



## Crocin attenuates methylglyoxal-induced osteoclast dysfunction by regulating glyoxalase, oxidative stress, and mitochondrial function



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### ABSTRACT

Methylglyoxal (MG), a highly reactive dicarbonyl compound, is a major cell-permeant precursor of advanced glycation end-products, which are associated with several conditions, including diabetes and degenerative diseases. Crocin, a constituent of saffron, is involved in many pharmacological activities. Recent studies have reported that crocin exerts protective effects against bone diseases. Osteoclasts are multinucleated cells derived from hematopoietic stem cells that are responsible for bone resorption. The up- or down-regulation of their proliferation and differentiation is often associated with many bone-related diseases. The present study aimed to investigate the effects of crocin on osteoclast differentiation and to clarify its mechanism of action in the presence of MG. We demonstrated that crocin reversed MG-induced inhibition of tartrate-resistant acid phosphatase activity and bone resorption activity in osteoclasts. Quantitative reverse transcription-polymerase chain reaction analysis indicated that crocin treatment decreased the expression of TNF receptor-associated factor-6 (*TRAF6*), *Akt2*, extracellular-signal-regulated kinase-1 (*ERK1*), osteopetrosis-associated transmembrane protein 1 (*OSTM1*), and matrix metalloproteinase 9 (*MMP-9*) genes in the presence of MG. Crocin pretreatment also reversed MG-induced changes in mitochondrial mass, mitochondrial membrane potential, mitochondrial superoxide, and glyoxalase I levels. Taken together, our data suggest that crocin may be a useful therapeutic agent for the treatment of diabetic bone disorders.

### 1. Introduction

Diabetic complications are induced by oxidative stress through advanced glycation end-products (AGEs) derived from the accumulation of methylglyoxal (MG), a highly reactive dicarbonyl compound, as a by-product of glycolysis (Han et al., 2007; Agalou et al., 2003). Diabetes patients are at a higher state of low-turnover osteoporosis due to abnormalities in bone metabolism (Kurra and Siris, 2011). Diabetic osteopathy leads to a higher incidence of bone fractures and delayed healing (Liang et al., 2011). The skeleton undergoes continuous maintenance through a coordinated cellular process known as bone remodeling. This complex process repairs microfractures and maintains bone quality (Martin and Seeman, 2008). Bone remodeling is a highly coordinated process responsible for bone formation and resorption. It is modulated by various factors including inflammation, hormonal changes and lack of mechanical stimulation. Bone remodeling is carefully regulated by clusters of bone-resorbing osteoclasts and bone-forming osteoblasts. Disruption of this balance can lead to pathological

conditions, such as osteoporosis, characterized by low bone density (Seeman and Delmas, 2006), or osteopetrosis, characterized by a lack of bone resorption resulting in increased bone density (Balemans et al., 2005). The osteoblasts express receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B) (RANK) ligand (RANKL) on their surface in response to various stimulating factors. RANKL directly engages a membrane receptor, RANK, on osteoclasts to trigger multiple signaling cascades that stimulate osteoclast function and survival (Asagiri and Takayanagi, 2007). Tartrate-resistant acid phosphatase (TRAP) is a key enzyme, which is highly expressed in osteoclasts and involved in osteoclast-mediated bone resorption.

Natural products have demonstrated potential for treating or preventing some bone disorders. In the search for naturally occurring anti-osteoporosis agents, Hosseinzadeh et al. (2009) identified saffron (*Crocus sativus* L.), which has been used as an herbal food additive, a flavoring, and a coloring ingredient in the food and drug industries. Crocin, a constituent of saffron, is a glycoside carotenoid responsible for its color and appears to possess various health-promoting properties

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**Abbreviations**

MG	methylglyoxal
AGEs	advanced glycation end-products
RANKL	receptor activator of nuclear factor kappa B (NF- $\kappa$ B) ligand
TRAF6	TNF receptor-associated factor-6
ERK1	extracellular-signal-regulated kinase-1
OSTM1	osteopetrosis-associated transmembrane protein 1
MMP-9	matrix metalloproteinase 9

(Hosseinzadeh and Nassiri-Asl, 2013; Alavizadeh and Hosseinzadeh, 2014). Crocin is involved in many pharmacological activities in the nervous system (Hosseinzadeh and Talebzadeh, 2005; Mehri et al., 2012; Hassani et al., 2014), reproductive organs (Hosseinzadeh et al., 2008), and the liver (Lin and Wang, 1986; Jalili et al., 2015). Crocin reduces inflammation (Xu et al., 2009) and ischemia (Hosseinzadeh et al., 2005). It seems that the various beneficial activities of crocin arise from its antioxidant properties (Bakhtiary et al., 2014; Bandegi et al., 2014; Rashedinia et al., 2015). Crocin revitalizes arthritis-induced cartilage and bone deterioration along with inflammation and oxidative damage (Hemshekhar et al., 2012). Considering that oxidative stress plays a critical role in the osteoporotic process (Manolagas, 2010), the antioxidant properties of crocin may improve osteoporosis. Cao et al. (2014) reported that the administration of crocin prevented ovariectomy-induced osteoporosis in rats without hyperplastic effects on the uterus, which may be attributed, at least partially, to the antioxidant properties of crocin. However, the effects of crocin on osteoclasts have not been investigated. Therefore, in the present study, we investigated the effects of crocin on receptor activator of nuclear factor kappa B (NF- $\kappa$ B) ligand (RANKL)-induced osteoclast differentiation in the presence of MG and further investigated the mechanism underlying this process.

**2. Materials and methods****2.1. Materials**

Crocin was purchased from ChromaDex Inc. (Irvine, CA, USA). Methylglyoxal was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest commercial grade available and purchased from Sigma (St. Louis, MO, USA).

**2.2. Osteoclast differentiation of RAW264.7 cells**

The RAW264.7 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin) and 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C in 5% CO<sub>2</sub> humidified air. The cells (2 × 10<sup>4</sup> cells/well) were seeded in 24-well plates and incubated to reach ~70% confluence. The cells were then cultured in DMEM

containing 50 ng/mL RANKL (R&D Systems, Minneapolis, MN, USA) and different concentrations of crocin and/or 200  $\mu$ M methylglyoxal for an additional 3 days (Suh et al., 2018).

**2.3. Tartrate-resistant acid phosphatase (TRAP) activity assay**

At the end of the culturing period, the medium was removed, and the cells were gently washed twice with cold phosphate-buffered saline (PBS). The cells were then lysed with 0.05% Triton-X100 at 4 °C. To measure TRAP activity, a marker of early phase of osteoclastogenesis, in the cell lysate, an Acid Phosphatase Assay Kit (BioVision, Inc., Milpitas, CA, USA) was used. The kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate, which turns yellow ( $\lambda_{\text{max}} = 405 \text{ nm}$ ) when it is dephosphorylated by acid phosphatase. Additionally, the total protein in the cell lysate was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA), and TRAP activity was adjusted by protein level. To confirm the generation of multinucleated osteoclasts, cells were fixed with 3.7% formalin (Sigma), permeabilized with 0.1% Triton X-100 and finally stained for TRAP with the leukocyte acid phosphatase kit (Sigma). The images of TRAP-positive cells were captured under a light microscope (Olympus, Japan).

**2.4. Cytotoxicity analysis**

The cytotoxic effects of crocin were detected using the WST-1 assay (Roche Diagnostics, Mannheim, Germany). RAW 264.7 cells were cultured under the same conditions used for the osteoclastogenesis assay. Then, WST-1 solution (10%) was added to each well and gently mixed. After a 3-h incubation at 37 °C in an atmosphere containing 5% CO<sub>2</sub>, the absorbance at 440 nm was measured in a microplate reader with a reference wavelength of 690 nm.

**2.5. Osteoclast bone resorbing activity**

RAW 264.7 cells were seeded onto an OsteoLyse plate and incubated to reach ~70% confluence. The cells were then cultured in DMEM containing 50 ng/mL RANKL and pretreated with different concentrations of crocin. After 1 h, 200  $\mu$ M MG was added to the wells, and the cells were cultured for 3 days, followed by RANKL treatment for an additional 3 days. Quantification of bone resorption by osteoclasts *in vitro* was measured using the OsteoLyse Assay Kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The OsteoLyse Assay Kit provides an easy-to-use protocol for quantitatively measuring *in vitro* osteoclast-mediated bone resorption in a high-throughput format. The assay directly measures the release of europium-labeled collagen fragments into the osteoclast cell culture supernatant via time-resolved fluorescence, indicating their resorption activity levels.

**2.6. RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After isolation, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies,

**Table 1**  
Primer sequences.

Genes	Forward primer	Reverse primer
AKT2	5'-CGA CCC AAC ACC TTT GTC A-3'	5'-GAT AGC CCG CAT CCA CTC T-3'
ERK1	5'-TGG AAG CCA TGA GAG ATG TTT-3'	5'-GCT CAG CTG CTG GCT TTT A-3'
HPRT	5'-TCC TCC TCA GAC CGC TTT T-3'	5'-CCT GGT TCA TCA TCG CTA ATC-3'
MMP-9	5'-ACG ACA TAG ACG GCA TCC A-3'	5'-GCT GTG GTT CAG TTG TGG TG-3'
OSTM1	5'-GGT CTC TGA GTT TTT CAA CAG CA-3'	5'-CCT CAC CAT TGT TTG TTA GGC-3'
Traf6	5'-TTG CAC ATT CAG TGT TTT TGG-3'	5'-TGC AAG TGT CGT GCC AAG-3'

Santa Clara, CA, USA). cDNAs were synthesized with a Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, GmbH Mannheim, Germany) and stored at  $-70^{\circ}\text{C}$  until further processing. All procedures were in accord with the manufacturer's instructions. Real-time (RT) PCR was performed to verify the differential expression of selected genes using a Roche LightCycler 480 system (Roche Diagnostics, GmbH Mannheim, Germany) and the Taqman method using a Roche Universal ProbeLibrary (UPL) kit. Relative gene expression was determined by employing the comparative CT method (Macdonald et al., 2001). All reactions were carried out in a total volume of 20  $\mu\text{L}$  of reaction mixture containing 10.0  $\mu\text{L}$   $2\times$  UPL master mix, 1.0  $\mu\text{L}$  5' primer (10 pmol/ $\mu\text{L}$ ), 1.0  $\mu\text{L}$  3' primer (10 pmol/ $\mu\text{L}$ ), 0.2  $\mu\text{L}$  UPL probe, 1.0  $\mu\text{L}$  cDNA, and 6.8  $\mu\text{L}$  sterile water. The thermal cycling conditions for PCR were an initial denaturation for 10 min at  $95^{\circ}\text{C}$ , followed by 40 cycles of  $94^{\circ}\text{C}$  for 10 s and  $60^{\circ}\text{C}$  for 30 s. The primers summarized in Table 1 were designed using the Roche ProbeFinder assay tool. The values obtained from each sample were normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression. The expression levels of each gene in all experimental groups were compared to the expression levels of the control group.

### 2.7. Mitochondrial mass detection using Mitotracker Green (MTG)

For relative mitochondrial function, staining with MTG from Invitrogen Molecular Probes (Invitrogen) was utilized. MTG fluoresces in the mitochondrial lipid environment regardless of the membrane potential and serves as the dye for mitochondrial physical mass quantification. RAW264.7 cells were seeded into black 96-well plates and incubated to reach  $\sim 70\%$  confluence. The cells were then cultured in DMEM containing 50 ng/mL RANKL and different concentrations of crocin and/or 200  $\mu\text{M}$  methylglyoxal for an additional 3 days. After two washes with warm  $1\times$  PBS, cells were stained with 200 nM MTG in DPBS. Cells were then incubated for 45 min at  $37^{\circ}\text{C}$  in standard culture conditions. The intensities of MTG (excitation, 490 nm; emission, 516 nm) were measured, which reflect mitochondrial mass.

### 2.8. Determination of mitochondrial membrane potential

The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) was used to demonstrate changes in the mitochondrial membrane potential (MMP) in cells. JC-1 is a lipophilic and cationic dye that permeates plasma and mitochondrial membranes. The dye fluoresces red when it aggregates in healthy mitochondria with a high membrane potential, whereas it appears in a monomeric form and fluoresces green in mitochondria with diminished membrane potential. RAW264.7 cells were seeded into black 96-well plates and incubated to reach  $\sim 70\%$  confluence. The cells were then cultured in DMEM containing 50 ng/mL RANKL and different concentrations of crocin and/or 200  $\mu\text{M}$  methylglyoxal for an additional 3 days. Cells were incubated with the MMP-sensitive fluorescent dye JC-1 for 20 min at  $37^{\circ}\text{C}$  and washed twice in PBS; red (excitation, 550 nm; emission, 600 nm) and green (excitation, 485 nm; emission, 535 nm) fluorescence were then measured using a fluorescence microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). Mitochondrial depolarization (i.e., loss of MMP) manifests as a decrease in the red/green fluorescence ratio.

### 2.9. Measurement of mitochondrial superoxide

Mitochondrial superoxide levels were detected using MitoSOX Red mitochondrial superoxide indicator (Invitrogen). MitoSOX Red (excitation, 510 nm; emission, 580 nm) is a fluorogenic dye for highly selective detection of superoxide in the mitochondria of cells (Schroeder et al., 2007). RAW264.7 cells were seeded into black 96-well plates and incubated to reach  $\sim 70\%$  confluence. The cells were then cultured in DMEM containing 50 ng/mL RANKL and different concentrations of

crocin and/or 200  $\mu\text{M}$  methylglyoxal for an additional 3 days. Cells were incubated with 2  $\mu\text{M}$  MitoSOX Red at  $37^{\circ}\text{C}$  for 20 min according to the manufacturer's instructions. After the cells were washed, the MitoSOX Red fluorescence was detected.

### 2.10. Measurement of glyoxalase I levels

RAW264.7 cells were seeded in 24-well plates and incubated to reach  $\sim 70\%$  confluence. The cells were then cultured in DMEM containing 50 ng/mL RANKL and different concentrations of crocin and/or 200  $\mu\text{M}$  methylglyoxal for an additional 3 days. Cells were lysed by repeating a freeze/thaw cycle three times. After centrifugation at  $10,000\times g$  for 15 min at  $4^{\circ}\text{C}$ , the supernatants of the cell lysates were assayed. Glyoxalase I was determined using a Mouse Glyoxalase I (GLO1) ELISA Kit (MyBioSource, Inc., San Diego, CA, USA), according to the manufacturer's instructions. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories).

### 2.11. Statistical analysis

Results are expressed as means  $\pm$  standard error of the mean (SEM). Statistical significance was determined using analysis of variance with subsequent application of Dunnett's *t*-test ( $P < 0.05$ ).

## 3. Results

### 3.1. Effects of crocin on RANKL-induced osteoclast differentiation

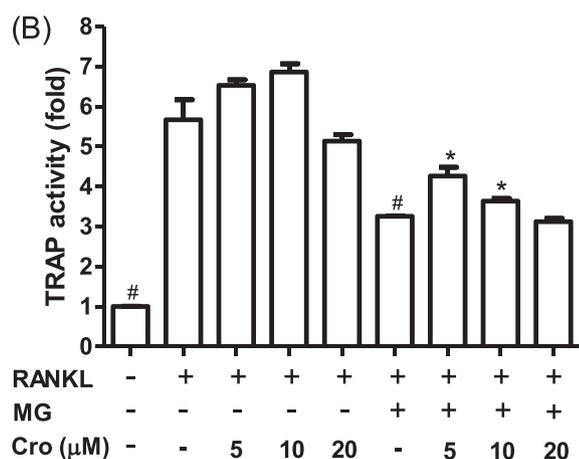
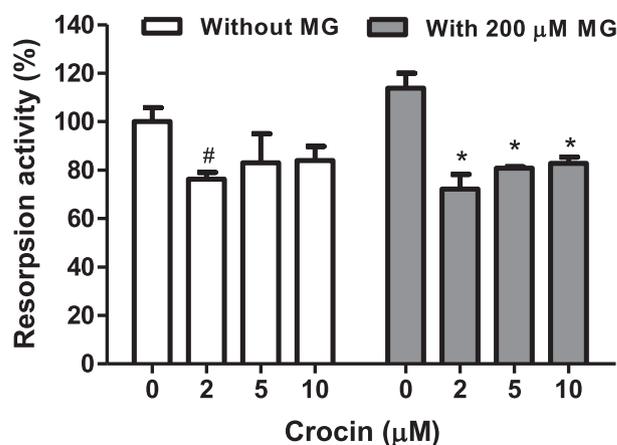
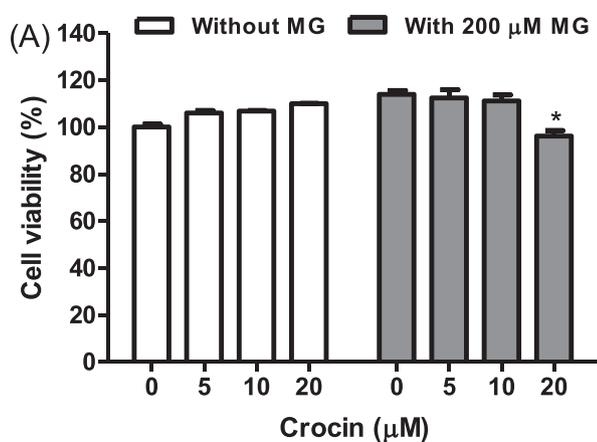
To examine the effects of crocin on cell viability during RANKL stimulation, RAW264.7 macrophages were treated with 5–20  $\mu\text{M}$  crocin in the presence of RANKL. As shown in Fig. 1A, crocin at a concentration of less than 20  $\mu\text{M}$  did not affect cell viability after 3 days. When 200  $\mu\text{M}$  MG was added, treatment with 20  $\mu\text{M}$  crocin decreased cell viability compared with cells treated with MG alone. We measured the activity of TRAP, a marker enzyme of osteoclasts, in RAW264.7 cells to determine the effects of crocin on osteoclastogenesis. As shown in Fig. 1B, MG at a concentration of 200  $\mu\text{M}$  inhibited TRAP activity in RANKL-stimulated RAW264.7 cells. However, crocin (2 and 5  $\mu\text{M}$ ) pretreatment inhibited MG-induced reductions in TRAP activity. The microscopic photographs are shown in Fig. 1C, and it is evident that the sizes of giant cells are extremely large in RANKL-stimulated RAW264.7 cells. However, the treatment with 200  $\mu\text{M}$  MG inhibited RANKL-induced formation of TRAP-positive cells and pre-treatment with crocin (5  $\mu\text{M}$ ) inhibited MG-induced reduction in the formation of TRAP-positive cells.

### 3.2. Effects of crocin on the bone resorbing activity of RANKL-induced osteoclasts

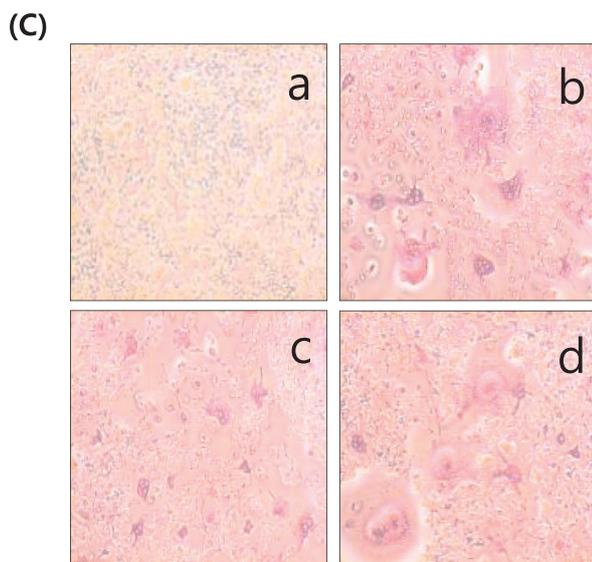
We then used an OsteoLyse Assay Kit to further examine whether MG has an effect on the ability of mature osteoclasts to resorb bone. RAW264.7 cells were incubated in media containing 50 ng/mL RANKL and pretreated with different concentrations of crocin. After 1 h, 200  $\mu\text{M}$  MG was added to the wells, and the cells were cultured for 3 days, followed by RANKL treatment for an additional 3 days. As shown in Fig. 2, treatment with 2  $\mu\text{M}$  crocin significantly reduced the bone resorbing activity of osteoclasts. Crocin treatment (2–10  $\mu\text{M}$ ) also decreased bone resorbing activity in the presence of 200  $\mu\text{M}$  MG.

### 3.3. Effects of crocin on osteoclast-specific gene expression in the presence of MG

We investigated the effects of crocin on the expression of key signaling molecules activated by RANKL signaling in the presence of MG. RAW264.7 cells were incubated to reach  $\sim 70\%$  confluence. The cells



RANKL	MG	Cro (μM)
-	-	-
+	-	-
+	-	5
+	-	10
+	-	20
+	+	-
+	+	5
+	+	10
+	+	20



**Fig. 1.** Effect of crocin on RANKL-induced osteoclast differentiation in RAW264.7 cells. RAW264.7 cells were incubated to reach ~70% confluence. Following that, the cells were cultured in DMEM containing 50 ng/mL RANKL. Cells were pretreated with different concentrations of crocin. After 1 h, 200 μM MG was added to wells and the cells were cultured for 3 days. (A) cell viability, (B) tartrate-resistant acid phosphatase (TRAP) activity. (C) Photograph of TRAP staining (100×). (a) vehicle; (b) RANKL (50 ng/mL); (c) RANKL (50 ng/mL) + MG (200 μM); (d) RANKL (50 ng/mL) + Crocin (5 μM) + MG (200 μM). #*P* < 0.05, control vs. MG; \**P* < 0.05, MG vs. crocin (Cro).

**Fig. 2.** Effect of crocin on osteoclast bone resorbing activity in RANKL-induced osteoclast differentiation. RAW264.7 cells were cultured in DMEM containing 50 ng/mL RANKL. Cells were pretreated with different concentrations of crocin. After 1 h, 200 μM MG was added to wells and the cells were cultured for 3 days, followed by RANKL treatment for an additional 3 days. Osteoclast bone resorbing activity was measured using OsteoLyse™ Assay Kit. #*P* < 0.05, control vs. MG; \**P* < 0.05, MG vs. crocin.

were pretreated with 2 μM crocin and cultured in media containing 50 ng/mL RANKL. After 1 h, 200 μM MG was added to the wells, and the cells were cultured for 3 days. As shown in Fig. 3, the expression levels of TNF receptor-associated factor-6 (*TRAF6*), *Akt2*, extracellular-signal-regulated kinase-1 (*ERK1*), and osteopetrosis-associated transmembrane protein 1 (*OSTMI*) genes were significantly increased following treatment with 200 μM MG. However, crocin treatment significantly reversed MG-induced expression of these genes. Additionally, 2 μM crocin decreased the expression of matrix metalloproteinase 9 (*MMP-9*) in the presence of MG.

#### 3.4. Effects of crocin on mitochondrial function and superoxide production in the presence of MG

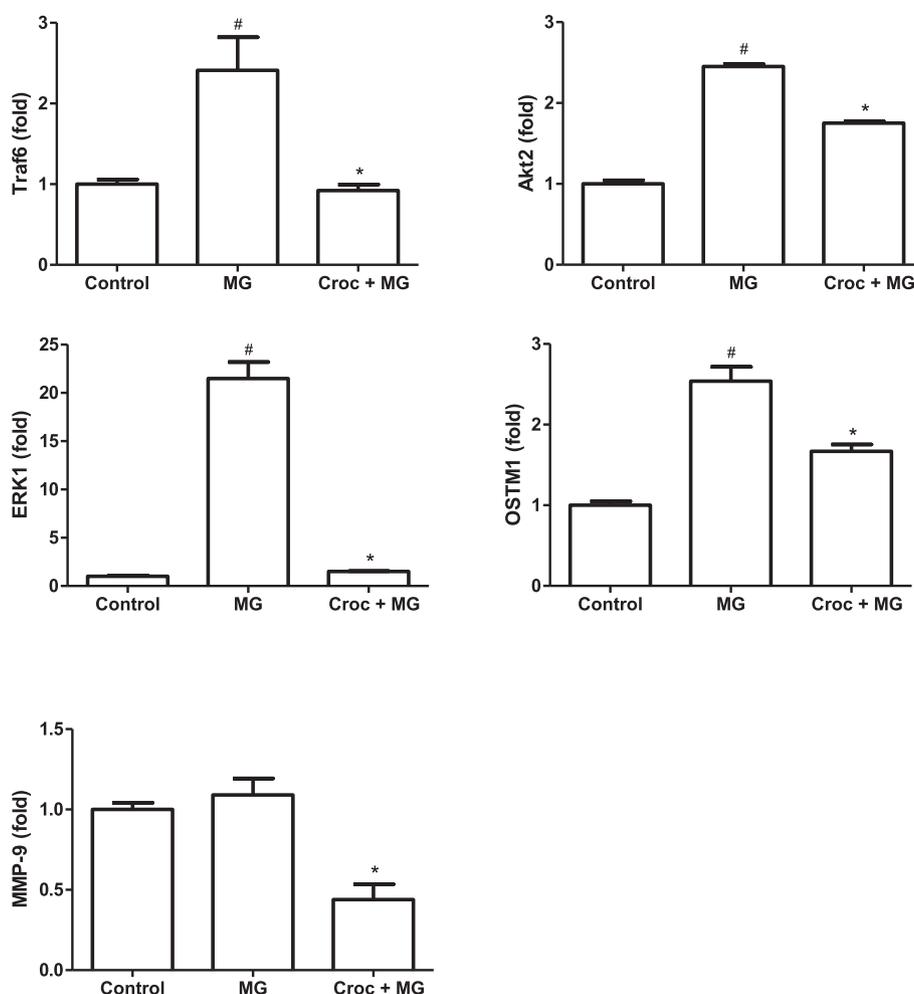
The mitochondrial mass and mitochondrial membrane potential were analyzed using an established method involving fluorescence staining with MitoTracker Green and JC-1. The mitochondrial mass (Fig. 4A) was lower in cells treated with 200 μM MG than in control group cells. However, crocin (2–10 μM) pretreatment reversed this reduction. Mitochondrial membrane potential was also decreased by treatment with 200 μM MG (Fig. 4B), which was reversed by crocin (2–10 μM) pretreatment. To examine mitochondrial superoxide levels, we used MitoSOX (Fig. 4C). Cells cultured with MG (200 μM) had significantly higher mitochondrial superoxide levels than untreated cells. However, crocin (2–10 μM) pretreatment decreased MG-induced superoxide levels.

#### 3.5. Effects of crocin on glyoxalase I levels in the presence of MG

The glyoxalase system is a set of enzymes that carry out the detoxification of MG. Glyoxalase I, the key enzyme in the anti-glycation defense, is part of the glyoxalase system. We explored the effects of crocin on the glyoxalase I levels of osteoclasts in the presence of MG. As shown in Fig. 5, treatment with 200 μM MG significantly reduced glyoxalase I levels in osteoclasts. However, pretreatment with crocin (2–10 μM) increased glyoxalase I levels.

## 4. Discussion

In the present study, crocin significantly reversed MG-induced inhibition of TRAP activity in the early phase of osteoclastogenesis. At the same time, crocin inhibited the bone resorption activity of osteoclasts.

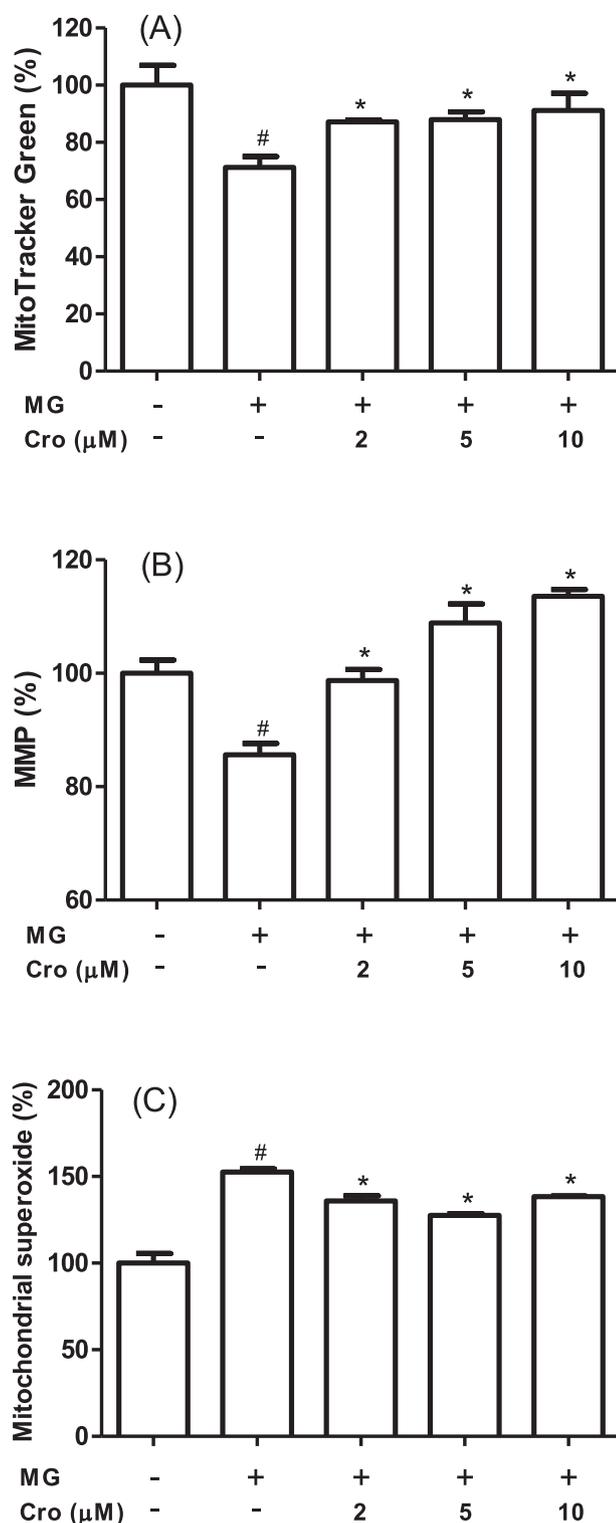


**Fig. 3.** Effect of crocin on the expression of key signaling molecules involved in osteoclastogenesis. RAW264.7 cells were incubated to reach ~70% confluence. Following that, the cells were cultured in DMEM containing 50 ng/mL RANKL. Cells were pretreated with 2  $\mu$ M crocin. After 1 h, 200  $\mu$ M MG was added to wells and the cells were cultured for 3 days. Gene expression was detected by real-time QPCR. <sup>#</sup> $P < 0.05$ , control vs. MG; <sup>\*</sup> $P < 0.05$ , MG vs. crocin.

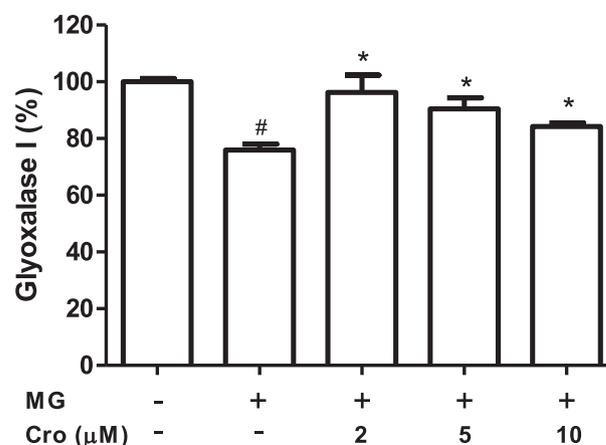
To elucidate the inhibitory mechanism of crocin on bone resorption, we examined its effects on the expression of signaling molecules. The binding of RANKL to its receptor RANK results in the recruitment of TRAF6, which activates mitogen-activated protein kinases (MAPKs). This subsequently induces the formation of mature, active osteoclasts (Lee and Kim, 2003). Kim et al. (2005) previously reported that TRAF6-deficient mice show severe osteopetrosis and defective osteoclast formation owing to abrogated RANK signaling. ERK and Akt have been reported to play critical roles in cell survival and may also be involved in the process of osteoclast differentiation (Miyazaki et al., 2000). Our results showed that crocin significantly inhibited MG-induced expression of TRAF6, ERK, and Akt. This suggests that the assembly of a multiprotein complex including RANK and TRAF6 may be facilitated by osteoclastogenic signals and that crocin may inhibit this multiprotein complex formation and consequently reduce RANK downstream signaling, possibly through the inhibition of TRAF6. Therefore, a reasonable explanation for anti-resorption may be that crocin suppresses upstream ERK and Akt expression. It has been shown in rat calvarial organ culture that treatment with PD98059, an ERK inhibitor, promotes the apoptosis of osteoclasts and the loss of ruffled borders (Nakamura et al., 2003), suggesting that ERK may be partially involved in osteoclast activity. As crocin inhibits ERK expression, it is also possible that crocin may influence osteoclast microstructures, such as the ruffled border, thereby influencing bone resorption. Based on our results, we suggest that crocin may inhibit bone resorption by inhibiting RANKL-induced activation of adaptor molecules, such as TRAF6, and osteoclast

survival-related signaling molecules, such as ERK/Akt.

Mature osteoclasts are also characterized by high expression of a series of osteoclast markers, of which OSTM1 and MMP-9 are the most prominent (Segovia-Silvestre et al., 2009). In this study, we showed that crocin also inhibits the expression of the above osteoclast-specific genes that play important roles in bone resorption. Moreover, the bone resorption assay revealed the inhibitory effects of crocin on bone resorption, suggesting that it may result from the potential of crocin to inhibit the RANKL-induced expression of bone resorption-related genes, such as *OSTM1* and *MMP-9*. *OSTM1* is important in the process of proton secretion. Mice deficient for the *OSTM1* gene develop severe osteopetrosis because their osteoclasts cannot secrete acid and thus cannot dissolve bone (Rousselle and Heymann, 2002). Thus, our data indicate that crocin regulates osteoclast acidification. Once mineralized compartments are dissolved by acidification, osteoclasts degrade the organic matrix in bone by secreting proteolytic enzymes, such as MMPs. Gelatinases, such as MMP-9, have been shown to be involved in cytokine-induced degradation of the bone matrix (Kusano et al., 1998). MMP-9 is an efficient collagenase that cleaves both collagen types I and II (Liu et al., 2003). Significant and dramatic reduction of *MMP-9* was also noted in crocin-treated cells in the presence of MG. Our observations demonstrate that crocin modulates the degradation of the organic matrix through the downregulation of *MMP-9*. This suggests that the inhibitory effects of crocin on bone resorption may result from its potential to inhibit the induction of bone resorption-related osteoclastic genes, such as *OSTM1* and *MMP-9*.



**Fig. 4.** Effects of crocin on mitochondrial function and ROS production in osteoclasts. RAW264.7 cells were seeded in black 96 well plate and incubated to reach ~70% confluence. Following that, the cells were cultured in DMEM containing 50 ng/mL RANKL. Cells were pretreated with different concentrations of crocin. After 1 h, 200 μM MG was added to wells and the cells were cultured for 3 days. The mitochondrial mass (A), mitochondrial membrane potential (MMP) (B), and mitochondrial superoxide levels (C) were analyzed using an established method involving fluorescence staining with MitoTracker<sup>®</sup> Green, JC-1, and MitoSOX<sup>™</sup> Red, respectively. <sup>#</sup>*P* < 0.05, control vs. MG; <sup>\*</sup>*P* < 0.05, MG vs. crocin.



**Fig. 5.** Effect of crocin on glyoxalase I levels in osteoclasts. RAW264.7 cells were incubated to reach ~70% confluence. Following that, the cells were cultured in DMEM containing 50 ng/mL RANKL. Cells were pretreated with different concentrations of crocin. After 1 h, 200 μM MG was added to wells and the cells were cultured for 3 days. Cellular glyoxalase I level was detected using ELISA kit. <sup>#</sup>*P* < 0.05, control vs. MG; <sup>\*</sup>*P* < 0.05, MG vs. crocin.

Osteoclasts are essential for physiological bone remodeling, a deficiency that can cause osteopetrosis. However, excessive osteoclasts can cause osteoporosis. Miyazaki (2013) revealed that ATP depletion following mitochondrial transcription factor A (Tfam) deficiency leads to increased bone-resorbing activity despite accelerated apoptosis, and the release of endogenous ATP negatively regulates osteoclast function through an autocrine/paracrine feedback loop. Mitochondrial dysfunction triggers specific mitochondrial stress signaling (Biswas et al., 2005). Complexes I and III have been suggested to be the major source of reactive oxygen species (ROS), although other membrane complexes and matrix enzymes also produce ROS, albeit at lower levels (Prabu et al., 2006). Mitochondrially generated ROS is known to damage the electron transport chain complexes, increase lipid peroxidation, inactivate TCA cycle enzymes, and eventually disrupt mitochondrial transmembrane potential. In this study we showed that crocin reverses MG-induced mitochondrial dysfunction and superoxide production. These data strongly indicate that crocin plays an important role in modulating ROS generation by reducing MG-mediated mitochondrial dysfunction.

The glyoxalase system, consisting of the enzymes glyoxalase I and glyoxalase II, is an integral component of cellular metabolism (Thornalley, 1998). A major function of the glyoxalase pathway is the detoxification of α-ketoaldehydes, particularly the cytotoxic metabolite MG. Glyoxalase I catalyzes the first step and hence is a key regulator of the cellular and extracellular levels of MG and MG-derived AGEs. According to Kawatani et al. (2008), glyoxalase I knockdown by siRNA and glyoxalase I inhibitor treatment in bone marrow macrophages inhibited osteoclastogenesis. MG is a highly reactive α-oxoaldehyde and can react with and modify both proteins and nucleic acids, leading to protein cross-linking, gene transcription, and cellular apoptosis (Thornalley, 1998). In the present study, MG treatment suppressed TRAP activity, suggesting that MG inhibits glyoxalase I, thereby allowing the accumulation of MG and resulting in the inhibition of TRAP activity. Crocin pretreatment reversed MG-inhibited glyoxalase I levels, thereby preventing the accumulation of MG. Therefore, crocin may inhibit the MG-modified proteins or nucleic acids that play critical roles in osteoclastogenesis.

## 5. Conclusions

In summary, the present study demonstrated that crocin inhibits the bone-resorbing activity of osteoclasts and suggested that its inhibitory activity may result from its potential to block the expression of

signaling molecules (Traf6, ERK, and Akt) that subsequently regulate the expression of osteoclast-associated genes (OSTM1 and MMP-9). We also demonstrated that crocin reverses MG-induced changes in TRAP activity, mitochondrial mass, mitochondrial membrane potential, mitochondrial superoxide, and glyoxalase I levels during osteoclastogenesis. Taken together, our data suggest that crocin may be a candidate natural compound for the regulation of diabetic bone diseases.

### Conflicts of interest

The authors declare that they have no conflict of interest.

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### Transparency document

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