Extracorporeal shock wave protects chondrocytes against interleukin-1β-induced apoptosis and promotes autophagy in vitro

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ABSTRACT: Osteoarthritis (OA) is a common joint disease, but there is no effective treatment except surgery at present. Extracorporeal shock wave (ESW) is an emerging therapy widely used in various medical fields. In this study, we mainly investigated the effects of ESW on apoptosis and autophagy of osteoarthritic chondrocytes by analyzing the cell apoptosis and the expression of autophagy markers' mRNAs and proteins, respectively. Primary chondrocytes were isolated from articular cartilage tissue of rats. Results showed that ESW effectively inhibited the interleukin-1β-induced (IL-1β-induced) chondrocyte apoptosis. Moreover, ESW treatment elevated the mRNA and the protein expression levels of Beclin 1, Atg5, LC3B, and Collagen II. Beclin 1 plays a key role in autophagy, positively regulating autophagic activity. Atg5 is a marker of autophagic activity, and LC3 is a specific marker of autophagosome. Collagen II is an important indicator to judge the functional activity of chondrocytes. In contrast, the mRNA and the protein expression levels of P62, which is a measure of autophagic flux and is thought to be negatively correlated with autophagic degradation, were decreased in the ESW treated cells. This study reveals the role of ESW in promoting chondrocyte autophagy and suppressing cell apoptosis. Thus, ESW may protect chondrocytes against IL-1β-induced apoptosis and promote autophagy in an \textit{in vitro} model of osteoarthritis.

KEYWORDS: extracorporeal shock wave, osteoarthritis, chondrocytes, apoptosis, autophagy

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease with progressive articular cartilage damage and pain, seen mostly in the middle-aged and the elderly\textsuperscript{[1]}. With the increasing global aging population, the incidence of the disease is increasing. Due to the slow progression of the disease, the lack of early diagnosis and treatment often leads to a delayed treatment\textsuperscript{[2]}. At present, the pathogenesis of OA is still unclear, but its main pathological changes are degeneration and damage of cartilage. Chondrocytes play an important role in the repair of cartilage damage. Related studies have shown that the progression of OA can be effectively delayed by inhibiting their apoptosis and improving the level of autophagy of chondrocytes\textsuperscript{[3–5]}.

In recent years, extracorporeal shock wave therapy (ESWT) has been widely used in the fields of physical therapy and rehabilitation medicine. Compared with other treatment methods, ESWT has the advantages of being non-invasive, painless, effective, safe, low cost, and low incidence of surgical complications. Recent studies have shown that ESWT can improve and delay the pathological process of OA\textsuperscript{[6]}. Moreover, ESWT has been shown in animal models and clinical trials to reduce OA pain and improve motor function\textsuperscript{[7, 8]}.

Zhao et al successfully constructed an \textit{in vitro} model of osteoarthritis chondrocytes using interleukin-1β (IL-1β)\textsuperscript{[9]}, so OA condition in chondrocytes was induced by treatment with IL-1β in this study. At present, there are few studies on the effects of ESW on chondrocyte apoptosis and autophagy. Thus, this study aimed to explore the effects of ESW on the apoptosis and autophagy in an \textit{in vitro} model of osteoarthritis chondrocytes, hoping to get more intuitive results from the cell level, paving the way for future \textit{in vivo} experiments.

MATERIALS AND METHODS

Cell isolation and culture

The rats were purchased from Beijing Huafukang Biotechnology Co., Ltd., license number: SCXK (Beijing) 2019-0008. The experiment was approved by the Animal Experiment Ethics Committee of China Rehabilitation Research Center (approval number: 2019-126-1). Hyaline cartilage from 1-week-old Sprague-Dawley rats was isolated and chopped to the size of 1 mm\textsuperscript{3}. After washing in phosphate-buffered saline (PBS), 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added, and the fragments were placed to be digested in a 37°C incubator with 5% CO\textsubscript{2} for 1–2 h. After washing with PBS, they were digested and incubated with 0.2% type II collagenase (Sigma-Aldrich). Via centrifugation, the supernatant was discarded, and the chondrocytes were obtained. The cells were collected and cultured in Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12) containing 10% fetal...
bovine serum (FBS) (Gibco Life Technologies, Grand Island, NY, USA). The cells were then, trypsinized with 0.25% trypsin and passaged at a ratio of 1:2 when the cell density reached 80%. The medium was changed every 2 days. Chondrocytes at passage three were utilized in the subsequent experiments.

**Interleukin-1β-induced cellular osteoarthritis model and experimental grouping**

The OA chondrocytes were stimulated with 10 ng/ml interleukin-1β (IL-1β, Peprotech, Suzhou, China) for 24 h to obtain the microenvironment of chondrocytes from osteoarthritis [9]. The chondrocytes were randomly divided into four groups: control, IL-1β, ESW, and IL-1β+ESW. Radial shock waves were applied using a STORZ device (STORZ Medical, Tägerwilen, Switzerland) with a dose of 500 impulses at 1.5 bars. The OA chondrocytes were stimulated with 10 ng/ml interleukin-1β (IL-1β, Peprotech, Suzhou, China) for 24 h to obtain the microenvironment of chondrocytes from osteoarthritis [9]. The chondrocytes were randomly divided into four groups: control, IL-1β, ESW, and IL-1β+ESW. Radial shock waves were applied using a STORZ device (STORZ Medical, Tägerwilen, Switzerland) with a dose of 500 impulses at 1.5 bars.

**Flow cytometry**

Cells in the logarithmic growth phase were collected and centrifuged at 4 °C for 5 min after 0.25% trypsin digestion. After washing with PBS, the cells were centrifuged again and resuspended in 100 µl 1 × binding buffer. Then, 5 µl of FITC-Annexin V and 5 µl of Propidium Iodide (Beyotime Biotech, Shanghai, China) working solutions were added to each tube. The cells were incubated at room temperature in the dark for 10–15 min, and 400 µl PBS buffer was added to each tube. Apoptosis was detected by a flow cytometer (BD, Influx, NJ, USA).

**RNA extraction and real-time PCR**

Total RNA from chondrocytes was extracted by the TRIzol method (Invitrogen, Carlsbad, CA, USA). An Ultrafine spectrophotometer was used to detect the RNA concentration and purity. Using a reverse transcription kit to reverse transcribe RNA to cDNA (Roche, Basel, Switzerland), the reaction system was set according to the instructions, and 400 ng mRNA was added to each retro transcription system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA, USA) was used as the standard, and the corresponding gene expression was analyzed by the 2−ΔΔCT method. Each sample had three replicates. Primer sequences are shown in Table S1.

**Western blot**

Protein was extracted from a protein lysate containing protease and phosphatase inhibitors, and the total protein concentration was measured using a BCA protein analysis kit (Beyotime, China). The samples were separated by SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membrane was incubated with 5% skim milk powder at room temperature for 1 h and, then, incubated overnight at 4 °C with primary antibody diluted containing anti-Atg5, LC3B, P62, Beclin 1, Collagen II, or GAPDH (as a loading control) and with secondary antibody for 1.5 h. (All the antibodies were purchased from Abcam, USA.) On the day after, the membranes were washed with PBS containing 0.05% Tween-20. An ECL detection kit (Beyotime, China) was used for chemiluminescence display. An Image J software system was used for photography and gray value measurement. After comparison with the corresponding GAPDH, the control group was set as 1, and the relative values of the other groups were calculated. The above experiments were separately repeated three times, and the results were averaged.

**Immunofluorescence**

The cells were inoculated onto sterile glass slides for 3 days. The cells slides were taken out, washed with PBS, and fixed with 4% paraformaldehyde for 30 min. After washing with PBS, 0.5% TritonX-100 was added for 15 min. Then, 5% goat serum was added for 30 min. After washing with PBS, the cells were incubated overnight in a wet box in a refrigerator at 4 °C with a primary antibody against Collagen II. On the second day, a fluorescent secondary antibody was added and incubated with the cells at room temperature in the dark for 1 h (all the following operations were in the dark). The cell nuclei were stained with DAPI for 5 min. After the excess dye solution had been washed away, the cells were sealed and photographed.

**Statistical analysis**

SPSS 22.0 statistical software was used (IBM Corp., Armonk, NY, USA). The measurement data were represented as the mean ± standard deviation. The differences among multiple groups of data were analyzed using one-way ANOVA, and factorial design ANOVA was used for multi-factor interaction.∗∗∗ p < 0.001, ** p < 0.01 and * p < 0.05 were considered statistically significant.

**RESULTS**

**ESW inhibited IL-1β-induced chondrocyte apoptosis**

Flow cytometry analysis showed that the apoptosis rate of the IL-1β group was significantly higher than the control group. Compared with the IL-1β group, the apoptosis rate of the IL-1β+ESW group was decreased. These results indicated that IL-1β-induced apoptosis of OA chondrocytes in vitro was increased, and ESW inhibited IL-1β-induced apoptosis of OA chondrocytes (Fig. 1).
ESW promoted IL-1β-induced chondrocyte autophagy

Compared with the control group, the Beclin 1, Atg5, and LC3B mRNA expressions in the IL-1β group were down-regulated; and the P62 mRNA expression was up-regulated. Compared with the IL-1β group, the Beclin 1, Atg5, and LC3B mRNA expressions in the IL-1β+ESW group were up-regulated, and the P62 mRNA expression was down-regulated (Fig. 2).

Compared with the control group, the expressions of Beclin 1, Atg5, and LC3B proteins in the IL-1β group was significantly down-regulated, and the expression of P62 protein was up-regulated. Compared with the IL-1β group, the expressions of Beclin 1, Atg5, and LC3B proteins in the IL-1β+ESW group were up-regulated, and the expression of P62 protein was down-regulated (Fig. 3). The results suggested that ESW promoted IL-1β-induced chondrocyte autophagy.

Expression of Collagen II in chondrocytes

Compared with the control group, the mRNA and protein expression levels of Collagen II in the IL-1β group were significantly down-regulated. Compared with the IL-1β group, the mRNA and protein expression levels of Collagen II in the IL-1β+ESW group were up-regulated (Fig. 4).

The qualitative analysis of Collagen II in chondrocytes by immunofluorescence staining

Compared with the IL-1β group, Collagen II was secreted significantly in the control group; and the IL-1β+ESW group significantly promoted the secretion of Collagen II. The results suggested that ESW promoted the synthesis of Collagen II in vitro chondrocytes (Fig. 5).

DISCUSSION

OA is a chronic joint disease characterized by cartilage damage and pain, which seriously affects the daily life of patients [10,11]. Cartilage, with cartilage cells and extracellular matrix (ECM), is rich in collagen and proteoglycan. Collagen II accounts for 80%–85% of the total collagen [12]. Expression of Collagen II plays a crucial role in cartilage repair. In this study, we found that ESW promoted the expression of Collagen II in an in vitro model of osteoarthritis. At present, the molecular mechanism of OA is unclear, but research has shown that ECM degradation, the expression of
inflammation, cell apoptosis, and the autophagy level could affect the progress of OA. However, an unbalance in the articular cartilage ECM homeostasis is one of the important reasons causing cartilage degeneration [13–15]. IL-1β is an inflammatory factor that regulates ECM component production in chondrocytes [16] and is widely used to induce the OA cell model [17]. Thus, in this study, IL-1β (10 ng/ml) was used to interfere with the chondrocytes of rats for 24 h in order to establish the osteoarthritic chondrocyte model.

Studies have shown that ESWT can reduce the chronic inflammatory activities of joints, relieve pain, and play a role in cartilage protection in the OA model [18]. At present, the mechanism by which ESW delays the progression of OA is still unclear. Related studies have shown that ESW may treat OA by promoting angiogenesis, reducing inflammation, inducing the expression of growth factors and cytokines related to tissue repair, and increasing the proliferation activity of chondrocytes and other factors [6, 19, 20]. The previous experiments confirmed that chondrocytes were highly sensitive to ESW. The related mechanism might involve a series of molecular signal cascade reactions that regulated cell growth, cell division, and protein

**Fig. 2** mRNA expression of cells detected by RT-PCR. The mRNA expression levels of Beclin 1 (A), Atg5 (B), P62 (C), and LC3 (D), as assayed by RT-PCR. Data are presented as mean ± S.D., n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 versus the IL-1β group.

**Fig. 3** Western Blot analysis of protein expression of cells. The expression levels of all proteins (A), Beclin 1 (B), Atg5 (C), P62 (D), LC3II/LC3I (E), and GAPDH as an internal control. Data are presented as mean ± S.D., n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 versus the IL-1β group.
Fig. 4  mRNA and protein expression levels of Collagen II detected by RT-PCR and Western blot. Band density (A), protein (B) and mRNA (C) showing expression levels of Collagen II, and GAPDH as an internal control. Data are presented as mean ± S.D., n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 versus the IL-1β group.

Fig. 5  Effect of ESW on the analysis of Collagen II in chondrocytes by immunofluorescence staining. Collagen II puncta were counted under confocal microscopy. Green indicates Collagen II, and blue indicates nuclei as stained by DAPI.

synthesis. In this study, flow cytometry showed that the apoptosis rate of chondrocytes in the IL-1β+ESW group was significantly lower than the IL-1β group, indicating that ESW could effectively inhibit the apoptosis of chondrocytes and thus delay the progression of OA.

Autophagy is an important protective response of eukaryotic cells to various types of stress, including the phagocytosis and recirculation of macromolecules and organelles to maintain cell metabolism [21]. It is widely involved in cell development, proliferation, apoptosis, and other processes, and plays an important role in chondrocyte maturation and homeostasis [22]. Bay-Jensen et al showed that maintaining the autophagy level in chondrocytes was important to prevent cartilage degeneration [23]. Green et al found that the occurrence of OA was related to a decreased level of autophagy in chondrocytes [24]. Therefore, improving the autophagy level of chondrocytes might be a new target for OA therapy. Wu et al [25] found that Sirt7 protected chondrogenic degeneration in OA by activating autophagy. In addition, Wang et al [26] found that metformin alleviated cartilage degradation by activating AMPK/SIRT1-mediated autophagy, and
Bao et al. [27] found that rapamycin therapy improved cartilage protection in OA by promoting autophagy. Thus, this study focused on the relationship between ESW and chondrocyte autophagy.

In order to confirm the effect of ESW on chondrocyte autophagy, we researched four autophagy-related proteins: Beclin 1, P62, Atg5, and LC3. Beclin 1 plays a key role in autophagy by regulating the formation of autophagosomes (mainly by forming a complex with phosphatidylinositol 3-kinase) which positively regulate autophagy activity. P62, as a measurement index of autophagy flux, is considered to be negatively correlated with autophagy degradation [28]. When the autophagy pathway is activated, Beclin 1 protein expression is increased, and P62 protein expression is decreased [29]. Autophagy pathway is regulated by more than 30 autophagy-related proteins (Atgs), of which Atg5 and Atg7 are the two most important regulatory proteins [30]. Autophagy-related gene 5 (Atg5) is a marker of autophagy activity [31]. LC3 is a specific marker of autophagosomes [32]. When autophagy occurs, the cytoplasmic form LC3-I binds to phospholipid amines through an ubiquitin-like enzyme reaction, converts it to the phospholipid-conjugated form LC3-II, and is continuously recruited into autophagic vacuoles to initiate the autophagy process [33, 34]. Autophagy-related signal transduction pathways are complex and interact with each other. Common autophagy-related signal transduction pathways include mTOR signal transduction pathway, PI3K/AKT signal transduction pathway, p38 MAPK signal transduction pathway, JNK signal transduction pathway, and nuclear factor-κB signal transduction pathway in bone and joint diseases. However, Bohensky et al. [35] believed that chondrocytes regulated autophagy mainly through the HIF-1/AMPK/mTOR signaling pathway. In this study, we found that ESWT could up-regulate the protein and gene expression levels of Beclin 1, Atg5, and LC3 in OA chondrocytes and reduce the protein and gene expression levels of P62. This may alter the transduction of autophagy-related protein signaling pathways during ESW intervention, thus changing the autophagy level of OA chondrocytes. These results revealed an important role of ESW in regulating chondrocyte autophagy.

There were several limitations to this study. The results of this study were from rats, which might differ from large animal models or human clinical trials. In addition, the dose of the shock waves was based on previous animal studies and cell-based therapeutic references, which might slightly differ from the optimal dose in human clinical trials. Although this study adopted the classical method to establish the OA model, it was different from the clinical development process of OA. ESW had an inhibitory effect on IL-1β-induced apoptosis of rat chondrocytes, and further investigations of possible molecular mechanism are needed. The purpose of this study was to investigate whether ESW, as a physical intervention, could affect the autophagy level of osteoarthritic chondrocytes. However, the specific receptors or pathways involved in the regulation process of ESW on autophagy and whether ESW would have the same effect on autophagy of human OA chondrocytes remain to be further verified.

In conclusion, ESW may protect chondrocytes against IL-1β-induced apoptosis and promote autophagy in an in vitro model of osteoarthritis. Meanwhile, ESW promotes the expression of Collagen II in vitro chondrocytes, which may provide a new target for cartilage repair.

Appendix A. Supplementary data
Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.105.

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REFERENCES
### Appendix A. Supplementary data

**Table S1** Primer sequence of real-time fluorescence quantitative PCR.

<table>
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<th>Gene</th>
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