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ORIGINAL RESEARCH

Cordycepin Alleviates Anterior Cruciate Ligament Transection (ACLT)-Induced Knee Osteoarthritis Through Regulating TGF-β Activity and Autophagy

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Introduction: Osteoarthritis is the most prevalent articular disease in the elderly. We aimed to explore the role of cordycepin (COR) in the progression and development of osteoarthritis and its correlation with TGF-β activity and autophagy.

Methods: Sprague Dawley rats were induced by anterior cruciate ligament transection (ACLT) to establish knee osteoarthritis model. To investigate the role of COR in knee osteoarthritis, rats were injected with 5, 10, and 20 mg/kg of COR before joint surgery. After surgery, paw withdrawal mechanical threshold (PWMT) was performed. HE staining and Alcian blue staining were carried out to detect cartilage damage. ELISA was used to detect the level of TGFβ in the serum. Protein expression was analyzed by Western blotting.

Results: In this study, we found that the PWMT of rats with osteoarthritis induced by ACLT was decreased significantly, accompanied by obvious histological and cartilage damage. After different doses of COR treatment, the PWMT of osteoarthritis rats induced by ACLT was increased in a dose-dependent manner. In addition, compared with the control group, COR treatment also reversed the effect of ACLT on cartilage injury in rats. Furthermore, the level of TGF-β in serum of ACLT rats was increased significantly, which may be related to the overexpression of TGF-β R1. However, the increase of serum TGF-β level in ACLT rats was reversed by COR treatment in a dose-dependent manner. It is worth noting that TGF-β overexpression reduced the proportion of autophagy-related protein LC3-II/I, thus inhibiting autophagy. In order to further confirm the effect of TGF-β on autophagy, TGF-β was overexpressed or the autophagy inhibitor 3-MA was applied. The results showed that TGF-β overexpression and 3-MA treatment reversed the effect of COR on autophagy.

Conclusion: In summary, our findings declared that COR alleviated ACLT-induced osteoarthritis pain and cartilage damage by inhibiting TGF-β activity and inducing autophagy in rat model with knee osteoarthritis.

Keywords: cordycepin, anterior cruciate ligament transection, osteoarthritis, TGF-β, autophagy, in vivo

Introduction

Osteoarthritis is the most common articular disease in the elderly.[1] The process is characterized by changes in the structure and tolerance of articular function, which is mainly caused by the degradation of articular cartilage.[2] Osteoarthritis affects nearly 70% of people and has a significant economic and social impact on patients and health-care systems.[3] Osteoarthritis can be intervened by non-pharmacological treatment such as exercise.[4] However, for patients who cannot stand high-intensity training, pharmacological treatment is still needed. The pathogenesis of

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osteoarthritis is elusive.[5] Therefore, there is an urgent need to develop drugs that can treat osteoarthritis. Cordycepin (3′-deoxyadenosine, COR), the main component of traditional Chinese herb *Cordyceps militaris*, has been proved to have many biological activities, such as selective interruption of nucleolar RNA synthesis, antibacterial, anti-inflammation, anti-adipogenesis, antifungal, anti-tumor, promoting cell differentiation and anti-apoptosis.[6–8] In particular, previous studies have indicated that COR plays an important role in the development of osteoarthritis. For example, Zhou et al found that COR suppressed IL-β-induced expression of inflammatory mediators in human osteoarthritis chondrocytes. Ashraf et al also suggested that administration of cordycepin before the onset of osteoarthritis caused by monosodium iodoacetate reduced cartilage damage and had significant protective effects on cartilage.[9]

Autophagy is a physiological cellular process in which cells use lysosomes to mediate self-digestion and recycling.[10,11] Autophagy, which can remove damaged organelles and long-acting macromolecules, is an indispensable mechanism to maintain homeostasis in cells. Indeed, it has been found that osteoarthritis is related to the decrease of autophagy level of chondrocytes.[12] There is increasing evidence that TGF-β plays an important role in the induction of autophagy, which increases the possibility of TGF-β inducing autophagy in the progression of osteoarthritis.[13–15]

In the current study, we investigated the role of COR in the progression and development of osteoarthritis and its association with TGF-β activity and autophagy. Our study showed that COR alleviated ACLT-induced pain and cartilage damage in knee osteoarthritis rats by inhibiting TGF-β activity and inducing autophagy. Overall, our findings provide a foundation for clinical application of COR in the treatment of osteoarthritis.

Materials and Methods

Groups

The rats were divided into eight groups (n = 10): control group, normal rats; sham-operated group, sham-operated rats; ACLT group, model rats; COR-5 + ACLT group, ACLT rats were given 5 mg/kg COR; COR-10 + ACLT group, ACLT rats were given 10 mg/kg COR; COR-20 + ACLT group, ACLT rats were given 20 mg/kg COR; COR-20 + ACLT + TGF-β1 group, ACLT rats were given 20 mg/kg COR and TGF-β1 overexpression; 3-MA + COR-20 + ACLT group, ACLT rats were given 20 mg/kg COR and 15 mg/kg 3-MA.

Animals and Treatment Protocol

The animal experiment protocol was carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the Ethics Committee of the Beijing Shijitan Hospital, Capital Medical University (SYXK (□) 2017–0025). Eighty male Sprague Dawley rats (8 weeks old, 220–280 g) were obtained from the Experimental Animal Center of Capital Medical University. COR (3′-Deoxyadenosine, from Cordyceps *militaris*) was purchased from Sigma-Aldrich. Before modeling, COR (5, 10, and 20 mg/kg) was injected into the joints of rats for three consecutive days. The control group, sham operation group and ACLT group used only the same amount of distilled water. For 3-MA treatment, rats were injected with 15 mg/kg of 3-MA intravenously. Under isoflurane-oxygen anesthesia, an ACLT rat model was established as previously described.[16,17] No arthrotomy was performed on the legs of rats in the sham operation group. After ACLT surgery, rats were injected with penicillin (400,000 U/d) intramuscularly for 3 consecutive days and treated with COR or 3-MA for 8 weeks. Rats can drink water freely and are kept in a room with controlled temperature and humidity, with a light-dark cycle of 12 hours (light-on time: 7:00 am to 7:00 pm). After 8 weeks, we evaluated the histological changes by HE staining and Alcian blue staining. Simultaneously, the medial femoral con was evaluated using the Osteoarthritis Research Society International (OARSI) scoring system and scored by an unsuspecting observer.[18] The OARSI score is obtained by multiplying the score by this stage.

TGF-β Overexpression in Rats

As described previously, an empty vector or an adenovirus containing an overexpression vector of TGF-β (Ad-TGF-$\beta^{223/225}$) was injected into rats intra-articularly (plaque-forming units [pfu] 10^7/6 μL).[19] Four days after infection, the knee tissue was harvested for histological analysis.

Histology Analysis

HE staining and Alcian blue staining were performed to detect cartilage damage. The isolated knee joints of rats were fixed in formalin containing phosphate buffer for 7 days, and then decalcified in 10% formic acid for 1 week. The tissue was dehydrated with an automatic tissue processing device (Miles Scientific Tissue-Tek VIP tissue

processor; Miles Scientific, USA) and embedded in paraffin. A total of 10 mm frontal sections were stained with corresponding dyes as previously described.[20,21]

Measurement of PWMT

At 1, 2, 4 and 8 h after ACLT, the mechanical allodynia of hind paw retraction was evaluated by the electrical mechanical analgesia tester (BME-404, Tianjin, China). In short, the rats were placed in a cage with a metal mesh floor. A linearly increasing force is applied through stainless steel wire (0.6 mm in diameter) to mechanically stimulate the sole surface of the rear paw. A critical value of 50 g was applied to prevent tissue damage. Each rat experiment was repeated three times.

Western Blotting

The protein was extracted from 10 mg articular cartilage using a protein isolation kit (ReadyPrep; GE Healthcare Life Sciences). The protein concentration was determined using a bicinchoninic assay kit (Thermo Fisher Scientific, Inc.). The protein (20 μg) was separated on 12% SDS PAGE gel and transferred to the nitrocellulose membrane. At room temperature, the membrane was sealed in 5% skimmed milk for 2 hours and incubated with the following primary antibodies at 4°C overnight: Anti-LC3 I, anti-LC3 II, anti-MMP13, anti-aggrecan, anti-collagen II and anti-TGFβ1, anti-β-actin (Cell Signaling Technology, Inc., Danvers, USA). The next day, the nitrocellulose membranes were washed three times and incubated with HRP labeled goat anti-rabbit IgG secondary antibody (1:10,000, cat. no. A16104SAMPLE; Thermo Fisher Scientific, Inc.) at 4°C for 2 h. The protein bands were detected by an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) scanned by ChemiDoc XRS (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression was normalized to β-actin and densitometric analysis was performed by ImageJ Software version 7.0 (National Institutes of Health, Bethesda, MD, USA).

Measurement of Serum Level of TGFβ

The activity of TGF β in serum samples was detected by ELISA (R&D Systems, Inc., Minneapolis, MN, USA). Before the measurement, the serum samples were treated with acid to convert the inactive form of TGFβ into the active form. After neutralizing the sample with sodium hydroxide, TGFβ was measured according to the manufacturer's instructions.

Statistical Analysis

All statistical analyses were using SPSS 16.0. Data are presented as mean ± standard deviation (SD). Statistical analyses were performed with Student's *t*-test (two-tailed) and one-way or two-way analysis of variance (ANOVA), followed by post hoc Tukey's tests for pair-wise comparisons. $P < 0.05$ was considered as significant.

Results

Establishment of Rat Model with Knee Osteoarthritis Induced by ACLT

PWMT values were detected at 1 h, 2 h, 4 h and 8 h after ACLT, respectively, and histology analysis and OARSI score were performed. As shown in Figure 1A, the OARSI score of ACLT group was significantly higher than that of sham group. Besides, PWMT values of ACLT group at 1 h, 2 h, 4 h and 8 h were lower than those of sham group and control group, and PWMT values of ACLT group increased in a time-dependent manner (Figure 1B). Histology analysis showed that the cartilage of the knee joint in the control group and the sham group was normal in histology. However, in ACLT group, the knee joint showed severe degeneration, accompanied by severe cartilage damage and large-scale cartilage fibrosis, indicating the severe cartilage damage caused by ACLT (Figure 1C). Further analysis showed that the expression of MMP13, aggrecan and collagen II in ACLT rat cartilage was inhibited, indicating that the swelling of chondrocytes was caused by a change towards a hypertrophic phenotype (Figure 1D–F). In conclusion, these findings indicate that ACLT-induced osteoarthritis of the knee in rats has been successfully established in this study.

COR Alleviated Bone-Arthrosis Pain and Cartilage Injury in Rat Model with Knee Osteoarthritis Induced by ACLT

Additionally, this study explored the effect of COR on ACLT-induced osteodynia and cartilage injury. As shown in Figure 2A, compared with the control group and sham group, the PWMT value of ACLT group rats was significantly lower. Surprisingly, COR treatment increased the PWMT value in a dose-dependent manner. Besides, HE staining and Alcian blue staining showed that COR treatment seemed to reduce ACLT-induced cartilage damage (Figure 2B). Moreover, as shown in Figure 2C and D, COR treatment unexpectedly reversed the low expression

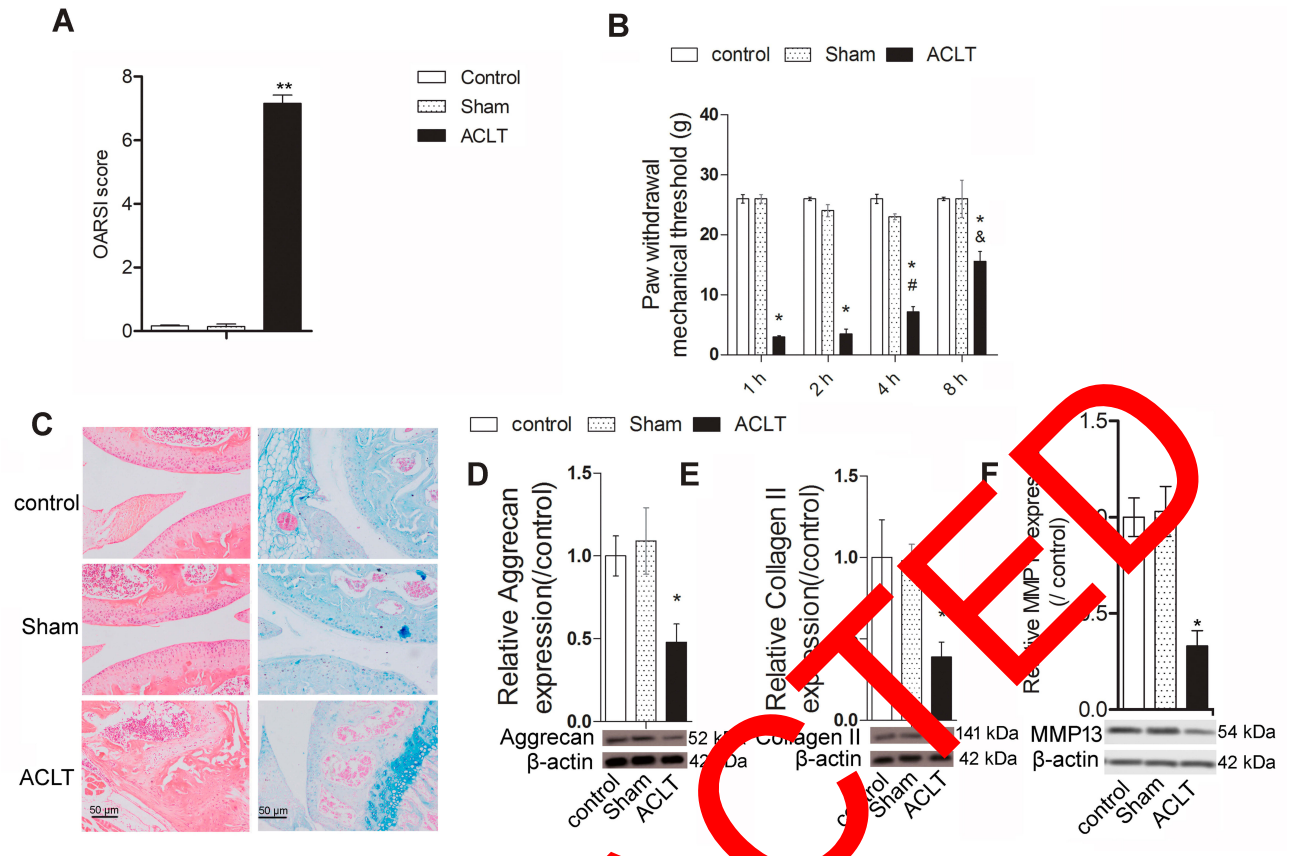

Figure 1 Establishment of rat model with knee osteoarthritis induced by ACLT. (**A**), OARSI score. (**B**), The value of PWMT in Control, Sham and ACLT groups at 1 h, 2 h, 4 h and 8 h after surgery, respectively. (**C**), Histological damage of isolated knee joint in control group, sham group and ACLT group was detected by HE staining and Alcian blue staining, respectively. (Magnification × 100) (**D**), the expression of Aggrecan in articular cartilage of rats in each group was detected by Western blotting. (**E**), the expression of collagen II in articular cartilage of rats in each group was detected by Western blotting. (**F**), the expression of MMP13 in articular cartilage of rats in each group was detected by Western blotting. Data are presented as mean ± standard deviation (SD). *P < 0.05, **P < 0.01 compared to Control group at same time. #P < 0.05 compared to ACLT group at 2 h after surgery. &P < 0.05 compared to ACLT group at 4 h after surgery.

of aggrecan and collagen II in the cartilage of ACLT rats, and the effect was dose-dependent. In conclusion, these studies show that COR can reduce the pain of osteoarthritis and cartilage damage of ACLT-induced knee osteoarthritis rats.

COR Decreased the Activity of TGF-β and Increased the Ratio of LC3-II/I in Rat Model with Knee Osteoarthritis Induced by ACLT

Further analysis showed that compared with the control group and sham group, the serum TGF-β level of ACLT group was increased significantly (Figure 3A). Notably, COR treatment reversed the increase of TGF-β in serum induced by ACLT. Moreover, compared with the control group and sham group, the expression of TGF-β R1 at mRNA and protein levels in ACLT group was also significantly promoted (Figure 3B–E), which further reduced the LC3-II/-I ratio. Surprisingly, COR treatment reversed the inhibitory effect of ACLT on autophagy. In general, these studies showed that COR reduced the activity of TGF-β and promoted autophagy in ACLT-induced knee arthritis rats.

TGF-β Overexpression Reversed the Effects of COR on ACLT-Induced Rat Model with Knee Osteoarthritis

TGF-β was overexpressed by adenovirus (Figure 4A). In addition, HE staining and Alcian blue staining showed that TGF-β overexpression reversed the effect of COR on ACLT-induced cartilage injury in rats (Figure 4B). Similarly, TGF-β overexpression reversed the effects of COR on autophagy and expression of aggrecan and type II collagen induced by ACLT (Figure 4C–F). In total, these results indicate that TGF-β overexpression reverses the effect of COR on ACLT-induced knee osteoarthritis in rats.

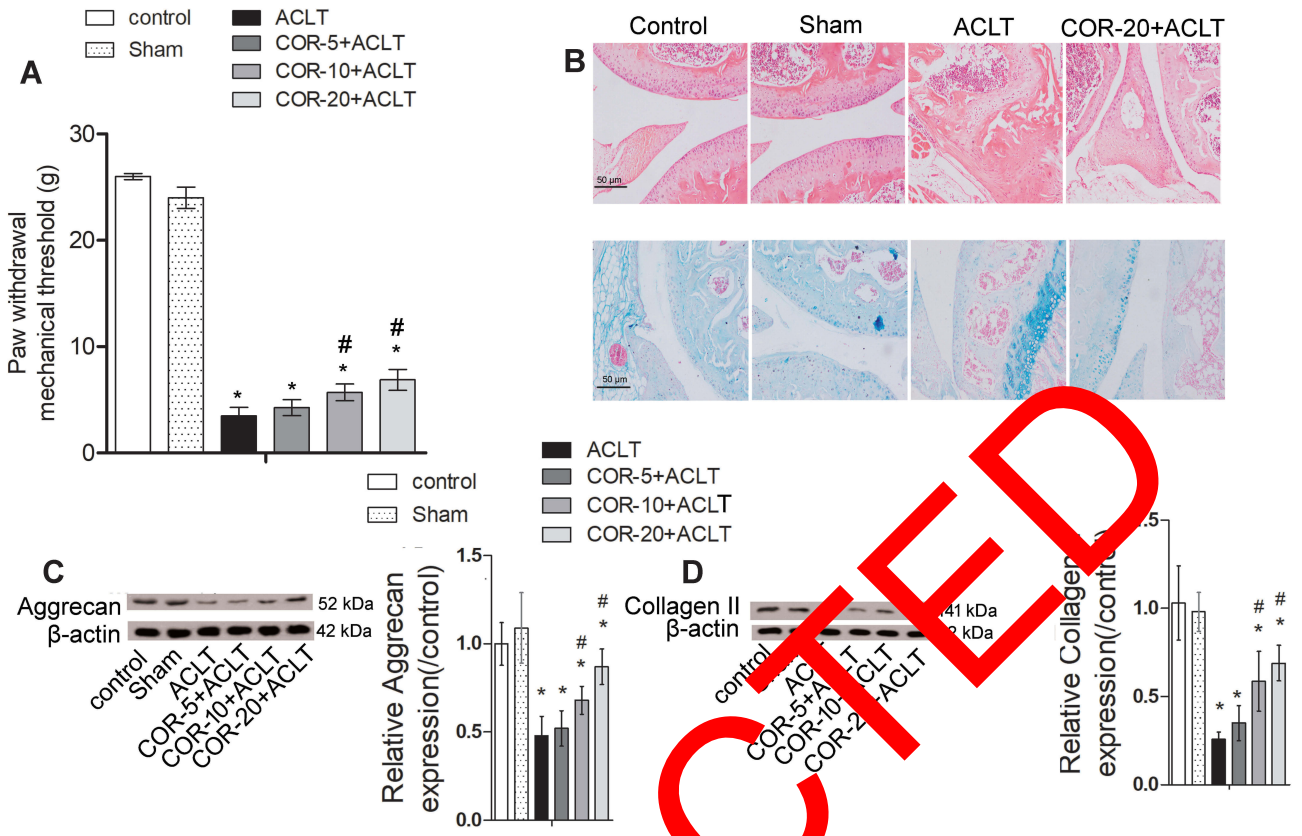

Figure 2 COR alleviated bone-arthrosis pain and cartilage injury in rat with knee osteoarthritis induced by ACLT. Before modeling, the rats were intraarticularly injected with COR (5, 10 and 20 mg/kg) for three consecutive days. (**A**), the value of PWMT in each group at 2 h after surgery. (**B**), HE staining and Alcian blue staining. (Magnification × 100) (**C**), the expression of Aggrecan in articular cartilage of rats in each group was detected by Western blotting. (**D**), the expression of collagen II in articular cartilage of rats in each group was detected by Western blotting. Data are presented as mean ± standard deviation (SD). *P < 0.05 compared to Control group. #P < 0.05 compared to ACLT group.

Autophagy Inhibitor 3-MA Reversed the Effects of COR on ACLT-Induced Rat Model with Knee Osteoarthritis

Finally, HE staining and Alcian blue staining showed that 3-mA treatment reversed the improvement of COR on cartilage injury (Figure 5A). Western blotting showed that 3-MA aggravated cartilage damage by increasing the expression of TGF-β R1 and decreasing the LC3-II/-I ratio and the expression of aggrecan and collagen II. (Figure 5B–D). These results showed that 3-MA reversed the effect of COR on ACLT-induced knee osteoarthritis in rats.

Discussion

ACLT is widely used in the surgical-induced osteoarthritis of rats. Therefore, this study constructed an ACLT rat model to explore the effect of COR on osteoarthritis in rats. First of all, we confirmed the successful establishment of osteoarthritis model by Score and histological staining. Secondly, the effect of COR on bone pain was evaluated by PWMT. PWMT is a pain-related behavioral test. Our results showed that ACLT reduced the value of PWMT in rats. Aggrecan and collagen II are well-known cartilage-specific genes, which are related to the grade of cartilage region.[22] In this study, we found that the expression of aggrecan and collagen II in ACLT rats decreased significantly, indicating that the cartilage of rats was obviously damaged. These results are consistent with previous studies.[23–25]

COR is a kind of natural nucleoside analog isolated from *Cordyceps militaris*, which has been proved to have many biological functions and broad clinical application prospects. Previous studies have also shown that COR reduced the pathological damage and pain of the rat model of osteoarthritis induced by sodium iodoacetate (MIA).[9] In order to explore the role of COR in ACLT-induced knee osteoarthritis, different doses of COR were injected into the joints of rats. The results showed that COR could reduce the pain and cartilage damage induced

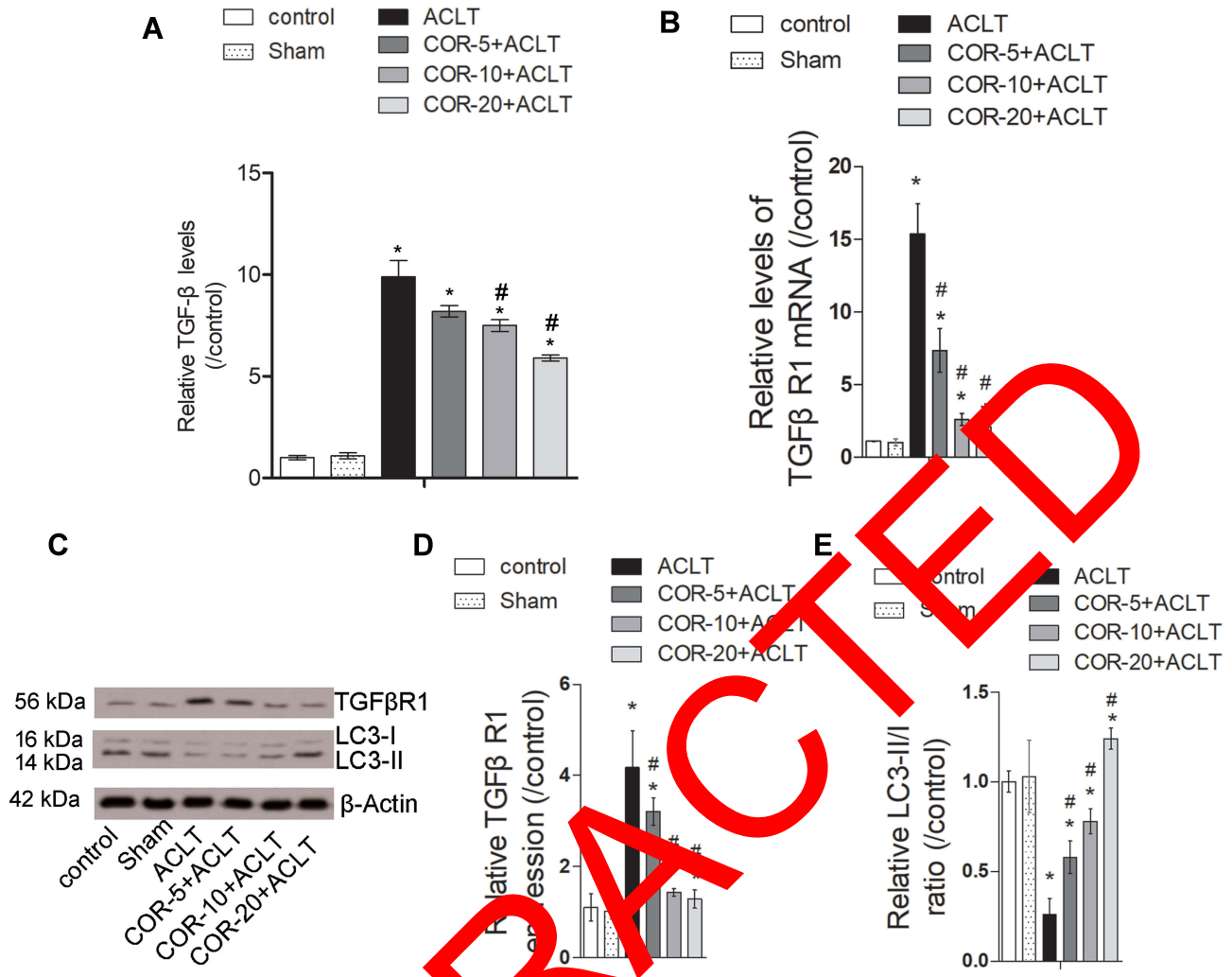

Figure 3 COR decreased the activity of TGF-β and [illegible]reased th[illegible]o of LC3-II/I in rat model with knee osteoarthritis induced by ACLT. Before modeling, the rats were intraarticularly injected with COR (5, 10 and 20 m[illegible]) for three co[illegible]utive days. (**A**), TGF-β level in serum in each group at 2 h. (**B**), the mRNA level of TGF-βR1 in each group was measured by RT-qPCR. (**C**–**E**), the [illegible]pres[illegible]n of TGF-βR1, [illegible]II and LC3 I in each group was measured by Western blotting. Data are presented as mean ± standard deviation (SD). *P < 0.05 compared to Contr[illegible]oup. #P < 0.05 compared to ACLT group.

by ACLT in a dose-d[illegible]pendent manner. MMP13 and a disintegrin and meta[illegible]oprote[illegible]ase with thrombospondin motifs-5 (ADAMTS-5) h[illegible] been i[illegible]ntified as the main enzymes[26,27] [illegible]at le[illegible] to c[illegible]ge degradation in the developme[illegible] of oste[illegible]rthritis. COR inhibited the expression of MM[illegible]3 and ADAMTS-5 in chondrocytes of advanced osteoa[illegible]itis induced by IL-1 β, indicating its potential role in preventing cartilage degradation.[28] Therefore, the expression of MMP13 was measured in this study. It was found that ACLT treatment caused overexpression of MMP13, and COR treatment reversed this effect.

Our results also showed that COR decreased the activity of TGF-β and promoted autophagy in ACLT-induced osteoarthritis rats. More and more evidence showed that TGF-β plays an important role in inducing autophagy.[13] In osteoarthritis, TGF-β is essential to maintain cartilage. Serum TGF-β 2 and TGF-β 3 were increased and positively correlated with the pain of osteoarthritis.[29] These studies show that the role of COR in the inhibitory effect of osteoarthritis pain and cartilage injury in osteoarthritis is at least partially achieved by inhibiting the expression of TGF-β. In addition, the overexpression of TGF-β reversed the regulatory effect of COR on the expression level of aggrecan and collagen II induced by ACLT. Further analysis showed that 3-MA could reverse the inhibitory of ACLT-induced autophagy by COR. Based on these studies, in this study, we can conclude that COR can reduce the pain and cartilage damage induced by ACLT by inhibiting TGF-β activity and inducing autophagy. Therefore, autophagy activation may be a new therapeutic target for osteoarthritis.

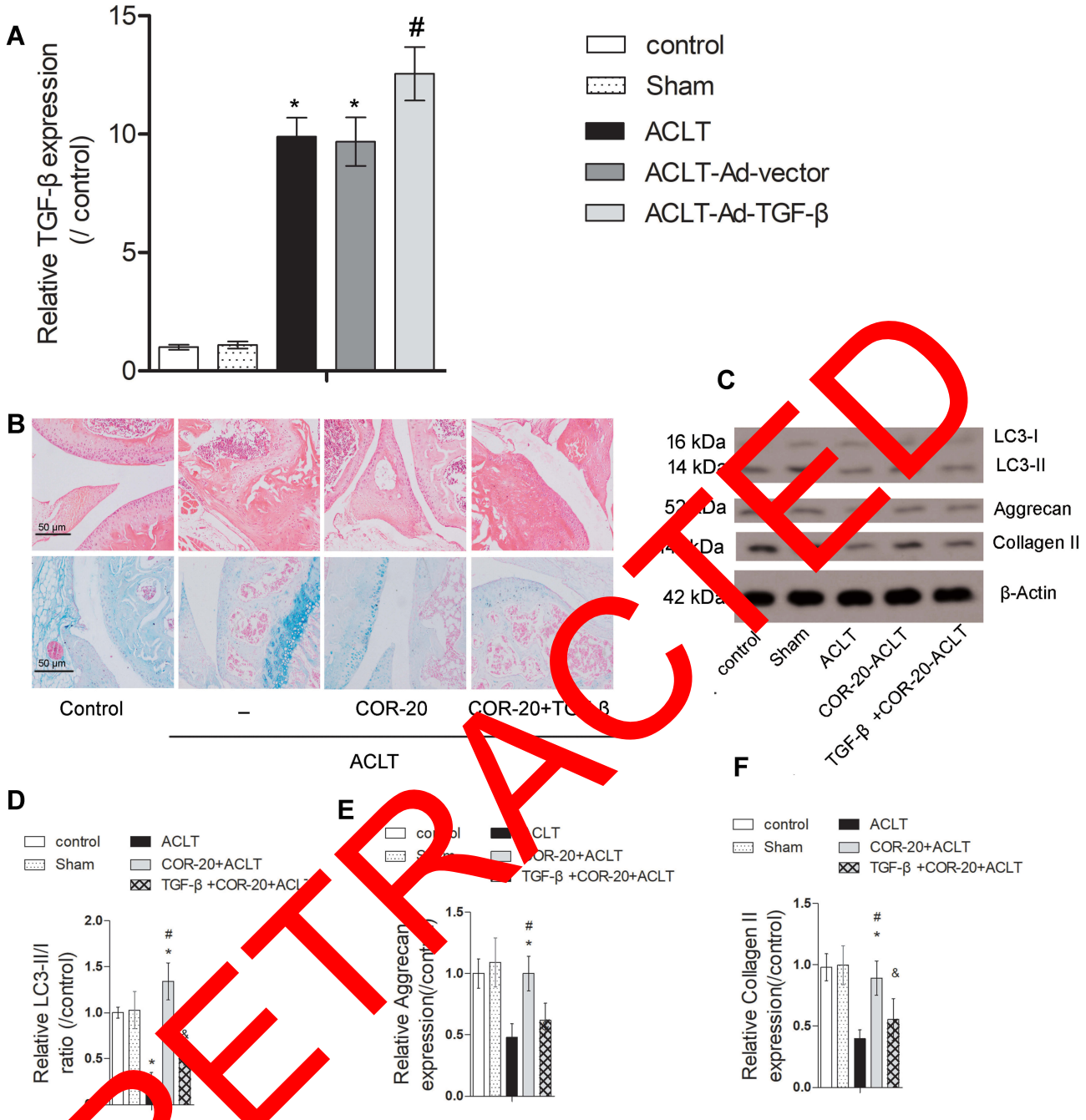

Figure 4 [illegible] of COR on ACLT-induced rat model with knee osteoarthritis. An empty vector or an adenovirus containing an overexpression vector of TGF-β (Ad-[illegible]$^{223/225}$) was injected into rats intra-articularly. (**A**), the mRNA level of TGF-β in serum. (**B**), HE staining and Alcian blue staining. (Magnification × 100) (**C**–**F**), the protein levels of LC3 II, LC3 I, Aggrecan and collagen II in each group were measured by Western blotting. Data are presented as mean ± standard deviation (SD). *P < 0.05 compared to Control group. #P < 0.05 compared to ACLT group. &P < 0.05 compared to COR-20+ACLT group.

Our study clarified the role of COR in improving cartilage matrix degradation and inducing autophagy in the ACLT-induced osteoarthritis rat model. TGF-β is involved in the development of most tissues and homeostasis,[30] including the induction of autophagy,[13] morphogenesis, cell differentiation and tissue remodeling. Our research shows that the role of COR in the ACLT-induced osteoarthritis rat model is achieved by inhibiting the activity of TGF-β and inducing autophagy. The specific underlying mechanism still needs further study.

Conclusion

To sum up, COR alleviated ACLT-induced osteoarthritis pain and cartilage damage in rats by inhibiting the activity of TGF-TGF and inducing autophagy. COR therapy or autophagic activation may be a new treatment for osteoarthritis. This

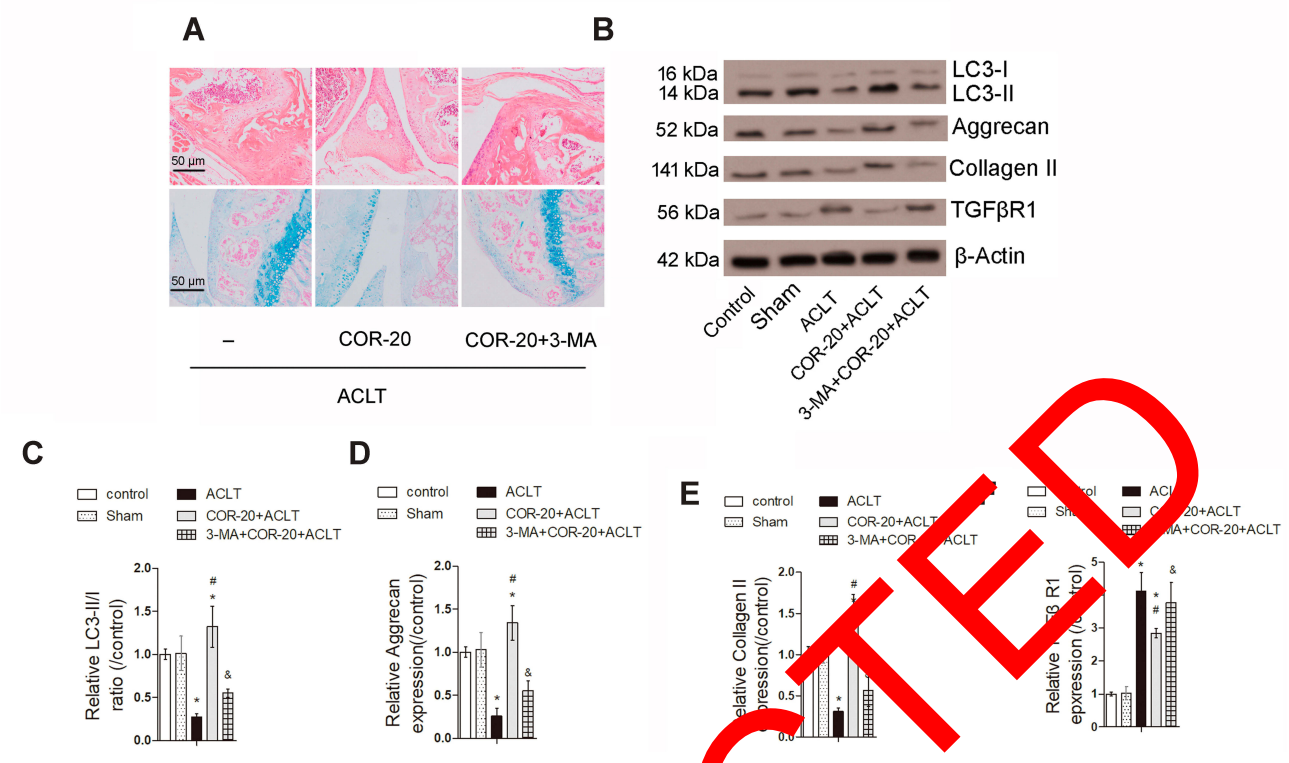

Figure 5 Autophagy inhibitor 3-MA reversed the effects of COR on ACLT-induced rat model with knee osteoarthritis. (**A**), the expression of TGF-β was measured by RT-qPCR. (**B**), HE staining and Alcian blue staining in each group. (Magnification × 100) (**C**–**F**), the protein levels of LC3 II, LC3 I, Aggrecan, collagen II and TGF-βR1 in each group were measured by Western blotting. Data are presented as mean ± standard deviation (n = 3). *P < 0.05 compared to Control group. #P < 0.05 compared to ACLT group. &P < 0.05 compared to COR-20+ACLT group.

study is a preliminary study, which provides a foundation for clinical application of COR in the treatment of osteoarthritis.

Abbreviations

COR, cordycepin; ACLT, anterior cruciate ligament transection; PWMT, paw withdrawal mechanical threshold.

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Disclosure

The authors report no conflicts of interest in this work.

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