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Ozone induces autophagy by activating PPAR γ /mTOR in rat chondrocytes treated with IL-1 β

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Abstract

Background: Osteoarthritis (OA) is the main cause of older pain and disability. Intra-articular injections of ozone (O₃) commonly have been found to have antioxidative and anti-inflammatory effects to reduce pain and improve function in knee osteoarthritis. It has been reported that reduced autophagy in chondrocytes plays an important role in the development of OA. This study aimed to probe the role of O₃ on the autophagy in chondrocytes treated with IL-1 β .

Methods: Primary chondrocytes were isolated from Wistar rats cartilage within 3 days. The OA chondrocytes model was induced via treatment with IL-1 β for 24 h. Then the cells were treated with O₃ and GW9662, the inhibitor of PPAR γ . 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: 30 μ g/ml O₃ improved the viability of chondrocytes treated with IL-1 β . The decreased level of autophagy proteins and the numbers of autophagosomes improved in IL-1 β -treated chondrocytes with O₃ via activating PPAR γ /mTOR. In addition, the qRT-PCR results showed that O₃ decreased the levels of IL-6, TNF- α and MMP-3, MMP-13 in chondrocytes treated with IL-1 β .

Conclusions: 30 μ g/ml O₃ improved autophagy via activating PPAR γ /mTOR signaling and suppressing inflammation in chondrocytes treated with IL-1 β .

Keywords: O₃, Osteoarthritis, PPAR γ /mTOR, Autophagy, Inflammation

Background

With the accelerating aging of the world population, the incidence of degenerative diseases, such as osteoarthritis (OA), is on the rise [1]. OA is the most common joint disorder and one of the most common sources of pain and disability in the elderly. The Global Burden of Disease Study has reported that the global age-standardized point

prevalence of OA was 3.75% in 2017, an increase of 9.3% from 1990 [2]. The pathological process of OA includes chronic inflammation and degeneration of cartilage [3]. Researchers have shown that the imbalance in chondrocyte homeostasis is associated with the occurrence and development of OA [4]. Therefore, it is particularly important to keep chondrocytes healthy for preventing the articular cartilage degeneration.

Autophagy is an important protective response of cells exposed to various stresses [5]. It also plays a critical role in articular chondrocytes as the primary mechanism for maintaining normal function and survival. Studies have linked the pathological process of OA with reduced autophagy in chondrocyte [6]. It has been confirmed that improving the level of autophagy can be beneficial in the

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treatment of OA [7]. In addition, excessive inflammatory cytokines can suppress autophagy activation, causing excessive matrix metalloproteinases (MMPs) expression, leading to cell death [8]. Thus, in the pathological process of OA, inflammatory cytokines also play a vital in cartilage degeneration.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a nuclear receptor involved in the regulation of many cellular processes [9, 10]. Some studies have indicated that PPAR γ activation reduces interleukin (IL)-1-induced expression of inflammatory mediators and MMPs in chondrocytes [11]. PPAR γ suppression by diacerein can alleviate oxidative stress and osteoarthritis in mice [12]. PPAR γ can also be expressed in different cell types and has been linked to autophagy in other diseases [13, 14]. Vasheghani et al. [15] demonstrated that PPAR γ plays a protective role in chondrocytes by regulating the mTOR autophagy signaling pathway. In addition, it was testified that PPAR γ is related to autophagy by interacting with the AKT/mTOR pathway. The study showed when AGEs were induced, loss of PPAR γ resulted in increased expression of mTOR, PPAR γ correlates with phosphorylation of mTOR and PPAR γ level was inversely proportion to the phosphorylation in human chondrocytes [16]. Thus, PPAR γ may play an important part in the development of OA.

O₃ was isolated for the first time in 1839 and used as a powerful oxidant in medicine for more than 150 years [17]. In clinical treatment, O₃ can be applied as a gas mixture of oxygen-ozone (medical- ozone), with different concentration [18–20]. O₃ can produce free radicals, such as reactive oxygen species (ROS) and lipid hydroperoxides (LOPs) when dissolved in the plasma/serum. The moderate oxidative stress caused by ROS induced an assortment of antioxidant enzymes concentration levels increased, such as superoxide dismutase, glutathione peroxidase, catalase, and NADPH quinone-oxidoreductase [21–23]. Some of these enzymes act as free radical scavengers which were testified to relevant to a wide variety of diseases clinically. It was reported that O₃ can activate antioxidant protection systems to attenuate chronic constriction injury (CCI)-induced neuropathic pain [24]. In recent decades, O₃ therapy has been extensively used in clinical practice for treatment of herniated discs, low back pain (LBP) and other chronic pains [25, 26]. The clinical application of O₃ therapy also produced good clinical therapeutic effects in the treatment of disc herniation and pain [27]. In addition, studies have found that O₃ at a suitable concentration can improve chondrocyte autophagy in OA by affecting the expression of mammalian target of rapamycin (mTOR) complexes [28]. A meta-analysis showed that repeating the intra-articular

injection using an appropriate concentration of 20–30ug/ml and a volume of 15–20 mL of ozone every 7 days for four consecutive weeks is advisable protocol in reducing pain, which produces an improvement of the symptoms at 1, 3 and 6 months after treatment [29]. Ozone therapy to alleviate the symptoms of chronic knee OA has been recognized by an ever-increasing number of physicians. Unfortunately, the evidence to elaborate specific mechanism of O₃ treatment is not sufficient, especially for OA. There are prejudices among some therapists toward O₃ treatment. These factors hindered the application of O₃ therapy in clinically. Thus, the aim of this study was to investigate whether PPAR γ /mTOR signaling affected autophagy in chondrocytes treated with IL-1 β following O₃ treatment. The study may reveal the possible underlying mechanisms of O₃ treatment in OA.

Materials and methods

Ethics statement

The rats were provided by the Experimental Animal Center of Shandong University. All rats were killed 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.

Isolation and culture of rat chondrocytes

Thirty male Wistar rats within three days of birth were used in the present study. The rats were killed for cervical dislocation under general anesthesia and disinfected with povidone iodine and 75% ethanol for 5 min. The cartilage tissue of the epiphyseal of the knee joint was separated by surgical scissors and washed twice with PBS. Then the knee cartilage was cut to about 1 mm³. The cartilage was digested with 0.25% trypsin containing ethylenediaminetetraacetic acid (EDTA) for 30 min and 0.2% type II collagenase for overnight at 37 °C. The following day, the digested cells were passed through a 75 μ m 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. In this study, chondrocytes from different individual rats were cultured together, it is deficient in cell homogeneity compared with single animal chondrocyte culture, and there may be differences in cell states from different individual animals,

which may have some influences on the results but is not decisive.

Experimental groups and drug administration

IL-1 β is a highly potent inducer of cartilage degradation, causing profound loss of proteoglycan from cartilage in vitro and in vivo which was deemed to disrupt the balance between catabolism and anabolism activity [30, 31]. IL-1 β -treated chondrocytes or cartilage tissues have been widely adopted as in vitro models to study OA. Then, the chondrocytes were treated with IL-1 β (10 ng/mL) (PeproTech) for 24 h in this present study. The incubated cells were randomly divided into the following groups: Control group, IL-1 β group, IL-1 β + O₃ group, IL-1 β + O₃ + 3MA group, IL-1 β + O₃ + GW9662 group and IL-1 β + GW9662 group. After being treated with IL-1 β for 24 h, the cells were exposed to O₃ for 30 min at a certain concentration which measured using the O₃ analyzer purchased from Perma Pure Inc (model MD-050-12-f-4, Perma Pure Inc). Then cells were pretreated with the 3-methyladenine (3MA, 100 nM) and PPAR γ inhibitor GW9662 for 12 h prior to O₃ 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 plates and incubated at 37 °C for 24 h. After being treated with IL-1 β for 24 h, the cells were exposed to O₃ at concentrations of 30, 50 or 70 μ g/mL for 30 min. Finally, 10 μ L 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 polyvinylidene difluoride (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-PPAR γ (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) 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 h 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 Tizol reagent (Takara, Shiga, Japan). Complementary DNA (cDNA) was amplified using a PrimeScript RT reagent kit (RR047A; Takara). mRNA expression was measured with qRT-PCR using a SYBR[®] GrenER[™] SuperMix (Takara) with the following specific primers for β -actin: forward, 5'-GGGAAATCGTGCCTGAC-3' and reverse 5'-AGGCTGGAAAAGAGCCT-3'; IL-6: forward, 5'-ATTGTA TGAACAGCGATGATGCAC-3', and reverse 5'-CCAGGTAGAAAACGGAAGCTCCAGA-3'; TNF- α : forward 5'-TTCCAATGGGCTTTCCGGAAC-3' and reverse 5'-AGACATCTTCAGCAGCCTTGTGAG-3'; MMP-13: forward 5'-TGATGATGAAACCTGGACAAGCA-3' and reverse 5'-GAACGTCATC ATCTGGGAGCA-3'; MMP-3: forward 5'-TGATGGGCCTGGAATGGTC-3' and reverse 5'-TTCATGAGCAGCAACCAGGAATAG-3'. β -actin was used as the internal control, and the level of gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Toluidine blue staining

Cells were fixed with 10% neutral buffered formalin at 25 °C for 10 min, washed with distilled water, incubated with 0.05% toluidine blue solution (Sigma, USA) for 30 min at 25 °C, and washed 3 times with distilled water. The stained chondrocytes were observed using the inverted phase contrast microscope.

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.

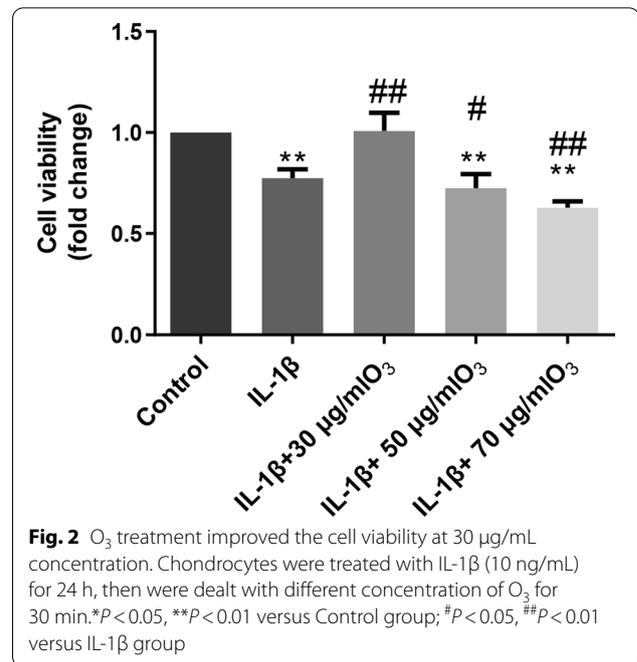
Statistical analysis

SPSS 22.0 software (IBM Corp. Armonk, NY, USA) was applied for data analysis. Measurement data were described as mean \pm standard deviation (SD). The differences among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test for two-group comparisons among the multiple comparisons. The $P < 0.05$ was considered to indicate a statistically significant difference. All measurements were carried out at least three times.

Results

Identification of chondrocytes

Toluidine blue staining result showed the cultured cells are long spindle shaped. The cell nucleus is round and regular, and the cytoplasm is purple-red with a few blue-purple heterochromatic particles, 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, supporting the above conclusion (Fig. 1B).



O₃ (30 μ g/ml) improves IL-1 β -treated chondrocyte viability

As shown in Fig. 2, 30 μ g/ml O₃ improved cell viability, while 50 and 70 μ g/ml O₃ obviously decreased cell viability relative to treatment with IL-1 β alone. Thus, the dose of 30 μ g/mL O₃ was selected for further experiments.

O₃ increases the expression of autophagy in chondrocytes treated with IL-1 β

The IL-1 β -stimulated chondrocytes were pretreated with the autophagy inhibitor 3MA (100 nM) before treatment with 30 μ g/mL ozone. As shown in Fig. 3A, IL-1 β

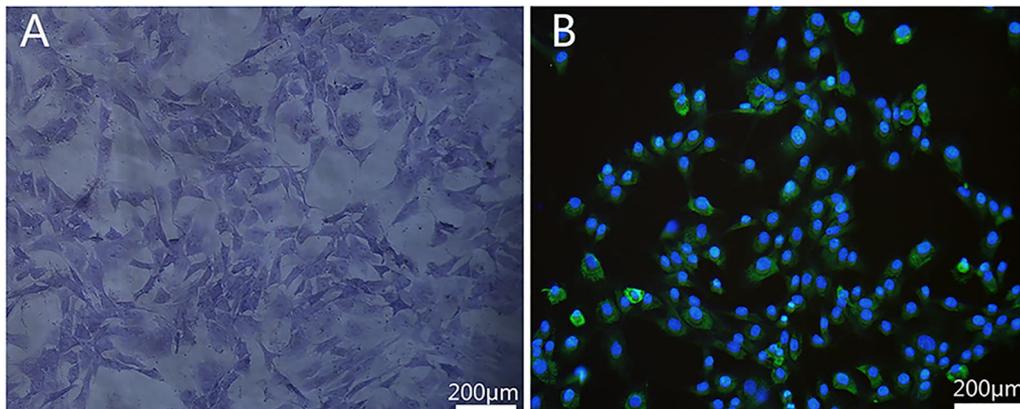
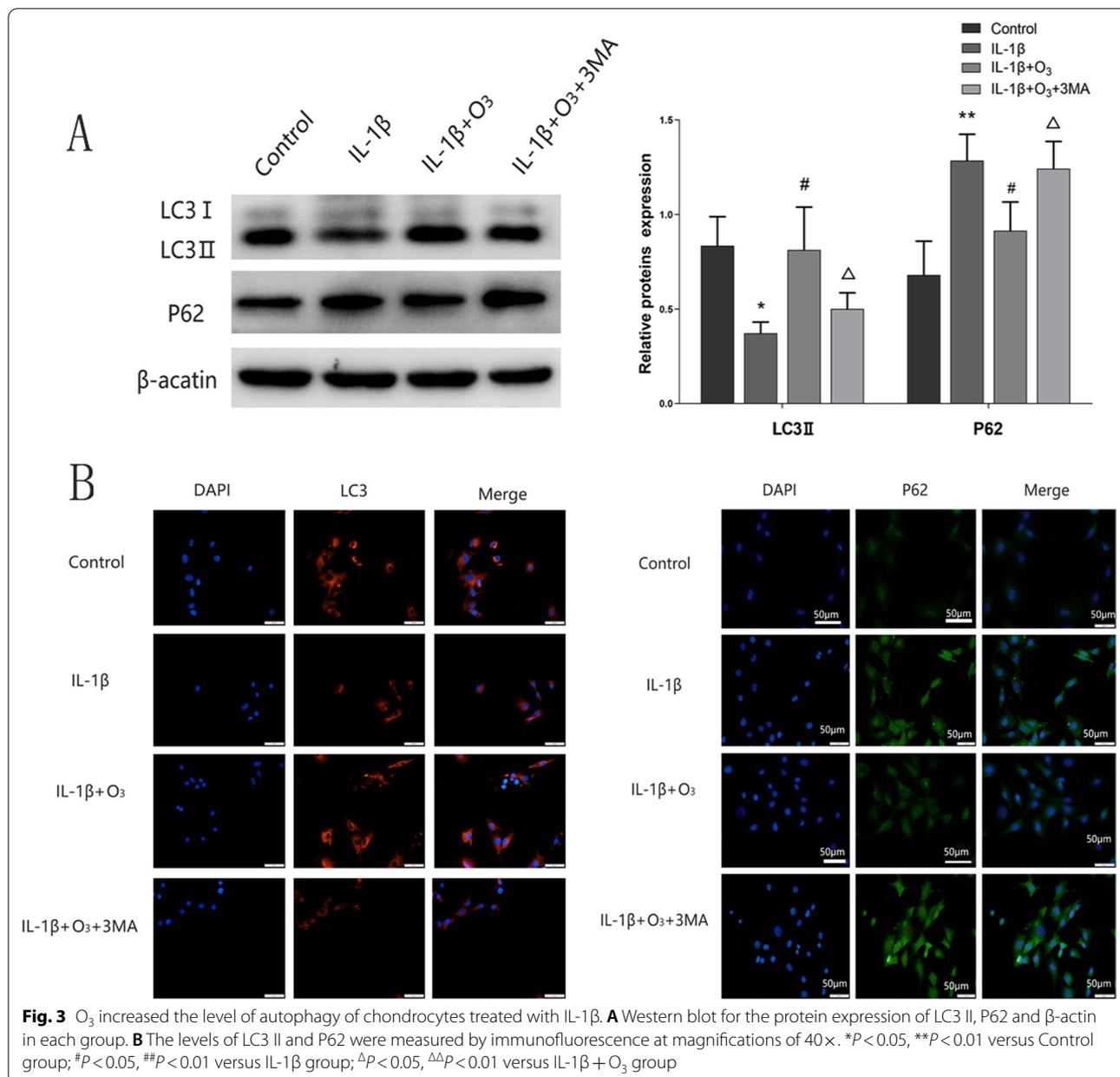


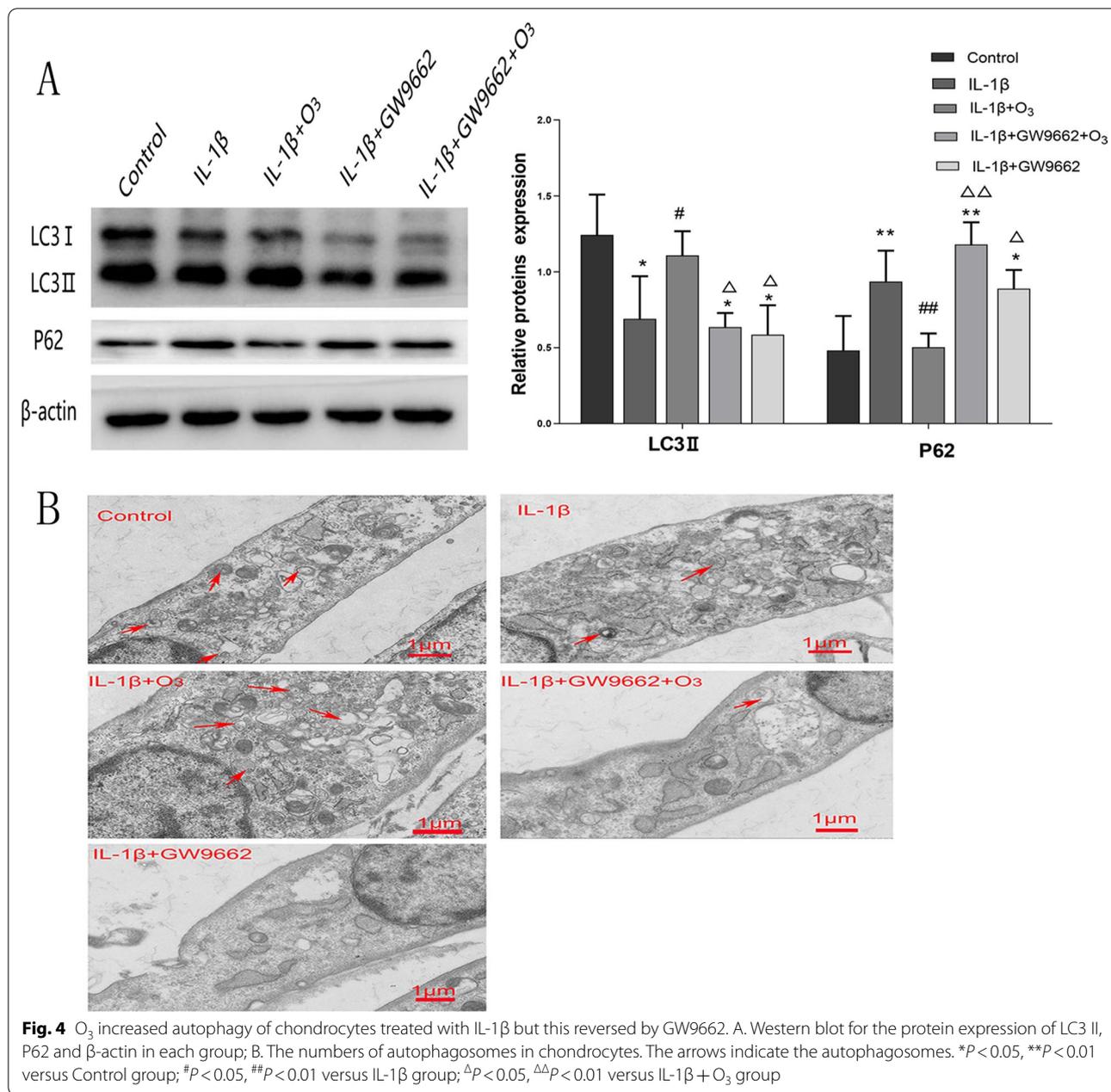
Fig. 1 Rat chondrocytes morphological observations. **A** Toluidine blue staining chondrocytes were observed under the inverted phase contrast microscope at magnifications of 20 \times ; **B** More than 95% of the cultured cells showed positive expression of type II collagen under a fluorescence microscope at 20 \times magnification



administration significantly attenuated the level of LC3 II and increased P62 in chondrocytes. 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 O₃. But O₃ did not obviously change the low level of autophagy in IL-1β-stimulated chondrocytes pretreated with 3MA (Fig. 3A). As shown in Fig. 3B, the result of immunocytochemical staining shows that compared with the IL-1β group, fluorescence intensities of LC3 enhanced while P62 decreased with O₃ treatment. While treated with 3MA, accordingly, led to reversed trends.

O₃ ameliorates autophagy via PPARγ/mTOR signaling in chondrocytes treated with IL-1β

The expression of PPARγ has been associated with autophagy. We detected the expression of biomarkers of autophagy in chondrocytes pretreated with GW9662 by Western blotting. The results showed that compared with the IL-1β + O₃ group, the level of LC3 II decreased and P62 increased in chondrocytes pretreated with GW9662 (Fig. 4A). Besides, TEM analysis revealed that the number of autophagosomes was decreased in chondrocytes treated with IL-1β and improved by O₃ treatment. In

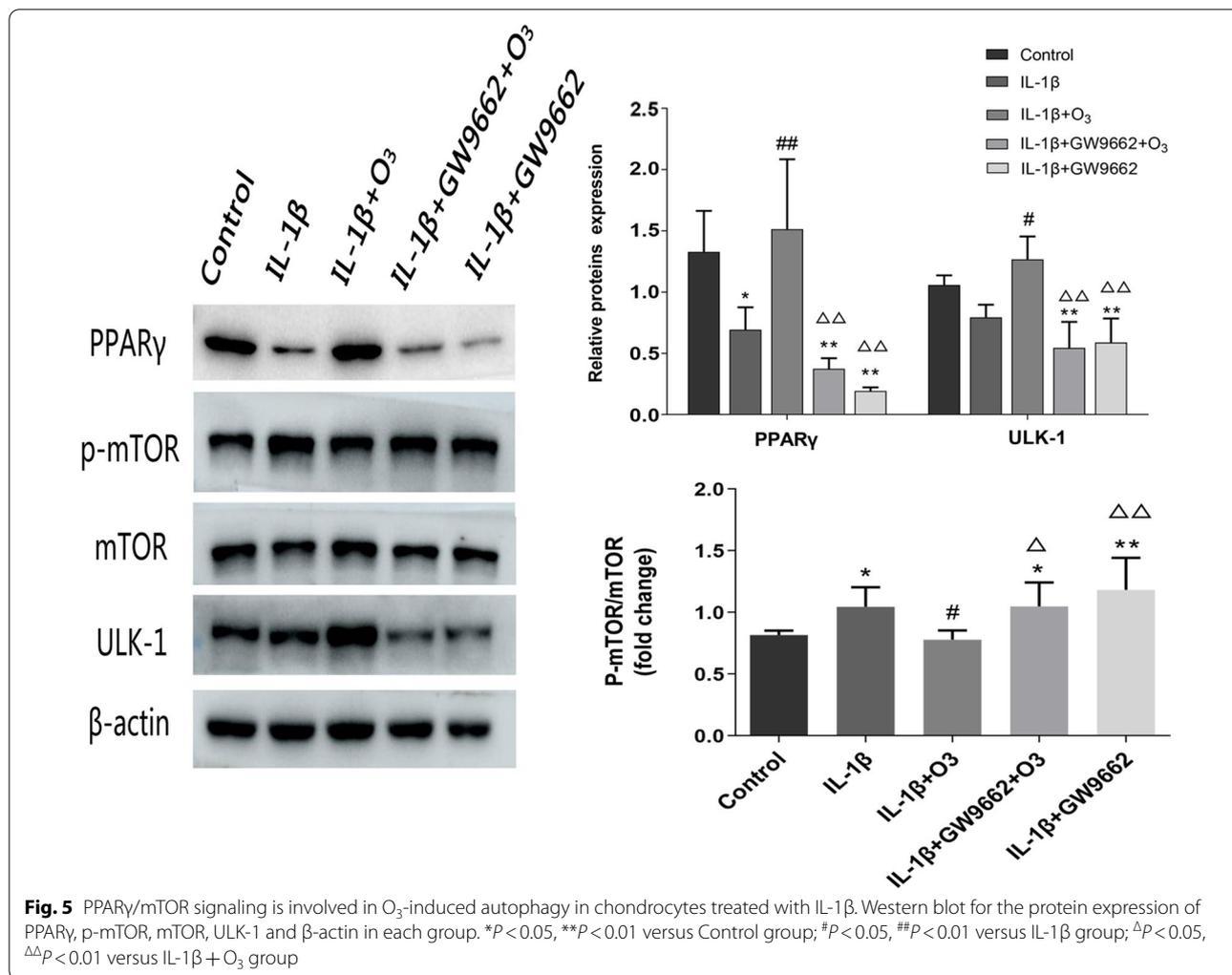


addition, the number of autophagosomes was decreased following treatment with GW9662 (Fig. 4B).

To further investigate the mechanism of O_3 -induced autophagy in chondrocytes treated with IL-1 β , the protein levels of phosphorylated mTOR (p-mTOR), ULK1 were assessed after treated with GW9662. The results showed that O_3 decreased p-mTOR and improved the levels of ULK1 in IL-1 β + O_3 group, compared with IL-1 β group. In the presence of GW9662, the level of ULK1 was declined significantly and the level of p-mTOR was up-regulated (Fig. 5).

O_3 decreases the inflammatory response in chondrocytes treated with IL-1 β

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 level mRNA of IL-6, TNF- α , MMP-3 and MMP-13 in IL-1 β + O_3 group, compared with IL-1 β group. While treated with GW9662, the level mRNA of IL-6, TNF- α , MMP-3 and MMP-13 was up-regulated significantly (Fig. 6).

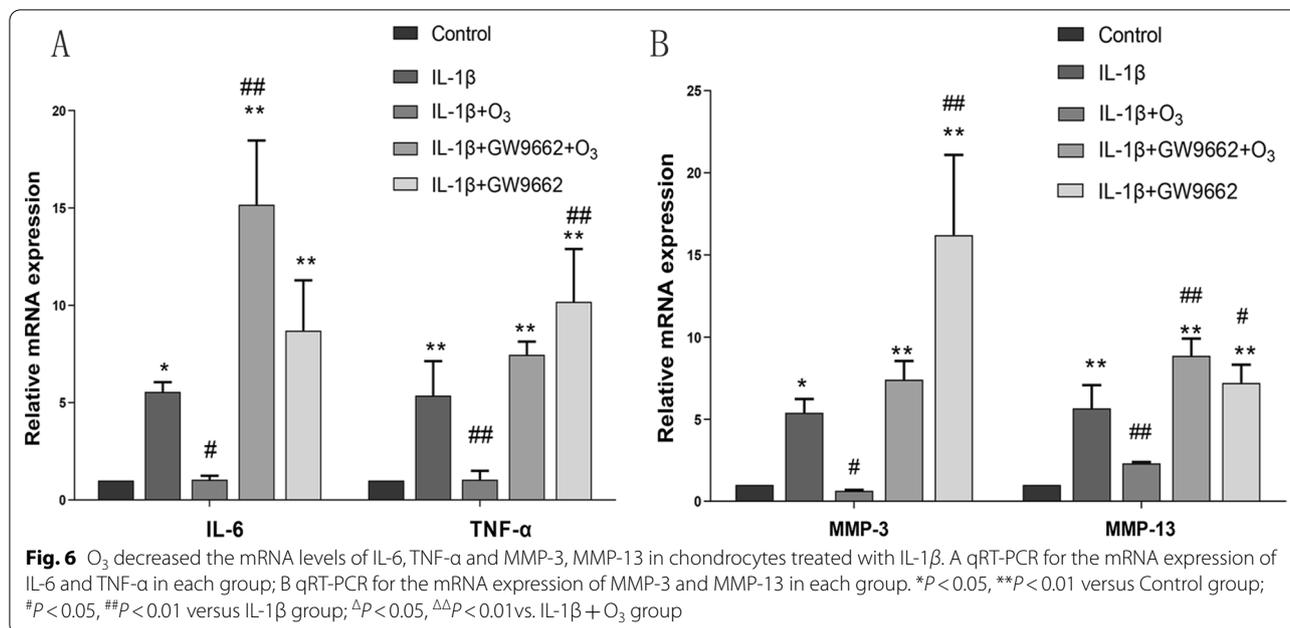


Discussion

OA is a multifactorial disease that is the result of the interaction of mechanical and biological factors [32]. In the progression of OA, inflammatory cytokines such as IL-1 β and TNF- α play important roles in promoting the degradation of the cartilage matrix and articular cartilage [32]. IL-1 β can induce the expression of many cytokines, changing the expression of various proteases related to the progression of OA [33]. Hence, the chondrocytes treated with IL-1 β can be used to simulate OA chondrocytes in vitro studies [34, 35]. 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 [36, 37]. Besides, it has been shown that O₃ can upregulate the reduced autophagy in OA chondrocytes and have

beneficial effects in the treatment of OA in clinically [38]. Based on the above, the aim of this study was to investigate the potential molecular biological mechanisms of O₃ on autophagy in chondrocytes treated with IL-1 β .

In this study, we investigated the appropriate concentration of O₃ for chondrocytes treated with IL-1 β using a CCK-8 kit. The results demonstrated that 30 μ g/mL O₃ improved cell viability. Thus, a dose of 30 μ g/mL O₃ was chosen to the treatment of chondrocytes treated with IL-1 β . In the present study, the results indicated that IL-1 β suppressed the level of autophagy which was reflected by reduced LC3II and increased P62 in chondrocytes. LC3 conversion (LC3-I to LC3-II) reflects the progression of autophagy, and detecting LC3 by immunoblot analysis is often used to monitor autophagic activity [39]. The p62 protein, also called sequestosome 1 (SQSTM1), binds directly to LC3 via a short LC3 interaction region, which degraded by autophagy and served as a marker to study autophagic flu [40]. After treatment with



O₃ (30 μ g/mL), the levels of autophagy were enhanced in IL-1 β -treated chondrocytes. But the ozone-induced autophagy was inhibited by 3MA, which is commonly used as autophagy inhibitor, selectively inhibiting class III PI3K to block autophagic activation. These findings indicate that O₃ regulates autophagy in IL-1 β -treated chondrocytes.

Previous studies have shown that the activation of PPAR γ has beneficial effects on the level of autophagy in type 2 diabetic mice. In addition, deletion of the PPAR γ gene can lead to cartilage-specific destruction leading to spontaneous OA [15]. To further explore the role of PPAR γ in O₃-induced autophagy, chondrocytes were treated with the PPAR γ inhibitor GW9662 before O₃ treatment. The potent, irreversible and selective PPAR γ antagonist GW9662 was proved to prevent the activation of PPAR γ and inhibit growth of human mammary tumor cell lines [41]. In this study, we found that the level of PPAR γ is sharply reduced following treatment with GW9662. Besides, the levels of LC3II decreased while the levels of P62 increased following treatment with GW9662. The O₃-induced autophagy was reversed by GW9662. Considering the negative effect of GW9662 on autophagy, it is logical that PPAR γ plays a key role in O₃-induced autophagy. Furthermore, the results of TEM on autophagosomes also elucidate this issue well.

As PPAR γ could induce aberrant mTOR signaling in chondrocytes, we suppose that in O₃-treated chondrocytes, PPAR γ 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 [42]. Moreover, our data demonstrate that GW9662 reversed the O₃-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 [43]. It was identified in HEK 293 cells where down-regulation of ULK1 was sufficient to inhibit autophagy [44]. Our data shows that ULK1 levels significantly decreased following an increase in p-mTOR in chondrocytes treated with GW9662. Therefore, the PPAR γ /mTOR pathway is activated in the chondrocytes in this study. But the mechanism of ozone action has not been clarified yet, and the study has shown that inhibiting the vicious cycle of TNF α -syndecan 4-TNF α may also play important roles in chondrocyte autophagy [45]. Therefore, ozone could induce autophagy in part by activating PPAR γ /mTOR signaling.

Since inflammation plays an important role in the development and progression of OA, the development of therapies focusing on inhibiting inflammation is increasing. 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 [46]. Certain studies have demonstrated that IL-1 β could suppress the autophagy pathway in chondrocytes [47]. Importantly, activation of PPAR γ has been shown to have beneficial effects in

alleviating inflammation in human OA chondrocytes [48]. In the present study, we found that O₃ inhibits the mRNA levels of the TNF- α and IL-6 in chondrocytes of treated with IL-1 β . However, this result was reversed by the suppression of PPAR γ . In addition, a number of studies have indicated that activation of PPAR γ can inhibit NF- κ B activation and decrease the levels of inflammatory cytokines [49]. The activation of autophagy can be suppressed by excessive release of inflammatory cytokines [50]. Considering that O₃ blocked IL-1 β -induced autophagy inhibition in chondrocytes, the result is likely due to its anti-inflammatory effect on chondrocytes. Moreover, study has shown that IL-1 β can induce the expression of MMPs, in turn inducing cartilage matrix degradation [51]. The result of qRT-PCR results revealed that the mRNA levels of MMP-13 and MMP-3 were decreased with O₃ treatment. These data suggest that inhibition of inflammatory cytokines through activation of PPAR γ has beneficial effects on IL-1 β -treated chondrocytes. Our findings suggest O₃ seems to be able to attenuated the IL-1 β -induced production TNF- α , IL-6, and MMPs. Taken together, O₃ PPAR γ activation plays an important role in the protective effect of O₃ in chondrocytes treated with IL-1 β .

There were some limitations to our study. We did not test whether administering PPAR γ agonist treatment had the protective effects in chondrocytes treated with IL-1 β . In this study, we observed the PPAR γ pathways is involved in the protective effects of osteoarthritis by reducing inflammatory reaction. However, the specific effects of these pathways in osteoarthritis are worth pursuing. In addition, the specific effects of these pathways in vivo remain to be investigated.

Conclusions

In this study, we revealed that O₃ can induce autophagy by activating the PPAR γ /mTOR signaling pathway and inhibit the inflammatory cytokines via the activation of PPAR γ in chondrocytes treated with IL-1 β .

Abbreviations

OA: Osteoarthritis; MMPs: Matrix metalloproteinases; PPAR γ : Peroxisome proliferator-activated receptor γ ; O₃: 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.

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Author contributions

PPS and WCX participated in the acquisition and culture of primary chondrocytes, literature research, experimental studies and manuscript writing and review. CZ, XZ, XWL and MXG participated in experimental studies, study design and coordination, analyzed the results. ZJF participated in the study design and manuscript review. All authors read and approved the final submitted manuscript.

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Data availability statement

All the data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the local ethics committee (Shandong University, Animal experiments local ethics committee chair. The ethics approval number is NSFC:2020-663).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H. Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies. *EClinicalMedicine*. 2020;29–30:100587–100587.
- Safiri S, Kolahi AA, Smith E, Hill C, Bettampadi D, Mansournia MA, Hoy D, Ashrafi-Asgarabad A, Sepidarkish M, Almasi-Hashiani A, et al. Global, regional and national burden of osteoarthritis 1990–2017: a systematic analysis of the Global Burden of Disease Study 2017. *Ann Rheum Dis*. 2020;79(6):819–28.
- Vinatier C, Domínguez E, Guicheux J, Caramés B. Role of the inflammation-autophagy-senescence integrative network in osteoarthritis. *Front Physiol*. 2018;9:706–706.
- Wang C, Shen J, Ying J, Xiao D, O'Keefe RJ. FoxO1 is a crucial mediator of TGF- β /TAK1 signaling and protects against osteoarthritis by maintaining articular cartilage homeostasis. *Proc Natl Acad Sci USA*. 2020;117(48):30488–97.
- Wang L, Ye X, Zhao T. The physiological roles of autophagy in the mammalian life cycle. *Biol Rev Camb Philos Soc*. 2019;94(2):503–16.
- Cheng N-T, Guo A, Meng H. The protective role of autophagy in experimental osteoarthritis, and the therapeutic effects of Torin 1 on

- osteoarthritis by activating autophagy. *BMC Musculoskelet Disord.* 2016;17:150–150.
7. Tang Y, Mo Y, Xin D, Zeng L, Yue Z, Xu C. β -ecdysterone alleviates osteoarthritis by activating autophagy in chondrocytes through regulating PI3K/AKT/mTOR signal pathway. *Am J Transl Res.* 2020;12(11):7174–86.
 8. Zhou H, Li G, Wang Y, Jiang R, Li Y, Wang H, Wang F, Ma H, Cao L. Microbial metabolite sodium butyrate attenuates cartilage degradation by restoring impaired autophagy and autophagic flux in osteoarthritis development. *Front Pharmacol.* 2021;12:659597–659597.
 9. Duszka K, Gregor A, Guillou H, König J, Wahli W. Peroxisome proliferator-activated receptors and caloric restriction-common pathways affecting metabolism, health, and longevity. *Cells.* 2020;9(7):1708.
 10. Feng S, Reuss L, Wang Y. Potential of natural products in the inhibition of adipogenesis through regulation of ppar expression and/or its transcriptional activity. *Molecules.* 2016;21(10):1278.
 11. Nebbaki S-S, El Mansouri FE, Aff H, Kapoor M, Benderdour M, Duval N, Pelletier J-P, Martel-Pelletier J, Fahmi H. Egr-1 contributes to IL-1-mediated down-regulation of peroxisome proliferator-activated receptor γ expression in human osteoarthritic chondrocytes. *Arthritis Res Ther.* 2012;14(2):R69–R69.
 12. Chen X, Zhu X, Dong J, Chen F, Gao Q, Zhang L, Cai D, Dong H, Ruan B, Wang Y, et al. Reversal of epigenetic PPAR γ suppression by diacerein alleviates oxidative stress and osteoarthritis in mice. *Am J Pathol.* 2022. <https://doi.org/10.1089/ars.2021.0219>.
 13. 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;9:851–851.
 14. Wang W, Li C, Zhang Z, Zhang Y. Arsenic trioxide in synergy with vitamin D rescues the defective VDR-PPAR- γ functional module of autophagy in rheumatoid arthritis. *PPAR Res.* 2019;2019:6403504–6403504.
 15. Vasheghani F, Monemdjou R, Fahmi H, Zhang Y, Perez G, Blati M, St-Arnaud R, Pelletier J, 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.
 16. Wang Z, Zhang H, Chen C, Huang H, Liang J. Effect of PPAR γ on AGES-induced AKT/MTOR signaling-associated human chondrocytes autophagy. *Cell Biol Int.* 2018;42(7):841–8.
 17. Elvis AM, Ekta JS. Ozone therapy: a clinical review. *J Nat Sci Biol Med.* 2011;2(1):66. <https://doi.org/10.4103/0976-9668.82319>.
 18. de Sire A, Agostini F, Lippi L, Mangone M, Marchese S, Cisaric C, Bernetti A, Invernizzi M. Oxygen-Ozone therapy in the rehabilitation field: state of the art on mechanisms of action, safety and effectiveness in patients with musculoskeletal disorders. *Biomolecules.* 2021;11(3):356. <https://doi.org/10.3390/biom11030356>.
 19. Sconza C, Leonardi G, Kon E, Respizzi S, Massazza G, Marcacci M. Oxygen-ozone therapy for the treatment of low back pain: a systematic review of randomized controlled trials. *Eur Rev Med Pharmacol Sci.* 2021;25(19):6034–46.
 20. Bonetti M, Zambello A, Princiotta C, Pellicanò G, Della Gatta L, Muto M. Non-discogenic low back pain treated with oxygen-ozone: outcome in selected applications. *J Biol Regul Homeostatic Agents.* 2020;34:21–30.
 21. Smith NL, Wilson AL, Gandhi J, Vatsia S, Khan SA. Ozone therapy: an overview of pharmacodynamics, current research, and clinical utility. *Med Gas Res.* 2017;7(3):212–9.
 22. Inal M, Dokumacioglu A, Özcelik E, Ucar O. The effects of ozone therapy and coenzyme Q10 combination on oxidative stress markers in healthy subjects. *Irish J Med Sci.* 2011;180(3):703–7. <https://doi.org/10.1007/s11845-011-0675-7>.
 23. Sagai M, Bocci V. Mechanisms of action involved in ozone therapy: is healing induced via a mild oxidative stress? *Med Gas Res.* 2011;1(1):29. <https://doi.org/10.1186/2045-9912-1-29>.
 24. Lu L, Pan C, Chen L, Hu L, Wang C, Han Y, Yang Y, Cheng Z, Liu W. AMPK activation by peri-sciatic nerve administration of ozone attenuates CCl₄-induced neuropathic pain in rats. *J Mol Cell Biol.* 2017;9(2):132–43.
 25. Braidy N, Izadi M, Sureda A, Jonaidi-Jafari N, Banki A, Nabavi S, Nabavi S. Therapeutic relevance of ozone therapy in degenerative diseases: focus on diabetes and spinal pain. *J Cell Physiol.* 2018;233(4):2705–14.
 26. Seyam O, Smith N, Reid I, Gandhi J, Jiang W, Khan S. Clinical utility of ozone therapy for musculoskeletal disorders. *Med Gas Res.* 2018;8(3):103–10.
 27. Migliorini F, Maffulli N, Eschweiler J, Bestch M, Tingart M, Baroncini A. Ozone injection therapy for intervertebral disc herniation. *Br Med Bull.* 2020;136(1):88–106.
 28. 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-1 β through the AMPK/mTOR signaling pathway. *J Pain Res.* 2018;11:3003–17.
 29. Oliviero A, Giordano L, Maffulli N. The temporal effect of intra-articular ozone injections on pain in knee osteoarthritis. *British Medical Bull.* 2019;132(1):33–44.
 30. Dingle JT, Page Thomas DP, King B, Bard DR. In vivo studies of articular tissue damage mediated by catabolin/interleukin 1. *Ann Rheumatic Dis.* 1987;46(7):527–33. <https://doi.org/10.1136/ard.46.7.527>.
 31. Fan Z, Söder S, Oehler S, Fundel K, Aigner T. Activation of interleukin-1 signaling cascades in normal and osteoarthritic articular cartilage. *Am J Pathol.* 2007;171(3):938–46.
 32. Martínez-Moreno D, Jiménez G, Gálvez-Martín P, Rus G, Marchal J. Cartilage biomechanics: A key factor for osteoarthritis regenerative medicine. *Biochim Biophys Acta Mol Basis Dis.* 2019;1865(6):1067–75.
 33. Ahmad N, Ansari MY, Bano S, Haqqi TM. Imperatorin suppresses IL-1 β -induced iNOS expression via inhibiting ERK-MAPK/AP1 signaling in primary human OA chondrocytes. *Int Immunopharmacol.* 2020;85:106612. <https://doi.org/10.1016/j.intimp.2020.106612>.
 34. Bai H, Zhang Z, Li Y, Song X, Ma T, Liu C, Liu L, Yuan R, Wang X, Gao L. L-Theanine reduced the development of knee osteoarthritis in rats via its anti-inflammation and anti-matrix degradation actions: in vivo and in vitro study. *Nutrients.* 2020;12(7):1988. <https://doi.org/10.3390/nu12071988>.
 35. Lu W, He Z, Shi J, Wang Z, Wu W, Liu J, Kang H, Li F, Liang S. AMD3100 attenuates post-traumatic osteoarthritis by maintaining transforming growth factor- β 1-Induced expression of tissue inhibitor of metalloproteinase-3 the phosphatidylinositol 3-kinase/Akt pathway. *Front Pharmacol.* 2019;10:1554.
 36. López de Figueroa P, Lotz M, Blanco F, Caramés B. Autophagy activation and protection from mitochondrial dysfunction in human chondrocytes. *Arthritis Rheumatol.* 2015;67(4):966–76.
 37. Lotz M, Caramés B. Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. *Nat Rev Rheumatol.* 2011;7(10):579–87.
 38. Manoto S, Maepa M, Motaung S. Medical ozone therapy as a potential treatment modality for regeneration of damaged articular cartilage in osteoarthritis. *Saudi J Biol Sci.* 2018;25(4):672–9.
 39. Jiang P, Mizushima NJM. LC3- and p62-based biochemical methods for the analysis of autophagy progression in mammalian cells. *Methods.* 2015;75:13–8.
 40. Komatsu M, Kageyama S, Ichimura Y. p62/SQSTM1/A170: physiology and pathology. *Pharmacol Res.* 2012;66(6):457–62.
 41. Seargent J, Yates E, Gill J. GW9662, a potent antagonist of PPAR γ , inhibits growth of breast tumour cells and promotes the anticancer effects of the PPAR γ agonist rosiglitazone, independently of PPAR γ activation. *Br J Pharmacol.* 2004;143(8):933–7.
 42. Zhang Y, Vasheghani F, Li Y, Blati M, Simeone K, Fahmi H, Lussier B, Roughley P, Lagares D, Pelletier J, et al. Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. *Ann Rheumatic Dis.* 2015;74(7):1432–40.
 43. Kim J, Kundu M, Viollet B, Guan K. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol.* 2011;13(2):132–41.
 44. Chan E, Kir S, Tooze S. siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. *J Biol Chem.* 2007;282(35):25464–74.
 45. He F, Ma Y, Li S, Ren H, Liu Q, Chen X, Miao H, Ye T, Lu Q, Yang Z, et al. Necroptotic TNF α -syndecan 4-TNF α vicious cycle as a therapeutic target for preventing temporomandibular joint osteoarthritis. *J Bone Miner Res.* 2022;37(5):1044–55. <https://doi.org/10.1002/jbmr.4542>.
 46. De Luna-Preitschopf A, Zwickl H, Nehrer S, Hengstschläger M, Mikula M. Rapamycin maintains the chondrocytic phenotype and interferes with inflammatory cytokine induced processes. *Int J Mol Sci.* 2017;18(7):1494.
 47. Yoshii S, Mizushima N. Monitoring and measuring autophagy. *Int J Mol Sci.* 2017;18(9):1865.
 48. Kang S, Zhang J, Yuan Y. Abietic acid attenuates IL-1 β -induced inflammation in human osteoarthritis chondrocytes. *Int Immunopharmacol.* 2018;64:110–5.
 49. Lim H, Lee E, Kim J, Park M, Kim D, Choi Y, Ha Y, Yoon J, Choi J, Yu B, et al. PPAR γ activation by baicalin suppresses NF- κ B-mediated inflammation in aged rat kidney. *Biogerontology.* 2012;13(2):133–45.

50. Qi Y, Zhang M, Li H, Frank J, Dai L, Liu H, Zhang Z, Wang C, Chen G. Autophagy inhibition by sustained overproduction of IL6 contributes to arsenic carcinogenesis. *Cancer Res.* 2014;74(14):3740–52.
51. Jia T, Cai M, Ma X, Li M, Qiao J, Chen T. Oridonin inhibits IL-1 β -induced inflammation in human osteoarthritis chondrocytes by activating PPAR- γ . *Int Immunopharmacol.* 2019;69:382–8.

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