

Research Article

# High-glucose environment accelerates annulus fibrosus cell apoptosis by regulating endoplasmic reticulum stress

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Diabetes mellitus (DM) is an important risk factor of intervertebral disc degeneration. However, how DM affects annulus fibrosus (AF) biology remains unclear. The present study was aimed to investigate the effects and mechanism of high glucose on AF cell biology. Rat AF cells were cultured in baseline medium and culture medium with 0.2 M glucose. The inhibitor 4-PBA was added along with the high glucose culture medium to study the role of endoplasmic reticulum (ER) stress in this process. Compared with the control cells, high glucose significantly increased cell apoptosis ratio and caspase-3/9 activity, up-regulated mRNA/protein expression of Bax and caspase-3/cleaved caspase-3, but down-regulated mRNA/protein expression of Bcl-2. Moreover, high glucose increased mRNA and protein expression of CHOP, ATF-6 and GRP78. However, once ER stress was inhibited by the inhibitor 4-PBA in the high glucose group, cell apoptosis ratio and caspase-3/9 activity were decreased, mRNA/protein expression of Bax and caspase-3/cleaved caspase-3 was down-regulated, but mRNA/protein expression of Bcl-2 was up-regulated. In conclusion, high glucose condition can promote AF cell apoptosis through inducing ER stress. The present study helps us understand the mechanism of disc degeneration in DM patients.

## Introduction

Intervertebral disc degeneration is the main cause of approximately 40% of low back pain in adults worldwide, which seriously affects social health care system [1]. The decrease in the number and viability of disc cells are responsible for disc degeneration [2–6]. Once disc cell density is declined, the micro-structure and composition of extracellular matrix (ECM) begin to change, with an imbalance between matrix anabolism and catabolism being induced [7]. Hence, more studies about the relationship between the etiology and disc cell apoptosis are essential to retard disc degeneration.

Diabetes mellitus (DM) is a public health issue that includes two main types: type I DM and type II DM. It is estimated that approximately 90% of all DM cases belong to type 2 DM [8]. DM often induces many complications including cardiovascular issue, renal failure and neuropathy [9]. Several studies have reported that DM is a potential etiological factor of intervertebral disc degeneration [10–15]. Moreover, some researchers have demonstrated that high glucose can promote apoptosis of disc cartilage endplate cells and notochordal cells, and ultimately accelerate the progression of disc degeneration [16,17]. Annulus fibrosus (AF) is the peripheral region of an intact intervertebral disc, which often exhibits tears and fissures in the degenerative discs [18]. Similarly, previous studies have shown that high glucose induces apoptosis or premature senescence of AF cells [19,20]. However, the mechanism behind the effects of high glucose on AF cell apoptosis remains unclear.

The endoplasmic reticulum (ER) is an important organelle that is essential for multiple cellular homeostasis. However, when cells perform its function to the greatest limit and the load imposed on the ER

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protein-folding machinery overwhelms its capability, ER stress begins to happen [21]. ER stress participates in various degenerative diseases, such as Parkinson's disease, Alzheimer's disease and chronic osteoarthritis [22,23]. Additionally, ER stress has also been proved to be involved in the progression of disc degeneration [24,25]. Furthermore, certain pathological factors induced ER stress often promotes disc nucleus pulposus (NP) cell apoptosis [24–27]. Though previous studies have demonstrated that high glucose induces ER stress in other studies, the relationship between ER stress and disc AF cell apoptosis in a high glucose environment remains unclear.

In the present study, we mainly aimed to investigate the effects of high glucose on disc AF cell apoptosis, and the role of ER stress in this process. AF cell apoptosis ratio, the activity of important caspases (i.e. caspase-3 and caspase-9), and mRNA/protein expression of apoptosis-associated molecules (bax, bcl-2, caspase-3, cleaved caspase-3) were used to evaluate AF cell apoptosis. The inhibitor 4-PBA was added along with culture media to verify the role of ER stress in this process.

## Materials and methods

### AF cell isolation and culture

The process of disc isolation was performed at the Animal Experiment Center of Liaocheng People's Hospital. After rats were killed by excessive carbon dioxide inhalation, the lumbar spine was cut, and then the individual disc was separated once the surrounding soft tissues were removed. Subsequently, AF tissue samples were obtained and digested by 0.20% collagenase type I for 3–4 h at 37°C. The isolated AF cells were subcultured in DMEM/F12 medium containing 15% fetal bovine serum (FBS, Gibco, U.S.A.). The culture media was refreshed every 2 days. The passage 3 (P3) AF cells were collected to use in the designed experiments. Briefly, the P3 AF cells were cultured in the baseline media (control cells) or the culture medium with 0.2 M glucose (conditioned media, experimental cells) for 3 days [15,16]. The baseline media is DMEM/F12 medium containing 15% fetal bovine serum, and the concentration glucose of the baseline media is approximately 0.017 M according to the product description. The inhibitor 4-PBA (5 mM) was added along with the culture media of the experimental cells to investigate the role of ER stress in this process.

### Flow cytometry analysis

The apoptosis ratio of AF cells was measured using the Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, China). After AF cells were cultured in the conditioned media for 3 days in six-well culture plates, the AF cells attached to the plate and AF cells floated in the media were collected by trypsinization and centrifugation, followed by suspending in the commixture containing 195 µl Annexin V-FITC binding buffer, 5 µl FITC-conjugated FITC and 10 µl propidium iodide (PI) for 20 min in the dark conditions. Then, the prepared AF cells were subjected to a FACalibration flow cytometer (Becton, Dickinson, U.S.A.). The double-positive stained AF cells were regarded as apoptotic cells.

### Caspase-3 and caspase-9 activity analysis

The commercial caspase-3 and caspase-9 activity detection kits (Beyotime, China) were used to measure caspase-3 activity and caspase-9 activity. After AF cells were cultured in the conditioned media for 3 days in six-well culture plates, the cells were lysed by lysis buffer provided in the kit. Then, the lysate was incubated with caspase-3 substrate and caspase-9 substrate for 6 h at 37°C in the dark conditions. Finally, caspase-3 activity and caspase-9 activity were calculated according to the absorbance at 405 nm and the calculation formula provided in the manufacturer's instructions.

### Real-time polymerase chain reaction analysis

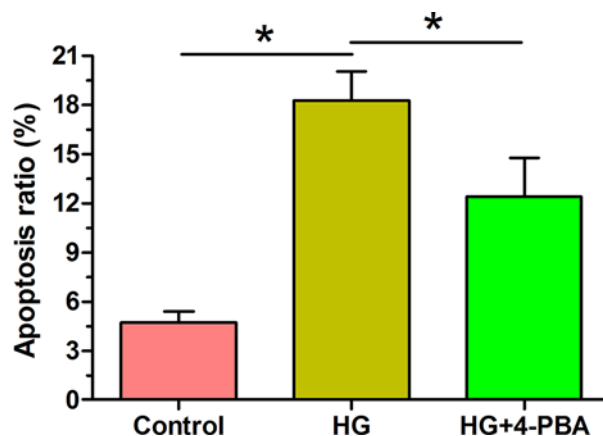
Total RNA was extracted using TRIzol reagent (TaKaRa, Japan) after AF cells were cultured in the conditioned media for 3 days. RNA concentration and purity were measured using a spectrophotometer. After 1 µg of total RNA was reverse-transcribed into cDNA, real-time polymerase chain reaction (PCR) was performed on a final reaction system containing cDNA templates, primers (Table 1) and SYBR Green Mix (TaKaRa, Japan). After reading the cycle threshold (Ct) values and normalizing them to the level of the internal β-actin, relative mRNA expression of target genes was calculated with the method of  $2^{-\Delta\Delta CT}$ .

### Western blot analysis

After AF cells were cultured in the conditioned media for 3 days, total protein was extracted using RIPA lysis buffer (Beyotime, China). Protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime,

**Table 1 Primers of target genes**

Gene	Forward (5'–3')	Reverse (5'–3')
β-actin	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
Bcl-2	GGGGCTACGAGTGGGATACT	GACGGTAGCGACGAGAGAAG
Bax	AGGGTTTCATCCAGGATCGAGCAG	ATCTTCCAGATGGTGAGCGAG
Caspase 3	GTACAGAGCTGGACTGCGGTATTG	AGTCGGCCTCCACTGGTATCTTC
CHOP	GGAGAAGGAGCAGGAGAATGA	AGACAGACAGGAGGTGATGC
ATF-6	CTCATGGACCAGGTGAAGACT	GGGCTCCATATGTCTGACTCC
GRP78	TCTGCTTGATGTGTCTCTCTT	GTCGTTACCTTCGTAGACCT



**Figure 1. AF cell apoptosis**

AF cells were cultured in a high glucose medium and 4-PBA was used to suppress ER stress. Data are showed as mean  $\pm$  SD ( $n = 3$ ). \*: Indicates a significant difference ( $P < 0.05$ ).

China). Then, equal protein samples in each group were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, U.S.A.). And thereafter, the PVDF membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-labeled secondary antibodies for 2 h at room temperature. Immunoreactive protein bands on the PVDF membrane were developed by BeyoECL Plus solution (Beyotime, China). Finally, densitometry analysis of protein bands was performed using the Image J software.

## Statistical analysis

Each experiment in the present study was performed in triplicate in the present study. All data in the present study were obtained from three independent assays and were expressed as mean  $\pm$  standard deviation values. Statistical analysis was performed by SPSS 19.0 software using the one-way analysis of variance.  $P < 0.05$  was considered significant.

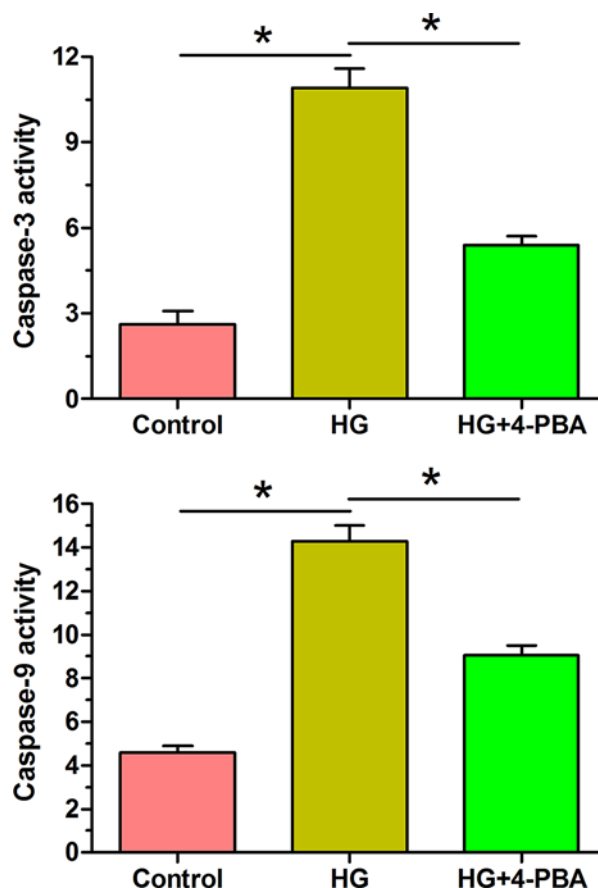
## Results

### Cell apoptosis ratio

To study the effects and mechanism of high glucose on AF cell apoptosis, we first used flow cytometry assay to measure AF cell apoptosis ratio. The results showed that after incubation with conditioned media, AF cell apoptosis ratio in the high glucose group was much higher than that in the control group. However, addition of 4-PBA in the high glucose group partly decreased AF cell apoptosis ratio (Figure 1).

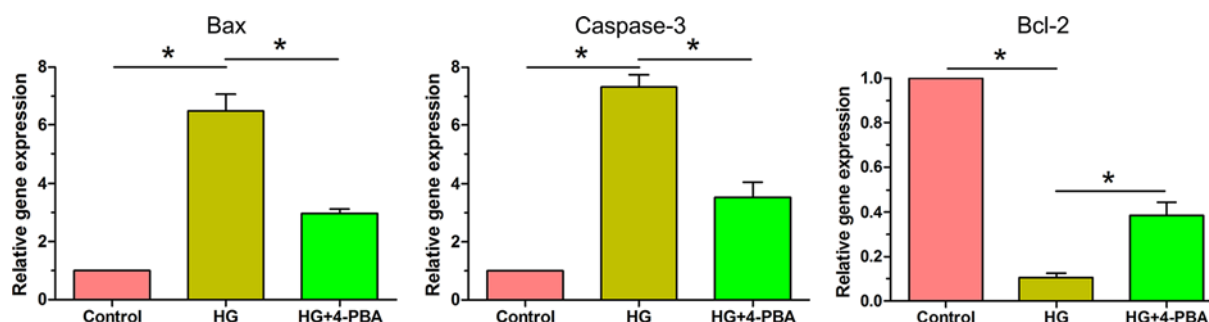
### Caspase-3/9 activity

Caspase-3 and caspase-9 are two important regulators in the apoptosis signal chain. We further measured their activity to evaluate AF cell apoptosis. The results showed that high glucose significantly increased their activity compared with the control group, whereas addition of 4-PBA decreased both caspase-3 activity and caspase-9 activity in the high glucose group (Figure 2).



**Figure 2. Analysis of caspase-3/9 activity of AF cells**

AF cells were cultured in a high glucose medium and 4-PBA was used to suppress ER stress. Data are showed as mean ± SD ( $n = 3$ ). \*: Indicates a significant difference ( $P < 0.05$ ).

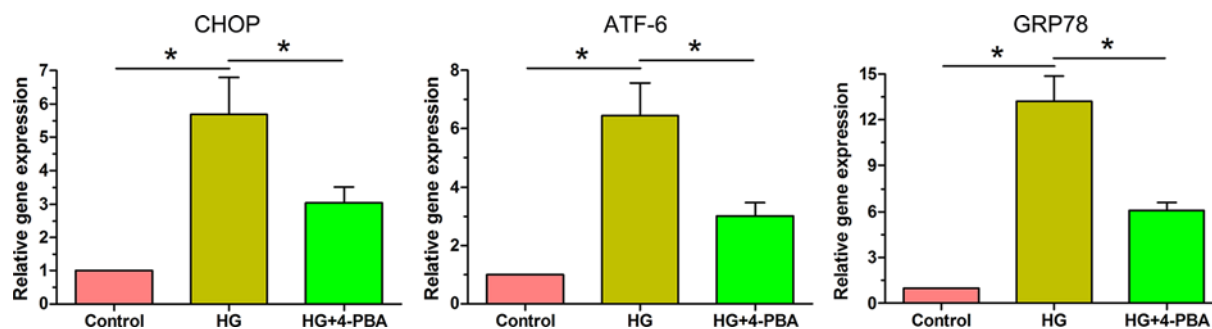


**Figure 3. Gene expression of apoptosis-related molecules of AF cells**

AF cells were cultured in a high glucose medium and 4-PBA was used to suppress ER stress. Real-time PCR was used to analysis mRNA expression of Bax, caspase-3 and Bcl-2. Data are showed as mean ± SD ( $n = 3$ ). \*: Indicates a significant difference ( $P < 0.05$ ).

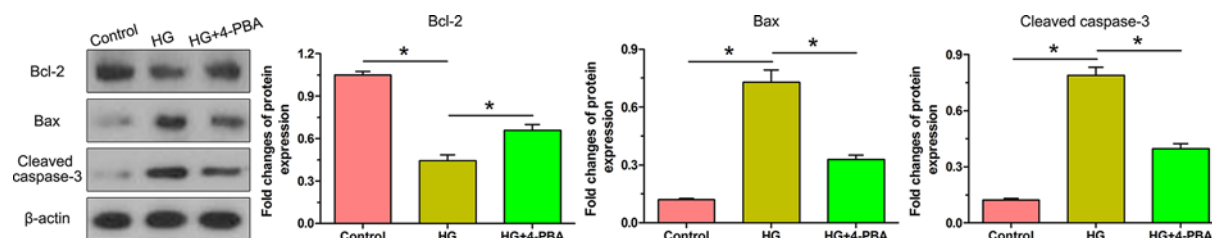
## mRNA expression of apoptosis-related molecules

In the present study, we analyzed mRNA expression of three relevant molecules (Bax, Bcl-2 and caspase-3) to assess cellular apoptosis. Compared with the control group, mRNA expression of Bax and caspase-3 was up-regulated but mRNA expression of bcl-2 was down-regulated in the high glucose group. On the contrary, 4-PBA obviously up-regulated mRNA expression of Bcl-2 and down-regulated mRNA expression of Bax and caspase-3 in the high glucose group (Figure 3).



**Figure 4. Gene expression of ER stress-related molecules of AF cells**

AF cells were cultured in a high glucose medium and 4-PBA was used to suppress ER stress. Real-time PCR was used to analysis mRNA expression of CHOP, ATF-6 and GRP78. Data are showed as mean $\pm$ SD ( $n = 3$ ). \*: Indicates a significant difference ( $P < 0.05$ ).



**Figure 5. Protein expression of apoptosis-related molecules of AF cells**

AF cells were cultured in a high glucose medium and 4-PBA was used to suppress ER stress. Western blot was used to analysis protein expression of Bcl-2, Bax and cleaved caspase-3. Data are showed as mean $\pm$ SD ( $n = 3$ ). \*: Indicates a significant difference ( $P < 0.05$ ).

## mRNA expression of ER-associated molecules

To evaluate the role of ER stress in the effects of high glucose on AF cell apoptosis, we analyzed mRNA expression of three markers (CHOP, ATF-6 and GRP78). The results showed that mRNA expression of these three markers were all significantly up-regulated compared with the control cells. Expectably, the inhibitor 4-PBA successfully down-regulated mRNA expression of CHOP, ATF-6 and GRP78 (Figure 4)

## Protein expression of apoptosis-related molecules

When cellular apoptosis happens, cleaved caspase-3 and Bax are often up-regulated, whereas Bcl-2 is down-regulated. The results showed that high glucose significantly increased protein expression of cleaved caspase-3 and Bax, whereas decreased protein expression of Bcl-2 compared with the control group. However, addition of 4-PBA decreased protein expression of Bax and cleaved caspase-3, and increased protein expression of Bcl-2 in the high glucose group (Figure 5).

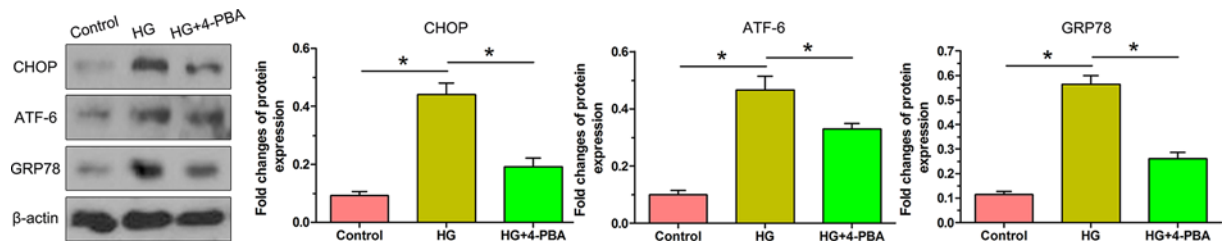
## Protein expression of ER markers

Similarly, we also detected protein expression of ER stress markers in the present study. The results showed that protein expression of CHOP, ATF-6 and GRP78 were all significantly increased compared with the control group. In line with the results of mRNA expression, the inhibitor 4-PBA partly decreased their protein expression in the high glucose group (Figure 6).

## Discussion

Disc degeneration is a chronic process characterized by excessive degradation of ECM, and it is also believed to be a leading cause of the lower back pain [1]. However, the accurate mechanism of disc degeneration is still not fully understood, and no effective therapies of disc degeneration were developed until now. Cellular loss resulted from excessive cellular apoptosis contributes to ECM degradation and decrease in ECM biosynthesis [7]. Hence, disc cell apoptosis has become a new research focus recently. AF cells residing in the disc AF tissue are responsible for synthesizing and secreting specific ECM macromolecules (i.e. aggrecan and collagen I), which ultimately supports





**Figure 6. Protein expression of ER stress-related molecules of AF cells**

AF cells were cultured in a high glucose medium and 4-PBA was used to suppress ER stress. Western blot was used to analysis protein expression of CHOP, ATF-6 and GRP78. Data are showed as mean  $\pm$  SD ( $n = 3$ ). \*: Indicates a significant difference ( $P < 0.05$ ).

disc AF biomechanical functions through regular structural arrangement [28]. In the present study, we mainly aimed to investigate the effects and mechanism of high glucose on disc AF cell apoptosis.

Diabetes is a systemic and complex metabolic disease that often causes other harmful complications, such as cardiovascular disease, renal failure and neuropathy [9]. A previous study has reported that DM patients have a higher incidence of disc degeneration than the non-DM patients [29]. The surgical outcome of disc degeneration-associated disease in DM patient is very poor compared with that in non-DM patients [30,31]. In other cell types, high glucose microenvironment can induce cellular apoptosis, such as retinal pigment epithelia cells [32], umbilical vein endothelial cells [33] and cardiomyocytes [34]. In the research field of disc degeneration, previous studies have also reported that high glucose promotes apoptosis of disc cartilage endplate cells, notochordal cells and nucleus pulposus cells [15–17]. In light of the key role of cellular apoptosis in the progression of disc degeneration, high glucose niche may accelerate disc degeneration through promoting disc cell apoptosis in DM patients. It is well known that disc cells are surrounded by an environment of nutrient and oxygen deprivation. The glucose transporters (i.e. GLUT 1, GLUT 3, GLUT 9) are responsible for the entry of glucose into intervertebral discs [35]. A previous study has shown that expression of GLUT 1 up-regulated as the grade of intervertebral disc degeneration increased [36]. This makes the disc cells vulnerable to deleterious effects of hyperglycemia even in insulinopenic or insulin resistant patients.

In the present study, we designed 0.2 M glucose concentration as a high glucose environment and cultured AF cells in this high glucose condition for 3 days. Our results showed that high glucose culture significantly increased cell apoptosis ratio and caspase-3/9 activity, up-regulated mRNA/protein expression of Bax, caspase-3/cleaved caspase-3, whereas down-regulated mRNA /protein expression of Bcl-2, indicating that this designed concentration of high glucose promotes disc AF cell apoptosis in the present study. In the degenerative disc tissue, AF region often exhibit tears and fissures-like structural changes [18]. Because normal AF cell viability is responsible for maintaining of the ECM in AF tissue, inhibiting high glucose-induced AF cell apoptosis may be a potential way to retard disc degeneration in DM patients. In line with us, several previous studies have shown that high glucose significantly affected biological behaviors of intervertebral disc cells, such as promoting cell apoptosis [15,19], accelerating autophagy [37], decreasing matrix biosynthesis [14] and inducing cellular senescence [15,38]. Together, these studies indicate that a high glucose environment is harmful to the healthy cellular activities of disc cells.

ER stress can lead to disturbance in many cellular homeostasis behaviors and thus slow protein folding process in the ER [39]. When the unfold proteins accumulates in the ER lumen, ER homeostasis is disrupted, and the ER stress, as well as the unfold protein response (UPR) is triggered [21]. The UPR can activate downstream regulators (i.e. c-Jun N-terminal kinase (JNK) and CHOP) and thus mediates caspase-12-participated cellular apoptosis [40]. ER stress plays a double edged sword-like role in some disease. For instance, ER stress protects cancer cells from apoptosis and increases drug resistance [41]. However, ER stress-induced disc cell apoptosis was important for progression of disc degeneration [25]. In the present study, we found that high glucose significantly increased expression of CHOP, ATF-6 and GRP78 compared with the control group, indicating that high glucose induces ER stress in AF cells. However, when ER stress was inhibited by the inhibitor 4-PBA, expression of CHOP, ATF-6 and GRP78 was decreased, and AF cell apoptosis was attenuated. These results indicate that high glucose promotes AF cell apoptosis through inducing ER stress.

However, several limitations also exist in the present study. First, the concentration of glucose used for ‘the high glucose group (0.2 M)’ is very high compared with the blood glucose level in diabetes patients, which affects the applicability of these results to clinical practice. Second, the isolated AF cells were two-dimensional cultured *in vitro*, which differs from the physiological conditions and may induce phenotypic changes during monolayer expansion.

## Conclusion

In the present study, we investigated the effects and mechanism of high glucose on AF cell apoptosis. The present results demonstrated that high glucose can promote AF cell apoptosis through inducing ER stress. The present study provides us new knowledge on the mechanism through which high glucose promotes AF cell apoptosis.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Author Contribution

Conception and design of the present study: LP and ZZ. Experiment performance: LP, KY and ZZ. Collection, analysis and explanation of experiment: LP, KY and ZZ. Drafting and critically revising of this article: LP, KY and ZZ. All authors approved the final submission.

## Ethics Approval

Forty-five Sprague-Dawley rats (11–14 weeks old, female) were purchased from the Animal Center of Liaocheng Clinical School of Taishan Medical University. All animal experiments were approved by the Ethics Committee at Liaocheng Clinical School of Taishan Medical University (EMY-2016-1273).

## Abbreviations

AF, annulus fibrosus; DM, diabetes mellitus; ECM, extracellular matrix; ER, endoplasmic reticulum; GLUT, glucose transporter; NP, nucleus pulposus; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; UPR, unfold protein response.

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