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# Neuroprotective effects of torularhodin against $H_2O_2$-induced oxidative injury and apoptosis in PC12 cells

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The neuroprotective effects of torularhodin against oxidative injury and apoptosis in PC12 cells, as well as the related mechanisms, were investigated. The results showed that torularhodin significantly reduced lactate dehydrogenase (LDH) release and malondialdehyde (MDA) production, meanwhile increased the activities of antioxidant enzymes, which were assessed by enzyme linked immunosorbent assay. The presence of torularhodin attenuated $H_2O_2$-induced apoptosis which was proven by flow cytometric detection of $Ca^{2+}$ influx inhibition and the mitochondrial membrane potential (MMP) reduction. Furthermore, the oxidative injury produced by $H_2O_2$ was mitigated by torularhodin pretreatment *via* down-regulation of GSK-3$\beta$ and Keap1 genes while up-regulating the expressions of Nrf2, HO-1 and NQO1 genes. The neuroprotective effects of torularhodin against oxidative injury and apoptosis appeared to be associated with the synergistic effect of mitochondria-mediated pathway and GSK-3$\beta$/ Nrf2 signaling pathway. These findings demonstrated that torularhodin could be considered as a neuroprotective agent against $H_2O_2$-induced oxidative stress.

## 1. Introduction

Injury of neuronal cells is a critical event in the development of neurodegenerative disease. Numerous studies have shown that oxidative stress is a feature among many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Argyri et al. 2014; Reeve et al. 2014; Tiwari and Chaturvedi 2014). Neurodegeneration can result from endogenous oxidative stress, and the production of reactive oxygen species (ROS) within neuronal cells has shown relevance to several neurodegenerative disorders in the vertebrate central nervous system (CNS) (Barja 2004; Hinerfeld et al. 2004; Melov 2004). Oxidative stress facilitating ROS production is an early and sustained event in neurodegenerative disease progression (Yuan et al. 2014). ROS can destroy neurons by perturbing their natural antioxidant defense system and inducing apoptosis, which has been implicated in several biological and pathological processes (Wu et al. 2014). Therefore, protecting neuronal cells from oxidative injury proves to be a promising strategy for the treatment of neurodegenerative disorders.

Some studies have indicated that carotenoids can effectively quench biofilm on $^1O_2$; capture $OH^-$, $NO_2^-$ and peroxynitrite, including scavenging oxygen free radicals and strongly clearing $H_2O_2$ (Sakaki et al. 2000; Brunet et al. 2001). Torularhodin, a, carotenoid with similar structure to that of lycopene consists of 14 conjugated double bounds which are important in radical-scavenging and has received much attention because of its potential antioxidant activity (Madhour et al. 2005). Despite the antioxidant activities of torularhodin have been demonstrated in chemical assays, there have been no intensive studies on an understanding of torularhodin neuroprotective effects mechanism *in vitro*, to the best of our knowledge. PC12 cells, which originate from a rat adrenal pheochromocytoma cell line, have been widely used in neurobiological and neurochemical studies (Westerink and Ewing 2008). $H_2O_2$, as a major source of ROS, could induce oxidative stress in cell lines, causing apoptosis and signalling pathway changes (Elias et al. 2008). The mitochondria-mediated pathway is one of the important signalling pathways in apoptosis, and is associated with the regulation of mitochondrial permeability transition pore opening and cytochrome c released which was related with a series of apoptosis chain reaction (Fernandes-Alnemri et al. 1994; Tewari et al. 1995). In the GSK-3$\beta$/ Nrf2 signaling pathway, glycogen synthesis kinase 3 (GSK-3$\beta$) adjusts the expression of NF-E2-related factor 2 (Nrf2) which is induced by various natural antioxidants resulting in the trans-activation of multiple antioxidant enzymes once it translocates from the cytosol to the nucleus (Kobayashi et al. 2004; Ben-Dor et al. 2005; Wang et al. 2008; Na and Surh 2008). Under normal conditions, Nrf2 localizes in the cytoplasm where it interacts with the actin binding protein, kelch-like ECH-associated protein1 (Keap1), and is rapidly degraded by the ubiquitin-proteasome pathway. However, in oxidative injured cells, antioxidants target the Nrf2-Keap1 complex, dissociating Nrf2 from Keap1, thereby inducing genes encoding antioxidant proteins and phase II detoxifying enzymes, such as hemeoxygenase 1 (HO-1) and NAD(P)H:quinone reductase (NQO1) (Venugopal and Jaiswal 1996).

In this study, a neuronal PC12 cell model was constructed to investigate neuroprotective effects of torularhodin *in vitro* as well as the related mechanisms.

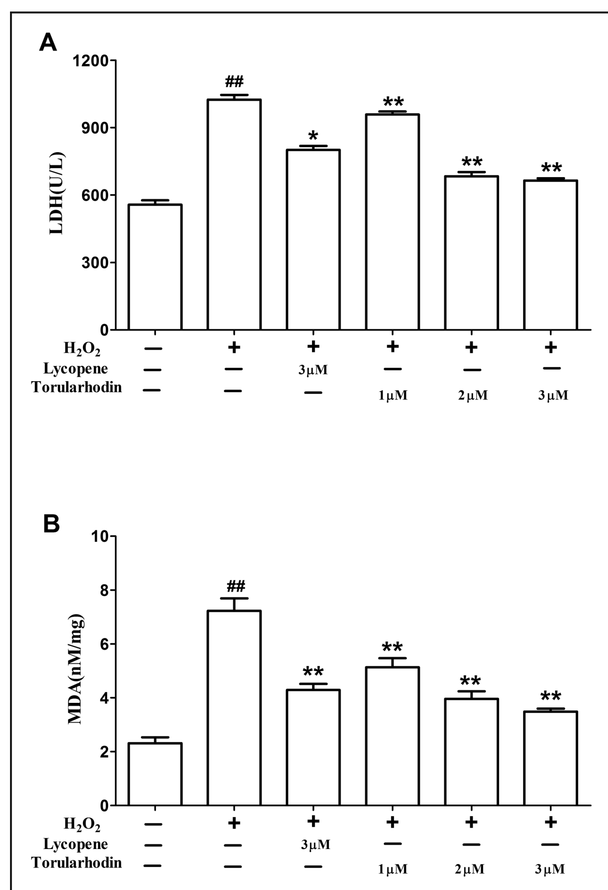


Fig. 1: Effects of torularhodin on LDH leakage (A) and MDA production (B) in oxidative injured PC12 cells. Data were representative of three independent experiments and presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the $H_2O_2$ group; $^{\#\#}p < 0.01$ compared to the control group.

## 2. Investigations, results and discussion

### 2.1. *Effects of torularhodin on LDH leakage and MDA production in oxidative injured PC12 cells*

MDA caused phospholipids and protein to denaturize and crosslink, which consequently led to cell membrane contracting (Liu et al. 2007). When cell membrane was damaged, LDH would be released into the supernatant. Therefore, the amount of LDH was assayed to evaluate the level of cell membrane injury. As illustrated in Fig. 1, the MDA production and LDH leakage of the $H_2O_2$ group increased significantly compared with those of the control group ($p < 0.01$), suggesting that $H_2O_2$ enhanced the production of MDA and destroyed the cell membrane system. However, in cells exposed to $H_2O_2$ alone, LDH leakage was observed to decrease from $1024.38 \pm 21.12$ to $958.76 \pm 12.9$, $683.41 \pm 19.01$ and $664.43 \pm 10.32$ with different concentrations (1 μM, 2 μM, 3 μM) of torularhodin, respectively ($p < 0.05$). In addition, the MDA levels of the torularhodin groups decreased dramatically compared with the $H_2O_2$ group ($p < 0.01$). These data indicated that torularhodin inhibited the lipid peroxidation by blocking reactive oxygen pouring into the cells and thus protected against $H_2O_2$-induced cell membrane damage.

### 2.2. *Effects of torularhodin on the activities of antioxidant enzymes in oxidative injured PC12 cells*

In general, T-AOC levels reflected the enzyme and nonenzyme antioxidase system; CAT helped to scavenge intracellular $H_2O_2$ and its activity indicated oxidative damage to cells; SOD levels indirectly indicated the level of intracellular radicals. Previous studies reported that polysaccharide isolated from cultivated *Cordyceps* mycelia could protect PC12 cells from $H_2O_2$-induced injury *via* increasing T-AOC, CAT and SOD levels (Breimer 1990). In this study, torularhodin altered the levels of T-AOC, CAT and SOD in the $H_2O_2$-induced PC12 cells, as shown in Table 2. Compared with the control group, treatment with $H_2O_2$ for 2 h decreased the levels of T-AOC, CAT and SOD by 76.83 %, 73.47 % and 50.13 % respectively, suggesting that the endogenous antioxidant system was interfered with $H_2O_2$. However, pre-incubation with different doses of torularhodin as well as lycopene significantly enhanced the T-AOC, CAT and SOD levels, compared with the $H_2O_2$ group. The results indicated that torularhodin could protect PC12 cells against oxidative damage by increasing the clearance rate of $H_2O_2$ and strengthening the activity of antioxidant enzyme system.

**Table 1: Primers used for quantitative real-time PCR**

| Target gene | Forward/reverse primers |
|---|---|
| $\beta$-Actin | Forward: 5′-GCTCCTTCGTTGCCGGTCC-3′ |
| | Reverse: 5′-CTCTTGCTCTGGGCCTCGTCA-3′ |
| GSK-3$\beta$ | Forward: 5′-CCTTATCCCTCCACATGCTC-3′ |
| | Reverse: 5′-ATTGGTCTGTCCACGGTCTC-3′ |
| Keap1 | Forward: 5′-ATGGCCACACTTTTCTGGAC-3′ |
| | Reverse: 5′-ATCAATTTGCTTCCGACAGG-3′ |
| Nrf2 | Forward: 5′-CCTCGCTGGAAAAAGAAGTG-3′ |
| | Reverse: 5′-GGAGAGGATGCTGCTGAAAG-3′ |
| HO-1 | Forward: 5′-CAGGTGATGCTGACAGAGGA-3′ |
| | Reverse: 5′-TCTCTGCAGGGGCAGTATCT-3′ |
| NQO1 | Forward: 5′-TTCTCTGGCCGATTCAGAGT-3′ |
| | Reverse: 5′-TCCAGACGTTTCTTCCATCC-3′ |

### 2.3. *Effects of torularhodinon on the nuclear staining apoptosis in oxidative injured PC12 cells*

$H_2O_2$, one of the main ROS, is known to elevate oxidative stress, resulting in apoptosis or necrosis of PC12 cells which was characterized by chromatin margination or condensation, DNA fragmentation, and nuclear collapse (Shaerzadeh et al. 2006). In Fig. 2A, apoptosis cells are brilliant blue, normal cells are light blue and dead cells are red. In the control group (Fig. 2Aa), almost no signs of morphological nuclear damage were observed. However, in the $H_2O_2$ group, cells turned to brilliant blue and red, meanwhile apoptotic body–like structures were frequently noticed, indicating that $H_2O_2$ could destroy the nucleus and induce cell apoptosis. Its number of apoptotic or necrotic cells was also significantly increased, compared with the control group, as shown in Fig. 2B. In marked contrast, the torualrhodin and lycopene groups showed significant attenuation in the number of apoptotic cells, which showed that torularhodin quenched free radicals and prevented the nuclei from being attacked by $H_2O_2$. Our results confirmed that torularhodin inhibited $H_2O_2$-activated apoptosis by eliminating ROS and reducing nuclear damage.

### 2.4. *Effects of torularhodinon on $Ca^{2+}$ influx in oxidative injured PC12 cells*

A major signaling molecule in neurons, intracellular $Ca^{2+}$, affected cellular function from many aspects and played a vital role in the induction of apoptosis (Ansari et al. 2006). As shown in Fig. 3, after incubating cells with $H_2O_2$ for 2 h (Peak 6), the peak drifted higher than that of the control group (Peak 1),

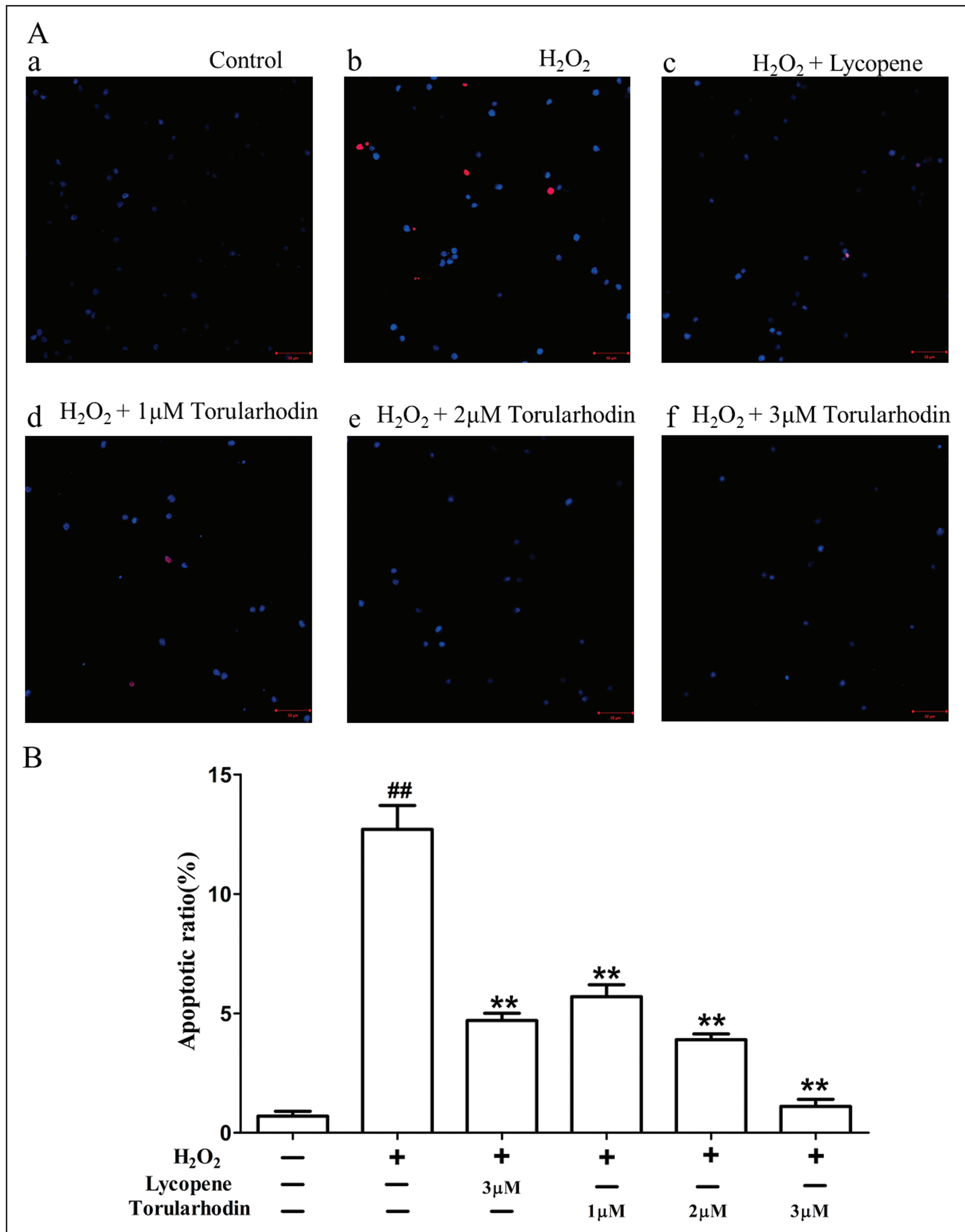


Fig. 2: Effects of torularhodin on the nuclear staining apoptosis in oxidative injured PC12 cells. (A) Morphological apoptosis was determined by staining with Hoechst 33342 and PI. (B) Apoptosis ratio was determined by counting under fluorescence microscope. Data were representative of three independent experiments and presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the $H_2O_2$ group; $^{\#\#}p < 0.01$ compared to the control group.

which suggested that the intracellular $Ca^{2+}$ level was elevated by $H_2O_2$. However, the peaks of the torularhodin groups (Peak 2, Peak 3 and Peak 5) and the lycopene group (Peak 4) drifted lower than that of the $H_2O_2$ group (Peak 6), which indicated that both torularhodin and lycopene obviously reduced the $H_2O_2$-induced level of intracellular $Ca^{2+}$. The intracellular $Ca^{2+}$ level rose rapidly with the release of cytochrome c to cytosol at the beginning of apoptosis (Koya et al. 2000). Our result was consistent with this theory (Fig. 3, Peak 6) and torularhodin markedly decreased the intracellular $Ca^{2+}$ level (Fig. 3, Peak 2, Peak 3 and Peak 5), illustrating that torularhodin could inhibit oxidative damage in the early stage of apoptosis.

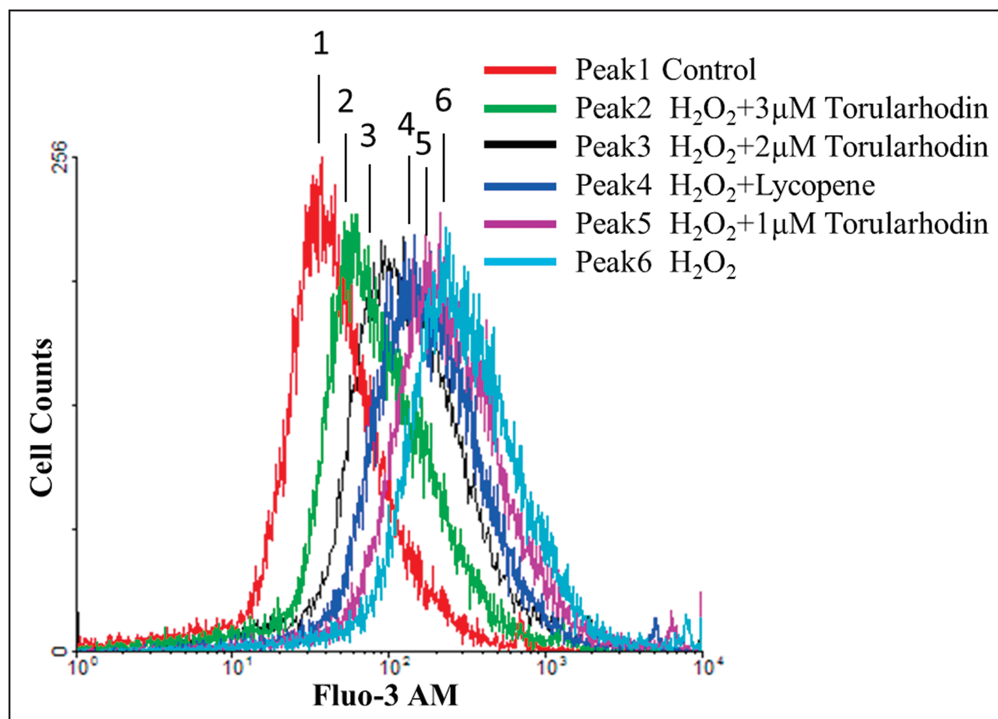


Fig. 3: Effects of torularhodin on $Ca^{2+}$ influx in oxidative injured PC12 cells.

### *2.5. Effects of torularhodin on the change of MMP in oxidative injured PC12 cells*

The level of MMP represented the membrane integrity of the mitochondria and is considered as an indicator of apoptosis induced by mitochondrial pathway (Arrigo et al. 2005). Therefore, apoptosis was well correlated with a drastic decrease in MMP (Wei et al. 2003; Orrenius et al. 2003). The effects of torularhodin on the change of MMP in PC12 cells are shown in Fig. 4. PC12 cells exposure to $H_2O_2$ led to a remarkable drop of 43.19 % in MMP (Fig. 4B) compared to the control group (Fig. 4A). However, when PC12 cells were pretreated with torularhodin (Fig. 4D-F) or lycopene (Fig. 4C), the mean values of JC-1 increased from 1.46 to 1.88, 1.97, 2.46 and 1.90, respectively. Our results revealed that torularhodin was able to inhibit the decrease of MMP and $H_2O_2$-induced cell apoptosis in PC12 cells.
The mitochondria-mediated pathway was known to be involved in specific aspects of mitochondrial dysfunctions such as the opening of the mitochondrial permeability transition pore that caused depolarization of the mitochondrial transmembrane potential, release of cytochrome c and increase of $Ca^{2+}$ influx (Zhao et al. 2011). Our results suggested that torularhodin reduced the change of MMP by controlling the opening of the mitochondrial permeability transition pores, eventually blocking the release of cytochrome c, maintaining the normal physiological functions of mitochondria and stabilizing the intracellular $Ca^{2+}$ level.
The results implied that the mitochondria-mediated pathway by which torularhodin exerted its neuroprotective effects against oxidative stress was through maintaining the MMP balance, which consequently inhibited the disruption of mitochondrial functioning and the apoptosis of cells.

### *2.6. Effects of torularhodin on the expressions of GSK–3β, Keap1, Nrf2, HO-1 and NQO1 mRNA in oxidative injured PC12cells*

To identify the mechanism of the neuroprotective effects of torularhodin against $H_2O_2$-induced oxidative injury in PC12 cells, the relative genes including GSK-3*β,* Keap1, Nrf2, HO-1 and NQO1were quantified. Compared to the mRNA expressions in the control group (Fig. 5, expressions of GSK-3*β* and Keap1 mRNA were high in the $H_2O_2$ group while expressions of Nrf2, NQO1 and HO-1 mRNA were low in $H_2O_2$–treated cells, which indicated that $H_2O_2$ induced the expressions of GSK-3*β* and Keap1 genes, but inhibited the expressions of Nrf2, NQO1 and HO-1 genes and thus led to oxidative stress. The results of the torularhodin groups was in contrast to those in the $H_2O_2$ group, which indicated that torularhodin significantly down-regulated the expressions of GSK-3*β* and Keap1 genes, and up-regulated the expressions of Nrf2, NQO1 and HO-1 genes. These findings suggested that torularhodin might be a vital factor to GSK-3*β*/Nrf2 signaling pathway in $H_2O_2$-induced PC12 cells. Oxidative stress in neurons was inhibited by phosphorylation of Ser-9 in GSK-3*β*which was thought to play a pivotal role in inactivation of GSK-3*β* and activation of Nrf2 resulting in Keap1 and Nrf2 dissociation (Luo 2009; Zhu et al. 2012). Based on the results in the current report, we speculated that the carboxyl of torularhodin might induce the phosphorylation of GSK-3*β*at Ser-9 which possibly was a crucial upstream signaling pathway which mediates the Nrf2-related neuroprotective effect. Torularhodin inhibited Keap1 gene as well, helping to release Nrf2 for translocation into the nucleus to enhance its transcriptional activity. With the activation of Nrf2, the expressions of HO-1 and NQO1 genes were also induced, which in turn led to an increase in antioxidant enzyme production, consequently resulting in the reduction of intracellular ROS and neuroprotection against oxidative stress.
In conclusion, pretreatment with torularhodin is capable of inhibiting $H_2O_2$-induced oxidative damage and apoptosis in PC12 cells. These neuroprotective effects can be attributed to neutralizing ROS damage by increasing intracellular antioxidase system load; inhibiting $H_2O_2$-activated apoptosis by maintaining $Ca^{2+}$ and MMP levels. Furthermore, torularhodin is essential in regulating the mitochondria-mediated pathway and GSK-3*β*/Nrf2 signaling pathway, which are involved in neuroprotection of oxidative stress. Our findings possibly

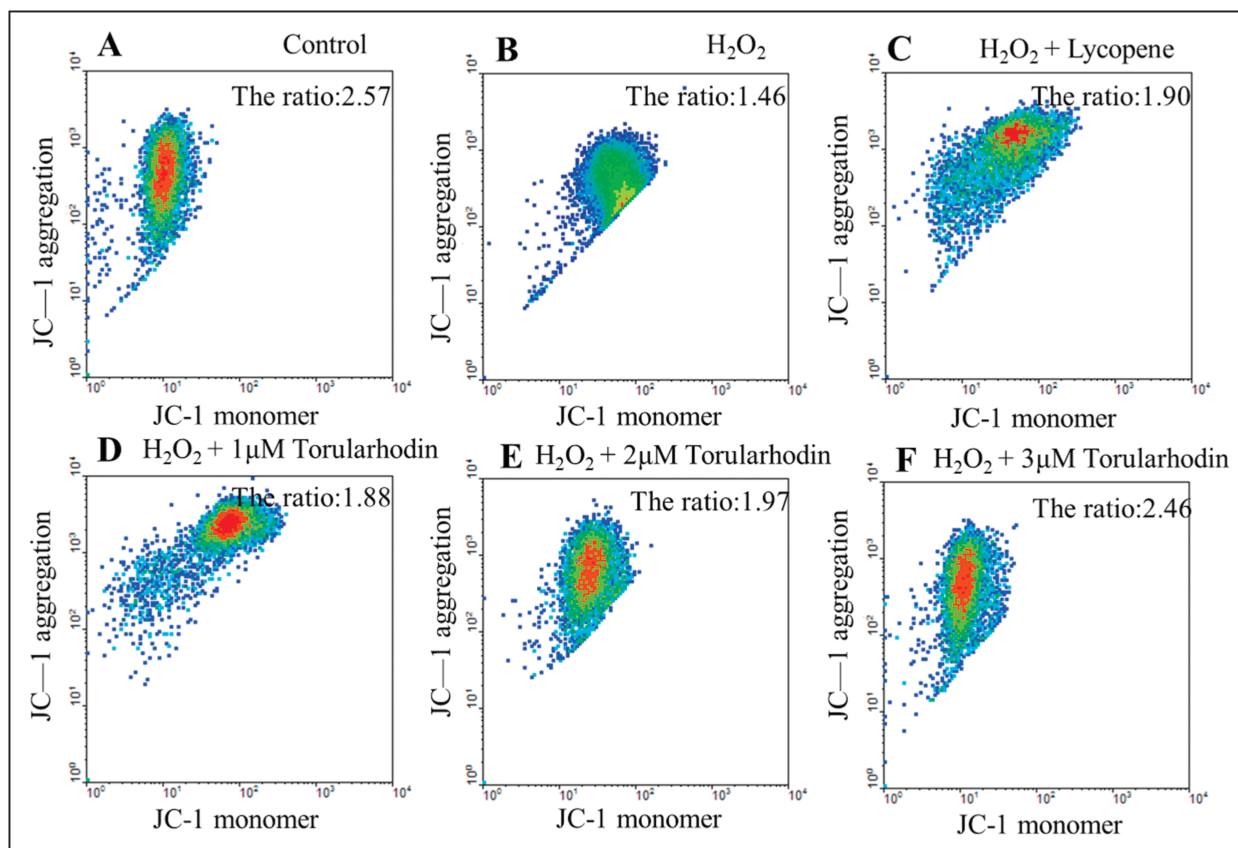


Fig. 4: Effects of torularhodin on the MMP change in oxidative injured PC12 cells.

give a new insight into the application of torularhodin in neurodegenerative disorders.

## 3. Experimental

### 3.1. Materials

Dulbecco's modified eagle's medium (DMEM), foetal bovine serum (FBS) and phosphate buffer saline (PBS) were obtained from Gibco (Grand Island, New York, the United States). Torularhodin was obtained and characterized by our lab. Lycopene and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, the United States). Trizol reagent was purchased from Sangon Biological Technology (Shanghai, China). Bicinchoninic acid (BCA) kit, assay kits of LDH, MDA, total antioxidant capacity (T-AOC), catalase (CAT) and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Propidium iodide (PI), Hoechst 33342, Fluo-3/AM and JC-1 were purchased from Beyotime Institute of Biotechnology (Haimen, China). Super-Script II reverse transcriptase kit was purchased from Shanghai Generay Biotech Co., Ltd (Shanghai, China). All other chemicals were of the highest analytical grade and purchased from commercial suppliers.

### 3.2. Cell culture and treatment

PC12 cells (Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China) were cultured in DMEM with 10 % (v/v) FBS, 100 U penicillin/mL and 100 mg streptomycin/mL aerated with 5 % $CO_2$ at 37 °C. $H_2O_2$ was freshly prepared prior to each experiment for the sake of oxidative stress. Earlier study found that 200 μM $H_2O_2$ was the most suitable concentration for use in the injury module, achieving around 50% cell survival rate (Zhang et al. 2012). In this study, PC12 cells were randomly divided into six groups: a normal control group (without $H_2O_2$ or pigments treatment), a $H_2O_2$ group (exposed to 200 μM $H_2O_2$ for 2 h without pigments treatment), a lycopene group (pretreated with 3 μM lycopene for 24 h and then exposed to 200 μM $H_2O_2$ for 2 h, used as a positive control) and three torularhodin groups (pretreated with various concentrations 1 μM, 2 μM, 3 μM for 24 h, and then exposed to 200 μM $H_2O_2$ for 2 h).

### 3.3. Analysis of LDH release and MDA production

Cells were inoculated into 96-well plates at a density of $5 \times 10^3$ cells/well. After the treatment, cells were washed with PBS (pH 7.4) twice then collected and lysed for 30 min at 4 °C. The cell lysates were then centrifuged at 12,000 g for 5 min at 4 °C. The protein content was measured using a BCA kit, with BSA as the standard. The LDH activity was determined by spectrophotometrically monitoring the decrease of pyruvic acid. The medium was collected to analyse the level of LDH. LDH leakage was calculated as the percentage of LDH in culture medium versus total LDH in cells. The content of MDA, a product of lipid peroxidation, was measured by assay kit. All procedures completely complied with the manufacture instructions. The content of MDA was determined at 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric product.

### 3.4. Measurements of T-AOC, CAT and SOD

For the determination of antioxidant capacity, PC12 cells were rinsed with PBS (pH 7.4) twice then lysed for 30 min at 4 °C. The cell lysates were then centrifuged at 15,000 g for 10 min at 4 °C, and the supernatants obtained were used for the measurements of T-AOC, CAT and SOD. The corresponding kits were used according to the manufacturers' protocol based on colorimetric methods. The levels of T-AOC, CAT and SOD were normalized with the corresponding protein content.

### 3.5. Assessment of apoptosis on nuclear staining

To observe the cell apoptosis, fluorescent DNA-binding dyes Hoechst 33342 and PI were used to define nuclear features as a quantitative index of apoptosis within the cell culture system (Aoki et al. 2001). PC12 cells were cultured as previously described, washed with PBS (pH 7.4) twice and then stained with 10 μL Hoechst 33342 (10 μg/mL) and 5 μL PI (10 μg/mL) at 37 °C for 10 min. Each group of cells was visualized at × 200 and photographed twice (Olympus Optical, Japan). The number of apoptotic cells was counted in five independent microscopic fields for each group (Giovannini et al. 2000).

### 3.6. Detection of intracellular $Ca^{2+}$ level

A $Ca^{2+}$ sensitive fluorescence probe, Fluo-3/AM, was used to monitor the intracellular $Ca^{2+}$ level. The treated cells were loaded with 10 μL Fluo-3/AM (5 μM) for 30 min at room temperature in the dark. After loading

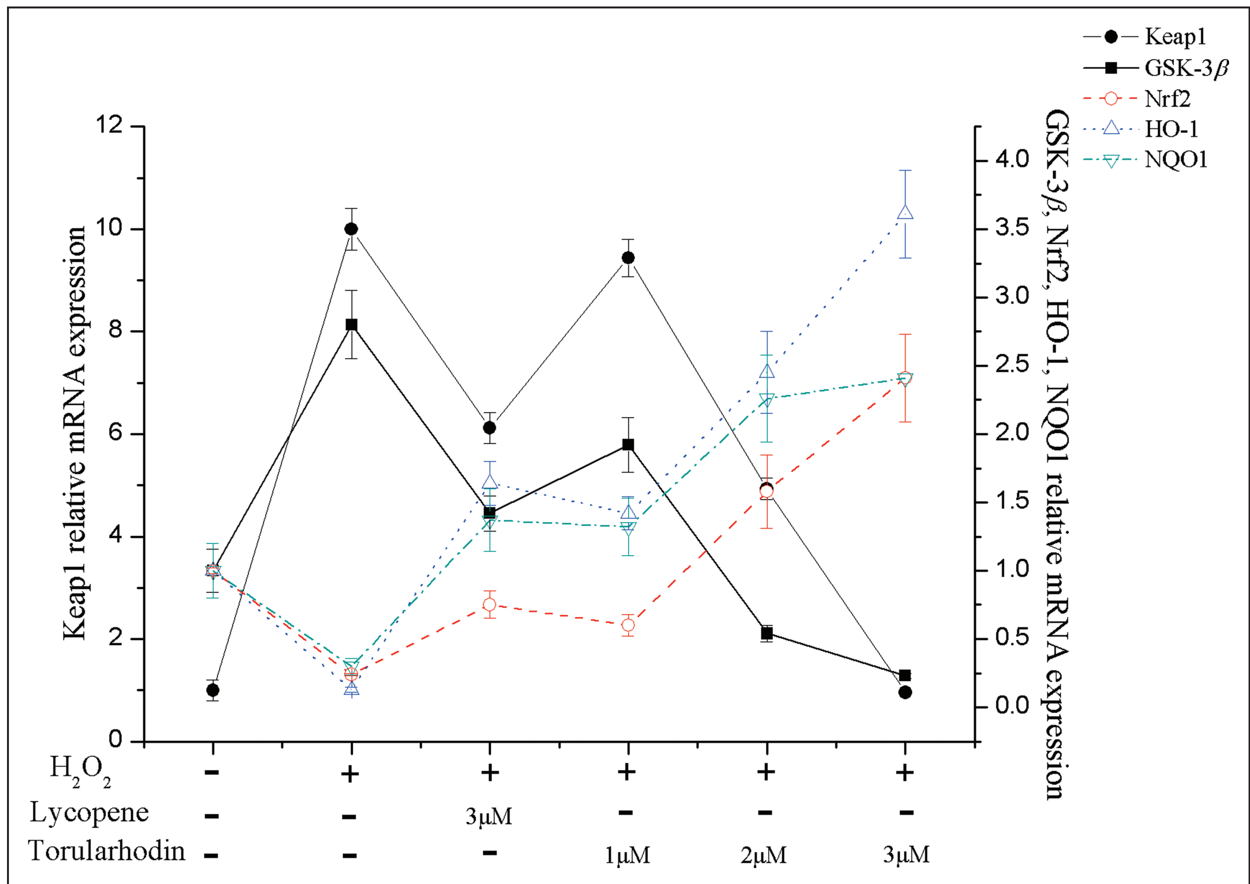


Fig. 5: Effects of torularhodin on the expressions of GSK-3β, Keap1, Nrf2, HO-1 and NQO1 mRNA in oxidative injured PC12 cells.

**Table 2: Effects of torularhodin on T-AOC, CAT and SOD levels in oxidative injured PC12 cells (mean ± SD)**

| | T-AOC (U/mg) | CAT (U/mg) | SOD (U/mg) |
|---|---|---|---|
| Control | 9.28 ± 0.26 | 21.37 ± 1.14 | 198.22 ± 3.89 |
| $H_2O_2$ | 2.15 ± 0.16$^{\#\#}$ | 5.67 ± 0.81$^{\#\#}$ | 98.86 ± 2.68$^{\#\#}$ |
| $H_2O_2$ + lycopene | 7.35 ± 0.20$^{*}$ | 13.46 ± 0.69$^{**}$ | 157.36 ± 4.35$^{*}$ |
| $H_2O_2$ + 1 μM torularhodin | 5.76 ± 0.24$^{**}$ | 8.79 ± 0.55$^{*}$ | 134.42 ± 3.46$^{*}$ |
| $H_2O_2$ + 2 μM torularhodin | 8.72 ± 0.10$^{**}$ | 17.02 ± 0.43$^{*}$ | 164.53 ± 4.77$^{**}$ |
| $H_2O_2$ + 3 μM torularhodin | 9.04 ± 0.17$^{**}$ | 18.58 ± 0.99$^{**}$ | 174.23 ± 2.48$^{**}$ |

$^{*}p < 0.05$, $^{**}p < 0.01$ compared to the $H_2O_2$ group; $^{\#\#}p < 0.01$ compared to the control group.

with Fluo-3/AM, cells were gently rinsed twice with PBS (pH 7.4) and further incubated for 30 min. The intracellular $Ca^{2+}$ level was measured *via* flow cytometry at $\lambda_{ex}$ 488 nm and $\lambda_{em}$ 525 nm and the values of fluorescence intensity were calculated.

### 3.7. Determination of mitochondrial membrane potential (MMP)

JC-1, a dual-emission potential-sensitive probe, was applied to measure MMP. The PC12 cells were harvested after treatment, and incubated with 10 μL JC-1 (10 μg/mL) for 10 min at 37 °C in the dark. Cells were washed twice with PBS, re-suspended in 1 mL PBS (pH 7.4) and analyzed by flow cytometry. The value of JC-1 was determined by the ratio of JC-1 aggregation versus JC-1 monomer in the cells.

### 3.8. Quantitative analysis of gene expression

Total cellular RNA was extracted using trizol reagent from cells following the procedure described by the manufacturer. cDNA was synthesized from the total RNA using Super-Script II reverse transcriptase kit essentially according the instructions of the manufacturer. The specific PCR primers for target genes, including GSK-3β, Keap1, Nrf2, HO-1 and NQO1 are listed in Table 1 β-Actin was used as an internal control. For each target mRNA, 2.5 μL of required cDNA was mixed with 10 μL SYBR Green I PCR Premix (TaKaRa, Dalian, China). Primers were added to a final concentration of 300 nM. The PCR amplification was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, USA). After an initial hold step at 95 °C for 2 min, the 2-step PCR was performed 40 cycles as follows: denaturation at 95 °C for 15 s, annealing at 62 °C for 15 s, followed by a final extension at 72 °C for 35 s. The levels of target genes were determined with comparative $C_T$ method by normalizing to β-actin and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of PCR products was verified by melting curves.

### 3.9. Statistical analysis

Values were expressed as means ± SD and analyzed using one-way ANOVA for comparisons of group means. For all analysis, differences were considered significant at $p < 0.05$. All statistical analyses were conducted using the Statistical Program for Social Sciences 13.0 software program (SPSS Inc, Chicago, IL).

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