• **Basic Research** •

High glucose: activating autophagy and affecting the biological behavior of human lens epithelial cells

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Abstract

● **AIM: To clarify the effect of autophagy on human lens epithelial cells (HLECs) under high glucose conditions.**

● **METHODS: HLECs were cultured with different concentrations of glucose and 3-methyladenine (3-MA); the expression of autophagy-related protein LC3B was detected by Western blotting and immunofluorescence histochemistry. The migration of HLECs was quantified by scratch wound assay and the expression of transforming growth factor-β (TGF-β) was measured by real-time polymerase chain reaction.**

● **RESULTS: Compared with 5 mmol/L normal glucose treatment, 40 mmol/L glucose treatment can significantly increase the generation of autophagosome in HLECs, which could be inhibited by 0.375 mmol/L 3-MA treatment. The migration of HLECs and the expression of TGF-β in HLECs induced by high glucose were significantly suppressed by 0.375 mmol/L 3-MA treatment.**

● **CONCLUSION: Autophagy promotes HLECs cell migration and increases the expression of TGF-β after exposed to high glucose, which may relate to the development of diabetic cataract.**

● **KEYWORDS:** high glucose; autophagy; lens epithelial cells

INTRODUCTION

Over 415 million people in the world have diabetes, and it is estimated that this number will reach around 642 million by 2040[1]. As the disease progresses, a significant proportion of patients with diabetes mellitus begin suffering from eye diseases. Diabetic cataract is a common complication and can lead to blindness[2-3]. At present, surgical treatment continues to be the most effective method to treat diabetic cataract. However, several studies suggest that the risk of postoperative complications, such as inflammation and retinopathy, is much higher in diabetic than non-diabetic patients[3-4]. What's more, in developing countries, diabetes treatment is insufficient and cataract surgery is often inaccessible[5]. Unfortunately, the pathogenesis of diabetic cataract remains poorly understood, limiting efforts to expand treatment. In this study, we aim to examine the effect of autophagy on human lens epithelial cells (HLECs) under high glucose conditions.

Autophagy, or "self-eating" in cells, is a regulatory defense and stress mechanism. Activation of the lysosome pathway and autophagy is critical to remove damaged, aging organelles and non-functional proteins, allowing reconstruction of damaged structures and recycling of degraded materials[6]. A base level of autophagy is essential for normal physiological function, but increased or decreased autophagy can induce damage and death in cells[7]. Recent studies show that dysfunction of autophagy is related to the generation and development of diseases such as cancer, neurodegeneration, immune disorders and infection[8-10]. Diabetes mellitus is a metabolism-associated disease, which is closely related to autophagy[11]. Autophagy plays a crucial role in the occurrence of diabetes and is involved in diabetic complications, such as diabetic nephropathy, diabetic peripheral neuropathy, and diabetic retinopathy[12-17]. However, evidence is still lacking on autophagy in high glucose-induced diabetic cataract. It is believed that protein glycosylation and oxidative stress play a major role in the pathogenesis of diabetic cataracts[4]. Today, the PI3K/Akt/mTOR class I pathway and the PI3K/Bcline class III pathway are the most currently recognized autophagy signaling pathways. In high glucose condition, the combination of advanced glycation end (AGE) products and the receptor of advanced glycation end (RAGE) products activates ERK, JNK, and P38 MAPK Signal pathways, inhibiting Akt activation[15]. In addition, oxidative stress damage may induce activation of the ERK/JNK/P53 pathway[18-19]. All of these show some cross with class III PI3K/Bcline1 and class I PI3K/Akt/mTOR pathways.

Table 1 Primer sequences

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
GAPDH	ACCACAGTCCATGCCATCA	TCCACCACCCTGTTGCTGTA
TGF-β	ACTCCCTCACTCCCCACTTT	AGCACCACAGAGGCATTCTT

In view of the above factors, in this study we aim to elucidate the pathogenesis of diabetic cataract as we investigate the regulation of autophagy by high glucose in HLECs. These results may provide an innovative perspective on preventing and mitigating diabetic complications.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (DMEM), trypsin, fetal calf serum (FCS), and phosphate buffered saline (PBS tablets) were obtained from Hyclone Co. (St Louis, MO, USA). The RNeasy Mini Kit and reverse transcription (RT) kit were purchased from Qiagen Co. (Germany). The RNA polymerase chain reaction (PCR) kit and DNA marker were purchased from TaKaRa (Dalian, China). SYBR® Premix Ex Taq™ was obtained from TaKaRa (Japan). Cell Lysis Buffer was purchased from Beyotime Biotechnology (Shanghai, China). The rabbit anti-LC3B antibody was obtained from Cell Signaling Technology (USA).

Cell Culture Obtained from Han Yin Biotechnology Co. Ltd. (Shanghai, China) and maintained in DMEM, the HLECs were supplemented with 10% FCS and incubated at 37℃ in a humidified incubator with 95% air and 5% CO_2. The media was changed every 3-4d. We used inverted phase contrast microscopy to observe cell morphology, and actively growing cells in logarithmic phase were selected for subsequent experiments.

Real-time Polymerase Chain Reaction The HLECs were treated with 5 mmol/L glucose media, 40 mmol/L glucose media, and 40 mmol/L glucose with 0.375 mmol/L 3-methyladenine (3-MA) media respectively for 6h and then collected by centrifugation. The total RNA was extracted from HLECs using the RNeasy Mini Kit; DNA impurities were removed by DNase treatment, and reverse transcription (RT) reactions were conducted according to the Reverse Transcription Kit manual. Finally, transcribed cDNA was stored at -20℃ before its use in real-time PCR. The cDNA was used as a template and we performed PCR amplification of the target gene transforming growth factor-β (TGF-β). GAPDH was used as the internal reference. The primer sequences applied are shown in Table 1.

Cell Scratch Test This test starved the HLECs with serum-free medium for 12h when they had grown to 90% confluence. Then two lines were scratched with a sterile 200 μL pipet tip perpendicular to the bottom of the well. The plate was then washed with sterile PBS three times to remove detached cells and to form a cell-free naked area. The 5 mmol/L glucose media, 40 mmol/L glucose media, and 40 mmol/L glucose with 0.375 mmol/L 3-MA media were added respectively. The 6-well plate was then incubated in a constant temperature incubator and observed with an inverted phase contrast microscope after 0, 24 and 48h. The naked areas of cell scratches were measured before and after the treatment with the ImageJ software's metrics tool. The area achieved by subtracting the post-treatment area (at 24 or 48h) from the pre-treatment area corresponds to the average migration distance. All processes were performed in triplicate.

Western Blot HLEC suspension in logarithmic phase was inoculated in a 6-well plate, with about 1×10^5 cells in each plate. Cells were cultivated to about 70% and the original cultivation solution was then removed with a sterile pipet. The 5 mmol/L glucose media, 40 mmol/L glucose media, and 40 mmol/L glucose with 0.375 mmol/L 3-MA media was then added respectively. After 24h, the cells were harvested and lased in lysis buffer with the protease inhibitor cocktail of the total protein extraction. Then the total protein was separated by SDS-PAGE (12%) and transferred to nitrocellulose membrane. The membrane was blocked in 5% skimmed milk at room temperature for 1h and then incubated with anti-GAPDH and anti-LC3 B antibodies overnight at 4℃. We washed it three times with phosphate buffered saline containing 0.05% Tween-20 (PBST) for 10min and incubated with secondary antibodies for 1h at room temperature. The protein bands were visualized by an enhanced chemiluminescence fluorescence detection system. GAPDH was used as the loading control. All processes were performed in triplicate.

Immunofluorescence Histochemistry HLEC suspension in logarithmic phase was inoculated in a 6-well plate, with about 1×10^5 cells in every plate, and then the original cultivation solution was removed with a sterile pipet. The 5 mmol/L glucose media, 40 mmol/L glucose media, and 40 mmol/L glucose with 0.375 mmol/L 3-MA media was then added respectively. After 24h, the original cultivation solution was removed. The cells were fixed with 4% paraformaldehyde for 10min, treated with 1% triton, blocked with 3% bovine serum albumin (BSA) for 1h at room temperature, and then incubated with LC3B antibodies overnight at 4℃. We washed it three times with PBST for 10min and incubated with secondary antibodies for 1h at room temperature. DAPI was then applied and the cells were photographed with an OLYMPUS BX51. All processes were performed in triplicate.

Statistical Analysis Experimental data are expressed as average values±the standard deviation (mean±SD). SPSS15.0
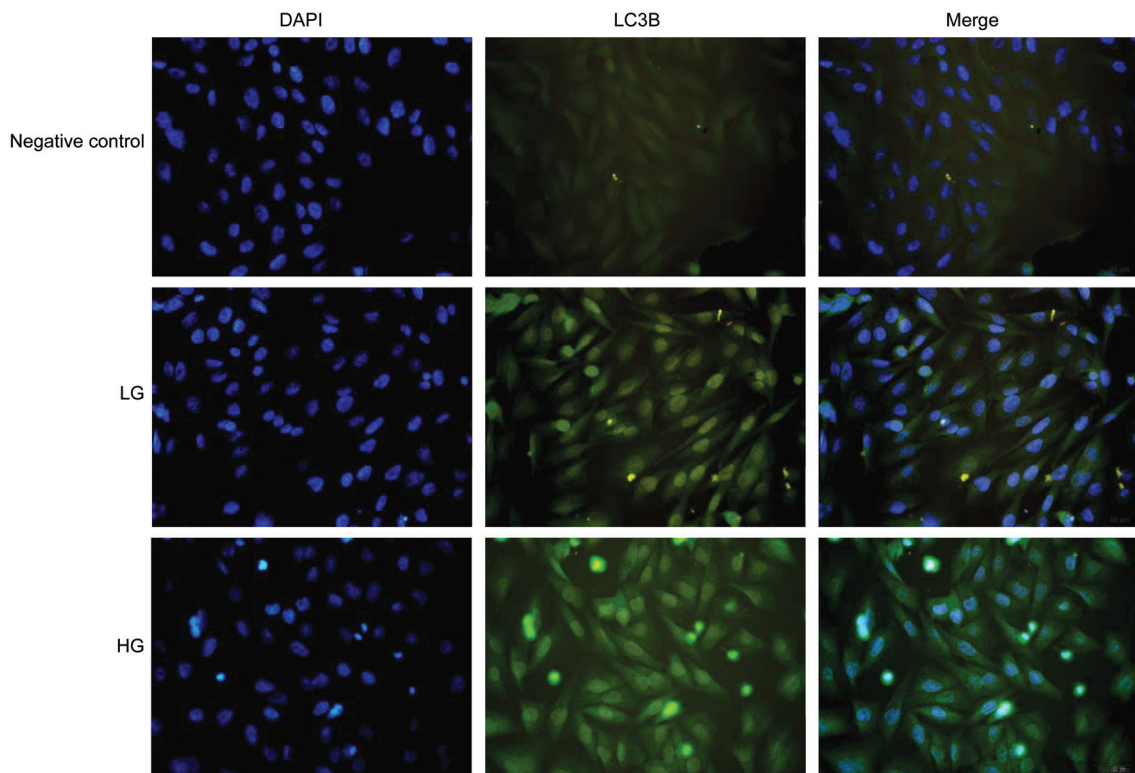
Figure 1 High glucose activates autophagy in HLECs The expression of LC3B in 5 mmol/L glucose group (LG) and 40 mmol/L glucose group (HG) group was detected by fluorescence histochemistry in HLECs. The negative control was treated only with the secondary antibody and no primary antibody was used. Green represent autophagosomes, blue represent the nucleus.

statistical software was utilized for statistical analysis. LSD-t test was used for comparison of multiple samples for the Western blot, cell migration, and real-time PCR experiments. P values less than 0.05 were considered statistically significant differences.

RESULTS

3-MA Inhibites Autophagy Activated by High Glucose in HLECs Normally, the autophagic protein LC3 is uniformly distributed, but aggregation of LC3 occurs when autophagy is induced. The results of fluorescence histochemistry showed that the number of LC3B puncta apparently increased in the 40 mmol/L glucose group compared to the 5 mmol/L glucose group, suggesting that high glucose could activate autophagy in HLECs (Figure 1).

The results of the Western blot showed that the relative expression of LC3B-II in the 5 mmol/L glucose group, 40 mmol/L glucose group, and 40 mmol/L glucose combined with 0.375 mmol/L 3-MA group were 0.883±0.059, 2.164± 0.624, and 0.631±0.535, respectively, and the differences were significant ($P<0.05$). The results indicated that high glucose could activate autophagy in HLECs, but this activation was reversed by the autophagy inhibitor 3-MA (Figure 2).

3-MA Prevents the HLECs Migration Induced by High Glucose The results of the cell scratch test showed that the ratio of migration area and the bare area for the 5 mmol/L glucose group, 40 mmol/L glucose group, and 40 mmol/L
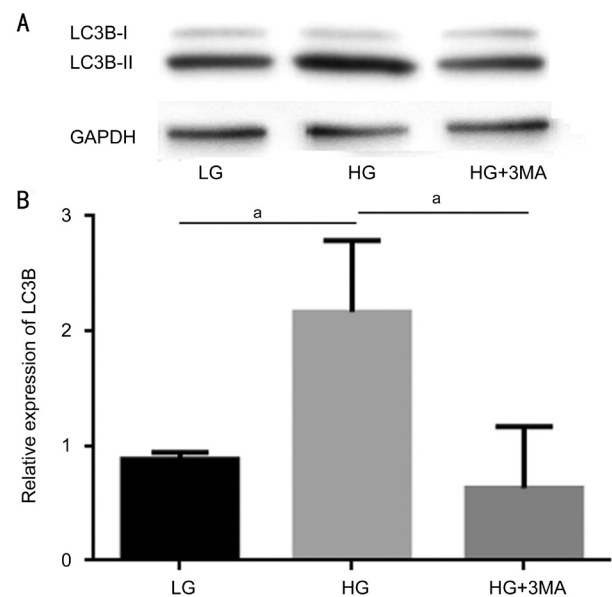
Figure 2 Protein of LC3B expression A: The expression of LC3B in 5 mmol/L glucose group (LG), 40 mmol/L glucose group (HG) group, and 40 mmol/L glucose with 0.375 mmol/L 3-MA group (HG+3MA) detected by Western blot in HLECs; B: The statistical results from three experiments performed under the same conditions ([a]$P<0.05$).

glucose with 0.375 mmol/L 3-MA group were 0.317±0.111, 0.314±0.007 and 0.371±0.111, respectively, after 24h; they became 0.441±0.094, 0.956±0.076, and 0.548±0.035, respectively, after 48h, in which the observed differences were significant ($P<0.05$). The results suggested that high glucose

enhanced HLEC migrate after 48h of scratching, and this effect was attenuated by 3-MA intervention (Figure 3).

3-MA Prevents the Increased Expression of TGF-β Induced by High Glucose The results of the real-time PCR showed that the relative expression of TGF-β in the 5 mmol/L glucose group, 40 mmol/L glucose group, and 40 mmol/L glucose with 0.375 mmol/L 3-MA group was 1.000, 1.407±0.060, and 1.134±0.026 respectively; the difference was significant ($P<0.05$). These findings suggest that the expression of TGF-β in high glucose was significantly increased relative to that in low glucose, which could be reversed by 3-MA treatment (Figure 4).

DISCUSSION

Lens epithelial cells are anterior subcapsular single-layered epithelial cells and are the most active cells in the lens. Mitosis of lens epithelial cells occurs continuously. After reaching the equator of the lens, the cells differentiate into lens fiber, which is important for lens transparency. The functional change of lens epithelial cells is thought to be the cellular basis of cataract development. Studies shows that increased migration of lens epithelial cells facilitates cataract development[20-21]. Meanwhile, many researchers demonstrate the effect of autophagy on functions of migration in cancer cells, smooth muscle cells, and epithelial cells[22-25]. Autophagy also plays a role in the development of chronic complications of diabetes[12-17]. However, its role in diabetic cataract development remains unclear. For this reason, we intend to study the regulation of autophagy in HLECs under high glucose conditions. LC3 is an autophagy-related protein. LC3A, LC3B and LC3C are the three subtypes of LC3, and LC3B is the most important one. When autophagy occurs, enzymolysis of a small fragment of polypeptide is induced by cytoplasmic LC3 to form LC3-I, which bonds with phosphatidyl ethanolamine and is then transformed into LC3-II. LC3-II is on the autophagosome's membrane and is regarded as a marker of autophagy[26]. In the pre-experiment, we treated HLECs with 5, 25, 40 mmol/L glucose. Western blot results showed that the expression of LC3B gradually increased with the increase of glucose concentration. In the following experiments, we selected 5 mmol/L glucose as normal glucose group and 40 mmol/L glucose as high glucose group. The PI3K-AKT-mTOR signal pathway is vital for autophagy. The 3-MA inhibits PI3K and blocks lysosome fusion with an autophagic vacuole. In the pre-experiment, we treated HLECs with 0.125, 0.250, and 0.375 mmol/L 3-MA under high glucose treatment. Western blot results showed that the expression of LC3B gradually decreased with the increase of 3-MA concentration. In further experiment, we selected 0.375 mmol/L 3-MA to inhibit autophagy in HLECs.

Within our study, the first conclusion we have reached is that high glucose activates autophagy in HLECs, which could
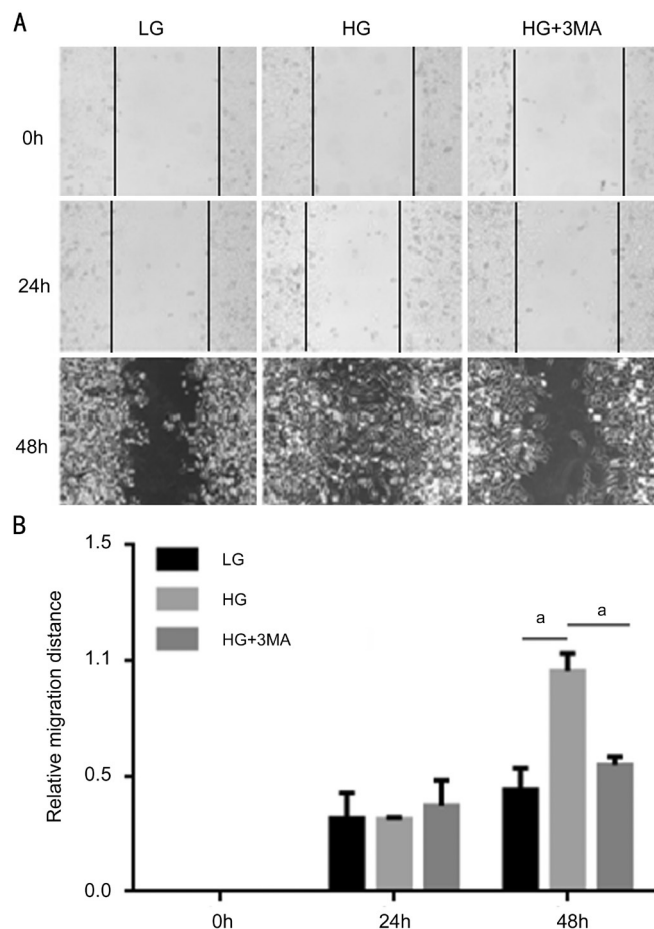
Figure 3 HLECs migration under high glucose A: The cell migration of HLECs after treatment for 24 or 48h with 5 mmol/L glucose media (LG), 40 mmol/L glucose media (HG), and 40 mmol/L glucose with 0.375 mmol/L 3-MA media (HG+3MA); B: The statistical results are from three experiments performed under the same conditions ([a]$P<0.05$).
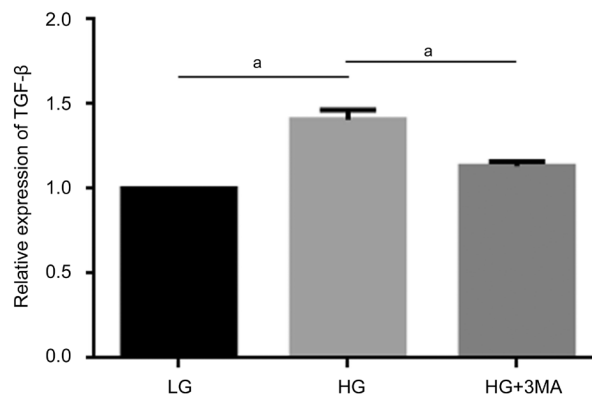
Figure 4 Increased TGF-β expression under high glucose The expression of TGF-β in HLECs after treatment with 5 mmol/L glucose media (LG), 40 mmol/L glucose media (HG), and 40 mmol/L glucose with 0.375 mmol/L 3-MA media (HG+3MA) by real-time PCR. The statistical results are from three experiments performed under the same conditions ([a]$P<0.05$).

be inhibited by the addition of 3-MA in protein level. In the cell wound scratch assay, we found that autophagy, induced by high glucose, can affect HLEC migration. As illustrated

above, the migration of HLECs plays an important role in the development of cataract. Therefore, we believe that high glucose may affect the migration of HLECs through inducing autophagy, thereby accelerating the development of cataract. Both *in vivo* and *in vitro* tests have shown that TGF-β expression is closely related with the occurrence of cataract. TGF-β receptors are on the surface of lens epithelial cells and facilitate the differentiation and fibrosis of lens epithelial cells. In animal experiments, high expression of TGF-β leads to cataract-like manifestations in lens epithelial cells, such as plaque-like aggregation and spindle changes[27-29]. In diabetes patients, TGF-β expression in lens epithelial cells is increased, possibly because high blood glucose and late glycosylation products activate TGF-β/Smad signal pathways. This activation affects biological functions, promoting diabetic cataract development[30-31]. To further explore the mechanism between autophagy and diabetic cataract, we measured TGF-β expression under different conditions. Real-time PCR analysis showed that autophagy, induced by high glucose, can affect the expression of TGF-β in HLECs. Thus, we speculate that high glucose may also affect expression of TGF-β in HLECs through inducing autophagy, once again accelerating the development of cataract.

Diabetes is a high-risk factor for cataract development, but the pathogenesis remains unclear. Although surgical treatment is an effective therapy for cataract, postoperative complications are higher in diabetes patients than in patients without diabetes. Therefore, studying the pathogenesis of the diabetic cataract is imperative for prevention and treatment. Our study indicated that autophagy, induced by high glucose, promotes the migration and increases the expression of TGF-β in HLECs, which may be related to the development of diabetic cataract.

ACKNOWLEDGEMENTS

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