Ozone Induces Autophagy by Activating PPARγ/mTOR in Rat Chondrocytes Treated with IL-1β

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Abstract

Background: Osteoarthritis (OA) is the main cause of older pain and disability, its dysfunction due to discomfort as well as the quality of life of patients having adverse effects. Medical ozone (O_3) has been found to have antioxidative and anti-inflammatory effects in the treatment of osteoarthritis. However, it is unclear whether O_3 can induces autophagy through the PPARγ/mTOR autophagy pathway in chondrocytes treated with IL-1β.

Methods: Primary chondrocytes were isolated from wistar rat cartilage within 3days. The OA chondrocyte model was induced via treatment with IL-1β to chondrocytes for 24 hours. Then the cells were treated with O_3 and GW9662, the inhibitor of PPARγ. Cell viability was assessed by CCK-8. Further, the cells subjected to western blot analysis, qRT-PCR and immunofluorescence assay. The numbers of autophagosomes were observed via transmission electron microscopy.

Results: 30μg/ml O_3 improved the viability of chondrocytes. O_3 significantly increased the level of autophagy proteins and the numbers of autophagosomes in chondrocytes treated with IL-1β via treated with O_3. qRT-PCR results showed that O_3 decreased the levels of IL-6,TNF-α and MMP-3,MMP-13 in chondrocytes treated with IL-1β.

Conclusions: 30μg/ml O_3 improved autophagy via activating PPARγ/mTOR signaling and suppressing inflammation in chondrocytes treated with IL-1β.

Background

Following the accelerated speed of population aging in the world, the incidence of degenerative diseases, such as osteoarthritis (OA), is on the rise. OA is an important arthritis and affects more than half of individuals over the age of 65 [1]. The pathological process of OA includes chronic inflammation and degeneration of cartilage [2]. Researchers have shown that an imbalance in chondrocyte homeostasis is associated with the occurrence and development of OA [3]. Therefore, to prevent articular cartilage degeneration, it is particularly important to keep chondrocytes healthy.

Autophagy is an important protective response of cells exposed to various stresses [4]. It also plays a critical role in articular chondrocytes as the primary mechanism for maintaining normal function and survival [5]. Studies have linked the pathological process of OA with reduced autophagy in chondrocytes [6–8]. It has been accepted that improving the level of autophagy can be beneficial in the treatment of OA. Furthermore, in the pathological process of OA, inflammatory cytokines also play a vital in cartilage degeneration in OA. Excessive inflammatory cytokines can suppress autophagy activationd, cause excessive matrix metalloproteinases (MMPs) expression, leading to cell death.

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor involved in the regulation of many cellular processes [7, 8]. Some studies have indicated that PPARγ activation reduces interleukin (IL)-1-induced expression of inflammatory mediators and MMPs in chondrocytes [9]. PPARγ can also be

expressed in different cell types and has been linked to autophagy in other diseases [10, 11]. Vasheghani et al [12] demonstrated that PPARγ plays a protective role in chondrocytes by regulating the mTOR autophagy signaling pathway. In addition, PPARγ was associated with autophagy in human chondrocytes through interactions with the AKT/mTOR pathway [13]. Thus, PPARγ may play an important part in the development of OA.

Ozone (O_3) is a controversial gas that produces free radicals, such as reactive oxygen species (ROS) and lipid hydroperoxides (LOPs) when dissolved in biological water. In recent decades, O_3 therapy has become accepted by some practitioners in Europe. Unfortunately, even today, given the paucity of controlled clinical studies, there are prejudices among some therapists towards O_3. These factors hindered the application of O_3 therapy in clinically. However, in recent years, the clinical application of O_3 therapy has gained more recognition, and has produced good clinical therapeutic effects in the treatment of disc herniation and pain [14]. Studies have also found that O_3 at a suitable concentration can improve chondrocyte autophagy in OA by affecting the expression of mammalian target of rapamycin (mTOR) complexes [15]. Moreover, the PPARγ/mTOR signaling pathway is closely related to changes in autophagy in OA. Thus, the aim of this study was to investigate whether PPARγ/mTOR signaling affected autophagy in chondrocytes treated with IL-1β following O_3 treatment.

Methods

Ethics statement

The rats were provided by the Experimental Animal Center of Shandong University. All rats were sacrificed by cervical dislocation after the experiment. The processes concerning animal use were in compliance with the relevant regulations of the National Institutes of Health and approved by the Animal Care and Use Committee of Shandong Provincial Hospital affiliated with Shandong University. Great efforts were made to minimize the suffering of conscious animals.

Establishment of osteoarthritis chondrocyte model

Cartilaginous tissue was separated from the Wistar rats borned within 3 days. The cartilage tissue was then digested with 0.2% type II collagenase overnight at 37°C. The following day, the digested cells were passed through a 75μm filter and centrifuged at room temperature at 800 rpm for 4 min. Finally, the chondrocytes were incubated in culture flasks with 10% fetal bovine serum in DMEM/F12 medium containing 10% fetal bovine serum (FBS) added. Chondrocytes were treated with IL-1β (10 ng/mL) (PeproTech) for 24 h to establish the OA chondrocyte model.

Experimental groups and drug administration

The incubated cells were randomly divided into the following groups: Control group, IL-1β group, IL-1β + O_3 group, Control + O_3 group, IL-1β + O_3 + GW9662 group and IL-1β + GW9662 group. The cells were exposed to of O_3 for 30 min at a certain concentration which measured using the O_3 analyzer purchased

from Perma Pure Inc (model MD-050-12-f-4, Perma Pure Inc). Then cells were pretreated with the PPARγ inhibitor GW9662 (20nM/mL, MCE Technologies) for 12 h prior to O_3 treatment.

Cell viability assay

The viability of cells was assessed using cell counting kit-8 assay (CCK-8 kit) (Dojindo Laboratories). Briefly, chondrocytes (6000/well) were seeded in 96-well platesand incubated at 37°C for 24 hours. After being treated with IL-1β for 24 hours, the cells were exposed to O_3 at concentrations of 30, 50 or 70μg/mL for 30 min. Finally, 10μL of CCK-8 kit solution was added to each well and plates were incubated at 37°C for 2 h. Absorbance was measured at 450 nm using a microplate reader.

Western blot analysis

All proteins were extracted using a radio immunoprecipitation assay (RIPA) lysis buffer with protease and phosphatase inhibitors. The protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China). Protein samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk solution for 1 h at room temperature. The membranes were then probed with anti-PPARγ (sc-7273, 1:200, Santa), anti-mTOR (#2983, 1:1000; Cell Signaling Technology), p-mTOR (#5536, 1:1000; Cell Signaling Technology), anti-ULK1 (#8054, 1:1000; Cell Signaling Technology), anti-LC3II (ab48394, 1:2000, Abcam), anti-P62 (ab56416, 1:2000; Abcam), anti-Beclin-1 (ab62557, 1:1000; Abcam) and anti-β-actin (1:2000; Zhongshan Golden Bridge Biotechnology) overnight at 4°C. The following day, membranes were washed three times with TBST and incubated with goat anti-rabbit or goat anti-mouse antibody IgG (H+L)-HRP (1:5000; Wuhan Sanying) for 1 hour at room temperature. Finally, the membranes were visualized using the enhanced chemiluminescence substrate LumiGLO (Millipore, MA, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from chondrocytes using TRIzol reagent (Takara, Shiga, Japan). cDNA was amplified using a PrimeScript RT reagent kit (RR047A; Takara). mRNA expression was measured with qRT-PCR using a SYBR® GrenERTM SuperMix (Takara) with the following conditions:denature at 95°Cfor 30 s, anneal at 60°Cfor 30s, extend at 95°Cfor 5 s. The primer sequences were as follows: β-actin, forward, 5'-GGGAAATCGTGCGTGAC-3' and reverse 5'-AGGCTGGAAAAGAGCCT-3'; IL-6,forward,5'ATTGTATGAACAGCGATGATGCAC-3', and reverse 5'-CCAGGTAGAAACGGA ACTCCAGA-3'; TNF-α,forward 5'-TTCCAATGGGCTTTCGGAAC-3' and reverse 5'-AGACATCT TCAGCAGCCTTGTGAG -3';MMP-13, forward 5'- TGATGATGAAACCTGGACAAGCA-3' and reverse5'- GAACGTCATCATCTGGGAGCA-3'; MMP-3, forward 5'TGATGGGCCTGGAAT GGTC-3'and reverse 5'-TTCATGAGCAGCAACCAGGAATAG-3'. β-actin was used as the internal control, and the level of gene expression was analyzed using the $2^{-\triangle\triangle Ct}$ method.

Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde for 30 min. Before blocking with goat serum, the cells were permeabilized with 0.3% Triton X-100. The chondrocytes were then incubated with anti-LC3 (ab48394, 1:100, Abcam) and anti-P62 (ab56416, 1:100; Abcam) overnight at 4°C. The following day, cells were incubated with secondary antibody (1:200; Wuhan Sanying) at room temperature for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with anti-fade medium. The stained chondrocytes were observed using immunofluorescence microscopy.

Transmission electron microscopy (TEM)

The cells were then gathered using a cell scraper and centrifuged to form cell clumps. The cell clumps were fixed with ice-cold 3% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in osmium tetroxide, embedded in Epon epoxy resin. The samples were then cut into ultrathin sections and further stained using uranyl acetate and lead citrate. Finally, the ultrathin sections were viewed using transmission electron microscopy.

Statistical analysis

SPSS 22.0 software (IBM Corp. Armonk, NY, USA) was applied for data analysis. Measurement data were described as mean ± standard deviation (SD). The differences among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by pairwise comparisons using Tukey's multiple comparisons test. The *P* value was calculated using a two-tailed test, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of chondrocytes

Toluidine blue staining result showed an intercellular matrix of cultured cells was colored purple-red, indicating that the cells were chondrocytes (Fig.1A). To further identify the isolated cells and determine cell purity, we performed immunofluorescence staining of type II collagen, which is specifically expressed in chondrocytes. The result indicated that more than 95% of the cultured cells were positive for collagen II staining, futher supporting the above conclusion (Fig.1B).

O_3 (30μg/ml) improves IL-1β-treated chondrocyte viability

As shown in Fig. 2, 30μg/ml O_3 improved cell viability ($P<0.05$), while 50 and 70 μg/ml O_3 obviously decreased cell viability relative to treatment with IL-1β alone ($P<0.05$, $P<0.05$). Previous studies have shown that 30 μg/ml of O_3 is safe and beneficial as a treatment for OA [16]. Thus, the dose of 30μg/mL O_3 was selected for further experiments.

O_3 increases the expression of PPARγ and autophagy in chondrocytes treated with IL-1β

The expression of PPARγ has been associated with autophagy and previous evidence has suggested that the expression of PPARγ in OA cartilage is decreased. To identify the effect of O_3, we detected the expression of PPARγ and biomarkers of autophagy in chondrocytes treated with IL-1β by western blotting. As shown in Fig. 3A, IL-1β decreased the production of PPARγ in chondrocytes (p<0.01), while O_3 increased the IL-1β-induced downregulation of PPARγ (p<0.01). Besides, IL-1β administration significantly attenuated the level of LC3 II and increased P62 in chondrocytes (p<0.05, p<0.05). When compared with IL-1β group, the autophagy marker proteins LC3 II obviously elevated and P62 declined in chondrocytes co-treated with IL-1β and O_3 (Fig. 3A, p<0.05, p<0.05). To explore whether the activation of PPARγ is linked to O_3-induced autophagy, the chondrocytes treated with IL-1β were dealed with GW9662, the specific inhibitor PPARγ. The results showed that compared with the IL-1β+O_3 group, the expression of the LC3II was decreased, while expression of P62 increased in chondrocytes pretreated with GW9662 (Fig. 3B, p<0.05, p<0.05). Beclin-1 levels changed slightly in chondrocytes treated with IL-1β, significantly increased in cells co-treated with IL-1β and O_3, but decreased sharply in chondrocytes treated with GW9662(Fig. 3B, p<0.05, p<0.05).

O_3ameliorates the decreased autophagy in chondrocytes treated with IL-1β

As shown in Fig. 4A, the result of immunocytochemical staining shows that compared with the IL-1β group, fluorescence intensities of LC3 enhanced while P62 increased with O_3 treatment. While treated with GW9662, accordingly, led to reversed trends. Besides, TEM analysis revealed that the number of autophagosomes was decreased in chondrocytes treated with IL-1β and improved by O_3 treatment. In addition, the number of autophagosomes was decreased following treatment with GW9662 (Fig. 4B).

O_3 ameliorates autophagy via PPARγ/mTOR signaling in chondrocytes treated with IL-1β

Some lines of evidence have shown that mTOR plays an important role in regulating cell growth and is also involved in autophagy[17]. Other studies have shown deficiency of PPARγ could induce aberrant mTOR signaling in chondrocytes[18]. To further investigate the mechanism of O_3-induced autophagy in chondrocytes treated with IL-1β, the protein levels of PPARγ, phosphorylated mTOR (p-mTOR) and ULK1 were assessed after treated with GW9662. The results of showed that O_3 improved the levels of PPARγ and ULK1, decreased p-mTOR levels in IL-1β+O_3 group, compared with IL-1β group (Fig.5, p<0.01, p<0.05, p<0.05). In the presence of GW9662, the level of ULK1and PPARγ were declined significantly (Fig.5, p<0.05, p<0.01) and the level of p-mTOR were up-regulated (Fig.5, p<0.05).

O_3decreases the inflammatory response in chondrocytes treated with IL-1β

There are various inflammatory cytokines involved in the pathologic process of OA. In this study, the levels mRNA of IL-6, TNF-α, MMP-3, and MMP-13 were measured by qRT-PCR. The results indicated that O_3 decreased the expression of IL-6, TNF-α, MMP-3 and MMP-13 in IL-1β+O_3 group, compared with IL-1β group (Fig. 6, p<0.05, p<0.01, p<0.01, p<0.05, respectively). While treated with GW9662, the level of IL-6,

TNF-α, MMP-3 and MMP-13 was up-regulated significantly (Fig. 6, $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, respectively).

Discussion

OA is a multifactorial disease that is the result of the interaction of mechanical and biological factors [19]. In the progression of OA, inflammatory cytokines such asIL-1β and TNF-α play an important role in promoting the degradation of the cartilage matrix and articular cartilage [20]. IL-1β can induce the expression of many cytokines, changing the expression of various proteases related to the progression of OA [21]. Hence, it is generally accepted that the chondrocytes treated with IL-1β can be used to simulate OA chondrocytes in vitro studies. Autophagy is a beneficial pathway for sustaining intracellular homeostasis by degrading damaged organelles and long-lived proteins. Previous reports have demonstrated that autophagy may be constitutively activated in normal chondrocytes and defective autophagy in chondrocytes is associated with the degradation of cartilage and the development of OA [22, 23]. It has been shown that O_3 can upregulate the reduced autophagy in OA chondrocytes and have beneficial effects in the treatment of OA in clinically [24]. Given that autophagy plays a crucial role in OA, the aim of this study was to investigate the potential molecular biological mechanisms of O_3 on autophagy in OA chondrocytes.

In this study, we investigated the appropriate concentration of O_3 for chondrocytes treated with IL-1β using a CCK-8 kit. The results demonstrated that 30 μg/mL O_3 improved cell viability. Thus, a dose of 30 μg/mL O_3 was choosen to the treatment of chondrocytes treated with IL-1β. Previous studies have shown that PPARγ exerts obvious anti-inflammatory effects by regulating the expression of several pro-inflammatory genes [25, 26]. In addition, deletion of the PPARγ gene can lead to cartilage-specific destruction leading to spontaneous OA [12]. In the present study, the results indicated that IL-1β suppressed the level of PPARγ and autophagy, which was reflected by reduced LC3II and increased P62 in chondrocytes. After treatment with O_3 (30 μg/mL), the levels of PPARγ and autophagy were enhanced in IL-1β-treated chondrocytes. The autophagy markers LC3II and P62 are autophagy-related proteins involved in key molecular signaling pathways. These findings indicate that O_3 likely regulates autophagy by activatingPPARγ.

Previous studies have shown that the activation of PPARγ has beneficial effects on the level of autophagy [27]. To further explore the role of PPARγ in O_3-induced autophagy, chondrocytes were treated with the PPARγ inhibitor GW9662 before ozone treatment. We found that the levels of LC3II and Beclin-1 decreased while the levels of P62 increased following treatment with GW9662. The O_3-induced autophagy was reversed by GW9662. Beclin-1 is a homolog of yeast Atg6, a multifunctional protein and a major positive regulator of autophagy. Considering the negative effect of GW9662 on autophagy, it is logical that PPARγ plays a key role in O_3-induced autophagy. Furthermore, immunofluorescence staining for LC3II and P62, as well as the results of TEM on autophagosomes also elucidate this issue well.

As PPARγ could induce aberrant mTOR signaling in chondrocytes, it is logical to suppose that in O_3-treated chondrocytes, PPARγ acts via the mTOR/autophagy signaling pathway. mTOR is the target molecule of rapamycin, which is the point of attachment between autophagy and the upstream signaling pathway. mTOR is usually considered a suppressor of autophagy and is involved in the inhibition of autophagy in chondrocytes, causing cartilage damage in OA [28]. Our data demonstrate that the level of PPARγ is sharply reduced following treatment with GW9662. Moreover, GW9662 reversed the O_3-mediated suppression of the phosphorylation of mTOR. ULK1, a homolog of yeast ATG1, is another key protein regulating autophagy. A previous study reported that ULK1 expression is negatively regulated by mTOR or activated directly by AMPK [29].Our data shows that ULK1 levels significantly decreased following an increase in p-mTOR in chondrocytes treated with GW9662. Therefore, the PPARγ/mTOR pathway is activated in the chondrocytes, this could be the mechanism by which O_3 promotes autophagy to some extent.

Certain studies have demonstrated that IL-1β could suppress the autophagy pathway in chondrocytes [30]. According to a study of inflammatory cytokines, the result indicated that some cytokines, such as TNF-α and IL-6, accelerated the progression of OA by promoting the catabolism of cartilage [31]. Importantly, activation of PPARγ has been shown to have beneficial effects in alleviating inflammation in human OA chondrocytes[32]. In the present study, we found that O_3inhibits the mRNA levels of the TNF-α and IL-6 in chondrocytes of treated with IL-1β.However, this result was reversed by the suppression of PPARγ. In addition, a number of studies have indicated that activation of PPARγ can inhibit NF-κB activation and decrease the levels of inflammatory cytokines [33].The activation of autophagy can be suppressed by excessive release of inflammatory cytokines [34]. Considering that O_3 blocked IL-1β-induced autophagy inhibition in chondrocytes, the result is likely due to its anti-inflammatory effect on chondrocytes. Moreover, studies have shown that IL-1β can induce the expression of MMPs, in turn inducing cartilage matrix degradation [35]. The result of qRT-PCR results revealed that the levels of MMP-13 and MMP-3 were decreased with O_3 treatment. These data suggest that inhibition of inflammatory cytokines through activation of PPARγ has beneficial effects on IL-1β-treated chondrocytes. Given the series beneficial effects of O_3, it may have further applications clinically. In future in vivo experiments, we would like to investigate the potential therapeutic effect of of O_3 treatment for OA.

Conclusions

This study showed that activation of PPARγ induced by O_3 treatment significantly altered the IL-1β-induced decrease in autophagy and attenuated the IL-1β-induced production of TNF-α, IL-6, and MMPs in chondrocytes. In addition, O_3 could improve autophagy by activating the PPARγ/mTOR signaling pathway in chondrocytes treated with IL-1β. These findings suggest that PPARγ activation plays an important role in the protective effect of O_3 in chondrocytes treated with IL-1β.

Abbreviations

OA
osteoarthritis; MMPs:matrix metalloproteinases; PPARγ:Peroxisome proliferator-activated receptor γ; O_3:Ozone; ROS:reactive oxygen species; LOPs:lipid hydroperoxides; mTOR:mammalian target of rapamycin; FBS:fetal bovine serum; qRT-PCR:quantitative real-time PCR; TEM:Transmission electron microscopy; ANOVA:one-way analysis of variance.

Declarations

Acknowledgments

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Authors' contributions

PP.S and WC.X participated in the study design and performed the histopathology studies. C.Z, X.Z, XW.L and MX.G carried out the PCR experiments, participated in the study design and coordination, analysed the results, performed the statistical analysis. All authors read and approved the final submitted manuscript.

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Ethics approval and consent to participate

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. We confirm that our study have been submitted to and approved by the Experimental Animal Ethics Committee and Biomedical Ethics Committee of Shandong Provincial Hospital affiliated with Shandong University.

Availability of data

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

All the authors of the manuscript have approved the publication of it. All the authors confirm that the work described has not been published before.

Competing interests

No conflict of interests.

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References

1. Shane Anderson A, Loeser RF. Why is osteoarthritis an age-related disease? Best practice research Clinical rheumatology. 2010;24(1):15–26.
2. Vinatier C, Dominguez E, Guicheux J, Carames B. Role of the Inflammation-Autophagy-Senescence Integrative Network in Osteoarthritis. Frontiers in physiology. 2018;9:706.
3. Yu D, Xu J, Liu F, Wang X, Mao Y, Zhu Z. Subchondral bone changes and the impacts on joint pain and articular cartilage degeneration in osteoarthritis. Clin Exp Rheumatol. 2016;34(5):929–34.
4. Mizushima N. Physiological functions of autophagy. Curr Top Microbiol Immunol. 2009;335:71–84.
5. Terman A, Kurz T, Navratil M, Arriaga EA, Brunk UT. Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging. Antioxid Redox Signal. 2010;12(4):503–35.
6. Li YS, Zhang FJ, Zeng C, Luo W, Xiao WF, Gao SG, Lei GH. Autophagy in osteoarthritis. Joint Bone Spine. 2016;83(2):143–8.
7. Chawla A, Schwarz EJ, Dimaculangan DD, Lazar MA. Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology. 1994;135(2):798–800.
8. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell. 1994;79(7):1147–56.
9. Nebbaki SS, El Mansouri FE, Afif H, Kapoor M, Benderdour M, Duval N, Pelletier JP, Martel-Pelletier J, Fahmi H. Egr-1 contributes to IL-1-mediated down-regulation of peroxisome proliferator-activated receptor gamma expression in human osteoarthritic chondrocytes. Arthritis research therapy. 2012;14(2):R69.
10. Chen W, Xi X, Zhang S, Zou C, Kuang R, Ye Z, Huang Y, Hu H. Pioglitazone Protects Against Renal Ischemia-Reperfusion Injury via the AMP-Activated Protein Kinase-Regulated Autophagy Pathway. Front Pharmacol. 2018;9:851.

11. Zhong J, Gong W, Chen J, Qing Y, Wu S, Li H, Huang C, Chen Y, Wang Y, Xu Z, et al. Micheliolide alleviates hepatic steatosis in db/db mice by inhibiting inflammation and promoting autophagy via PPAR-gamma-mediated NF-small ka, CyrillicB and AMPK/mTOR signaling. Int Immunopharmacol. 2018;59:197–208.
12. Vasheghani F, Monemdjou R, Fahmi H, Zhang Y, Perez G, Blati M, St-Arnaud R, Pelletier JP, Beier F, Martel-Pelletier J, et al. Adult cartilage-specific peroxisome proliferator-activated receptor gamma knockout mice exhibit the spontaneous osteoarthritis phenotype. Am J Pathol. 2013;182(4):1099–106.
13. Wang ZJ, Zhang HB, Chen C, Huang H, Liang JX. Effect of PPARG on AGEs-induced AKT/MTOR signaling-associated human chondrocytes autophagy. Cell Biol Int. 2018;42(7):841–8.
14. Magalhaes FN, Dotta L, Sasse A, Teixera MJ, Fonoff ET. Ozone therapy as a treatment for low back pain secondary to herniated disc: a systematic review and meta-analysis of randomized controlled trials. Pain Physician. 2012;15(2):E115–29.
15. Zhao X, Li Y, Lin X, Wang J, Zhao X, Xie J, Sun T, Fu Z. Ozone induces autophagy in rat chondrocytes stimulated with IL-1beta through the AMPK/mTOR signaling pathway. Journal of pain research. 2018;11:3003–17.
16. Raeissadat SA, Rayegani SM, Forogh B, Hassan Abadi P, Moridnia M, Rahimi Dehgolan S. Intra-articular ozone or hyaluronic acid injection: Which one is superior in patients with knee osteoarthritis? A 6-month randomized clinical trial. Journal of pain research. 2018;11:111–7.
17. Tee AR. **The Target of Rapamycin and Mechanisms of Cell Growth**. *International journal of molecular sciences* 2018, 19(3).
18. Vasheghani F, Zhang Y, Li YH, Blati M, Fahmi H, Lussier B, Roughley P, Lagares D, Endisha H, Saffar B, et al. PPARgamma deficiency results in severe, accelerated osteoarthritis associated with aberrant mTOR signalling in the articular cartilage. Ann Rheum Dis. 2015;74(3):569–78.
19. Martinez-Moreno D, Jimenez G, Galvez-Martin P, Rus G, Marchal JA. Cartilage biomechanics: A key factor for osteoarthritis regenerative medicine. Biochimica et biophysica acta Molecular basis of disease. 2019;1865(6):1067–75.
20. Marks PH, Donaldson ML. Inflammatory cytokine profiles associated with chondral damage in the anterior cruciate ligament-deficient knee. Arthroscopy: the journal of arthroscopic related surgery : official publication of the Arthroscopy Association of North America the International Arthroscopy Association. 2005;21(11):1342–7.
21. Aigner T, McKenna L, Zien A, Fan Z, Gebhard PM, Zimmer R. Gene expression profiling of serum- and interleukin-1 beta-stimulated primary human adult articular chondrocytes–a molecular analysis based on chondrocytes isolated from one donor. Cytokine. 2005;31(3):227–40.
22. Benderdour M, Martel-Pelletier J, Pelletier JP, Kapoor M, Zunzunegui MV, Fahmi H. Cellular Aging, Senescence and Autophagy Processes in Osteoarthritis. Curr Aging Sci. 2015;8(2):147–57.
23. Loeser RF, Collins JA, Diekman BO. Ageing and the pathogenesis of osteoarthritis. Nature reviews Rheumatology. 2016;12(7):412–20.

24. Manoto SL, Maepa MJ, Motaung SK. Medical ozone therapy as a potential treatment modality for regeneration of damaged articular cartilage in osteoarthritis. Saudi journal of biological sciences. 2018;25(4):672–9.
25. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998;391(6662):82–6.
26. Ji JD, Cheon H, Jun JB, Choi SJ, Kim YR, Lee YH, Kim TH, Chae IJ, Song GG, Yoo DH, et al. Effects of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) on the expression of inflammatory cytokines and apoptosis induction in rheumatoid synovial fibroblasts and monocytes. J Autoimmun. 2001;17(3):215–21.
27. Zhong J, Gong W, Lu L, Chen J, Lu Z, Li H, Liu W, Liu Y, Wang M, Hu R, et al. Irbesartan ameliorates hyperlipidemia and liver steatosis in type 2 diabetic db/db mice via stimulating PPAR-gamma, AMPK/Akt/mTOR signaling and autophagy. Int Immunopharmacol. 2017;42:176–84.
28. Zhang Y, Vasheghani F, Li YH, Blati M, Simeone K, Fahmi H, Lussier B, Roughley P, Lagares D, Pelletier JP, et al. Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. Ann Rheum Dis. 2015;74(7):1432–40.
29. Kim J, Kundu M, Viollet B, Guan KL: **AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1**. *Nature cell biology* 2011, **13**(2):132–141.
30. Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. Int J Mol Sci. 2017;18(9):1865.
31. De Luna-Preitschopf A, Zwickl H, Nehrer S, Hengstschlager M, Mikula M. **Rapamycin Maintains the Chondrocytic Phenotype and Interferes with Inflammatory Cytokine Induced Processes**. *International journal of molecular sciences* 2017, 18(7).
32. Kang S, Zhang J, Yuan Y. Abietic acid attenuates IL-1beta-induced inflammation in human osteoarthritis chondrocytes. Int Immunopharmacol. 2018;64:110–5.
33. Lim HA, Lee EK, Kim JM, Park MH, Kim DH, Choi YJ, Ha YM, Yoon JH, Choi JS, Yu BP, et al. PPARgamma activation by baicalin suppresses NF-kappaB-mediated inflammation in aged rat kidney. Biogerontology. 2012;13(2):133–45.
34. Qi Y, Zhang M, Li H, Frank JA, Dai L, Liu H, Zhang Z, Wang C, Chen G. Autophagy inhibition by sustained overproduction of IL6 contributes to arsenic carcinogenesis. Cancer research. 2014;74(14):3740–52.
35. Jia T, Cai M, Ma X, Li M, Qiao J, Chen T: **Oridonin inhibits IL-1beta-induced inflammation in human osteoarthritis chondrocytes by activating PPAR-gamma**. *International immunopharmacology* 2019, **69**:382–388.
36. Captions.

Figures

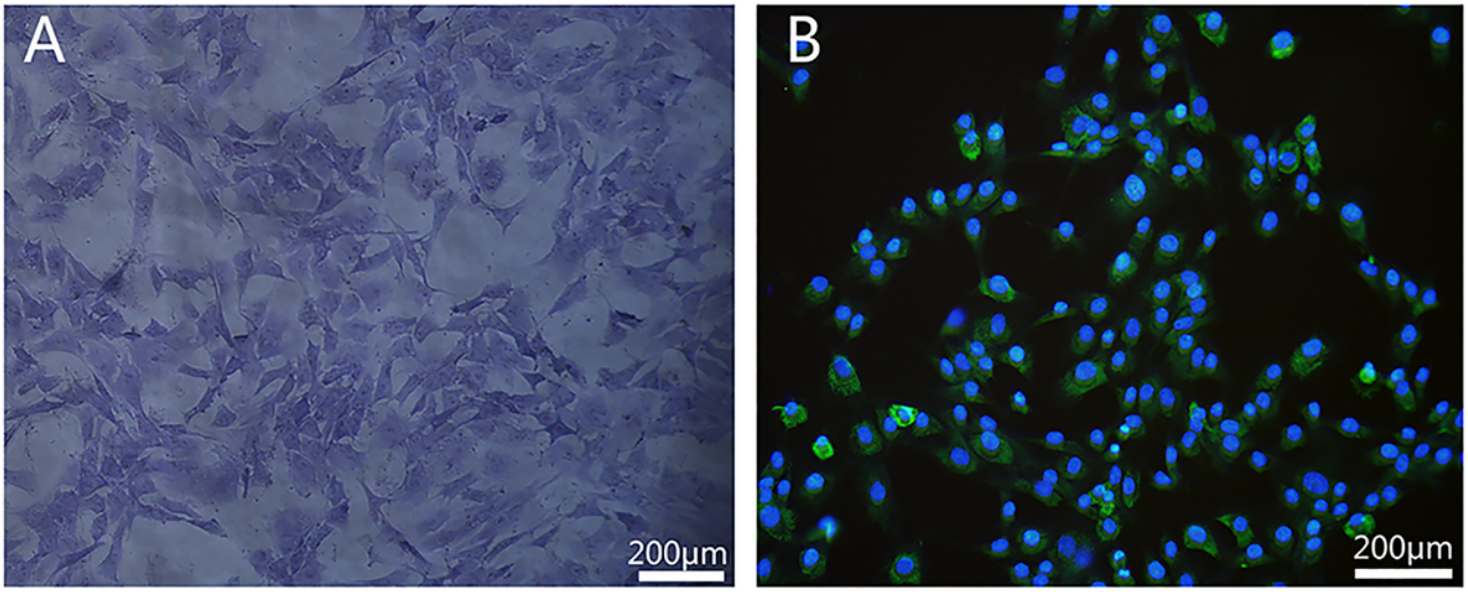

Figure 1

Rat chondrocytes morphological observations. A Toluidine blue staining chondrocytes were observed under the inverted phase contrast microscope at magnifications of 20×; B More than 95% of the cultured cells showed positive expression of type II collagen under a fluorescence microscope at 20×magnification.

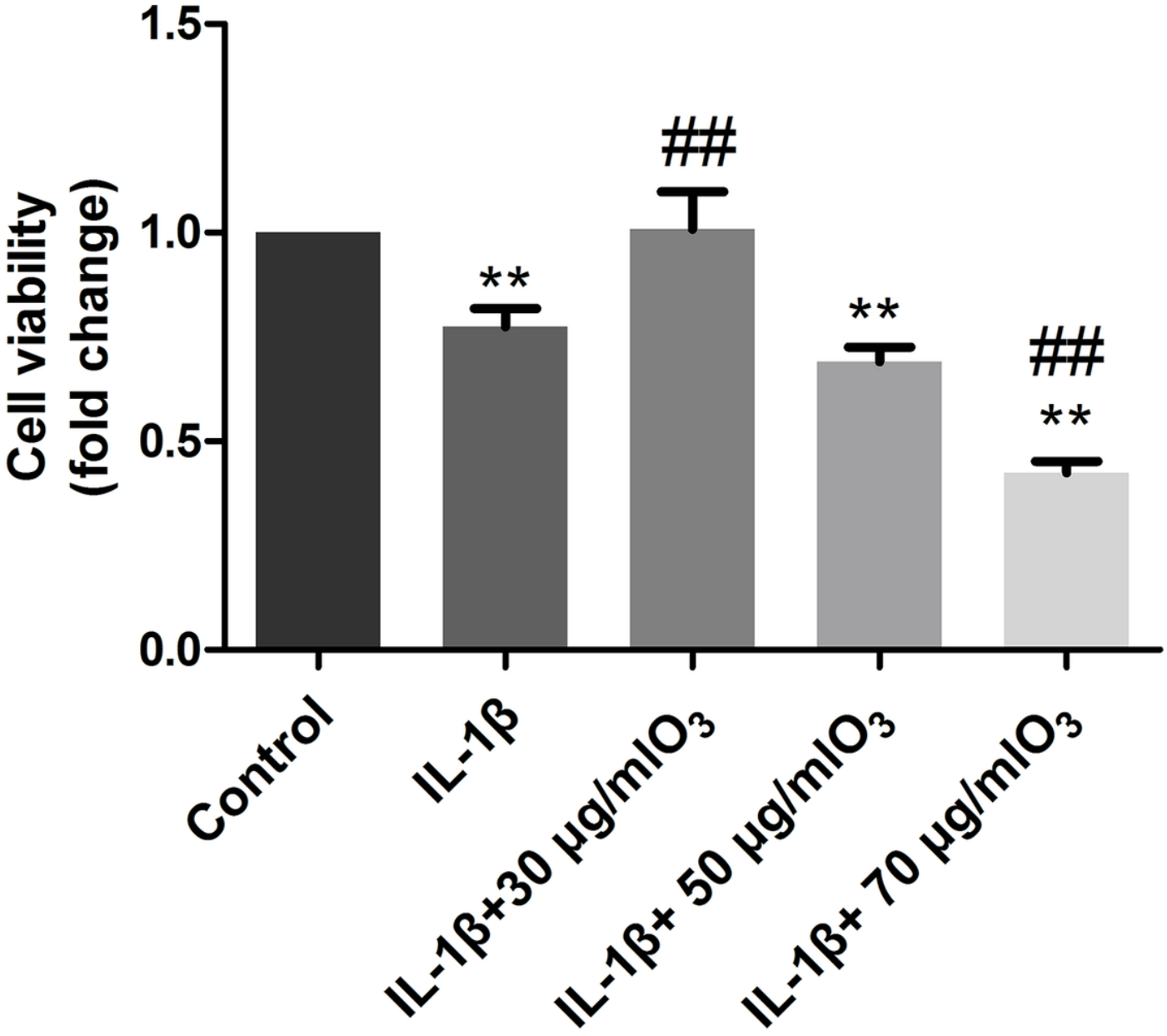

Figure 2

O3 treatment improved the cell viability at 30 μg/mL concentration. Chondrocytes were treated with IL-1β (10 ng/mL) for 24 hours, then were dealed with different concentration of O3 for 30 minutes.*P<0.05, **P<0.01compared with control group; #P<0.05, ##P<0.01,copmared with IL-1β group; Replicates = 3 in each group.

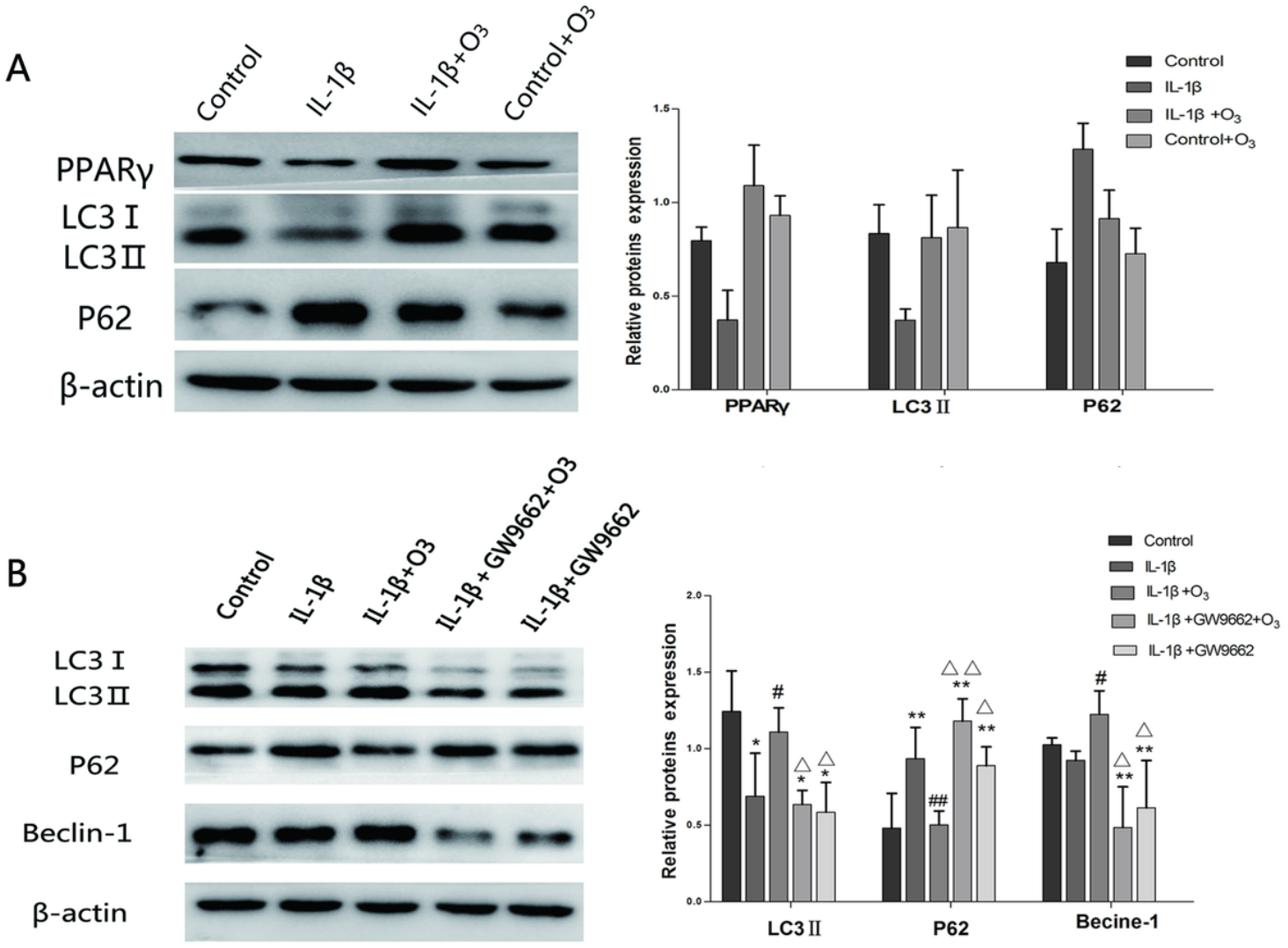

Figure 3

O3 increasesd the level of autophagy of chondrocytes treated with IL-1β but this reversed by GW9662.Western blot for the protein expression of LC3 II,P62,Beclin-1 and β-actin in each group. *P<0.05, **P<0.01vs. Control group; #P<0.05, ##P<0.01vs. IL-1β group; △P<0.05, △△P<0.01vs. IL-1β +O3 group; Replicates = 3 in each group.

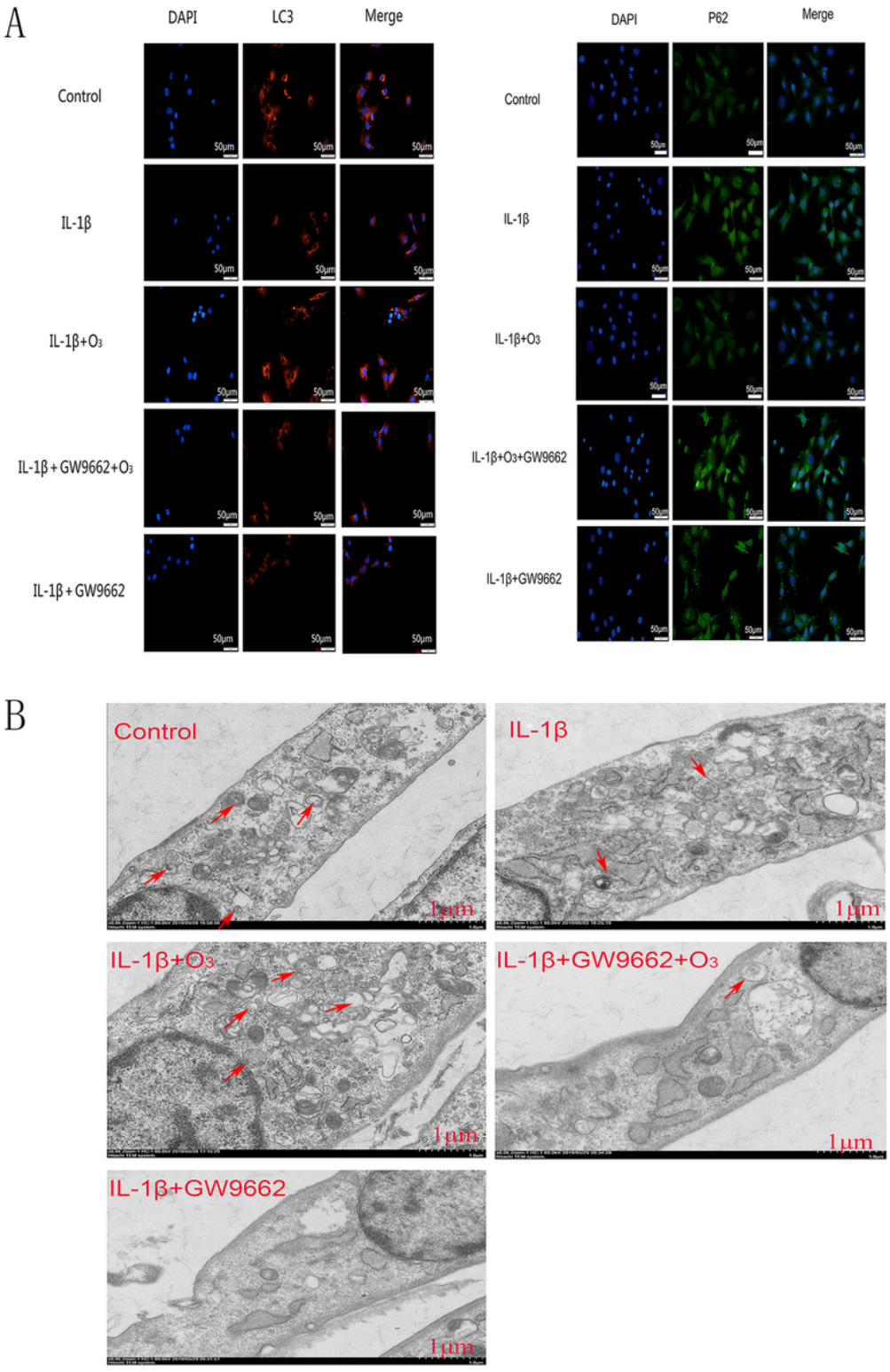

Figure 4

O3 increasesd autophagy of chondrocytes treated with IL-1β but this reversed by GW9662.A The levels of LC3 II and P62 were measured by immunofluorescence at magnifications of 40×; B The numbers of autophagosomes in chondrocytes. The arrows indicate the autophagosomes; Replicates = 3 in each group.

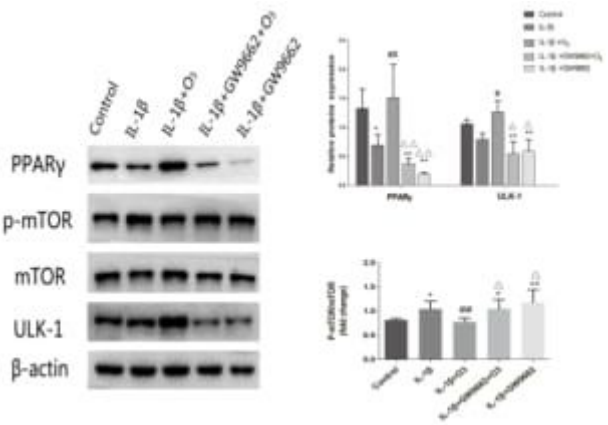

Figure 5

PPARγ/mTOR signaling is involved in O3-induced autophagy in chondrocytes treated with IL-1β. Western blot for the protein expression PPARγ, p-mTOR, mTOR and ULK-1 and β-actin in each group. *P<0.05, **P<0.01vs. Control group; #P<0.05, ##P<0.01vs. IL-1β group; △P<0.05, △△P<0.01vs. IL-1β +O3 group; Replicates = 3 in each group.

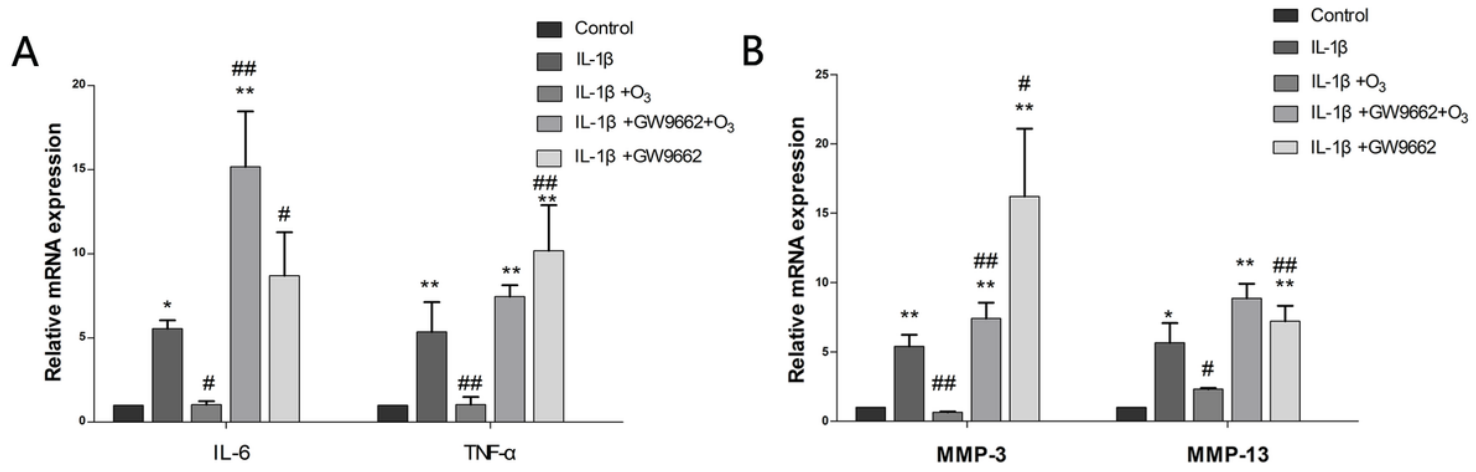

Figure 6

O3 decreased inflammatory cytokines and reduced extracellular matrix degradation of chondrocytes treated with IL-1β. A qRT-PCR for the mRNA expression of IL-6 and TNF-α in each group; B qRT-PCR for the mRNA expression of MMP-3 and MMP-13 in each group. *P<0.05, **P<0.01vs. Control group; #P<0.05, ##P<0.01vs. IL-1β group; △P<0.05, △△

Supplementary Files

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