4-Octyl itaconate protects chondrocytes against IL-1β-induced oxidative stress and ferroptosis by inhibiting GPX4 methylation in osteoarthritis

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Research Article

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Abstract

The role of oxidative stress and ferroptosis in the pathogenesis of osteoarthritis (OA) is significant. 4-Octyl Itaconate (O1) has been shown to have protective effects against oxidative stress and inflammatory response, and has been identified as a potential therapeutic agent for OA. However, the specific effects of O1 on the regulation of chondrocyte degeneration, oxidative stress, and ferroptosis require further investigation. The objective of our study was to examine the effects of O1 on IL-1β-induced chondrocytes and an OA mouse model. Our results demonstrate that O1 effectively mitigates IL-1β-induced chondrocyte degeneration in a dose-dependent manner. Additionally, O1 exhibited a significant inhibitory effect on ROS production and mitigated the decline in Recombinant Glutathione Peroxidase 4 (GPX4) levels induced by IL-1β and Erastin, a ferroptosis activator. As a result, O1 demonstrated potential in attenuating the degenerative effects of IL-1β and Erastin on chondrocytes, possibly through the reduction of ferroptosis. The molecular mechanism underlying O1’s regulation of GPX4 expression in chondrocytes was found to involve the repression of GPX4 methylation. Furthermore, the inhibition of GPX4 methylation was observed to improve IL-1β-triggered degeneration, oxidative stress, and ferroptosis in chondrocytes. Comparable outcomes were obtained in animal models of osteoarthritis (OA). The utilization of O1 and Ferrostatin-1 (Fer-1) mitigated the progression of OA and cartilage degeneration in the mouse model of OA, which was established by destabilization of medial meniscus (DMM). Consequently, it can be inferred that O1 alleviates degeneration, oxidative stress, and ferroptosis in IL-1β-treated chondrocytes by inhibiting GPX4 methylation both in vitro and in vivo. These findings indicate that O1 represents a promising new therapeutic modality for OA.

Introduction

Osteoarthritis (OA) is a degenerative disease that affects the articular cartilage and is a leading cause of chronic disability in the elderly population[1]. As a high incidence and disability rate of OA is observed among the elderly, it poses a huge burden on society[2]. Progressive degradation of articular cartilage is a key pathological feature of OA[3]. Currently, therapeutic interventions for OA mainly focus on pain alleviation but fail to delay the progression of OA [4–6]. Although treatments such as non-steroidal anti-inflammatory drugs (NSAIDs), physiotherapy, and glucocorticoids can alleviate symptoms, they do not reverse the loss of articular cartilage[7, 8]. Therefore, identifying effective treatment strategies for OA is still a challenge. The chondrocyte is the only cell in articular cartilage, and it maintains cartilage integrity by maintaining the balance between synthesis and degradation of extracellular matrix (ECM)[9]. Therefore, excessive chondrocyte death has destructive effects on articular cartilage[10]. Moreover, the impairment of chondrocytes is a critical factor in the progression of OA[11]. The chondrocytes can be damaged by necrosis, apoptosis, and autophagy, according to previous studies[12].

Ferroptosis is a form of cell death that is associated with oxidative stress, characterized by iron-dependent accumulation of lipid hydroperoxides[13–16]. Extensive research has been conducted on ferroptosis, which is involved in cell metabolism, oxidative and electrophilic stress responses, and immune responses[17–19]. Recently, ferroptosis has been linked to many degenerative diseases,
including brain injury, cerebral hemorrhage, Alzheimer's disease, cancer, stroke, and others[20–24]. Similarly, ferroptosis has been identified as a key player in the development of osteoarthritis (OA)[15]. Literatures have demonstrated that Recombinant Glutathione Peroxidase 4 (GPX4) is a crucial molecule in the signal transduction of ferroptosis, and decreased GPX4 activity leads to significant reduction in lipid peroxide clearance[12, 25–29].

Itaconate, a metabolite of the tricarboxylic acid cycle (TAC), is derived from the mitochondrial matrix of macrophage and produced during macrophage activation[30]. Apart from decreasing the generation of reactive oxygen species (ROS), itaconate also blocks proinflammatory cytokine release, it has been identified as a negative regulator of oxidative stress and immune inflammation[31]. In studies, itaconate has been shown to suppress the production of reactive oxygen species (ROS) and the oxidation of lipids which are major indicators of ferroptosis[32, 33]. 4-Octyl Itaconate (OI), an itaconate derivative that is cellular permeable, can be hydrolyzed to itaconate by esterase[34]. OI, being a molecule with higher cellular permeability, is more appropriate for investigating cellular and molecular mechanisms than itaconate[35]. Emerging evidence shows that OI can improve OA by inhibiting inflammatory response and ampoptosis, and enhancing autophagy [36, 37]. Nevertheless, to date, the effects of this compound on the regulation of chondrocyte degeneration, oxidative stress, and ferroptosis remain unknown. The purpose of this study is to investigate the impact of OI on the regulation of chondrocyte degeneration, oxidative stress, and ferroptosis in OA.

Materials and Methods

Chondrocyte isolation, culture and treatment

Primary chondrocytes were isolated from 3-day-old SD rats as previously described [38]. In a humidified environment (37°C; 5% CO2), chondrocytes were cultured in DMEM/F12 (10% FBS). To simulate OA in vitro, IL-1β (10 ng/ml, Beyotime, China) was applied to induce OA-like phenotypes in chondrocytes and the duration was 24 hours.

CCK-8 assay

To determine the viability of chondrocytes, Cell Counting Kit-8 (CCK8; Beyotime, China) was used in this study. Chondrocytes were seeded at a density of 1×10^4 cells/well in 96-well plates. After treatment and cultivation for the indicated time, CCK-8 reagent (10 µL/well) was added and incubated for 2 hours at room temperature. The optical density at 450 nm was measured using a microplate reader (BioTek, USA) to assess cell viability.

Immunofluorescence

First, the chondrocytes were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton-X100 (Millipore Sigma). Next, the chondrocytes were rinsed with PBS and blocked in 10% normal goat serum with 0.1% Triton X-100 for 1 hour. Following this, the chondrocytes were incubated overnight at 4°C with a primary antibody against COL2A1, rinsed extensively with PBS, and then incubated at room temperature
for 1 hour with a secondary antibody. Finally, the chondrocytes were stained with DAPI (300 nM) for 5 minutes and rinsed with PBS. The images were captured using a fluorescence microscope (Leica DM4000 B, Solms, Germany).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

To extract RNA from chondrocytes, we used TRIzol reagent (Invitrogen) and synthesized cDNA through reverse transcription. Subsequently, we utilized Takara SYBR® Premix Ex Taq™ kit (Takara, Japan) to perform qRT-PCR on a ViiA TM 7 Real-Time PCR system (Applied Biosystems). The expression levels of mRNA were normalized to GAPDH, and we employed the $2^{-\Delta\Delta CT}$ method to calculate the relative expression levels. To ensure the validity of our results, we performed three independent experiments. The primer sequences we used are listed below: ADAMTS5 F: 5′-AGT ACA GTT TGC CTA CCG CC-3′, ADAMTS5 R: 5′-GAT TTG CCG TTA GGT GGG CA-3′; SOX9 F: 5′-GGA TGT CAA AGC AAC AGG CG-3′, SOX9 R: 5′-ATG TGC GTT CTC TGG GAC TG-3′; MMP13 F: 5′-CCT GGA GCC CTG ATG TTT C-3′, MMP13 R: 5′-TGG TGC ACA CTT CTC TGG TG-3′; Aggrecan F: 5′-GCA GCA CAG ACA CTG CAG GA-3′, Aggrecan R: 5′-CCC ACT TTC TAC AGG CAA GC-3′; GAPDH F: 5′-TCT CTG CTC CTC CCT GTT CT-3′, GAPDH R: 5′-ATC CGT TCA CAC CGA CCT TC-3′; GPX4 F: 5′-CGG TCT CGT GAG CCG CTT CCT ATT GAA-3′, GPX4 R: 5′-ACA CGC AAC CCC TGT ACT TA-3′; GPX4-M F: 5′-AAA TAA TTT AAG GTT GTT TGG ACG A-3′, GPX4-M R: 5′-AAC TAA CCC TTA CCG AAT ATC GAA A-3′; GPX4-U F: 5′-AAA TAA TTT AAG GTT GTT TGG ATG A-3′, GPX4-U R: 5′-CAA CTA ACC CTT ACC AAA TAT CAA A-3′.

**Western blotting**

To extract total protein from chondrocytes, RIPA lysis buffer (Beyotime, China) was used. The protein samples were separated into aliquots using SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Germany). Specific primary antibodies, including anti-ADAMTS5 (1:1000, ABclonal), anti-SOX9 (1:1000, proteintech), anti-MMP13 (1:2000, proteintech), anti-Aggrecan (1:1000, ABclonal), anti-Collagen II (1:250, proteintech), and anti-Gpx4 (1:2000, proteintech) were incubated with the membranes overnight at 4°C, followed by 1 hour of incubation with secondary antibodies at room temperature. GAPDH was used as an internal control. Protein blots were visualized using an ECL system (Amersham Pharmacia, UK). The secondary antibodies used were goat anti-rabbit IgG (1:2000, FRDBIO) or goat anti-mouse IgG (1:2000, FRDBIO).

**Methylation-specific PCR (MSP)**

MSP was applied to evaluate the methylation status of GPX4. Briefly, genomic DNA was extracted from chondrocytes with DNA Extraction Kit (GE Healthcare, UK) and modified via bisulfite conversion using EZ DNA Methylation Kit (Zymo Research, USA). Next, bisulfite-converted genomic DNA was applied for PCR with primers specific to GPX4 methylation.

**Bisulfite-specific PCR (BSP)**

To analyze the methylation status of BSP, bisulfite sequencing was conducted on genomic DNA that had undergone bisulfite conversion. The BSP-specific primers were used in PCR amplification, as previously...
described[39]. The PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV light. The PCR products were then purified using a Gel Extraction Kit and cloned into the pESI-T vector system (Yeasen) before sequencing. Ten clones were selected for sequencing from each group.

**Detection of reactive oxygen species (ROS)**

To evaluate ROS production in chondrocytes, flow cytometry was performed with Reactive Oxygen Assay Kit (Beyotime, China). Briefly, chondrocytes were incubated with 10 μM DCFH-DA for 30 minutes in darkness and rinsed with PBS. Then, the fluorescence intensity was measured using 488nm laser with FACS Calibur Flow Cytometry (Becton Dickinson, USA).

**Measurement of lipid peroxidation**

The lipid peroxidation was detected using flow cytometry analysis by detecting the shift from green to red in fluorescence emission .. In brief, chondrocytes were seeded in 12-well plates (1×10^5 cells/well) and incubated with 1 μM C11 BODIPY™ 581/591 (Invitrogen, USA) at 37°C for 30 min, followed by collection via trypsinisation. Finally, chondrocytes were resuspended in PBS and analyzed with BD LSRFortessa™ flow cytometer (BD, USA) using 488nm laser.

**Mice and surgery**

Male C57BL/6J mice, weighing between 20–25 g and aged 8 weeks, were obtained from Zhejiang Vital River Laboratory Animal Technology Co., LTD (China) and housed under specific-pathogen-free conditions with a 12-hour light/dark cycle. The mice were randomly divided into four groups, each consisting of 6 mice. For the surgical groups, mice were anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneally) and underwent unilateral destabilization of the medial meniscus (DMM)[40], while the sham group received a skin incision and suturing without joint manipulation. Two treatment groups were administered Fer-1 (1 mg/kg) (meilunbio, China, MB4718) and OI (25 mg/kg), respectively, via intra-articular injection on the 1st, 4th, and 7th day of each week. Mice were sacrificed for analyses 8 weeks after surgery. The animal protocol was approved by the Ethics Committee of Jiaxing Second Hospital (JXEY-2020JX051).

**Safranin O & Fast Green Staining**

We dissected the distal femur, fixed it with 4% PFA, and decalcified it. The paraffin section was stained quickly with safranin O-fast green. A dark red stain was used to visualize the cartilage matrix, while green staining was used to showcase the cytoplasm, muscles, collagen fibres, and bone tissue directly.

**Immunohistochemistry**

The remaining thin sections were subjected to immunohistochemical staining. To block endogenous peroxidase activity, 3% H₂O₂ was added for 10 min, followed by incubation with trypsin for 20 min and 5% BSA. Unspecific antigens were blocked with 1% Tween-20 in PBS for 30 min. Primary antibodies against Aggrecan, Col-II, and MMP13 were added and incubated overnight at 4°C. Then, corresponding HRP-conjugated secondary antibodies (FRDBIO, 1:2000) were added to the sections, followed by
counterstaining with hematoxylin. Positive cells were quantified in a random selection of five fields of view from each section. Three independent researchers counted the cells in each field of view. The results were averaged from at least three thin sections of each specimen.

**Osteoarthritis calculation**

A score system based on the Osteoarthritis Research Society International (OARSI) was used to grade the histology of articular cartilage in mice. There were three criteria used to determine score: the presence or absence of safranin O staining, the fracture depth of articular cartilage, and the degree of cumulative articular surface damage.

**Statistical analyses**

The data were presented as mean values along with their corresponding standard deviation. We utilized SPSS 23.0 and GraphPad Prism 6 software for conducting statistical analyses, and multiple comparisons were performed using ANOVA. To ensure reliability, duplicate samples were tested independently on a minimum of three occasions. Results were considered statistically significant at a level of \( P < 0.05 \), with asterisks denoting the degree of significance (\(*P < 0.05; **P < 0.01; ***P < 0.001\) and "NS" indicating no significant difference.

**Results**

**OI improved IL-1\( \beta \)-triggered chondrocyte degeneration**

To estimate the cytotoxic effects of OI on chondrocytes, cells were cultured for 48 hours at concentrations of 0, 10, 50, 100, 200, 300, 400, and 500 \( \mu \)M of OI. The inhibitory rate of OI on chondrocytes was evaluated by CCK-8 assay. As shown in Fig. 1A, the OD450 value of OI on chondrocytes was decreased significantly at 200 \( \mu \)M after 48 h, suggesting that OI exerted almost no cytotoxic effects on chondrocytes below 200 \( \mu \)M. Therefore, different OI concentrations (50, 100 and 200 \( \mu \)M) were used in subsequent experiments.

In order to study how OI affected IL-1\( \beta \)-induced chondrocyte degeneration, chondrocytes were treated with OI (50, 100 and 200 \( \mu \)M) for 24 h followed by IL-1\( \beta \) (10 ng/mL) treatment for 48 h. IL-1\( \beta \) treatment downregulated type II collagen synthesis, while OI at different concentrations altered the above changes, as shown in the immunofluorescence analysis (Fig. 1B-C). In addition, OI upregulated Aggrecan and SOX9 expression but downregulated MMP13 and ADAMTS5 expression in IL-1\( \beta \)-treated chondrocytes at both gene and protein levels (Fig. 1D-I). These results indicated that OI could protect chondrocytes against IL-1\( \beta \)-triggered chondrocyte degeneration.

**OI inhibited oxidative stress and upregulated the expression of GPX4 in chondrocytes**
According to the above experiments, OI showed inconsistent effect on chondrocytes at the concentration of 200 µM. We selected the 100µM concentration as the optimum concentration of OI for further experiments. Furthermore, we identified the molecular mechanism by which OI ameliorated the degeneration of chondrocytes. As reported in a previous study, OI acts as an activator of GPX4 in sepsis-induced acute lung injury [32]. In addition, GPX4 has been identified as an important regulator of OA progression [12]. Hence, we speculated that OI might improve chondrocyte degeneration by upregulating GPX4 expression in chondrocytes. As illustrated in Western blotting results, GPX4 was downregulated in IL-1β-challenged chondrocytes and OI upregulated this decline (Fig. 2A-C). Moreover, the ROS detection confirmed the decrease of oxidative stress induced by OI after the addition of IL-1β in cells (Fig. 2D and E). According to reports, erastin plays a crucial role in activating ROS to induce ferroptosis[16]. Similarly, OI counteracted the increase in ROS caused by erastin. The above results suggested that OI upregulated GPX4 expression and reduced the production of ROS may be related to ferroptosis in OA chondrocytes.

**Chondrocytes degeneration was prevented by OI, through inhibition of ferroptosis**

It has been reported that OI serves as an inhibitor of macrophage ferroptosis in sepsis-induced acute lung injury [32]. Interesting, chondrocyte ferroptosis has been widely accepted as an essential factor contributing to OA progression [41]. To investigate whether ferroptosis was involved in OI-mediated inhibition of chondrocyte degeneration, Erastin (a ferroptosis activator) was applied to stimulate ferroptosis in chondrocytes. As shown in Fig. 2F-L, IL-1β or Erastin treatment downregulated GPX4, SOX9 and Aggrecan expression but upregulated MMP13 and ADAMTS5 expression in chondrocytes at both gene and protein levels, which was greatly improved by OI. Therefore, OI might ameliorate chondrocyte degeneration by inhibiting ferroptosis.

**OI inhibited GPX4 methylation in chondrocytes**

Methylation is negatively correlative to GPX4 expression and acts as an important promoting factor of ferroptosis [39, 42]. Considering the observed increase in GPX4 expression in chondrocytes upon exposure to OI, it was postulated that OI could potentially elevate GPX4 expression by inhibiting GPX4 methylation. Western blotting results showed that OI mitigated the increase in the protein levels of methylases (DMNT1, DMNT3a, and DMNT3b) caused by IL-1β or Erastin (Fig. 3A-B). Correspondingly, OI decreased GPX4 gene hypermethylation in chondrocytes treated with IL-1β or Erastin, as illustrated by Methylation Specific PCR (MSP) results (Fig. 3C and D). Moreover, the Bisulfite Sequencing PCR (BSP) findings revealed that IL-1β treatment induced hypermethylation of the promoter region of GPX4 in chondrocytes, which was significantly alleviated by OI treatment (Fig. 3E-G). There were nearly 92.64% of the nucleotides methylated in IL-1β group, compared with control group which was 76.16%, while about 81.5% of the nucleotides were methylated with OI rescued group (Fig. 3E-G). All these findings indicated that OI could inhibit GPX4 methylation in chondrocytes.
OI protected against chondrocytes degeneration by suppressing ferroptosis, inhibiting GPX4 methylation and reducing oxidative stress

In order to reconfirm the effects of GPX4 methylation on OI-mediated inhibition of IL-1β-triggered degeneration, ferroptosis and oxidative stress of chondrocytes, 5-AZA (a methylase inhibitor) was used to inhibit GPX4 methylation. As shown in Fig. 4E and F, OI and 5-AZA reversed IL-1β-stimulated increase in methylases expression; Besides, OI and 5-AZA could also rescue IL-1β-induced low expression of GPX4 at protein level (Fig E and F), implying the inhibitory effects of OI and 5-AZA on GPX4 methylation may result in GPX4 upregulation in IL-1β-treated chondrocytes. It was also found that OI and 5-AZA treatment enhanced type II collagen synthesis in IL-1β-challenged chondrocytes, as shown by increased fluorescence intensity (Fig. 4A and B). In addition, OI and 5-AZA treatment upregulated Aggrecan and SOX9 expression but downregulated MMP13 and ADAMTS5 expression in IL-1β-treated chondrocytes at protein levels (Fig. 4C and D). IL-1β-induced increase of ROS was rescued by OI and 5-AZA (Fig. 4G and H). Altogether, OI attenuated degeneration, oxidative stress and ferroptosis of IL-1β-induced chondrocytes may by inhibiting GPX4 methylation and increasing GPX4 expression.

OI alleviated OA in DMM mouse model

To evaluate the potential therapeutic efficacy of OI in treating OA, we established an OA mouse model (DMM mouse model) and administered OI through intra-articular injection into the knee cavity. Subsequently, we utilized immunohistochemical staining, HE staining, and safranin O/fast green staining to examine the pathological morphology of the cartilage.

Immunohistochemical results showed that in the OA model group, the expression of the cartilage degradation-related marker of ADAMTS5 and MMP13, was significantly enhanced (Fig. 5A, C, F, H), while the expression of the chondrogenesis-related markers of Aggrecan and SOX9 was significantly decreased (Fig. 5B, D, G, I). However, treatment with OI and ferrostatin-1 (Fer-1), a highly effective and specific first-generation inhibitor of ferroptosis, appeared to ameliorate these changes. The results also showed that the ferroptosis-related markers of GPX4 expression was significantly lower in DMM group than that in DMM + OI and DMM + Fer-1 group (Fig. 5E, J). Additionally, the safranin O/fast green and HE staining revealed that the cartilage surface of DMM-induced OA mice improved upon OI and Fer-1 injection (Fig. 6A, B). These findings were further supported by the Osteoarthritis Research Society International (OARSI) score (Fig. 6C). Taken together, these results suggest that OI could potentially delay the progression of DMM-induced OA in mice by suppressing ferroptosis.

Discussion

With increasing research studies being conducted, it is becoming increasingly evident that cartilage damage caused by osteoarthritis is irreversible[8]. Inflammatory factors alter the intraarticular
environment, resulting in increased matrix metalloproteinases, which leads to accelerated degradation of cartilage matrix and further aggravation of cartilage injury[43]. Some studies have revealed the protective effects of OI in various degenerative diseases, including cartilaginous endplate degeneration[44], intervertebral disc degeneration[45], and OA[36, 37]. OI may have different mechanisms that have a positive impact on OA[34]. The impact of OI on the regulation of chondrocyte degeneration, oxidative stress, and ferroptosis has not been extensively studied to date.

In the current study, chondrocytes were subject to IL-1β treatment to induce OA-like phenotypes in vitro[46]. First of all, we treated chondrocytes with increasing concentrations and found that OI dose-dependently improved anabolism and catabolism of IL-1β-challenged chondrocytes, suggesting OI improved IL-1β-induced chondrocyte degeneration. According to the previous studies, OI could reduce cell death by inhibiting oxidative stress and ferroptosis [32, 47, 48]. In this study, Erastin (a ferroptosis activator) or Fer-1 (a ferroptosis inhibitor) was applied to stimulate or inhibit ferroptosis in chondrocytes. OI treatment reversed Erastin-mediated downregulation of GPX4 and SOX9 as well as upregulation of MMP13 and ADAMTS5 expression in chondrocytes at both mRNA and protein levels, indicating OI might ameliorate chondrocyte degeneration by inhibiting ferroptosis. In addition, OI treatment abated IL-1β-induced increases of ROS production and lipid peroxidation in chondrocytes. Fer-1 also exerted similar effects. Besides, OI also inhibited ROS production and lipid peroxidation in chondrocytes treated by Erastin. Therefore, OI attenuated oxidative stress and ferroptosis, thereby ameliorating degeneration in IL-1β-stimulated chondrocytes.

Then, we applied OI to treat IL-1β-induced chondrocytes to explore the molecular mechanism of OI in OA in vitro. As previously reported in a study, OI acts as an activator of GPX4 in sepsis-induced acute lung injury [32]. In addition, GPX4 has been identified as an important regulator of OA progression [12]. Furthermore, GPX4 methylation is negatively correlative to GPX4 expression and acts as an important promoting factor of ferroptosis [39, 42]. Consistent with above studies, OI reversed IL-1β-caused GPX4 inhibition in chondrocytes. Besides, we demonstrated that OI treatment reversed the high expression of methylases in IL-1β-treated chondrocytes. MSP and BSP results also confirmed that OI treatment substantially abolished the hypermethylation of GPX4 promoter region in IL-1β-challenged chondrocytes, suggesting OI increased GPX4 expression in chondrocytes by suppressing GPX4 methylation.

To verify whether GPX4 methylation is involved in OI-mediated inhibition of IL-1β-triggered degeneration, ferroptosis and oxidative stress of chondrocytes, 5-AZA as a methylase inhibitor was used to inhibit GPX4 methylation. It was shown that 5-AZA played a similar role as OI in reducing the expression of methylases and GPX4 and attenuating IL-1β-induced degeneration, ferroptosis and oxidative stress of chondrocytes, implying OI attenuates degeneration, oxidative stress and ferroptosis of IL-1β-induced chondrocytes by inhibiting GPX4 methylation and increasing GPX4 expression.

**Conclusion**
Our study found that OI could upregulate GPX4 expression by inhibiting GPX4 methylation, thereby improving IL-1β-induced degeneration, oxidative stress, and ferroptosis of chondrocytes. These results suggest that OI may serve as a potential therapeutic agent for the treatment and intervention of OA. Further in vivo studies are required to validate the findings of our *in vitro* experiments. In the mouse OA model, OI demonstrated superior anti-osteoarthritis effects compared to Fer-1, indicating that the reparative effects of OI on OA may not solely be limited to inhibiting ferroptosis. This highlights the need for further exploration of other biological mechanisms that underlie the potential therapeutic effects of OI in treating OA.

**Declarations**

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**Availability of data and materials**

The data used to support the findings of this study are available from the corresponding author upon request.

**Compliance with Ethics Requirements**

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Jiaxing Second Hospital Ethics Committee (JXEY-2020JX051). The committee approved the final manuscript.

**Competing Interests**

All authors declared no potential conflicts of interest with respect to the research, author-ship, and publication of this article.

**Author Contributions**

Huigen Lu, Mige Wang and Gang Chen designed and supervised the project. Xuekang Pan, Xiangjia Kong and Zheyuan Jin performed the experiments and collected the data. Xuekang Pan and Xiangjia Kong contributed equally to this manuscript. Xuekang Pan, Zheyuan Jin and Xiangjia Kong analysed and interpreted the data, contributed to the writing of the manuscript, discussed the results and implications, and edited the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

Not applicable.

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Figures
Figure 1

OII inhibited the IL-1β induced inflammatory reaction in chondrocytes. Over a 48-hour treatment period, chondrocytes were exposed to various concentrations of OII (ranging from 50 to 200 μM), following a 24-hour pre-treatment with 10 ng/mL of IL-1β. (A) The graph illustrates the results of a CCK-8 assay conducted to measure the viability of chondrocytes, which was quantified by the absorbance at 450 nm. (B) Col2α1 protein expression levels in chondrocytes treated with OII and IL-1β. (C) The mean fluorescence
intensity is quantified. (D-G) A comparison between the gene expression levels of ADAMTS5, MMP13, SOX9, and Aggrecan in qPCR of OI and IL-1β treated chondrocytes. (H) ADAMTS5, MMP13, SOX9, Aggrecan proteins in western blot of OI and IL-1β treated chondrocytes. (I) The quantification of the western blot results is presented as mean±SD from three independent experiments. It is also possible to determine statistical significance by using NS (not significant, p>0.05), *, (p<0.05), ** (p<0.01), or *** (p<0.001). Standard deviation (SD) is represented by the error bars.
Figure 2

O1 alleviated oxidative stress and decreases ferroptosis of chondrocytes. Chondrocytes were treated by IL-1β (10 ng/mL) with Erastin (5 μM) for 24 hours and O1 (100 μM) rescued for 48 h. (A) The levels of protein expression of GPX4 in the IL-1β and O1 treated chondrocytes. (B) An analysis of the blot's quantitative data. (C) The GPX4 gene expression levels of IL-1β and O1 treated chondrocytes. (D) Reactive Oxygen Species detection of IL-1β(10ng/ml) or Erastin (5 μM) treated chondrocytes with O1 (100 μM) rescue. (E) A mean fluorescence intensity statistic for ROS. (F) As measured by western blot, ADAMTS5, MMP13, SOX9, Aggrecan, and GPX4 protein expression levels were found in IL-1β (10ng/ml) or Erastin (5 μM) treated chondrocytes with O1 (100 μM) rescue. (G) An analysis of the blot's quantitative data. (H-L) The gene expression level of ADAMTS5, MMP13, SOX9, Aggrecan and GPX4 in Western blot of IL-1β(10ng/ml) or Erastin (5 μM) treated chondrocytes with O1 (100 μM) rescue. Each of the three experiments is presented as a mean ± SD. It is also possible to determine statistical significance by using NS (not significant, p>0.05), *, (p<0.05), ** (p<0.01), or *** (p<0.001). Standard deviation (SD) is represented by the error bars.
Figure 3

Ol inhibited GPX4 methylation in chondrocytes. Chondrocytes were treated by IL-1β (10 ng/mL) with Erastin (5 μM) for 24 hours and Ol (100 μM) rescued for 48 h. (A) The DNMT1, DNMT3a, DNMT3b protein expression levels in western blots of IL-1β (10ng/ml) or Erastin (5 μM) treated chondrocytes with Ol (100 μM) rescue. (B) An analysis of the blot's quantitative data. (C) A Methylation-specific PCR was used to identify methylation of the GPX4 promoter: M represented the methylated GPX4 and U represented the un-
methylated GPX4. (D) Quantification of the blot. (E) The GPX4 promoter region was identified via bisulfite-specific PCR (BSP). CpG islands are indicated by the sequence of the BSP sequenced region, which is numbered 1 to 20. Un-methylated and methylated CpGs were indicated in the GPX4 promoter region of chondrocytes rescued by IL-1(10ng/ml) and OI (100 μM). In each group, five bacteria monoclonal antibodies selected for sequencing are represented by a different row. (F) Quantification of methylation ratio. (G) Gene sequencing of GPX4 promoter region. Each of the three experiments is presented as a mean ± SD. It is also possible to determine statistical significance by using NS (not significant, p>0.05), *, (p<0.05), ** (p<0.01), or *** (p<0.001). Standard deviation (SD) is represented by the error bars.
Figure 4

Ol rescued the degeneration of chondrocytes caused by IL-1β. Chondrocytes were treated by IL-1β (10 ng/ml) for 24 h with Ol (100 μM) and 5-AZA (0.1 μM and 0.2 μM) rescued for 48 h. (A) The amount of Col2α1 protein expressed in the immunofluorescence assay of chondrocytes from mice treated with IL-1β (10 ng/mL), Ol (100 μM), and two different concentrations of 5-AZA (0.1 μM and 0.2 μM) showed significant rescue effects (scale bar = 100 μm). (B) Quantification of Mean Fluorescence intensity. (C) In
the western blot analysis of chondrocytes treated with IL-1β (10 ng/mL), OI (100 μM), and two different concentrations of 5-AZA (0.1 μM and 0.2 μM), significant rescue effects were observed for the protein expression levels of ADAMTS5, MMP13, SOX9, and Aggrecan. (D) Quantification of the blot. (E) The Western blot analysis of chondrocytes treated with IL-1β (10 ng/mL), OI (100 μM), and two different concentrations of 5-AZA (0.1 μM and 0.2 μM) showed significant rescue effects on the protein expression levels of DNMT1, DNMT3a, DNMT3b, and GPX4. (F) An analysis of the blot’s quantitative data. (G) Reactive Oxygen Species detection of IL-1β (10 ng/ml) treated with OI (100 μM) and 5-AZA (0.1 μM and 0.2 μM) rescued chondrocytes. (H) The mean fluorescence intensity statistics for ROS were calculated and presented as the mean ± SD of three independent experiments. It is also possible to determine statistical significance by using NS (not significant, p>0.05), *, (p<0.05), ** (p<0.01), or *** (p<0.001). Standard deviation (SD) is represented by the error bars.
Figure 5

Modulation of cartilage phenotype and inflammation in the OA model. Representative immunohistochemical staining of (A) ADAMTS5, (B) Aggrecan, (C) MMP13, (D) SOX9 and (E) GPX4 in knee joint (scale bar = 100-200 μm). (F-J) The quantification of ADAMTS5, Aggrecan, MMP13, SOX9 and GPX4 expression (n=5). Each of the three experiments is presented as a mean ± SD. It is also
possible to determine statistical significance by using NS (not significant, p>0.05), *, (p<0.05), ** (p<0.01), or *** (p<0.001). Standard deviation (SD) is represented by the error bars.

**Figure 6**

**Histological evaluation of OA therapy.** (A) Representative Safranin O staining in knee joint. Representative hematoxylin-eosin staining in knee joint. (B) Representative hematoxylin-eosin staining in
knee joint. (C) The Safranin O/fast green staining was used to evaluate the Osteoarthritis Research Society International (OARSI) score. Values are the means ± SD (n=6). It is also possible to determine statistical significance by using NS (not significant, p>0.05), *, (p<0.05), ** (p<0.01), or *** (p<0.001). Standard deviation (SD) is represented by the error bars.