

# Pioglitazone Protect PC12 Cells Against Oxidative Stress Injury: Involvement of Anti-apoptotic Effect and PPAR $\gamma$ Activation

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## Research

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# Abstract

**Objectives:** Using a PC12 cell model with Hydrogen peroxide, we investigated the neuronal apoptotic gene expression and neuronal apoptosis after oxidative stress damage. We further explored protective effect of pioglitazone and its mechanisms.

**Methods:** Taking  $H_2O_2$  treated PC12 cells as oxidative stress damaged neuron models, MTT and flow cytometry methods were performed to measure the influence of  $H_2O_2$  on neuronal apoptosis and the protective effect of pioglitazone. Neuronal apoptosis was detected by TUNEL staining. Real-time PCR and Western blot methods were performed to investigate the expression of PPAR $\gamma$ , Bax, Bcl-2 and Caspase-3.

**Results:**  $H_2O_2$  can induce the apoptosis of PC12 cells in an dose- and time-dependent manner. And  $H_2O_2$  (100 $\mu$ mol/L, 24h) can induce the expression of PPAR $\gamma$  mRNA and protein ( $p < 0.01$ ). Pioglitazone significantly up-regulated the protein expression of Bax, caspase-3 ( $p < 0.01$ ) and decreased the expression of Bcl-2 ( $p < 0.01$ ). Pioglitazone can dose-dependently decrease the apoptotic ratio of  $H_2O_2$ -damaged PC12 cells.  $1.0 \times 10^{-6}$  mol/L pioglitazone can induce PPAR $\gamma$  mRNA and protein expression. Pioglitazone decreased Bax, caspase-3 protein expression ( $p < 0.01$ ) and increased Bcl-2 protein expression ( $p < 0.01$ ), thus down-regulated the expression ratio of Bax/Bcl-2 ( $p < 0.01$ ) and decreased the apoptotic ratio of PC12 cells ( $p < 0.01$ ). GW9662, the antagonist of PPAR $\gamma$ , and PPAR $\gamma$  siRNA can offset the protective effect of pioglitazone on PC12 cells to different degrees ( $p < 0.01$ ).

**Conclusions:** hydrogen peroxide can induce apoptosis of PC12 cells in dose- and time-dependent manner. PPAR $\gamma$  activation by pioglitazone can significantly decreased expression of Bax/Bcl-2 and Caspase-3, thus plays a part in neuron protective effects on  $H_2O_2$ -treated PC12 cells. The antagonist and RNAi of PPAR $\gamma$  can offset protective effect of pioglitazone to different degree, which indicates PPAR $\gamma$  activation exerts protective role in decreasing the apoptosis of PC12 cells.

## 1 Introduction

Progressive neuronal loss is a major pathological feature of neurodegenerative diseases, in which oxidative stress plays an important role in neuronal apoptosis<sup>[1]</sup>. Neurons contain polyunsaturated fatty acids which are sensitive to free radicals. They are easily attacked by free radicals and have low content of antioxidant enzymes. Therefore, the antioxidant capacity of neurons is significantly reduced. These factors together promote the sensitivity of neurons to oxidative stress injury<sup>[2, 3]</sup>. How to effectively reduce oxidative stress injury on neurons has become a hot topic in recent years. PC12 cells which have the properties of neurosecretory cells and neurons, and have high stability, homogeneity and high degree of differentiation are currently widely used in the study of nerve cell function, differentiation, development and death of the cell model<sup>[4]</sup>. Previous studies have confirmed that  $H_2O_2$  can induce PC12 cell injury<sup>[5]</sup> and the expression and activation of apoptosis-related gene Caspase-3<sup>[6]</sup>. Thiazolidinediones (TZD) are ligands known to bind to and activate the nuclear peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), and are currently used as insulin sensitizers in type 2 diabetes. Our previous studies have shown

that PPAR $\gamma$  agonist, rosiglitazone may protect neuronal microenvironment and preserve nerve cells in the CA1 subfield of hippocampi of SHRs through antioxidative and anti-apoptotic pathways, which was independent of blood pressure control<sup>[7]</sup>. However, the underlying mechanism needs to be further studied. In this study, the oxidative stress injury model of PC12 cells induced by H<sub>2</sub>O<sub>2</sub> was used to observe whether pioglitazone has neuroprotective effect and to detect the underlying mechanism.

## 2 Materials And Methods

### 2.1 Cell culture and preconditioning protocols

PC12 rat pheochromocytoma cells were obtained from the institute of neurobiology, school of medicine, xi'an Jiaotong university. The cells were plated at a density of 3x10<sup>5</sup> cells/well in 6-well plates maintained in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C under an atmosphere of 5% CO<sub>2</sub> and 95% air. The culture media were changed three times per week. PC12 cells were preconditioned with 0, 6h, 12h, 24h, 36h and 48h exposure of different concentrations (0, 50, 100, 200 and 400  $\mu$ mol/L) of H<sub>2</sub>O<sub>2</sub>.

### 2.2 MTT assay

The viability of cell cultures was estimated using MTT. The MTT assay relies primarily on mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state. After incubation, Cells were incubated with MTT solution (0.5mg/ml in PBS) for 4h at 37°C. Following this incubation, the MTT solution was removed from the plate and left to dry. DMSO (100  $\mu$ l) was then added to each well to dissolve the formazan crystals before the optical density was measured at 570nm. Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

### 2.3 Detection of apoptotic cells with flow cytometry

Apoptosis was assessed by annexin V-FITC and PI staining followed by analysis with flow cytometry (Beckman-Coulter, USA). The methodology followed the procedures as described in the annexin V-FITC/PI detection kit. The cultured cells were given treatment. Eventually, the cells were resuspended in a 400 $\mu$ l 1xbinding buffer solution with a concentration of 1x10<sup>6</sup> cells/ml, and the cells were stained with 5 $\mu$ l annexin V-FITC and 10 $\mu$ l PI for 15 min at room temperature in the dark. Then the cell suspension was ready for the analysis by the flow cytometry.

### 2.4 Real time Q-PCR

Total RNA was extracted from PC12 cells of different groups with the use of Trizol reagent (Invitrogen) and the RNA samples were transcribed to cDNA with the PrimeScript RT Master Mix kit (TaKaRa) according to the manufacturer's instructions. Real-time RT-PCR was performed with SYBR ExScript RT-PCR Kit (TaKaRa) on an iQ Multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, CA). The primers for rat PPAR $\gamma$  (forward: 5'- GGAGCCTAAGTTTGAGTTTGCTGTG-3', reverse: 5'- TGCAGCAGGTTGTCTTGGATG-3'); Caspase-3 (forward: 5'-GAGACAGACAGTGGAAGTACGATG-3',

reverse: 5'-GGCGCAAAGTACTGGATGA-3'); Bax (forward: 5'- GCGTCCACCAAGAAGCTGA-3', reverse: 5'- ACCACCCTGGTCTTGGATCC-3'); Bcl-2 (forward: 5'- GACTGAGTACCTGAACCGGCATC-3', reverse: 5'- CTGAGCAGCGTCTTCAGAGACA-3') and B-actin (forward: 5'-GGAGATTACTGCCCTGGCTCCTA-3', reverse: 5'- GACTCATCGTACTCCTGCTTGCTG-3') were designed and synthesized by TaKaRa Biotechnology. Amplification was performed at 95°C for 30s, followed by 40 cycles of 95°C for 3s and 60°C for 30s. Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard Edition optical System software (Bio-Rad). Relative quantification by the comparative method ( $2^{-\Delta\Delta C_t}$ ) was used and data was presented as the mean  $\pm$  SD of three separate experiments done in triplicate.

## 2.5 Western blot

PC12 cells were harvested by scraping into ice-cold phosphate buffered saline (PBS), and centrifuged at 800 rpm for 8 min at 4°C. The protein concentrations were determined using a BCA kit (Beyotime Inc, China). Different samples with an equal amount of protein (20 $\mu$ g) were separated on 12% SDS polyacrylamide gels, transferred to PVDF membranes, and blocked in 10% non-fat milks at room temperature for 2h. Membranes were incubated overnight at 4°C with a rabbit polyclonal antibody against PPAR $\gamma$ (1:400, Abcam, UK); rabbit polyclonal antibodies against Bax, Bcl-2 and caspase-3 (1:800, Bioworld, USA). Then they were washed with 1 $\times$ TBST for 3 times, and incubated with horse radish peroxidase (HRP)-coupled secondary antibody for 2h at room temperature. After washing, protein bands were detected with chemiluminescent HRP substrate (SuperSignal West Pico; Thermo scientific Inc, USA) for 5 min at room temperature in dark room and exposed to X-ray film (Fujifilm Inc, JPN). The signal intensity of primary antibody binding was analyzed using Quantity One software 4.6.2 (Bio-Rad Laboratories Inc, USA) and normalized to a loading control  $\beta$ -actin.

## 2.6 TUNEL staining

For TUNEL assay, a cell death detection kit was used ( Promega Inc, USA). PC12 cells were stained after fixation with 4% paraformaldehyde, then incubated with proteinase-K for 20 min at room temperature followed by three washes in PBS. Then sections were covered with equilibration buffer for 10 min at room temperature, followed by incubation with the rTdT incubation buffer (50 $\mu$ l) in dark for 1 h at 37°C. Then slides were immersed in 2 $\times$ SSC buffer for 15 min to stop the reaction and then washed with PBS. Sections were counterstained with 4', 6'-diamino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich) and washed in PBS and mounted. Fluorescence microscopy was carried out using an Olympus BX51 microscope equipped with a mercury lamp power supply. Neurons with bright green nuclei were identified as TUNEL-positive neurons. TUNEL positive cells were normalized to DAPI stained cells. Immunoreactive cells from 9 random fields (3 samples per group, 3 fields per sample) were counted using a 20 objective lens by an observer blind to the treatment groups.

## 2.7 Statistical analysis

Statistical analysis was carried out by the SPSS16.0 software. The quantitative data were represented as mean  $\pm$  SD and analyzed using SPSS 16.0 software. Statistical comparisons between groups were carried out using Student's t test and one-way ANOVA.  $p < 0.05$  was considered to be significant.

## 3 Results

### 3.1 PC12 cell apoptosis induced by H<sub>2</sub>O<sub>2</sub>

In order to establish the appropriate concentration of H<sub>2</sub>O<sub>2</sub> in the PC12 cell damage model, different concentrations of H<sub>2</sub>O<sub>2</sub> (25 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L, 400 μmol/L) were used. PC12 cell viability were detected by MTT assay after cultured with H<sub>2</sub>O<sub>2</sub> for 0, 6, 12, 24, 36 hours. MTT results showed that cell viability of PC12 cells decreased with the increase of H<sub>2</sub>O<sub>2</sub> concentration and in a time-dependent manner (Fig. 1A). In addition, the degree of cell damage is related to the density of cell seed plate. In order to simulate the internal condition, we aimed to select a treatment method with slow action and a certain degree of damage. The selected H<sub>2</sub>O<sub>2</sub> concentration, action time, and cell plate density were: 100 μmol/L, 24h and 1×10<sup>5</sup>/ mL.

### 3.2 Effects of preconditioning with different concentrations of pioglitazone on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in PC12 cells

MTT results showed that H<sub>2</sub>O<sub>2</sub> can significantly reduce the cell viability of PC12 cells ( $p < 0.01$ ). Pioglitazone plays a protective role on H<sub>2</sub>O<sub>2</sub> treated PC12 cells and the effect was in concentration-dependent manner. The maximum action concentration of pioglitazone was 1×10<sup>-5</sup> mol/L (Fig. 1B).

### 3.3 Pioglitazone significantly elevated PPAR $\gamma$ mRNA and protein expression

Real time Q-PCR and Western blot results were shown in Fig. 2. Compared with control group, H<sub>2</sub>O<sub>2</sub> induced PPAR $\gamma$  mRNA and protein expression ( $p < 0.01$ ). Pioglitazone pretreatment also significantly increased PPAR $\gamma$  mRNA and protein expression ( $p < 0.01$ , vs control and H<sub>2</sub>O<sub>2</sub> group). Compared with the Pioglitazone group, PPAR $\gamma$  siRNA and PPAR $\gamma$  antagonist GW9662 group significantly reduced PPAR $\gamma$  mRNA and protein expression ( $p < 0.01$ ).

### 3.4 The neuroprotective effect of pioglitazone on H<sub>2</sub>O<sub>2</sub> treated PC12 cells

In order to investigate the protective effect of PPAR $\gamma$  on H<sub>2</sub>O<sub>2</sub> treated PC12 cells, we used MTT, TUNEL staining and flow cytometry to observe the neuroprotective effect of pioglitazone.

#### 1) MTT assay

As shown in Fig. 3, 100μmol/L H<sub>2</sub>O<sub>2</sub> significantly reduced the PC12 cell viability to 56.8% ( $p < 0.01$ , vs control group). 1×10<sup>-5</sup> mol/L pioglitazone increased PC12 cell viability to 80.2%, which shows significant difference with control group and H<sub>2</sub>O<sub>2</sub> group ( $p < 0.01$ ), suggesting that the neuroprotective effect of pioglitazone is not enough to completely protect PC12 cells from oxidative damage. Both PPAR $\gamma$  siRNA

and PPAR $\gamma$  antagonist GW9662 reversed the neuroprotective effects of pioglitazone to different degrees. The cell viability of PC12 cells decreased to 59.6% and 59.1%, respectively, which both shows significant difference with pioglitazone group ( $p < 0.01$ ), suggesting that pioglitazone plays a neuroprotective role through the PPAR $\gamma$  activation pathway.

## 2) Flow cytometry results

As shown in Figure.4, the percentage of apoptotic cells in the control group was approximately 6.72% (Fig. 4A). After H<sub>2</sub>O<sub>2</sub> treatment, the percentage of apoptotic cells increased significantly to 25.8% ( $p < 0.01$ , vs control group) (Fig. 4B). Pioglitazone decreased the percentage of apoptotic PC12 cells to 11.8% (Fig. 4C), which was significantly different with H<sub>2</sub>O<sub>2</sub> group and control group ( $p < 0.01$ ,  $p < 0.05$ , respectively), suggesting that the neuroprotective effect of pioglitazone is not enough to completely reverse the oxidative damage of H<sub>2</sub>O<sub>2</sub>. PPAR $\gamma$  siRNA (Fig. 4D) and PPAR $\gamma$  antagonist GW9662 (Fig. 4E) pretreatment decreased the apoptotic ratio of PC12 cells to 23.2% and 21.6% respectively, which both shows significant difference with pioglitazone group ( $p < 0.01$ ). It suggested that PPAR $\gamma$  inhibition all reversed the neuroprotective effect of pioglitazone to different degrees and the neuroprotective effect of pioglitazone was through PPAR $\gamma$  pathway.

## 3) TUNEL staining assay

Considering that the cell growth density is not exactly the same, we used the ratio of TUNEL and DAPI dual-positive cells to DAPI positive cells to calculate the apoptotic rate of PC12 cells as the experimental indicator. As Fig. 5 showed, the control group showed complete cell morphology, low-intensity fluorescence, larger nuclei, more DAPI positive and fewer TUNEL positive cells. The TUNEL/DAPI ratio was approximately 5.2% (Fig. 5A). After treated with H<sub>2</sub>O<sub>2</sub> for 24h, the number of DAPI-positive nuclei decreased and high-intensity concentrated fluorescence appeared in the nucleus. The nuclei became smaller, concentrated and fragmented, showing the characteristics of apoptotic cells (Fig. 5B). The TUNEL/DAPI ratio increased to 26.8% ( $p < 0.01$ , vs control group) (Fig. 5F), suggesting that H<sub>2</sub>O<sub>2</sub> promotes cell apoptosis in PC12 cells. Pioglitazone preserved the number and the morphology of PC12 cells to some extent (Fig. 5C). The TUNEL/DAPI ratio decreased to 11.9%, which showed difference with H<sub>2</sub>O<sub>2</sub> group and control group ( $p < 0.01$ ,  $p < 0.05$ , respectively), suggesting that pioglitazone can protect PC12 cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative damage, but the protective effect was not enough to completely reverse oxidative damage. PPAR $\gamma$  antagonist GW9662 and PPAR $\gamma$  siRNA increased the TUNEL/DAPI ratio to 22.3% and 23.3%, respectively, which were significantly different from pioglitazone group ( $p < 0.01$ ). It suggested that pioglitazone exerts neuroprotective effect through PPAR $\gamma$  activation pathway.

## 3.5 Pioglitazone decreased H<sub>2</sub>O<sub>2</sub>-induced elevation of Bax/Bcl-2 ratio and caspase-3 expression

As shown in Fig. 6, after H<sub>2</sub>O<sub>2</sub> treatment, Bax, Bcl-2 and Caspase-3 protein expression increased by 4.03, 2.28 and 3.14 times, respectively ( $p < 0.01$ , vs control group) (Fig. 6A,B,D). The Bax/Bcl-2 ratio of H<sub>2</sub>O<sub>2</sub>

group was significantly higher than that of control group ( $p < 0.01$ ) (Fig. 6C). Compared with the  $H_2O_2$  group, pioglitazone reduced the expression levels of Bax and Caspase-3 proteins to 46.9% and 59.4%, respectively ( $p < 0.01$ ), and increased Bcl-2 protein expression by 1.53 times ( $p < 0.01$ ), thus significantly reduced the Bax/Bcl-2 expression ratio ( $p < 0.01$ ). Compared with pioglitazone group, GW9662 and PPAR $\gamma$  siRNA groups both significantly increased Bax and Caspase-3 protein expression levels, reduced Bcl-2 protein expression level and elevated Bax/Bcl-2 expression ratio ( $p < 0.01$ ). But there was no significant difference between the GW9662 and PPAR $\gamma$  siRNA group. It suggested that the PPAR $\gamma$  agonist can increase the expression of antiapoptotic protein Bcl-2 and decrease the expression levels of proapoptotic protein Bax and caspase-3, thus protect PC12 cells from  $H_2O_2$ -induced oxidative damage and pioglitazone mainly exerts its anti-apoptotic effect through the PPAR $\gamma$  pathway.

## 4 Discussion

PC12 cell is a tumor cell line isolated from rat adrenal pheochromocytoma. The differentiated PC12 cell has typical neuronal characteristics in morphology and function, and is widely used as a cell model to study apoptosis and differentiation of nerve cells<sup>[8]</sup>. Hydrogen peroxide ( $H_2O_2$ ) is thought to be the major precursor of ROS and is widely used to induce oxidative stress injury. Exogenous  $H_2O_2$  can easily immerse into cells through cell membrane, producing a large number of ROS, thus is commonly used to simulate cell peroxidation damage in vitro<sup>[9]</sup>. There are many studies on the apoptosis of PC12 cells induced by  $H_2O_2$ , and the conclusions are consistent<sup>[10, 11]</sup>, which can reflect the pathological changes of nerve cells. However, the effective concentration and action time are different. In general, low and moderate concentrations (50–500 micromol/L) of  $H_2O_2$  can induce oxidative stress, while high concentrations can rapidly cause cell necrosis. In this study, we found that PC12 cells treated with  $H_2O_2$  for a certain period of time, the cells did not stretch well, the process disappeared, the cell body retracted, a large number of particles accumulated in rough cytoplasm, and some cells did not adhere to the wall, which suggesting that  $H_2O_2$  may induce cell apoptosis by interfering with cell metabolism and changing cell adhesion. The apoptosis of PC12 cells was analyzed by morphological observation and MTT. The results showed that the apoptosis of PC12 cells could be induced by 100  $\mu$ mol/L  $H_2O_2$  for 24 hours.

The pathway of  $H_2O_2$  induced apoptosis is not uniform. Dumont et al found that  $H_2O_2$  induced apoptosis of T cells mainly depended on mitochondrial ROS and NF- $\kappa$ B activation<sup>[12]</sup>.  $H_2O_2$  promotes apoptosis through activating Caspase-3 of HL-60 cells<sup>[13]</sup>. Kitamura et al found that Bcl-2 and Bax did not increase significantly after treated with  $H_2O_2$ , but mediated apoptosis by increasing P53 expression<sup>[14]</sup>.  $H_2O_2$  induced apoptosis of hepatoblastoma cells, not only up-regulated the expression of P53, but also decreased the levels of Bcl-2 protein and Bax protein<sup>[15]</sup>. In this study, we found that  $H_2O_2$  can up-regulate the expression of Bax, Caspase-3 and down-regulate the expression of Bcl-2. It is suggested that the mechanism of apoptosis of PC12 cells induced by  $H_2O_2$  may involve increasing pro-apoptotic protein and

decreasing anti-apoptotic protein, thus changing PC12 cell apoptotic environment. This is consistent with recent research that H<sub>2</sub>O<sub>2</sub> can increase Bax and cleaved caspase-3 expression in PC12 cells<sup>[16]</sup>.

The process of neuronal apoptosis is similar to that of other cells. After death signals, apoptosis-promoting proteins such as Bax and Bid are translocated to the extracellular membrane of mitochondria and interact with anti-apoptotic proteins such as Bcl-2, which deprive the anti-apoptotic protein of its inhibitory effect on apoptosis, increase the permeability of mitochondrial membrane, release cytc into cytoplasm and activate Caspase-3, which eventually leads to apoptosis<sup>[17]</sup>. Bax/Bcl-2 ratio plays an decisive role in determining whether cells enter the apoptotic state, so the expression ratio of two genes is often used to study the degree of apoptosis<sup>[18]</sup>. The results of Realtime Q-PCR and Western blot showed that the expression levels of pro-apoptotic factors Bax and Caspase-3, anti-apoptotic factor Bcl-2 and Bax/Bcl-2 were significantly increased after treated with 100 mmol/L H<sub>2</sub>O<sub>2</sub> for 24 hours. TUNEL staining also confirmed this view. PC12 cells treated with 100 micromol/L H<sub>2</sub>O<sub>2</sub> could obviously induce apoptosis of PC12 cells, the apoptosis rate was 26.8%.

PPAR $\gamma$  is a ligand-activated nuclear transcription factor. After being activated by its ligand, PPAR $\gamma$  can combine with specific DNA response elements to regulate the transcription and expression of genes<sup>[19]</sup>. Our previous studies demonstrated that PPAR $\gamma$  agonist rosiglitazone can upregulate PPAR $\gamma$  mRNA and protein expression in aged SHR, which was accompanied by markedly decreased expression of oxidative stress markers (iNOS and gp47<sup>phox</sup>) and pro-apoptotic markers (Bax and caspase-3)[7]. Karen et al. demonstrated that rosiglitazone up-regulates the Bcl-2 protein in neurons and induces mitochondrial stabilization and protection against oxidative stress and apoptosis<sup>[20]</sup>. These results indicated that the PPAR $\gamma$  agonist, rosiglitazone, may exert neuroprotective effects through antioxidative and anti-apoptotic mechanisms. To confirm the protective effect of PPAR $\gamma$  agonist on PC12 cells injured by H<sub>2</sub>O<sub>2</sub>, PC12 cells were incubated with different concentrations of pioglitazone before exposure to H<sub>2</sub>O<sub>2</sub> for one hour. MTT results showed that pioglitazone concentration-dependently increased the survival rate of PC12 cells and played a neuroprotective role. The results of flow cytometry and TUNEL staining also confirmed the conclusion of MTT experiment. The early and late apoptotic cells were significantly less in the pioglitazone-protected group than in the H<sub>2</sub>O<sub>2</sub>-injured group, and the apoptotic rate was significantly lower. Realtime Q-PCR and Western blot assays confirmed that pioglitazone significantly increased PPAR $\gamma$  expression in PC12 cells, which was 4.4 times higher than that in the control group. This confirms previous reports that neuroprotective doses of pioglitazone can induce a five-fold increase in PPAR $\gamma$  expression, thereby maintaining the responsiveness to cortical neurons by increasing the expression of its receptors<sup>[21]</sup>.

In addition, we also observed that hydrogen peroxide could also induce the increased expression of PPAR $\gamma$ , which may be a compensatory protective mechanism for the cells damaged<sup>[22]</sup>. Western blot was used to detect the expression of Bax, Bcl-2 and Caspase-3 proteins. The results showed that 100 micromol/L H<sub>2</sub>O<sub>2</sub> could increase the expression of Bax and Caspase-3 proteins, decrease the expression of Bcl-2 protein and increase the ratio of Bax to Bcl-2. Pioglitazone could down-regulate the expression of

Bax and Caspase-3 protein and up-regulate Bcl-2 protein expression, thus reducing the ratio of Bax to Bcl-2. These results suggest that pioglitazone can attenuate the pro-apoptotic environment of PC12 cells induced by H<sub>2</sub>O<sub>2</sub>. To further explore whether rosiglitazone can play a role in the activation of PPAR $\gamma$ . We used PPAR $\gamma$  antagonist GW9662 and PPAR $\gamma$  siRNA to block the expression of PPAR $\gamma$  in PC12 cells. The results demonstrated that pretreatment with GW9662 significantly decreased the increased Bax/Bcl-2 ratio and Caspase-3 expression in PC12 cells injured by H<sub>2</sub>O<sub>2</sub>. PPAR $\gamma$  siRNA had the same effect. So GW9662 and PPAR $\gamma$  siRNA can offset the protective effect of pioglitazone on PC12 cells injured by H<sub>2</sub>O<sub>2</sub>.

In conclusion, the neuronal apoptosis model induced by oxidative stress was established in vitro, and the neuroprotective effect of pioglitazone was studied. The results showed that pioglitazone could increase the activity of PC12 cells damaged by H<sub>2</sub>O<sub>2</sub>, increase the expression of Bcl-2, decrease the expression of Bax and Caspase-3, thus decreased the pro-apoptotic environment and the apoptotic rate of PC12 cells. PPAR $\gamma$  antagonist or PPAR $\gamma$  siRNA blocked the expression of endogenous PPAR $\gamma$ , inhibited the protective effect of pioglitazone on PC12 cells injured by H<sub>2</sub>O<sub>2</sub>, suggesting that pioglitazone could protect PC12 cells from oxidative stress by reducing the expression ratio of Bax/Bcl-2 and Caspase-3 through PPAR $\gamma$  activation pathway. In conclusion, pioglitazone can exert anti-apoptotic effect and promote the survival of PC12 cells in the presence of oxidative stress injury. This effect is through PPAR $\gamma$  activation pathway. This study thus suggests that PPAR $\gamma$  activation might have potential for intervention in neurodegenerative disorders.

## **Declarations**

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### **Author contributions**

Yali Li, Xiaolin Niu and Jianbo Yu conceived the study and wrote the manuscript. Libo Li, Lijuan Liu, Bin Hou and Yanjing Liang collected data. Jun Long and Ziyao Yu analyzed and interpreted the data. Yali Li drafted the article. All authors read and approved the final manuscript.

### **Competing Interests statement**

The authors declare that we have no competing financial, professional or personal interests that might influenced the performance or presentation of the work described in this manuscript.

### **Approval and accordance statement**

The methods of specimen collection were conducted in accordance with the guidelines of National Institutes of Health. The experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine and followed the guidelines of the declaration of Helsinki.

## Data and material availability

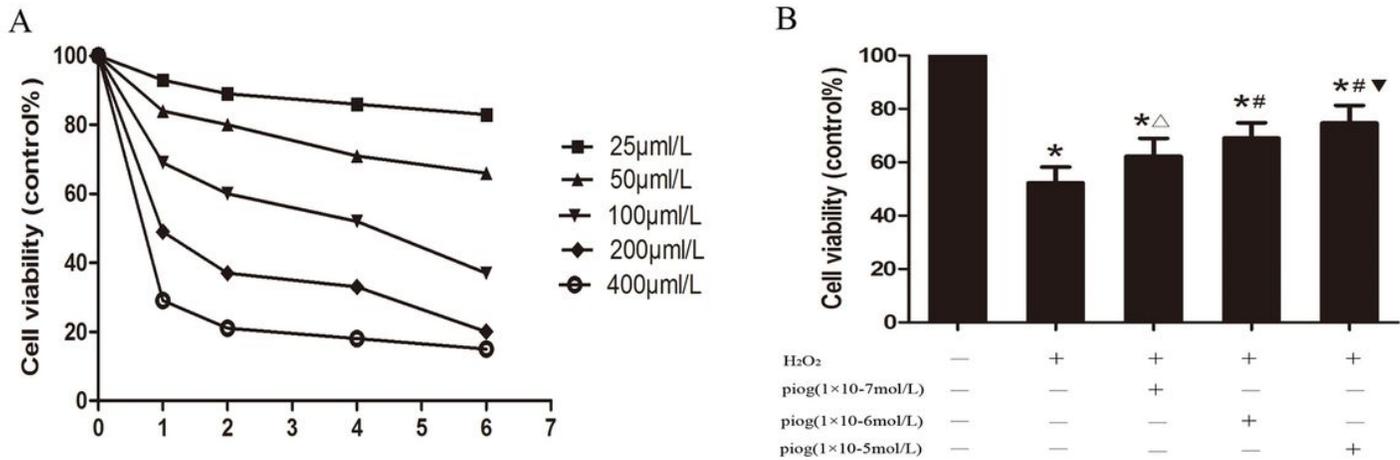
Most of the raw/processed data required to reproduce our findings was submitted on line.

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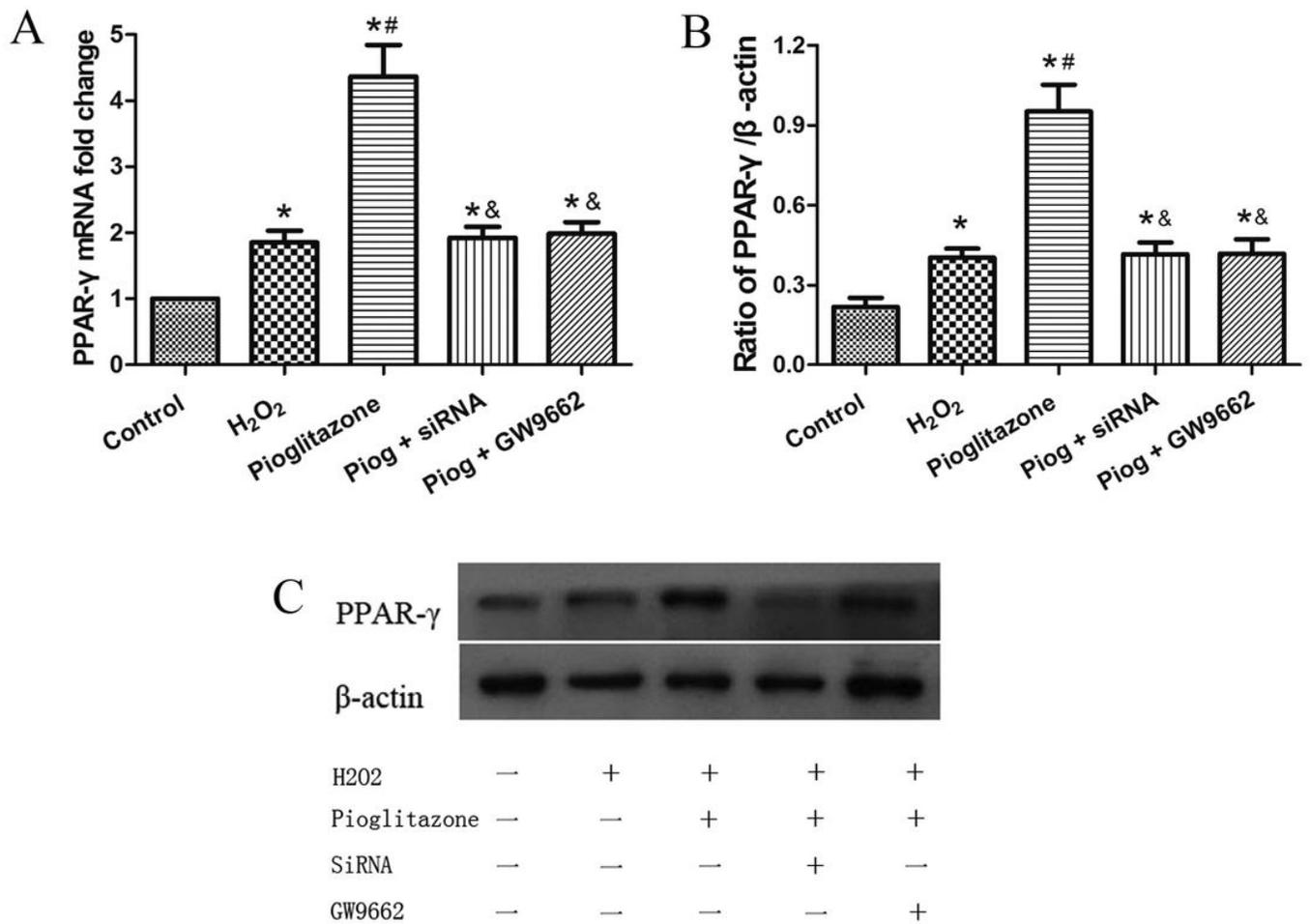
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## Figures



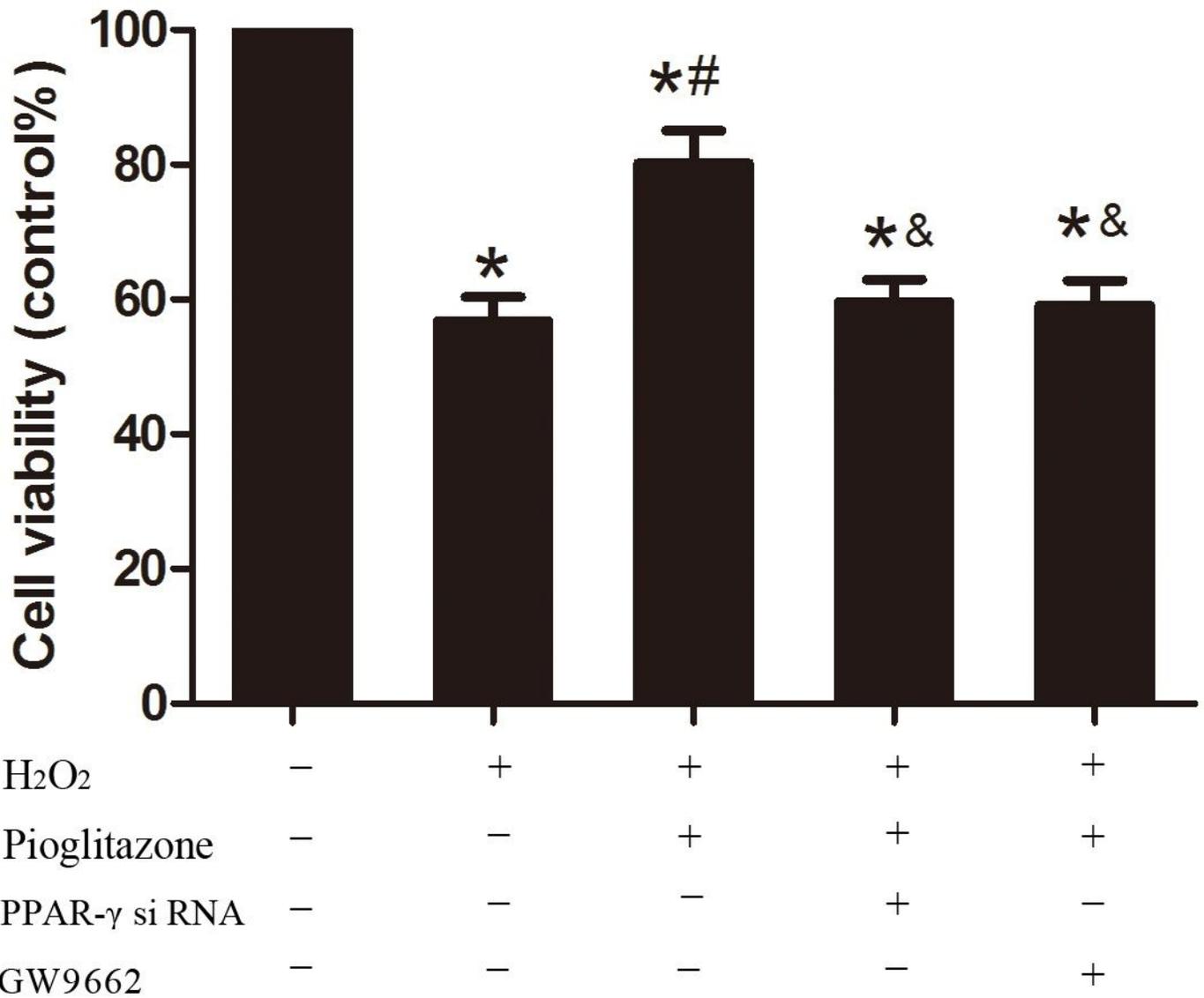
**Figure 1**

MTT results of cell viability changes in PC12 cells Fig.1A: The cell viability of PC12 cells decreased with the increase of H<sub>2</sub>O<sub>2</sub> concentration and in a time-dependent manner. The appropriate H<sub>2</sub>O<sub>2</sub> concentration, action time, and cell plate density were: 100 μmol/L, 24 h and 1×10<sup>5</sup>/mL. Fig.1B shows cell viability changes of PC12 cells treated by H<sub>2</sub>O<sub>2</sub> after preconditioning with pioglitazone at different concentrations. \*p<0.01 vs control group; #p<0.01, Δp<0.05 vs H<sub>2</sub>O<sub>2</sub> group, ▼p<0.05 vs Pioglitazone group (1×10<sup>-7</sup>mol/L).



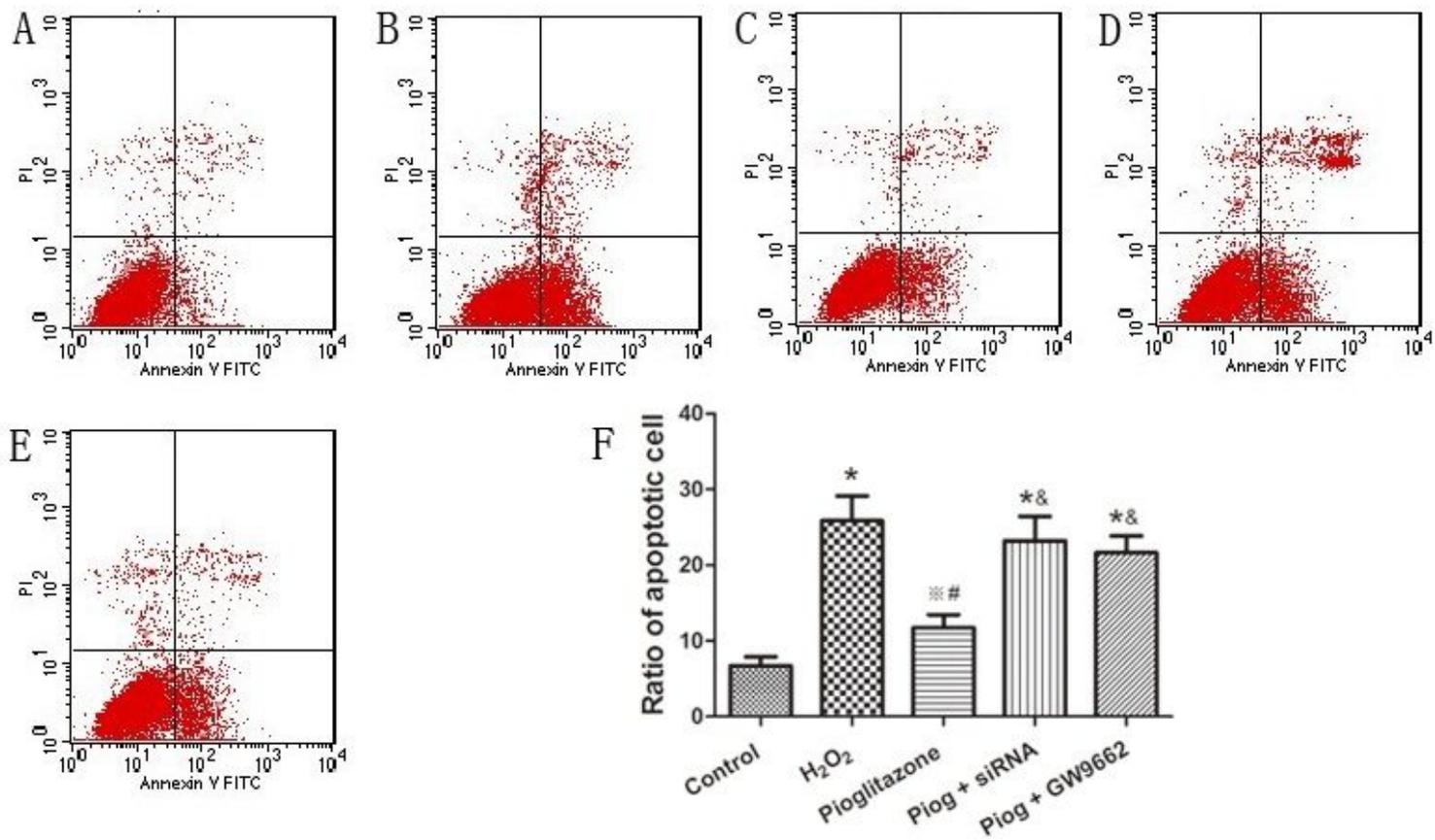
**Figure 2**

Effects of H<sub>2</sub>O<sub>2</sub>, Pioglitazone, GW9662 and PPAR $\gamma$  siRNA on PPAR $\gamma$  mRNA and protein expression. (A) PPAR $\gamma$  mRNA expression in five groups. (B) PPAR $\gamma$  protein expression in five groups. (C) Representative western blotting image displaying PPAR $\gamma$  protein expression. \* $p < 0.01$ , \* $p < 0.05$  vs control group; # $p < 0.01$  vs H<sub>2</sub>O<sub>2</sub> group, & $p < 0.01$  vs pioglitazone group.



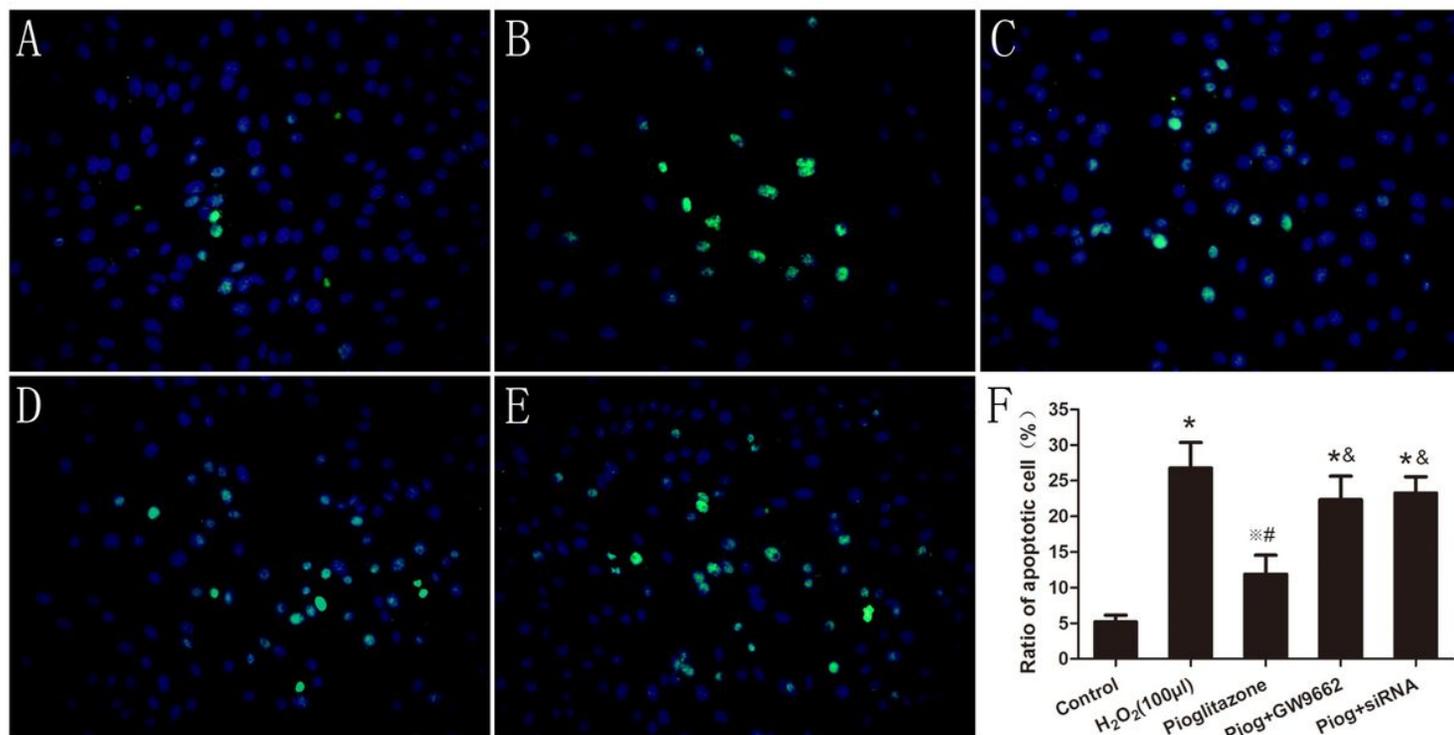
**Figure 3**

MTT assay results of five groups. \* $p < 0.01$  vs control group; # $p < 0.01$  vs H<sub>2</sub>O<sub>2</sub> group, & $p < 0.01$  vs pioglitazone group.



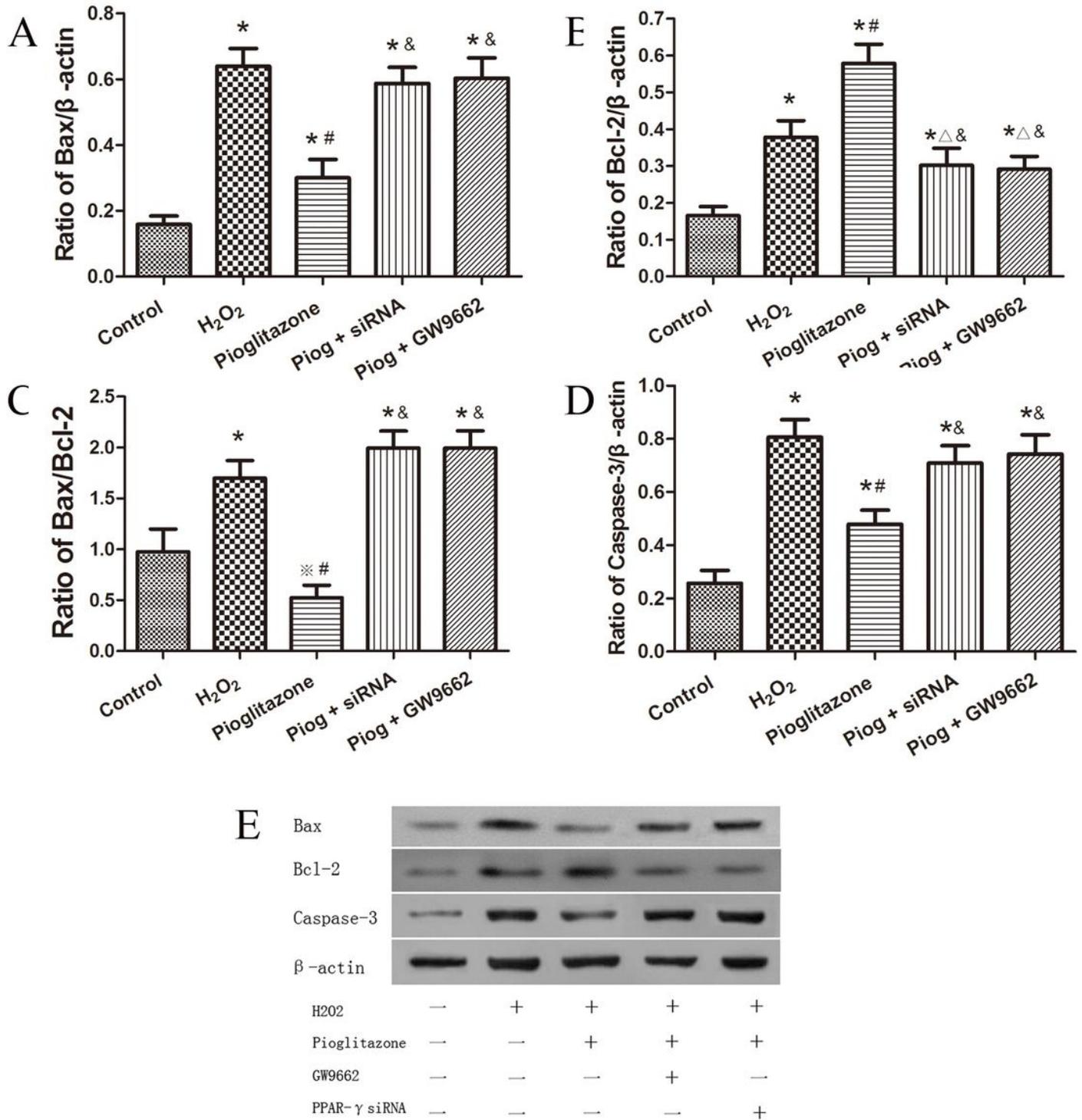
**Figure 4**

Flow cytometry results of five groups. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: Pioglitazone+H<sub>2</sub>O<sub>2</sub> group; D: Pioglitazone+GW9662+H<sub>2</sub>O<sub>2</sub> group; E: Pioglitazone+PPAR $\gamma$  siRNA+H<sub>2</sub>O<sub>2</sub> group. F: \*p<0.01, \*\*p<0.05 vs control group; #p<0.01 vs H<sub>2</sub>O<sub>2</sub> group, &p<0.01 vs pioglitazone group.



**Figure 5**

TUNEL staining results of five groups. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: Pioglitazone+H<sub>2</sub>O<sub>2</sub> group; D: Pioglitazone+GW9662+H<sub>2</sub>O<sub>2</sub> group; E: Pioglitazone+PPAR $\gamma$  siRNA +H<sub>2</sub>O<sub>2</sub> group ; E: Analysis of TUNEL staining. \*p<0.01, ※p<0.05 vs control group; #p<0.01 vs H<sub>2</sub>O<sub>2</sub> group; &p<0.01 vs pioglitazone group.



**Figure 6**

Effect of Pioglitazone on expression of Bax, Bcl-2 and Caspase-3 proteins in PC12 cells. \*p<0.01, ※p<0.05 vs control group, #p<0.01, Δp<0.05 vs H2O2 group; &p<0.01 vs pioglitazone group.