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Suppression of puerarin on polymethylmethacrylate-induced lesion of peri-implant by inhibiting NF-κB activation *in vitro* and *in vivo*

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ABSTRACT

Puerarin (PR), a natural isoflavone isolated from Chinese traditional plant pueraria lobata, has attracted considerable attention due to its important biological and pharmacological activities. However, its effects on lesion of peri-implant and related mechanism of action are still not clear, which require further investigation. In this study, we evaluated the effects of PR on polymethylmethacrylate (PMMA)-induced lesion of peri-implant *in vitro* and *in vivo*, and explored its possible mechanism of action. Our results indicated that PR could inhibit PMMA-induced osteoclastogenesis in RAW264.7 cells with a dose-dependent manner *in vitro* and effectively down-regulate mRNA and protein expressions of matrix metalloprotein 9 (MMP-9), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and receptor activator of nuclear factor (NF)-κB (RANK), primarily via the suppression of NF-κB signaling. Furthermore, we found that PMMA induction could directly cause the phosphorylation of IκB and significantly promote the nuclear translocation of p65 in RAW264.7 cells. In other words, PR was able to dose-dependently attenuate the PMMA-induced nuclear translocation of p65 in RAW264.7 cells. *In vivo*, PR was observed to attenuate PMMA-induced osteoclastogenesis, osteolysis, mRNA expressions of receptor activator of nuclear factor (NF)-κB ligand (RANKL) and RANK, as well as protein levels of MMP-9, TNF-α, IL-6, and p65 in a murine calvarial osteolysis model. These findings suggested that PR might be a potential therapeutic drug to lesion of peri-implant, and provided new insights for understanding its possible mechanism.

1. Introduction

Lesion of peri-implant, known as peri-implant tissue inflammation, refers to inflammatory damage that occurs in soft and hard tissues around the implant, including peri-implant mucositis and peri-implantitis [5]. Peri-implant mucositis is limited to the surrounding soft tissues, peri-implantitis is however the inflammation that goes deep into the implant bone bed and causes severe bone resorption [17]. In response to the lesion of peri-implant, macrophages would produce inflammatory cytokines (TNF-α, IL-1β, PGE2), chemokines (CCL2, CCL3, IL-8) and growth factors (M-CSF, GM-CSF, VEGF) [7,10], which directly support osteoclastogenesis and osteoclast function. Moreover, inflammatory mediators derived from macrophages could increase the production of receptor activator of nuclear factor-κB ligand (RANKL) and decrease that of osteoprotegering (OPG) [18]. These all promote the development of a microenvironment that is conducive to osteoclastogenesis and osteolysis, leading to prosthesis loosening and even fall off [19]. Therefore, it has vital clinical significance to develop methods to reduce the lesions around the implant and delay loosening of the implant.

Puerarin (PR), a isoflavone component, is isolated from pueraria lobata known as Gegen in Chinese traditional medicine. There is mounting evidence that indicates PR has a variety of biological activities, such as anti-inflammation [27], anti-tumor [28], anti-oxidation [2], anti-diabetes [16], and so on. It was found that PR could inhibit iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells by suppressing NF-κB activation [11]. Some studies reported that PR is able to inhibit osteoclast formation in many disease models, for instance, PR could attenuate bone loss in ovariectomized mice [29], and prevent LPS-induced osteoclast formation as well as bone resorption by

Abbreviations: PR, Puerarin; PMMA, polymethylmethacrylate; MMP-9, matrix metalloprotein 9; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; NF-κB, nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand

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inhibiting Akt activation [31]. On the other hand, recent studies demonstrated that the NF-κB signaling pathway is activated in macrophages after exposure to implant debris, suggesting that NF-κB signaling may play an important role in particle-directed osteoclastogenesis [1,23].

Taken together, all of the above findings indicate the excellent biological activities of PR and the strong correlation between NF-κB signaling pathway and osteoclastogenesis. However, it is poorly understood the interaction between PR and the lesion of peri-implant. Therefore, it is necessary to evaluate the effects of PR on lesion of peri-implant and get a better understanding of its possible mechanism of action.

In our study, RAW264.7 cells were induced lesion of peri-implant by polymethylmethacrylate (PMMA) while treated with PR and their physiological-biochemical indexes were analyzed. To illustrate its underlying molecular mechanism, we further studied NF-κB signaling in PR treated RAW264.7 cells with the presence or absence of PMMA. *In vivo*, PMMA was implanted into parietal bones of C57BL/J6 mice, and then the mice were treated with PR. We found that PR could significantly reduce PMMA-induced lesion of peri-implant *in vivo* and *in vitro*. Hence, we here propose that PR has promising therapeutic effect in the lesion of peri-implant, but how this occurs still needs further study.

2. Materials and methods

2.1. Cell culture

RAW264.7 cells were obtained from Shanghai Zhong Qiao Xin Zhou Biotechnology (Shanghai, China) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, BI, Kibbutz Beit Haemek, Israel) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells in the logarithmic growth phase were used in the experiments.

2.2. Cell viability

CCK-8 assay was used to determine the cell viability. Briefly, RAW264.7 cells were seeded into 96-well plates at a density of 3×10^4 cells/ml. After incubating at 37 °C for 24 h, cells were treated with PR (0, 25, 50, 75, and 100 μM) for 48 h. PR was purchased from Dalian Meilun Biotechnology (MB6183, Dalian, China). CCK-8 (10 μl) subsequently was added to each well. After 1 h incubation at 37 °C, the absorbance at a wavelength of 450 nm was recorded by a microplate reader (BIOTEK, USA).

2.3. Tartrate-resistant acid phosphatase (TRAP) staining

Cells were fixed in 4% paraformaldehyde (pH 7.4) at room temperature for 15 min and stained for TRAP using leukocyte Acid Phosphatase Kit (387 A, SIGMA, USA) according to the manufacturer's protocol. The number of osteoclasts was calculated.

2.4. RNA extraction and real-time polymerase chain reaction (PCR) assay

The total RNA was extracted using a Total RNA Fast Extraction Kit (BioTeke, Beijing, China) according to the manufacturer's instructions. Super M-MLV reversetranscriptase (BioTeke, Beijing, China) and SYBR Green (Solarbio, Beijing, China) were used to synthesize cDNA and amplify target gene, respectively. Amplification was preceded using Exicycler™ 96 (Bioneer, Daejeon, Korea). The real-time PCR primers were shown as below: MMP-9 forward, 5′-GGGACCATCATAACATC ACA-3′; MMP-9 reverse, 5′-ATGACAATGTCCGCTTCG-3′; TNF-α forward, 5′-AGAAAGCATGATCCGCGAC-3′; TNF-α reverse, 5′-TTGTGAG TGTGAGGGTCTGG-3′; IL-6 forward, 5′-ACTTCCATCCAGTTGCCTT CTT-3′; IL-6 reverse, 5′-TCATTTCCACGATTTCCCAGA-3′; RANKL forward, 5′-ATGAAAGGAGGGAGCACGAA-3′; RANKL reverse, 5′-AAG GGTTGGACACCTGAATG-3′; RANK forward, 5′-CATCATCTTCGGCGTT TACT-3′; RANK reverse, 5′-CACCGTCTTCTGGAACCATC-3′. The mRNA relative expression levels of target gene were normalized to β-actin and calculated using the $2^{-\Delta\Delta CT}$ method.

2.5. Western blotting

Total proteins were extracted from the cells using lysis buffer, and then separated by 10% SDS polyacrylamide gels and transferred to PVDF membrane. Then membrane was blocked with 5% non-fat dried milk proteins at room temperature for 1 h, followed by incubation with primary antibodies at 4 °C overnight. Primary antibodies included MMP-9 (bs-4593R, Bioss, China, 1:400 dilution), TNF-α (#11948, CST, USA, 1:1000 dilution), IL-6 (#12912, CST, USA, 1:1000 dilution), RANK (#4845, CST, USA, 1:1000 dilution), P-IκB (bs-2513R, Bioss, China, 1:400 dilution), IκB (bs-1287R, Bioss, China, 1:400 dilution), P65 (D221030, Sangon Biotech, China, 1:1000 dilution), Histone H3 (17168-1-AP, proteintech, China, 1:500 dilution), β-actin (60008-1-Ig, proteintech, China, 1:2000 dilution). The second day, membrane was washed with TBST three times for 5 min each time, followed by incubation with the conjugated secondary antibodies at room temperature for 1 h.

2.6. Immunofluorescence staining for NF-κB localization

RAW264.7 cells were fixed in 4% paraformaldehyde for 15 min, and then washed with PBS for three times, followed by permeabilization with 0.1% tritonX-100 for 30 min. After PBS washing, goat serum (SL038, Solarbio, China) was applied to the cells for 15 min. Cells were then stained with the specific fluorescent antibody (Cy3-labeled goat anti-rabbit IgG, A0516, Beyotime, China) and DAPI (C1002, Beyotime, China), respectively. The nuclear translocation of NF-κB/p65 was observed under fluorescence microscopy (BX53, OLUMPUS, Japan).

2.7. Animal experiments

A total of 24 healthy male C57BL/J6 mice (6–8 weeks old) were obtained from Liaoning changsheng biotechnology co., LTD. (Liaoning, China). Animal care and experiments were conducted in accordance with guidelines and procedures authorized by the animal care of the first affiliated hospital of Zhengzhou University. In brief, mice were randomly divided into 4 groups including control, PMMA, PMMA + PR0.5 and PMMA + PR1 group. For the last three groups, we performed PMMA (30 mg/300 μL) on the surface of the parietal bone, carefully suture the skin, and do not let the particles in the homogenate flow out. For the control, they were treated with same dose of normal saline by daily intraperitoneal injection for 14 days. And PMMA + PR0.5 and PMMA + PR1 groups were injected intraperitoneally with different doses of PR (0.5 mg/kg/d and 1 mg/kg/d, respectively) for 14 days. After injection, mice were treated with sodium pentobarbital (200 mg/kg) and then bone tissue samples were collected for further biochemical measurements.

2.8. Histological analysis

Bone tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections were then cut to a thickness of 5 μm and then stained with hematoxylin and eosin (H&E) for histological evaluation. The samples were photographed using light microscope (OLUMPUS, Japan).

2.9. Statistical analysis

Results were expressed as mean ± SD. One-way analysis of variance (ANOVA) using SPSS ver. 20.0 software was used for multiple

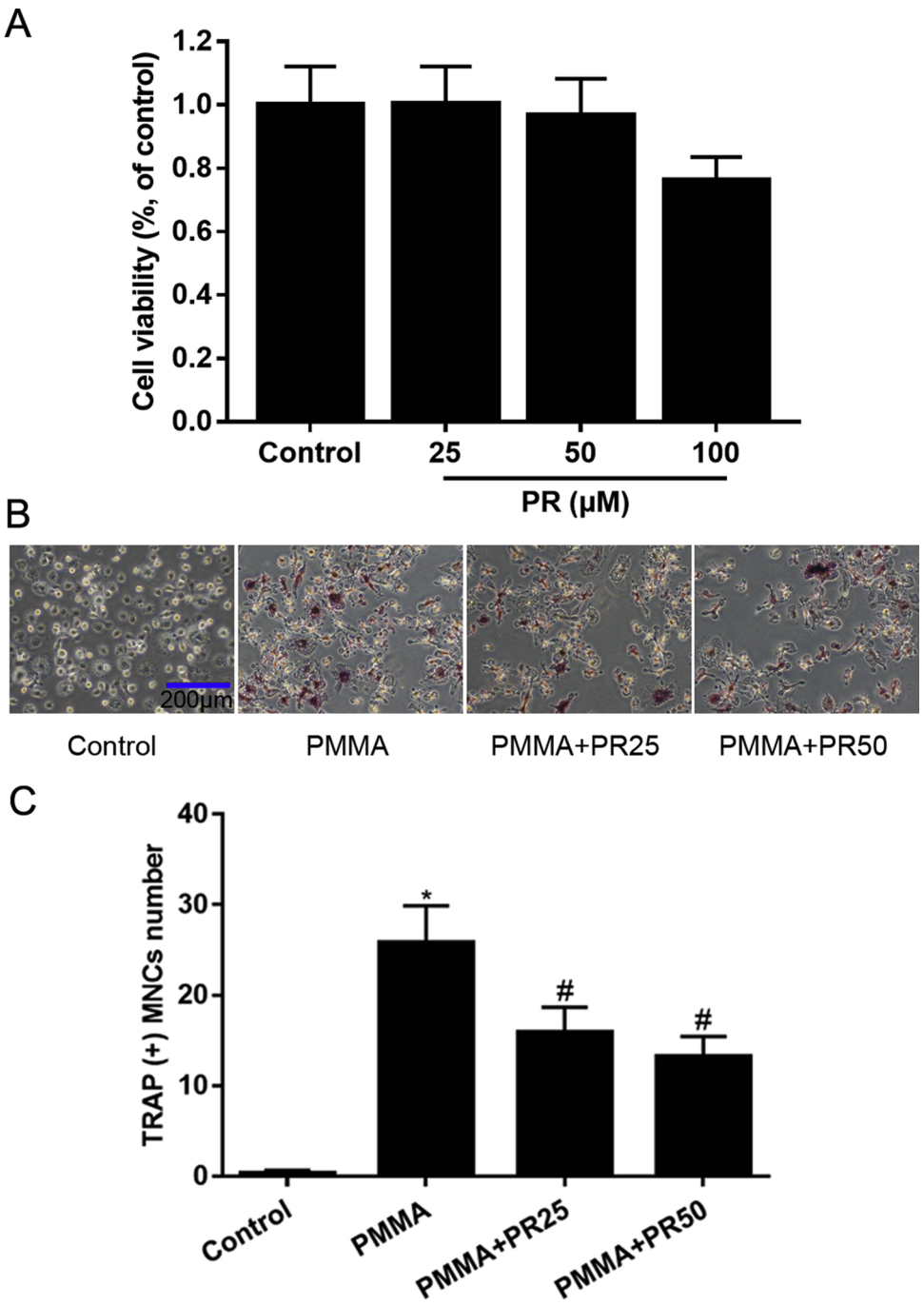

Fig. 1. PR inhibited PMMA-induced osteoclastogenesis. (A) The cell viability was detected via CCK-8 assay after incubated 48 h. (B) Tartrate-Resistant Acid Phosphatase (TRAP) Staining. Cells were stained with TRAP after 48 h. (C) Quantification of TRAP (+) multinucleated cells (MNCs). Data were expressed as mean ± SD (n = 3 per group), and $P < 0.05$ indicated a significant difference. * and # represented the difference compared to Control and PMMA respectively.

comparisons followed by Bonferroni's *post-hoc* test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. PR inhibited PMMA-induced osteoclast formation

The cytotoxicity test was performed via CCK-8 assay. As shown in Fig. 1A, the PR (1–50 μM) had no cytotoxicity in RAW264.7 cells. The following studies were performed within the safe doses (25 and 50 μM).

RAW264.7 cells were treated with PR (25 μM and 50 μM separately) in the presence of PMMA, and then stained with TRAP for identifying the effect of PR on PMMA-induced osteoclastogenesis. There was a significant difference in terms of TRAP (+) multinucleated cells (MNCs) numbers between PMMA treated groups and control, which indicated that PMMA could significantly stimulate RAW264.7 cells differentiation into TRAP (+) MNCs. Results also showed statistical significances between PR treated and non-treated groups, which suggested PR could dose-dependently inhibite PMMA-induced osteoclast formation (Fig. 1B, 1C). In addition, this inhibitory effect was not due to the cytotoxicity of PR itself. These results once again illustrated that treatment with PR (25 μM or 50 μM) has preventive effects on osteoclastogenesis in PMMA-induced Raw264.7 cells.

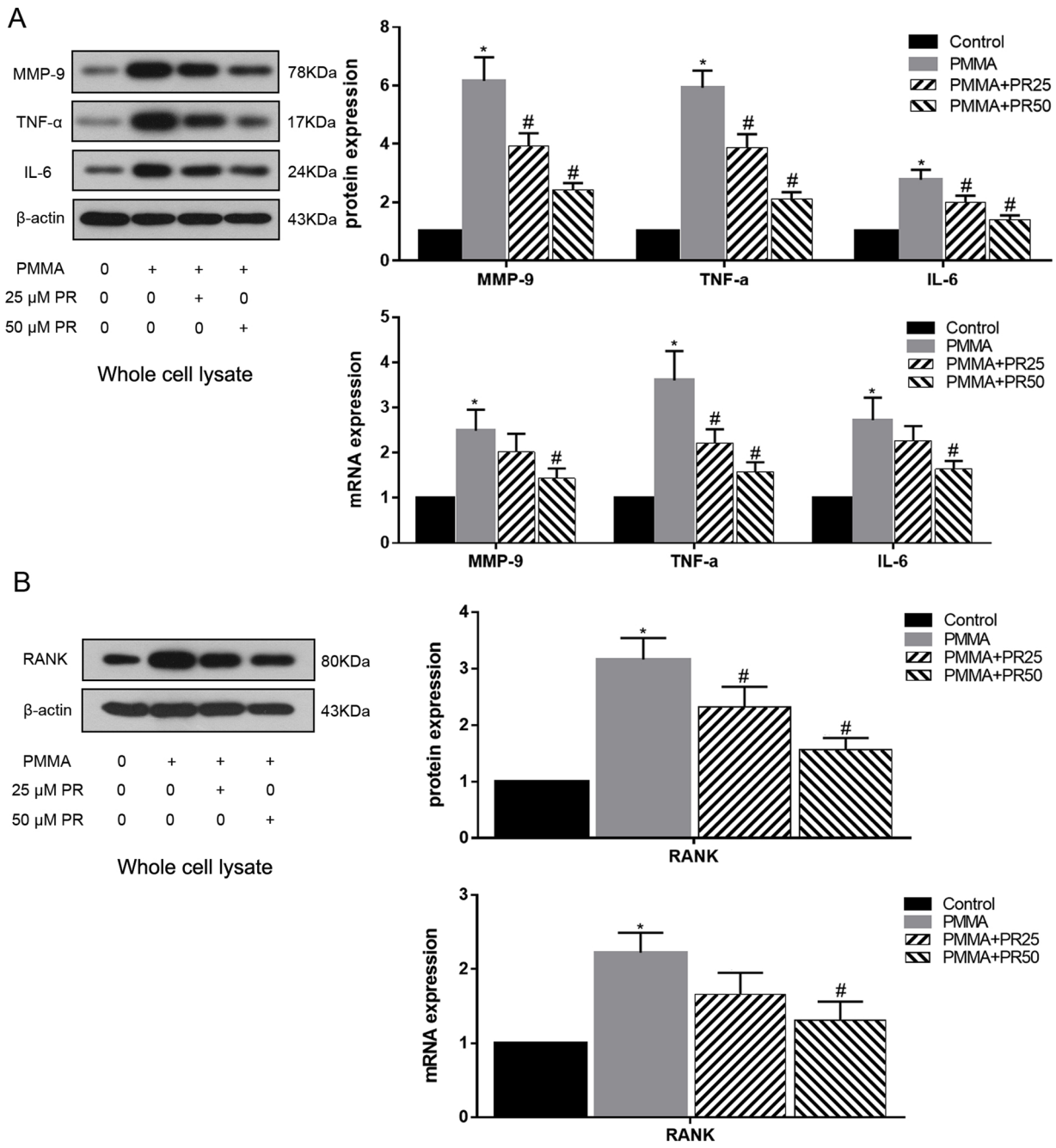

Fig. 2. The expression levels of mRNA and protein. PR attenuated pro-inflammatory factors expression (MMP-9, TNF-a, and IL-6) (A) and the expression of key regulator of osteoclast differentiation and bone resorption (RANK) (B). Results were showed as mean ± SD (n = 3 per group). P < 0.05 indicated a significant difference. * different compared to Control; # different compared to PMMA.

3.2. PR down-regulated mRNA and protein expressions of osteoclastogenic genes

Exposure of macrophages to PMMA induces their activation and secretion of pro-inflammatory cytokines, which further result in osteoclast formation and migration in bone destructive diseases. In this study, the levels of mRNA and protein expression of MMP-9, TNF-α, IL-6 and RANK were assessed. We found that it was significantly different between PMMA treated groups and control. More importantly, PR treatment was observed to be able to weaken PMMA-induced the production of these cytokines with a dose-dependent manner on both mRNA and protein expression levels (Fig. 2A, 2B).

3.3. PR suppressed PMMA-triggered activation of NF-κB

To elucidate the molecular mechanisms underlying the inhibitory effect of PR on PMMA-induced osteoclast differentiation, we assessed the protein levels of different components involved in related signaling pathway by western blot (Fig. 3A, 3B). We found that NF-κB pathway-related proteins, including inhibitor of NF-κB (IκB) and p65, were activated or phosphorylated by PMMA stimulation. The activation and phosphorylation of these proteins was observed to be suppressed by 25/50 μM PR with a concentration-dependent manner ($p < 0.05$). Furthermore, immunofluorescence staining was performed to show p65 nuclear translocation (Fig. 3C). Images analysis exhibited that PMMA stimulation promoted p65 translocation from cytoplasm to nucleus, however, PR inhibited nuclear translocation. As we expected, PMMA-

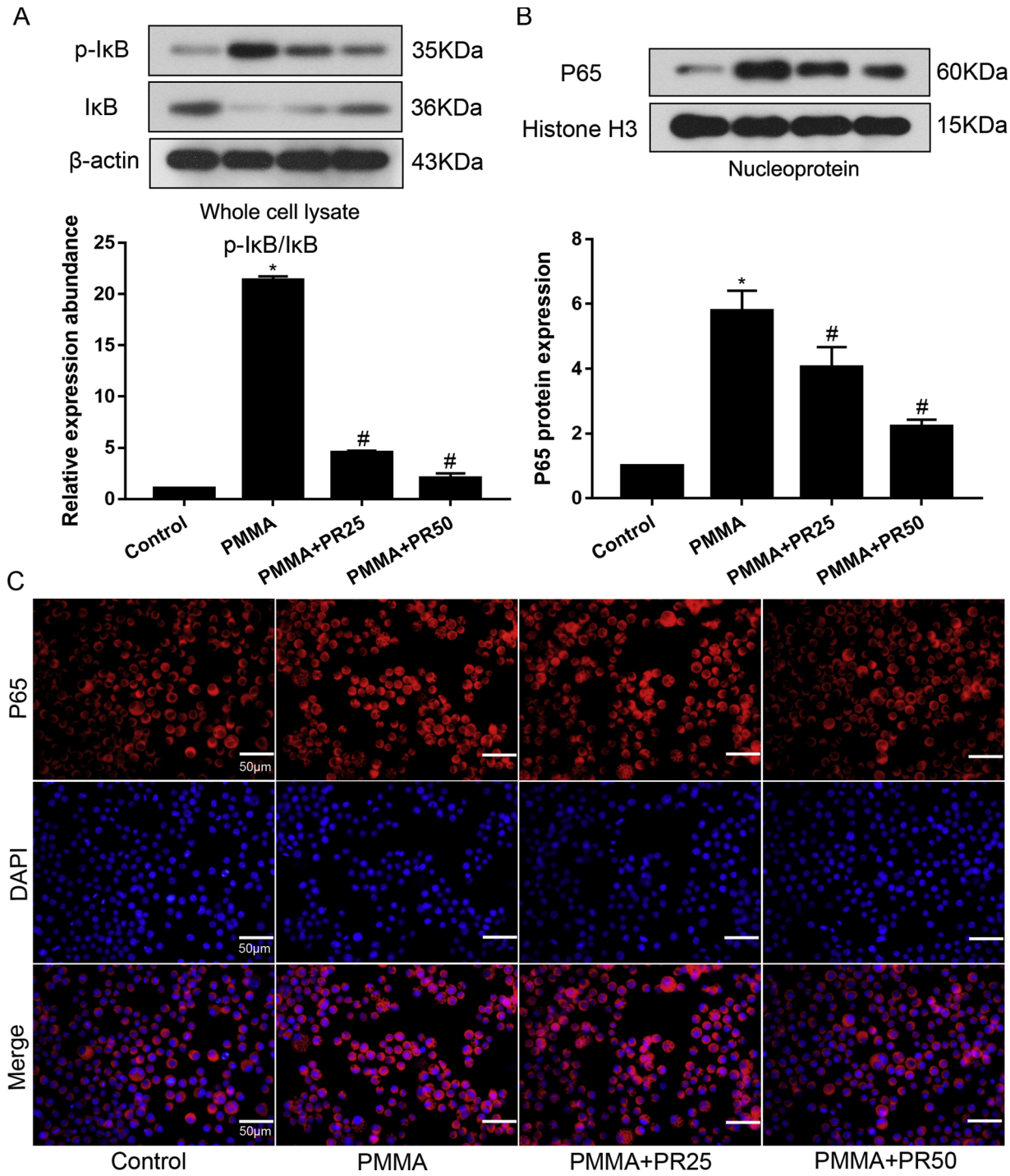

Fig. 3. Effect of PR on NF-κB signaling pathway. The expression levels of P-IκB, IκB (A) and nucleoprotein (P65) (B). The localization of P65 was observed by immunofluorescence microscopy. Cell nuclei were stained with DAPI (C). The values presented were the mean ± SD of three independent experiments. $P < 0.05$ indicated a significant difference. * different compared to Control; # different compared to PMMA.

triggered NF-κB activation was significantly inhibited by PR, and the level of p-IκB was significantly decreased after PR treatment.

3.4. PR prevented PMMA-induced osteolysis in vivo

To evaluate anti-inflammatory effect of PR *in vivo*, the murine osteolysis model was established. It was observed that the mice had no fatalities after PMMA and PR administration, and remained normal activity throughout the duration of the experiment. HE and TRAP staining of interfacial membrane around the implant were carried out to validate the anti-inflammatory effect of PR (Fig. 4A). Little inflammatory reaction and resorption were observed in control group. There was a pronounced inflammatory reaction with highly vascularized granulation containing macrophages in PMMA group. In addition, marked osteolysis was found to locate adjacent to these inflammatory reaction tissues, and the bone volume of murine calvarium was reduced extensively resulting from osteolysis in this group. Bone destruction and resorption in PR (0.5 and 1 mg/kg) treated groups were more conspicuous than that in control group, however, less remarkable than that in PMMA group. The osteoclast level was assessed from the HE staining

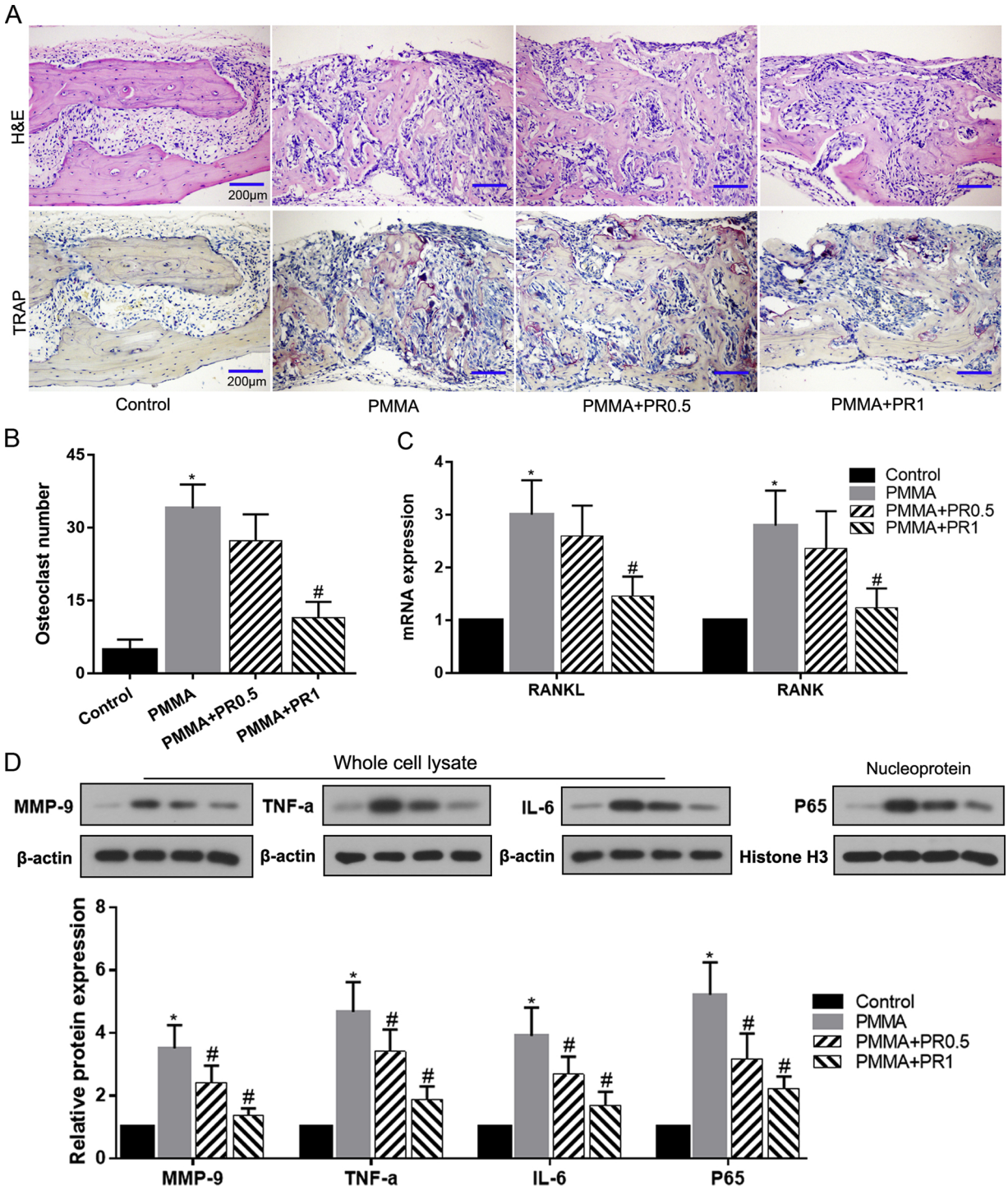

Fig. 4. PR prevented PMMA-induced osteoclastogenesis and osteolysis *in vivo*. PMMA was evenly applied to the parietal surface, and PR was locally intraperitoneal injection as described in Materials and Methods. Two weeks later, mice were sacrificed and calvaria were collected. Histological sections were processed and stained for H&E and TRAP (A). The osteoclast number was counted (B). The effects of mRNA expression on RANKL and RANK (C) in PMMA-induced mice bone tissue. The expression levels of MMP-9, TNF-a, IL-6 and nucleoprotein (P65) were detected by western blot (D). The data were expressed as mean ± SD (n = 6 per group). $P < 0.05$ indicated a significant difference. * different compared to Control; # different compared to PMMA.

(Fig. 4B). The results showed that there was little osteoclast in the control group, but significant increasing in PMMA group. Moreover, there was a significant difference on osteoclast level between PR treated and non-treated PMMA group, suggesting that PR treatment could attenuate PMMA-induced the production of osteoclasts with a dose-dependent manner ($p < 0.05$).

To further assess the effects of PR from molecular level, the expression levels of RANKL, RANK, MMP-9, TNF-α, IL-6, and p65 in bone tissue near each group of implants was examined. The mRNA levels of RANKL and RANK in the high dose of PR group (1 mg/kg) were found to be reduced significantly compared to those in the non-PR treated PMMA group ($p < 0.05$, Fig. 4C), confirming that PR could down-regulate mRNA expression of osteoclastogenic genes (RANKL and RANK). Meanwhile, the protein levels of MMP-9, TNF-α, IL-6 and p65 were decreased markedly after treated with PR.

4. Discussion

In order to meet the needs of people, implant technology is increasingly advanced. Meanwhile, it is accompanied by the occurrence of lesions around the implant, which attracts more and more attention. One of the common complications is particle wear-induced implant damage [12]. In recent years, great progress has been made in the treatment of the related diseases; however it is not satisfactory because of the side effects and single effect. Therefore, it is necessary to actively develop new drugs against implant related diseases.

Large multinucleated TRAP-positive cells can be found in peri-implant tissues [9,30]. TRAP is a marker that is widely used to identify osteoclasts. Macrophages that have phagocytosed prosthetic particles can differentiate into cells that express TRAP and resorb bone [22]. Wang et al [25] had found that wear particles could induce multinucleated cells to differentiate into osteoclasts, thereby increasing bone resorption. TRAP positive cells often contained particles of prosthetic material and had the appearance of giant cells. In this study, we found that PMMA could induce significant accumulation of TRAP-positive cells in bone tissues, and this elevation was inhibited by PR. These data indicate that PR exhibits inhibitory effects on PMMA-induced osteoclastic differentiation.

In response to wear particles, inflammatory cytokines will be produced, and subsequentially participate in osteolysis [20,24]. TNF-α and IL-6, as major proinflammatory cytokines in the osteolytic process, can induce aseptic inflammation and differentiation of osteoclast precursors into mature osteoclasts in direct and indirect manners [3,14]. MMP-9 over-generation was found to facilitate osteoclastogenesis [6]. Hence, it is very meaningful to study whether PR can inhibit the release of these pro-inflammatory cytokines. We found that PR could not only suppress the production of pro-inflammatory factors (MMP-9, TNF-α, IL-6), but also restrain the production of RANK, a key regulator of osteoclast differentiation and bone resorption *in vitro* and *in vivo*. Such findings demonstrate the effectiveness of PR in attenuating lesion of peri-implant. Similar anti-inflammatory effects of PR have been reported before [26].

NF-κB, an important transcription factor, is involved in the regulation of pro-inflammatory cytokines, chemokines and adhesion molecules [15]. It has been found that PMMA particles could accelerate osteoclastogenesis through amplification of various signals including NF-κB, MAP kinases and nuclear factor of activated-T-cells (NFAT) signal transduction pathways [21]. NF-κB is mainly composed of the p65 subunit and the p50 subunit, which remains in the cytoplasm of resting cells via binding to its inhibitor IκB [8]. Upon stimulation, IκB is phosphorylated and subsequently degraded, and then NF-κB is rapidly translocated into nucleus, regulating the transcription of NF-κB-mediated genes [4,8]. Thus, the activation of NFκB was assessed in RAW264.7 in our present study by measuring the quantities of IκB and p65 protein expression. We found that PMMA induction touched off the phosphorylation of IκB directly and distinctly increased the nuclear translocation of p65 in RAW264.7 cells. Namely, PMMA could regulate the inflammation factors (MMP-9, TNF-α and IL-6) and key regulator of osteoclast differentiation and bone resorption (RANK) via NF-κB signaling pathway. It was identified that PR could dose-dependently attenuate the PMMA-induced nuclear translocation of p65 in RAW264.7 cells, and then modulated its binding activity. In sum, these findings suggested that PR is able to affect the regulation of MMP-9, TNF-α, IL-6, RANK and p65 induced by PMMA via NF-κB signaling pathway.

Rats administrated with NPI-028 (puerarin content of NPI-028 was 26 mg/g dry weight) of 2.0 g/kg/d for 3 months displayed normal growth, with no change in hematologic or hepatic parameters being found [13]. In our study, 0.5 or 1 mg/kg/d of PR was used to treat mice. The experimental animals treated with PR remained normal activity throughout the experiment, and no facility was observed. Therefore, we believed that this relatively low doses of PR had little toxicity *in vivo*.

5. Conclusion

In summary, PR could inhibit PMMA-induced lesion of peri-implant through inhibition of NF-κB activation signaling *in vitro* and *in vivo*. Our findings suggested that PR might be a novel therapeutic approach to control lesion of peri-implant, which allow broad application in future clinical therapies to bone loss-related diseases.

Conflicts of interest

The authors declare no competing financial interest.

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