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# Myriocin alleviates Oleic/Palmitate induced chondrocyte degeneration *via* the suppression of ceramide

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**Abstract. – OBJECTIVE: Abnormal lipid metabolism plays a role that cannot be ignored in articular cartilage bone marrow lesions, synovial inflammation, and the destruction of chondrocytes (CHs). Ceramide is one of the key constructions of membrane lipid bilayers, which is an intracellular lipid mediator regulating varieties of cellular behaviors. The purpose of this study was to explore the role of ceramide and its inhibitor in the development of the CHs degeneration.**

**PATIENTS AND METHODS: CHs were isolated from the cartilage collecting from the osteoarthritis (OA) patients, and oleic acid/palmitic (O/P) acid was used to induce CHs lipid disordered. Then, myriocin was used to inhibit the accumulation of ceramide. After that, the apoptosis, cell viability, glucose uptake, oxidative stress, and the chondrogenic gene expression were tested to evaluate the degenerated degree of CHs.**

**RESULTS: Results revealed that O/P induced CH apoptosis, ceramide accumulation, a higher level of oxidative stress, IL-1β and MMP-13, but it also decreased the collagen-II and SOX-9 expressions and affected the glucose uptake of CHs. After the stimulation of myriocin, the side effects induced by O/P was partly reversed.**

**CONCLUSIONS: O/P induces the accumulation of ceramide and the degeneration of CHs, and myriocin can reject the harmful effect caused by O/P *via* the suppression of ceramide.**

*Key Words:*
Myriocin, Chondrocyte degeneration, Lipid metabolism disorder, Ceramide.

## Introduction

Osteoarthritis (OA), a degenerative disease of the joint mainly occurring in old age, is characterized by local degradation of cartilage with complex etiology. Metabolic syndrome (MetS) refers to a morbid syndrome grouped by multiple metabolic abnormalities such as insulin resistance, hypertension, lipid metabolism disorders, and obesity in the same person. 10% to 30% of the world's population have MetS, but about 59% of them have OA at the same time. Recently, Le Clanche et al[1] have proposed the link between OA and MetS and introduced the concept of metabolic OA. Lipid metabolism in MetS is very complicated. Many metabolites produced by lipids and lipid metabolism disorder can cause a variety of diseases[2]. Liu et al[3] found that high low-density cholesterol (LDLC), high total cholesterol (TC), high triglycerides (TG), and dyslipidemia appear to be associated with OA in women's knees. The importance of lipids in the pathogenesis of OA is that OA chondrocytes (CHs) exhibit intracellular lipid deposition, and the amount of lipid deposition in CHs is positively correlated with the severity of OA[4]. However, the specific mechanism of lipid metabolism in OA is not clear.

Sphingolipids, primary constituents of lipoproteins, together with cholesterol and phospholipids are key elements of cellular membrane lipid bilayers, mediating the lipid synthesis and lipoprotein metabolism[5,6]. Ceramide, an intracellular lipid mediator and the building blocks of sphingolipids, regulates a great variety of cellular responses[7], acting as second messengers of cell signaling in cell cycle regulation, differentiation, senescence, and apoptosis[8]. Villasmil et al[9] reported that ceramide accumulation leads to the cell cycle arrest of yeast. Ceramide is proved to mediate the embryonic stem (ES) cell differentiation[10]. Besides, ceramide channels play a vital



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role in cell oxidative stress[11] and glucose uptake[12], which is closely connected with OA[13,14]. However, the effect of ceramide in the progression of OA remains unknown.

Too many sphingolipids contribute to the damage of cell metabolism, so the application of myriocin the inhibitor of ceramide is widely used in the intervention of lipid metabolism[15,16]. The purpose of this study was to elucidate the effect of ceramide in the lipid excess treated CHs and whether myriocin would play a protective function within this injure to CHs. Here, an oleic acid/palmitic acid (O/P) mixture was used to treat human CHs as simulating lipid disorder *in vitro*. Besides, the cell viability, apoptosis, oxidative stress status, glucose uptake, and the chondrogenic gene levels were focused to indicate the effect of myriocin on the O/P induced CHs degeneration.

## Patients and Methods

### Patient Tissue Samples

This project was approved by the Ethics Committee of The Affiliated Hospital of Xuzhou Medical University. The articular cartilage was collected from 5 OA patients, including 3 males and 2 females with a mean age of years old (39-73 years old) who underwent total knee replacement in our hospital. The outer side of the articular surface was chosen, and the degenerated degree was mild as the source of CHs isolation. Then, the samples were conserved in cold Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 1% penicillin/streptomycin for the following investigations.

### CHs Isolation

Cartilage was removed from the articular, cut into small pieces and washed with phosphate-buffered saline (PBS). Then, the fragment was digested with 0.2% trypsin and 0.2% type XI collagenase at 37°C overnight. CHs were obtained after filtrating from a cell strainer and resuspend with DMEM containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Finally, cells were seeded in 75 mm flasks and passaged to the first generation for the cell treatments.

### Oleic Acid/Palmitic Acid (O/P) Preparation and Cell Treatments

Before use, 640 μL or 320 μL oleic acid (0.25 M, Sigma-Aldrich, St. Louis, MO, USA) and palmitic acid (0.25 M, Sigma-Aldrich, St. Louis, MO, USA) stock were diluted into 20 mL 5% bovine serum albumin (BSA) to make 8 mM and 4 mM solution, respectively. Then, equal volumes of oleic acid and palmitic acid solution were mixed with the culture medium to a total concentration of from 10 μM to 1 mM (2:1, M/M). Next, CHs were treated with 0.5 mM O/P solution from 6 hours to 48 hours or treated different concentrations of O/P for 24 h. For the myriocin treatments, CHs were treated with or without O/P (0.5 mM) for 24 h and then treated with myriocin (2 mM) for another 24 hours.

### Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from CHs with or without treatments using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was reversely transcribed to complementary deoxyribose nucleic acid (cDNA) by PrimeScript Master (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using SYBR Green (Invitrogen, Carlsbad, CA, USA) to quantify collagen-II, COX-9, IL-1β, MMP-13, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expressions. The sequences used for RT-PCR are listed as Table I. Relative mRNA expression levels were calculated by the normalization to GAPDH by the $2^{-\Delta\Delta Ct}$ methods.

**Table I.** Primer sequences of the genes for RT-PCR.

| Gene name | Forward (5′>3′) | Reverse (5′>3′) |
|---|---|---|
| IL-1β | ATGATGGCTTATTACAGTGGCAA | GTCGGAGATTCGTAGCTGGA |
| MMP-13 | ACTGAGAGGCTCCGAGAAATG | GAACCCCGCATCTTGGCTT |
| Collagen-II | TGGACGATCAGGCGAAACC | GCTGCGGATGCTCTCAATCT |
| SOX-9 | AGCGAACGCACATCAAGAC | CTGTAGGCGATCTGTTGGGG |
| CAT | TGGAGCTGGTAACCCAGTAGG | CCTTTGCCTTGGAGTATTTGGTA |
| GSH | GGTGGGCCAAAGGATGAAGAG | CCACAAGCCAAACGACTTCC |
| GPX1 | CAGTCGGTGTATGCCTTCTCG | GAGGGACGCCACATTCTCG |
| GAPDH | ACAACTTTGGTATCGTGGAAGG | GCCATCACGCCACAGTTTC |

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

### Western Blot (WB) Analysis

Protein extraction of CHs was using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) according to the manufacturer's protocol. Concentrations of protein were determined by the enhanced bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). After that the protein was subjected to electrophoresis on a polyacrylamide gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% milk for 1 h at room temperature and incubated with anti-SOD1 (ab13498, Abcam, Cambridge, MA, USA), anti-SOD2 (ab13534, Abcam, Cambridge, MA, USA), and anti-β-actin (179467, Abcam, Cambridge, MA, USA). After washing, the membrane was incubated with secondary antibody for 2 h at room temperature. Finally, protein bands were detected using the enhanced chemiluminescence (ECL) solution (Beyotime, Shanghai, China).

### Immunofluorescence (IF) Staining

CHs were fixed with 4% formaldehyde for 15 min and blocked with 5% BSA for 1 h. Then, the cells were incubated with a primary antibody against collagen-II (ab34712, Abcam, Cambridge, MA, USA) or caspase-9 (ab32539, Abcam, Cambridge, MA, USA) overnight at 4°C, followed by goat anti-rabbit IgG antibody and 4',6-diamidino-2-phenylindole (DAPI, Beyotime, Shanghai, China) incubation for 1 hour in the dark. Finally, the fluorescence intensity of CHs was visualized with a fluorescence microscope.

### Flow Cytometry

Total reactive oxygen species (ROS) production of CHs was using diacetyl dichlorofluorescein staining (Sigma-Aldrich, St. Louis, MO, USA). The apoptosis of CHs was determined by flow cytometry using Annexin V-FITC/PI double-stained apoptosis detection kit (Keygen, Nanjing, China) according to the manufacturer's instructions.

### Cell Viability Assay

Cells viability was assessed with the cell counting kit-8 (CCK-8) assay (C0009, Beyotime, Shanghai, China). Cells were cultured ($1\times10^4$ cells/well) in 96-well plates and added with 10 μL of CCK-8 reagent in a total volume of 100 μL, and the plates were incubated for 3 hours. After that, the absorbance was measured using a microtiter plate reader at 450 nm.

### 2-Deoxyglucose (2-DOG) Uptake

CHs were starved (without FBS) for 2 h, washed with Krebs-Ringer phosphate (KRP) buffer, and treated with cytochalasin B (CB, Sigma-Aldrich, St. Louis, MO, USA) as blank for 3 h at room temperature. [$^3$H]2-DOG mixture [hot 2-deoxyglucose (GE Healthcare, Amersham, UK) dissolved in cold 2-DOG (Sigma-Aldrich, St. Louis, MO, USA)] was added in the CHs for another 30 min. Radioactivity was counted in the presence of 3 mL of Ultima Gold scintillation liquid (Packard BioScience, Kennesaw, GA, USA), and normalized to the protein content determined in Bradford analysis.

### Ceramide Quantitation

The total ceramide in CHs was determined by liquid chromatography-mass spectrometry (LC-MS). Sphingolipid extracts were prepared by Bligh-Dyer method and analyzed as previously described[17]. Quantification of ceramide was performed by the extracted ion chromatogram using a triple quadrupole mass spectrometer as reported[18].

### Statistical Analysis

All data were presented by means ±SD (standard deviation). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p$-value $<0.05$ was considered statistically significant.

## Results

### The Effect of O/P Acid on the Viability of CHs

To explore the effect of O/P on the viability of CHs, CCK-8 assay was used at different time points up to 48 h under the stimulation of 0.5 mM O/P and different concentrations of O/P up to 1 mM after 24 h treatments. As shown in Figure 1A, 0.5 mM O/P caused the cell viability continuous declination until 24 h treatments. There was no significant difference between the time point at 48 hours and 24 h. In addition, the concentration of O/P ranging from 0.01 mM to 1 mM was changed and the cell viability was found to gradually declined compared to the control (Figure 1B). Besides, the chondrogenic genes and

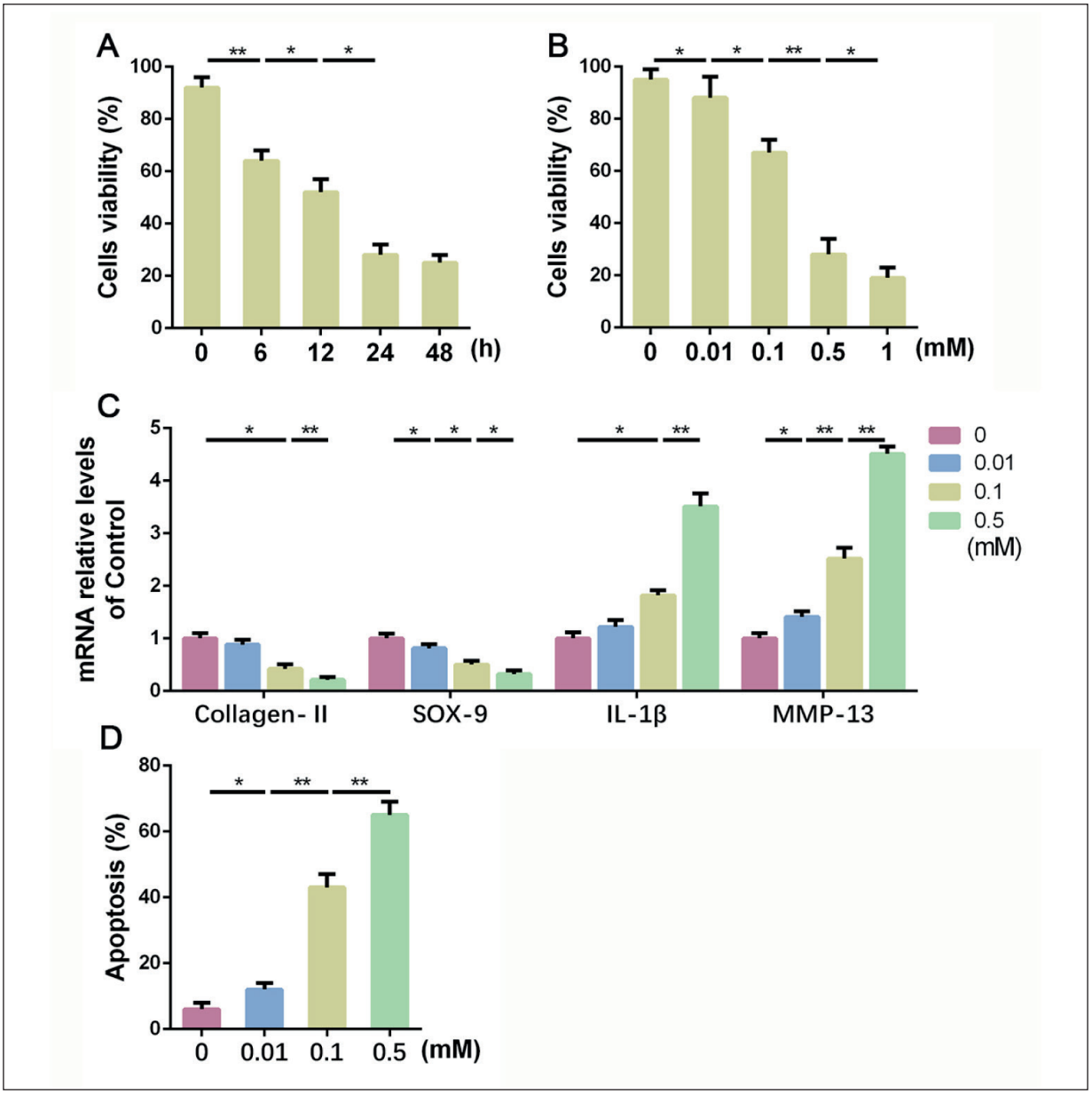


**Figure 1.** CHs are treated with 5 μM O/P solution from 6 hours to 48 hours or treated with different concentrations from 0.01 mM to 1 mM for 24 hours. **A, B,** The viability of CHs is determined by CCK-8 assay. **C,** The mRNA expression levels are assayed by RT-PCR. **D,** The ratio of apoptotic CHs is determined by flow cytometry. Data are presented as mean ± SD of three independent experiments. (*$p$<0.05, **$p$<0.01).

inflammatory genes level such as collagen-II, SOX-9, IL-1β, and MMP-13 were also measured, and the result indicated O/P affected these genes expression with a dose-dependent (Figure 1C). The result of flow cytometry suggested that O/P accelerated the apoptotic speed of CHs, especially at 0.5 mM (Figure 1D). Therefore, O/P acid is a harmful stimulus to CHs with a time-dependent and dose-dependent.

### *O/P Acid Treatment Increased Ceramide, Oxidative Stress Level, and Affected Glucose Uptake of CHs*

To determine the mechanism that O/P treatment degrades the CHs, the ceramide level, glucose uptake, and the oxidative stress level in the CHs were analyzed. As a result of Figure 2A, the content of ceramide arose resulting from the stimulation of O/P at a dose-dependent

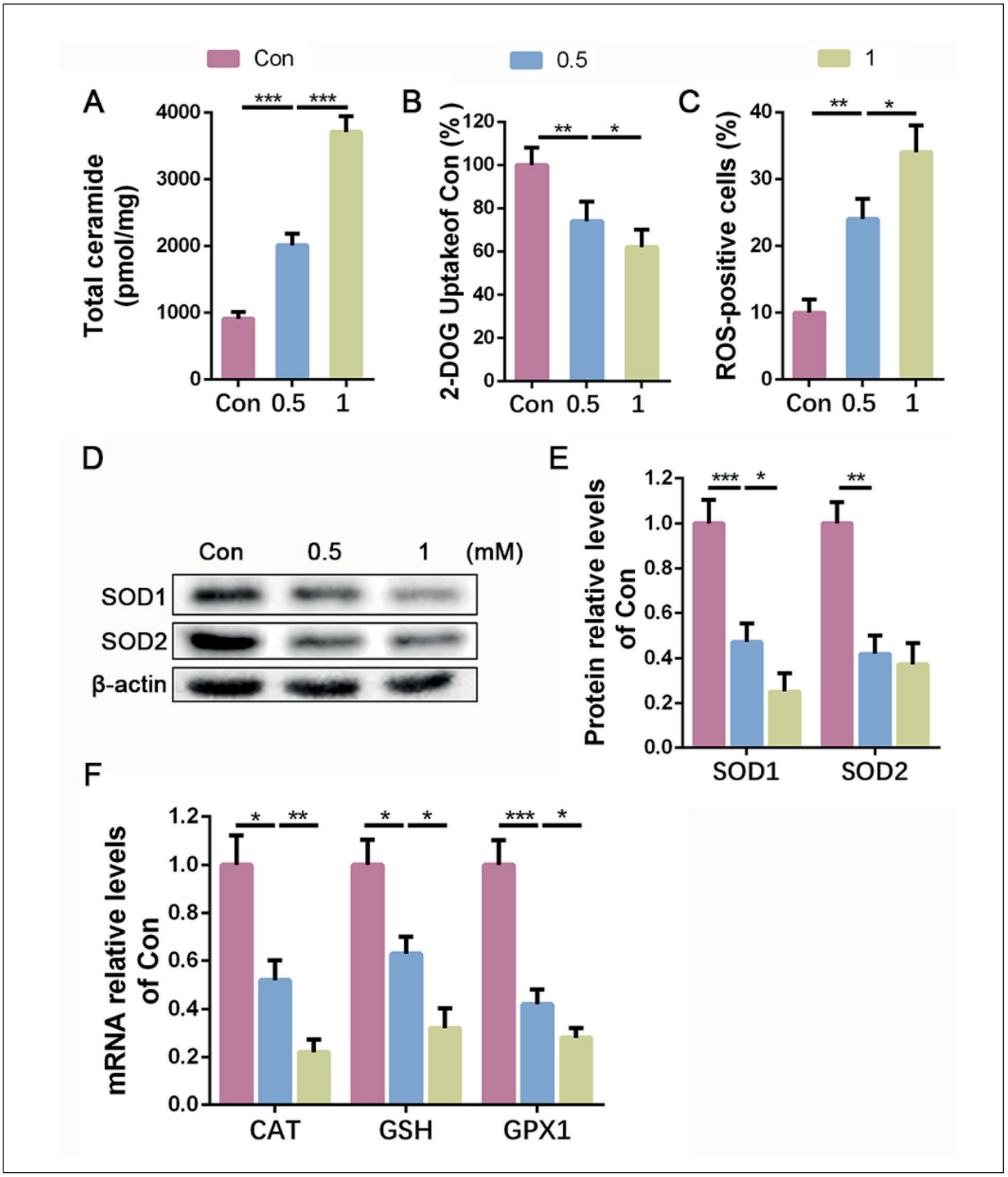


**Figure 2.** CHs are incubated with 0.5 mM or 1 mM O/P for 24 hours. **A,** Total ceramide quantitation by LC-MS. **B,** Glucose uptake of CHs. **C,** The ratio of ROS positive CHs is determined by flow cytometry. **D,** The protein expressions are assayed by WB, and **E,** quantification analysis. **F,** The mRNA expression levels are assayed by RT-PCR. Data are presented as mean ± SD of three independent experiments. (*$p$<0.05, **$p$<0.01, ***$p$<0.001).

compared to the control. It has been known that lipid accumulation would affect glucose uptake and glycogen synthesis[19]. So, the condition of glucose uptake of each group was also measured, and the data suggested that O/P decreased the glucose uptake of the CHs compared to the

control (Figure 2B). As for the interaction between oxidative stress and lipid metabolism, it was found that the O/P significantly increased the ROS level of CHs (Figure 2C). In addition to the total ROS positive cell, O/P also affected the antioxidative enzymes. As shown in Figure 2D, the protein expressions of SOD1 and SOD2 were decreased caused by O/P, especially the SOD reduced with a dose-effect manner. Besides, the other antioxidative enzymes containing CAT, GSH, GPX1 in the mRNA level were also analyzed, and the result was similar to the SOD indicated that O/P affected the expression of these enzymes as well. Collectively, the treatments of O/P markedly increased the ceramide content and ROS level of CHs but decreased the glucose uptake and the antioxidative enzymes.

### Myriocin Rejected O/Pacid-Induced CHs Degeneration

Ceramide is the regulator of the cellular lipid metabolism, so whether the suppression of ceramide by myriocin would reverse the effect of O/P on CHs was investigated. The supply of myriocin did no harm to the viability of CHs, and myriocin protected the CHs viability after the stimuli of O/P (Figure 3A). Apart from this, myriocin also decreased the apoptotic CHs population compared to the O/P-treated group (Figure 3B). As we presented before, O/P affected the collagen II and SOX-9 expressions that were reversed by myriocin, so were the expressions of IL-1β and MMP-13 (Figure 3C). Next, the collagen II level was measured by IF staining, and the tendency was the same as the RT-PCR. The staining of caspase-9 suggested that O/P contributed to the accumulation of caspase-9 positive cells but it was inhibited by the supplement of myriocin (Figure 3D, 3E). To sum up, myriocin is efficient to reject the harmful effect brought by O/P containing the decreased viability, chondrogenic gene expression, and increased apoptosis.

### Myriocin Protected O/P-Treated CHs Oxidative Stress Level and Glucose Uptake

As e inhibitor of ceramide, myriocin significantly reduced the accumulation of ceramide compared to the O/P treated group (Figure 4A). O/P decreased the glucose uptake compared to the control group, but myriocin reversed this side effect caused by O/P (Figure 4B). As expected, the ROS content was significantly reduced after the suppression of ceramide, comparing to the O/P treated group (Figure 4C). Finally, the levels of the antioxidative enzymes were detected. The result of the WB showed that myriocin contributed to the expression of SOD2 compared to the control, and myriocin protected the SOD1 and SOD2 expressions under the treatment of O/P (Figure 4D, 4E). Apart from this, the mRNA level of CAT, GSH, GPX1 were all recalled resulting from the stimulation of myriocin compared to the O/P treated group (Figure 4F). In this part, it was detected that myriocin was effective to protect the glucose uptake and the balance of oxidative stress of CHs by the suppression of ceramide.

## Discussion

Disturbances of lipid metabolism in the body such as serum cholesterol, low-density lipoprotein, and free fatty acids cause CHs to show intracellular lipid deposition leading to the cartilage break down[4]. Sphingomyelin is the second most abundant phospholipid in the body, accounting for 18% of phospholipids in serum. Sphingomyelin is present in all major lipoproteins, including very-low-density lipoproteins and low-density lipoproteins. It plays an important role in lipid metabolism[20]. Ceramide is a member of the sphingolipid family abundant in cell membranes, consisting of sphingosine long-chain bases and fatty acids[21]. Ceramide is the central molecule of sphingomyelin metabolism, function mainly as inducing apoptosis, regulating cell differentiation, regulating cellular immunity, and participating in inflammatory responses[22]. In this study, the overexpression of ceramide caused by O/P acid influenced the CHs viability and apoptosis, which is corresponding with the previous studies. However, the inhibition of ceramide by myriocin protected the CHs viability and decreased apoptotic CHs. Zulueta et al[23] found myriocin suppresses ceramide *de novo* synthesis decreasing the cigarette smoke-induced damages in airways epithelia. Kasumov et al[24] reported that myriocin reduces apoptosis, atherosclerosis, and hepatic steatosis by increasing ceramides and sphingomyelin.

Ceramide production pathway mainly includes the *de novo* synthesis pathway that occurs in the endoplasmic reticulum, the neurophospholipase pathway that can occur in multiple organelles, and the rescue pathway that oc-

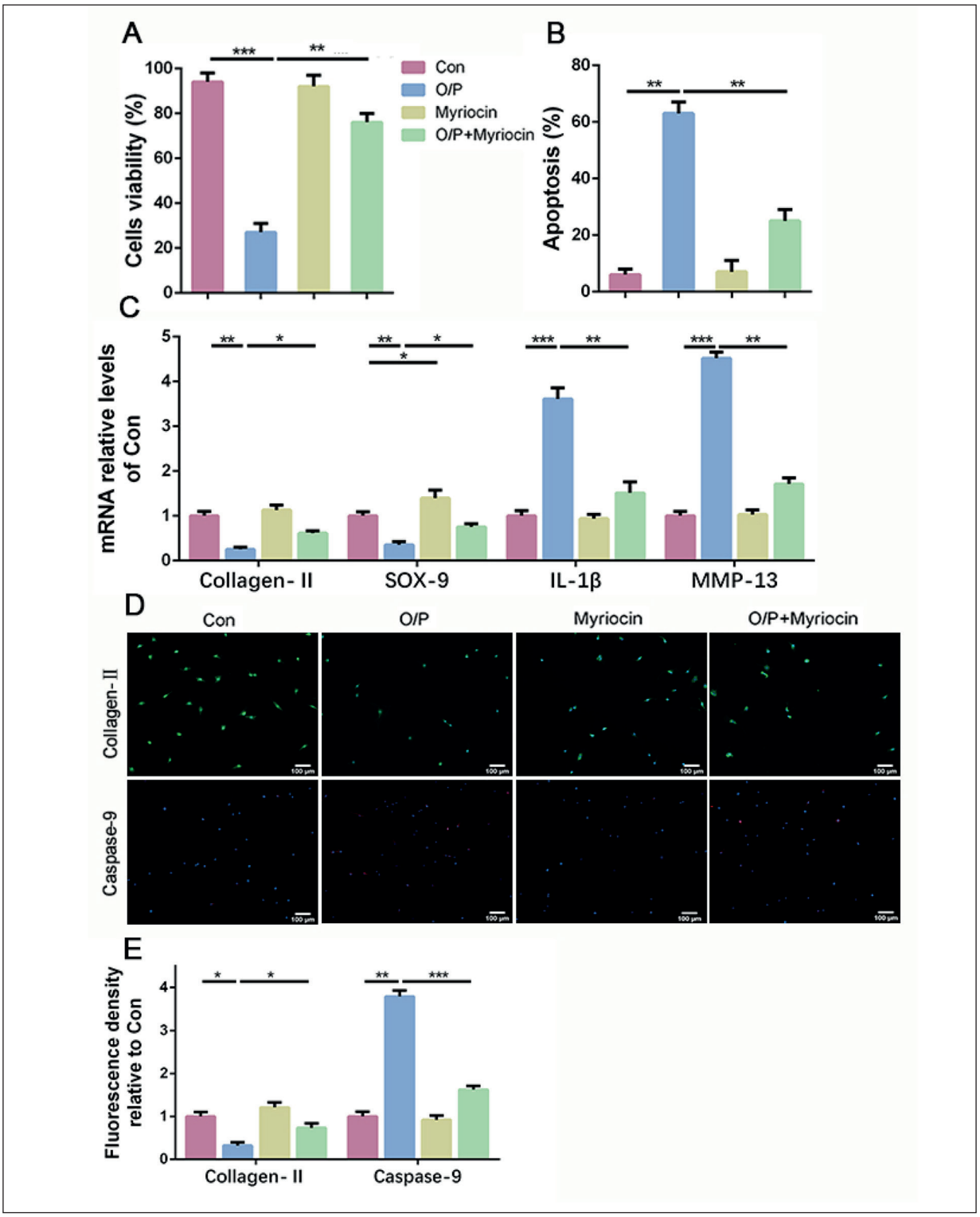


**Figure 3.** CHs are treated with myriocin (20 mM) for 24 h as a negative control or O/P (1 mM) for 24 h as a positive control. Besides, CHs are treated with O/P (1 mM) for 24 h and then changed to myriocin (20 mM) for another 24 h. **A,** The viability of CHs is determined by CCK-8 assay. **B,** The ratio of apoptotic CHs is determined by flow cytometry. **C,** The mRNA expression levels are assayed by RT-PCR. **D,** IF staining of collagen-II and caspase-9, and **E,** quantification analysis. Data are presented as mean ± SD of three independent experiments. (magnification: 400×) (*$p$<0.05, **$p$<0.01, ***$p$<0.001).

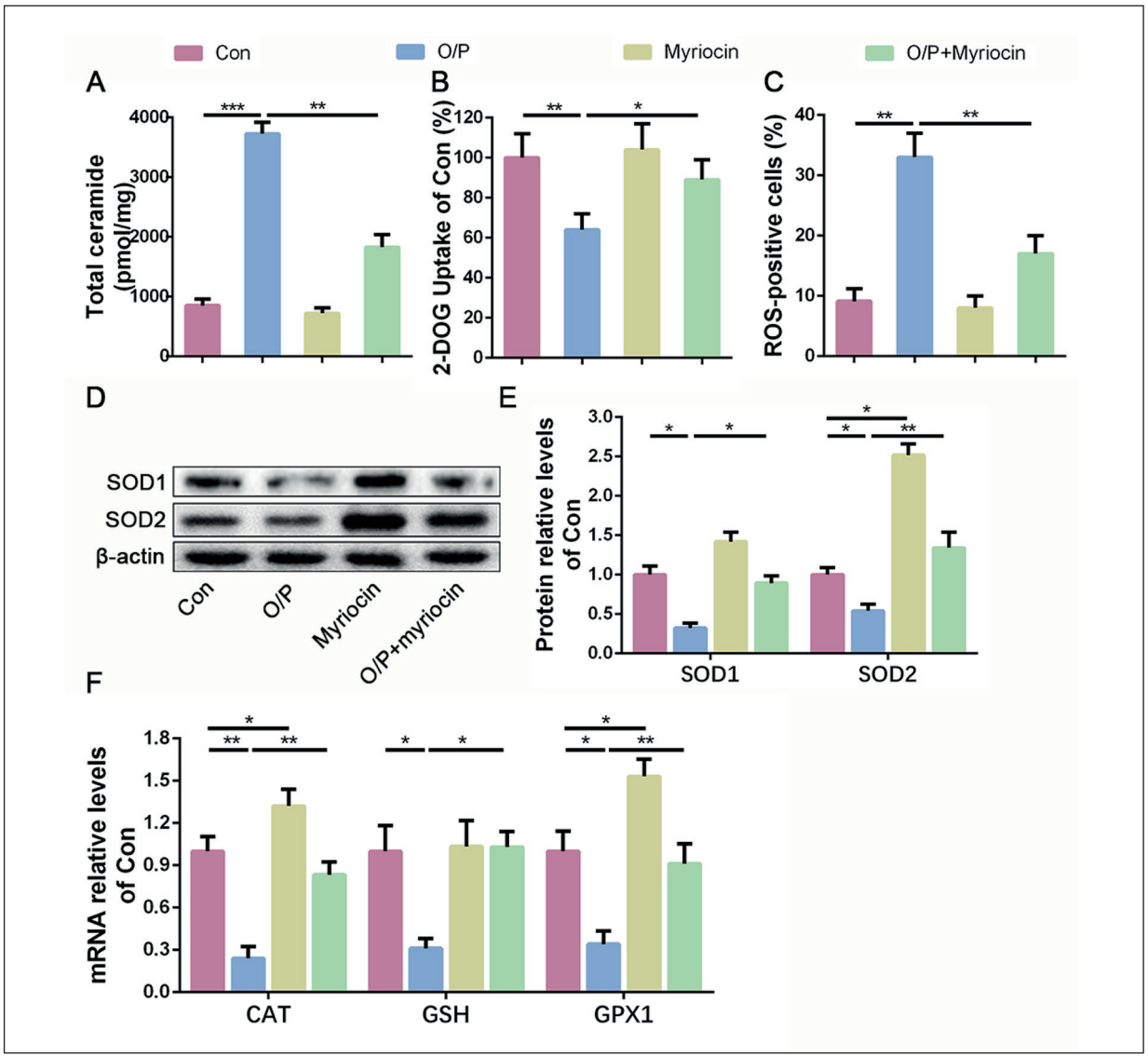


**Figure 4.** CHs are treated with myriocin (20 mM) or O/P (1 mM) for 24 h. Besides, CHs are treated with O/P (1 mM) for 24 h and then changed to myriocin (20 mM) for another 24 h. **A,** Total ceramide quantitation by LC-MS. **B,** Glucose uptake of CHs. **C,** The ratio of ROS positive CHs is determined by flow cytometry. **D,** The protein expressions are assayed by WB, and **E,** quantification analysis. **F,** The mRNA expression levels are assayed by RT-PCR. Data are presented as mean ± S.D. of three independent experiments. (*$p$<0.05, **$p$<0.01, ***$p$<0.001).

curs in lysosomes and endosomes, among which myriocin can prevent ceramide synthesis by the de novo pathway. It was found that the myriocin could improve the glucose uptake, which is very meaningful to the stable of CHs. Glucose is the raw material for normal anabolic metabolism of CHs, and the weakening of glucose uptake will affect CHs proliferation and synthesis of the extracellular matrix such as proteoglycans[25,26]. It has been reported that ceramide can disrupt insulin signaling by reducing the sensitivity of cells to insulin and inhibiting the translocation of glucose transporter 4, so as to affect the glucose uptake[27,28]. Furthermore, the accumulation of ceramide results in a detrimental increased in ROS production, which plays an important role in the destruction of cartilage. However, it was found that myriocin could inhibit the ROS level by supporting the expression of the antioxidative enzymes, which should be one of the reasons that alleviated the CHs degeneration. In the previous studies, myriocin reduces the hepatic oxidative stress and regulates the endoplasmic reticulum stress pathway by the inhibition of ce-

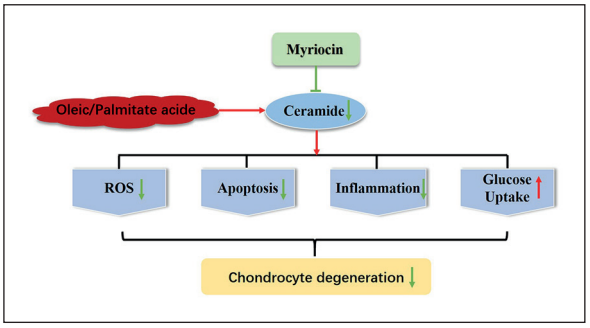


**Figure 5.** A proposed diagram depicting the mechanism of myriocin protecting O/P-induced CHs degeneration *via* the suppression of ceramide.

ramide[29]. Reforgiato et al[30] also found myriocin suppresses ceramide *de novo* and decreases the ROS and inflammation in myocardial reperfusion injury.

## Conclusions

We elucidated that ceramide accumulates in the progression of O/P induced CHs degeneration, and myriocin is an effective drug that can inhibit the expression of ceramide in the abnormal lipid environment. Of note, the mechanism that myriocin protecting O/P-induced CHs degeneration *via* the suppression of ceramide is related to the decreased apoptosis, glucose uptake improvement, and the decreased ROS and inflammation level (Figure 5). The etiology of OA is complex, and myriocin or other ceramide inhibitors may become a new direction for the prevention and treatment of MetS associated OA.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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