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ORIGINAL PAPER

Neuroprotective Effects of Deuterium-Depleted Water (DDW) Against H_2O_2-Induced Oxidative Stress in Differentiated PC12 Cells Through the PI3K/Akt Signaling Pathway

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Abstract

Oxidative stress plays an important role in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease. Induction of endogenous antioxidants to act against oxidative stress-mediated neuronal damage seems to be a reasonable strategy for delaying the progression of such diseases. In this study, we investigated the neuroprotective effect of deuterium-depleted water (DDW) against H_2O_2-induced oxidative stress in differentiated PC12 cells and the possible signaling pathways involved. The differentiated PC12 cell line was pretreated with DDW containing different concentrations (50–100 ppm) of deuterium and then treated with H_2O_2 to induce oxidative stress and neurotoxicity. We assessed cell survival, reactive oxygen species (ROS) generation, TUNEL assay, catalase (CAT), copper and zinc-containing superoxide dismutase (CuZn-SOD) and superoxide dismutase (SOD) activity and performed Western blot analysis to investigate the neuroprotective effect of DDW. The results indicated that DDW could attenuate H_2O_2-induced apoptosis, reduce ROS formation, and increase CAT, CuZn-SOD and SOD activity in H_2O_2-treated PC12 cells. Western blot analysis revealed that DDW treatment significantly increased the expression of p-Akt, Bcl-2 and GSK-3β. However, the protective effect of DDW on cell survival and the DDW-mediated increases in p-Akt, Bcl-2 and GSK-3β were abolished by pretreatment with the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002. In summary, DDW may protect differentiated PC12 cells against H_2O_2-induced oxidative stress through the PI3K/Akt signaling pathway.

Keywords Deuterium-depleted water (DDW) · PC12 cells · Neuroprotective effect · Oxidative stress

Introduction

The term oxidative stress refers to an imbalance between the oxidation system and the antioxidant system in favor of oxidants, which potentially leads to cellular injury. Oxidative stress leads to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and induces neuronal injury involved in the pathogenesis of a variety of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease and atherosclerosis [17]. ROS are formed as normal products of aerobic metabolism at relatively low levels in living tissues. At these low levels, ROS play important roles in diverse physiological processes, including intracellular signal transduction and gene expression. However, excessive production of ROS may cause DNA damage, protein oxidation, and lipid peroxidation, eventually resulting in cellular dysfunction and apoptosis [2, 7, 25]. Induction of endogenous antioxidants to act against oxidative stress-mediated neuronal

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damage seems to be a reasonable strategy for delaying the progression of related diseases.

Water is the origin of life and is composed of oxygen and hydrogen atoms. Water is the most abundant component of organisms; indeed, the human body contains up to 70% water. Deuterium is the naturally stable isotope of hydrogen, and the ratio of deuterium to protium (D/H) in natural water is approximately 1:6600; thus, the natural percentage of deuterium is approximately 0.0139–0.0151% [3, 15]. When the deuterium concentration is lower than 0.015% (150 ppm), water is known as deuterium-poor water, low-deuterium water, superlight water, or deuterium-depleted water (DDW). The mass of water can be significantly different depending on the concentrations of the two isotopes, resulting in critical differences in physical and chemical properties. Several studies on the biological activity of DDW have mainly focused on antitumor properties [4, 27, 28]. However, little is known about the neuroprotective effect of DDW. Recently, Ávila showed that DDW with 90 ppm deuterium can significantly reverse Mn^{+}-induced reductions in nematode lifespan and restore DAF-16 and SOD-3 levels in *Caenorhabditis elegans*, suggesting that DDW can improve antioxidant ability in model animals to prevent Mn^{+} neurotoxicity [1]. Syroeshkin showed that the dynamics of the cell doubling index in the deuterium depleted water-based growth medium showed higher proliferation potential compared to the water with normal isotopic composition [24]. In this study, we investigated the neuroprotective effect of DDW in PC12 cells with H_2O_2-induced oxidative injury and explored the possible signaling pathways involved.

Materials and Methods

Materials and Reagents

Fetal calf serum, horse serum and RPMI 1640 medium were purchased from Gibico (Carlsbad, CA, USA). A rat adrenal pheochromocytoma cell line (PC12) was obtained from the cell collection of the Shanghai Institute for Cell Research, Chinese Academy of Sciences. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and a Cell Counting Kit-8 (CCK8) were purchased from Boster Biological Technology (Wuhan, Hubei, CHN). Monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). A goat anti-mouse IgG (H + L)/TRITC antibody was purchased from Invitrogen (Carlsbad, CA, USA) and was used as the secondary antibody. Alexa Fluor 594-conjugated goat anti-mouse IgG (H + L) was purchased from Invitrogen. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore. ROS, TUNEL assay, catalase (CAT), copper and zinc-containing superoxide dismutase (CuZn-SOD) and superoxide dismutase (SOD) analysis kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

PC12 Cell Culture and Drug Treatment

PC12 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 5% horse serum, 100 U/ml penicillin, and 100 mg/l streptomycin at 37℃ with 5% CO_2. For experiments on the effects of DDW in PC12 cells, cells were deprived of serum for 12 h prior to experiments and then cultured in RPMI 1640 dry powder culture medium dissolved in water containing various concentrations of deuterium (50, 75, 100, and 150 ppm) and supplemented with 10% FBS, 5% horse serum, 1% sodium carbonate and 1% penicillin–streptomycin for 24 h. Then, the PC12 cells were exposed to H_2O_2 at a final concentration of 100 μM for an additional 2 h. The cells were preincubated for 30 min in the presence or absence of LY294002 before DDW treatment.

Cell Viability Assay

Cell viability was determined by MTT and CCK8 assays. For the MTT assay, cells in the logarithmic growth phase were seeded in 96-well plates at a density of 1×10^5 cells/ml. After treatment, MTT solution (5 mg/ml) was added, and the plates were continuously incubated for an additional 4 h. Then, the medium was removed, and the colored formazan was dissolved in 150 ml of dimethyl sulfoxide (DMSO) with shaking for 10 min. The absorbance was measured using a spectrophotometric microtiter plate reader (BioTEK, USA) at 570 nm. The viability of the treated PC12 cells compared to the control cells is presented as a percentage. For the CCK8 assay, after treatment, CCK8 solution (10 μl) was added, and the plates were continuously incubated for an additional 4 h. The absorbance was measured using a spectrophotometric microtiter plate reader at 450 nm.

TUNEL Assay

TUNEL assay was carried out according to the instructions of a TUNEL kit (Beyotime Biotechnology, Shanghai, China) to observe the status of cell apoptosis.

Measurement of ROS

Intracellular ROS levels were assessed by using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). In brief, 1×10^5 cells/ml were pretreated for 24 h with media containing various concentrations of deuterium (150, 100, 75 and 50 ppm) at 37℃ with 5% CO_2, and the cells were then incubated with 10 μl of DCF-DA for 30 min at 37℃. Next, the cells were incubated with 100 μM H_2O_2 for 2 h. Finally, fluorescence was measured using a spectrophotometric

microtiter plate reader with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Measurement of CAT, SOD and CuZnSOD Activity

PC12 cells in six-well plates were incubated under control and experimental conditions. After treatment, the cells were lysed with RIPA buffer supplemented with 1% phenylmethylsulfonyl fluoride and 1% tyrosine phosphatase inhibitor and then centrifuged at 12,000 rpm for 10 min at 4 °C. The proteins were assayed using a NanoDrop spectrophotometer and stored at − 80 ℃ until tested. CAT, SOD and CuZnSOD activity in the proteins was determined with a CAT analysis kit, a CuZnSOD kit and a SOD analysis kit, respectively, according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China).

Western Blot Analysis

Briefly, PC12 cells were plated in 6-cm culture dishes in FBS-free RPMI 1640 for 12 h and then cultured with media containing different concentrations of deuterium (150, 100, 75 and 50 ppm) for 24 h. Then, the cells were exposed to H_2O_2 at a final concentration of 100 μM for an additional 2 h. In addition, to confirm the role of the PI3K/Akt pathway in the protective effects of DDW in neurocytes, cells were pretreated with LY294002 (20 μM) 30 min before exposure to H_2O_2 in some experiments. The cells were then washed twice with ice-cold PBS and lysed in 150 μl of RIPA buffer containing 1% phenylmethylsulfonyl fluoride and 1% tyrosine phosphatase inhibitor. The protein concentrations were determined using a NanoDrop spectrophotometer (Davis, CA, USA). Equal amounts of proteins (100 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 2.5% nonfat dry milk in PBST (400 ml PBS + 100 μl Tween 20) for 60 min and then incubated overnight at 4℃ with anti-Akt (1:1000), anti-p-Akt (1:1000), anti-Bcl-2 (1:750), anti-PTEN (1:1000), anti-p-PDK (1:1000), and anti-GSK-3β (1:750) monoclonal antibodies and an anti-β-actin antibody (1:3000). Following incubation with a goat anti-mouse IgG (H + L)/TRITC or Alexa Fluor 594 goat anti-mouse IgG (H + L) secondary antibody for 1 h at 37℃, the membranes were washed thoroughly for 3 × 10 min with PBST. The bands were detected using a LI-COR Odyssey Infrared imaging system (Lincoln, USA).

Statistical Analysis

The data are presented as the mean ± standard deviation. One-way ANOVA and the LSD test were used for comparisons between different groups. Differences were considered to be statistically significant at $P < 0.05$. All analyses were performed using SPSS version 19.0.

Results

DDW Attenuates H_2O_2-Induced Cytotoxicity in PC12 Cells

First, PC12 cells were incubated for 24 h in media with different concentrations of deuterium to confirm the safety of DDW for the cells. As shown in Fig. 1a, the different concentrations of deuterium (150, 100, 75 and 50 ppm) had no cytotoxic effects with regards to cell viability. Then, we treated PC12 cells with 20–200 μM H_2O_2 to induce oxidative stress and neurotoxicity. Cell viability gradually decreased as the concentration of H_2O_2 increased from 20 to 200 μM (Fig. 1b). After treatment with 100 μM H_2O_2 for 2 h, the cell viability had decreased to 55.80%. To evaluate the protective effect of DDW, cells were treated with DDW containing different concentrations of deuterium for 24 h and then exposed to 100 μM H_2O_2 for 2 h. After treatment, the cells were assayed for cell viability by using MTT and CCK8 assays. As shown in the MTT results in Fig. 1c, pretreatment with DDW containing different concentrations of deuterium dose-dependently protected cells against H_2O_2-induced cell damage, recovering the cell viability from 56.61 to 69.00%, 78.50% and 88.06%, respectively. The CCK8 assay results also showed the same protective effect of DDW against H_2O_2-induced cytotoxicity in PC12 cells (Fig. 1d). Then, we adjusted the order of administration. Cells were first exposed to 100 μM H_2O_2 for 2 h and then treated with DDW containing different concentrations of deuterium for the next 24 h. The results of the MTT and CCK8 assays are shown in Fig. 1e, f. Similar to DDW pretreatment, administration of DDW after H_2O_2-induced cell damage DDW protected cells and restored cell viability. These results suggest that DDW can not only protect PC12 cells from oxidative injury but also promote the repair of PC12 cells after oxidative injury.

DDW Inhibits Increases in Intracellular ROS and Attenuate H_2O_2-Induced Apoptosis in PC12 Cells

To further investigate whether DDW protects cells against oxidative stress, ROS accumulation in PC12 cells after treatment with DDW containing different concentrations of deuterium was measured utilizing a conversion reaction of DCFH-DA to DCF. ROS accumulation as determined by measurement of DCF fluorescence was approximately 85.27% higher in cells treated with 100 μM H_2O_2 than in control cells. Preincubation of cells with media containing different concentrations of deuterium (100, 75, and 50 ppm) clearly suppressed the increases in ROS caused by H_2O_2

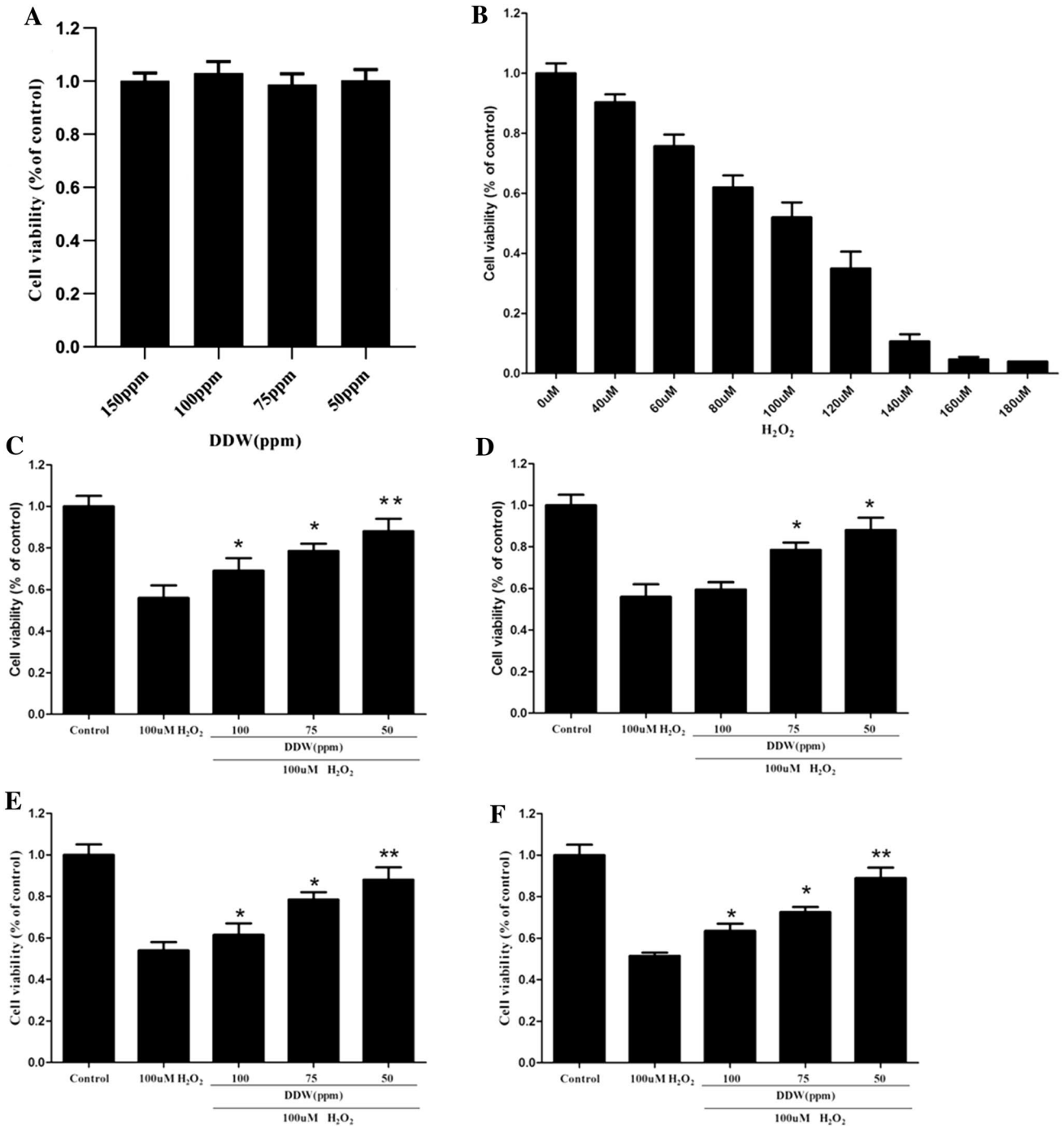
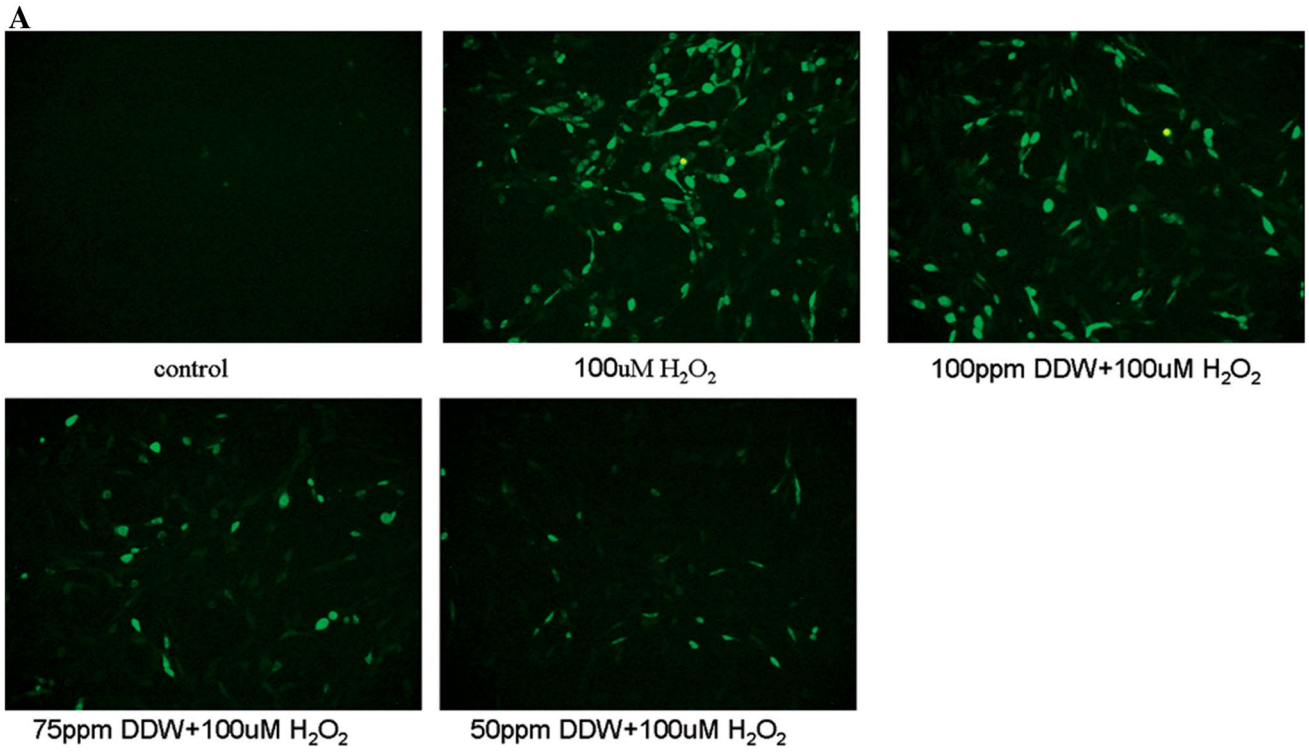


Fig. 1 Cell viability was protected by DDW (100, 75, and 50 ppm) under conditions of oxidative injury. **a** The MTT assay results showed that DDW (100–50 ppm deuterium) had no toxic effects on PC12 cells. **b** Viability of PC12 cells exposed to various concentrations of H_2O_2. **c** and **d** PC12 cells were pretreated with DDW (100–50 ppm deuterium) for 24 h and then cocultured with H_2O_2 (100 μM) for 2 h. **e** and **f** To investigate the effect of DDW on cell repair, PC12 cells were cocultured with H_2O_2 (100 μM) for 2 h and then treated with DDW (100–50 ppm deuterium) for 24 h (**c** and **e**: MTT assay results, **d** and **f**: CCK8 assay results; *, $P \leq 0.05$ vs model; **, $P \leq 0.01$ vs model)

treatment alone (the 100 μM H_2O_2 group) (Fig. 2, $P < 0.05$ or $P < 0.01$). The effect was most obvious in the group treated with medium containing 50 ppm deuterium, whose DCF fluorescence was similar to that of the control group in the



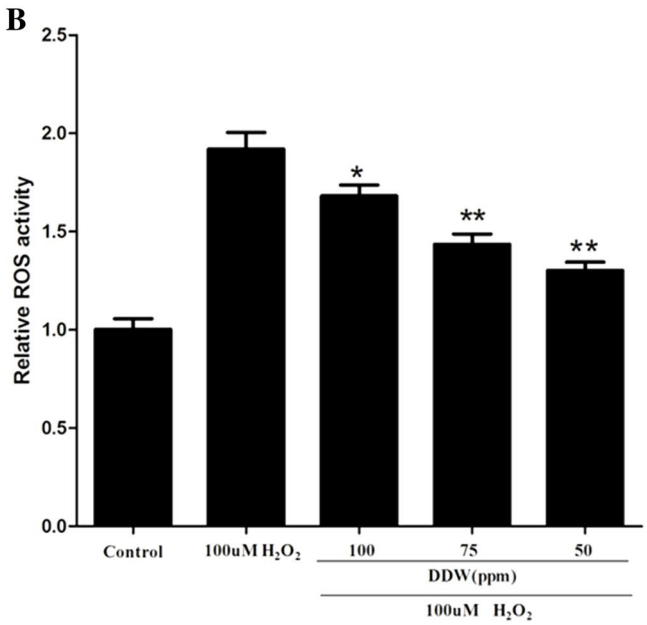


Fig. 2 Effect of DDW (100, 75, and 50 ppm deuterium) on intracellular ROS formation in H_2O_2-treated PC12 cells. **a** ROS accumulation was monitored by fluorescence microscopy for control cells without any treatment, cells treated with 100 μM H_2O_2, and cells pretreated with DDW (100, 75, 50 ppm deuterium) and then treated with 100 μM H_2O_2. Fluorescence images were obtained using a ZEISS fluorescence microscope under 20×magnification **b** Bar graph of DCF fluorescence. Compared with H_2O_2 alone, DDW (100, 75, and 50 ppm deuterium) clearly suppressed ROS accumulation. Fluorescence intensity is expressed relative to the control value (% of control). The results are presented as the mean ± SD (n = 3) for each concentration (*, $P \leq 0.05$ vs model; **, $P \leq 0.01$ vs model)

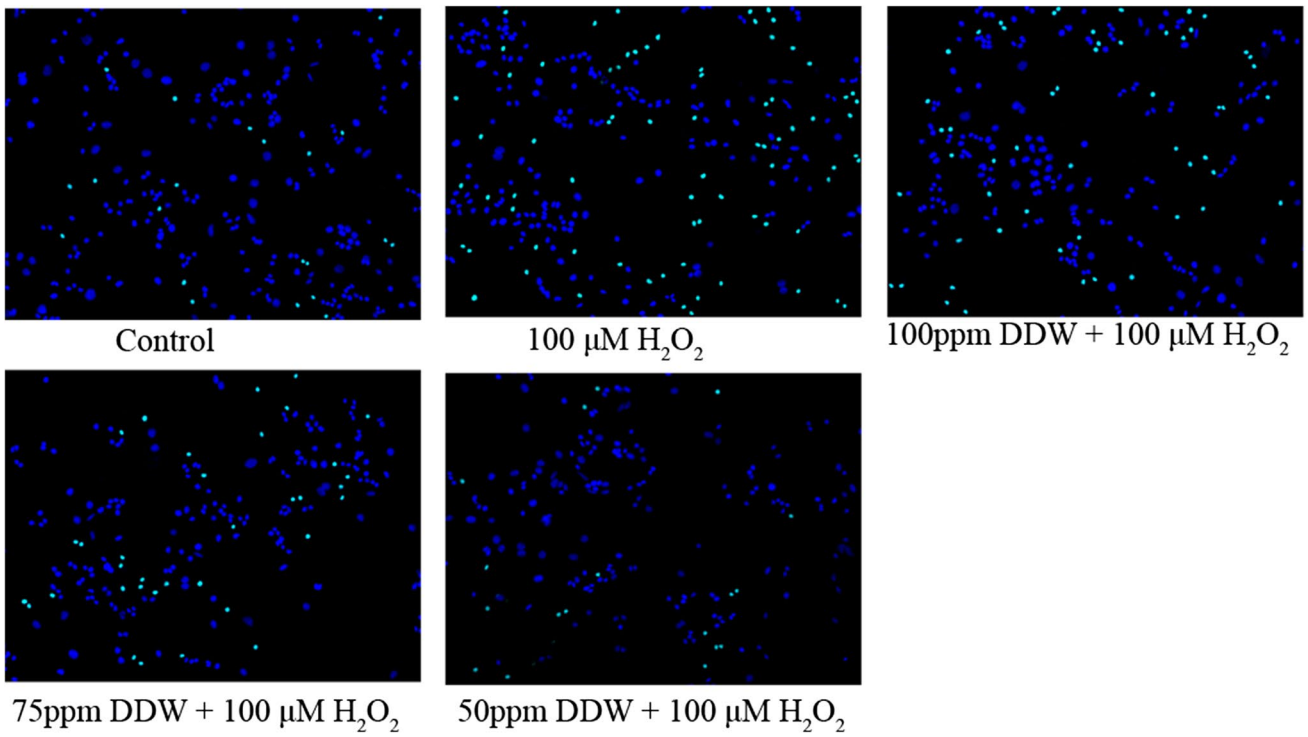


Fig. 3 DDW could attenuate H_2O_2-induced apoptosis in H_2O_2-treated PC12 cells (TUNEL staining). The apoptotic cells (green fluorescence) and the nuclei (blue fluorescence) was monitored by fluorescence microscopy for control cells without any treatment, cells treated with 100 μM H_2O_2, and cells pretreated with DDW (100, 75, 50 ppm deuterium)

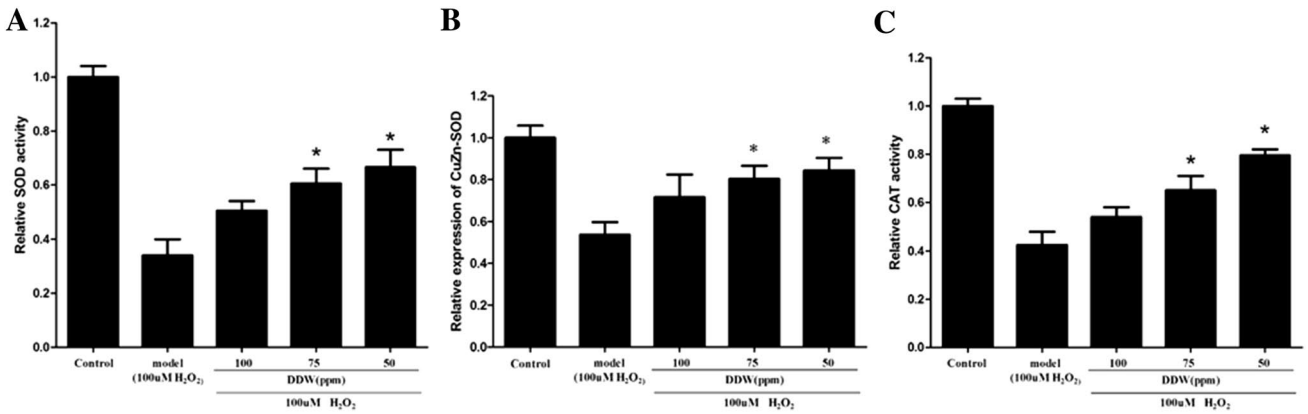


Fig. 4 DDW can markedly increase the activity of the antioxidant enzymes SOD (**a**), CuZn-SOD (**b**) and CAT (**c**) in H_2O_2-treated PC12 cells (*, $P \leq 0.05$ vs model)

absence of H_2O_2. The TUNEL assay results were similar to the ROS results(Fig. 3).

DDW Increases the Activity of Antioxidative Enzymes in PC12 Cells

The effects of DDW on the activity of the antioxidant enzymes SOD and CAT against H_2O_2 toxicity are presented in Fig. 4. Significantly lower antioxidant enzyme (CAT, SOD and CuZnSOD) activity was observed in the H_2O_2-treated groups than in the control group. However, treatment with 75 ppm and 50 ppm deuterium-containing DDW markedly attenuated these differences.

DDW Modulates the Expression of Some Proteins Associated with the PI3K-Akt/PKB Signaling Pathway in H_2O_2-Treated PC12 Cells

The PI3K/Akt signaling pathway is an important intracellular regulatory pathway. Activation of the PI3K pathway

elicits survival signals to counteract multiple types of proapoptotic injury [11]. To investigate whether the PI3K/Akt pathway is involved in DDW-mediated protection against H_2O_2-induced apoptosis, select proteins associated with the PI3K/Akt signaling pathway were examined by Western blot analysis. The results showed that after H_2O_2-induced injury in PC12 cells, the protein levels of PTEN, p-PDK, Akt, p-Akt, Bcl-2, and GSK-3β were markedly decreased compared to control levels. With decreasing deuterium concentrations, the protein levels of PTEN, p-PDK and Akt did not significantly change compared to those in the model (H_2O_2-only) group, but the protein levels of p-Akt, GSK-3β and Bcl-2 gradually increased. The 50 ppm deuterium group exhibited the most significant difference from the model group (Fig. 5, $P < 0.05$ or $P < 0.01$).

Then, we tested whether LY294002, a selective inhibitor of PI3K, abolished the effect of DDW on p-Akt, Bcl-2 and GSK-3β. As shown by the representative image in Fig. 6, preincubation with LY294002 (20 μM) attenuated the increase in p-Akt expression induced by DDW. Similar to the case for p-Akt, the DDW-induced increases in Bcl-2 and GSK-3β were abolished by pretreatment with LY294002.

Discussion

The human body is very sensitive to changes in deuterium concentrations in water. High deuterium concentrations can cause various types of damage. When deuterium enters the body, it combines easily with certain bioactive substances and then influences physiological synthesis and metabolism processes. For example, deuterium can affect the structure of DNA, leading to DNA damage or mutation. Deuterium can also influence enzymes, resulting in abnormal activity and ultimately affecting normal physiological function [5, 6, 23]. In contrast, low deuterium concentrations can stimulate growth. The diameters of low-deuterium water molecules are similar to those of hydrophilic channels on the cell membrane,thus, DDW has higher permeability than normal water and is more likely to pass through membrane water channels. In addition, DDW has a 30% greater ability to act as a solvent than normal water, enabling DDW to effectively dissolve nutrients, accumulated fat, cholesterol and other substances that are not fully absorbed by cells. DDW can also promote insulin secretion, improve glycolipid metabolism, and prevent and delay insulin resistance and type 2 diabetes [14]. As described previously, DDW can significantly reverse Mn^+-induced reductions in nematode lifespan by restoring DAF-16 and SOD-3 levels, suggesting that DDW can improve antioxidant ability [1]. However, the antioxidant effect of DDW on the nervous system has not been confirmed, and the potential mechanism remains unclear.

The pathophysiology of neurodegenerative diseases is dependent upon dysfunction of the oxidant and antioxidant systems. Oxidative stress occurs when the production of oxidant species exceeds the ability of endogenous antioxidants to destroy them. Accumulating evidence has shown that oxidative stress plays an important role in the development of neuronal cell injury and leads to apoptosis in many neurological diseases [13, 18, 22]. Differentiated PC12 cells have characteristics of sympathetic neurons, with structures and functions very similar to those of neurons [19]. In this study, we investigated the neuroprotective effect of DDW against H_2O_2-induced oxidative stress in differentiated PC12 cells and the possible signaling pathways involved.

First, we administered DDW before or after H_2O_2 was used to induce cell damage. The MTT and CCK8 results revealed that as the concentration of deuterium decreased, cell viability gradually increased under both conditions. This finding suggests that DDW can not only protect cells from oxidative injury but also promote the repair of PC12 cells after oxidative injury.

To obtain more evidence about the antioxidative effect of DDW, we further examined the level of ROS and apotosis in H_2O_2- and DDW-treated PC12 cells. Measurement of DCF fluorescence revealed that the relative ROS levels in H_2O_2-treated PC12 cells were significantly increased, but with decreasing deuterium concentrations, the relative ROS levels decreased markedly compared to those in the model group. ROS can damage cell components, such as lipids, proteins and DNA, and then induce nerve cell necrosis or apoptosis [9]. The TUNEL assay results were similar to the ROS results. By reducing intracellular ROS, DDW can play an important role in antioxidation and cell protection. Both CAT, CuZn-SOD and SOD are endogenous antioxidant enzymes that can directly scavenge excessive ROS in the body and protect organisms from oxidative damage [12]. We examined the effects of different concentrations of deuterium on intracellular CAT, CuZn-SOD and SOD activity. H_2O_2 significantly reduced intracellular CAT, CuZn-SOD and SOD activity, but DDW with different concentrations of deuterium increased intracellular CAT, CuZn-SOD and SOD activity. These findings show that DDW can increase antioxidant enzyme activity and protect organisms from oxidative damage. Moreover, the protective effect became more obvious with decreasing deuterium concentrations.

The PI3K/Akt signaling pathway is an important signal transduction pathway in vivo [26]. PI3K family members are proto-oncogenes, and PI3K itself is a heterodimer composed of a catalytic subunit, p110, and a regulatory subunit, p85. PI3K, a phosphatidylinositol kinase that phosphorylates the third hydroxyl group in inositol rings, can phosphorylate inositol on the cell membrane and produce phosphatidylinositol-3-phosphate (PIP), phosphatidylinositol-3,4-bisphosphate (PIP2), and phosphatidylinositol-3,4,5-trisphosphate

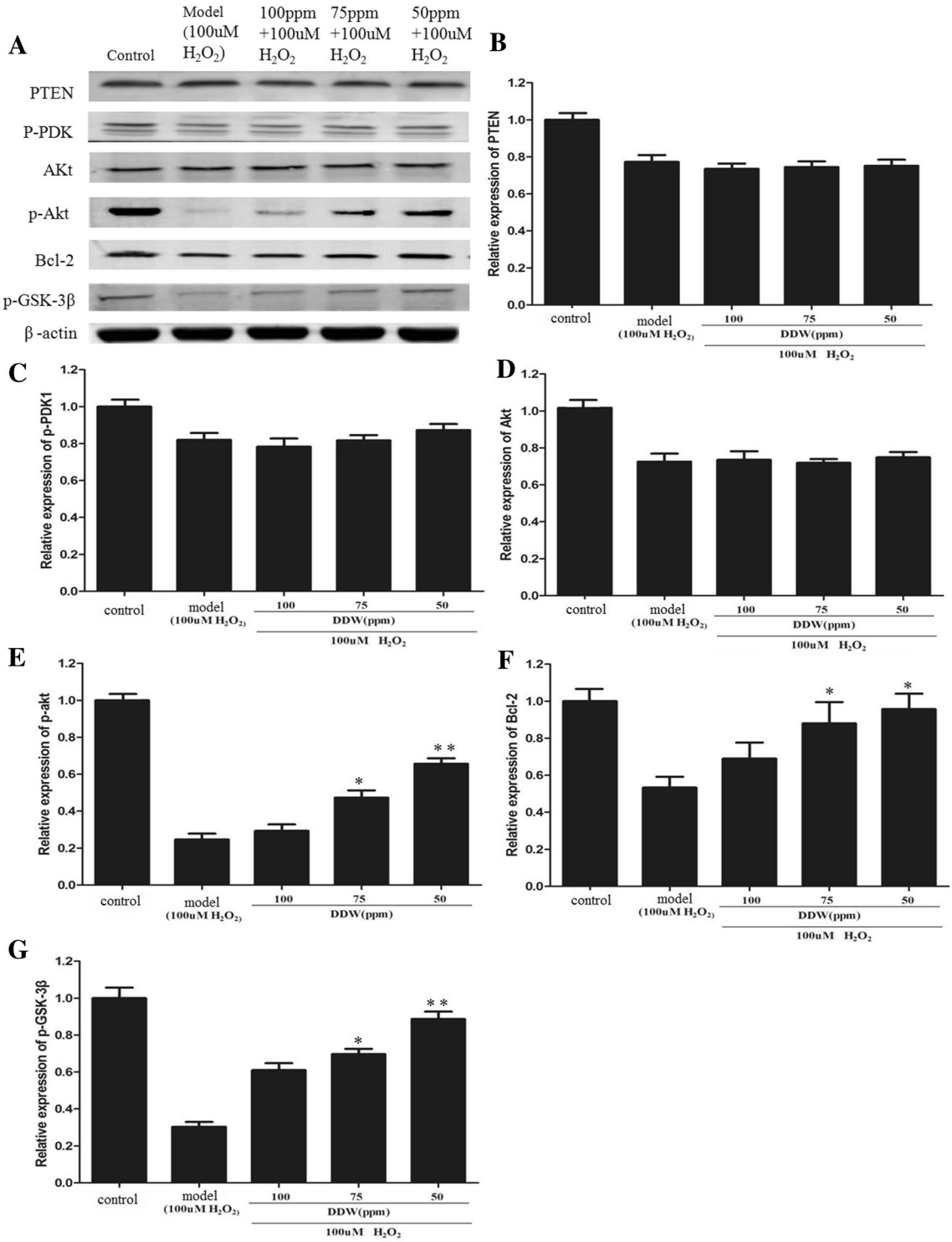


Fig. 5 DDW modulates the expression of proteins associated with the PI3K-Akt/PKB signaling pathway in H_2O_2-treated PC12 cells (**a**), including PTEN (**b**), p-PDK1 (**c**), Akt (**d**), p-Akt (**e**), Bcl-2 (**f**) and p-GSK-3β (**g**). With decreasing deuterium concentration, the protein levels of PTEN, p-PDK and Akt did not significantly change, but the protein levels of p-Akt, Bcl-2 and GSK-3β gradually increased compared to those in the model group (n = 4 for PTEN, p-PDK and Akt; n = 3 for p-Akt, Bcl-2 and GSK-3β; *, $P \leq 0.05$ vs model; **, $P \leq 0.01$ vs model)

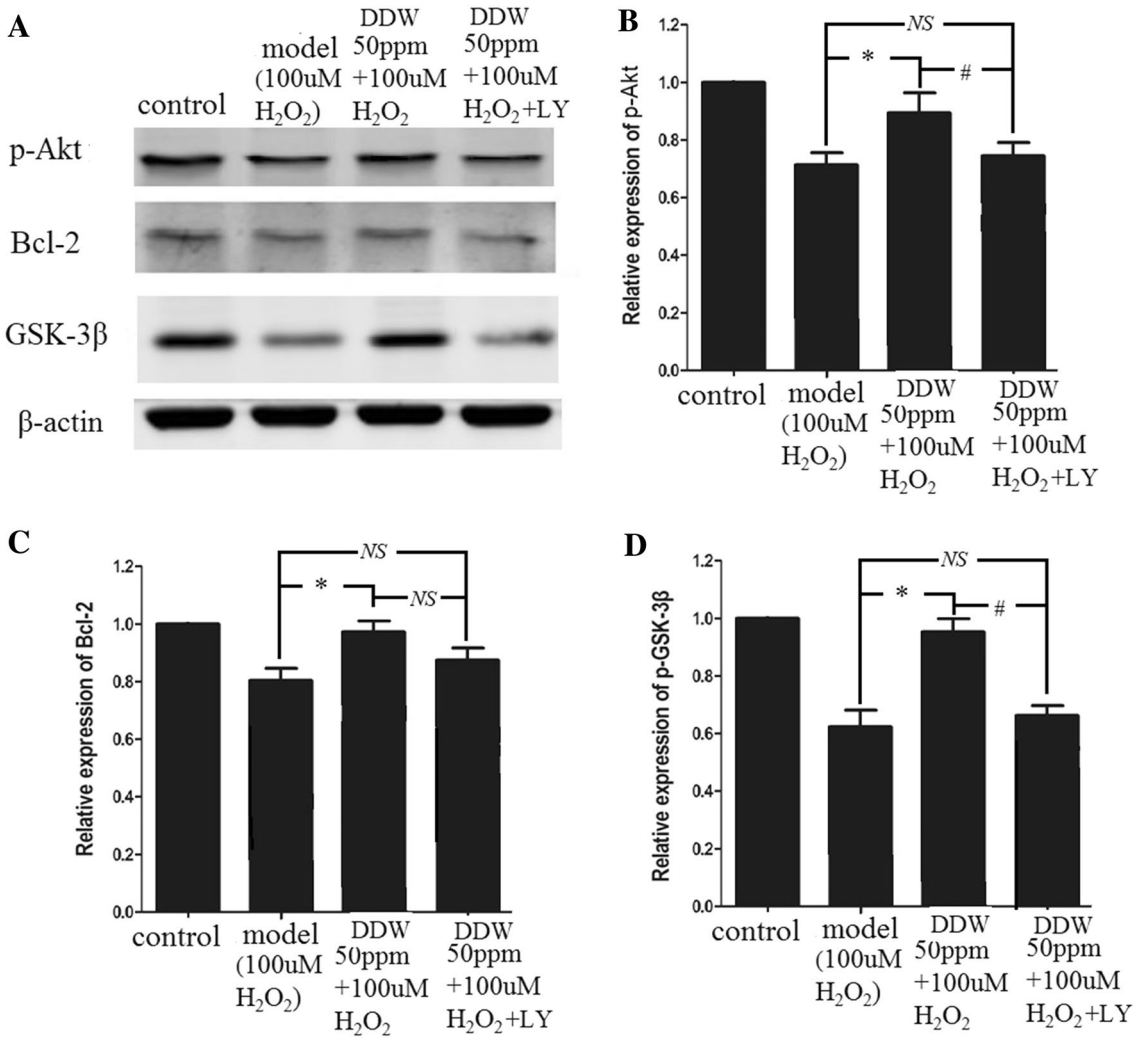


Fig. 6 DDW-mediated increases in p-Akt, Bcl-2 and GSK-3β were abolished by pretreatment with LY294002 (LY). **a** Representative image showing that DDW-mediated increases in p-Akt, Bcl-2 and GSK-3β were abolished by pretreatment with LY294002. **b**, **c**, **d**, Bar graphs showing p-Akt (**b**), Bcl-2 (**c**) and GSK-3β (**d**) protein levels (% of control). Compared with the H_2O_2 alone, DDW (50 ppm deuterium) increased the protein levels of p-Akt, Bcl-2 and GSK-3β. However, preincubation with LY294002 before DDW abolished the increases in p-Akt, Bcl-2 and GSK-3β induced by DDW. Protein expression is expressed relative to the control values (% of control). The results are presented as the mean ± SD (n = 4) for p-Akt, Bcl-2 and GSK-3β (*, $P \leq 0.05$ vs model; #, $P \leq 0.05$ vs DDW with 50 ppm deuterium + 100 μM H_2O_2; *NS* not significant)

(PIP3). The latter two, in combination with the pH region of Akt, partially activate Akt and translocate it to the inner surface of the cell membrane. Then, PIP3 activates phosphoinositide-dependent kinase 1 (PDK1), which is located on the inner surface of the cell membrane and further activates Akt. Akt can cause phosphorylation of serine/threonine (Ser/Thr) sites and produce biological effects.

The PI3K/Akt signaling pathway plays an important role in protection against oxidative stress by inhibiting apoptosis and promoting cell survival [21]. Under certain conditions, activation of the PI3K/Akt signaling pathway can effectively inhibit neuronal apoptosis. In this study, we first confirmed the antioxidative effects of DDW, including increased cell viability and antioxidant enzyme activity and decreased ROS levels. However, the mechanism remained unclear. To investigate whether the PI3K/Akt signaling pathway participated in the antioxidative effect of DDW, we assessed the effect of DDW on related proteins in the PI3K/Akt signaling pathway, including PTEN, Akt, p-Akt, p-GSK3β and Bcl-2. The results demonstrated that after exposure to H_2O_2 for 2 h, the expression levels of these proteins were decreased compared with control levels. Pretreatment with DDW, especially DDW containing 50 ppm deuterium, increased the protein levels of p-Akt, Bcl-2 and GSK-3β compared to H_2O_2 alone but did not significantly change the protein levels of PTEN, p-PDK and Akt. PTEN, p-PDK and Akt are all upstream

proteins in the PI3K/Akt signaling pathway. PTEN is a phosphatase whose function is to dephosphorylate phosphatidylinositol triphosphate. Therefore, PTEN is an important regulatory gene that antagonizes PI3K and downregulates the Akt pathway. p-PDK is an upstream gene of Akt that can activate Akt and exert biological effects. Our results suggested that DDW did not affect the expression of these upstream proteins in the PI3K/Akt signaling pathway but did increase the expression of some downstream proteins, such as p-Akt, Bcl-2 and GSK-3β. Pretreatment with LY294002, a selective inhibitor of PI3K, abolished the DDW-induced increases in p-Akt, Bcl-2 and GSK-3β, further confirming that DDW may affect only downstream proteins in the PI3K/Akt signaling pathway. Bcl-2, a member of a family of antiapoptotic proteins, can inhibit cell death caused by many cytotoxic factors. Much evidence has demonstrated that increased Bcl-2 and/or lowered Bax expression plays a critical role in preventing ROS-induced oxidative damage in cells [10]. GSK-3 is a highly conserved serine/threonine kinase that is ubiquitous in mammalian cells. GSK-3β not only regulates the activity of glycogen synthetase (GS) but also potentially acts on many structural proteins, transcription factors and signaling proteins. In addition, GSK-3β has been confirmed to play an important role in the regulation of cell differentiation, proliferation, survival and apoptosis [16]. As a therapeutic target in many diseases, such as cancer and neurodegenerative diseases, GSK-3β has been paid increasing attention by researchers [8, 20]. Based on our findings, it can be inferred that DDW may affect downstream proteins in the PI3K/Akt signaling pathway, increasing the expression levels of p-Akt, Bcl-2 and GSK-3β and further promoting cell proliferation, inhibiting apoptosis and protecting PC12 cells from oxidative damage.

In conclusion, the results of this study suggest that DDW has a protective effect against oxidative stress injury in neurons and that the potential mechanism may be related to activation of the PI3K-Akt/PKB signaling pathway.

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Compliance with Ethical Standards

Conflicts of interest All authors declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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