

Upregulation of caspase-3 by high glucose in chondrocyte involves the cytoskeleton aggregation

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Abstract. – OBJECTIVE: The hyperglycemic environment of diabetes promotes chondrocyte (CH) apoptosis and is closely related to the occurrence and development of osteoarthritis (OA). This present study aimed to elucidate the relation between the cytoskeleton and the caspase-3 expression of human CHs in high glucose *in vitro*.

PATIENTS AND METHODS: We used different concentrations of glucose medium to test the effect of glucose on the CHs viability. Cytochalasin D and colchicine were used to prevent the aggregation of F-actin and β -tubulin. Besides, Z-DEVD-FMK (ZDF) or Apoptosis Activator 2 was used to inhibit or activate the caspase-3 expression. The intensity of F-actin and β -tubulin, cell viability, apoptosis, and caspase-3 expression were analyzed.

RESULTS: Three days of treatment of 30 mM or 40 mM glucose significantly decreased the CHs viability compared to the 10 mM but increased the caspase-3, apoptosis, collagen, and the aggregation of the F-actin and β -tubulin. However, the cytochalasin D and colchicine partly rejected the high-glucose induced caspase-3 upregulation, apoptosis, and CHs disability. Besides, these two anti-aggregation drugs also suppressed the Apoptosis Activator 2 induced caspase-3 upregulation and apoptosis. Furthermore, the application of ZDF could only prevent the F-actin aggregation, but not the β -tubulin.

CONCLUSIONS: Long-term high glucose triggers the caspase-3 expression and leads to the CH apoptosis involving cytoskeleton aggregation. Inhibition of cytoskeleton aggregation through the F-actin or β -tubulin could alleviate the high glucose-induced caspase-3 upregulation.

Key Words:

Caspase-3, Chondrocyte, Diabetes, F-actin, β -tubulin, Cytoskeleton.

Introduction

Osteoarthritis (OA) results in joint deformities and mobility disorders of patients, loss of labor, and even inability to take care of themselves, which brings tremendous mental stress and substantial economic burden on families and society¹. At present, patients with advanced OA are mostly treated by various surgical methods, such as knee replacement. Surgical treatment has shortcomings including massive trauma, high cost, and many postoperative complications². The pathogenesis of OA is not clear. Possible risk factors include age, metabolism, injury, inflammation, and so on, of which the relationship between metabolism and OA is a hot topic in recent years³. Epidemiological studies have found that diabetes is closely related to OA, which not only promotes the onset of OA but also hurts the treatment of OA patients⁴. Schett et al⁵ first conducted a 20-year follow-up study of 927 participants aged 40 to 80 years, which proved that diabetes was an independent risk factor for OA, excluding gender, age, BMI, and other risk factors, and explicitly proposed the concept of diabetic OA. Eymard et al⁶ found that the degree of joint space stenosis in patients with diabetic OA was more pronounced than that in patients with non-diabetic OA⁷.

The mechanism by which diabetes promotes the development of OA has not been clarified, which should be a complicated pathological process involving multiple factors such as inflammation, oxidative stress, and hyperglycemia. The accumulation of glucose in CHs induces the disorder of glucose metabolism and promotes the apoptosis and changes of the extracellular matrix (ECM). The main pathway of apoptosis is the caspase-dependent pathway, of which caspase-3

is downstream of the apoptotic enzyme chain reaction and directly performs the task of causing cell death^{8,9}. High glucose can cause caspase-3 upregulation by oxidative stress production in cultured CHs¹⁰.

The cytoskeleton plays a vital role in various physiological processes of cells, including changes in cell morphology, organelle transport, cell migration and adhesion, cell secretion and swallowing, and formation of ECM. In addition to the above characteristics, the cytoskeleton also has essential significance for the phenotypic maintenance, differentiation, and mechanical properties of CHs¹¹. Cytoskeletal remodeling can induce CHs apoptosis¹². Jin et al¹³ reported sodium nitroprusside induced CHs apoptosis by the cytoskeleton aggregation. However, it is still unclear how the high extracellular glucose concentration affects the cytoskeleton and the apoptotic function of CHs. In our study, we aimed to explore the relationships among high glucose, caspase-3, and cytoskeleton to uncover a novel insight into the diabetic OA.

Patients and Methods

CHs Isolation

We isolated the CHs from the knee joint cartilage from the patients undergoing amputation of the distal fibula chondrosarcoma. These patients (two males, one female, from 24 to 42 years) were diagnosed without OA history. We separated the cartilage from the joint and conserved it in the cell culture medium for CHs isolation. All the steps were approved by the Ethics Committee of the Jinhua Municipal Hospital and conducted by the Declaration of Helsinki. The cartilage was digested with a mixture of 0.25% trypsin and type I collagenase (Gibco, Rockville, MD, USA) in the incubator overnight. After centrifuge, CHs pellets were re-suspended and seeded in 6-well plates with Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium (containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, Invitrogen, Carlsbad, CA, USA).

CHs Treatments

We split the first generation CHs into the first generation and cultured in the DMEM/F12 with different concentration of glucose (10 mM to 40 mM) for one day or three days; to inhibit the aggregation of F-actin and tubulin, we used cytochalasin D (2 μ M) and colchicine (3 nM;

Sigma-Aldrich, St. Louis, MO, USA) for incubation; To suppress or activate the expression of caspase-3, we used Z-DEVD-FMK (ZDF) or Apoptosis Activator 2 (AA2, 4 μ M) (Selleck, Houston, TX, USA) for incubation.

Cell Viability Assay

We used the CCK-8 test to evaluate cell viability. CHs were seeded at a density of 5000/well in 96-well plates and treated as designed. After treatments, CHs were incubated with cell counting kit-8 (CCK-8) kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The intensity of the CCK-8 product was measured at an optical density (OD) of 450 nm using a microplate reader. We set the OD of 10 mM glucose treatment as a control group, and the other cell viability was shown relative to the control.

Flow Cytometry of Apoptosis Analysis

The apoptosis of CHs after each treatment was determined by Annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA). We collected the CHs into single-cell suspension and incubated with Annexin V-FITC reagent according to the manufacturer's instructions. Relative fluorescence was detected by flow cytometry to present the percentage of apoptotic cells.

Immunofluorescence (IF) of F-Actin and Tubulin

We determined the F-actin and tubulin density of CHs by the IF method. After treatment, CHs were treated with 4% paraformaldehyde for fixation, 0.2 % Triton-X for permeabilization. The F-actin filament was stained with Phalloidin-iFluor 488 reagent (green, ab176753, Abcam, Cambridge, MA, USA), and tubulin filament was stained with anti-tubulin antibody (ab6046, Abcam, Cambridge, MA, USA) and IgG (red). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The staining intensity was measured using the Image-J software (NIH, Bethesda, MD, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

We measured the content of caspase-3, collagen II, and collagen I secreted by CHs by the ELISA method according to the manufacturer's instructions (MBS283970; MBS263555; MBS703198, MyBioSource, San Diego, CA, USA). Briefly,

CHs were seeded and treated in a 96-well plate at 5000 /well. After fixation and permeabilization, CHs were incubated with primary antibody and followed by the addition of a labeled secondary antibody. The content of the desired detection was fluorescent for a single target.

Statistical Analysis

Data were analyzed by the Statistical Product and Service Solutions (SPSS) 17.0 software package (SPSS Inc., Chicago, IL, USA) and expressed as mean \pm standard deviation (SD). A comparison between multiple groups was done using one-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). The statistical difference between groups was considered significant when the p -value < 0.05 .

Results

High Glucose Triggered the Apoptosis of CHs

To simulate the diabetic cartilage microenvironment, we used ranged concentration (10 mM to 40 mM) of glucose to treat CHs. We used a low dose of 10 mM glucose in the DMEM medium as control. After 24 h treatment, we found no significant difference in cell viability between the higher dose (20 mM to 40 mM) glucose compared to the control (Figure 1A). However, the cell viability of 30 mM and 40 mM group was decreased compared to the control after 72 hours of treatment (Figure 1B). Therefore, short-term glucose treatment did not affect the CHs viability, but long-term high dose glucose affected the CHs viability. Besides, the apoptosis in 30 mM and 40 mM group also significantly increased compared to the 10 mM group (Figure 1C). Although glu-

cose is an essential substrate for the metabolism of CH, long-term exposure to a high level of glucose was indicated to reduce the viability of CH to trigger apoptosis.

Inhibition of Cytoskeleton Aggregation Decreased High Glucose-Induced CHs Apoptosis

The changes in the cytoskeleton regulate glucose catabolic¹⁴. Whether glucose affects the cytoskeleton of CHs remains to discovery. Compared to the control, we found the density of F-actin and β -tubulin increased, resulting from the high-dose of glucose treatment. Therefore, the anti-aggregation drug for actin and tubulin named cytochalasin D and colchicine were used to co-incubate with 40 mM glucose for 72 hours. As shown in Figure 2A and 2B, cytochalasin D suppressed the density of F-actin, and colchicine contained the density of β -tubulin. The high-dose affected the cell viability of CHs, but the anti-aggregation of the cytoskeleton by cytochalasin D and colchicine did not contribute to the survival of CHs compared to the 40 mM group (Figure 2C). Of note, the apoptotic cell ratio decreased after the supplement of cytochalasin D and colchicine compared to the 40 mM group (Figure 2D). In addition to this, the caspase-3 content also reduced after the aggregation of cytoskeleton compared to the 40 mM treated group (Figure 2E). To value the activity of CHs, we tested the cellular collagen II and collagen I expression of CHs. The result said high glucose inhibited the collagen II but promoted collagen I expression, and the supplement of cytochalasin D and colchicine rejected this effect caused by high glucose in different degrees, suggesting the inhibition of cytoskeleton aggregation played a protective role in the CHs' function.

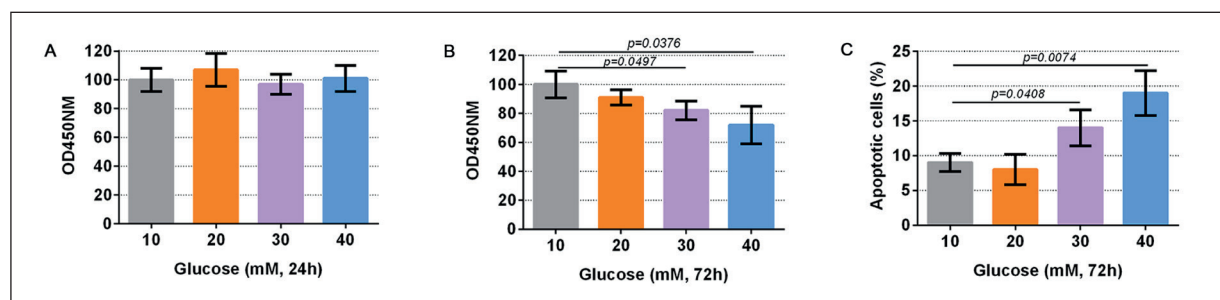


Figure 1. High glucose triggered the apoptosis of CHs. **A**, Cell viability of CHs with the treatment of 10-40 mM glucose for 24 hours. **B**, Cell viability of CHs with the treatment of 10-40 mM glucose for 72 hours. **C**, Apoptotic rate of CHs with the treatment of 10-40 mM glucose for 72 hours. The values are mean \pm SD of three independent experiments.

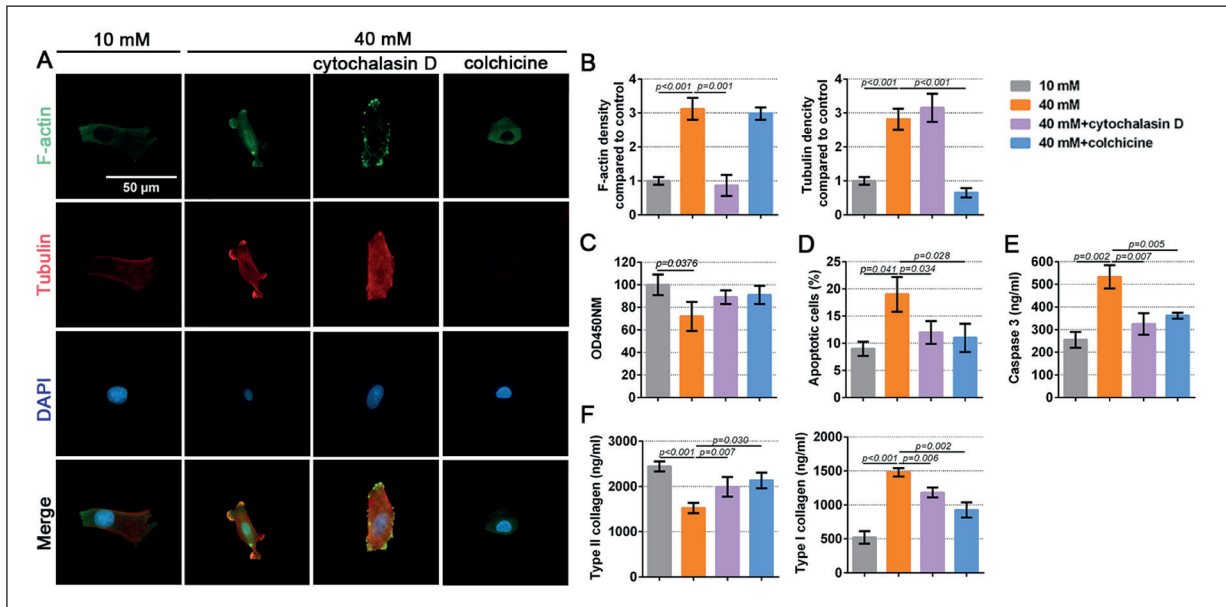


Figure 2. Inhibition of cytoskeleton aggregation decreased high glucose-induced CHs apoptosis. **A**, IF staining of F-actin (green) and β -tubulin (red) (magnification: 400 \times), and **(B)** quantification analysis of intensity; **(C)** Cell viability of CHs; **(D)** Apoptotic rate of CHs. The content of **(E)** caspase-3, **(F)** collagen II and collagen I. The values are mean \pm SD of three independent experiments.

Inhibition of Cytoskeleton Aggregation Decreased Caspase-3 Induced CHs Apoptosis

The inhibition of cytoskeleton aggregation could suppress the caspase-3 expression caused by high glucose. To further confirm the antagonistic effect of caspase-3, we also applied the caspase-3 activator AA2 to upregulated the caspase-3 expression and co-treated with cytochalasin D and colchicine. CHs cultured with 10 mM DMEM/F12 also were set as a control group. We treated CHs with AA2 for 24 hours to upregulate the caspase-3 expression. Meanwhile, the other two groups were treated with cytochalasin D and colchicine along with AA2 and 10 mM glucose for 24 hours. Compared to the control, AA2 aggregated the cytoskeleton with the increased density of F-actin and β -tubulin, which was also reversed by cytochalasin D or colchicine, respectively (Figure 3A, 3B). However, only colchicine could prevent the CHs' viability from the injury caused by AA2 (Figure 3C). As an activator of caspase-3, AA2 increased the caspase-3 level and apoptotic cell number compared to the control (Figure 3D, 3E). As expected, the inhibition of cytoskeleton aggregation partly reversed the AA2 induced apoptosis and caspase-3 upregulation. Additionally, the variation tendency of collagen II and collagen I was coincident with the caspase-3 expression (Figure 3F). Therefore,

the inhibition of cytoskeleton aggregation was certain to suppress the caspase-3 expression, no matter caused by the high glucose or the AA2.

Inhibition of Caspase-3 Decreased F-Actin Aggregation in High Glucose Condition

The findings mentioned above indicated the caspase-3 and cytoskeleton could affect each other under the high glucose condition. We wondered whether the suppression of caspase-3 in the high glucose condition could suppress the cytoskeleton aggregation. Therefore, we supplied the specific inhibitor of caspase-3 named ZDF in the 40 mM glucose medium and analyzed the parameters above. The results indicated that ZDF suppressed the F-actin intensity compared to the 40 mM group, but played no role in the β -tubulin concentration (Figure 4A, 4B). Similarly, the ZDF also did not improve the cell viability affected by the high glucose. As a result of the inhibition of caspase-3, the apoptosis also decreased after the ZDF treatment compared to the 40 mM group (Figure 4C-4E). Beyond that, the collagen II level was higher than that without ZDF, and collagen I was lower than the 40 mM group (Figure 4F). Therefore, the suppression of caspase-3 under the high glucose protected the collagen II production and the F-actin aggregation, but not the β -tubulin gathering.

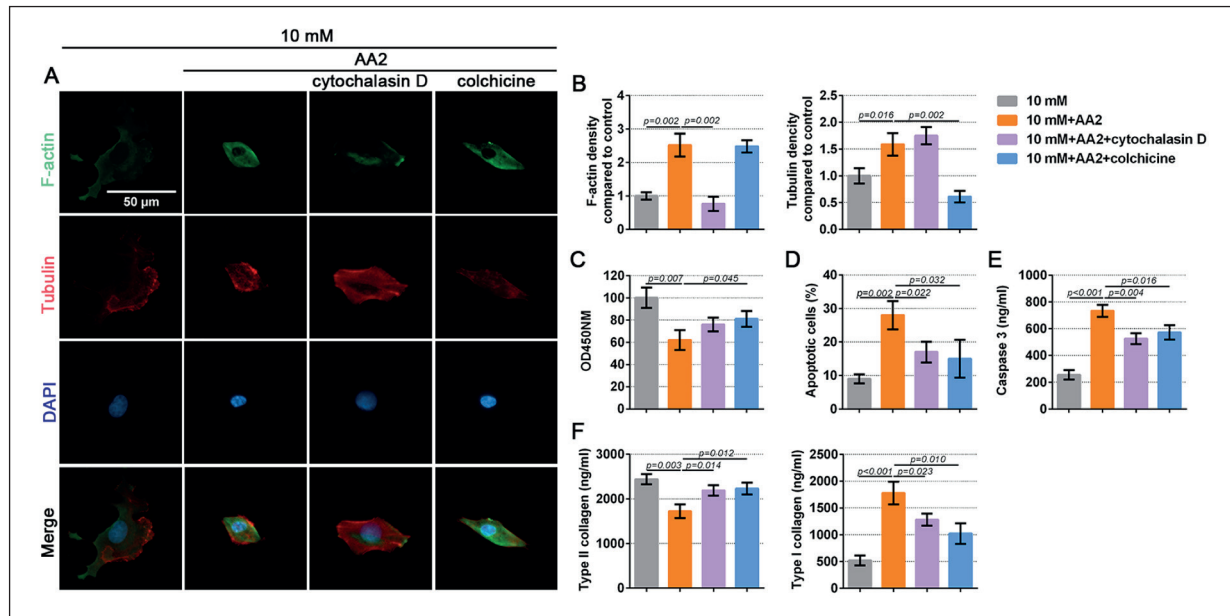


Figure 3. Inhibition of cytoskeleton aggregation decreased caspase-3 induced CHs apoptosis. (A) IF staining of F-actin (green) and β -tubulin (red) (magnification: 400 \times), and (B) quantification analysis of intensity. C, Cell viability of CHs; D, Apoptotic rate of CHs. The content of (E) caspase-3, (F) collagen II and collagen I. The values are mean \pm SD of three independent experiments.

Discussion

Glucose plays a massive part in the source of energy for CHs, which is also the main component of synthetic proteoglycans and the precursor

of glycosaminoglycans¹⁵. Therefore, glucose contributes to the regulation in the physiological function of CHs to synthesize ECM. However, the high-glucose environment is more likely to cause CHs to produce excessive matrix metal-

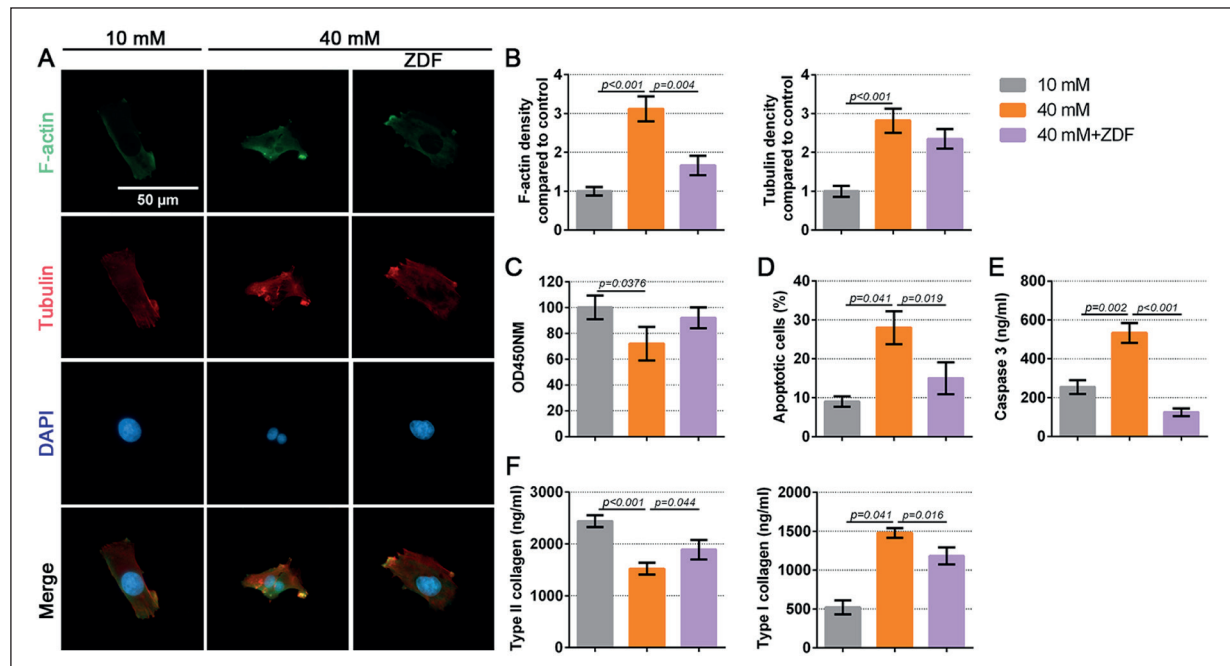


Figure 4. Inhibition of caspase-3 decreased F-actin aggregation in high glucose condition. A, IF staining of F-actin (green) and β -tubulin (red) (magnification: 400 \times), and (B) quantification analysis of intensity. C, Cell viability of CHs. D, Apoptotic rate of CHs. The content of (E) caspase-3, (F) collagen II and collagen I. The values are mean \pm SD of three independent experiments.

loproteinases (MMPs), induce the secretion of interleukin-1 (IL-1) and tumor necrosis factor (TNF), cause degradation of type II collagen and loss of proteoglycans, change the mechanical properties of cartilage, disrupt the stability of the cartilage matrix, and ultimately promote the formation of OA^{16,17}. Persistent hyperglycemia caused by diabetes leads to non-enzymatic glycosylation of various proteins in the body and the accumulation of advanced glycation end products (AGEs). It increases the hardness and fragility of cartilage, affects the metabolism of CHs, and accelerates the occurrence and development of OA¹⁸. Huang et al¹⁹ has shown that the accumulation of AGEs in cartilage leads to a decline in the synthesis capacity of type II collagen and proteoglycans, and also promotes the release of iNOS and COX2, the proinflammatory mediators of CHs, leading to enhanced catabolic activity and destruction of the anabolic and catabolic balance. From our experiment, short-term high-glucose treatment did not significantly affect the cell viability of CHs. However, three days' high-glycemic treatment resulted in a reduction of cell viability and a significant increase in cell apoptosis.

The cytoskeleton structure mainly includes three components: actin, vimentin, and tubulin. There are two forms of cellular actin, containing the monomeric globules called G-actin and polymeric filaments called F-actin. Besides, among the six members of the tubulin superfamily²⁰, the polymerization of α - and β -tubulin, especially β -tubulin, constitutes the dynamic microtubules. In the physiological state, the polymerization and depolymerization of F-actin and β -tubulin monomers in the cytoskeleton are in a dynamic balance. The destructive stimulus will change the structure and expression of the internal skeleton of the CH, and cause a variety of cellular biological responses²¹. Studies have found that CHs gradually lose the normal phenotype during two-dimensional (2D) culture, of which reason is 2D culture on the surface of the hard material promotes cell dispersion. It increases the expression of actin stress fibers causing normal CHs to differentiate into fibroblast-like CH²². However, destroying actin with cytochalasin D restores the typical phenotype of CHs, suggesting that actin plays an essential role in maintaining a normal CH phenotype²³. Can the destruction of the cytoskeleton induce apoptosis? Leadsham et al²⁴ and Desouza et al²⁵ explained this question. In their experiments, the excessive polymerization

of monomeric actin induced the apoptosis of cells in a short time, and the apoptosis phenomenon was alleviated after using cell cytochalasin D. Therefore, we hypothesized that the excessive polymerization of actin leads to the destruction of the cytoskeleton integrity, which leads to the CH apoptosis, and maintaining a stable cytoskeleton is also a method for preventing and treating CH apoptosis.

Caspase-3 is a member of the CPP32 subfamily and one of the most critical executors of apoptosis in the caspase family. It is activated from an inactivated zymogen form to an activated form under the stimulation of a variety of apoptotic signals. Some characteristic markers of apoptosis, such as chromosome condensation and DNA fragmentation, are directly related to the activation of caspase-3²⁶. Under the high-glucose condition, AGEs could activate the caspase-3 pathway and induce apoptosis in rat osteoblast cells²⁷ or retinal endothelial cells²⁸. In our research, long-term high glucose exposure increased the caspase-3 expression and the apoptosis level, as well as the aggregation of the cytoskeleton. Furthermore, the anti-aggregation of F-actin and β -tubulin also played a role in the suppression of the caspase-3. We also found that activation of caspase-3 is related to the aggregation of the cytoskeleton containing F-actin and β -tubulin, whether in a high glucose environment or not. But inhibiting the expression of caspase-3, it would affect the polymerization of F-actin, but no changes in β -tubulin.

Conclusions

In summary, our study, for the first time, proves that under the high glucose environment of diabetes, the polymerization of the CH cytoskeleton participates in the upregulation of caspase-3, and inhibiting the polymerization of the cytoskeleton can suppress caspase-3 expression and inhibit apoptosis. Diabetic OA is a complex process involving multiple factors. The novelty of our findings provides a new perspective about the CH cytoskeleton and apoptosis to better understand the interaction between this disease, which might be a new way for the treatment of OA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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