PGAM1 knockdown is associated with busulfan-induced hypospermatogenesis and spermatogenic cell apoptosis

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Abstract. Phosphoglycerate mutase 1 (PGAM1) is reported to be involved in spermatogenic dysfunction. However, the association between PGAM1 and busulfan-induced hypospermatogenesis and spermatogenic cell apoptosis remains unclear. The aim of the current study was to investigate the association between PGAM1 expression and busulfan-induced hypospermatogenesis, and the effect of PGAM1 expression on spermatogenic cell apoptosis. PGAM1 expression was detected in mouse models of busulfan-induced hypospermatogenesis by western blotting, reverse transcription-quantitative polymerase chain reaction and immunohistochemistry. Then, spermatogenic cell apoptosis in mouse models of busulfan-induced hypospermatogenesis was assessed by TUNEL assay. The effect and potential mechanism of PGAM1 downregulation on spermatogenic cells were further investigated. The results indicated that PGAM1 expression was significantly downregulated in the mouse models of busulfan-induced hypospermatogenesis, compared with those with normal spermatogenesis (P<0.05). Furthermore, the TUNEL assay revealed that the apoptosis of spermatogenic cells was accelerated in the mouse model of busulfan-induced hypospermatogenesis. In addition, PGAM1 knockdown promoted the apoptosis of spermatogenic cells *in vitro*, which was associated with the P53/Caspase 3/Caspase 6/Caspase 9 signaling pathway. In conclusion, these data indicate that PGAM1 knockdown is associated with busulfan-induced hypospermatogenesis and contributes to spermatogenic cell apoptosis by regulating the P53/Caspase 3/Caspase 6/Caspase 9 signaling pathway.

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Introduction

Phosphoglycerate mutase 1 (PGAM1) is an important glycolytic enzyme that catalyzes the conversion of 3-phosphoglycerate into 2-phosphoglycerate in the glycolytic pathway (1,2). PGAM1 is normally expressed in the brain, liver and kidney tissues (3,4). PGAM1 overexpression has previously been observed in multiple human cancer types, including breast carcinoma, lung cancer, hepatocellular carcinoma, oral squamous cellular carcinoma and urothelial bladder cancer (5-9). Furthermore, PGAM1 has been reported to be associated with the migration, proliferation and apoptosis of tumor cells (10-13).

PGAM1 has also been reported to be associated with autoimmune central nervous system diseases (14). The current group reported that PGAM1 is involved in spermatogenic dysfunction and affected cell proliferation, apoptosis and migration (15). However, the effect and mechanism of PGAM1 knockdown on spermatogenic cells remains unclear. In the current study, the aim was to investigate the association between PGAM1 expression and busulfan-induced hypospermatogenesis and the effect of PGAM1 knockdown on spermatogenic cell apoptosis. Firstly, PGAM1 expression was detected in a mouse model of busulfan-induced hypospermatogenesis by western blotting, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC). Then, spermatogenic cell apoptosis was assessed by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. Finally, the effect and potential mechanism of PGAM1 downregulation on spermatogenic cells was explored.

Materials and methods

Animal model construction. A total of 10 C57 male mice weighing 18-21 g were purchased and raised in Guangzhou Medical University Animal Center (Guangzhou, China). All mice were housed under standard conditions (21±2˚C, 40% humidity and 12-h light/dark cycle), and had free access to water and food. The procedures were performed as described previously (15,16). Briefly, mouse models of hypospermatogenesis (N=5) were established by a single intraperitoneal injection of busulfan (30 mg/kg). Normal controls (N=5) didn't

receive any treatment. Following 2 weeks, fresh testis tissues were collected and stored in liquid nitrogen or fixed with 1.25% Bouin solution for 6 h at room temperature. The current study was approved by the Guangzhou Medical University Institutional Animal Care and Use Committee.

Hematoxylin and eosin (HE) staining. Testis tissues were fixed with Bouin's solution for 6 h at room temperature and embedded in paraffin. Sections (2-μm-thick) were heated at 65°C for 4 h, deparaffinized with dimethylbenzene, rehydrated in gradient ethanol series and stained with HE for 5 min at room temperature. Under the light microscope, reduced production of spermatozoid in seminiferous tubules was easily observed in mouse models of hypospermatogenesis by counting the number of sperm cells.

RT-qPCR. Total RNA was extracted from testis tissues and spermatogenic cells using RNA TRIzol reagent (Takara Bio, Inc., Otsu, Japan) and synthesized using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.) at 37°C for 15 min. qPCR analysis was performed using the SYBR Green RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's protocol. The primers of PGAM1 and GAPDH were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The following primers were used: PGAM1, forward (F) 5'-ATG CTAAGCCATGACCAGTGAG-3' and reverse (R) 5'-ATC ACCACGCAGGTTACATTCG-3'; GAPDH, F 5'-AGGTCG GTGTGAACGGATTTG-3' and R 5'-TGTAGACCATGTAGT TGAGGTCA-3'. Cycling conditions were 95°C for 5 min, followed by 40 cycles at 95°C for 40 sec, 60°C for 30 sec and 72°C for 30 sec. Experiments were repeated three times for each reaction. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative mRNA expression levels (16).

Western blot analysis. Total protein lysates were extracted from testis tissues and spermatogenic cells using cell lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology, Shanghai, China) containing a protease inhibitor cocktail (cat. no. P1006; Beyotime Institute of Biotechnology) and 100 nM phenylmethanesufonyl fluoride. Protein concentration was determined by bicinchoninic acid assay. Subsequently, 50 μg protein was separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were dipped into PBS with 0.1% Tween 2000 (BestBio, Shanghai, China) solution and blocked with 5% skimmed milk at room temperature for 2 h, and then incubated with rabbit monoclonal PGAM1 antibody (cat. no. ab129191; 1:1,000; Abcam, Cambridge, MA, USA), rabbit polyclonal P53 antibody (cat. no. ab16033; 1:2,000; Abcam), rabbit polyclonal Caspase 3 antibody (cat. no. ab13847; 1:500; Abcam), rabbit polyclonal Caspase 6 antibody (cat. no. ab231349; 1:500; Abcam), rabbit poly-clonal Caspase 9 antibody (cat. no. ab52298; 1:500; Abcam) and monoclonal tubulin antibody (cat. no. ab6160; 1:2,000; Abcam), respectively. Finally, polyvinylidene difluoride membranes were incubated with a rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (cat. no. ZB-2306; 1:1,000; OriGene Technologies, Inc., Beijing, China) at room temperature for 30 min. Signals were detected using an EasyBlot ECL kit (cat. no. C506668-0050; Sangon Biotech

Co., Ltd., Shanghai, China) and an enhanced chemilumines-cence detecter (ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

IHC. Testis tissues were fixed with Bouin's solution for 6 h at room temperature and embedded in paraffin. Sections (2-μm-thick) were deparaffinized with dimethylbenzene and rehydrated with gradient ethanol. Antigen retrieval was performed with 0.01 mol/l sodium citrate buffer (pH 7.0). The sections were incubated with rabbit monoclonal PGAM1 antibody (cat. no. ab129191; 1:150; Abcam) for 2 h, followed by incubation with 50 μl horseradish peroxidase-labeled goat anti-mouse/rabbit IgG polymer (cat. no. PV-6000; OriGene Technologies, Inc.) for 30 min at room temperature. Finally, sections were reacted with diaminobenzidine at room temper-ature for 1-3 min and stained with hematoxylin for 3 min at room temperature. Negative controls were performed by replacing the primary antibody with PBS. Sections with posi-tive expression of PGAM1 were treated as positive controls.

TUNEL assay. The procedures were performed as described previously (17). Testis tissues were fixed with Bouin's solu-tion for 12 h at room temperature and embedded in paraffin. Sections (3-μm-thick) were deparaffinized with dimethylben-zene and rehydrated with gradient ethanol. Then, sections were incubated with 1% proteinase K (20 mg/ml) at 37°C for 30 min. Following washing with PBS, sections were incu-bated with TUNEL mix (45 μl Equilibration Buffer, 1.0 μl biotin-11-dUTP, 4.0 μl TdT enzyme and 50 μl reaction buffer) at 37°C for 60 min. Finally, sections were stained with DAPI for 5 min at room temperature and mounted in 50% glycerin diluted in water. A total of 10 randomly chosen microscopic fields were analyzed under a fluorescence microscope (magni-fication, x40). The nuclei of apoptotic cells were marked as red under fluorescence microscopy at 570 nm.

Cell culture and transfection. GC-1 spg cells (a mouse sper-matogonia cell line) and GC2 cells (a mouse spermatocyte cell line) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO_2 incubator. Cells at 40-60% confluence were transfected with PGAM1 short hairpin (sh)RNA (sense, 5'-ATGCTAAGCCATGACCAGTGAG-3' and anti-sense, 5'-ATCACCACGCAGGTTACATTCG-3') lentivirus and recombinant PGAM1 lentivirus, respectively. The negative control was transfected with scrambled shRNA lentivirus (sense, 5'-UUCUCCGAACGUGUCACGU-3' and anti-sense, 5'-AAGAGGCUUGCACAUGCA-3').

Apoptosis analysis. At 48 h following transfection, 1x10^6 GC1 and GC2 cells were collected and washed with PBS. Then, cells were incubated with propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and Annexin V-FITC (BestBio) at room temperature for 15 min. Cell apoptosis was analyzed using a BD FACSCalibur™ flow cytometer and BD CellQuest™ software version 5.1 (BD Biosciences, Franklin Lakes, NJ, USA).
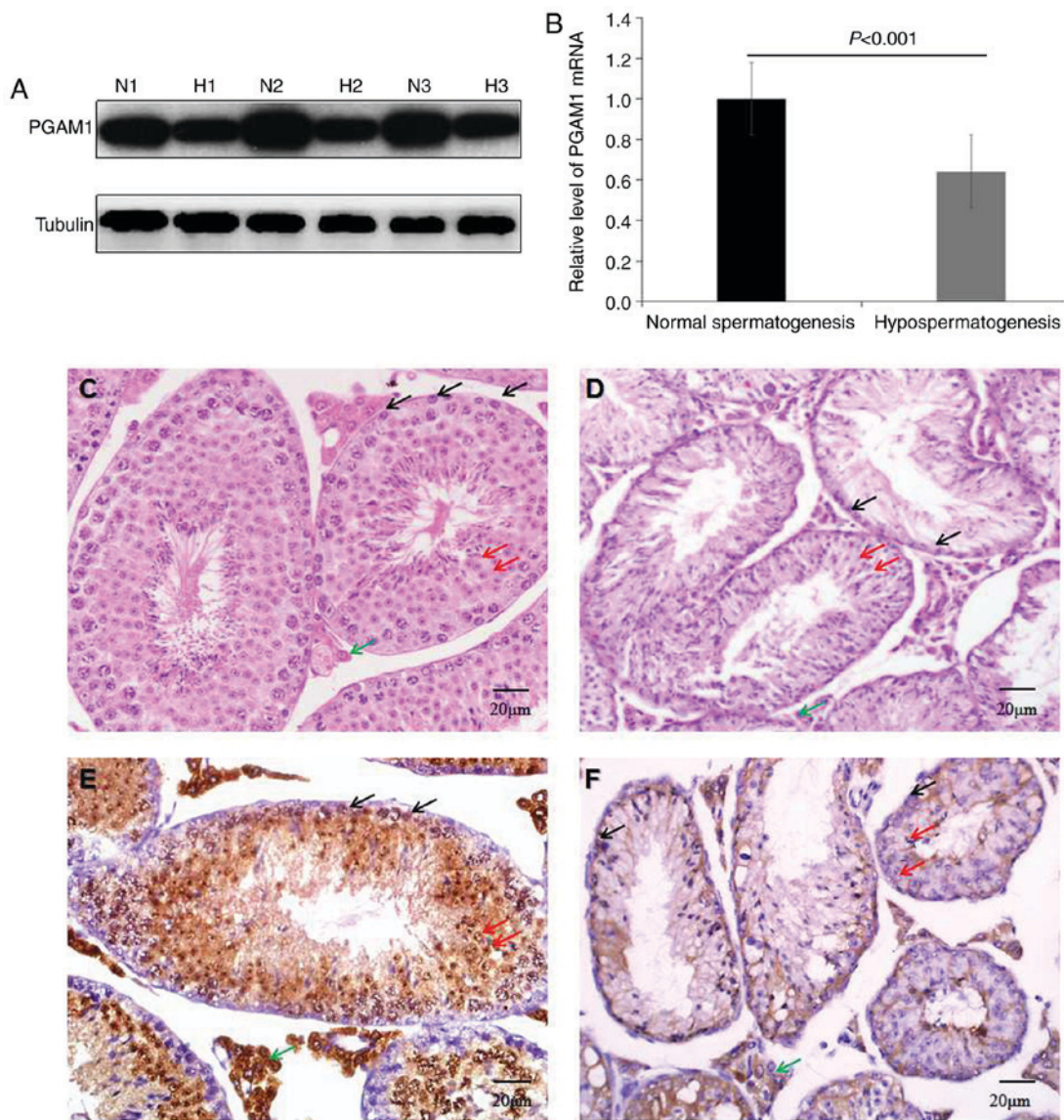
Figure 1. PGAM1 downregulation was associated with busulfan-induced hypospermatogenesis. (A) Representative western blots of PGAM1 expression in testis tissues with hypospermatogenesis and normal spermatogenesis. (B) PGAM1 expression in testis tissues with hypospermatogenesis and normal spermatogenesis was detected by reverse transcription-quantitative polymerase chain reaction. (C) HE staining of test tissues with normal spermatogenesis (magnification, x40). (D) HE staining of test tissues with hypospermatogenesis (magnification, x40). (E) PGAM1 expression in testis tissues with normal spermatogenesis was detected by immunohistochemistry (magnification, x40). (F) PGAM1 expression in testis tissues with hypospermatogenesis was detected by immunohistochemistry (magnification, x40). Black arrows indicate spermatogonia. Red arrows: Spermatocytes. Green arrows: Leydig cells. HE, hematoxylin and eosin; PGAM1, phosphoglycerate mutase 1. N, negative control; H, hypospermatogenesis.

Statistical analysis. All experiments were repeated three times and the data are presented as the mean ± standard deviation. All data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Independent samples t test was used to compare the difference between two groups. All tests were two-sided and P<0.05 was considered to indicate a statistically significant difference.

Results

PGAM1 downregulation is associated with busulfan-induced hypospermatogenesis and spermatogenic cell apoptosis. To investigate the association between PGAM1 expression and hypospermatogenesis, the expression of PGAM1 was detected in mouse models of busulfan-induced hypospermatogenesis by

western blotting, RT-qPCR and IHC (Fig. 1). Western blotting (Fig. 1A) and RT-qPCR (Fig. 1B) results revealed that PGAM1 expression levels were significantly decreased compared with normal controls (P<0.001). HE staining demonstrated that the number of spermatogonia, spermatocytes and spermatozoid in seminiferous tubules was decreased in mouse models of busulfan-induced hypospermatogenesis compared with the controls (Fig. 1C and D). IHC results demonstrated that positive PGAM1 expression, visible as brown staining, was present in spermatogonia, spermatocytes and Leydig cells in the testis tissues with normal spermatogenesis (Fig. 1E), while expression was hardly detected in mouse models of busulfan-induced hypospermatogenesis (Fig. 1F). These data indicated that PGAM1 downregulation was associated with busulfan-induced hypospermatogenesis.
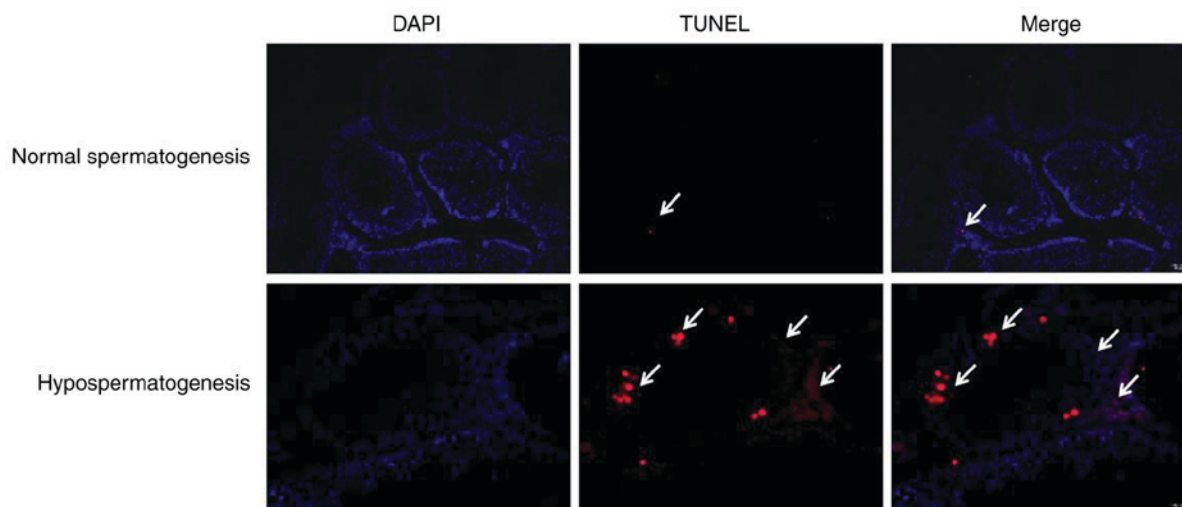
Figure 2. Apoptosis of testis tissues with hypospermatogenesis and normal spermatogenesis was evaluated by TUNEL assay. TUNEL, Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.
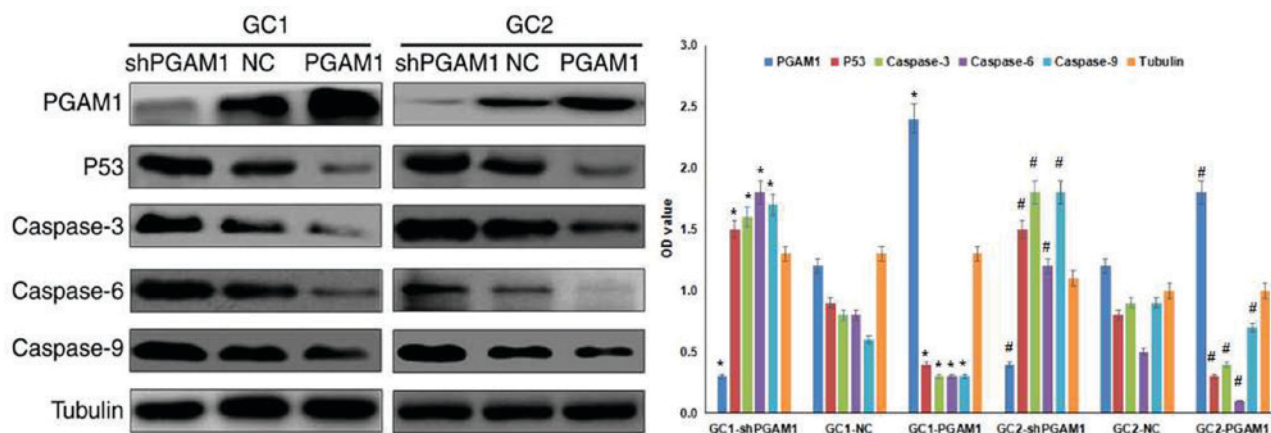

Figure 3. PGAM1 knockdown activated the P53/Caspase 3/Caspase 6/Caspase 9 signaling pathway. PGAM1, phosphoglycerate mutase 1; shRNA, short hairpin RNA; NC, negative control; PI propidium iodide. *P<0.001 vs. GC1-NC; #P<0.001 vs. GC2-NC.

To investigate the association between PGAM1 expression and spermatogenic cell apoptosis in a mouse model of busulfan-induced hypospermatogenesis, a TUNEL assay was performed. As presented in Fig. 2, the apoptosis rate of spermatogenic cells was accelerated in mouse models of hypospermatogenesis compared with normal spermatogenesis. These data indicated that PGAM1 downregulation may be associated with spermatogenic cell apoptosis in mouse models of busulfan-induced hypospermatogenesis.

PGAM1 knockdown promotes spermatogenic cell apoptosis by regulating the P53/Caspase 3/Caspase 6/Caspase 9 pathway. To further investigate the association between PGAM1 expression and spermatogenic cell apoptosis *in vitro*, a flow cytometry assay was performed. Firstly, spermatogenic cells were infected with PGAM1 shRNA lentivirus or recombinant PGAM1 lentivirus. As presented in Fig. 3, western blotting results indicated that PGAM1 was successfully knocked down and upregulated following transfection, respectively. Then, cell apoptosis was measured by flow cytometry (Fig. 4). The rate of apoptosis was 15.7±2.3% in GC1 cells with PGAM1

downregulation, which was significantly higher compared with the negative controls (4.8±0.9%; P<0.001). The rate of apoptosis was 2.2±0.6% in GC1 cells with PGAM1 upregulation, which was significantly reduced compared with negative controls (4.8±0.9%; P<0.001). In addition, the rate of apoptosis was 16.4±2.8% in GC2 cells with PGAM1 knockdown, which was significantly greater compared with negative controls (5.9±1.1%; P<0.001). When PGAM1 expression was upregulated in GC2 cells, the rate of apoptosis was 2.3±0.5%, which was significantly reduced compared with negative controls (5.9±1.1%; P<0.001). These data demonstrated that PGAM1 knockdown contributed to spermatogenic cell apoptosis.

To investigate the mechanism of PGAM1, the expression levels of P53, Caspase 3, Caspase 6 and Caspase 9 were detected by western blotting. As presented in Fig. 3, the expression levels of P53, Caspase 3, Caspase 6 and Caspase 9 were significantly elevated following PGAM1 knockdown. However, PGAM1 upregulation produced the opposite effects. These data indicated that PGAM1 knockdown activated the P53/Caspase 3/Caspase 6/Caspase 9 signaling pathway, which may result in spermatogenic cell apoptosis.
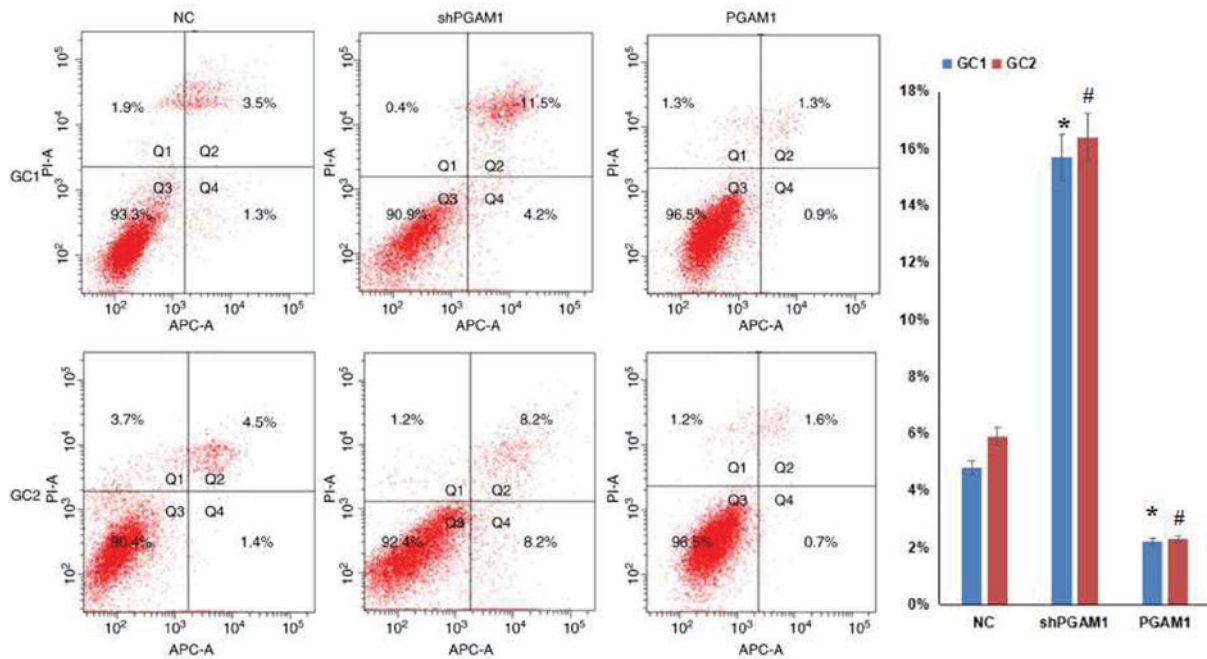
Figure 4. PGAM1 expression affected the apoptosis of spermatogenic cells. Apoptosis of spermatogenic cells was measured by flow cytometry. PGAM1, phosphoglycerate mutase 1; shRNA, short hairpin RNA; NC, negative control. [*]P<0.001 vs. GC1-NC; [#]P<0.001 vs. GC2-NC.

Discussion

Hypospermatogenesis is prevalent in azoospermic patients and is associated with male infertility. It is characterized by a lower production of spermatozoids in seminiferous tubules (18,19). It is well known that abnormal apoptosis of spermatogenic cells is implicated in hypospermatogenesis (20-23). PGAM1, an important glycolytic enzyme, is reported to be associated with the migration, proliferation and apoptosis of tumor cells (10-13). Furthermore, PGAM1 is involved in spermatogenic dysfunction and affects cell proliferation, apoptosis and migration (15). Therefore, it has been demonstrated that PGAM1 may be associated with hypospermatogenesis and spermatogenic cell apoptosis. However, the mechanism of PGAM1 in cell apoptosis remains unclear.

In the current study, the association between PGAM1 expression and busulfan-induced hypospermatogenesis was investigated. First, PGAM1 expression was detected in a mouse model of busulfan-induced hypospermatogenesis by western blotting, RT-qPCR and IHC. Western blotting and RT-qPCR results revealed that PGAM1 expression was reduced in mice with busulfan-induced hypospermatogenesis compared with those with normal spermatogenesis. IHC results indicated that PGAM1 was weakly expressed in the mouse model of hypospermatogenesis, which was consistent with a previous report (15). These data supported the proposal that PGAM1 downregulation was associated with busulfan-induced hypospermatogenesis. To investigate the association between PGAM1 and hypospermatogenesis and spermatogenic cell apoptosis, a TUNEL assay was performed. The results demonstrated that spermatogenic cell apoptosis was accelerated indicating that PGAM1 downregulation may be associated with spermatogenic cell apoptosis in a mouse model of busulfan-induced hypospermatogenesis. To further validate the association between PGAM1 expression

and spermatogenic cell apoptosis, PGAM1 expression was successfully knocked down and upregulated in spermatogenic cells. Subsequently, flow cytometry results demonstrated that PGAM1 downregulation promoted the apoptosis of spermatogenic cells. Furthermore, apoptotic proteins, including P53, Caspase 3, Caspase 6 and Caspase 9, were activated when PGAM1 expression was knocked down. P53, an important apoptotic protein, participates in cell cycle arrest, senescence and apoptosis of spermatogenic cells (24-26). The activation of p53 signaling may induce mitochondria-associated apoptotic cell death and disrupt sperm production and fertility (27,28). In addition, caspase-mediated apoptosis is involved in normal spermatogenesis (17). Caspase 3 and Caspase 6 may be activated by Caspase 9 to cause cell apoptosis (29,30). Therefore, these data indicated that PGAM1 knockdown promoted spermatogenic cell apoptosis by regulating the P53/Caspase 3/Caspase 6/Caspase 9 pathway.

In conclusion, the current data indicates that PGAM1 knockdown is associated with busulfan-induced hypospermatogenesis and spermatogenic cell apoptosis. Furthermore, spermatogenic cell apoptosis caused by PGAM1 knockdown is associated with the P53/Caspase 3/Caspase 6/Caspase 9 pathway. However, the current data are not sufficient to explain the detailed mechanism of PGAM1 in spermatogenic cell apoptosis. Therefore, further investigations in this area are required.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and SZ designed the experiments; YZ performed in the experiments; SZ analyzed the data and wrote the paper.

Ethics approval and consent to participate

The present study was approved by the Guangzhou Medical University Institutional Animal Care and Use Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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