatory responses induce the massive production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 by macrophage[8,9], which can lead to tissue injury and multiple organ failure[10].

The present study was designed to determine the effect of PNN on LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase -2 (COX-2) gene expression and to clear up its mechanism of action in RAW264.7 macrophages. The effects of PNN on the secretion of LPS-induced pro-inflammtory cytokines was assessed by ELISA, and iNOS and COX-2 expression were monitored by immunoblot analysis. As a result, we found that PNN significantly inhibited production of NO and pro-inflammatory cytokines, in parallel with the suppression of iNOS and COX-2.

Ⅱ. Materials and Methods

1. Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol

eum and LPS (Escherichia coli 026:B6) were obtained from Sigma (St. Louis, MO, USA). The fetal bovine serum (FBS) and antibiotics were purchased from Gibco/BRL (Eggenstein, Germany). The antibodies of iNOS, COX-2 and actin were obtained from BD Bioscience (San Jose, CA, USA), Cayman (Ann Arbor, Mi, USA) and Santa cruz (Santacruz, CA, USA), respectively. The NC paper was obtained from Schleicher & Schuell (Dassel, Germany). The TNF-α, IL-6 and IL-1β ELISA kits were purchased from Pierce endogen (Rockford, IL, USA). The PGE2 assay kit was obtained from R&D Systems (Minneapolis, MN, USA).

2. Preparation of Methanol Extract of PNN

PNN was pick in Niji (meaning mud pond) of Gyeongsan city at Aug. 2008. PNN (300 g) was prepared by extracting with 1000 ml of methanol (MeOH) at room temperature for 72 h. The extract was filtered through a 0.2 μm filter (Nalgene, New York, NY, USA). The solvent filtrates were condensed using rotary evaporator (EYELA, Tokyo, Japan), put into the freeze dry machine (Nihon freezer, Japan), and dried in freeze-dryer (Labconco, USA) by lyophilization.

The amount of PNN MeOH extract was estimated by the dried weight of lyophilized MeOH extract of PNN. The yield of lyophilized PNN MeOH extract was 2.65%. The lyophilized extract was stored at -20℃ until needed.

3. Cell Culture

Raw264.7 cell, which is a murine macrophage cell line, was obtained from Korean Cell Line Research Foundation (KCLRF, Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Gibco, Germany) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. For all experiments, the cells were grown to 80~90% confluence, and were subjected to no more than 20 cell passages. The cells were maintained at 37℃ in a humidified atmosphere containing 5% CO_2 incubator (Sanyo, Japan). The Raw264.7 cells were plated at a density of $2\sim3\times10^6$/ml and pre-incubated at 37℃ for 24 h. After serum starvation for 24 h, the cells were exposed to either LPS (1 μg/ml) or LPS+PNN for 24 h. PNN was dissolved in DMSO + DMEM and added to the incubation medium 1 h prior to adding the LPS.

4. Assay of NO Production

The level of NO production was monitored by measuring the nitrite concentration in the cultured medium. Briefly, the samples were mixed with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid) and incubated for 10 min at room temperature in dark. The absorbance was measured at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA).

5. Cell Viability

The Raw264.7 cells were plated at a density of 1×10^4cells/well in a 24 well plate to determine the cytotoxic concentrations of PNN. Cells were serum-starved for 24 h, and then treated with LPS (1 μg/ml) or LPS+PNN for the next 24 h. After incubation of the cells, viable cells were

stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml, 4 h). The media were then removed, and produced formazan crystals in the wells were dissolved by addition of 200 µl dimethylsulphoxide. Absorbance was measured at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA). Cell viability was defined relative to untreated control cells (that is viability; Fold Increase = absorbance of treated sample /absorbance of control).

6. Immunoblot Analysis

The obtained cells were washed two times with ice PBS and were lysed in the buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 µg/ml leupeptin. The total cell lysate was prepared by centrifuging the cells at 10,000×g for 10 min and collecting the supernatant. The expression of iNOS and COX-2 was immunochemically monitored with the total lysate fraction using anti-rabbit iNOS and COX-2 antibodies, respectively. The secondary antibody was horseradish peroxidase -conjugated anti-rabbit antibody. The bands for the iNOS and COX-2 proteins were visualized using ECL western blotting detection reagents (Amersham Biosciences, New Jersey, USA) according to the manufacturer's instructions. Equal loading of proteins was verified by actin protein immunoblotting.

7. Measurement of Cytokines Production

For the cytokine immunoassays, the cells (1×10^4/ml) were pre-incubated with PNN for 1 h and further cultured for 24 h with 1 µg/ml of LPS in 24-well plates. The supernatants were removed at the allotted times and the levels of TNF-α, IL-6 and IL-1β production were measured using an ELISA Kit (Pierce endogen, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, 50 µl of biotinylated antibody reagent and the samples were added to the anti-mouse TNF-α, IL-6 and IL-1β pre-coated 96-well strip plates. The plates were covered and kept at room temperature for 2 h and washed three times in a prepared washing buffer. This was followed by the addition of 100 µl of Streptavidin-HRP Concentrate. After 30 min incubation at room temperature, the wells were washed three times, and 100 µl of TMB Substrate Solution was then added and developed in the dark at room temperature for 30 min. The reaction was quenched by adding 100 µl of TMB Stop Solution, and the absorbance of the plates was measured at 450 nm to 550 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-6 and IL-1β in serial dilutions. The level of TNF-α, IL-6 and IL-1β was quantified from standard curve.

8. Measurement of PGE_2 Production

PNN was treated into culture medium 1 h before the addition of 1 µg/ml LPS. LPS-treated cells were further cultured with vehicle or PNN for 24 h. The cultured medium was collected and assayed with ELISA kit (RnD Systems, Minneapolis, MN, USA). Cultured medium was incubated in goat anti-mouse IgG coated plate with acetylcholinesterase linked to PGE_2 and PGE_2 monoclonal antibody for 2 h at room temperature. The plate was emptied and rinsed four times with washing buffer contained

in the kit. And then, 200 μl of substrate reagent was added to each well and incubated for 30 min at room temperature. The developed plate was read at 450 nm and the PGE_2 concentration of each sample was determined according to the standard curve.

9. Scanning Densitometry

Scanning Densitometry of the immunoblots of protein bands was performed with a UVP Epi Chemi BioImaging System (UVP, Inc., CA, USA).

10. Statistical Analysis

The data were expressed as a mean ± S.D. of the results obtained from a number of experiments. One-way analysis of variance (ANOVA) was used to assess the significant differences between the treatment groups. For each significant effect of treatment, the Tukey test was used to compare the multiple group means. A P value <0.05 was considered significant.

III. Results

1. Inhibition of LPS-inducible NO production by PNN

First, we determined the effect of PNN on the production of NO, a hallmarker of macrophage activation. NO is a gaseous molecule synthesized from L-arginine the presence of NOS enzyme and is involved in inflammation, immune function, bone metabolism, and apoptosis[11]. As expected, treatment of LPS significantly induced NO level in culture medium(Fig. 1). However, pretreatment of PNN (0.1 mg/ml) was markedly abrogated the ability of LPS to produce NO.

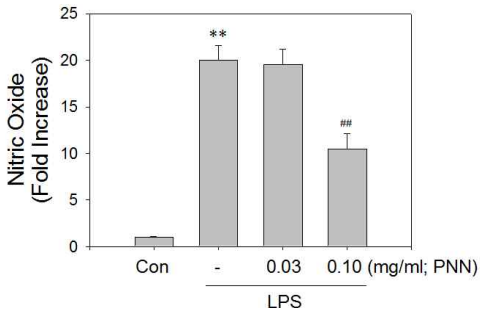

Fig. 1. Effects of PNN on the production of NO in LPS stimulated RAW 264.7 cells.

RAW 264.7 cells were treated with 0.03 and 0.10 mg/ml concentrations of PNN dissolved in DMEM for 1h prior to the addition of LPS (1μg/ml), and the cells were further incubated for 24 h. Control cells were incubated with vehicle alone. The concentrations of nitrite and nitrate in culture medium were monitored as described in the materials and methods. Data represent the mean ± S.D. with eight separate experiments. One-way ANOVA was used to compare the multiple group means followed by tukey test. (significant as compared to control, $^{**}P<0.01$, significant as compared to LPS alone, $\#P<0.05$, $\#\#P<0.01$).

2. Effect of PNN on cell viability

Next, we confirmed the effect of PNN on cell viability. LPS treatment for 24 h slightly, but significantly, decreased cell viability in RAW264.7 cells (Fig. 2A). However, PNN+LPS did not further increase cell death. Considering the effect of LPS and/or PNN on cell viability, we verified the PNN inhibition of NO production. The increase in ratio of NO/cell viability by LPS was significantly decreased by treatment of PNN(Fig. 2B).

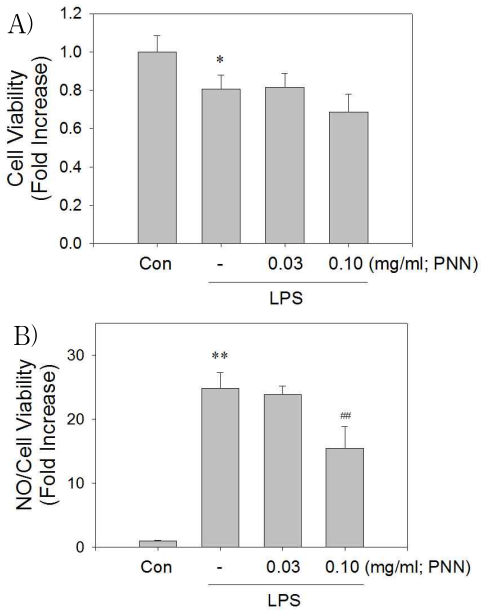

Fig. 2. Effect of PNN on the cell viability in LPS stimulated RAW 264.7 cells.

RAW 264.7 cells were treated with 0.03 and 0.10 mg/ml concentrations of PNN dissolved in DMEM for 1h prior to the addition of LPS (1μg/ml), and the cells were further incubated for 24h. Control cells were incubated with vehicle alone. Panel A is cell viability and B is nitric oxide per cell viability. Each bar shows the mean ± S.D. of three independent experiments performed in triplicate. One-way ANOVA was used to compare the multiple group means followed by tukey test. (significant as compared to control, *P<0.05, **P<0.01, significant as compared to LPS alone, ##P<0.01).

3. Inhibition of LPS-inducible cytokine production by PNN

As PNN inhibited expression of LPS-inducible NO production, we analyzed the effects of PNN on representative pro-inflammatory cytokine, TNF-α. TNF-α is recognized to be a important mediator in the development of endotoxicity or septic shock. TNF-α is considered to be a major mediator in the systemic inflammatory response syndrome observed during gram-negative sepsis[12]. LPS treatment induced TNF-α production, which was abrogated by 0.1 mg/ml of PNN(Fig. 3A).

IL-1 has pro-inflammatory effects, by increasing synthesis of potent mediators and by up-regulation of the expression of adhesion molecules in immune cells. IL-1β is co-stimulator of activation of T-cells, maturation of B-cells, activity of natural killer cells, and expression of adhesion molecules[13]. As expected, LPS increased IL-1β level in medium. But, 0.03 and 0.1 treatment of PNN almost inhibited the ability of LPS to produce IL-1β(Fig. 3B).

IL-6 is one of a family of cytokines that act via the gp130 receptor, and is an important cytokine in the regulation of inflammation and immunity. IL-6 stimulates activation and proliferation of lymphocyte. IL-6, secreted primarily by monocytes and macrophages cytokine, is always found in increased levels at the inflammatory region[14,15]. So, we measured the effect of PNN on LPS-inducible IL-6 production.

As shown in figure 5, the production of the IL-6 at the indicate time were dramatically increased by LPS stimulation, which was decreased by PNN treatment(Fig. 3C).

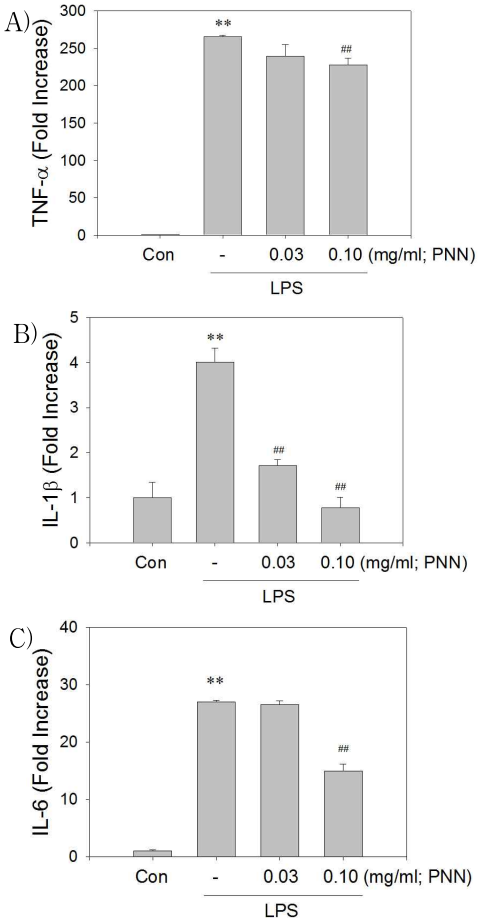

Fig. 3. The effect of PNN on LPS-stimulated Cytokine production.

Production of cytokines (A; TNF-α, B; IL-1β, IL-6 were measured in the medium of RAW 264.7 cells cultured with LPS (1μg/ml) in the presence or absence of PNN for 24 h. The amount of cytokines were measured by immunoassay as described in materials and methods. Data represent the mean ± S.D. with three separate experiments. One-way ANOVA was used to compare the multiple group means followed by tukey test. (significant as compared to control, **P<0.01, significant as compared to LPS alone, ##P<0.01).

4. Inhibition of LPS-inducible PGE_2 production by PNN

To assess whether PNN could inhibit LPS-induced PGE2 production in the RAW264.7 cells, we assayed PGE2 one of the crucial inflammatory mediator in the culture media of cells pretreated with PNN and subsequently treated with LPS for 24 h.

LPS stimulated PGE2 production in macrophage. But PNN pretreatment (0.03 and 0.10 mg/ml) significantly decreased PGE2 production in LPS-added cells(Fig. 4).

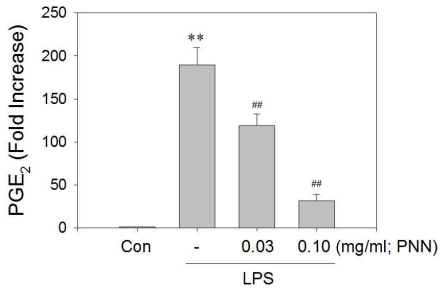

Fig. 4. Inhibition of LPS-activated PGE_2 production by PNN.

RAW264.7 cells were cultured with LPS (1 ug/ml) in the presence or absence of PNN for 24 h to determine the level of PGE_2. The cultured medium was collected and directly assayed for PGE_2 as described in materials and methods. The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means followed by tukey test. (significant compared with the control, **P<0.01, significant compared with the LPS alone, ##P<0.01).

5. Inhibition of LPS-inducible iNOS and COX-2 expression by PNN

In order to examine the inhibitory mechanism of PNN on cytokine production, we next determined the expression of iNOS and COX-2 protein. As expected, treatment of LPS for 24 h significantly increased iNOS and COX-2 protein level(Fig. 5A). However, PNN (0.1 mg/ml) inhibited the ability of LPS to induce iNOS and COX-2 protein level(Fig. 5B and C).

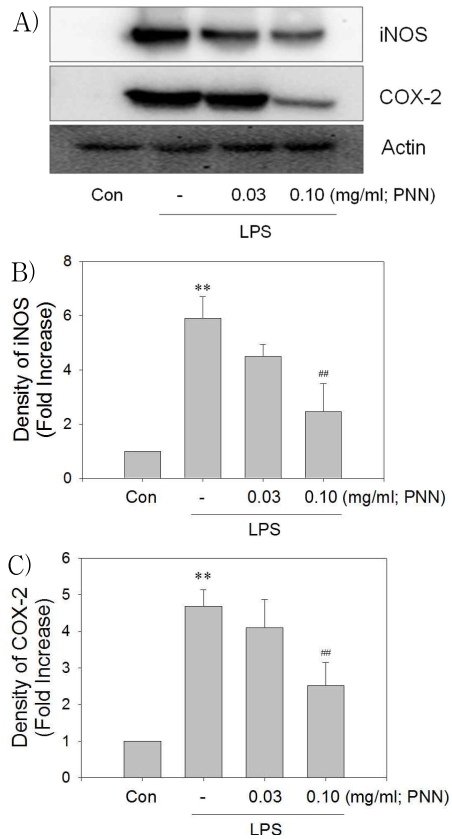

Fig. 5. Effect of PNN on the expression of iNOS and COX-2 protein.

RAW 264.7 cells were treated with 0.03 and 0.10 mg/ml concentrations of PNN dissolved in DMEM for 1h prior to the addition of LPS (1μg/ml), and the cells were further incubated for 18h. Control cells were incubated with vehicle alone. Protein bands were monitored 18 h after treatment with LPS with or without PNN (A). The relative protein levels were measured by scanning densitometry as described in the materials and methods (B and C). The data represent the mean ± SD of three separate experiments. One-way ANOVA was used to compare the multiple group means followed by tukey test. (significant compared with the control, **P<0.01, significant compared with the LPS alone, ##P<0.01).

Ⅳ. Discussion

The study was designed to determine the effect of PNN on LPS-induced iNOS and COX-2 gene expression and to clear up its mechanism of action in RAW264.7 macrophages. The pistil of nelumbo nucifera (PNN) is frequently used for the treatment of nocturnal pollution, hematemesis, epistaxis, metrorrhagia and diarrhoea in traditional medicine.

Sepsis is one of the most common factors of death in units of patients demanding intensive care. In every year, some 215,000 mortalities are attributed to sepsis in the United States[16]. Although lots of new antibiotics and anti-inflammatory drugs have developed, death rate has remained unbelievably high (i.e. 30 % ~ 50 %) in patients with severe sepsis[17,18]. The pathology of sepsis is triggered by complex progress stimulated by the microbial pathogens. LPS can directly activate the macrophages, endothelial cells and complement triggering production of inflammatory mediators such as NO, PGE2, TNF-α and ILs[6,7,19].

NO is produced from L-arginine by NO synthase (NOS) and plays both beneficial and detrimental roles. It is known that there are three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS)[20]. nNOS and eNOS are mainly expressed in neurons and endothelial cells, separately. They produce little NO in a pulsative manner. On the other hand, iNOS produces a lot of NO induced by cytokines in various tissues[21]. Although physiological production of NO has profitable effects, excessive NO produced by iNOS is considered an essential mediator of inflammatory diseases and causes cell injury[21,22]. In this study, we confirmed PNN strong inhibition of LPS-inducible iNOS expression.

PGE2 have a line of function in the body such as contraction and relaxation of smooth muscle

and blood vessels, and modulation of inflammation[20,23]. This is released by blood vessel in case of infection or inflammation inducing fever. Therfore, PGE2 is another inflammatory mediator in the process of septic shock. COX-2 is main modulator of PGE2 production in the inflammatory process. In this study, PNN significantly inhibited LPS-inducible COX-2 expression as well as iNOS, indicating PNN has significant anti-inflammatory effects.

The pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are small secreted proteins which mediate inflammation. TNF-α is a toxic cytokine involved in inflammatory and other pathological processes such as rheumatoid arthritis[23]. At the high concentration of LPS, TNF-α is thought to be a principal mediator in LPS-inducible tissue injury and shock. TNF-α promoter-reporter gene assay demonstrated that activation of NF-κB largely contributes to the induction of TNF-α[24]. IL-1β is a crucial inflammatory cytokine, which is found in the circulation following gram negative sepsis, and a mediator of the host inflammatory response in innate immunity[25]. IL-6 is another inflammatory cytokine mainly synthesized by macrophages, and plays a key role in the acute phase response having various clinical and biological features such as the production of acute inflammatory phase proteins[26]. Here, we demonstrated that PNN significantly inhibited LPS-induced TNF, IL-1β and IL-6 secretions in RAW 264.7 cells at the indicated time.

In summary, PNN inhibits LPS-inducible NO and PGE2 production, iNOS and COX-2 gene expression, and pro-inflammatory cytokine secretion including TNF-α, IL-1β and IL-6 in RAW 264.7 macrophages. The observation that PNN has anti-inflammatory effects *in vitro* model provides a possible therapeutic approach to the treatment of severe inflammatory diseases.

Ⅴ. Conclusion

Based on the above results, we determined that PNN can inhibit the production of nitrite and nitrate, TNF-α, IL-6, IL-1β and the PGE2 in Raw 264.7 cells activated by LPS. In addition to, PNN significantly suppressed iNOS and COX-2 expression induced by LPS stimulation. These results demonstrated that PNN could be an attractive candidate for the treatment of inflammation-related disease by reducing pro-inflammatory mediators.

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