

Research Article

Ghrelin Inhibit the Synovial Cells Apoptosis and Aoutphagy Via ADORA2B/ PI3K/Akt/mTOR Axis

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Abstract

Objectives: To explore the regulatory role of Ghrelin in synovial cells from OA patient apoptosis and autophagy in association with ADORA2B and PI3K/Akt/mTOR signaling pathway.

Methods: Ghrelin was detected by elisa kit in OA patient. And the cell proliferation was determined by CCK-8. The protein expression such as LC3 II/I, Becline-1, Bax, Bcl-2, p-PI3K, p-Akt, p-mTOR, PI3K, Akt, mTOR, GAPDH was detected by western blot.

Results: In the OA patients, ghrelin was high expression in the serum. Ghrelin could promote the proliferation of the synovial cells, and inhibit the synovial cells apoptosis and aoutphagy. The LC3 II/I, Becline-1 and Bax was lower and the Bcl-2 was higher in the ghrelin group than the control group. And this processing was regulated by ADORA2B. And the ADORA2B could regulated the ghrelin inhibiting the synovial cells apoptosis and aoutphagy though PI3k/Akt/mTOR pathway.

Conclusion: Ghrelin could inhibit the synovial cells apoptosis and aoutphagy via ADORA2B/ PI3K/Akt/mTOR axis.

Keywords: Apoptosis, Aoutphagy, ADORA2B, Ghrelin, Osteoarthritis

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Osteoarthritis (OA) is a chronic pain disease with whole arthropathies such as synovitis, osteophyte formation et al.^[1-4] OA give a big social burden on public health.^[5, 6] In worldwide, 303 million adults were affected. OA has high prevalence, however there is no drug to treat it.^[7-9] Present drug only can relieve the OA symptoms, long-term using is given more side effects and toxicities.^[10-12]

As an inflammation, OA, especially synovitis, can be regulated through the extracellular adenosine.^[7, 10, 13, 14] Adenosine receptors (Adora) (Adora1, Adora2a, Adora2b, Adora3) can show adenosine function. Adora2b can realize the pain behavior.^[15-18] ADORA2B plays a major role

in chronic pain by promoting immune-neuronal interaction.^[19, 20]

Ghrelin a gut-derived peptide hormone, first isolated from the stomach, is capable of regulating osteoblast differentiation and function as well as bone structure.^[21-23] Ghrelin is one of the endogenous ligands of growth hormone secretagogue receptor, which can promote secretion of growth hormone and is proven to be with the orexigenic and adipogenic effects.^[24-28] we tried to explore the regulatory role of ghrelin in synovial cells from OA patient apoptosis and autophagy in association with ADORA2B and PI3K/Akt/mTOR signaling pathway.

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Methods

Unless stated otherwise, Sigma Chemical Company (Missouri, USA) was the source for all reagents and chemicals used in the study. Cell Signaling Technology (USA) was used as the source for the antibodies to LC3II/I, Beclin-1, Bax, Bcl-2, p-PI3K, p-Akt, p-mTOR, PI3K, Akt, mTOR, GAPDH.

Patient Sample Collection

For this study, 100 samples of OA patients serum and 100 samples of normal serum were collected from patients who permitted us to use their tissues via written informed consent. Additionally, we and the patients followed and were aware of institutional protocols approved by the Institutional Ethical Review Board of Inner Mongolia Medical University (NO. YDK2020021025).

Ghrelin Assay

The ghrelin in the serum was tested by using human ghrelin Elisa kits. The experiments were carried out in triplicate.

Cell Culture

Synovial cells from OA patients took place at 37°C, 5% CO₂, and in humidified air. These are considered standard conditions.

Cell Proliferation

We used a CCK-8 (Dojindo Laboratories, Kumamoto, Japan) to evaluate cell proliferation and viability. Absorbance at 450 nm was measured using Bio-Tek Instruments (Vermont, USA). The following equation was used to calculate cell growth rate: % growth rate = (mean experimental absorbance/mean control absorbance) × 100. Each test was conducted five times.

Levels of miRNA Determined Using qRT-PCR

Tiagen reagent was used to isolate total RNA, which served as a guide for complementary DNA (cDNA) synthesis conducted with a universal reverse transcription kit (M-MLV) and reverse transcriptase. RNA quantification was performed with a SYBR-Green PrimeScript miRNA RT-PCR kit (Takara, Japan). We assessed RNA expression using the 2-ΔΔCt method.^[29, 30]

Western Blotting

To obtain protein, we used RIPA lysis buffer (Beyotime, China), and a BCA kit was used to determine the protein content (Beyotime, Haimen, China). We isolated protein samples using 10% SDS-PAGE. Then, the results were transferred to a PVDF membrane, followed by 5% nonfat milk blocking for 1h at 37°C. After blocking, we added primary antibodies (incubated overnight at 4°C). Then,

we washed the membranes. Next, secondary antibodies (species-compatible, peroxidase-conjugated) were added. Membrane-bound antibodies were detected with ECL (Pierce, Illinois, USA).^[30] Protein quantification was performed using Clinx Chemi Analysis software (ChemiScope 6000, Shanghai, China). Experiments were performed in triplicate.

Cell Transfection

ShRNA of ADORA2B were sourced from GenePharma (Shanghai, China). According to standard protocols, plasmids or oligonucleotides were transfected into cells using Lipofectamine 2000.

Statistical Analysis

GraphPad Prism version 5 software (GraphPad Software, CA, USA) was employed to make comparisons between groups. Differences in levels of gene expression were determined using one-way analysis of variance (ANOVA) with the Newman-Keuls posthoc test. We included replicates in the statistical model. A 95% confidence level ($p < 0.05$) was accepted as statistically significant. All data are presented as mean ± SD.

Results

Ghrelin Low Expression in OA Patients and Promote the Proliferation of Synovial Cells from OA Patients

To explore the content of ghrelin in the OA patient serum, the OA patient blood and normal people blood was obtained. The ghrelin in the OA patient serum was lower than the normal person (Fig. 1a, $p < 0.05$). And to show the optimal concentration effecting on the synovial cells, the different concentration ghrelin effected on the synovial cells from OA patients. And the cells proliferation at 12h, 24h and 36h was obtained. The results showed that the optimal ghrelin concentration was 600ng/ml at 36h (Fig. 1B).

Ghrelin Promote ADORA2B Expression and Inhibit the Synovial Cell Apoptosis and Autophagy Via PI3k/akt/mTOR Pathway

ADORA2B expression in mRNA and protein level was detected by qPCR and western blot. The ADORA2B expression in mRNA and protein level in synovial cells was higher in the ghrelin group (Fig. 2a and b). The effects on the synovial cell apoptosis and autophagy by ghrelin also detected. The results showed that the LC3II/I, Beclin-1 and Bax was lower in the ghrelin group than the control group (Fig. 2c). However, the Bcl-2 in the ghrelin group was higher than the control group (Fig. 2c). To show the changes of PI3k/akt/

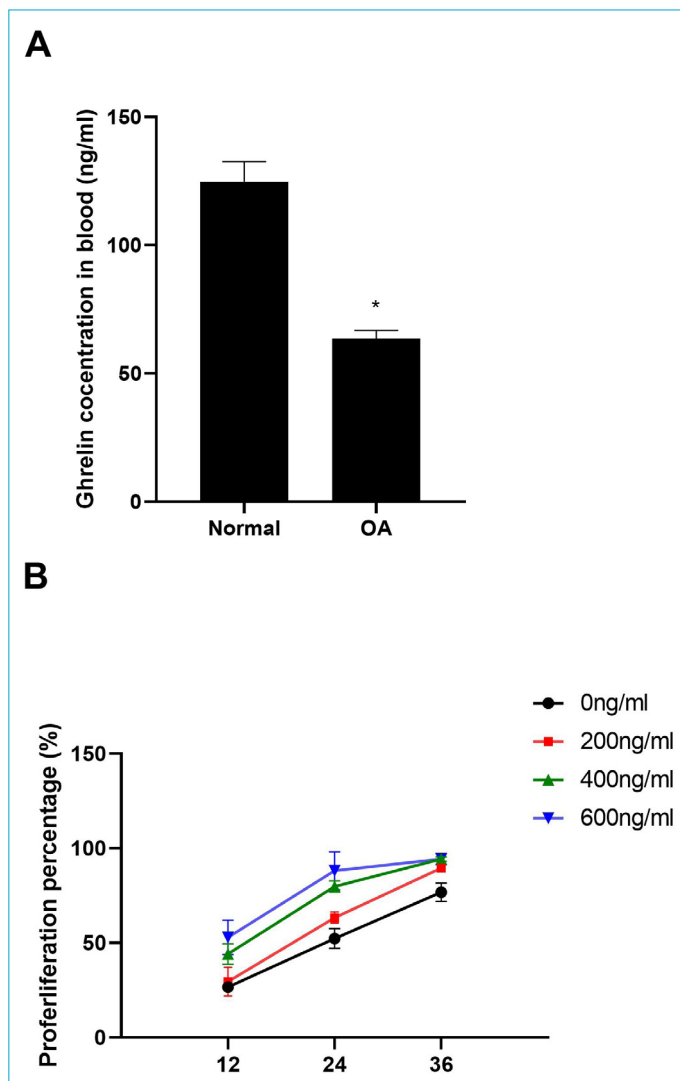


Figure 1. Ghrelin concentration in blood of OA patients and Synovial cells from patient proliferation effected by ghrelin. A, Ghrelin concentration in blood of OA patients. B, Synovial cells from patient proliferation effected by ghrelin. Comparison of data between groups at different time points was performed using two-way ANOVA. *, $p < 0.05$. All experiments were repeated three times.

mTOR pathway effected by grhelin, the phosphorylation of the PI3K, Akt and mTOR was explored. The phosphorylation of the PI3K, Akt and mTOR was high in the ghrelin group (Fig. 2d).

The Influence of Ghrelin on Synovial Cells After ADORA2B Silenced

To show the effect of ADORA2B on the Synovial cells, the ADORA2B was silenced. And when the ADORA2B was silenced by siRNA, the expression of ADORA2B in mRNA and protein level was lower with or without ghrelin added (Fig. 3a and b). Meanwhile, the LC3II/I, Becline-1 and Bax was higher in the siRNA group than the without siRNA group.

The Bcl-2 was lower in the siRNA group (Fig. 3c). The phosphorylation of the PI3K, Akt and mTOR was lower in the siRNA group (Fig. 3d). And the cell viability was lower in the siRNA group (Fig. 3e).

The grhelin effect on the Synovial cells after ADORA2B silenced was also showed. The LC3II/I, Becline-1 and Bax was higher in the siRNA+ghrelin group than the ghrelin group. The Bcl-2 was lower in the siRNA+ghrelin group (Fig. 3c). The phosphorylation of the PI3K, Akt and mTOR was lower in the siRNA+ghrelin group than the ghrelin group (Fig. 3d). And the cell viability was lower in the siRNA+ghrelin group (Fig. 3e).

The Influence of Ghrelin on Synovial Cells After mTOR Silenced by Rapamycin

To explore the function of the PI3k/akt/mTOR pathway on the Synovial cells after ghrelin added, the mTOR was inhibited by rapamycin. When the rapamycin added, the expression of ADORA2B in mRNA and protein level was no changed. And when also added ghrelin, the expression of ADORA2B in mRNA and protein level was also no changes. However, the expression of ADORA2B in mRNA and protein level was higher in ghrelin added groups (Fig. 4a and b). The LC3II/I, Becline-1 and Bax was higher in the rapamycin group than the control group. The Bcl-2 was lower in the rapamycin group. And when grelin added, this changes was more (Fig. 4c). The phosphorylation of the PI3K and Akt was no changes with or without the rapamycin. And when ghrelin added, the phosphorylation of the PI3K and Akt was no changes, but was higher than the without ghrelin groups (Fig. 3d). And the cell viability was lower in the rapamycin group than the control group. And the cell viability was also lower in the rapamycin group than the control group with ghrelin added (Fig. 3e).

Discussion

OA is a chronic disease. Osteoarthritis is showed the articular cartilage degeneration and persistent pain, causing disability, loss of function, decreased quality of life.^[9, 31, 32] Synovia inflammation in joint occurs in disease progression.^[33] Hence, synovial cells from the OA patients are always the research model in vitro. In this study, the synovial cells from the OA patients were used to show the ghrelin function on the synovial cells apoptosis and aoutphagy. In the OA patients, ghrelin was high expression in the serum. To show the ghrelin function on the synovial cells, we used ghrelin added in the synovial cells from the OA patients. And we found ghrelin could promote the proliferation of the synovial cells. Ghrelin, a gut-derived peptide hormone, first isolated from the stomach,^[34-36] is capable of regulat-

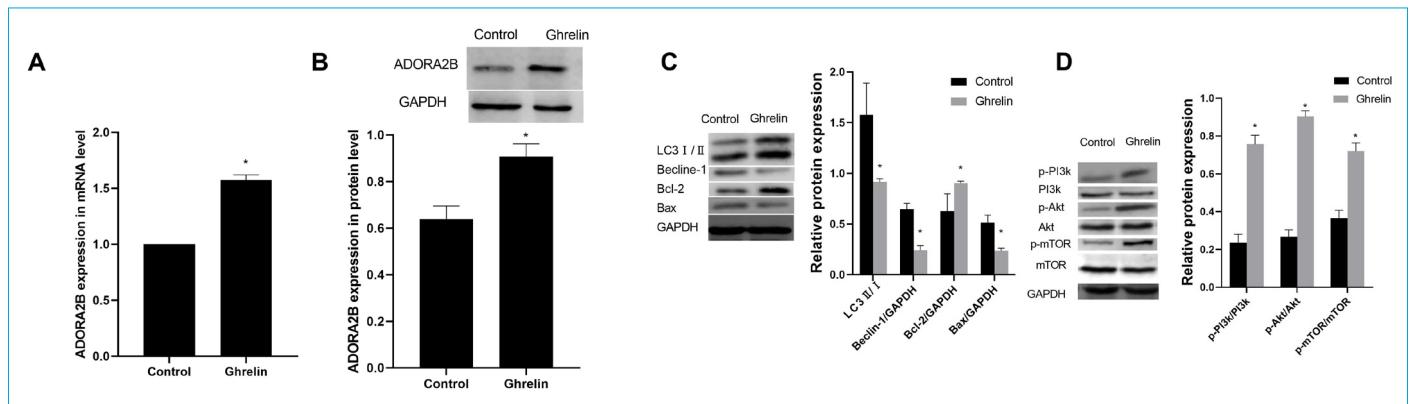


Figure 2. Ghrelin decelerate Synovial cells autophagy and apoptosis via ADORA2B and PI3K/Akt/mTOR pathway. A, ADORA2B expression in mRNA level effected by ghrelin. B, ADORA2B expression in protein level effected by ghrelin. C, The protein expression of becline-1, LC3I/II, Bcl-2 and Bax. D, The protein expression of p-PI3K, PI3K, p-Akt, Akt, p-mTOR and mTOR. *, $p < 0.05$.

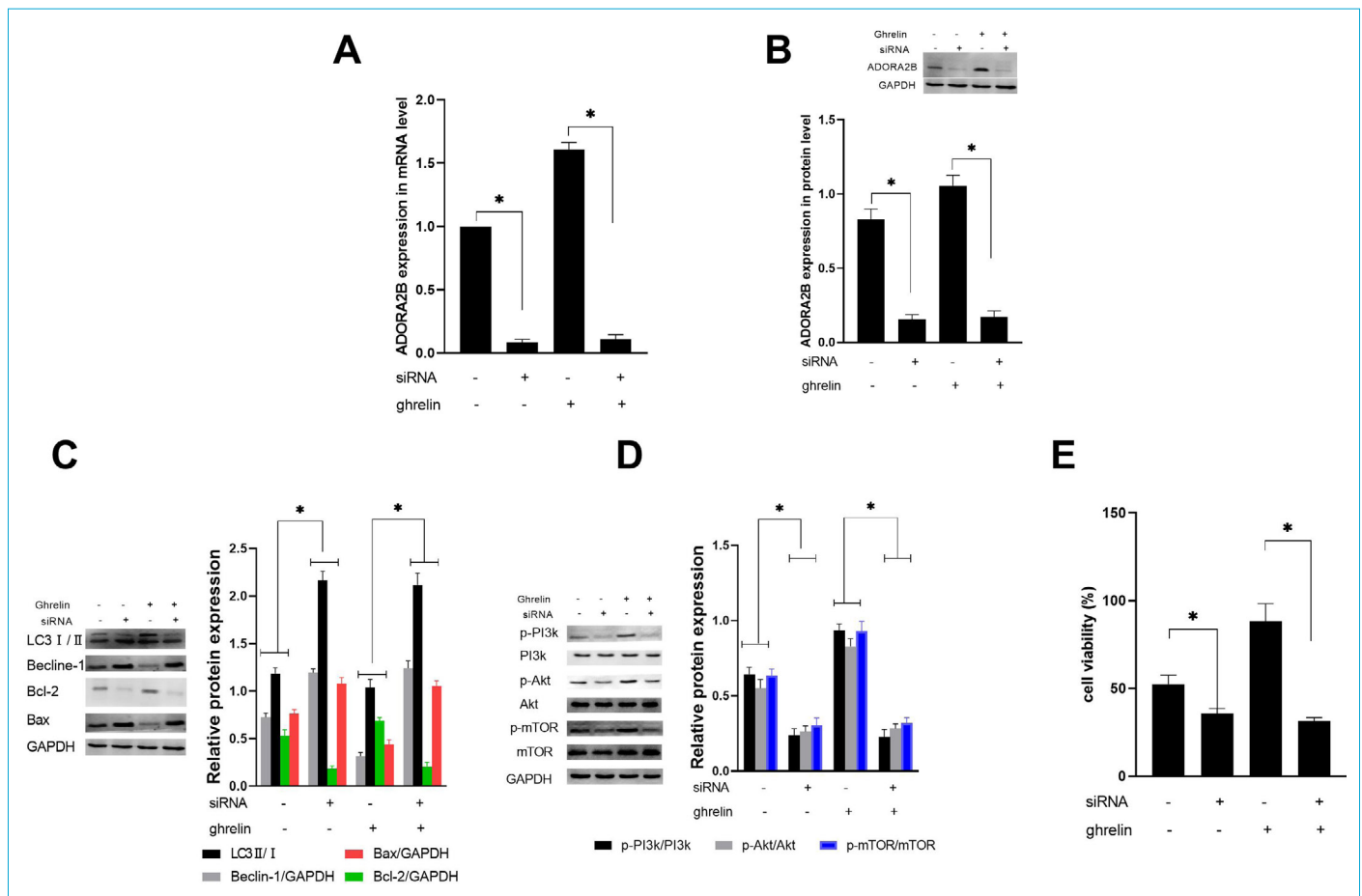


Figure 3. The influence of ghrelin on Synovial cells after ADORA2B silenced. A, ADORA2B expression in mRNA level effected by ghrelin. B, ADORA2B expression in protein level effected by ghrelin. C, The protein expression of becline-1, LC3I/II, Bcl-2 and Bax. D, The protein expression of p-PI3K, PI3K, p-Akt, Akt, p-mTOR and mTOR. E, Cell viability effected by ghrelin. *, $p < 0.05$.

ing osteoblast differentiation and function as well as bone structure.^[37-39] Hence, ghrelin was important on the joint.

In this study, ghrelin could inhibit the synovial cells apoptosis and autophagy. The LC3I/II, Beclin-1 and Bax was lower and the Bcl-2 was higher in the ghrelin group than

the control group. And this processing was regulated by ADORA2B. ADORA2B was the adenosine receptors which could show the antithrombotic and antiinflammatory effects.^[40-42] Adora2a is expressed on immune cells, and has been shown to dampen harmful inflammation.^[40, 41, 43] In

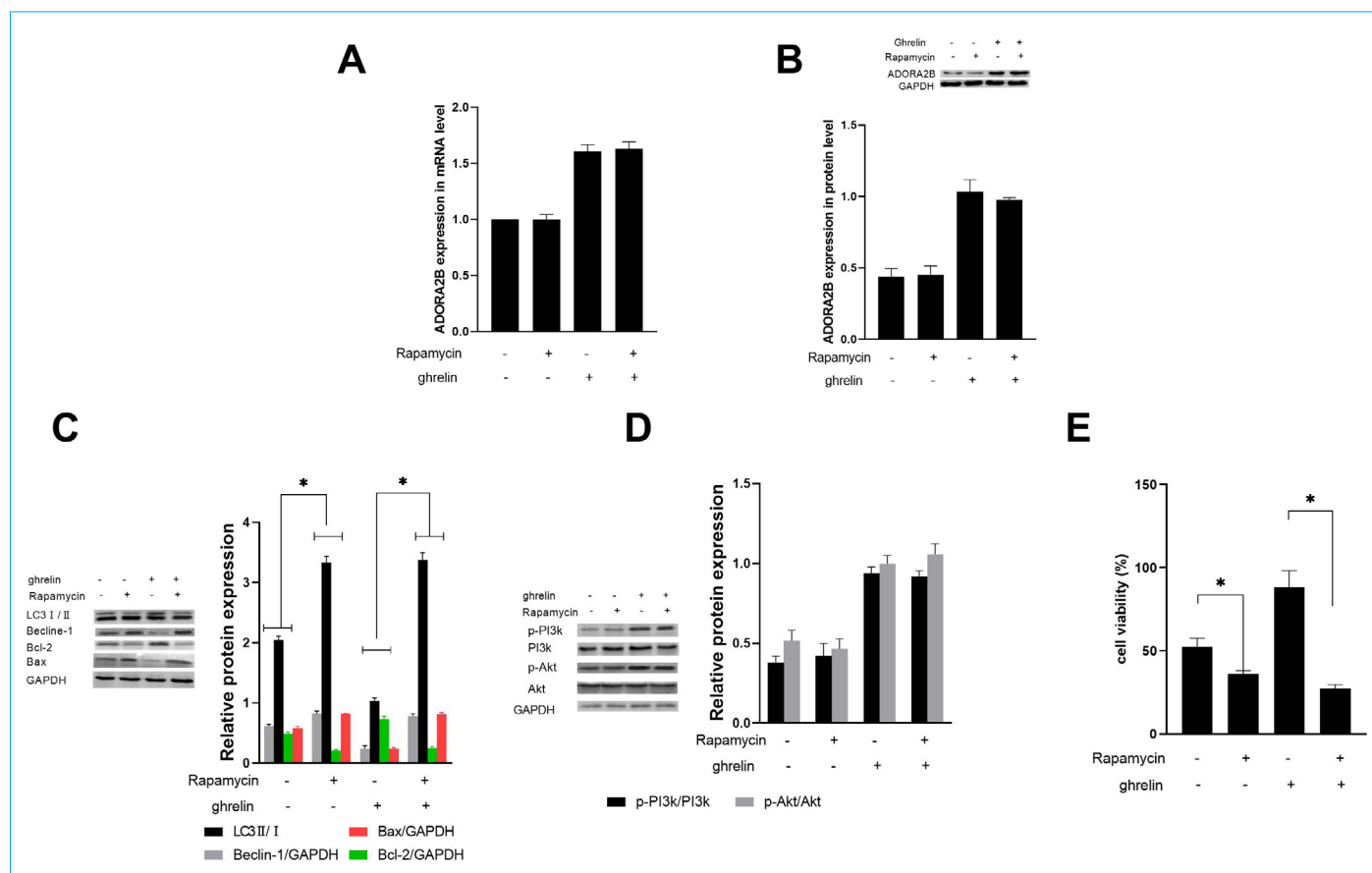


Figure 4. The influence of ghrelin on Synovial cells after mTOR silenced by rapamycin. A, ADORA2B expression in mRNA level effected by ghrelin. B, ADORA2B expression in protein level effected by ghrelin. C, The protein expression of becline-1, LC3I/II, Bcl-2 and Bax. D, The protein expression of p-PI3K, PI3K, p-Akt and Ak. E, Cell viability effected by ghrelin. *, $p < 0.05$.

this study, the ghrelin could regulated the synovial cells apoptosis and aoutphagy via ADORA2B. And the ADORA2B could regulated the ghrelin inhibiting the synovial cells apoptosis and aoutphagy though PI3k/Akt/mTOR pathway. PI3K/Akt/mTOR pathway is a key intracellular signal transduction pathway that is associated with cell proliferation, apoptosis, autophagy.^[44-46] Rapamycin was the first known mTOR inhibitor and was originally isolated from *Streptomyces hygroscopicus*.^[47] In this study, the mTOR was inhibited by rapamycin. And the ghrelin regulation on the synovial cells apoptosis and aoutphagy was disappeared. And cell proliferation was also no difference.

Conclusionly, Ghrelin could inhibit the synovial cells apoptosis and aoutphagy via ADORA2B/ PI3K/Akt/mTOR axis.

Disclosures

Ethics Committee Approval: All patients provided informed consent during this study, which was conducted with the approval of the Institutional Ethical Review Board of Inner Mongolia Medical University (NO. YDK2020021025).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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Availability of Data and Materials: All data generated or analyzes during this study are included in this published article.

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