

Continuous infusion of dexamethasone aggravates the damage of cartilage via upregulating p-AKT and impairing articular autophagy in experimental OA model

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Abstract

Objective

To explore the effect of dexamethasone (Dex) infusion on articular cartilage and the underlying mechanisms *in vitro* and *in vivo*.

Methods

Destabilization of medial meniscus (DMM)-induced OA mouse model was used in this study. The mice with Dex treatment were sacrificed and then the knee joint samples were obtained for pathological analysis. Mouse primary chondrocytes were isolated and cultured in the presence or absence of Dex, which were used for calcification analysis and western blot assay.

Results

Dex accelerated the loss of articular cartilage matrix in mice, while it aggravated the damage of cartilage in DMM-induced OA model at the late stage. The calcium content in calcified cartilage layer in the joints from Dex treated OA mice was significantly higher than that from control mice. Dex treatment enhanced mineralization of articular cartilage matrix and led to massive apoptosis of chondrocytes in OA model. In addition, Dex caused autophagy of chondrocytes in the early stage, which was decreased at the late stage of Dex treatment. Moreover, we found that the effect of Dex on the mineralization of articular cartilage matrix in mice was related to AKT activation.

Conclusions

Continuous infusion of Dex can enhance the calcification of cartilage via AKT activation and increase chondrocyte apoptosis through inhibiting autophagy, which aggravates the damage of articular cartilage and accelerates the progression of OA *in vivo*.

Introduction

Osteoarthritis (OA) is the most common joint disease and leading cause of disability worldwide [1]. Through decades of research, OA is currently accepted as a whole joint disease [2] and even whole-person disease [3] developing along a continuum from early to late stages. The epidemiology of this disorder is complex and multifactorial, with genetic [4], biological [5], and biomechanical [6] components. However, the therapeutic effect is still unsatisfactory in clinic until now.

Corticosteroids [7] have been widely used as a first-line anti-inflammatory and immune-modulating drug for many immune-mediated conditions or as an adjunctive therapy for some infectious or malignant

diseases. However, high doses or prolonged use leads to a number of side-effects including joint injury [8–11]. In the past 10 years, several professional societies including Osteoarthritis Research Society International (OARSI), National Institute for Health and Care Excellence (NICE) in 2014 and American College of Rheumatology (ACR) in 2012 recommended glucocorticoids for patients with knee OA [12]. Long acting corticosteroids including dexamethasone (Dex) are recommended for the treatment of flare of knee pain in clinic mainly due to its anti-inflammation effect [13]. However, the American Academy of Orthopedic Surgeons (AAOS) found a lack of compelling evidence to support the use of glucocorticoids for the treatment of OA, as well as an unclear balance between the benefits and potential harms of this treatment [14]. Moreover, more and more studies have suggested that corticosteroids such as Dex could aggravate the damage of articular cartilage [15–19]. One study showed that patients who had been treated with 2 years of intra-articular triamcinolone suffer from significantly greater cartilage volume loss and no significant improvement of knee pain can be found [18]. Yihui Tu et al. reported that Dex can induce the apoptosis and significantly upregulate the expression of apoptotic gene Fas/FasL in human articular chondrocytes [19]. However, the detailed mechanisms of Dex-mediated cartilage damage are still not well known.

In this study, we explored the effect of dexamethasone infusion on articular cartilage in vivo and the potential mechanisms. Our study revealed that continuous infusion of Dex can enhance the calcification of cartilage via AKT activation and increase chondrocyte apoptosis through inhibiting autophagy, which aggravates the damage of articular cartilage and accelerate the progression of OA in vivo. Our study provided a new perspective to understand the mechanisms of Dex related side effect on joint, which will be beneficial for future treatment in OA.

Materials And Methods

Sample collection and processing

This study was conducted in compliance with the regulations of the ethics committee of the Daping Hospital (Chongqing, China). In OA group, human articular cartilage samples were collected from 3 primary knee OA (grade IV in The Kellgren Lawrence grading system) patients with multiple intra-articular corticosteroid injections and Varus deformity receiving knee arthroplasty. In control group, human articular cartilage samples were collected from 3 patients who had amputations due to trauma. All of them were confirmed without inflammatory arthritis or prior knee surgery history. All samples of full thickness articular cartilage were cut from patient's tibial plateau and fixed in 4% PFA. Half of them were decalcified in 15% EDTA for wax embedding, and remaining samples underwent hard tissue embedding.

Primary chondrocyte isolation and culture

Mouse primary chondrocytes were isolated and cultured according to our previous report [20]. Briefly, the cartilage was isolated from knee joints of 3 ~ 5-day-old C57BL/6J mice and treated with 0.25% trypsinase/DMEM at 37°C for 15 min to remove the soft tissues (including muscles, ligaments and bone

tissues). After further incubation with 0.1% collagenase II/DMEM overnight in a CO₂ incubator at 37 °C, the chondrocytes were collected and cultured in DMEM/F12 supplemented with 10% FBS.

Methylmethacrylate embedding and sectioning

The undecalcified specimens were dehydrated in ascending concentrations of ethanol (from 70–100%) and then embedded in methylmethacrylate according to the instructions of manufacturer. The embedded specimens were trimmed with a hard tissue cutting device (Leica RM2265; Germany) to expose the target area. Finally, the cut specimens were sanded down with sequential #2000, #4000, #8000 and #10000 grit lapping plastic sandpaper (3M; Japan). Each specimen was placed in an ultrasonic bath after the polishing steps.

Scanning Electron Microscope Imaging and Energy Dispersive Spectrometer detection

After vacuum drying, the samples were sputter-coated with gold and palladium and then observed by Hitachi SU8010 scanning electron microscope (SEM) under 5 keV accelerating voltage, 10 μA probe current, a 10-mm working distance, and an image resolution of 1560 × 1920. The surface calcium element analysis of the target area was analyzed with line scan by Oxford X-max50 Energy Dispersive Spectrometer (EDS).

Surgical model of OA in mice and Dex deliver

Adult male C57BL/6J mice (10 ~ 12 weeks) were purchased from purchased from the Beijing HFK Bioscience Co.Ltd. and maintained in the animal facility (specific pathogen free) of the Daping Hospital (Chongqing, China). The mice were randomly divided into six groups: Control groups (vehicle group and Dex group), Sham groups (Sham + vehicle group, Sham + Dex group), and DMM groups (DMM + vehicle group, DMM + Dex group) (n = 36 ~ 45 per group). DMM surgery was performed on the right knee joints to establish experimental OA model according to the described methods [21]. In short, after anesthetization (1% pentobarbital sodium), joint capsule was incised, then the medial meniscotibial ligament was sectioned with microsurgical scissors. As a control, sham operation was performed on the left knee joint, but the ligaments remained intact. After 2 days, dissolved dexamethasone with saline was injected intraperitoneally (5 mg/Kg, 3 times per week). The vehicle group was injected intraperitoneally with saline. Then the effect of dexamethasone on the progression of osteoarthritis was observed at week 4, week 8, and week 12. All experiments were performed according to protocols approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (Chongqing, China).

Histological analysis

The knee joints were fixed with 4% paraformaldehyde for 24 h, decalcified with 0.5M EDTA at pH 7.4 for 2 weeks and embedded in paraffin. Five-micrometer-thick sagittal serial sections were made across the knee joints. Sections from the medial tibial plateau and medial femoral condyle of knee joints were stained with Safranin-O/Fast Green and scored on a scale of 0–6 according to the recommendation of the Osteoarthritis Research Society International (OARSI) [22]. Each section was assessed by two blinded,

independent graders (Jinfan Zhang, Junlan Huang) and the average score was used for statistical analysis.

Immunohistochemical staining (IHC)

Immunohistochemical staining was performed using Histostain-Plus Kits (ZSGB-BIO, PV-9004). Briefly, the knee joint sections were deparaffinized, quenched with hydrogen peroxide, trypsin treated, blocked with goat serum and then orderly stained with primary (CST, LC3B Antibody #2775; CST, Phospho-Akt (Thr308) Antibody #13038) and secondary antibodies. Signals were visualized using DAB (ZSGB-BIO, ZLI-9019) and counterstained with methyl green. Positive cells from three random high-power fields were calculated for statistical analysis.

Western Blot assay

Cells lysate were lysed with RIPA lysis buffer (Beyotime, P0013B). Equal amount of protein samples were resolved on 10–12% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane. After blocking with 5% nonfat milk, the membrane was probed with indicated primary (CST, LC3B Antibody #2775) and secondary antibodies. The signals were detected using chemiluminescence (Pierce, NCI4106) according to the manufacturer's instruction. Images were captured by ChemiScope (Clinx, Shanghai, China).

Calcification analysis

For chondrocyte calcification analysis, cells were cultured for 7 days in complete Fitton-Jackson Modified (BJGb) medium (Gibco) (10% FBS, 50 mg/mL ascorbic acid, 20 mM β -glycerol phosphate), stimulated with Dex as well as AKT inhibitor: LY294002 (10 nM, CST). Medium was changed for the last 4 days. Alizarin red staining and Alizarin red absorbance at 405 nm were used to evaluate the degree of calcification.

Tunel assay

Apoptosis of articular chondrocytes in cartilage tissues was determined by TUNEL assay using a kit from Invitrogen (Thermo Fisher, USA) according to the manufacturer's instructions. Specimens were visualized under a fluorescence microscope. The number of apoptotic chondrocytes in relation to the total number of cells was quantified in tissue sections. More than three fields of microscopic view in each section, and multiple sections (more than three) from 4 different experimental animals in each experimental group were used.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA). All numerical values were presented as mean values \pm the standard deviation (SD). Student's t-test or two-way ANOVA analysis was used to determine significance. Difference was considered significant when $p \leq 0.05$.

Results

Continued use of Dex leads to the loss of articular cartilage matrix in mice

Firstly, we study the effect of Dex on the articular cartilage of control mice. The mice were sacrificed at the time points of 4 weeks, 8 weeks and 12 weeks after continuous intraperitoneal Dex injection. Then the knee joint samples were obtained and stained with Safranin O-fast green (Fig. 1A) to assess the extent of articular cartilage degeneration by OARSI scoring system (Fig. 1B). The results showed that intraperitoneal Dex injection led to significant progressive loss of extracellular matrix (Fig. 1A) of knee joint cartilage in the middle and late stage (8 weeks, 12 weeks) of OA, including the femur side and the tibial side. The sum score of medial femoral condyle (MFC) and medial tibial plateau (MTP) (Fig. 1B) was also decreased after Dex treatment. These data indicate that continued intraperitoneal use of Dex lead to the loss of articular cartilage matrix in normal adult mice.

Long-term continuous Dex application aggravates DMM-induced cartilage damage

Next, we further evaluated the effect of long-term continuous use of Dex on the cartilage damage in an experimental OA model. Surgical DMM in mice is a well-established OA model, which is commonly used to study and evaluate the effects of drugs on OA. DMM surgery was applied in adult mice to create a mechanically unstable OA model. Safranin O-fast green staining and OARSI scoring system were taken to assess the extent of articular cartilage degeneration. The data in Fig. 2A showed that continuous injection of Dex for 12 weeks significantly aggravated cartilage destruction in mice leading to deficiency of large areas of surface articular cartilage in the calcified cartilage layer. Meanwhile, the total score and maximum score of MTP and MFC were significantly increased in Dex-treated group (Fig. 2F-I). These data suggest that long-term Dex treatment can accelerate cartilage damage in OA mice.

Continuous Dex results in decreased subchondral bone mass and bone density and alleviated synovial inflammation

OA is considered as a disease of the whole joint. In recent years, a large number of studies have shown that dysregulated subchondral bone remodeling and synovial inflammation are also involved in OA process [23]. Therefore, we further observed the effect of Dex on subchondral bone and synovial inflammation in DMM OA model. Continuous Dex injection resulted in decreased bone mineral density (BMD) and bone mass/volume ratio (BV/TV) of subchondral bone in both DMM and Sham groups (Fig. 3C and D) at 12 weeks. These results showed that the local Dex application could lead to loss of subchondral bone mass and decreased bone density, which was consistent with previous study [24]. Meanwhile, the data from synovitis scoring showed that Dex attenuated the severity of synovitis following DMM surgery compared with the sham mice (Fig. 3A-B), suggesting that Dex, as a classical anti-

inflammatory drug, has a remarkable inhibitory effect on synovial inflammation in DMM-induced OA model.

Dex increases calcium content in calcified cartilage layer

The calcification of cartilage is highly involved in the pathological changes of OA [25]. To further investigate the mechanisms of Dex-worsened cartilage damage, we detect the calcium content in each layer of articular cartilage of mouse knee joint by SEM and EDS. There were significant calcium concentration gradients in each layer of cartilage in the knee joint of normal adult mice (Fig. 4A-B). The calcium content in the non-calcified cartilage layer was extremely low, while it increased successively in the calcified cartilage and subchondral bone plate (Fig. 4B). After use of Dex, the abnormally increased calcium content in the calcified cartilage layer (CCL) was detected from 4 weeks to 12 weeks (Fig. 4B), which disturbed the original calcium distribution pattern. Next, we assessed the changes of cartilage calcification in DMM model (Fig. 4C-D). We found that the calcium content in CCL was gradually increased during the OA process (from 8 to 12 weeks). Dex group, however, showed an abnormally sudden increase of calcium content in the CCL at the early period (after 4 weeks). Moreover, the samples from OA patients with a history of Dex treatment presented a high calcium content in calcified cartilage (Fig. 5B and D) and a large number of horizontal clefts in the junction between calcified and non-calcified cartilage (arrowheads in Fig. 5), which suggested to be closely related to the rapid degeneration and peeling of articular cartilage[26].

Dex induces calcification of ECM by partially activating AKT

Previous study showed that AKT could positively regulate the calcification of chondrocytes in vitro, and calcified osteophyte formation was prevented in the Akt1^{-/-} joints in mice with surgically induced OA. Therefore, we suspect that AKT signal contributes to Dex-mediated cartilage calcification. As shown in Fig. 6A, the effects of Dex at different concentration gradients (0, 1 nM, 10 nM and 100 nM) on extracellular matrix calcification were observed, and the OD value of alizarin red was detected at 405 nm (Fig. 6B). As shown in Fig. 6B, Dex dose-dependently promoted the calcification of extracellular matrix of primary chondrocytes. Next, we detected the change of AKT signal after Dex treatment. The AKT pathway inhibitor LY294002 reversed Dex-induced increase of extracellular matrix calcification (Fig. 6C and D), suggesting that AKT pathway plays an important role in Dex-mediated chondrocyte calcification in vitro. In addition, the protein expression of AKT in Dex group was slightly increased, while the expression of P-AKT³⁰⁸ rather than PAKT⁴⁷³ was significantly higher (Fig. 6E), suggesting that Dex may partially lead to calcification of extracellular matrix of chondrocytes through activation of P-AKT³⁰⁸. As the activation of AKT contributes to Dex-induced chondrocyte calcification in vitro, we further examined the activation state of AKT in the joint sample from Dex-treated mice. After 4 weeks of Dex treatment, the percentage of P-AKT³⁰⁸ positive cells was significantly increased in articular cartilage of DMM mice (Fig. 6F). The percentage of P-AKT³⁰⁸ positive cells was almost 90%, which mainly appeared in the deep layer of cartilage in Dex-treated group (Fig. 6G). These results suggest that Dex may partially, at the early stage,

activate the AKT pathway to cause abnormal calcium deposition in calcified articular cartilage layer of OA.

Dex promotes the apoptosis of articular chondrocytes in vivo

The increase of chondrocyte apoptosis is an important reason for the destruction and degeneration of articular cartilage [27]. Previous studies have revealed that Dex could enhance apoptosis in multiple types of cells [28, 29]. Therefore, we further estimated the effect of Dex on the apoptosis of articular chondrocytes in sham and DMM mice. As shown in Fig. 7C, the number of apoptotic cells in Dex group was significantly increased in the non-calcified layer in sham group. For the sham group, the numbers of cells in the non-calcified cartilage layer, calcified cartilage layer and subchondral bone plate were not changed significantly in the Vehicle group and the Dex group (Fig. 7B, E, H). The number of apoptotic cells in Dex group was significantly increased in the non-calcified layer (Fig. 7C), while the number and proportion of the apoptotic cells in the calcified layer was significantly increased (Fig. 7F, G). It is suggested that long-term use of Dex promotes apoptosis of chondrocytes, especially in calcified chondrocytes. Similarly, in the DMM + Vehicle group, we also found that apoptotic cells were concentrated in the calcified cartilage layer (Fig. 7F). In the DMM group, Dex significantly decreased the cell number of chondrocytes in both non-calcified and calcified cartilage layers (Fig. 7B and E). Both the number and proportion of apoptotic cells were increased in the non-calcified cartilage layer (Fig. 7C and D). While the number of apoptotic cells in the calcified cartilage layer was not significantly changed, but the proportion of apoptotic cells was significantly increased due to the decreased total number of cells (Fig. 7F and G). These results suggest that continued use of Dex promotes apoptosis of articular chondrocytes in both OA and non-OA models, while the apoptotic chondrocytes were mainly located in the calcified and non-calcified cartilage layer in OA models.

Continuous Dex impaires chondrocyte autophagy at the late stage of DMM surgery

As autophagy could protect the cells from apoptosis in various conditions [30], and Dex was reported to induce autophagy in cultured chondrocytes [31], we deduced whether autophagy is involved in the Dex-induced chondrocyte apoptosis in DMM model. Our results showed that the LC3-positive cells in articular cartilage at 4 weeks and 8 weeks after DMM operation were significantly higher in Dex group than those in the Vehicle group (Fig. 8A-C). At 12 weeks after DMM, the numbers of LC3-positive cells were few in Dex-treated groups and there is no significant difference between control and Dex-treated groups (Fig. 8D), indicating the stress-response autophagy was eliminated after long time processing of Dex. As autophagy could either promote cell survival or cell death, we further investigated the effect of Dex-induced autophagy on chondrocyte apoptosis using autophagy inhibitor Baf1. We found that Baf1 could enhance the apoptosis induced by Dex alone (Fig. 8E), suggesting that Dex could induce a protective autophagy of primary chondrocytes to prevent their apoptosis.

Collectively, our data revealed that Dex could enhance the calcification of cartilage via AKT activation and increase chondrocyte apoptosis through inhibiting autophagy, which may further promote the lesion of articular cartilage and accelerate OA progression (Fig. 8F).

Discussion

Corticosteroids has been widely used in the management of osteoarthritis, rheumatoid arthritis and some sports injuries for decades [32–34]. However, there has been more and more evidence showed that corticosteroids had a degenerative-effect on several collagen-producing tissues, such as bone, tendons [35, 36]. Accordingly, interests have focused on how corticosteroids led to degeneration in these tissues. However, the mechanism of corticosteroids induced degeneration of cartilage is not well known. Chrysis and his colleagues revealed that Dex induces apoptosis of chondrocytes in a caspases-dependent manner [37]. In addition, Dex significantly increased ATP-induced mineralization in articular chondrocytes in vitro [38], suggesting a potential role of Dex in the pathologic mineralization and loss of cartilage in OA.

The calcified cartilage layer (CCL) forms an important interface between compliant cartilage and stiff bone for transmitting force, attaching cartilage to bone, and limiting material diffusion [39]. It is separated from the other zones of cartilage by the tidemark, and the CCL borders the subchondral bone with the cement line, which forms a highly interdigitated interface with subchondral bone [40]. Contributing to the stiffness gradient in the soft-hard tissue junction, the CCL is about 100 times stiffer than the overlying hyaline cartilage and 10 times softer than the underlying bone [41]. However, this stiffness gradient is altered during the OA process, during which the stiffness of the calcified cartilage zone is gradually increased from superficial layers of cartilage to subchondral bone [42]. The mechanical properties of cartilage could be affected by the extent of its mineralization. For calcified cartilage, the nanoindentation modulus is positively related to the local mineral content [43, 44]. In healthy human knee specimens, the percentage of inorganic compound in CCL is less than that of subchondral bone [45]. More detailed analyses of bovine tibiofemoral joints find that the articular cartilage zone was mineral free, whereas the mineral content of calcified cartilage zone increases exponentially but is still significantly lower than that of the normal bone [46]. Interestingly, horizontal splits at the interface between the uncalcified and the calcified layers of the articular cartilage have previously been described in degenerative joint disease of humans, mice and hamsters, and have been suggested to be related with the shearing damage at the uncalcified calcified cartilage interface [47]. In OA, extremely hyper-mineralization was found in calcified cartilage zone [44]. The above studies suggest that changes in the mineral content of calcified cartilage zone may lead to changes in its mechanical properties, which damages the role of calcified cartilage zone as the middle buffering zone between cartilage and bone.

AKT plays an important role in the maintenance of cartilage homeostasis and the progression of OA. In mice with surgically induced OA, calcified osteophyte formation was prevented in the Akt1^{-/-} joints. Calcification was suppressed in cultured Akt1^{-/-} chondrocytes or ATDC5 cells with Akt1 knockdown, but enhanced in ATDC5 cells overexpressing constitutively active Akt1[48]. The forced expression of

constitutively active AKT rescued the expression of phenotypic markers and the apoptosis induced by CXCR2 blockade, indicating CXCR2-dependent chondrocyte homeostasis was mediated by AKT signaling [49]. IL-1 β -mediated activation of NF- κ B and apoptosis in chondrocytes was inhibited by IGF-1 and PDGF-bb, which could be related to the suppression of Src/PI-3K/AKT pathway [50]. Similarly, Tenuigenin inhibits IL-1 β -induced inflammation in human osteoarthritic chondrocytes by suppressing PI3K/AKT/NF- κ B pathway [51]. The AKT is necessary for the synergistic induction of MMP-1 and MMP-13 and the cartilage breakdown stimulated by IL-1 in combination with oncostatin M. Moreover, C.Shen et al. reported that Dex increased the expression of Akt in human chondrocytes, which was related to the degenerative process in cartilage[52]. However, some studies reported that the AKT signaling was inhibited by Dex in other models, indicating the complicated mechanisms of the effects of Dex on different cells. More studies are needed to investigate the details.

Autophagy contributes to the maintenance of homeostasis of chondrocytes, whose impairment greatly aggravates OA. Autophagy is constitutively active in normal cartilage, which would be compromised with aging and precedes cartilage cell death and structural damage [53]. Rapamycin could improve severity of cartilage degradation as well as synovitis in mouse OA model via inducing autophagy, indicating pharmacological activation of autophagy may be an effective therapeutic approach for OA [54]. Besides, cartilage-specific ablation of mTOR results in increased autophagy level and reduced articular cartilage degradation, apoptosis and synovial fibrosis in DMM OA model [55]. In addition, Boudierlique, T. et al reported that targeted deletion of Atg5 in chondrocytes promotes age-related OA by facilitating chondrocyte survival, suggesting that autophagy is beneficial to the age-related OA [56]. In this study, Dex treatment increased the autophagy activity of chondrocytes at the early stage, which was gradually decreased with the extension of processing time. We speculate that autophagy may be one of the adaptive protective responses for chondrocytes under the stimuli of Dex. The long-time application of Dex, however, weakened the autophagy-mediated protective effect and ultimately aggravated the damage of cartilage. More research are needed to investigate the details.

In brief, our present study revealed that Dex could enhance the calcification of cartilage via AKT activation and increase chondrocyte apoptosis through inhibiting autophagy, which aggravates the damage of articular cartilage and accelerate the progression of OA in vivo. Our data provided a new perspective to understand the effect of Dex on cartilage maintenance and degeneration, which may be beneficial for the clinical use of Dex for OA treatment in future.

Conclusions

Continuous infusion of Dex can enhance the calcification of cartilage via AKT activation and increase chondrocyte apoptosis through inhibiting autophagy, which aggravates the damage of articular cartilage and accelerates the progression of OA in vivo.

Abbreviations

Dex
Dexamethasone
DMM
Destabilized medial meniscus
OA
Osteoarthritis
OARSI
Osteoarthritis Research Society International
NICE
National Institute for Health and Care Excellence
ACR
American College of Rheumatology
AAOS
American Academy of Orthopedic Surgeons
SEM
Scanning electron microscope
EDS
Energy Dispersive Spectrometer
IHC
Immunohistochemical
MFC
Medial femoral condyle
MTP
Medial tibial plateau
BMD
Bone mineral density
CCL
Calcified cartilage layer
IL
Interleukin
MMP
Matrix metalloproteinase
AKT
Protein kinase B
p-AKT
Phosphorylated protein kinase B
DMSO
Dimethylsulfoxide
LC3
Microtubule-associated protein light chain 3

ANOVA

Analysis of variance

SD

Standard deviation

Declarations

Availability of data and materials

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

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Contributions

Study design: Lin Chen.

Experimental performance: Liang Chen, Zhenhong Ni, Jinfan Zhang, Junlan Huang, Yangli Xie, Bin Zhang, Liang Kuang, Xianding Sun, Dali Zhang, Nan Su, Huabing Qi, Qiaoyan Tan, Jing Yang.

Data analysis: Liang Chen, Min Jin, Fengtao Luo, Hangang Chen, Siru Zhou.

Data interpretation: Liang Chen, Xiaolan Du, Junjie Ouyang, Zuqiang Wang.

Drafting of the manuscript: Liang Chen and Zhenhong Ni.

Ethics declarations

Ethics approval and consent to participate

All animal experiments were performed according to protocols approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (Chongqing, China). The clinical specimen study was approved by the ethics committee of the Daping Hospital (Chongqing, China), and informed consent was obtained from each donor.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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Figures

Figure 1

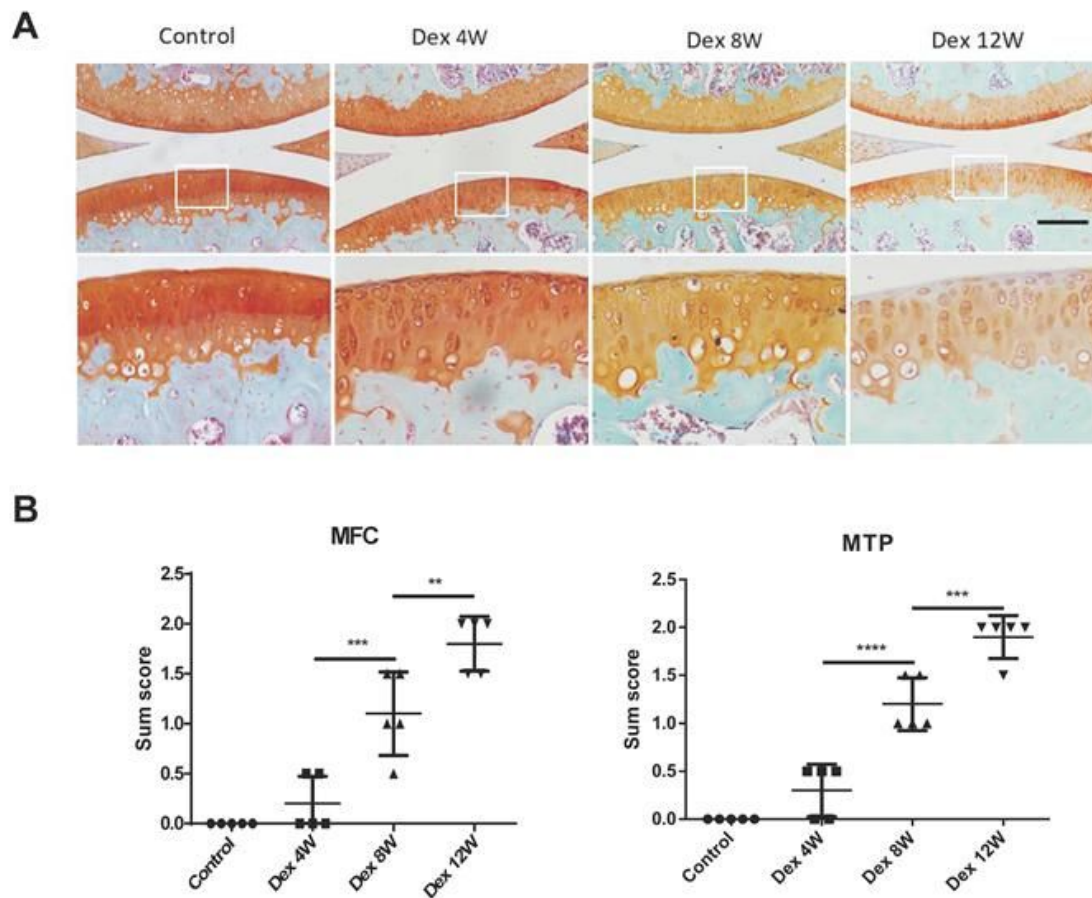


Figure 1

Histologic analysis of structural damage in the articular cartilage of continuously intraperitoneal Dex injection. (A) The articular cartilage was stained with Safranin O-fast green at 4 weeks, 8 weeks and 12 weeks after continuously intraperitoneal Dex injection to assess the extent of articular cartilage degeneration. (B) The OARSI scoring system showed that with the prolonged use of Dex, the degree of cartilage degeneration increased, mainly manifested by the loss of extracellular matrix and without structural changes (n=5 mice per group). Scale bar: 100 μ m. Data were expressed as the mean \pm 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

Figure 2

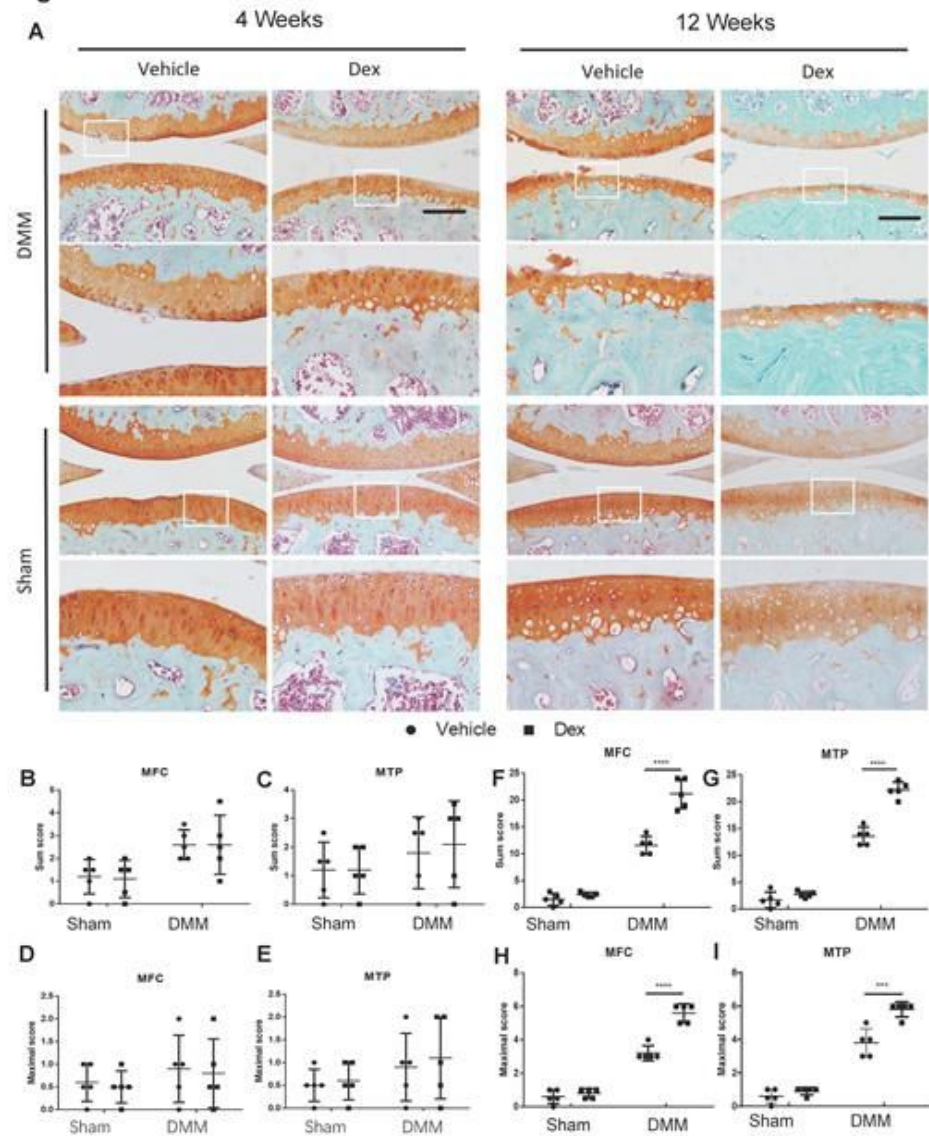


Figure 2

Histologic analysis of structural damage in the articular cartilage of continuously intraperitoneal Dex injection in DMM mice. (A) Vehicle and Dex groups were used to observe the degeneration of articular cartilage with Safranin o-fast green staining at 4 and 12 weeks after DMM and sham group, respectively. (B-E) Severity of articular cartilage damage in Vehicle and Dex groups at 4 weeks after sham operation or DMM surgery were evaluated using the Osteoarthritis Research Society International (OARSI) scoring system (n=5 mice per group). Total (sum) scores (B and C) and maximal scores (D and E) were calculated for the medial femoral condyle (MFC) (B and D) and medial tibial plateau (MTP) (C and E). (F-I) Severity of articular cartilage damage in Vehicle and Dex groups at 12 weeks after sham operation or DMM surgery were evaluated (n=5 mice per group). Total (sum) scores (F and G) and maximal scores (H and I) were calculated for the medial femoral condyle (MFC) (F and H) and medial tibial plateau (MTP) (G

and I). Scale bar: 100 μ m. Data were expressed as the mean \pm 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

Figure 3

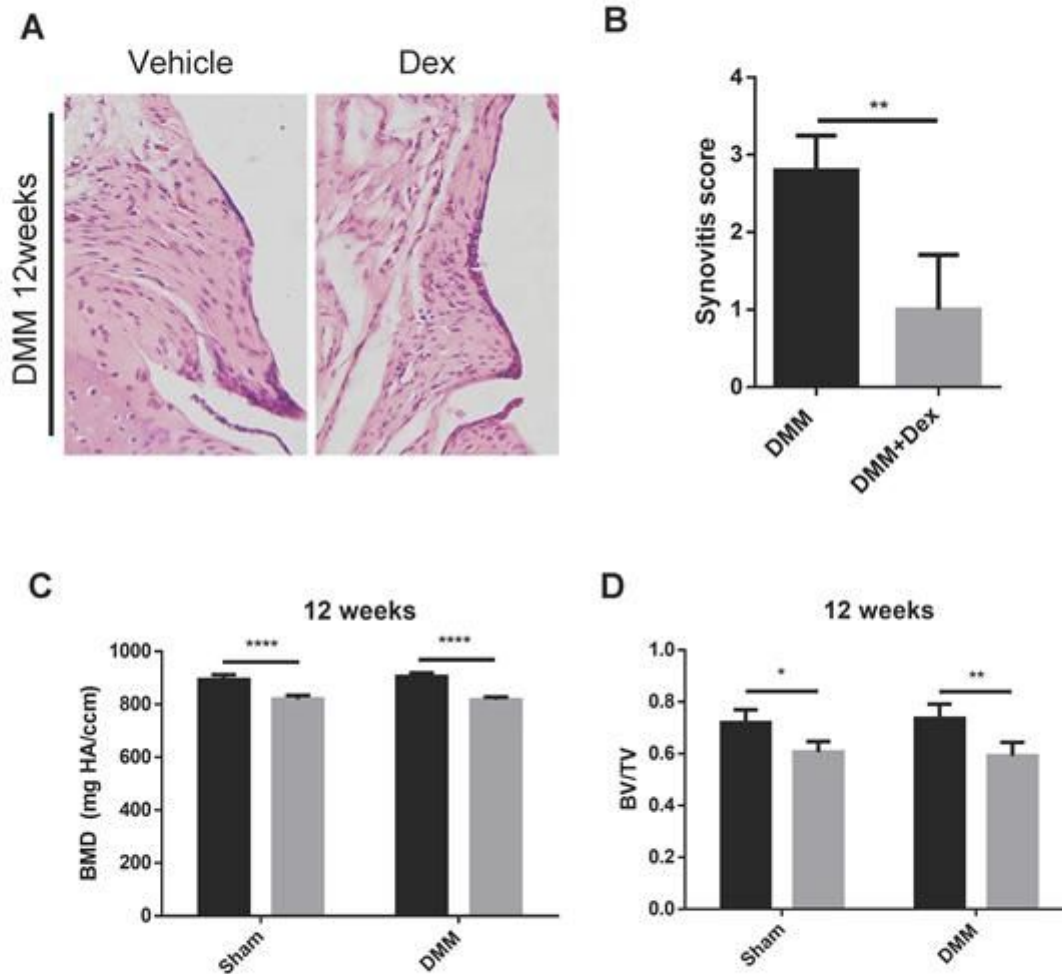


Figure 3

Continuously intraperitoneal injection of Dex promoted the loss of bone mass and relieved the synovitis. (A-B) H&E staining and scoring of synovitis showed that continuous Dex use can attenuate the severity of synovitis after DMM surgery compared with vehicle (n=5 mice per group). Scale bar: 100 μ m. (C-D) Evaluation of the effect of continuous Dex use on subchondral bone of DMM and sham group by μ CT three-dimensional reconstruction (n=4 mice per group). (C) Continuously Dex use resulted in a decrease in bone mass density (BMD) of subchondral bone in the DMM and sham groups. (D) Continued use of Dex resulted in a decrease in the ratio of subchondral bone volume (BV) to total volume (TV) in the DMM and

sham groups. Data were expressed as the mean \pm 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

Figure 4

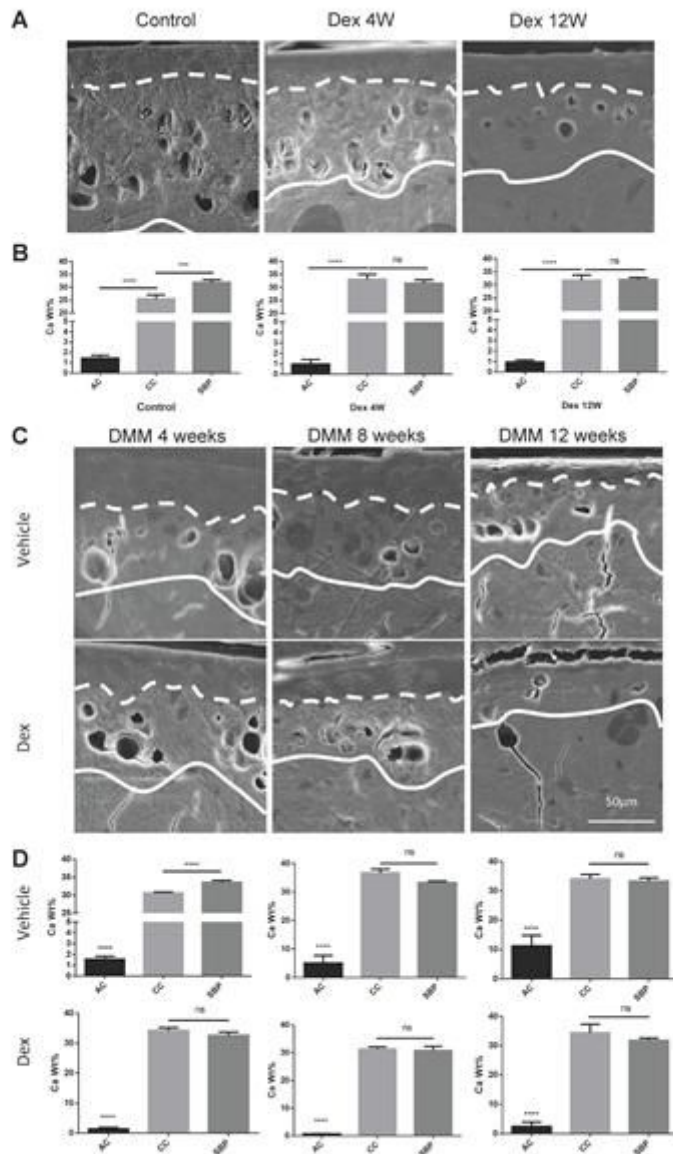


Figure 4

Dex significantly enhances cartilage calcium deposition during normal and DMM condition (A) Scanning electron microscopy of articular at 4 weeks and 12 weeks after continuously intraperitoneal Dex injection. (B) Calcium content in each layer of articular cartilage (non-calcified cartilage layer, calcified cartilage layer, subchondral bone plate) was determined by EDS analysis at 4 weeks and 12 weeks compared with control group after continuously intraperitoneal Dex injection (n=3 mice per group). (C) Scanning electron microscopy of articular cartilage layers at 4 weeks, 8 weeks and 12 weeks after DMM surgery with continuously intraperitoneal Dex or vehicle injection, respectively. (D) Calcium content in each layer of

articular cartilage (non-calcified cartilage layer, calcified cartilage layer, subchondral bone plate) was determined by Energy Dispersive Spectrometer (EDS) analysis at 4 weeks, 8 weeks and 12 weeks after DMM with continuous Dex or vehicle injection (n=3 mice per group). The dotted line marks the boundary between the noncalcified cartilage layer and the calcified cartilage layer, while the solid line marks the boundary between the calcified cartilage layer and the subchondral bone. Scale bar: 50µm. Data were expressed as the mean ± 95% confidence intervals. * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.

Figure 5

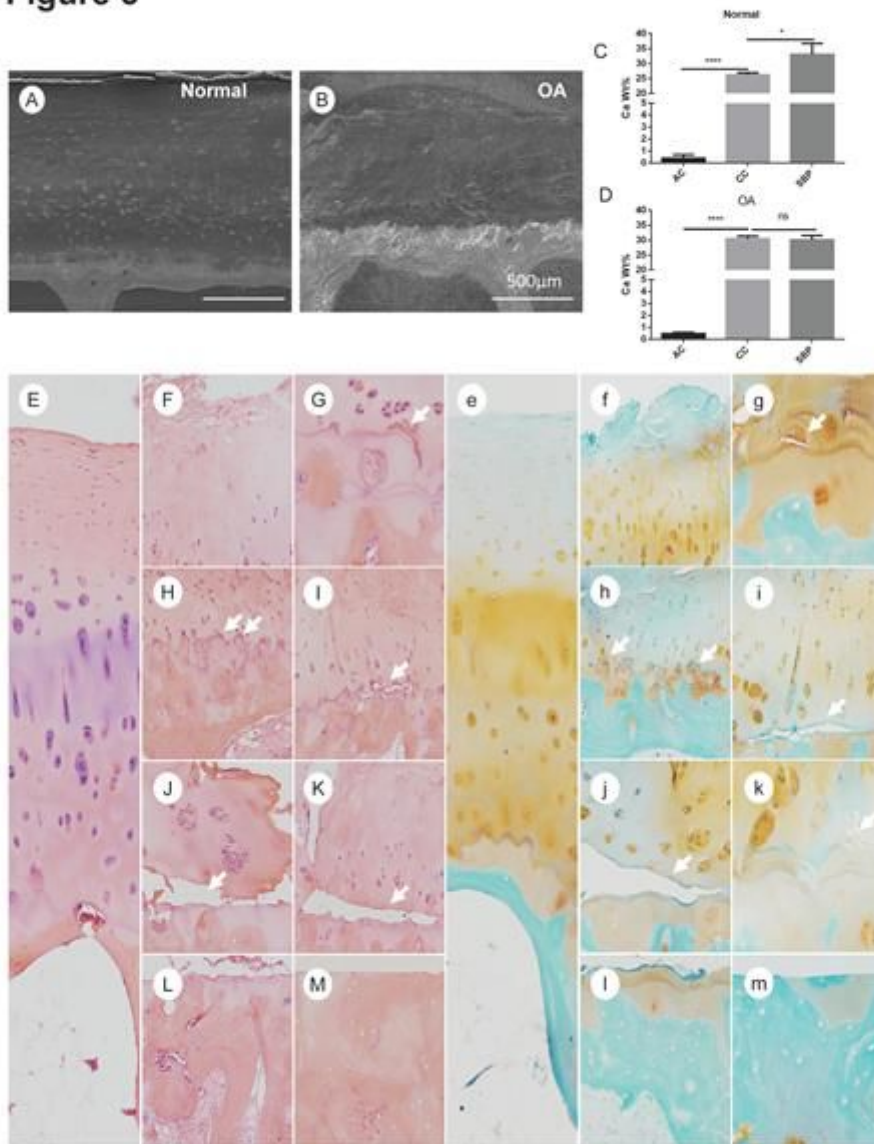


Figure 5

Histologic and SEM features of articular in OA patients with a history of repeated use of Dex. (A-B) SEM of articular in normal(A) and OA(B) patients. (C-D) Calcium content in each layer of articular cartilage (non-calcified cartilage layer, calcified cartilage layer, subchondral bone plate) was determined by EDS in

normal(C) and OA(D) patients (n=3 per group). The articular cartilage was stained with HE (E-M) and Safranin O-fast green(e-m) in normal(E and e) and OA(F-M and f-m) patients. Lots of horizontal clefts (arrowheads) can be found in sections of OA patients' samples. Scale bar: 500µm. Data were expressed as the mean ± 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

Figure 6

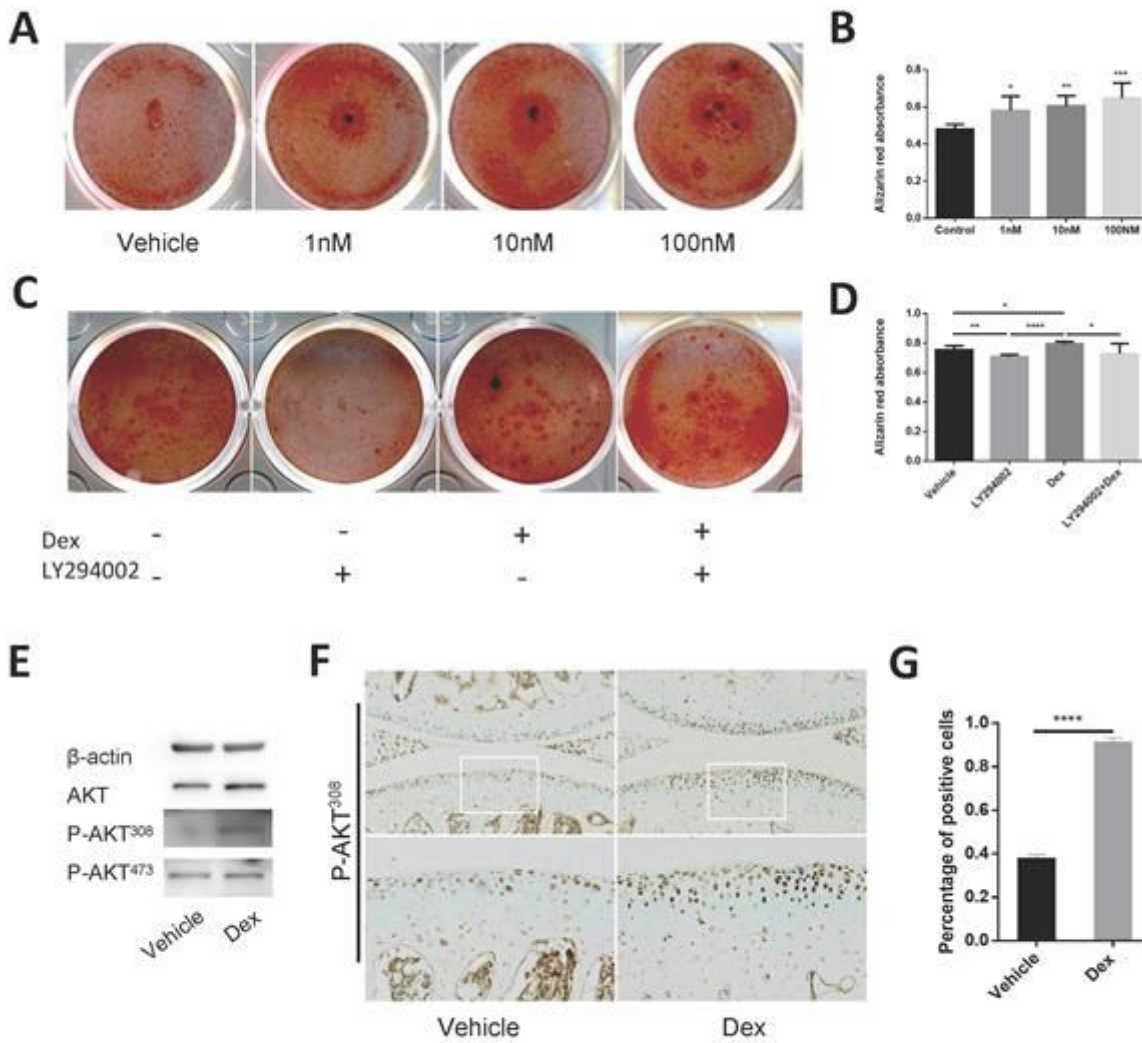


Figure 6

The calcification was obviously increased by Dex partially dependent on AKT (A and C) Alizarin red staining was used to observe the effect of different concentration gradient Dex(A) and AKT signaling inhibitor LY294002(C) on extracellular matrix calcification. (B and D) Alizarin red absorbance was detected by microplate reader at 405nm (n=3). (E) Cell lysates of primary chondrocytes were analyzed by western blotting using antibodies of AKT, P-AKT308, P-AKT473 (n=3). (F) Immunohistochemistry of P-

AKT308. (G) Statistical results of P-AKT308 positive cells in articular cartilage after continuously intraperitoneal Dex injection at 4 weeks compared with control group (n=4 per group). Data were expressed as the mean \pm 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

Figure 7

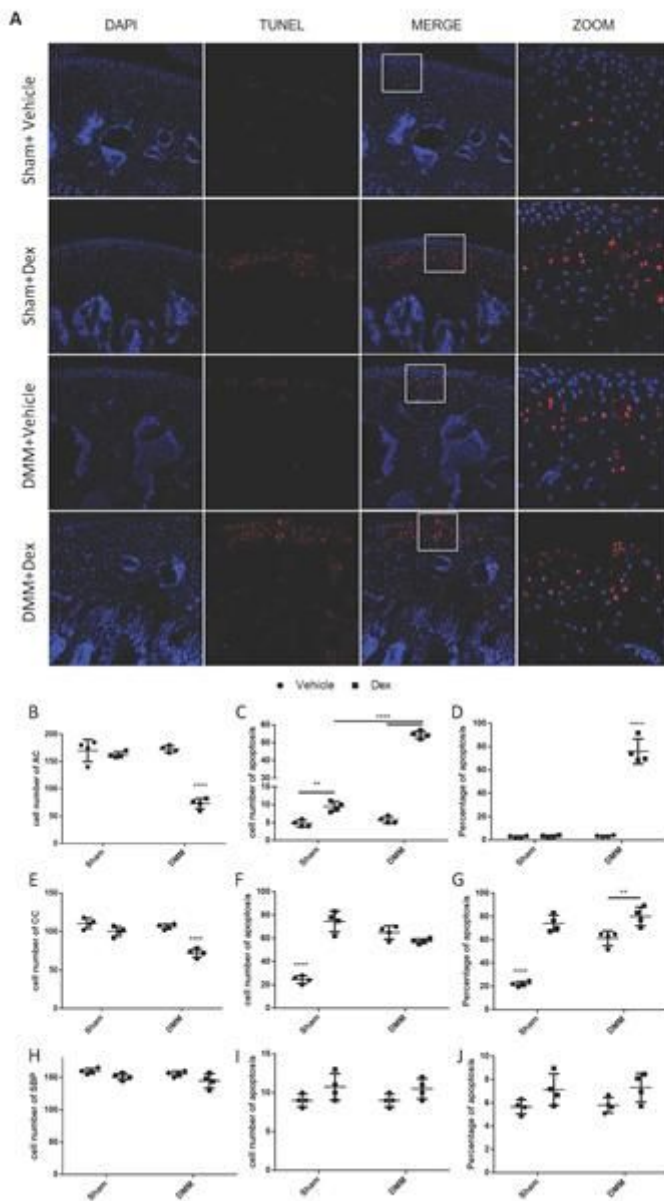


Figure 7

Effects of continuous Dex on apoptosis of articular chondrocytes (A) TUNEL was used to assess the effects of continuous Dex on apoptosis of articular chondrocytes after DMM. (B-J) The total number of cells (B, E, H), the number of apoptotic cells (C, F, I) and the proportion of apoptotic cells (D, G, J) in non-calcified cartilage layer (B-D), calcified cartilage layer (E-G) and subchondral bone plate (H-J) were statistically analyzed by cell count in each group (n=4 per group). Scale bar: 500 μ m. Data were expressed as the mean \pm 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

Figure 8

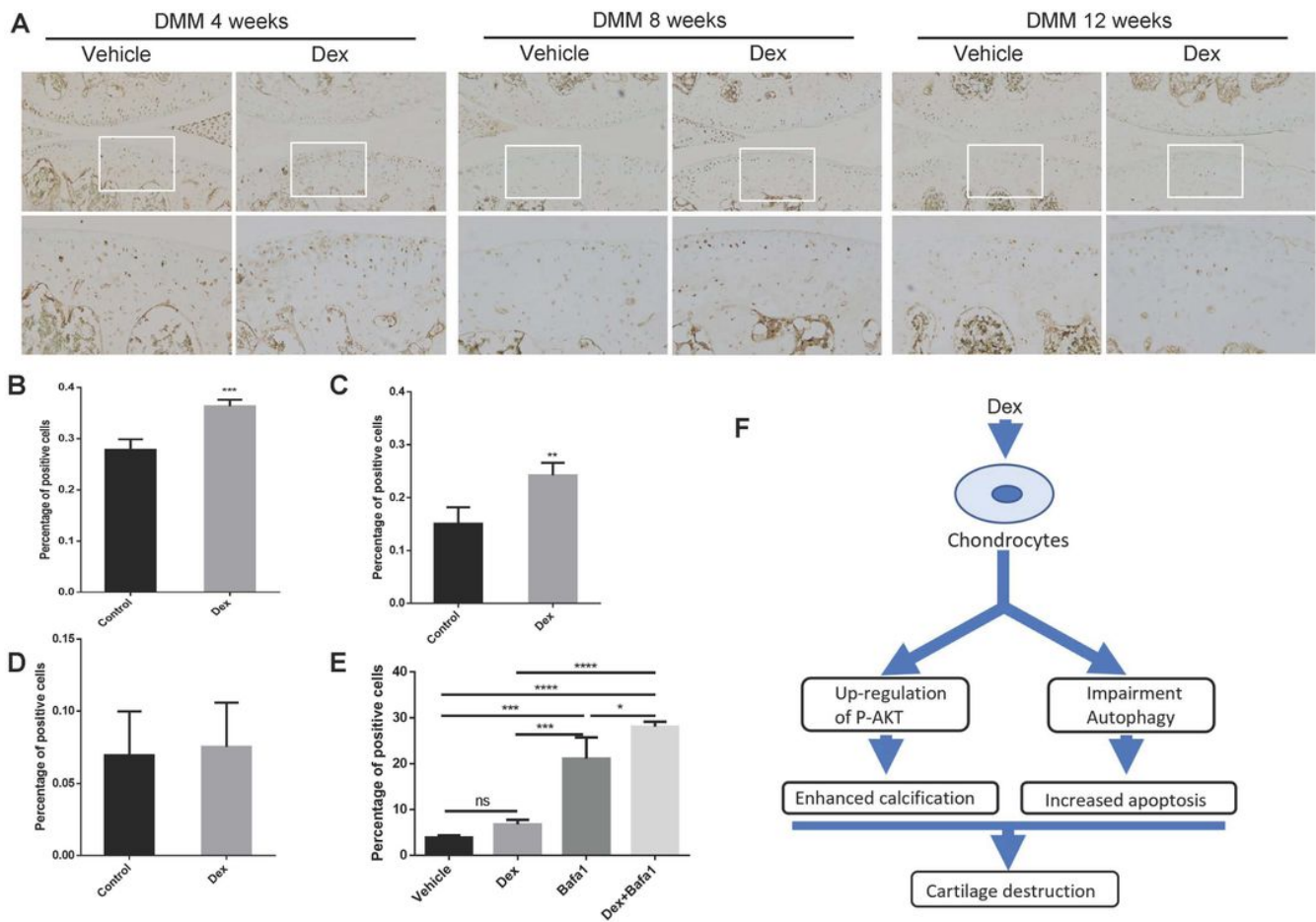


Figure 8

Effects of continuous Dex on LC3 expression in articular cartilage in DMM mice model (A) The effect of Dex on LC3 expression in articular cartilage of DMM mice was analyzed by immunohistochemistry. (B-D) Statistical results of LC3 positive cells in articular cartilage in vehicle and Dex group were obtained at 4 weeks(B), 8 weeks(C) and 12 weeks (D) after DMM surgery, respectively (n=4 per group). (E) Trypan blue staining was used to analyze the influence of autophagy inhibitors Bafa1 and Dex on the apoptosis rate of primary chondrocytes (n=3). (F) Working model. Dex aggravates the damage of cartilage via upregulating p-AKT and impairing articular autophagy in OA. Scale bar: 100µm. Data were expressed as the mean ± 95% confidence intervals. *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.