

Glucocorticoid induced autophagy in N1511 chondrocyte cells

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Abstract. – OBJECTIVE: To determine whether autophagy was involved in chondrocyte cells post Glucocorticoids (GCs) treatment.

MATERIALS AND METHODS: LC3-GFP reporter plasmid transfection and western blotting analysis were conducted to determine the autophagic vesicles and autophagy-associated molecules in the N1511 chondrocyte cells post dexamethasone (Dex) treatment. And the N1511 cell viability was also determined by MTT assay.

RESULTS: We found that autophagy was induced in the N1511 chondrocyte cells post treatment with Dex of 5 μ M to 1 mM, and the autophagy-induction by Dex could be inhibited by 3 MA and RU486, a GC antagonist. And the autophagy induced by the high dose of Dex (200 μ M or 1 mM) was associated with a reduction of N1511 cell viability.

CONCLUSIONS: These results suggested that GCs could induce autophagy, as might contribute to the viability reduction of chondrocyte cells.

Key Words:

N1511 cells, Autophagy, Cell viability.

growth plate responsible for longitudinal bone growth⁶. And the decreased growth is accompanied by morphological changes in the growth plate: it becomes thinner^{3,7}, which has been attributed to decreased proliferation of the chondrocytes^{7,8}. Detailed researches have revealed that decreased proliferation⁷⁻⁹ and increased apoptosis^{10,11} of epiphyseal chondrocytes contributing to the thinner cartilage. But the molecular mechanism of GCs-induced growth plate impairment was unclear.

Autophagy is essential for cell growth, survival, differentiation, development, and homeostasis¹². It helps to maintain a balance among the synthesis, degradation, and subsequent recycling of cellular products. But it is uncertain whether autophagy is involved in chondrocytes post GC treatment.

In this study, we determined whether GC treatment induced the autophagy in chondrocytes, and then evaluated the inhibitors against the GC-induced autophagy. Our observations offer novel findings that autophagy is an important mechanism of GC-induced cell chondrocytes impairment.

Introduction

Glucocorticoid (GCs) is widely used for the local and systemic treatment in orthopedic diseases. The former is mainly subject to chronic sports injury, lumbar disc herniation and ankylosing spondylitis; the latter is mainly used for acute spinal cord injury, to reduce edema and secondary lipid peroxidation, to avoid further damage to the spinal cord. Prolonged use of GCs always results in metabolic disorders, reduction of bone mineral density (BMD) and other various side-effects¹⁻³. Administration of GCs leads to decreased generation of osteoblasts and osteocytes, accompanied by a prolonged lifespan of osteoclasts¹. Permanent growth impairment has also been noted in children receiving alternate-day GC treatment^{4,5}. It is well known that it's the

Materials and Methods

Reagents and Antibodies

Dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO, USA) was resolved in alpha-MEM (minimum essential medium eagle) with 0.5% ethanol, RU486 (Sigma-Aldrich, St. Louis, MO, USA) and rapamycin (Sigma-Aldrich, St. Louis, MO, USA) was resolved in dimethyl sulfoxide (DMSO), 3-methyladenine (3-MA) and polyclonal antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and LC3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Beclin 1 polyclonal antibody was purchased from Santa Cruz Biotechnology (San-

ta Cruz, CA, USA). The MAP1-LC3 (Major Antigenic Protein 1- Light Chain 3) fusion with Green Fluorescent Protein (GFP) was conducted by our lab.

Cell Culture

The N1511 mouse chondrocytes was provided by the Cell Resource Center of Chinese Academy of Medical Sciences. The cells were cultured in alpha-MEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 0.2% L-glutamine, 0.2% penicillin/streptomycin. All cells were incubated at 37°C, 5% CO₂.

Quantitative GFP-LC3 Analysis and Electron Microscopy

To determine the autophagic vacuoles specific for autophagy, quantitative GFP-LC3 light assays were performed in N1511 cells with various treatments. N1511 Cells grown to 80% confluency were transfected with a GFP-LC3-expressing plasmid. 24 h post transfection, cells were treated with rapamycin (200 nM), Dexamethasone (Dex) (5 µM), Dex (40 µM), Dex (200 µM), 3MA (100 µM) or RU486 (50 µM) for another 24 h and analyzed by fluorescence microscopy.

Protein Isolation and Western Blot Analysis

Whole cell extracts were prepared by a standard protocol, and proteins were detected by western blot analysis using polyclonal (mouse) anti-Beclin 1 antibody, polyclonal (mouse) anti-Atg 5 antibody, or polyclonal (rabbit) anti-LC3 or GAPDH antibody (Sigma-Aldrich). Goat anti-mouse IgG or goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (Super Signal West Femto; Pierce, Rockford, IL, USA) were used for detection.

Cell Viability Assay

Cell viability was determined by MTT assay. N1511 cells were seeded in 96-well plates, and after 24 h, the medium was changed with alpha-MEM medium containing 1% FBS; Dex (0 µM, 5 µM, 40 µM, 200 µM or 1 mM), 3 MA (0 µM, 100 µM, 200 µM or 400 µM) or (and) RU486 (0 µM, 10 µM, 50 µM or 300 µM) was added into the medium and continued incubation for up to 0h, 12h, 24 h or 48 h. Then, the incubation medium in the test wells was replaced with 50 µl 1x MTT solution, and the cells were incubated for 2 h at 37°C. After incubation, the MTT solution

was discarded, and 150 µl DMSO was added to dissolve the precipitate completely at room temperature. The optical density was then measured at 570 nm using a spectrophotometer.

Statistical Analysis

For LC3 dot number analysis, MTT measurements, statistical evaluations are presented as mean ± SE. Data were analyzed using the Student's *t* test. *p* < 0.05 was considered statistically significant.

Results

Dex Induces Autophagy and Autophagy-Associated Molecules Expression in N1511 cells

To observe the characterized acidic vesicular organelles (AVOs)¹³ of autophagy, GFP-LC3 report vector was utilized to report the acidic vesicular organelles with a fluorescence microscope. Untreated N1511 cells showed limited AVOs in the cytoplasm. The rapamycin-treated (200 nM) cells and cells post Dex treatment of 5 µM, 40 µM or 200 µM demonstrated a significant increase in AVOs (Figure 1). In addition, it has been reported that converse of LC3-II to LC3-I during autophagy. To detect the expression of LC3-II, we performed western blotting with lysates from N1511 cells subject to rapamycin or Dex (0 µM, 5 µM, 40 µM, 200 µM) (Figure 3A). The expression of LC3-II/LC3-I ratio increased in N1511 cells treated with rapamycin or Dex. The autophagy gene beclin 1 is part of a type III PI3 kinase complex that is required for the formation of the autophagic vesicles¹⁴ in autophagy. Figure 3B showed that the expression levels of Beclin 1 and Atg 7 in protein levels increased post Dex treatment. These results indicate that the Dex treatment induces autophagy in N1511 cells.

Dex-induces Autophagy-Related Molecules Expression in N1511 Cells Could be Inhibited by Glucocorticoid Antagonist RU486

The glucocorticoid antagonist RU486 with various concentrations was utilized to re-confirm the stimulatory effect of Dex on autophagy, RU486 was pre-subject to the N1511 cells following Dex treatment. And it was shown that pretreatment with RU486 completely reversed the stimulatory effect of Dex on autophagy: The

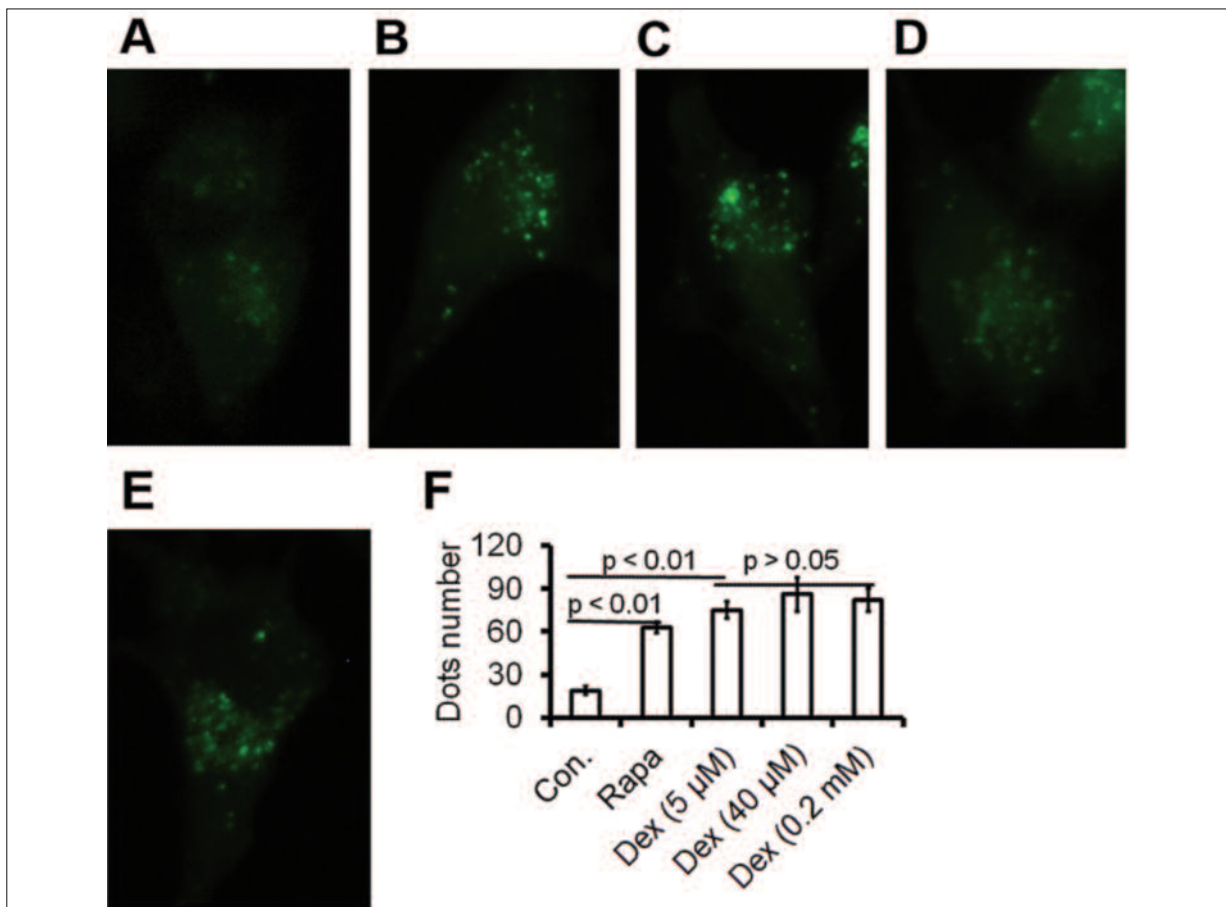


Figure 1. Autophagic vesicles in N1511 cells post Dex treatment. N1511 cells were transfected with a GFP-LC3 reporter vector and incubated at 37°C for 24 h; then, cells were incubated for another 24 h at 37°C in alpha-MEM containing 2% FBS with 1/10,000 DMSO (Con., **A**), 200 nM rapamycin (Rapa, **B**), or Dex (5 μM, 40 μM or 200 μM, **C** to **E**). Following fixation, the number of punctate GFP-LC3 dots in each cell was counted by fluorescence microscopy, and at least 100 cells were included for each group. The data were normally distributed and were statistically analyzed using the Student-Newman-Keuls test.

GFP-LC3 reporter vector revealed a decreased microphotographs of AVOs in cells, treated with 3 MA or RU486 (Figure 2). The LC3-II/LC3-I ratio (Figure 3C) and the expression levels of Beclin 1 and Atg 5 in protein levels decreased (Figure 3D). These results re-confirmed the stimulatory effect of Dex on autophagy in N1511 cells.

N1511 Cells Decreased in Viability Because of Dex-induces Autophagy

To further determine the influence of Dex-induced autophagy on the cell viability, the MTT assay was conducted. Firstly, because the Dex utilized in this study was dissolved in 0.5% ethanol, the influence on cell viability of ethanol was evaluated by MTT assay, Figure 4A showed that there was no influence of ethanol, less than 5%, on the cell viability. Next, the N1511 cell vi-

ability post treating with various concentration of Dex was assayed, and it was shown that there was no significance in the viability of N1511 cells post treating with Dex of 1 μM or 40 μM, for 24-48 h, while the cells treated with 200 μM or 1mM Dex decreased significantly in viability (Figure 4B). Furthermore, to evaluate the autophagy on the viability reduction by Dex, the autophagy inhibitor, 3 MA was utilized to discriminate the cell viability with or without autophagy blockage, Figure 4C demonstrated that there was no significant difference in the N1511 cell viability among groups with various 3 MA treatments; but the cell viability reduction induced by Dex of 200 μM or Dex of 1 mM was ameliorated by 3 MA treatment of 100 μM to 400 μM. These results suggest that the viability reduction by Dex was caused by the autophagy.

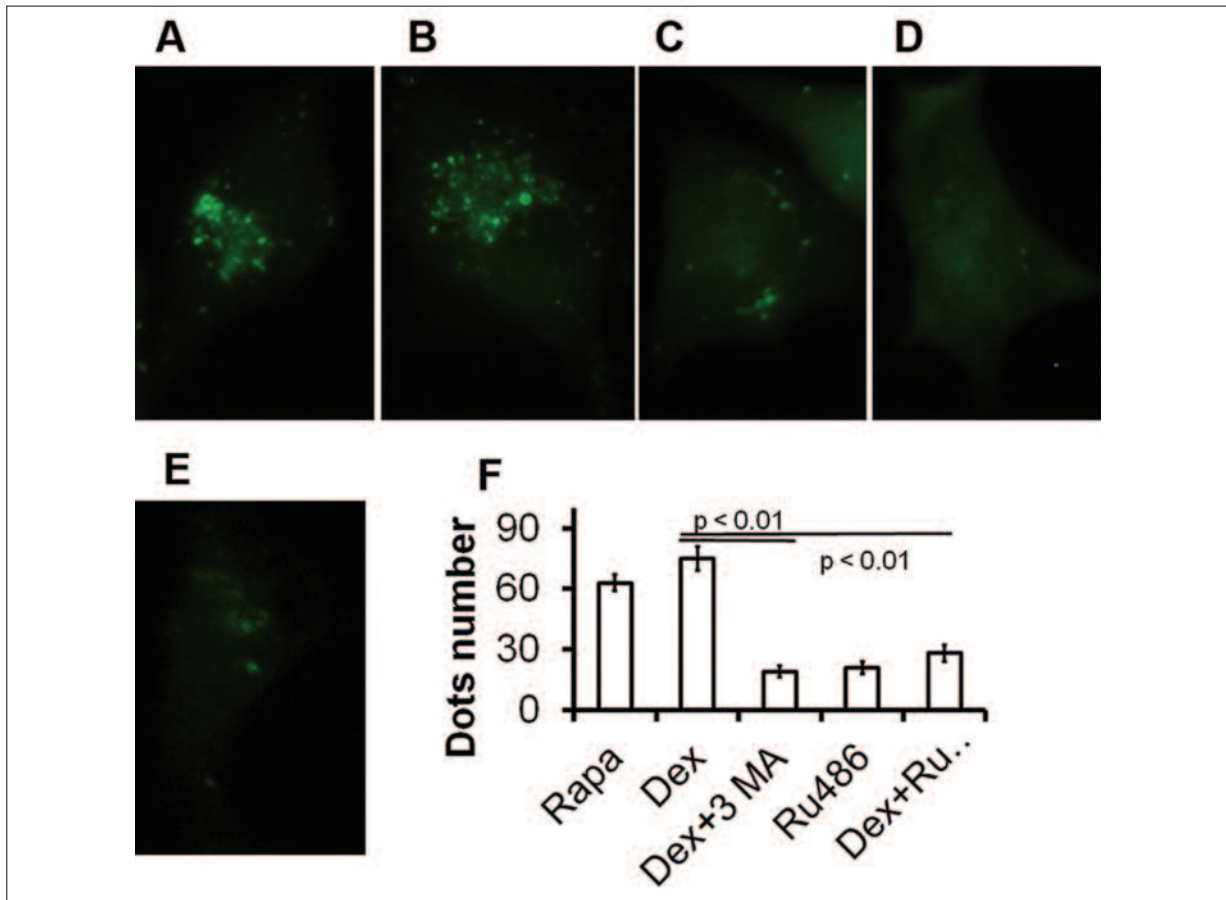


Figure 2. Dex-induced Autophagy in N1511 cells was inhibited by 3 MA or RU486. N1511 cells were transfected with a GFP-LC3 reporter vector and inoculated with at 37°C for 24 h; then, cells were incubated for another 24 h at 37°C in alpha-MEM containing 2% FBS with 200 nM rapamycin (Rapa), **(A)**, Dex (200 μM), **(B)**, Dex (200 μM) + 3 MA (100 μM) **(C)**, RU486 (10 μM), **(D)** or Dex (200 μM) + RU486 (10 μM) **(E)**. The number of punctate GFP-LC3 dots in each cell was also counted by fluorescence microscopy, and at least 100 cells were included for each group. The data were normally distributed and were statistically analyzed using the Student-Newman-Keuls test.

Discussion

It has been shown that growth and skeletal development are impaired post prolonged treatment with GCs¹⁻³. Children subject to such treatment is influenced by a number of different mechanisms, such as disrupting GH secretion, altering GH/IGF-1 sensitivity^{3,13}. Although catch-up growth often follows cessation of GC therapy, children with long-term GCs may have reduced final height^{14,15}. Also, permanent growth impairment has also been noted in children receiving alternate-day GC treatment^{4,5}. It is well known that the growth plate is responsible for longitudinal bone growth⁶. And the decreased growth is accompanied by a thinner growth plate^{3,7}, which has been attributed to de-

creased proliferation^{7,8} or increased apoptosis^{10,11} of the chondrocytes.

As one of the two main degradative pathways in eukaryotes, autophagy is involved in cell growth, survival, development and death, the levels of autophagy must be tightly regulated to maintain a balance among the synthesis, degradation, and subsequent recycling of cellular products¹⁶. Autophagy has been proposed to protect the cells from apoptosis by removing oxidatively damaged organelles or to destroy cellular components. Autophagy, therefore, can be protective or, alternatively, can be a self-destructive process that leads to cell death¹⁷.

In this study, increased autophagic activity were detected in N1511 chondrocytes induced by Dex. Autophagy-specific fluorescent GFP-LC3

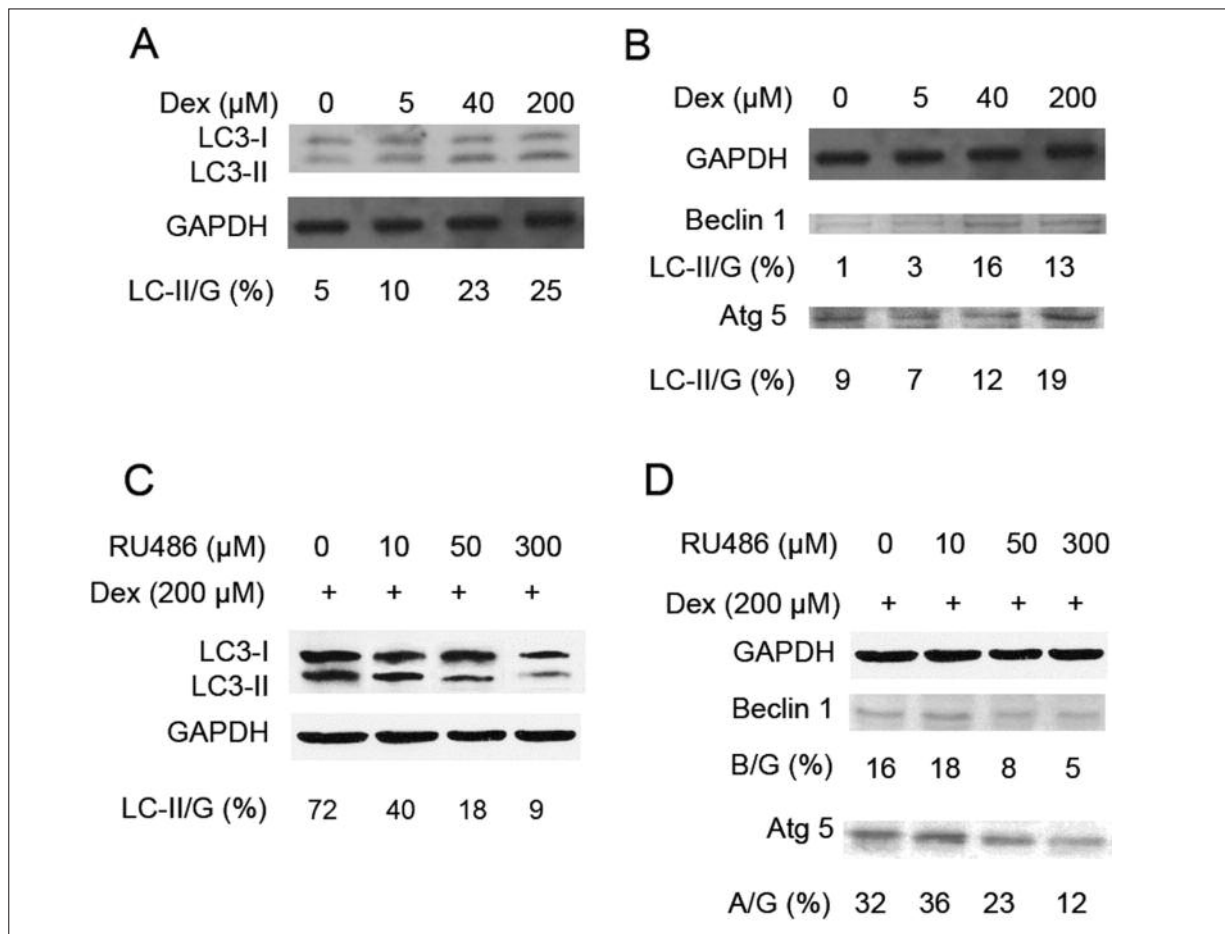


Figure 3. Dex increases the conversion of LC3-I to LC3-II and the expression of beclin 1 and Atg 5. Following 0 to 200 μM Dex treatment for 24 h, cells were lysed and subjected to western blotting with the antibodies indicated. Densitometry was performed for quantification. The ratio of LC3-II to GAPDH (**A**) is presented below the blots. **B**, Western blotting assay of Beclin 1 and Atg 5 expression in protein level of N1511 cells 24 h post 0 to 200 μM Dex treatment. **C**, RU486 inhibited the conversion of LC3-I to LC3-II by Dex. Conversion of LC3-I to LC3-II induced by Dex significantly reduced post 10 μM to 300 μM RU486 treatment. **D**, Beclin 1 and Atg 5 expression protein level in N1511 group post Dex was inhibited by RU486.

dots and autophagy-associated molecules expression were determined to observe autophagy. Results showed significant increase in the autophagy-specific AVOs via GFP-LC3 report vector with a fluorescence microscope in rapamycin-treated (200 nM) cells or cells post Dex treatment of various concentration. Further autophagy-associated molecules determination also confirmed the autophagy induced by Dex: the expression of LC3-II/LC3-I ratio increased in cells post Dex treatment; the beclin 1 and Atg 5 expressed higher in N1511 cells post Dex treatment in protein level. Taking together, autophagy could be induced by Dex in N1511 cells *in vitro*.

To reverse the Dex-induced autophagy in N1511 cells, RU486 and 3MA were utilized to

inhibit the Dex-induced autophagy. As supposed, the RU486 also could inhibit autophagy induction of Dex (Figure 2), as the inhibition of 3 MA. The blockage of autophagy induction by RU486 was confirmed by the determination of autophagy-associated molecules too (Figure 3).

Dex with doses of 5 μM or 200 μM could induce autophagy in N1511 cells, but the cell viability influenced by them was quietly different. Dex of 5 μM or 40 μM had no effect on the cell viability, without significance compared to the control. But the high dose of Dex (200 μM or 1 mM) could significantly inhibit the N1511 cell viability (Figure 4). And the inhibition could be reversed by 3 MA or RU486. Therefore, the autophagy induced by high dose of Dex could de-

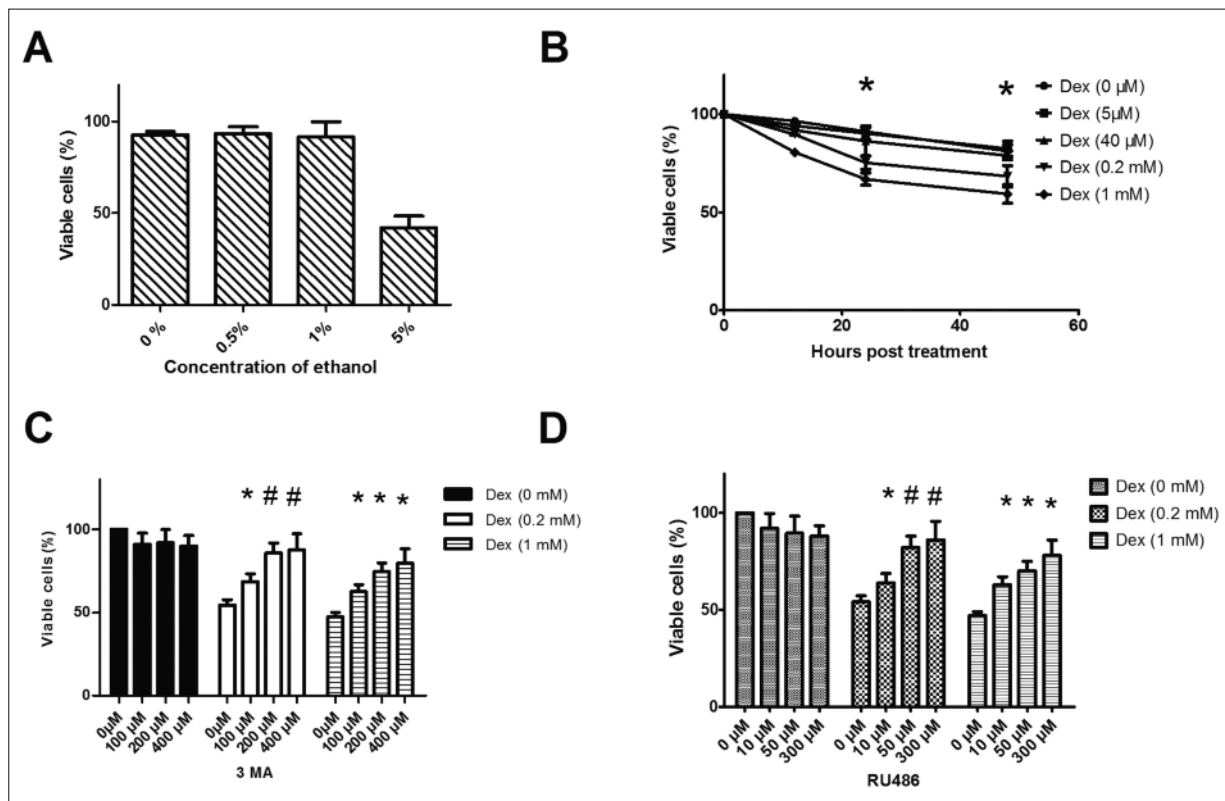


Figure 4. N1511 cell viability decreased post high dose of Dex treatment. **A**, Viability of N1511 cells with 0.5% to 5% ethanol, no significance between 0.5% and 1% ethanol groups. **B**, Viability of N1511 cells post treatment with 0 to 1 mM Dex. N1511 cells 24 h or 48 h post inoculation with 200 μ M or 1 mM Dex decreased significantly in viability. **C** and **D**, 3 MA and RU486 could ameliorate the Dex-induced N1511 cell viability reduction. * $p < 0.05$; ** $p < 0.01$.

stroy cellular components, leading to cell death. And it may be a novel mechanism involved in the decreased proliferation of the chondrocytes post sustained treatment of GC of high dose.

Conclusions

Glucocorticoids induce autophagy in N1511 chondrocyte cells. A high dose of GC may induce excess autophagy and reduces the cell viability.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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