Anti-inflammatory effect of polydeoxyribonucleotide on zoledronic acid-pretreated and lipopolysaccharide-stimulated RAW 264.7 cells

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Abstract. Bisphosphonates are generally used as therapeutic agents for bone diseases. However, previous reports on bisphosphonates-related osteonecrosis of the jaw (BRONJ) demonstrated that inflammation triggers and worsens the disease. Recently, polydeoxynucleotide (PDRN), an A_{2A} receptor agonist, has been suggested for the treatment of various diseases and broadly studied for its anti-inflammatory effect. The present study aimed to measure the effect of PDRN on macrophage cells treated with zoledronic acid (ZA) and lipopolysaccharide (LPS). Macrophage cells were cultured with ZA for 24 h, following which they were stimulated with LPS in the presence or absence of varying concentrations of PDRN for 24 h. The cell viability and nitric oxide (NO) production of the cells were analyzed. In addition, protein expression levels were quantified by western blotting. Cell viability was compromised and NO was overexpressed by ZA and LPS stimulation. However, under ZA and LPS stimulation cell viability was enhanced, and NO production, and inducible nitric oxide synthase, interleukin (IL)-1β, -6, and tumor necrosis factor-α overexpression were suppressed on exposure to PDRN. A_{2A} receptor and vascular endothelial growth factor (VEGF) expression levels increased following PDRN treatment. These results indicate that PDRN treatment of macrophages inhibits the inflammatory cytokines induced by ZA and LPS stimulation. It was hypothesized that the inflammatory cytokines were inhibited through A_{2A} activation by PDRN. In addition, increased VEGF expression may contribute to increased vascularization and subsequently improve the pathological condition in BRONJ. As inflammation and LPS may stimulate the occurrence of BRONJ, the present study postulated that PDRN is possibly a candidate for the therapeutic management of BRONJ by decreasing inflammation and increasing vascularization.

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Abbreviations: BRONJ, bisphosphonate-related osteonecrosis of the jaw; PDRN, polydeoxynucleotide

Key words: bisphosphonate-related osteonecrosis of the jaw, zoledronic acid, lipopolysaccharide, polydeoxynucleotide, adenosine A2 receptor

Introduction

Zoledronic acid (ZA) is a nitrogen-containing bisphosphonate, administered intravenously to treat several bone diseases, including osteoporosis, hypercalcemia, Paget's disease, bone metastasis of a tumor and myeloma (1-3). ZA is selectively accumulated in bone tissue due to its high affinity to bone (4). ZA induces osteoclast apoptosis and consequently inhibits bone resorption as well as bone remodeling (5,6). One of the side effects of bisphosphonates is bisphosphonate-related osteonecrosis of the jaw (BRONJ), first noted in 2003 and now recognized as a well-known complication (7,8). It is occasionally necessary to treat BRONJ, which is associated with the consequent suffering of patients, by performing resective surgery (9). Although the mechanism of action of bisphosphonates in BRONJ remains to be fully understood, the inflammatory process from a bacterial infection and trauma are suspected to cause and intensify the severity of the affliction (10,11).

Lipopolysaccharide (LPS), a component from the cell walls of Gram-negative bacteria, induces immune responses of macrophages, thereby increasing the production of nitric oxide (NO) as well as various cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-6 (12,13). NO regulates various pathophysiological processes and its overproduction damages the surrounding tissue resulting in increased inflammation (14). A previous study revealed that the induction of proinflammatory cytokines by LPS is enhanced by pretreatment of ZA and functions due to the reduction of suppressor of cytokine signaling-1 (15).

Polydeoxyribonucleotide (PDRN), consisting of a mixture of deoxyribonucleotide polymers, is easily extracted from the sperm of trout and highly purified by a classified extractive process (16). Following the cleavage of PDRN by active cell membrane enzymes, it provides a source for purine and pyrimidine deoxynucleosides and deoxyribonucleotides (17). Adenosine, a purine nucleoside, activates adenosine A_{2A} receptor and the stimulation of the receptor is suspected to decrease inflammatory cytokine secretion (16,18,19). Previous studies have demonstrated that PDRN significantly reduces the clinical signs of arthritis in mice, by decreasing the circulating levels of several inflammatory cytokines (20) and increasing vascular endothelial growth factor (VEGF) production, indicating decreased ischemic tissue damage and an enhanced oxygen supply (21). Ramanathan *et al* (22) also reported that the adenosine A_{2A} receptor and LPS increased VEGF expression through transcriptional regulation of the VEGF promoter. VEGF acts as a main regulatory factor for endothelial cell proliferation and increases neovascularization (23). Therefore, VEGF may contribute to enhanced vascularization in BRONJ, which was compromised by increased endothelial cell apoptosis (24). Subsequently, the pathological condition in BRONJ can be improved through increased vascularization with VEGF. Thus, the present study aimed to evaluate whether PDRN regulates the expression of inflammatory cytokines in macrophages pretreated with ZA and LPS.

Materials and methods

Cell culture. The macrophage cell line, RAW 264.7, was purchased from the Korea Cell Line Bank (Korean Cell Line Research Foundation, Seoul, Korea). The cells were maintained in high glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin (Lonza Group, Ltd., Basel, Switzerland) and 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a 37˚C humidified incubator with 5% CO_2 for all experiments. At the occurrence of BRONJ, which may be caused by dental procedures or intraoral trauma, bisphosphonates accumulate in bone tissue, subsequently, a bacterial infection occurs and causes an increase in LPS. In the present study, in order to mimic the aforementioned situation, ZA was incubated with RAW 264.7 cells, which were then exposed to LPS. Control cells were maintained without ZA and LPS.

Cell viability analysis. Cytotoxicity was assessed by the MTT assay. RAW 264.7 cells were seeded in 96-well plates at a density of $1x10^5$ cells/well containing 200 μl high glucose DMEM supplemented by 100 μg/ml streptomycin, 100 U/ml penicillin and 10% heat-inactivated fetal bovine serum, and incubated with 1, 10 or 100 μM ZA (Novartis International AG, Basel, Switzerland) for 24 h. The cells were then exposed to 0.01, 0.1 or 1 μg/ml LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and various concentrations of PDRN (1, 10 or 100 μg/ml; PharmaResearch Products Co., Ltd., Seongnam, Republic of Korea) in serum free medium at 37˚C for 24 h. MTT solution (Sigma-Aldrich; Merck KGaA) was added to a final concentration of 0.5 mg/ml and the plates were incubated at 37˚C for 1 h in a humidified incubator. The medium was then removed, the formazan precipitate was solubilized in dimethylsulphoxide and the solution was measured at 570 nm by a microplate reader.

Measurement of NO production. NO production was measured using Nitric oxide (NO) Detection kit (cat. no. 21021; Intron Biotechnology, Sungnam, Republic of Korea). RAW 264.7 cells were plated at a density of $1x10^5$ cells/well in 96-well culture plates and incubated with 10 μM ZA for 24 h at 37˚C, followed by exposure to 0.1 μg/ml LPS and various concentrations of PDRN (1, 10 or 100 μg/ml) in serum free medium at 37˚C for 24 h. Nitrite levels in the culture medium were measured for NO estimation. The supernatant collected from the culture plate (100 μl) and N1 buffer (50 μl) were added to a new 96-well plate in triplicate. The plate was maintained at room temperature for 10 min, and N2 buffer (50 μl) was subsequently added. The absorbance of the content of each well was measured at a wavelength of 540 nm with a microplate reader. The nitrite concentration was calculated using a nitrite standard curve, according to the manufacturer's protocol.

Western blot analysis of inflammatory factors in whole cell lysate. Cell proteins of RAW 264.7 cells were collected. Briefly, cells were plated at a density of $2x10^5$ cells/dish in a 100 mm culture dish and incubated with 10 μM ZA for 24 h, followed by incubation with 0.1 μg/ml LPS and various concentrations of PDRN (1, 10 or 100 μg/ml) in serum free medium at 37˚C for 24 h. Cells were harvested and gently homogenized in radioimmunoprecipitation assay buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin (Cell Signaling Technology, Inc., Danvers, MA, USA) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Subsequently, the lysates were incubated for 20 min at 4˚C, centrifuged at 16,000 x g for 20 min at 4˚C, following which they were rapidly frozen. The lysates were measured for protein quantification using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 30 μg/lane protein was subjected to western blot analysis using 12% SDS-PAGE gel and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were incubated in a blocking buffer containing 5% skim milk for 1 h at room temperature. They were subsequently incubated overnight at 4˚C with the following primary antibodies: Mouse β-actin, inducible NO synthase (iNOS), TNF-α and VEGF (cat. nos. SC-4778, SC-7271, SC-52746 and SC-7269, respectively), rabbit A_{2A} (cat. no. 13937; all 1:1,000; Santa Cruz Biotechnology Inc., Dallas, TX, USA), IL-1β and IL-6 (cat. nos. bs-6319r and bs-0782r, respectively; both 1:1,000; Bioss Antibodies, Inc., Woburn, MA, USA). The membranes were then incubated at room temperature for 1 h with anti-mouse and anti-rabbit secondary horseradish peroxidase-conjugated antibodies (cat. no. PI-2000 and PI-1000, respectively; both 1:1,000; Vector Laboratories, Inc., Burlingame, CA, USA). Bands were detected using an enhanced chemiluminescence detection kit (Bio-Rad Laboratories, Inc.). Densitrometric analysis of the resultant bands was performed using Molecular Analyst™ software, version 1.4.1 (Bio-Rad Laboratories, Inc.).

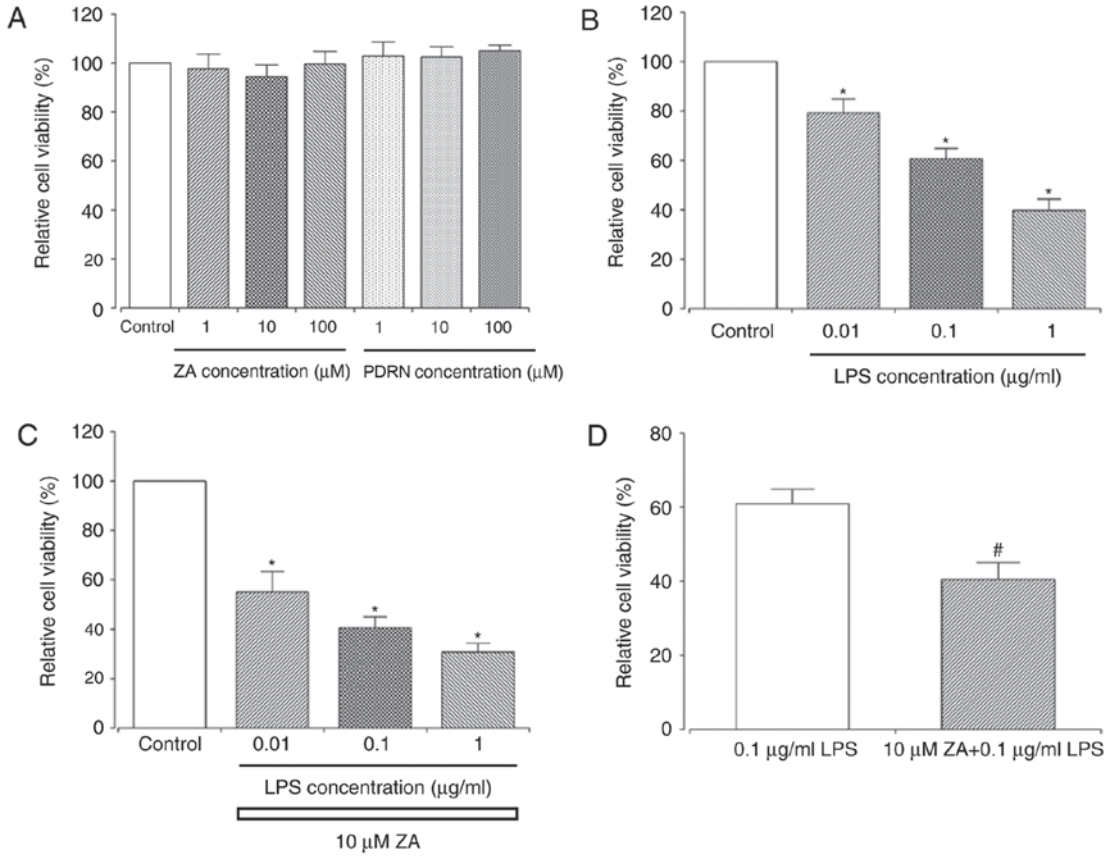

Figure 1. Cell viability is decreased by LPS and ZA+LPS stimulation. Relative cell viabilities were compared with the control group, which was set to 100%. Cell viability was measured in RAW 264.7 cells cultured with (A) ZA for 48 h, (B) LPS for 24 h and (C) ZA for 24 h, followed by LPS for 24 h. (D) Cell viability was measured in RAW 264.7 cells pretreated with 10 μM ZA and stimulated 0.1 μg/ml LPS compared with 0.1 μg/ml LPS-stimulated cells. The experiments were conducted in triplicate. Data are presented as the mean + standard error of the mean. *P<0.05 compared with the control group. $^{\#}$P<0.05 compared with the LPS group. ZA, zoledronic acid; LPS, lipopolysaccharide.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by Duncan's post hoc test using SPSS software (version 23.0; IBM Corp., Armonk, NY, USA). Data are expressed as the mean + standard error of the mean. P<0.05 indicated that the difference between groups was statistically significant.

Results

ZA+LPS decreases and PDRN increases cell viability. Single treatment with either ZA or PDRN had no significant effect on the viability of RAW 264.7 cells at any concentration (1, 10 or 100 μM ZA; 1, 10 or 100 μg/ml PDRN; Fig. 1A). However, there was a significant decrease in cell viability when cells were exposed to LPS alone (0.01, 0.1 or 1 μg/ml; Fig. 1B) or ZA+LPS (10 μM ZA + 0.01, 0.1 or 1 μg/ml LPS; Fig. 1C) compared with control cells. Treatment with ZA+LPS (10 μM + 0.1 μg/ml) significantly decreased the cell viability by more than 20% compared with the cells treated with LPS alone (0.1 μg/ml; Fig. 1D). Treatment with 1, 10 or 100 μg/ml PDRN significantly increased cell viability of the ZA+LPS-stimulated RAW 264.7 cells compared with ZA+LPS-stimulated cells (Fig. 2). However, the cell viabilities in ZA+LPS-stimulated cells with or without PDRN treatment were significantly low compared with the control group.

PDRN decreases NO production. ZA+LPS (10 μM + 0.1 μg/ml, respectively)-stimulated RAW 264.7 cells demonstrated significantly enhanced NO production compared with the control group (Fig. 3). NO production significantly decreased in what appeared to be in a dose-dependent manner following 10 and 100 μg/ml of PDRN treatment compared with ZA+LPS-stimulated RAW 264.7 cells.

PDRN decreases the expression of inflammatory cytokines. iNOS, IL-1β, IL-6, and TNF-α inflammatory cytokine expression levels were significantly increased following ZA+LPS-stimulation of RAW 264.7 cells compared with cells in the control group (Fig. 4). However, as PDRN concentration (1, 10 or 100 μg/ml) increased, iNOS expression decreased compared with the ZA+LPS-stimulation of RAW 264.7 cells. Similarly, IL-1β and IL-6 expression significantly decreased following PDRN treatment (1, 10 or 100 μg/ml) compared with ZA+LPS-stimulation of RAW 264.7 cells. Although 1 μg/ml PDRN demonstrated no significant differences, 10 or 100 μg/ml PDRN significantly suppressed TNF-α expression compared with ZA+LPS-stimulated RAW 264.7 cells.

PDRN increases A_{2A} and VEGF expression. VEGF expression was significantly increased by ZA+LPS-stimulation compared with control cells, and the expression was further and significantly increased by PDRN treatment

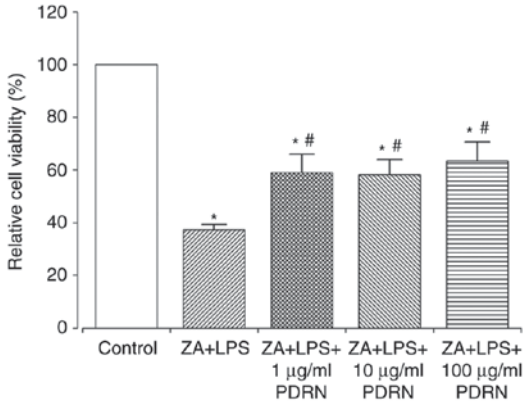

Figure 2. PDRN increases cell viability of RAW 264.7 treated with ZA+LPS. Relative cell viabilities were compared with the control group, which was set to 100%. Cell viability was measured in RAW 264.7 cells treated with 10 μM ZA for 24 h, and then with 0.1 μg/ml LPS and PDRN (1, 10, and 100 μg/ml) for 24 h. The experiments were conducted in triplicate. Data are presented as mean ± standard error of the mean. *P<0.05 compared with the control group. #P<0.05 compared with the ZA+LPS group. ZA, zoledronic acid; LPS, lipopolysaccharide; PDRN, polydeoxynucleotide.

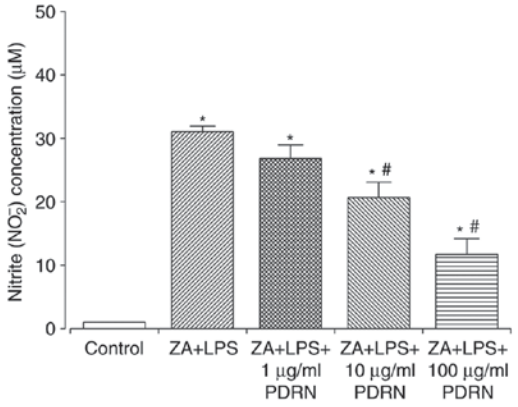

Figure 3. PDRN decreases ZA+LPS-induced NO production. NO production in the culture medium was analyzed with an NO detection kit. RAW 264.7 cells were cultured with 10 μM ZA for 24 h, and then with 0.1 μg/ml LPS and PDRN (1, 10, and 100 μg/ml) for 24 h. The experiments were conducted in triplicate. Data are presented as mean ± standard error of the mean. *P<0.05 compared with the control group. #P<0.05 compared with the ZA+LPS group. ZA, zoledronic acid; LPS, lipopolysaccharide; PDRN, polydeoxynucleotide.

in a marked dose-dependent manner compared with the ZA+LPS-stimulation of RAW 264.7 cells (Fig. 4). PDRN (100 μg/ml) treatment demonstrated the most potent increase in VEGF expression. ZA+LPS-stimulation significantly lowered the expression of A_{2A} compared with the control group. However, the expression of A_{2A} was enhanced by PDRN treatment in what appeared to be a dose-dependent manner compared with control and ZA+LPS-stimulated cells.

Discussion

Several factors are associated with BRONJ, including dental procedures, trauma and inflammation. Previous studies have elucidated the development of BRONJ along with bacterial infection (25-28). LPS, which is found in the cell wall of Gram negative bacteria, induces inflammatory cytokines and NO via nuclear factor-κB activation and NOS. Furthermore, Muratsu *et al* (15) previously suggested that ZA enhances LPS-stimulated proinflammatory reactions in macrophages, with a consequent increase in LPS-induced apoptosis. PDRN has demonstrated effectiveness in decreasing the circulating levels of several inflammatory cytokines and increasing VEGF production in previous studies (20,21). The present study was therefore undertaken to analyze the effect of PDRN on RAW 264.7 cells pretreated with ZA and stimulated with LPS, by measuring inflammatory mediators.

In the present study, the cell viability of RAW 264.7 was increased by ZA pre-treatment and LPS stimulation. This is due to nitrogen-containing bisphosphonates causing cell apoptosis by inhibiting the mevalonate pathway (4) and LPS stimulation increasing inflammatory cytokine expression levels (15). Notably, the viability of RAW 264.7 cells stimulated by ZA and LPS were significantly enhanced by PDRN treatment.

Western blotting revealed that PDRN treatment significantly decreased the NO production and iNOS expression of RAW 264.7 cells. iNOS catalyzes the synthesis of NO from the amino acid L-arginine as an immune defense mechanism in macrophages; overproduction of NO causes oxidative damage to the surrounding tissue, with a consequent increase of the inflammatory response (29). Furthermore, proinflammatory cytokines, including IL-1β, IL-6 and TNF-α were decreased by PDRN treatment in what appeared to be a dose-dependent manner. Overexpressed TNF-α stimulated the release of free radicals such as NO, inducing cell injury (30,31). Administration of nitrogen-containing bisphosphonate induced the release of IL-1β and IL-6 by macrophages or monocytes, resulting in an acute phase response; one of the known adverse effects of bisphosphonates (32). The results of the current study therefore indicate that PDRN downregulates inflammatory cytokines, and may help in prevention and management of BRONJ.

The present study also demonstrated that PDRN increased A_{2A} receptors and VEGF expression on ZA-pretreated and LPS-stimulated RAW 264.7 cells in what appeared to be a dose-dependent manner. As ZA and LPS induce cell apoptosis, it was hypothesized that adenosine A_{2A} receptor expression decreased in the ZA and LPS-treated cells, and that the expression of the receptor recovered following PDRN treatment. However, the mechanism of the recovered adenosine A_{2A} receptor expression should be further evaluated in a future study. ZA and LPS treatment (10 μM and 0.1 μg/ml, respectively) increase VEGF expression without PDRN. Koide *et al* (33) suggested that LPS induces VEGF overproduction in macrophages, and Ben-Av *et al* (34) explained that inflammatory mediators, including IL-1β, prostaglandin E2 and TNF-α, increase VEGF expression.

The activation of adenosine A_{2A} receptors also indicated an increase of VEGF release through the signaling pathways activated by LPS treatment (35). This suggested that PDRN, an A_{2A} receptor agonist, is capable of restoring blood flow and tissue architecture by modulating VEGF expression (36). Additionally, the PDRN-stimulated increase of A_{2A} receptors caused VEGF production during pathological conditions characterized by low tissue perfusion (16,21). Thus, it was hypothesized that PDRN can be used to counteract the inflammatory process inducing osteonecrosis of the jaw. Although

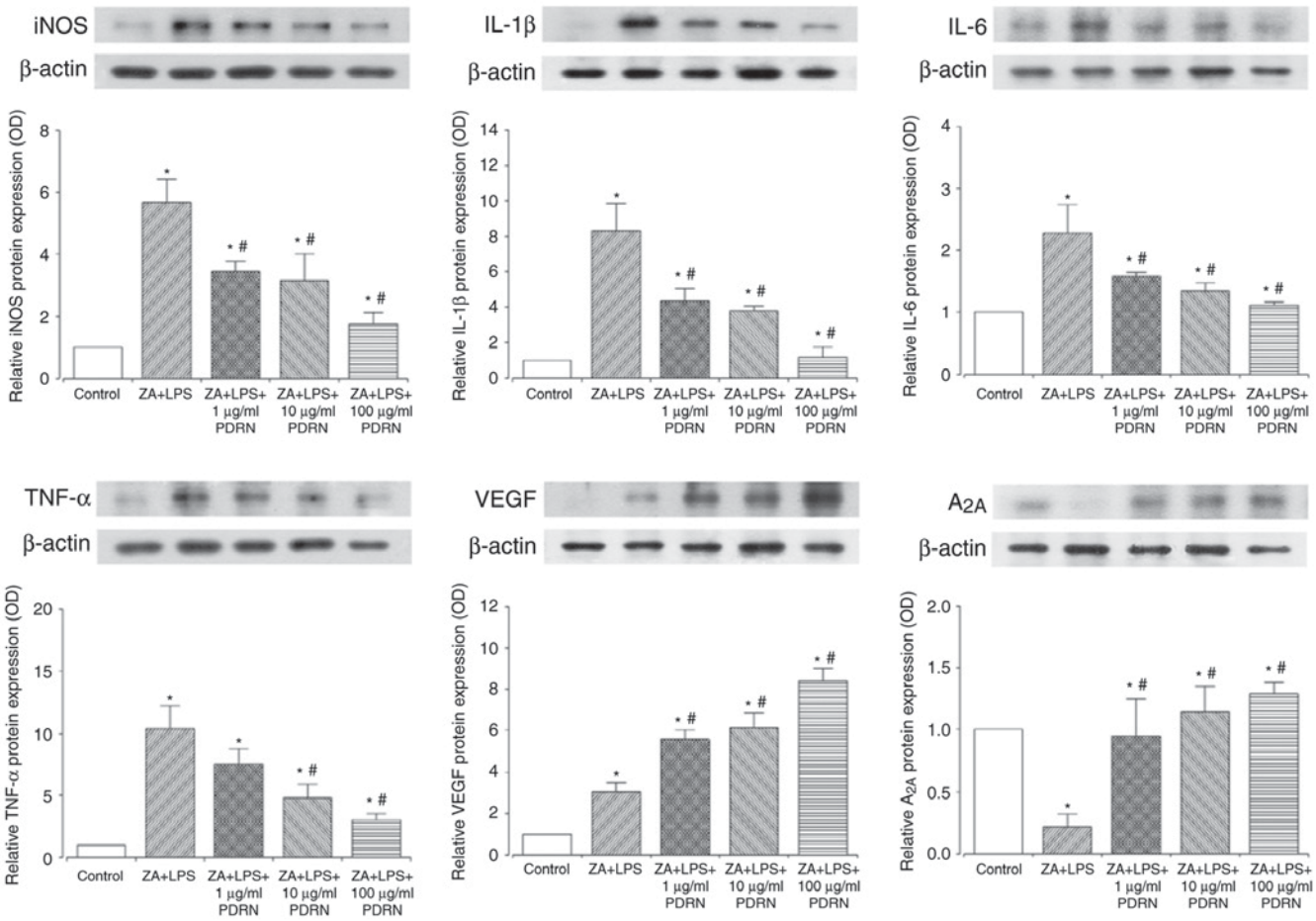

Figure 4. PDRN decreased inflammatory cytokine expression and increased A_{2A} and VEGF expression. The expression levels of iNOS, IL-1β, IL-6, TNF-α, A_{2A} and VEGF were measured in RAW 264.7 cells treated with 10 μM ZA for 24 h, and then with 0.1 μg/ml LPS and PDRN (1, 10, and 100 μg/ml) for 24 h. Data are presented as mean ± standard error of the mean. *P<0.05 compared with the control group. #P<0.05 compared with the ZA+LPS group. ZA, zoledronic acid; LPS, lipopolysaccharide; PDRN, polydeoxynucleotide; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

other mechanisms may also be involved, these results demonstrate that PDRN increased cell viability and suppressed the inflammatory process.

In conclusion, the present study demonstrated that LPS potently accelerated inflammation in RAW 264.7 cells, following pretreatment with ZA, and that PDRN acted as a suppresser of inflammatory cytokines. Additionally, increased VEGF expression, either directly by PDRN or indirectly by other cytokines and A_{2A} receptors, may have contributed to increased vascularization and subsequently improved the pathological condition of BRONJ. As inflammation and LPS may stimulate the occurrence of BRONJ, the authors of the present study postulated that PDRN is possibly a candidate for the therapeutic management of BRONJ.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

J-HH, JJ and LH wrote the manuscript. JJ, LH and I-GK performed the experiments. OHN and MSK analyzed the data. J-HH, JJ, J-WL and B-JC interpreted the data and revised the manuscript. D-WL conceived and managed the study design, and gave final approval of the version to be published.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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