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# 3,3′-Diindolylmethane attenuates experimental arthritis and osteoclastogenesis

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ABSTRACT

3,3′-Diindolylmethane (DIM) is a natural compound formed during the autolysis of glucobrassicin present in *Brassica* food plants. This study aimed to investigate the therapeutic efficacies of DIM on experimental arthritis. The effects of DIM on experimental arthritis were examined on a rat model of adjuvant-induced arthritis (AIA), with daily AIA paw swelling observation and histological/radiographic analysis. To elucidate the possible mechanisms of its action, serum cytokine levels as well as the expression of receptor activator for nuclear factor κ B ligand (RANKL) in infected tissues were subsequently analyzed. The impact of DIM on osteoclastogenesis was further investigated on a mouse model of endotoxin-induced bone resorption (EIBR) and *in vitro* cultures of fibroblast-like cells and osteoblasts, with RANKL expression being evaluated with great interest. The administration of DIM was demonstrated to attenuate AIA in animal models, as judged by clinical and histologic indices of inflammation and tissue damage. On the one hand, DIM could reduce the expression of several inflammatory cytokines, which was, however, not adequate to prevent the development of the arthritis. On the other hand, DIM was shown to effectively inhibit the expression of RANKL, leading to the blockade of osteoclastogenesis and consequently an alleviation of experimental arthritis. Further *in vitro* and *in vivo* studies confirmed the inhibition of RANKL by DIM. DIM has shown anti-arthritis activity in animal models via inhibiting the expression of RANKL, and thus may offer potential treatments for arthritis and associated disorders.



## 1. Introduction

3,3′-Diindolylmethane (DIM) is a natural product formed during the autolytic breakdown of glucobrassicin present in food plants of the *Brassica* genus, including the common vegetables cabbage, Brussels sprouts, cauliflower, and broccoli. DIM is a promising anti-tumor agent that is widely studied in laboratory and clinical trials [1–3]. Moreover, recent studies demonstrated that DIM had the ability to suppress inflammatory response in macrophages and in murine animal models of inflammatory bowel disease [4,5]. It is rational that DIM can modulate immunity and be used as therapeutic agent for certain inflammatory diseases because DIM is considered as an antagonist of aryl hydrocarbon receptor (Ahr) which is involved in various cell signal transduction pathways including of some important inflammatory signals' activation [6–8].

The aryl hydrocarbon receptor (Ahr) is a cytosolic transcription factor that can be activiated by some specific ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), leading to changes in gene transcription [9]. DIM is also a ligand of Ahr but have an antagonism on the Ahr activated by TCDD [6]. Some recent studies reported that one of the Ahr ligand, 3-methylcholanthrene inhibited the formation of osteoclasts [10]. Osteoclasts are multinuclear cells that are responsible for resorption of bone [11]. In arthritis, excessive osteoclastogenesis is a key pathological process that is responsible for bone destructions [12]. Inhibition of osteoclastogenesis is always a potential therapeutic strategy for arthritis [13].

In the present study, we examined the anti-arthritis property of DIM in a rat model of adjuvant-induced arthritis (AIA). Furthermore, we investigated the possible mechanisms involved in DIM's anti-arthritis activity in both aspects: anti-inflammation activity and anti-osteoclastogenesis effects.

## 2. Materials and methods

### 2.1. Reagents

DIM and TCDD were purchased from Ruima Chem Co. (Nanjing, China) (purity >99%). DIM and TCDD are difficult to

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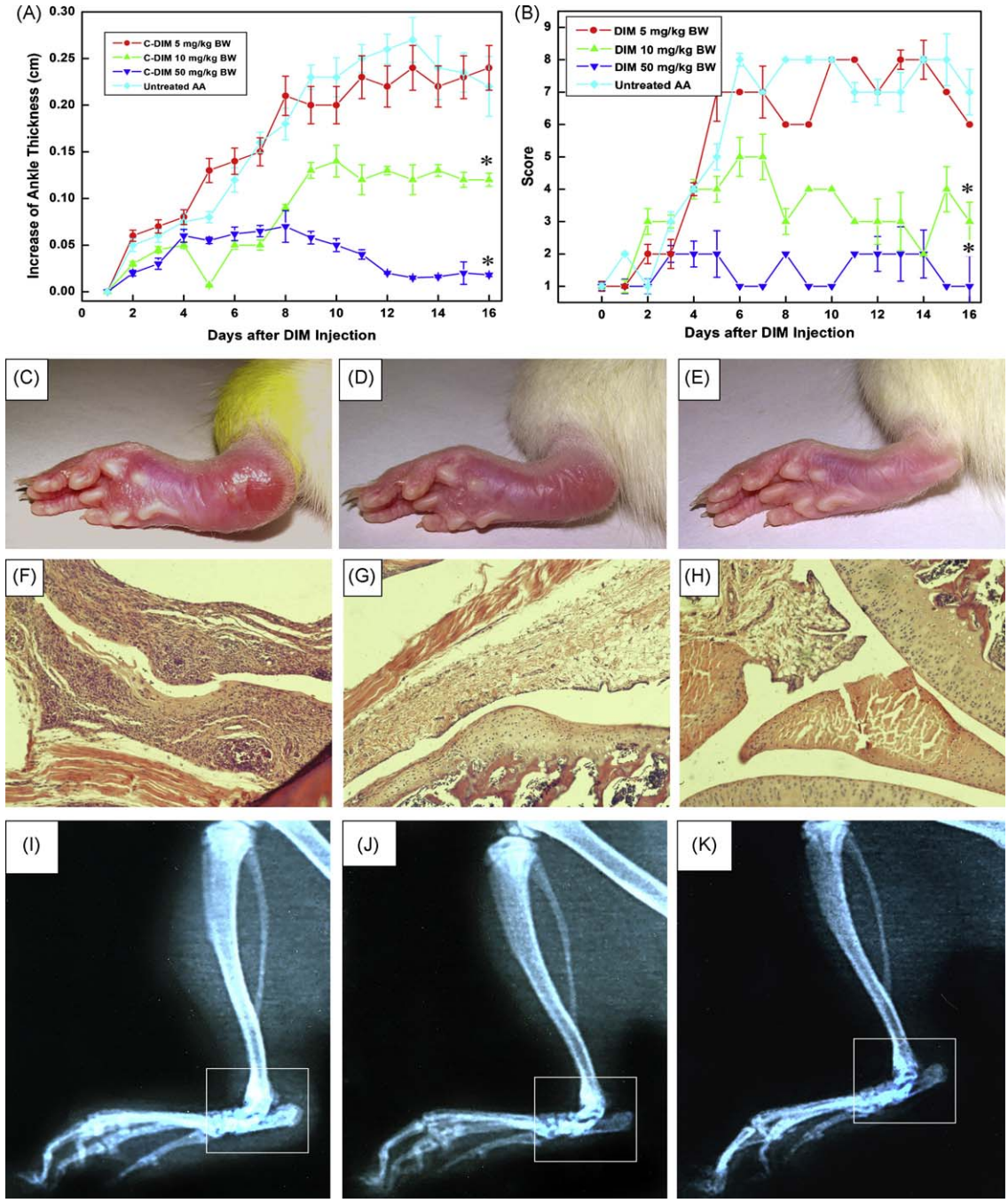


**Fig. 1.** Anti-arthritis effect of DIM. (A) AIA animals were given DIM at 7th day after the adjuvant was injected. The ankle thickness increases of AIA animals were measured every day from the day ASO was given until the 16th day. (B) The arthritis score of the animals. (C) Photograph of a representative foot from AIA model. (D) A representative foot from AIA model treated with DIM for 16 days. (E) Healthy foot. (F) Tissue section of footpad from AIA model. (G) Tissue section of footpad from AIA model treated with DIM for 16 days. (H) Tissue section of footpad from healthy animal. (I) The result of X-ray examination of the AIA foot; (J) AIA model treated with DIM for 16 days; (K) Healthy foot. The boxes in (I) (J) (K) indicated the joint-spaces of the ankle joints. Every experimental group contained 10 animals and the experiment was triplicated.

be solved in water. To obtain a stable aqueous solution, they were dissolved in a 2-hydroxypropyl-β-cyclodextrin (Sigma–Aldrich, St. Louis, MO) solution (molar ratio = 1:10; prepared at 40 °C for 20 min). These stock solutions were serially diluted in sterile water to give final concentrations on the day of use. DMEM, penicillin/streptomycin antibiotic mixture, and fetal bovine serum were purchased from Invitrogen (Shanghai, China). Freund's complete adjuvant (CFA) and lipopolysaccharide were purchased from Sigma–Aldrich (St. Louis, MO). Other chemical reagents were purchased from Sangon Biotech (Shanghai, China).

### 2.2. Animals and cells

Female SD rats and C57/Bl6 mice were purchased from the Experimental Animal Centre of Nanjing Medical University (Nanjing, China) and maintained under barrier conditions and were pathogen free as assessed by regular microbiologic screening.

### 2.3. Cell culture

Fibroblast-like cells and osteoblasts were isolated from 7 day old neonatal mouse calvaria according to a reported method [14].

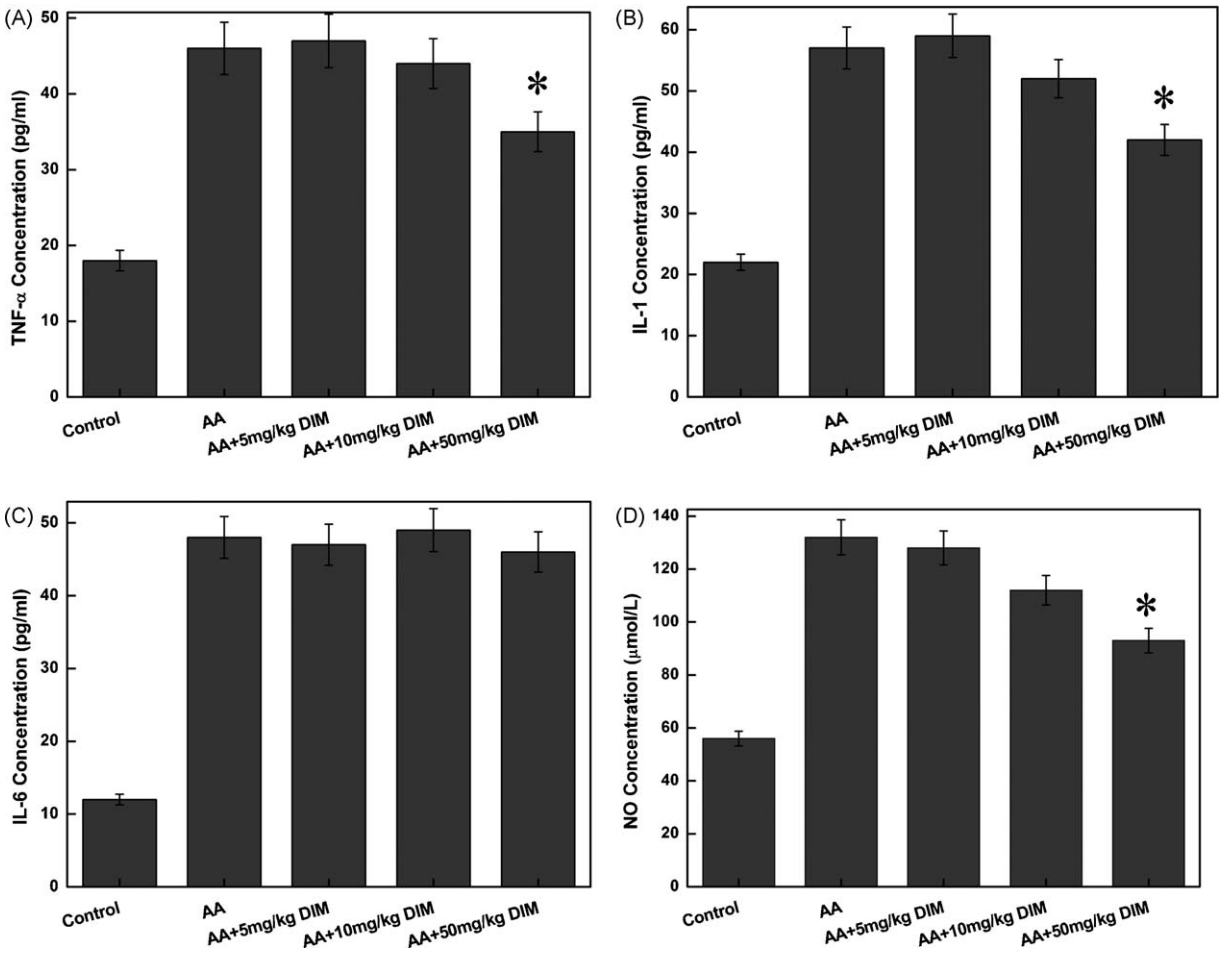


**Fig. 2.** The influences of DIM on the secretion of inflammatory mediators. The AIA models treated with DIM for 16 days before the plasma concentration of the mediators were examined. The plasma from healthy animals was used as the control. Results were expressed as mean ± SEM. $^{*}p \leq 0.05$ ($n = 10$ and the experiment was triplicated).

Briefly, the calvaria were chopped and digested with collagenase A (Sangon Biotech, Shanghai, China) for 40 min then centrifuged for 3 min at 1500 rpm to collect cells (fraction 1). The supernatant collagenase solution was then reapplied to the calvaria fragments for another 20 min (fraction 2) and cells were collected as described above. Subsequent 20 min digestions were performed and labeled fractions 3–5. Fraction 1 and 2 cells were used as fibroblasts, as normally fraction 3–5 cells are treated as osteoblasts. The purity of the fibroblast-like cells reached at least 75%, which was examined by the morphological identification in our experiments. The purity of the osteoblast was also examined by alkaline phosphatase staining to ensure their quality.

### 2.4. Induction and assessment of rat AIA

Experiments were performed in 7–8-week-old male rats. Experimental procedures were performed in accordance with local policy for animal experiments. For statistical analysis, a minimum of 10 animals per group were used; all experiments were triplicated to assure reproducibility of response. Rat AIA was induced according to reported methodology [15]. Briefly, animals were injected subcutaneously (SC) with CFA into the footpads (100 μl per rat).

Animals were evaluated daily for arthritis development. Arthritis was assessed by the clinical scores of the infected foots. The clinical score ranged from 0 to 4 was determined by the degree of the inflammation, 0 for non-inflammation and 4 for the most serve inflammation in the experiments [16]. Statistical analysis was performed using unpaired one-way analysis of variance. In all cases, $p$ values less than 0.05 were considered statistically significant.

DIM were given every day at different doses via intraperitoneal injection with the 2-hydroxypropyl-β-cyclodextrin at the same concentration as the control.

### 2.5. Endotoxin-induced bone resorption mouse model (EIBR)

Endotoxin-induced bone resorption mouse model was performed according to an established method [17,18]. Mice of 7–8-week-old were administered a local calvarial injection of lipopolysaccharide at 25 mg/kg ($n = 10$). DIM were given every day via intraperitoneal injection with the 2-hydroxypropyl-β-cyclodextrin at the same concentration as the control. After 7 days, we measured osteoclast number per square millimetre of trabecular bone surface [18].

### 2.6. Histologic assessment

Animals were sacrificed at the indicated time points after the induction of arthritis. Foot pads were dissected, fixed in neutral buffered formal saline, and embedded in paraffin. Serial sections (5 μm thickness) were cut and stained with hematoxylin and eosin (H&E). The slides were scored by three independent observers who were blinded to the experimental procedure. The sections were graded subjectively using various parameters. Synovial hyperplasia (pannus formation), cellular exudate, and cartilage depletion/

bone erosion were each scored from 0 (normal) to 4 (severe); synovial infiltrate was scored from 0 to 4. All parameters were subsequently summed to give an arthritis index.

### 2.7. Radiographic analysis

Radiographic severity of AIA was assessed blindly on day 28 by high resolution digital radiographs (48 kV, 2 mAs). Rats were given a score of 0–4 for the most suffered limb based on the extent of soft tissue swelling, joint space narrowing, bone destruction, and periosteal new bone formation [19].

### 2.8. Cytokine analysis

TNF-α, IL-1, IL-6 concentration in animal plasma were evaluated by using ELISA kits (R&D Systems, MN). Nitric oxide (NO) and prostaglandin E2 (PGE2) concentrations in plasma were also analyzed by commercial kits (Jiancheng Biothech, Nanjing, China). Receptor activator for nuclear Factor κ B ligand (RANKL) expression level in animal tissues and cell cultures were analyzed by real-time PCR. GAPDH were used as the internal reference (Primers for RANKL: 5′-CACCATCAGCTGAIAGATAGT-3′, 5′-CCAIA-GATCTCTAIACATGACG-3′, and GAPDH: 5′-AIACGACCCCTTCATT-GAC-3′, 5′-TCCACGACATACTCAGCAC-3′). Tartrate resistant acid phosphatase (TRAP) was quantified by test kits (Tissue TRAP Quantification Kit from Jian Cheng Biotech, Nanjing, China).

### 2.9. Statistical analysis

Data are expressed as means ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA) with multiple comparison of Tukey, where $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Anti-arthritis effects of DIM in AIA models

DIM was given to AIA models 7 days after the injection of CFA, when the acute inflammation induced by CFA was relieved and the chronic inflammation was about to start. The effects of DIM were shown in Fig. 1. Daily administration of DIM at the dose of 10 mg/kg bodyweight exhibited notable anti-arthritis effects and 50 mg/kg bodyweight almost inhibited the development of the arthritis. As shown in Fig. 1A, ankle swelling caused by adjuvant-induced inflammation was suppressed by DIM. Clinical scoring gave a more comprehensive evaluation of the AIA arthritis, including edema, erythema and possible joint dysfunction of the infected animal feet. In Fig. 1B, the score of AIA was successfully reduced by DIM at the dose of 10 mg/kg BW and 50 mg/kg BW. In DIM-treated animals, erythema and edema of the infected ankle joints was relived. More importantly, the joint dysfunction caused by AIA arthritis in late stage of the disease was alleviated, that was observed in the experiments. Fig. 1C, D, E show the footpad photographs of controlled AIA model, DIM-treated AIA model and healthy animal, respectively. It was observed that animals suffering experimental arthritis lost their body weights while the body weights of the animals given DIM keep increasing.

Animals were sacrificed 16 days after DIM administration. Fig. 1F–H shows the histologic sections of the footpads. AIA model exhibited massive inflammatory cell invasion and hyperplasia of synovial tissue while DIM dramatically reduced these pathological changes. The bone damages caused by AIA were examined by radiographic analysis. AIA caused obvious bone destructions at the ankle joint which resulted in joint-space narrowing (Fig. 1I) and disappearance. DIM prevented this in AIA model (Fig. 1J).

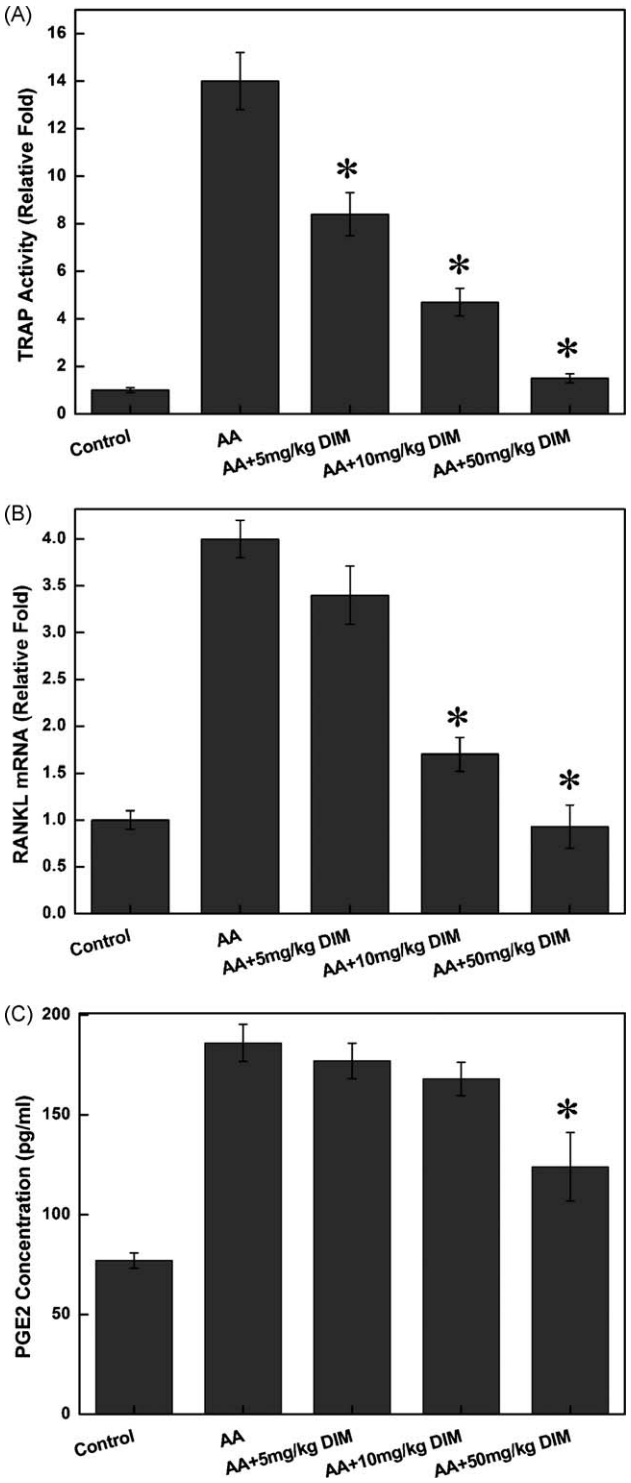


**Fig. 3.** AIA models treated with DIM at different doses before the TRAP activity, RANKL expression level and PGE2 concentration in the footpads were examined. Results were expressed as mean ± SEM. $^*p \leq 0.05$ ($n = 10$ and the experiment was triplicated).

It was been observed that the body weight of the arthritic animals given DIM increased while the control animal models lost their bodyweight during the development of experimental arthritis.

### 3.2. Plasma cytokines analysis

Cytokines in animal plasma were quantified 16 days after DIM were given. The results were demonstrated in Fig. 2. Concentra-

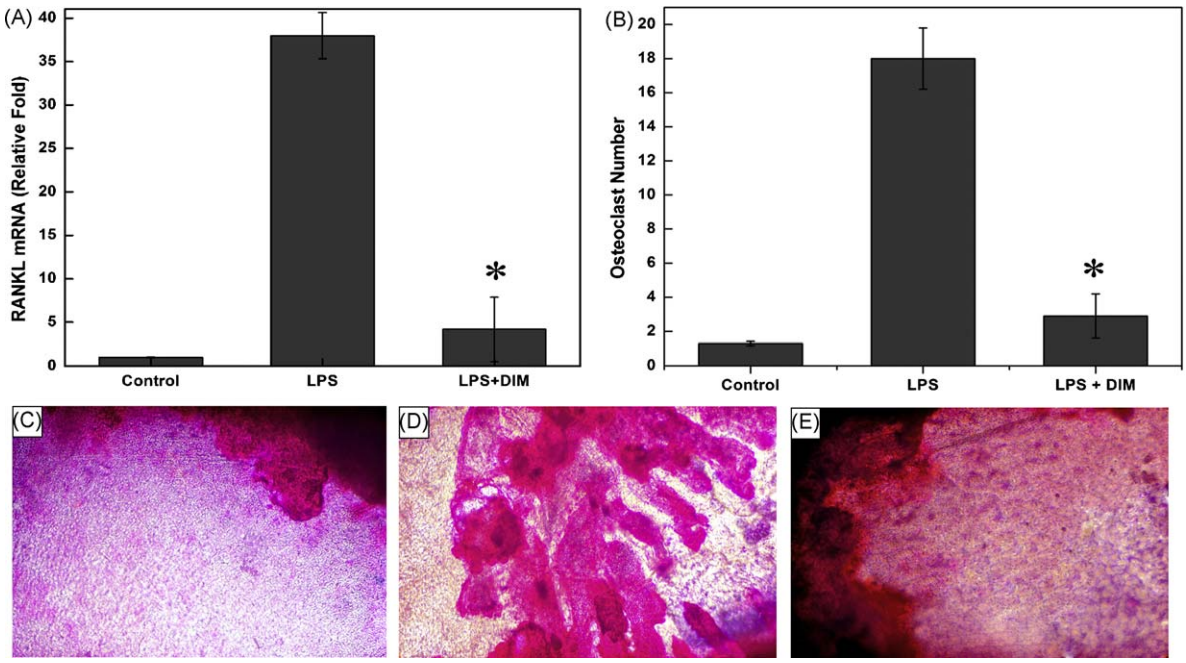


**Fig. 4.** DIM inhibited osteoclastogenesis in EIBR model. After treatment with 10 mg/kg body weight DIM (A) RANKL expression level in EIBR models. (B) Osteoclasts numbers in EIBR models. (C)–(E) TRAP stained mouse calvaria of healthy animal, EIBR model and DIM-treated EIBR model. $^{*}p \leq 0.05$ ($n = 10$ and the experiment was triplicated).

tions of TNF-α, IL-1 and NO were reduced about 30% by DIM at the dose of 50 mg/kg BW. 10 mg/kg BW DIM did not significantly influence the cytokine's concentrations ($p > 0.05$). The concentration of IL-6 did not change by DIM.

### 3.3. Osteoclastogenesis-related elements analysis

TRAP is the main marker of the osteoclasts. Its activity reflects the activity and the amount of osteoclasts. The tissues separated from AIA models' ankles were analyzed for their TRAP activity. Results shown in Fig. 3A exhibited more than 60% inhibition of TRAP activity in AIA models by 10 mg/kg and 90% by 50 mg/kg BW DIM daily injection. RANKL expression levels in these tissues were examined by real-time PCR. Results in Fig. 3B demonstrated that excessive expression of RANKL in the inflammatory tissues was 60% inhibited by 10 mg/kg BW DIM and totally inhibited by 50 mg/kg BW DIM administration. PGE2 in AIA models' plasmas were analyzed. DIM of 50 mg/kg BW inhibited about 30% of PGE2 in AIA model (Fig. 3C). DIM of 10 mg/kg BW did not significantly reduce the concentration of PGE2.

### 3.4. DIM inhibited osteoclastogenesis in endotoxin-induced bone resorption mouse model

DIM at the dose of 10 mg/kg BW inhibited the osteoclastogenesis in endotoxin-induced bone resorption mouse models. Results shown in Fig. 4 demonstrated that DIM suppressed the excessive RANKL expression (Fig. 4A) and subsequently reduced the osteoclast number (Fig. 4B). Fig. 4C–E is the TRAP staining calvaria of the mouse models treated by DIM, controlled animal model and healthy animal's calvarium, respectively. DIM was shown to inhibit the generation of osteoclasts on the calvaria.

### 3.5. PGE2-stimulated expression of RANKL in fibroblast-like cells and osteoblasts could be suppressed by DIM and reestablished by TCDD

Fibroblast-like cells and osteoblasts separated from mouse calvaria express high level of RANKL when stimulated by 40 μmole/ml PGE2 for 12 h. In both kinds of cells, pre-incubation with DIM at a dose of 100 μg/ml for 6 h inhibited most of the expression of the RANKL (Fig. 5A and B). In PGE2-activated fibroblast-like cells, RANKL expression inhibited by 100 μg/ml DIM was reestablished by 20 μg/ml TCDD (Fig. 5C).

## 4. Discussion

The AhR is a cytosolic transcription factor which belongs to the family of basic-helix–loop–helix transcription factors [20]. The AhR is normally inactive, bound to some co-chaperones. Upon ligand binding to chemicals such as TCDD, the chaperones dissociate resulting in AhR translocating into the nucleus and dimerizing with AhR nuclear translocator, leading to changes in gene transcription [21]. IL-1, IL-6, TNF-α and other pro-inflammatory cytokines were reported to be up regulated when macrophages were challenged with AhR ligands such as benzo(a)pyrene and TCDD [22–24]. *In vivo* investigation found that TCDD could exacerbate RA pathophysiology via AhR ligand activation and subsequently stimulation of inflammation cytokines' expression [25]. These results suggest that AhR ligands can promote inflammatory reaction and induce or exacerbate inflammatory diseases.

However, recent studies indicated that another group of AhR ligands such as DIM and 3-methylcholanthrene (3MC) which were considered to combine AhR in a much less compact way had totally different effects on the inflammatory reaction [9]. Some kinds of inflammatory cytokines expressed by macrophages stimulated by LPS could be suppressed by DIM [4]. Moreover, 3MC was reported to have the effects of inhibiting osteoclastogenesis via suppressing RANKL expression in osteoblastic cells [10]. In our present studies, DIM at high concentrations suppressed the expression of inflammatory cytokines (TNF-α, IL-1 and NO). However, we believe that the suppression of cytokines by DIM is too limited to interfere with the progress of the experimental RA. So we examined the inhibitory effects of DIM on the osteoclastogenesis in the EIBR animal model. Results demonstrated that osteoclastogenesis induced by LPS could be dramatically inhibited by the administration of DIM. This effect was not resulted from the suppression of DIM on the expression of inflammatory cytokines because DIM at the given dose had little suppression effects on

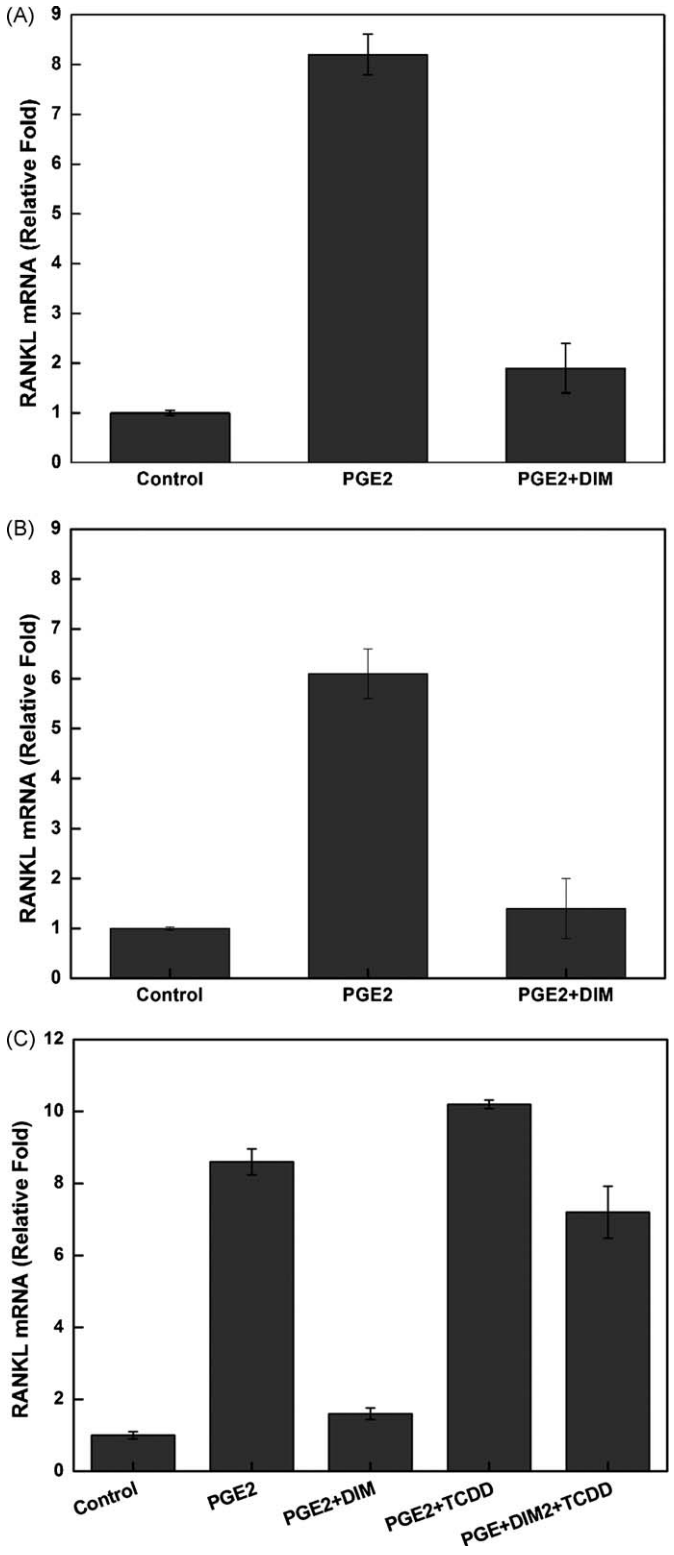


**Fig. 5.** DIM suppressed the expression of RANKL in fibroblast-like cells and osteoblasts stimulated by PGE2. Pre-incubation with 100 μg/ml DIM for 6 h inhibited PGE2 stimulated RANKL expression in (A) fibroblast-like cells, (B) osteoblasts and (C) TCDD at a concentration of 20 μg/ml treated fibroblast-like cells for 6 h after the cells were incubated with 100 μg/ml DIM abolished the inhibition effects of DIM on the PGE2-induced expression of RANKL.

TNF-α or IL-1. We examined the effect of DIM on the expression of RANKL in fibroblast-like cells and osteoblastic cells. We found that DIM could reduce PGE2-induced RANKL expression in a very effective way. These results indicated that the effect of DIM on the inhibition of osteoclastogenesis could be due to its ability to suppress the expression of RANKL. Osteoclastogenesis is one key step in the pathogenesis of RA. Osteoclast was responsible for bone degradation as well as exacerbation of the inflammatory symptoms. Block of osteoclastogenesis can inhibit the development of RA, which is considered as a potential therapeutics for RA [26].

There was report demonstrating TCDD had no effects on osteoclastogenesis [27]. We also tested TCDD in our experiments. Intriguingly, TCDD was not only exhibiting any inhibiting effect on RANKL expression and osteoclastogenesis but enhanced the expression of RANKL by PGE2-stimulated osteoblastic cells and accelerate the experimental osteoclastogenesis. Moreover, suppression of RANKL expression and osteoclastogenesis by DIM was completely abrogated by the addition of TCDD. These results in present studies proved that different AhR ligands had quite different (even contrary) effects. This may be because they have different ways of combination with AhR. There is evidence that DIM and 3MC had different dissociation constant (Ka value) [9]. However, the possibility that DIM and 3MC had other receptors cannot be excluded.

DIM is not a water-soluble compound. In most of the studies, DIM was dissolved in vegetable oil such as corn oil [28]. There is no problem when this oil-dissolved DIM was used *in vitro*. Oil emulsion can help DIM to enter cell membrane. However, when this kind of formulation was used *in vivo*, the DIM-contained oil drops will be captured mainly by cells of reticuloendothelial system and activate the innate immunity to secrete some cytokines. There was report that DIM could stimulate the expression of IFN-γ when tested in animal [29]. In this case, DIM can hardly enter other tissue and cells because it was infiltrated by the macrophages and endothelial cells. To avoid this problem, in our present studies, DIM was formulated in 2-hydroxypropyl-β-cyclodextrin to enhance its water-solubility. In pharmaceutical research and industry, 2-hydroxypropyl-β-cyclodextrin is widely applied to the treatment of water-insoluble agents [30]. The drug-in-2-hydroxypropyl-β-cyclodextrin formulation is a hydrophilic formulation which can avoid the infiltration of reticuloendothelial system. We believe that the 2-hydroxypropyl-β-cyclodextrin formulation of DIM has the advantage compared to the oil formulations when used in animal experiments.

DIM is a natural product from the *Brassica* genus and is taken by people as a health product for years that proved it is safe for people, and there are some clinical trials to use DIM for the treatment of cancers. Our present study indicate DIM is a potential anti-arthritis agent that is safe, cost-effective and easy to obtain. At least, it can be used as an adjuvant therapy for RA patients.

## Acknowledgments

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