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High glucose promotes prostate cancer cells apoptosis *via* Nrf2/ARE signaling pathway

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Abstract. – OBJECTIVE: To explore the influences of high glucose on the proliferation and apoptosis of prostate cancer cells and analyze its possible mechanism of action.

MATERIALS AND METHODS: Human prostate cancer cell line LNCaP was divided into control group, mannitol group, and high glucose group. Then, the proliferation in each group was detected via methyl-thiazolyl-tetrazolium (MTT) assay. Hoechst staining assay was performed to determine the apoptosis level in each group. Western blotting was employed to measure the expression levels of apoptosis-related proteins and nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and γ-glutamylcysteine synthetase (γ-GCS) proteins. The cellular reactive oxygen species (ROS) level was measured through 2,7-dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. Enzyme-linked immunosorbent assay (ELISA) was carried out to detect the content of lactate dehydrogenase (LDH) and inflammatory factors.

RESULTS: High glucose significantly promoted the proliferation of prostate cancer cells LNCaP ($p<0.01$) and increased the apoptosis level of cells ($p<0.01$). In high glucose group, the expression level of Caspase-3 protein was overtly increased ($p<0.01$), while that of B-cell lymphoma-2 (Bcl-2)/Bcl-2 associated X protein (Bax) was significantly decreased ($p<0.01$). High glucose group had clearly increased the content of ROS ($p<0.01$), LDH ($p<0.01$), and interleukin-6 (IL-6) ($p<0.01$), but decreased the content of IL-10 ($p<0.01$). High glucose notably lowered the protein expression levels of Nrf2, HO-1, and γ-GCS in the cells ($p<0.01$).

CONCLUSIONS: High glucose represses the activation of the Nrf2/anti-oxidation response element (ARE) signaling pathway in prostate cancer cells and increases the content of ROS, IL-6, and the expression of apoptotic proteins in the cells, thus promoting the apoptosis of prostate cancer cells.

Key Words:
Prostate cancer, High glucose, Oxidative stress, Apoptosis.

Introduction

Prostate cancer, a common malignancy of the male urinary system, has a strong impact on the life and health of patients, and based on epidemiological investigations, its incidence rate is evidently elevated with age, and clearly higher in Western developed countries than that in China[1,2]. In addition, the pathogenic factors of prostate cancer remain unclear. In China, the number of patients with prostate cancer is notably increased due to a high-fat diet, reduced exercise, and continuous advances in detection techniques[3,4]. As for the treatment of prostate cancer, chemotherapy, androgen ablation therapy, and operation are most commonly used at present, and they can significantly prolong the survival of some patients with androgen-dependent prostate cancer, while the metastasis of tumor cells is a leading cause of the death among patients with androgen-independent prostate cancer[5,6]. Therefore, the knowledge of the key cytogenetic changes in the pathogenesis of prostate cancer and the finding of new treatment methods are of great urgency.

Slob et al[7] found that high-dose glucose activates the protein kinase C pathway to suppress the activity of Na^+-K^+-ATPase, thereby reducing the cell basement membrane turnover and affect-

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ing cell growth and adhesion. A large number of researches have manifested that high glucose is able to bind to proteins to form glycosylation end products, induce an oxidative stress response, promote the expression of apoptotic proteins, regulate the expression of inflammatory factors, and affect the proliferation, migration and adhesion of cells[8,9]. Nuclear factor erythroid 2-related factor 2 (Nrf2) inhibits the anti-oxidation response elements (AREs) and regulates the oxidation-reduction reaction of cells, thus modulating the proliferation and apoptosis of the cells[10]. However, the influences of high glucose on the Nrf2/ARE signaling pathway and the proliferation and apoptosis of prostate cancer cells are still unclear. Therefore, this study aims to evaluate the effects of high glucose on the Nrf2/ARE signaling pathway and the proliferation and apoptosis of prostate cancer cell line LNCaP by establishing *in vitro* models of high glucose.

Materials and Methods

Materials and Instruments

The main reagents used were: human prostate cancer cell line LNCaP (Kunming Cell Bank of Chinese Academy of Sciences, Kunming, China), dimethyl sulfoxide (DMSO) and 5-diphenyltetrazolium bromide [(methyl thiazolyl tetrazolium (MTT)] (Sigma-Aldrich, St. Louis, MO, USA), glucose, mannitol, Hoechst 33258 kit, lactate dehydrogenase (LDH) enzyme-linked immunosorbent assay (ELISA) kit, interleukin-6 (IL-6) ELISA kit, and IL-10 ELISA kit (Boster Biological Technology Co., Ltd., Wuhan, China), Dulbecco's Modified Eagle's Medium (DMEM) low-glucose medium (Heclone, South Logan, UT, USA), RIPA lysis solution, phosphorylation protease inhibitor, and Hoechst assay kit (ServiceBio, Wuhan, China), antibodies (Abcam, Cambridge, MA, USA), an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan), a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA), a confocal microscope (Nikon, Tokyo, Japan), electrophoresis apparatus (Corning, Corning, NY, USA) and a pipettor (Eppendorf, Hamburg, Germany).

Detection of Cell Proliferation Via MTT

MTT was employed to detect the role of high glucose in the proliferation of prostate cancer cell line LNCaP. After thawing and passage, LNCaP cells in the logarithmic growth phase were collected and inoculated into a 96-well plate, and the cell density was 10^4 cells/well after counting. Then, the cells were added with the serum-free low-glucose medium for culture in a 5% CO_2 incubator at 37°C for 24 h. Thereafter, they were divided into control group, mannitol group, and high glucose group. Next, 5.5 mmol/L glucose, 15 mmol/L mannitol, and 15 mmol/L glucose were added to the cells in the control group, mannitol group, and high glucose group, respectively, followed by 48 h of culture. After that, the supernatant was discarded, and the complete medium containing 5 mg/mL MTT was added to each well for 4 h of incubation. Then, the supernatant was discarded. DMSO was added and mixed well. Lastly, the wavelength of the microplate reader was adjusted to 490 nm, and the proliferation of the cells in each group was determined.

Determination of Apoptosis Through Hoechst 33258 Staining

Hoechst 33258 staining assay was conducted to detect the effect of high glucose on the apoptosis of prostate cancer cell line LNCaP. LNCaP cells were firstly thawed and passaged, and then those in the logarithmic growth phase were collected and inoculated into a 6-well plate. Finally, the cell density was adjusted to 1×10^5 cells/well. Thereafter, the serum-free low-glucose medium was added for culture in the 5% CO_2 incubator at 37°C for 24 h. Different concentrations of mannitol and glucose were added to the cells in the control group, mannitol group, and high glucose group for culture for 48 h, respectively. After that, the supernatant was discarded, and freshly-prepared 4% paraformaldehyde was added to fix the cells for 30 min. Next, the cells were washed with Phosphate-Buffered Saline (PBS), added with 50 μL/well Hoechst 33258 stain and incubated in a dark place at room temperature for 10 min. Lastly, the fluorescence microscope was used to observe the cells. Highlighted blue in nuclei indicated apoptosis, and the cells with highlighted blue nuclei were recorded as apoptotic cells.

Detection of Cellular Reactive Oxygen Species (ROS) Level by 2,7-Dichloro-Dihydrofluorescein Diacetate (DCFH-DA) Assay

The influence of high glucose on ROS content in the cells was detected by DCFH-DA assay. LNCaP cells in the logarithmic growth phase were collected and inoculated in a 6-well plate after thawing and passage, and the cell density was adjusted to 1×10^5 cells/well. Next, the cells were

cultured with the serum-free low-glucose medium in the 5% CO_2 incubator at 37°C for 24 h. Thereafter, the cells in the control group, mannitol group, and high glucose group were separately added with different concentrations of mannitol and glucose for culture for 24 h and then 10 μM DCFH-DA for incubation in the dark at 37°C for 20 min. Then, the medium was discarded, and the cells were washed with PBS for 3 times. The confocal microscope (excitation wavelength: 488 nm, and emission wavelength: 525 nm) was applied to measure the fluorescence intensity to evaluate the ROS level in the cells. The higher was the ROS level in cells, the brighter was the nucleus.

Determination of Content of Lactate Dehydrogenase (LDH) and Inflammatory Factors via ELISA

The content of LDH and inflammatory factors IL-6 and IL-10 in the cells of each group was detected using the corresponding ELISA kits. After thawing and passage, LNCaP cells in the logarithmic growth phase were collected and inoculated in a 6-well plate (the cell density was adjusted to 1×10^5 cells/well), followed by addition of serum-free low-glucose medium and culture in the 5% CO_2 incubator at 37°C for 24 h. Next, the different concentrations of mannitol and glucose were added to the cells in the control group, mannitol group, and high glucose group, respectively. After 48 h of culture, the cells were extracted for later use. Standard curves of LDH, IL-6 and IL-10 were plotted separately. 100 μL standard solution of each group of cell solution was added in an antibody-coated 96-well plate, followed by blocking with a sealing membrane and incubation at 37°C for 1 h. Next, the liquid in the plate was removed by patting, and 100 μL biotin-labeled antibodies (anti-LDH, IL-6, or IL-10 antibodies, respectively) were added, blocked with the sealing membrane, and incubated at 37°C for 1 h. Thereafter, each well was added with 250 μL diluted washing solution and washed 3 times. Then, 100 μL ABC working solution was added and incubated at 37°C for 30 min after blocking with the sealing membrane. Next, 250 μL washing solution was added to wash the plate 3 times. After that, 90 μL TMB coloring solution was added, blocked with the sealing film, and incubated at 37°C for 30 min. Then, the TMB stop solution was added and shaken well. Lastly, the microplate reader was employed to measure the absorbance at 450 nm, the CurveExpert 1.4 software was used to plot standard curves, and the content of LDH, IL-6, and IL-10 in the cells in each group was calculated.

Measurement of Related Protein Expression Levels Through Western Blotting

LNCaP cells were thawed and passaged. Then, those in the logarithmic growth phase were collected and seeded in a 6-well plate, and the cell density was adjusted to 1×10^5 cells/well. Next, they were incubated with the serum-free low-glucose medium in the 5% CO_2 incubator at 37°C for 24 h. Thereafter, the cells in the control group, mannitol group, and high glucose group were separately cultured with different concentrations of mannitol and glucose for 48 h. After that, the medium was discarded, and the total protein was extracted after adding radioimmunoprecipitation assay (RIPA) lysis solution (Beyotime, Shanghai, China). The bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA) was used to determine the protein concentration in each group of cells. Next, the loading buffer in equal concentration was prepared after adding protein dilution solution, boiled for 15 min and loaded, followed by electrophoresis at a constant voltage of 80 V. After that, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) at 90 V for 100 min by wet transfer. Thereafter, the PVDF membranes were immersed in freshly-prepared 5% skim milk powder for 1 h of blocking. The strips were cut according to the size of target bands, separately incubated with Rabbit-Nrf2, Rabbit-heme oxygenase-1 (HO-1), Rabbit-γ glutamylcysteine synthetase (γ-GCS), Rabbit-Caspase3, Rabbit-B-cell lymphoma-2 (Bcl-2), Rabbit-Bcl-2 associated X protein (Bax), and Rabbit-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 4°C overnight, washed with Tris-Buffered Saline-Tween 20 (TBST) for 3 times and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h, followed by washing with TBST for 3 times (5 min/time). Next, the developing solution was prepared, and the strips were placed in it. Lastly, the expression levels of corresponding proteins were calculated using Nrf2/GAPDH, HO-1/GAPDH, γ-GCS/GAPDH, Caspase3/GAPDH, and Bcl-2/Bax.

Statistical Analysis

All data in this study were expressed as mean ± standard deviation and analyzed by the Statistical Product and Service Solutions (SPSS) 22.0

software (IBM Corp., Armonk, NY, USA). The comparison between groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). The data with homogeneity of variance were subjected to pairwise comparison *via* Bonferroni method, while those with the heterogeneity of variance were analyzed by the Welch method. $p<0.05$ suggested that the difference was statistically significant.

Results

High Glucose Inhibited the Proliferation of Prostate Cancer Cells

The results of the detection of the effect of high glucose on the proliferation of prostate cancer cell line LNCaP *via* MTT showed that the proliferation of the cells in the mannitol group was comparable to that in the control group ($p>0.05$), while the proliferation of the cells in high glucose group was overtly higher than that in the control group ($p<0.01$) (Figure 1).

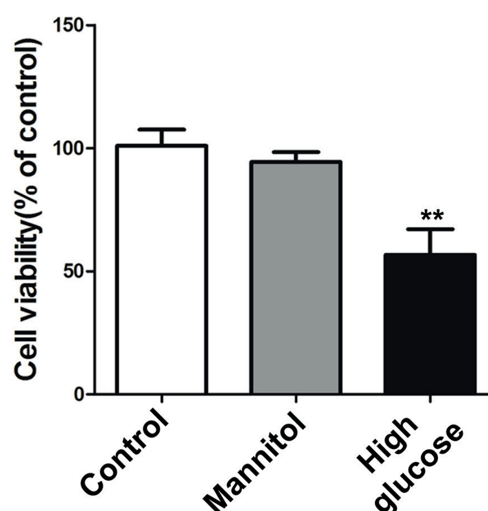

Figure 1. Proliferation of cells in each group detected *via* MTT. Compared with that in the control group, the proliferation of the cells is notably enhanced in high glucose group, $^{**}p<0.01$ *vs.* control group.

High Glucose Increased the Level of Apoptosis in Prostate Cancer Cells

The influence of high glucose on the apoptosis of prostate cancer cell line LNCaP was detected through Hoechst 33258 staining assay, and it was found that the apoptosis level of the cells was clearly increased in high-glucose LNCaP ($p<0.01$), and the high-concentration mannitol had no effect on the apoptosis of LNCaP ($p>0.05$) (Figure 2).

Effects of High Glucose on Apoptosis-Related Proteins in Prostate Cancer Cells

The expression levels of apoptosis-related proteins in each group were measured *via* Western blotting. The results (Figure 3) revealed that high glucose group exhibited a significantly increased expression level of Caspase-3 ($p<0.01$) and an overtly lowered Bcl-2/Bax expression level ($p<0.01$) in the cells in comparison with the control group, and mannitol had no influence on the expression levels of Caspase-3 and Bcl-2/Bax in cells ($p>0.05$).

High Glucose Elevated ROS Level in Prostate Cancer Cells

The results of DCFH-DA assay, used to determine the influence of high glucose on ROS level, showed that the intracellular ROS level was clearly higher in high glucose group than that in the control group ($p<0.01$), while it was comparable in the mannitol group to that in the control group ($p>0.05$) (Figure 4).

Influences of High Glucose on LDH and Inflammatory Factors in Prostate Cancer Cells

The content of LDH and inflammatory factors in each group of cells was determined through ELISA. The results (Figure 5) showed that the high glucose group had significantly increased the content of LDH ($p<0.01$) and IL-6 ($p<0.01$), and overtly decreased the content of IL-10 ($p<0.01$), while there was no significant difference in the content of LDH, IL-6, and IL-10 in the cells between the mannitol group and control group ($p>0.05$).

Impact of High Glucose on the Nrf2/ARE Signaling Pathway in Prostate Cancer Cells

Western blotting was carried out to measure the expression levels of the Nrf2/ARE signaling pathway-related proteins in each group of cells, and it was found that, compared with those in control group, the protein expression levels of Nrf2, HO-1, and γ-GCS remarkably declined in high glucose group ($p<0.01$), while they were comparable in the mannitol group to those in the control group ($p>0.05$) (Figure 6).

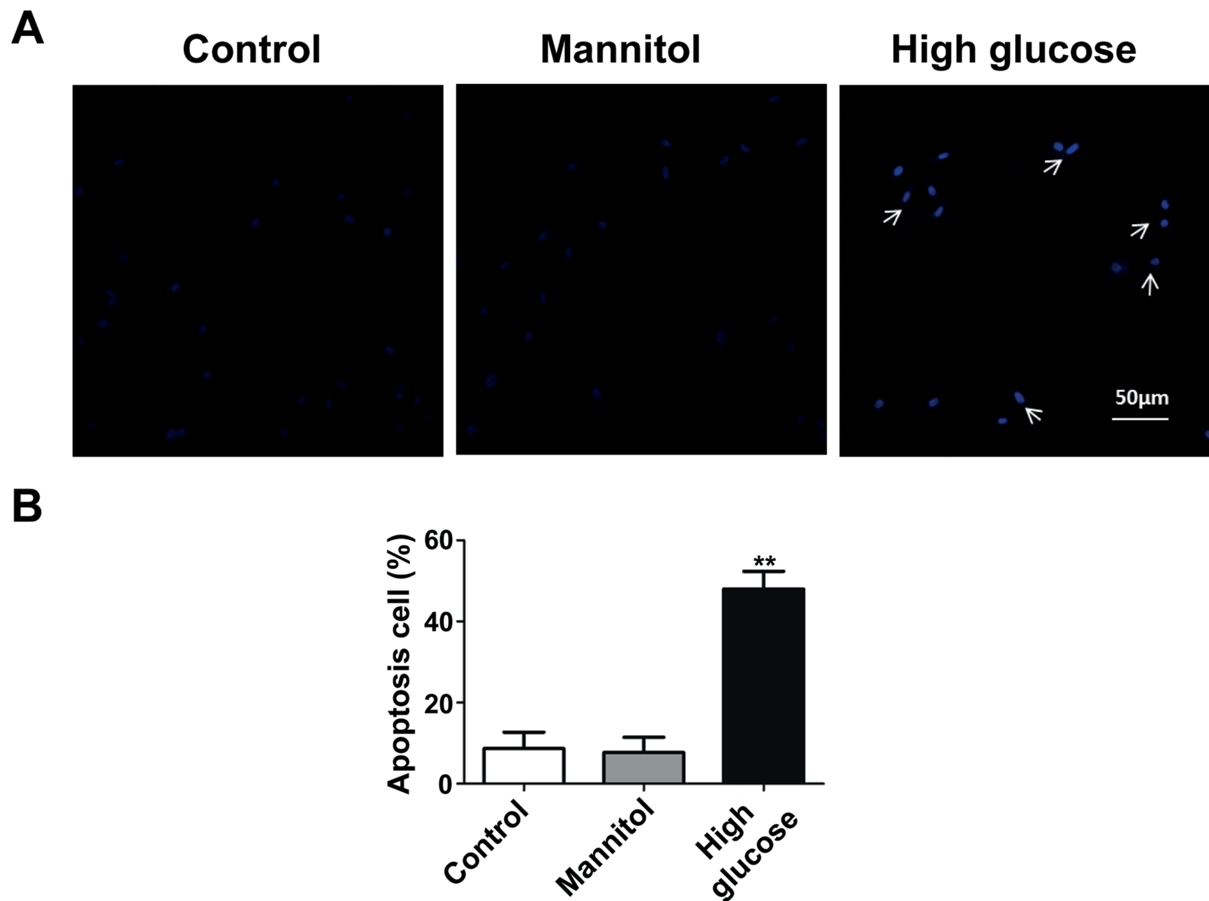

Figure 2. Apoptosis level in cells detected through Hoechst 33258 staining assay. ***(A)*** Micrographs (magnification: 100×), and ***(B)*** histogram. Compared with that in control group, the apoptosis level in the cells is evidently elevated in high glucose group, scale bar=50 µm, $^{**}p<0.01$ *vs.* control group.

Discussion

The Nrf2/ARE signaling pathway, a hotspot in the studies of the mechanism of the proliferation and apoptosis of tumor cells, is a crucial endogenous antioxidant pathway, which regulates the expression and roles of peroxides and antioxidants *in vivo* and participates in the antioxidant damage process of multiple cells[11,12]. Nrf2 triggers the expression of the target genes of the antioxidant enzymes, induces the production of the downstream detoxification enzymes, and regulates the oxidative damage of the body[13]. High glucose, which is metabolized by glycolysis in the body, affects energy metabolism in the body, increases the ROS level, destroys the stability of cellular DNA, and also affects the proliferation of the cells[14]. Zu et al[15] proved that the proteomic research of high-glucose-activated endothelial microparticles and related proteins is associated with Alzheimer's disease.

In this study, *in vitro* high glucose prostate cancer cell line LNCaP was established to explore the effects of high glucose on the proliferation and apoptosis of the cells. The results showed that high glucose significantly promoted the apoptosis of prostate cancer cells and inhibited cell proliferation, while isotonic mannitol had no influence on cell proliferation. This study further demonstrated that high glucose induction treatment overtly elevated the expression levels of apoptosis-related proteins Caspase-3 and Bax in prostate cancer cells and decreased the expression level of Bcl-2 protein. Besides, it was found in this study that high glucose promoted the release of ROS in the cells, so the LDH content in the cells was significantly increased. Zhang et al[16] have revealed that high glucose induces retinal pigment epithelium

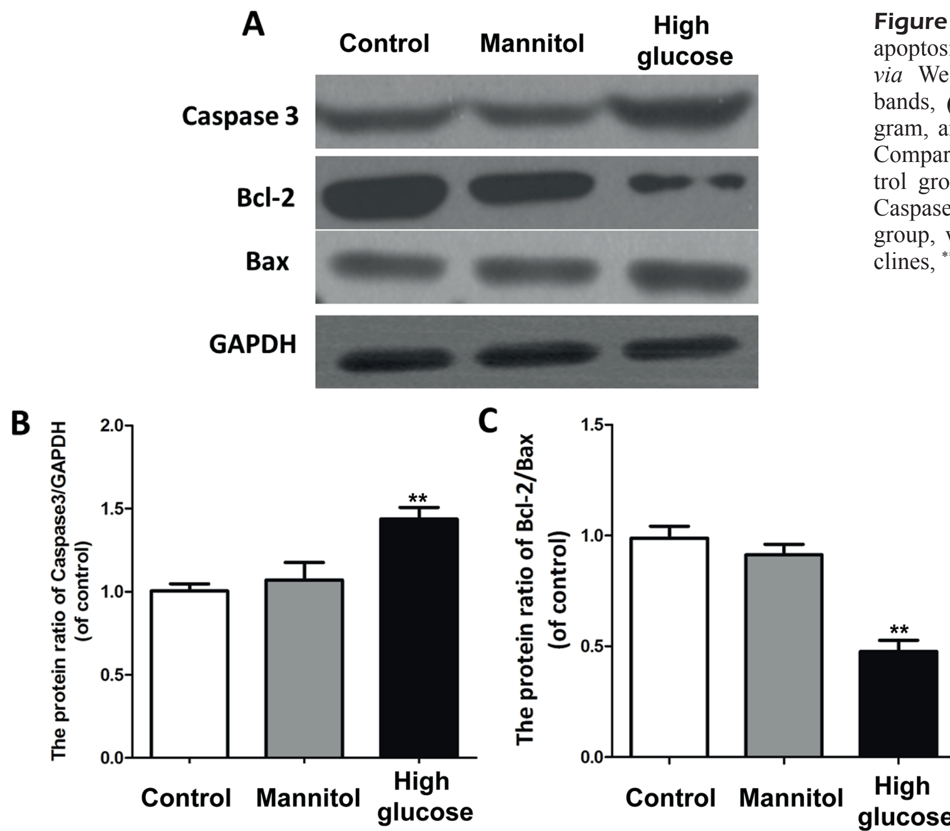

Figure 3. Expression levels of apoptosis-related proteins measured *via* Western blotting. ***(A)*** Protein bands, ***(B)*** Caspase3 protein histogram, and C: Bcl-2/Bax histogram. Compared with those in the control group, the expression level of Caspase3 is raised in high glucose group, while that of Bcl-2/Bax declines, $^{**}p<0.01$ *vs.* control group.

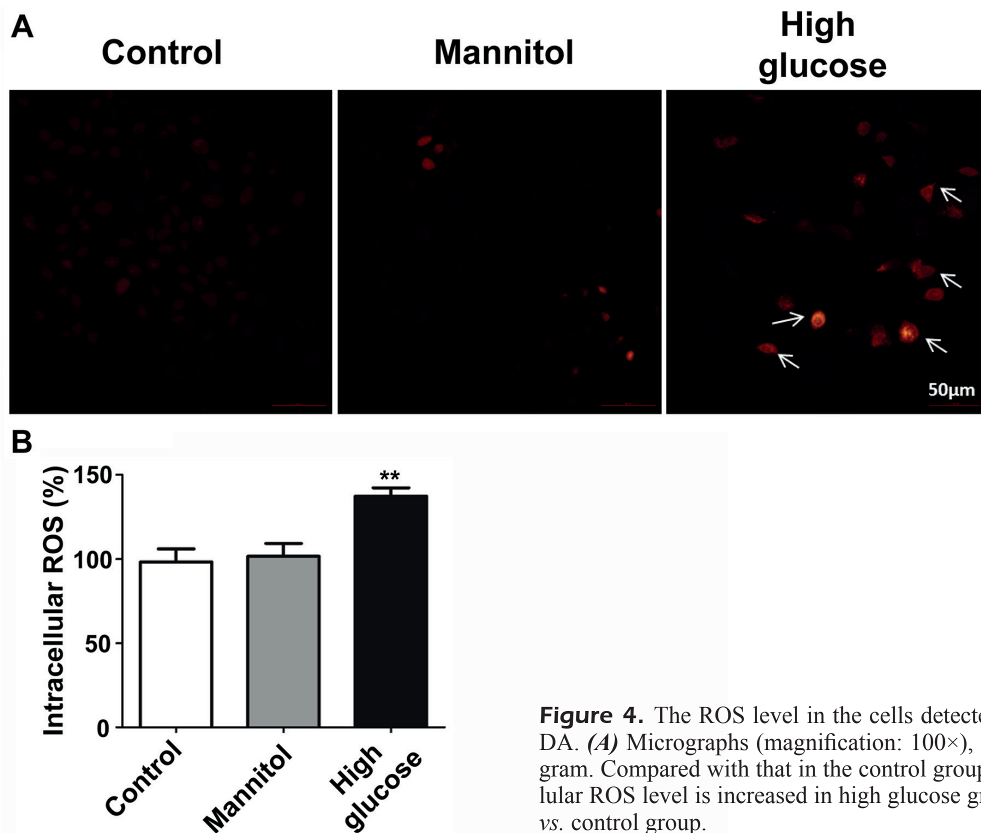

Figure 4. The ROS level in the cells detected *via* DCFH-DA. ***(A)*** Micrographs (magnification: 100×), and ***(B)*** histogram. Compared with that in the control group, the intracellular ROS level is increased in high glucose group, $^{**}p<0.01$ *vs.* control group.

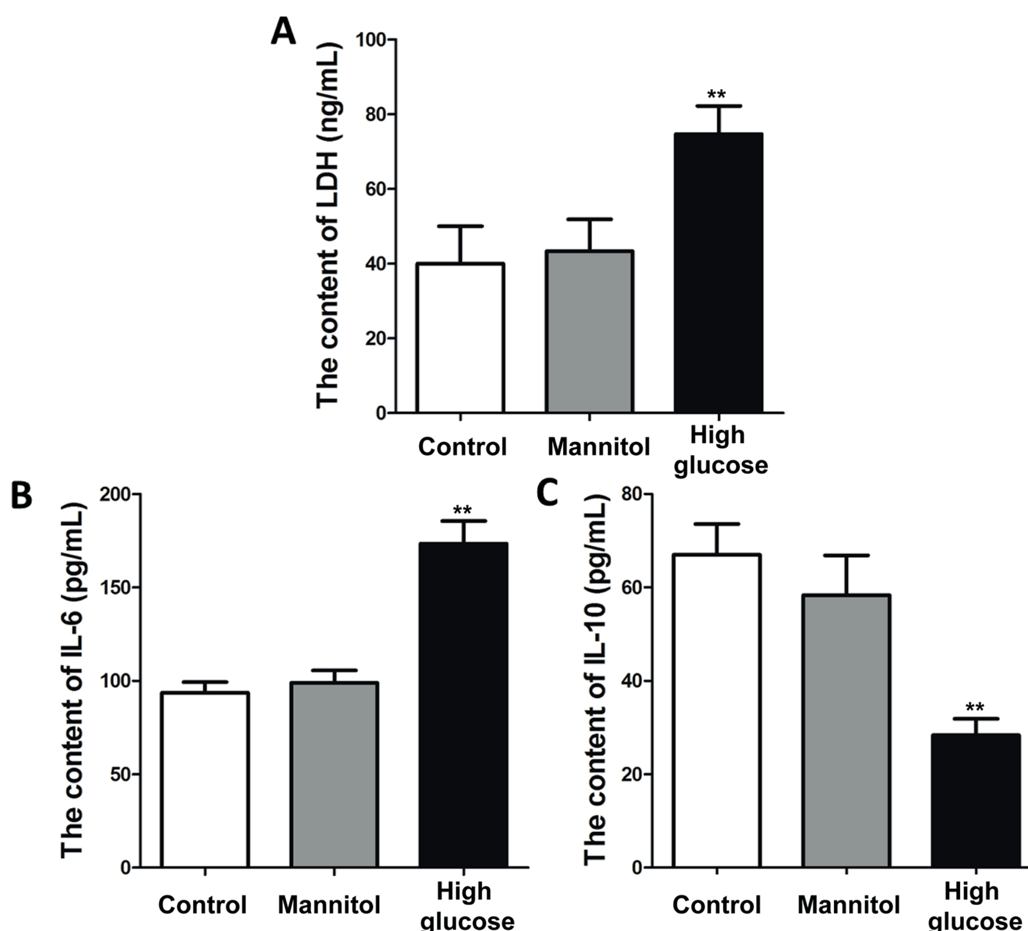

Figure 5. The content of LDH, IL-6, and IL-10 in the cells determined through ELISA. ***(A)*** The content of LDH, ***(B)*** the content of IL-6, and ***(C)*** the content of IL-10. Compared with that in the control group, the content of LDH and IL-6 in the cells is increased in high glucose group, while that of IL-10 is lowered, $^{**}p<0.01$ *vs.* control group.

mitochondrial pathways of apoptosis and inhibits mitophagy by regulating ROS/PINK1/Parkin signal pathway. Luo et al[17] have discovered that ROS is closely related to the occurrence of the inflammatory response *in vivo*, and that the high-concentration ROS in the cells triggers the occurrence of the downstream inflammatory responses, promotes the expression of the inflammatory factors IL-6 and IL-1β, and reduces the content of anti-inflammatory factor IL-10, which is consistent with the results of this study.

Many studies[18] have manifested that the natural products including curcumin and resveratrol are capable of activating the Nrf2/ARE signaling pathway, relieving oxidative tissue damage, and playing an anti-inflammatory role. Shi et al[19] suggested that HO-1 and γ-GCS protect the cells through antioxidant reaction, that is, Nrf2/ARE signal is closely related to the proliferation and apoptosis of the cells. Activated Nrf2/ARE signal promotes the expression of the downstream protective gene γ-GCS and serves as a cell protector. HO-1, an antioxidant enzyme, is an important participant in the role of the Nrf2/ARE signaling pathway, and the increased HO-1 expression can significantly suppress oxidative stress response[20]. This study found that high glucose promoted the apoptosis of tumor cells, and inhibited cell proliferation and Nrf2 expression, further leading to significant decreases in the expression levels of the downstream target proteins HO-1 and γ-GCS. In this study, mannitol isotonic to high glucose was selected as a control, and the results showed that the proliferation and apoptosis of cells and the expression levels of Nrf2/ARE signaling pathway-related proteins in the mannitol group were

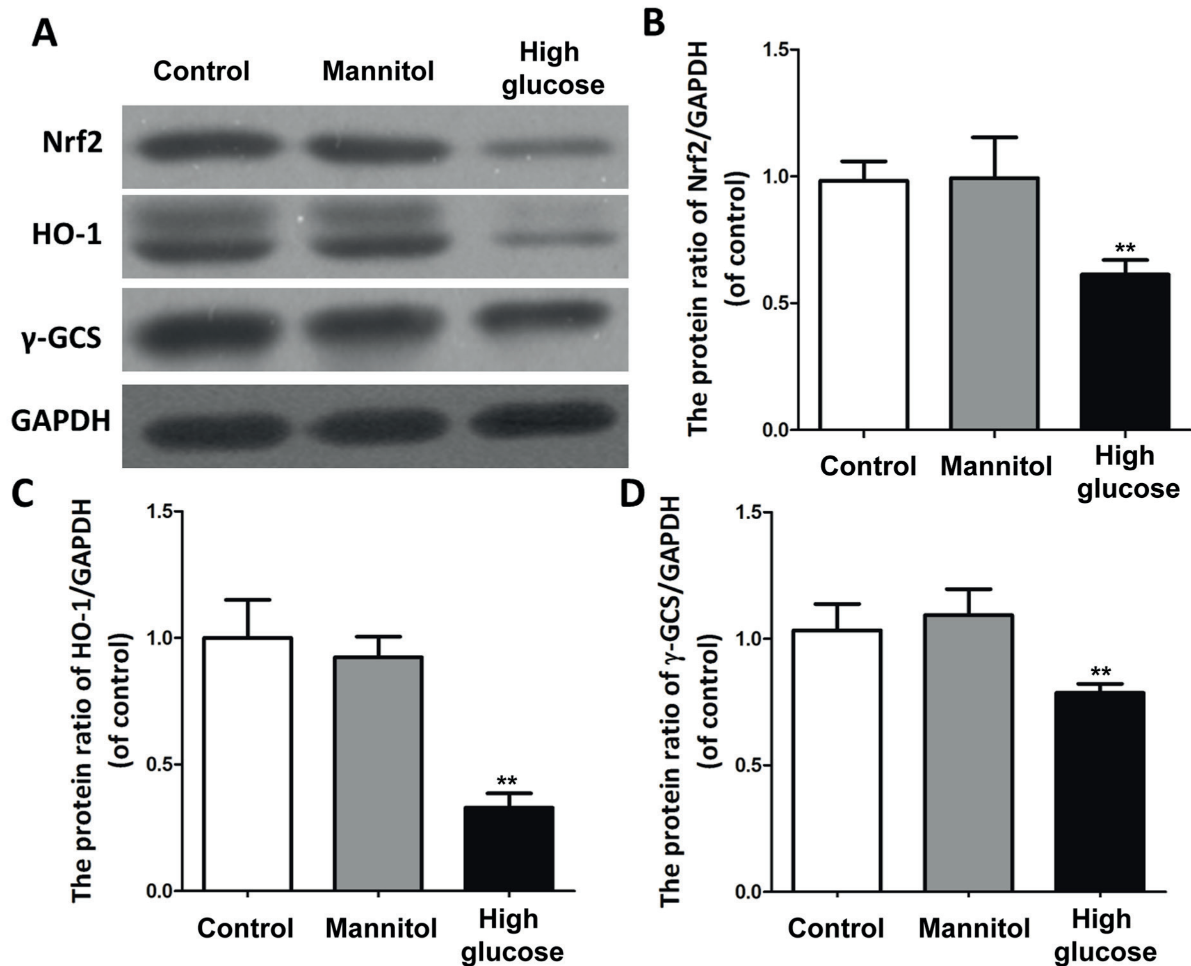

Figure 6. The expression levels of the Nrf2/ARE signaling pathway-related proteins measured *via* Western blotting. ***A,*** Protein bands, ***(B)*** Nrf2 protein histogram, ***(C)*** HO-1 protein histogram, and ***(D)*** γ-GCS protein histogram. High glucose notably lowers the protein expression levels of Nrf2, HO-1, and γ-GCS, $^{**}p<0.01$ *vs.* control group.

comparable to those in the control group. The above results imply that high glucose represses the activation and the anti-oxidant and anti-inflammatory effects of the Nrf2/ARE signaling pathway in prostate cancer cells, thus resulting in the apoptosis of prostate cancer cells.

Conclusions

We proved from multiple aspects that high glucose was able to inhibit the activation of the Nrf2/ARE signaling pathway in prostate cancer cells, increased the content of intracellular ROS and the pro-inflammatory cytokine IL-6, decreased the content of IL-10, and elevated the expression levels of the apoptotic proteins in the cells, thereby facilitating the apoptosis of prostate cancer cells, which provides a theoretical basis for the treatment of prostate cancer in clinical practice.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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