Original Article
Curculigoside regulates proliferation, differentiation, and pro-inflammatory cytokines levels in dexamethasone-induced rat calvarial osteoblasts

Fang-Bing Zhu[1], Jian-Yue Wang[1], Ying-Liang Zhang[1], Ren-Fu Quan[1], Zhen-Shuang Yue[1], Lin-Ru Zeng[1], Wen-Jie Zheng[1], Qiao Hou[1], Shi-Gui Yan[2], Yun-Gen Hu[1]

[1]Department of Orthopaedic Surgery, Xiaoshan Traditional Chinese Medical Hospital, Hangzhou 311200, China; [2]Department of Orthopaedic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, China

Abstract: Background: Curculigoside (CCG), one of the main bioactive phenolic compounds isolated from the rhizome of *Curculigo orchioides* Gaertn., is reported to prevent bone loss in ovariectomized rats. However, the underlying molecular mechanisms are largely unknown. Therefore, we investigated the effects of CCG on proliferation and differentiation of calvarial osteoblasts and discussed the related mechanisms. Materials and methods: Osteoblasts were incubated with dexamethasone (DEX) in the absence or presence of CCG concentrations for 24-72 h. Cell proliferation was evaluated by Cell Counting Kit-8 assay. Mitochondria membrane potential (MMP) and reactive oxygen species (ROS) were assessed by flow cytometry. We assessed the anti-inflammatory responses of CCG on DEX-induced osteoblasts by an enzyme-linked immunosorbent assay (ELISA). Relative protein expression of BMP-2, β-catenin, RANKL, OPG and RANK was measured using Western blotting. Results: It was found that osteoblasts proliferation decreased significantly after treated with 1 μM of dexamethasone (DEX), compared with untreated osteoblasts and the cytotoxic effect of DEX was reversed remarkably when pretreatment with 25-100 μg/ml of CCG. Pretreatment with 25-100 μg/ml of CCG increased MMP level and decreased ROS production in osteoblasts induced by DEX. In addition, DEX-induced inhibition of differentiation markers such as alkaline phosphatase (ALP), OPG, BMP-2, β-catenin, IGF-1 and M-CSF level, and promotion of differentiation markers such as RANKL and RANK was significantly reversed in the presence of CCG. CCG also reversed DEX-induced production of pro-inflammatory cytokines. Conclusions: These results provide new insights into the osteoblast-protective mechanisms of CCG through inducing proliferation and differentiation and reducing the inflammatory responses, indicating that CCG may be developed as an agent for the prevention and treatment of osteoporosis.

Keywords: Curculigoside, calvarial osteoblasts, osteoporosis, proliferation, pro-inflammatory cytokines

Introduction

Osteoporosis, a systemic skeletal disorder characterized by low bone mineral density (BMD) and deteriorating bone microarchitecture, is prevalence by increased longevity and a changing lifestyle [1]. In bone, osteoblasts and osteoclasts are responsible for bone formation and resorption, respectively. Increased bone resorption over bone formation, the imbalance between osteoblastic bone formation and osteoclastic bone resorption, can result in bone loss, leading to osteoporosis. After aging and sex steroid deficiency, the therapeutic use of glucocorticoids is the most common cause of osteoporosis [2]. Fractures triggered by

osteoporosis are an important cause of morbidity and mortality [3, 4], particularly in elderly women [5].

Glucocorticoids modify the proliferation and metabolism of bone cells [6, 7]. They inhibit osteoblastogenesis and osteoclastogenesis and reduce the lifespan of osteoblast [8]. These changes lead to glucocorticoid-induced osteoporosis via reducing bone formation, which has been demonstrated histomorphometrically and clinically [9]. Dexamethasone (Dex), a synthetic glucocorticoid, is well known to promote progenitor cells, such as bone marrow stromal cells, differentiates to the osteoblastic phenotype [10]. Chronic treatment of osteoblast cul-
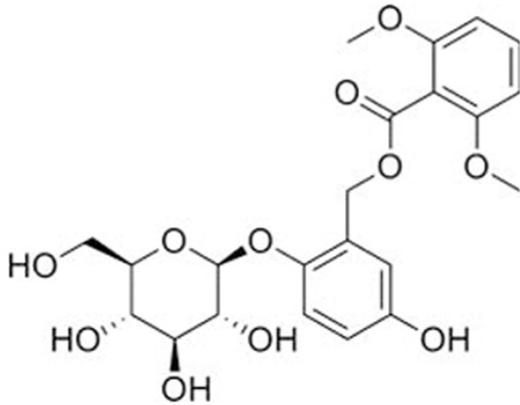
Figure 1. The chemical structure of curculigoside (CCG, $C_{22}H_{26}O_{11}$).

tures with Dex increases the number of mineralized bone nodules in calvarial osteoblast cultures of primary fetal rat [11]. McLaughlin et al., showed diminished bone formation and turnover in DEX-treated mice [8], and Alm et al., reported decreases in serum osteocalcin in patients given DEX treatment [10]. Glucocorticoids promote markers of osteoblast differentiation, such as ALP, osteopontin, bone sialoprotein and osteocalcin, while they suppress insulin-like growth factor-I (IGF-1) and type I collagen production by osteoblasts. However, the relevant molecular mechanisms remain unknown.

Curculigoside (CCG) shown in **Figure 1**, one of the main bioactive compounds in the rhizome of *Curculigo orchioides* Gaertn., has been shown to have significant anti-osteoporotic activity in rat plasma [12] and anti-apoptotic activity in H_2O_2-treated vascular endothelial cells [13]. Previous study showed that CCG increased the proliferation and differentiation of osteoblasts by stimulating ALP activity, while inhibited osteoclast bone resorption and osteoclast formation [14, 15]. However, there is no direct evidence to certify the relativity between anti-osteoporotic activity and CCG, and the molecular mechanisms remain unclear.

In this study, we investigated the effects of CCG on calvarial osteoblasts treated with DEX, and found that CCG protected osteoblasts against DEX-induced damage and promoted osteoblastic differentiation via increasing differentiation markers expression and inhibiting pro-inflammatory cytokines expression under DEX conditions, implying that it has a potential therapeutic value for the treatment of osteoporosis.

Materials and methods

Regents

Dulbecco's minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, USA). Type I collagenase was purchased from Hyclone (Utah, USA). Rhodamine-123 (Rho-123) dye and DEX was purchased from Sigma (St Louis, USA). Kits for ALP activity and ELISA measurement were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies of OPG, β-catenin, BMP-2, and GAPDH obtained from Abcam (Cambridge, UK); antibodies of RANKL and RANK obtained from Santa Cruz (Santa Cruz, USA) were used for Western blot analysis.

Isolation and cell culture of osteoblasts

Osteoblasts were isolated from calvariae of newborn (3 days) Sprague-Dawley rats by sequential enzymatic digestion as described previously. Briefly, calvariae were minced and incubated in an enzymatic solution containing 0.4% type I collagenase at 37°C for 20 min with shaking, and then incubated with 0.4% type I collagenase for 90 min before stop digesting by 10% FBS. The cells were cultured separately in DMEM containing 10% FBS and antibiotics (100 mg/ml of penicillin G and 100 IU/ml of streptomycin). After reaching 80-90% confluent, the cells were removed from each flask and combined together as osteoblasts.

Cell treatment

Cells were harvested and randomly divided into five groups: control group (untreatment), DEX treatment group, and CCG treatment groups (25, 50 and 100 μg/ml). The cells in the DEX treatment group were incubated with a medium containing 1 μM DEX. In the CCG groups, the cells were pre-incubated for 24 h with different concentrations of CCG, then incubated with 1 μM DEX.

Cell proliferation assay

Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay as described previously with some modifications. In brief, the cells were seeded at density of $5×10^4$ cells/well in 96-well plates and cultured in an incubator at 37°C for 12 h. At the indicated time points, the culture supernatant
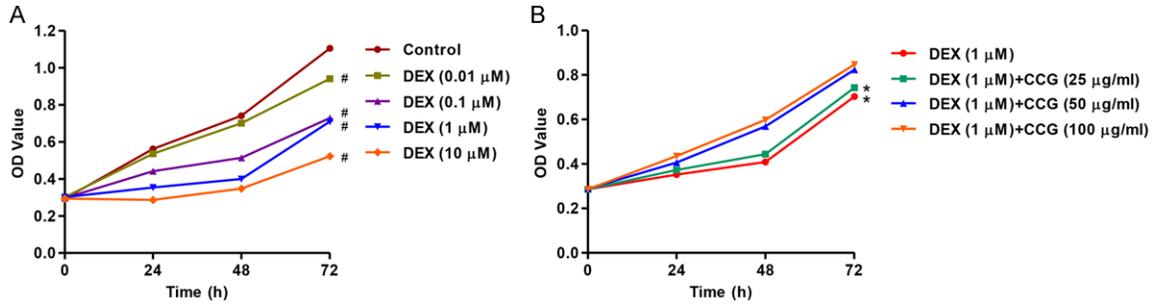
Figure 2. Protective effects of CCG on DEX-induced cell injury in osteoblasts. A. Dose-dependent effect of DEX on cell proliferation. DEX at 1 μM significantly reduced cell proliferation after 72 h of incubation. B. Pretreatment with CCG (25, 50 and 100 μg/ml, 24 h) alleviated DEX-induced cell injury. The data were presented as the mean ± SD (n=3); #$P<0.05$ vs. control without any treatment group, *$P<0.05$ vs. the DEX only group.
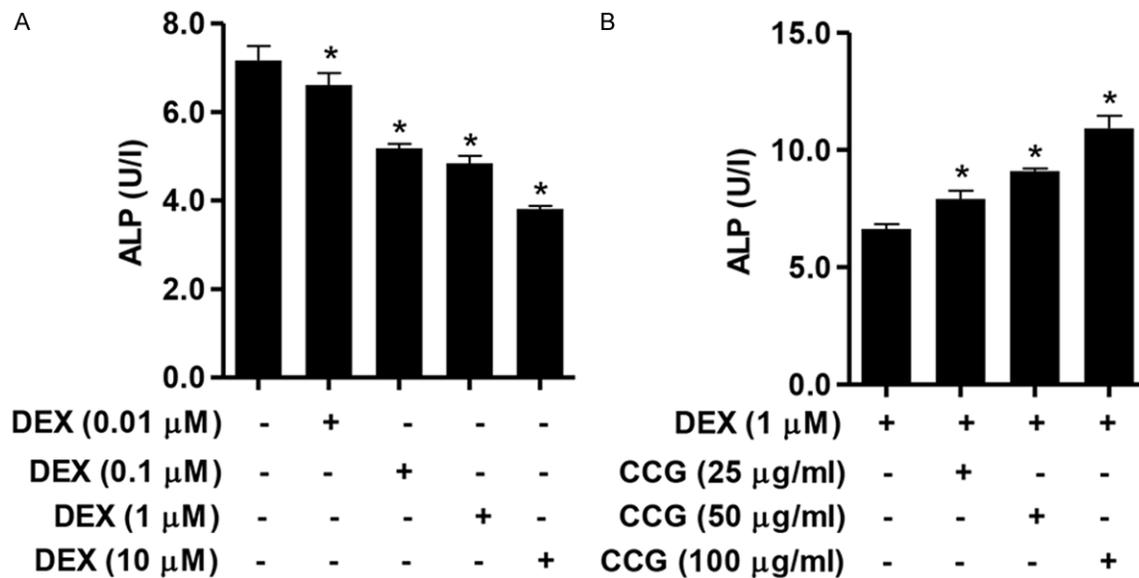
Figure 3. The activity of ALP is altered by CCG under DEX condition. A. The cells were treated with medium containing 0, 0.01, 0.1, 1 or 10 μM of DEX and the activity of ALP was decreased. B. The cells were pretreated with 25, 50 and 100 μg/ml of CCG for 24 h, and then incubated with an additional 1 μM of DEX. The data were presented as the mean ± SD (n=3); #$P<0.05$ vs. control without any treatment group, *$P<0.05$ vs. the DEX only group.

was removed, and cells were washed with PBS, and then 100 μl of fresh medium mixed with CCK-8 solution was added to each well. After incubation at 37°C for 1 h, the culture medium containing CCK-8 solution was removed. The absorbance at 450 nm was measured using a spectrophotometric microplate reader (Bio-Tek, Winchester, USA).

Assay of ALP activity

The induction of ALP is an unequivocal marker for bone cell differentiation. At the end of the treatment, ALP activity was measured by using ALP assay kit following the manufacturer's pro-

tocol. Absorbance of the samples and standards were measured at 520 nm.

Mitochondria Membrane Potential (MMP)

Mitochondria Membrane Potential (MMP) was detected by using Tetramethyl rhodamine methyl ester (TMRM) dye. Cells (1×10^6 cells/well) were cultured in 6-well plate. After a period of treatment (24 h) with various concentrations of CCG (25, 50 and 100 μg/ml), cells were washed with PBS, incubated with TMRM (10 nM) and subsequently subjected to flow cytometry.
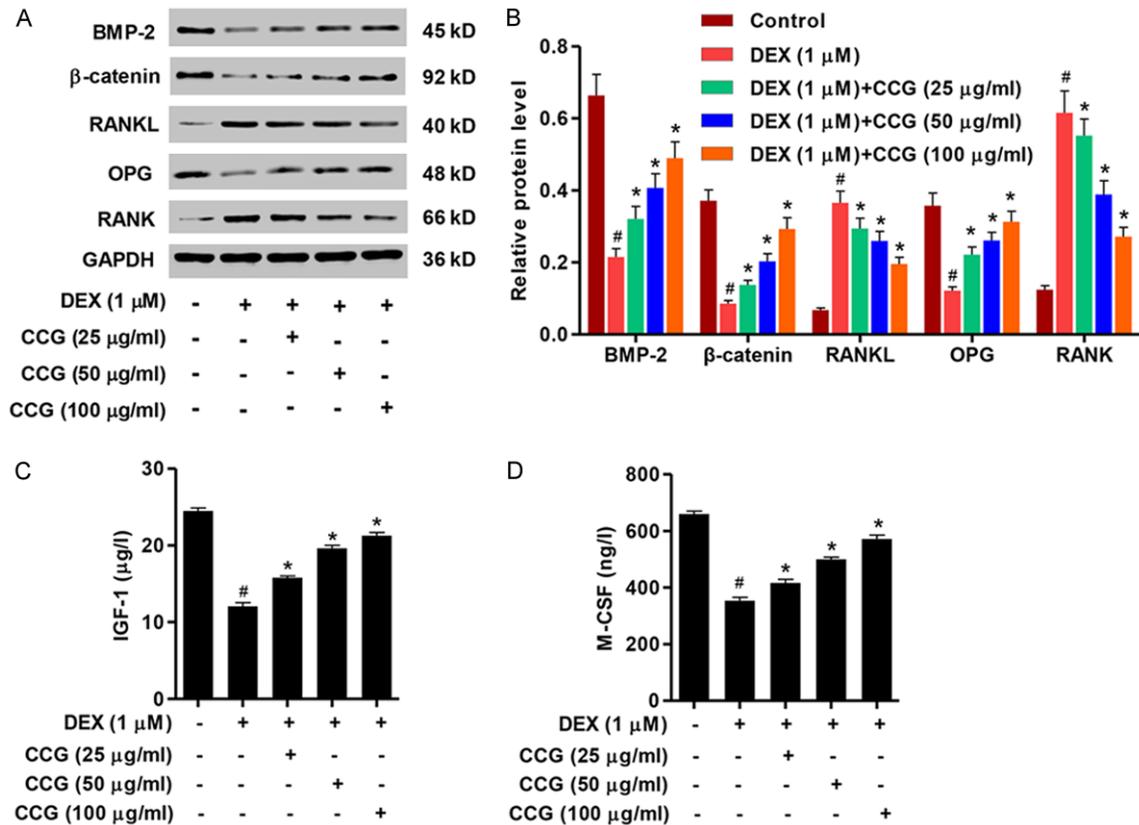
Figure 4. The effects of CCG on the expression of osteoblastic differentiation associated markers. A, B. Western blotting (left) and quantification (right) of BMP-2, β-catenin, RANKL, OPG and RANK expression profiles in five treatment groups. C, D. ELISA analyzed IGF-1 and M-CSF protein expression in five treatment groups. The data were presented as the mean ± SD (n=3); $^{#}P<0.05$ vs. control without any treatment group, $^{*}P<0.05$ vs. the DEX only group.

Detection of reactive oxygen species (ROS)

Detection of ROS was performed by flow cytometry analysis as described previously. In brief, ($5×10^4$ cells/well) were cultured in 6-well plate, after a period of treatment (12 h) with various concentrations of CCG (25, 50 and 100 μg/ml), cells were washed with PBS and resuspended in complete medium followed by incubation with 10 μM DCFH-DA for 20 min at 37°C. ROS fluorescence intensity was determined by cytometry with excitation at 480 nm and emission at 525 nm.

ELISA

TNF-α, IL-1β, IL-6, COX-2, IGF-1 and M-CSF levels present in osteoblasts were determined using commercially available murine-specific sandwich enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol.

Protein extraction and western blotting

Cells were harvested and lysed on ice for 30 min in RIPA buffer (Beyotime) supplemented with 1 μM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of cell lysates were subjected to electrophoresis using SDS-PAGE and subjected to Western blot analysis with appropriate primary and horseradish peroxidase-conjugated secondary antibodies. Blots were incubated with the primary antibodies of BMP-2 (1:1000), β-catenin (1:1000), RANKL (1:500), RANK (1:1000), OPG (1:1000) and GAPDH (1:1000) at 4°C overnight.

Statistical analysis

Experimental data were presented as mean ± SD of at least three independent replicates through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and assessing comparisons between different groups by
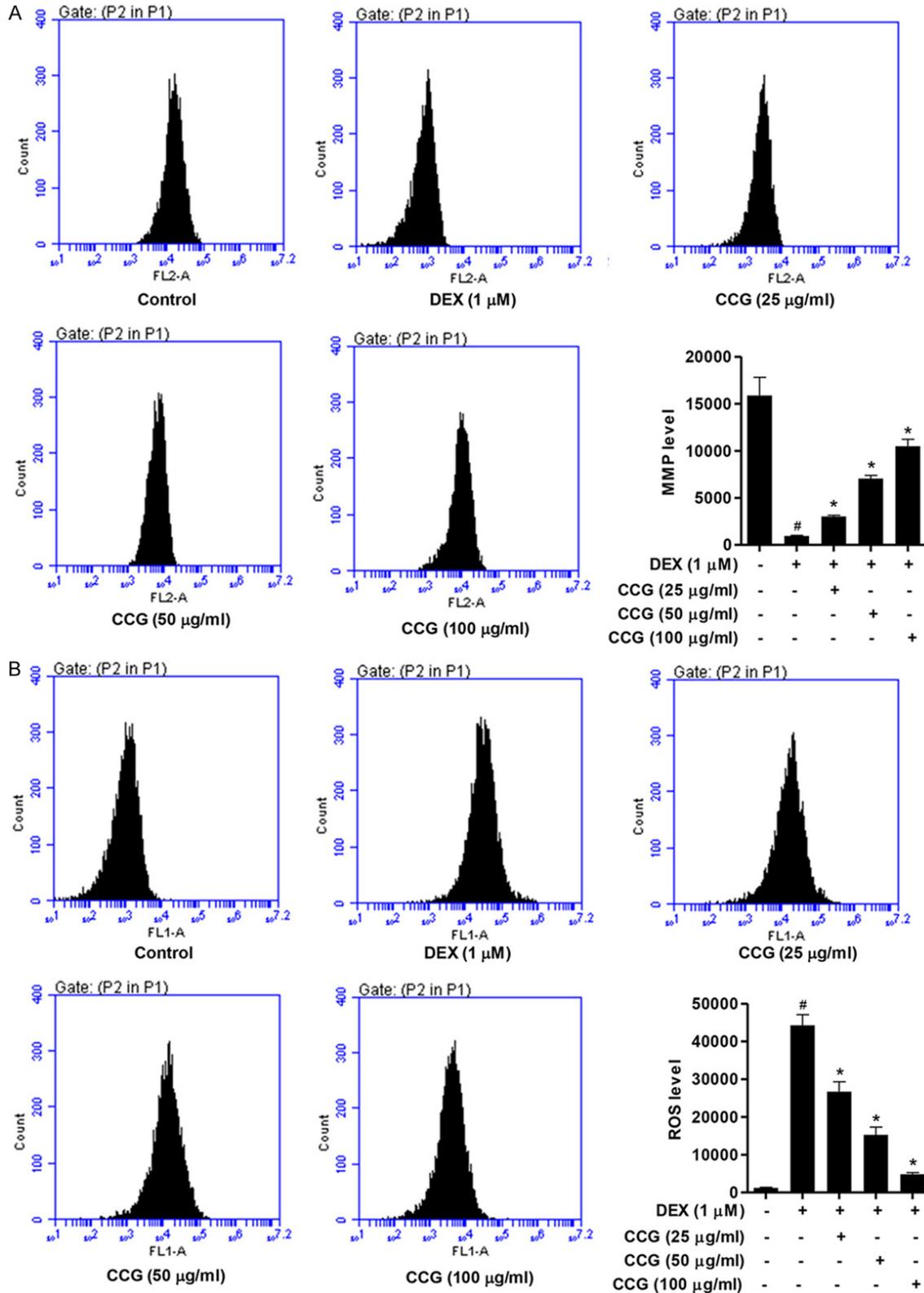
Figure 5. The effects of CCG on MMP and ROS in DEX-induced osteoblasts. A. DEX-induced osteoblasts were pretreated with CCG for 24 h at 25, 50 and 100 μg/mL respectively, then incubated with TMRM and analyzed by flow cytometry. B. DEX-induced osteoblasts were pretreated with CCG for 24 h at 25, 50 and 100 μg/mL respectively, and fluorescence probe DCFH-DA was used to determine the levels of ROS production. The data were presented as the mean ± SD (n=3); #$P<0.05$ vs. control without any treatment group, *$P<0.05$ vs. the DEX only group.
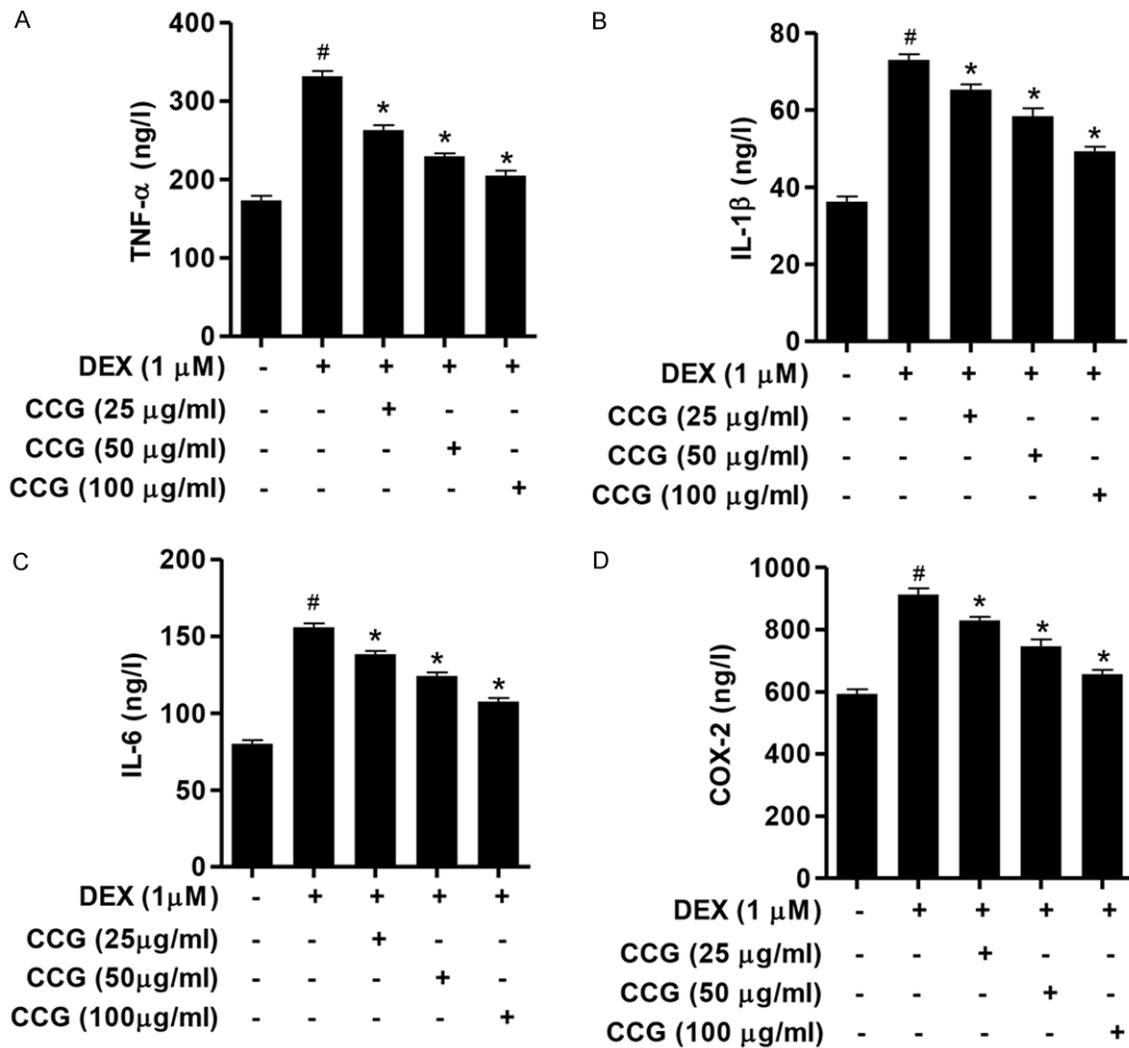
Figure 6. The effects of CCG on the expression of pro-inflammatory cytokines. ELISA analysis of TNF-α (A), IL-1β (B), IL-6 (C) and COX-2 (D) protein expression in five treatment groups. The data were presented as the mean ± SD (n=3); $^{\#}P<0.05$ vs. control without any treatment group, $^{*}P<0.05$ vs. the DEX only group.

the Student's t test. Differences were considered significant at values of $P<0.05$.

Results

CCG protects osteoblasts against DEX-induced cell injury

The dose-dependent effects of CCG and DEX on cell proliferation were first investigated. Treatment with DEX (0.01-10 μM) significantly decreased cell proliferation in dose- and time-dependent manners and treatment with 1 μM of DEX decreased cell proliferation to 35.7% of the control (**Figure 2A**). Interestingly, pretreatment with CCG at dose of 25, 50 and 100 μg/ ml for 24 h notably protected osteoblasts against DEX (1 μM)-induced cell injury and showed dose-dependent manners (**Figure 2B**).

CCG attenuates DEX-suppressed cell differentiation

ALP is the earliest marker of osteoblasts differentiation. As shown in **Figure 3A**, there was a slight decrease in ALP activity of DEX-induced osteoblasts. Interestingly, pretreatment of osteoblasts with CCG at dose of 25, 50 and 100 μg/ml for 24 h significantly attenuated DEX down-regulated the activity of ALP (**Figure 3B**). To further investigate the protective effects of CCG on osteoblasts differentiation, we also

examined the effects of CCG on the expression of differentiation associated markers under DEX condition. As shown in **Figure 4**, treatment with DEX (1 µM) stimulated the expression of RANKL and RANK, and inhibited the expression of OPG, BMP-2, β-catenin, IGF-1 and M-CSF. However, CCG at dose of 25, 50 and 100 µg/mL significantly down-regulated DEX-stimulated expression of RANKL and RANK and DEX-suppressed expression of OPG, BMP-2, β-catenin, IGF-1 and M-CSF in osteoblasts, suggesting that CCG may protect osteoblasts against injury and promote proliferation and differentiation.

CCG inhibits DEX-induced apoptosis in the mitochondrial pathway

Loss of MMP is related to the mitochondrial apoptotic pathway. To assess the effect of CCG on the changes of MMP in osteoblasts, FCM analysis was carried out to detect the fluorescence intensity of Rho-123. As shown in **Figure 5A**, treatment of osteoblasts with DEX (1 µM) caused a moderate depolarization of MMP. However, treatment with CCG at indicated concentrations for 24 h significantly increased the MMP levels in a dose-dependent manner.

On the other hand, ROS generation is also linked to mitochondria. Fluorescence probe DCFH-DA was used to determine the levels of ROS production in osteoblasts. As shown in **Figure 5B**, osteoblasts treatment with DEX (1 µM) caused a significant increase in the intracellular accumulation of ROS. However, treatment with CCG at indicated concentrations for 24 h significantly decreased the ROS accumulation in a dose-dependent manner.

Effects of CCG on the expression of pro-inflammatory cytokines in DEX-induced osteoblasts

To elucidate the effect of CCG on inflammatory responses under DEX condition, we investigated the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and COX-2. As shown in **Figure 6**, treatment with DEX (1 µM) increased the expression of TNF-α, IL-1β, IL-6 and COX-2. Pretreatment with CCG at indicated concentrations reversed DEX-induced production of TNF-α, IL-1β, IL-6 and COX-2 in osteoblasts compared with DEX treatment group. These data suggest that CCG may protect osteoblasts against inflammation.

Discussion

As we know, glucocorticoids are well known to be a potent stimulator of osteoblastic differentiation, so we utilized DEX, a synthetic glucocorticoid, to induce osteoblasts [9, 16, 17]. In this study, we first investigated the effect of various concentrations of DEX on cell proliferation. Treatment cells with DEX significant proliferation inhibition in a dose-dependent manner. In preliminary experiments, we found that when osteoblasts were treated simultaneously with CCG and H_2O_2, the proliferation of osteoblasts has significant improvement compared with H_2O_2-treated alone [18]. Similarly, pretreatment with CCG for 24 h significantly protected osteoblasts against DEX-induced cell injury with dose-dependent manners. This result suggests that CCG protects against osteoporosis through enhancing cell proliferation not only in H_2O_2-induced but also in DEX-induced osteoblasts.

Osteoblastic bone formation is thought to be mediated by the formation of new osteoblasts and the activity of osteoblasts to produce bone matrix [19, 20]. ALP is an important enzyme in the process of bone remodeling [21], which responsible for mineralization of the matrix, thus increasing the phosphorous concentration. Previous studies showed that DEX suppressed the differentiation process of osteoblasts in bone marrow-derived human mesenchymal stem cells and MC3T3-E1 cells [22, 23]. In this study, we found that DEX significantly suppressed activity of ALP, which was reversed by CCG. These results demonstrate that CCG may promote osteoblasts differentiation.

To confirm this, we further measured the expression of differentiation associated markers, such as bone morphogenetic protein (BMP-2), β-catenin, receptor activator of NF-κB (RANK), receptor activators of NF-κB ligand (RANKL), osteoprotegerin (OPG), insulin-like growth factor-I (IGF-1) and macrophage-colony stimulating factor (M-CSF). BMP-2 and β-catenin, produced and secreted by osteoblasts, have been shown to have independent effects in regulating osteoblasts proliferation and mineralization [24, 25]. Osteoblasts are not only involved in bone formation but also modulate the formation, differentiation and bone resorbing activity of osteoclasts by production of RANKL and OPG [26]. RANKL pro-

vides a signal to combine with RANK, thus activating osteoclast differentiation and function, however, OPG blocks the interaction between RANKL and the RANK receptor. In other words, OPG inhibits osteoclastogenesis while RANKL supports bone resorption of osteoclast [27]. Therefore, the relative ratio of OPG to RANKL can be an assessment for bone remodeling. In the present study, CCG significant increase in the expression of BMP-2, β-catenin, IGF-1 and M-CSF, and the relative ratio of OPG to RANKL, indicating that CCG may act on osteoclasts to alter RANKL and inhibit osteoclastogenesis.

Mitochondria play a crucial role in the complex process of cell apoptosis [28]. During this process, mitochondrial membrane pores are opened, resulting in the loss of mitochondrial membrane potential (MMP) [29]. ROS are chemically reactive molecules obtained from oxygen, and are capable of causing oxidative damage to biomacromolecules [30, 31]. We examined the MMP and the intracellular generation of ROS, and found that CCG increased DEX-suppressed MMP and reduced DEX-stimulated ROS production. In the primary mouse BMSCs and human osteoblast-like MG63 cell line, the elevation of ROS level stimulated RANKL expression [32] and ethanol-induced RANKL expression relied on increased intracellular levels of ROS in osteoclast precursors from BMSCs [33].

Osteoclasts are influenced by a variety of pro-inflammatory osteoclastogenic and anti-osteoclastogenic cytokines that can either stimulate or suppress their activity [34]. Ralston reported that the expression of osteoclastogenic cytokines TNF-α, IL-1β and IL-6 is more remarkably in women with osteoporosis fractures than in normal postmenopausal women [35]. Our results indicated that higher level of TNF-α, IL-1β, IL-6 and COX-2 in DEX-induced osteoblasts suggest enhanced osteoclastogenesis, leading to increased bone loss [36-38]. Interestingly, pretreatment with CCG reversed the higher level of the osteoclastogenic cytokines, suggesting that CCG may protect against osteoporosis via inhibition of pro-inflammatory osteoclastogenic cytokines expression.

In summary, CCG reversed the dysfunction induced by DEX in osteoblasts and its effect was associated with promotion of bone formation. In addition, our study suggests that these effects mediated by CCG via differentiation associated markers and pro-inflammatory cytokines may play key roles in the protection of osteoblasts. These results provide new insights into the osteoblast-protective mechanisms of CCG, implying that CCG may be developed for the prevention and treatment of osteoporosis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yun-Gen Hu, Department of Orthopaedic Surgery, Xiaoshan Traditional Chinese Medical Hospital, 152 Yucai Road, Xiaoshan Distract, Hangzhou 311200, China. Tel: +86-571-83815098; Fax: +86-571-83815098; E-mail: hu_yungen@163.com

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