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# Serum containing *Tongqiaohuoxue* decoction suppresses glutamate-induced PC12 cell injury<sup>☆</sup>

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## Abstract

Glutamate application is an established method of inducing PC12 cell injury. PC12 cells were cultured with serum containing *Tongqiaohuoxue* decoction consisting of *moschus*, *Carthamus tinctorius*, *Rhizoma chuanxiong*, *Semen pruni persicae*, and *Radix Paeoniae Rubra*. After 24 hours of co-cultivation, glutamate (12.5 mM) was added to the culture medium. We found that serum containing *Tongqiaohuoxue* decoction prevented the increase in reactive oxygen species, and the decreases in superoxide dismutase and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, induced by glutamate. It also reduced the concentration of malondialdehyde, enhanced the mitochondrial transmembrane potential, inhibited the elevation of cellular calcium, and decreased phosphorylation of calmodulin-dependent protein kinase II. Thus, serum containing *Tongqiaohuoxue* decoction had protective effects on cell proliferation and membrane permeability in glutamate-injured PC12 cells.

## Key Words

serum pharmacology of Chinese medicine; *Tongqiaohuoxue* decoction; PC12 cells; glutamate; neural regeneration

## Abbreviations

ATP, adenosine triphosphate; CaMKII, calmodulin-dependent protein kinase II; DCF, 2'-7'-dichlorodihydrofluorescein; MDA, malondialdehyde; MMP, mitochondrial membrane potential; P-CaMKII, phosphorylated CaMKII; Rh-123, rhodamine 123; ROS, reactive oxygen species; SOD, superoxide dismutase; TQHXD, *Tongqiaohuoxue* decoction

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## INTRODUCTION

*Tongqiaohuoxue* decoction (TQHXD) is a classical prescription in traditional Chinese medicine, established by Wang Qingren—the distinguished doctor of the Qing Dynasty. TQHXD is made of *moschus*, *Carthamus tinctorius*, *Rhizoma chuanxiong*, *Semen pruni persicae*, and *Radix Paeoniae Rubra*. TQHXD promotes blood circulation by removing blood stasis, and induces resuscitation by dredging the channels. TQHXD has marked efficacy in the treatment of stagnation of blood in the head

and face channel<sup>[1]</sup>, has preventive and therapeutic effects on stroke, and can ameliorate symptoms of the apoplectic<sup>[2-3]</sup>. We have previously shown that TQHXD has beneficial effects in cerebral ischemia<sup>[4-6]</sup>. The method of serum pharmacology of Chinese medicine can be used to study the effects of a decoction of Chinese Materia Medica *in vitro* with minimal influence of the preparation procedure. Blood serum obtained from rats treated with TQHXD has been shown to have a protective effect on cellular proliferation and membrane permeability in cultured PC12 cells subjected to glutamate toxicity<sup>[7]</sup>. In this

study, we investigated in more detail the mechanisms of this neuroprotective effect. With the aim to inform clinical application, we considered three aspects of cellular function: (1) oxidative stress, involving reactive oxygen species (ROS), superoxide dismutase (SOD), and maleic dialdehyde (MDA); (2) energy metabolism involving adenosine triphosphatase (ATP); and (3) intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) and phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII).

## RESULTS

### Influence of TQHXD-containing serum on intracellular accumulation of ROS

Cultures exposed to glutamate for 20 minutes (model group) displayed increased intensity of 2'-7'-dichlorodihydrofluorescein (DCF)-labeled cells compared with untreated control cultures ( $P < 0.01$ ; Table 1), indicating increased ROS levels. Cultures pretreated with 5% or 10% TQHXD-containing serum showed significantly reduced fluorescence intensity compared with the model group ( $P < 0.01$ ).

### Influence of TQHXD-containing serum on MDA content, SOD activity, and $Na^+-K^+-ATPase$ activity

Glutamate treatment resulted in a decrease in the activity of SOD and  $Na^+-K^+-ATPase$ , and an increase in the

concentration of MDA ( $P < 0.05$ ; Table 1). TQHXD-containing serum at 5% and 10% partially restored these changes ( $P < 0.05$ ; Table 1).

### Influence of TQHXD-containing serum on the mitochondrial transmembrane potential

Rhodamine 123 (Rh-123) staining resulted in regions with fortified fluorescence in cells in the control group (Figure 1A). The staining intensity was considerably decreased in the glutamate-treated model group in the presence of 5%, 10%, and 20% control serum (Figure 1B). The red fluorescence intensity in the TQHXD group at all serum concentrations was higher than that of the model group and a minority of cells showed very intense fluorescence (Figure 1C).

Flow cytometry demonstrated that the proportion of cells with reduced yellow-green fluorescence intensity was significantly higher in the model group compared with the control group, and restored towards control levels in the TQHXD group (Figure 2, Table 1). These results indicated that TQHXD enhanced the mitochondrial transmembrane potential (MMP) in PC12 cells.

### Influence of TQHXD on the glutamate-induced elevation of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$  was significantly increased in cells exposed to glutamate ( $P < 0.01$ ), and TQHXD-containing serum at different concentrations significantly inhibited this increase ( $P < 0.05$ ; Table 1).

Table 1 Neuroprotective effects of serum containing *Tongqiaohuoxue* decoction (TQHXD) against glutamate-induced neurotoxicity in PC12 cells

Group	Serum concentration (%)	Fluorescence intensity	SOD (U/mL)	Malondialdehyde ( $\mu$ M)	$Na^+-K^+-ATPase$ ( $\mu$ molPI/mg/h)	$\Delta\Psi_m$ ↓ cell rate (%)	$[Ca^{2+}]_i$ (nM)	Gray value ratio of P-CaMK II/CaMK II
Control	5	70.89±12.46	85.59±9.36	0.93±0.04	3.70±0.15	23.87±1.28	394.81±36.23	0.38±0.01
	10	114.10±14.31	92.34±6.32	1.28±0.09	4.34±0.24	30.43±2.86	200.50±28.21	0.27±0.03
	20	110.70±24.35	105.30±9.24	1.30±0.07	4.35±0.48	32.79±1.38	136.82±47.23	0.29±0.03
Model	5	93.56±11.40 <sup>b</sup>	76.64±8.46 <sup>a</sup>	1.41±0.09 <sup>b</sup>	2.56±0.24 <sup>b</sup>	50.32±1.89 <sup>b</sup>	864.72±14.25 <sup>b</sup>	0.59±0.03 <sup>b</sup>
	10	146.5±20.54 <sup>b</sup>	83.46±6.25 <sup>a</sup>	1.48±0.05 <sup>b</sup>	3.05±0.18 <sup>b</sup>	53.66±1.98 <sup>b</sup>	755.60±42.68 <sup>b</sup>	0.55±0.03 <sup>b</sup>
	20	134.10±17.43 <sup>b</sup>	88.26±7.19 <sup>b</sup>	1.62±0.07 <sup>a</sup>	3.64±0.33 <sup>a</sup>	61.83±2.39 <sup>b</sup>	447.64±35.79 <sup>b</sup>	0.45±0.03 <sup>b</sup>
TQHXD	5	81.78±10.95 <sup>d</sup>	80.36±6.44 <sup>c</sup>	1.18±0.06 <sup>d</sup>	3.66±0.14 <sup>d</sup>	45.89±2.79 <sup>c</sup>	588.87±64.35 <sup>d</sup>	0.48±0.03 <sup>d</sup>
	10	137.60±26.53 <sup>c</sup>	89.25±9.34 <sup>c</sup>	1.36±0.04 <sup>c</sup>	4.02±0.17 <sup>d</sup>	48.15±1.02 <sup>c</sup>	224.04±37.98 <sup>d</sup>	0.50±0.03 <sup>c</sup>
	20	132.30±16.89	91.35±8.37	1.38±0.04 <sup>c</sup>	4.26±0.17 <sup>c</sup>	53.11±3.29 <sup>d</sup>	233.43±25.76 <sup>c</sup>	0.46±0.03

Data are presented as mean ± SD ( $n = 6$ ) and were analyzed by one-way analysis of variance and Fishers least significant difference *post hoc* test. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs. control group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , vs. model group. SOD: Superoxide dismutase; CaMK II: calmodulin-dependent protein kinase II; P-CaMK II: phosphorylated CaMKII;  $[Ca^{2+}]_i$ : intracellular calcium concentration.

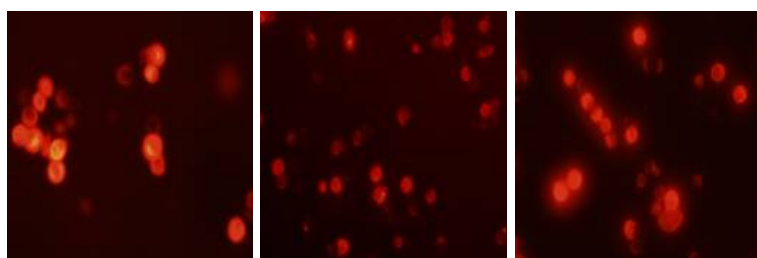


Figure 1 Effect of 5% serum with *Tongqiaohuoxue* decoction (TQHXD) on the intensity of fluorescence in PC12 cells stained with Rhodamine 123 (Rh-123) ( $\times 400$ ). PC12 cells stained by Rh-123 displayed red fluorescence.

(A) Cells in the control group showed intense fluorescence intensity. (B) Fluorescence intensity in the model group was weak. (C) The fluorescence intensity of cells in the TQHXD group was enhanced compared with the model group (B).

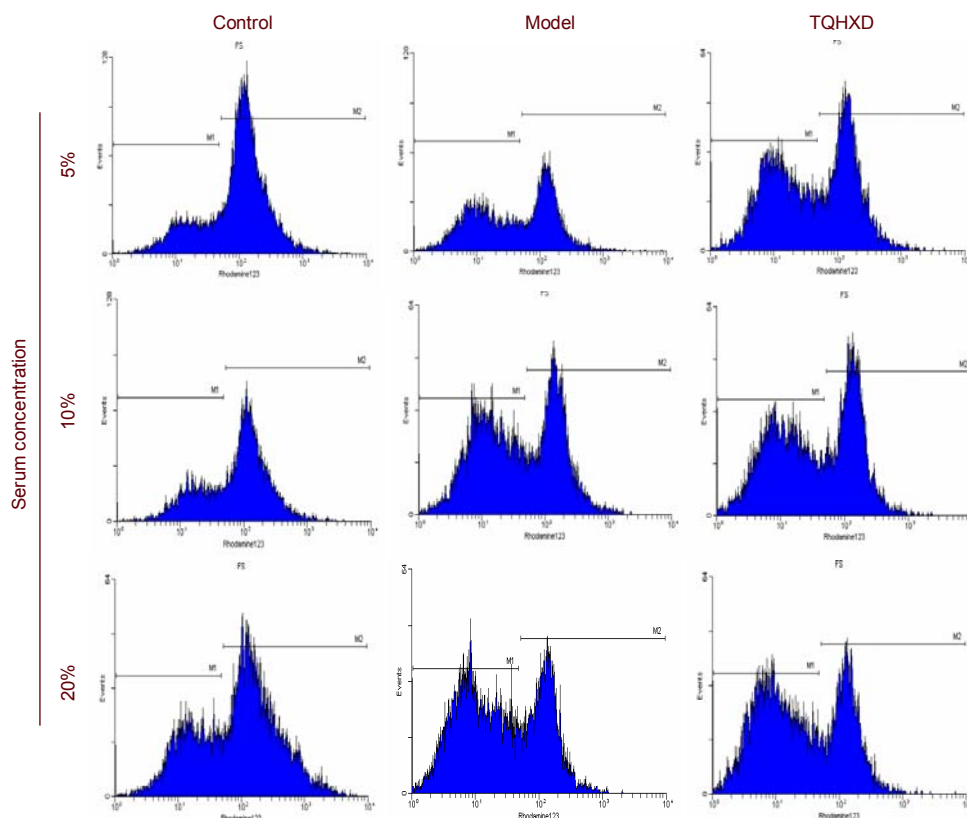


Figure 2 Effect of serum with *Tongqiaohuoxue* decoction (TQHXD) on the fluorescence intensity of PC12 cells stained with Rh-123, detected by flow cytometry. The proportion of cells with reduced fluorescence intensity ( $\Delta\Psi_m\downarrow$ ) was lower in the TQHXD group than in the model group. M1: The cells with decreased  $\Delta\Psi_m$ ; M2: The cells with normal  $\Delta\Psi_m$ .

### Influence of TQHXD-containing serum on phosphorylation of CaMKII

The ratio of phosphorylated to total CaMKII (P-CaMKII/CaMKII) determined by western blot analysis represents the degree of phosphorylation of CaMKII. We found that P-CaMKII/CaMKII was significantly higher in the model group compared with the control group, and that TQHXD-containing serum decreased CaMKII phosphorylation (Figure 3, Table 1).

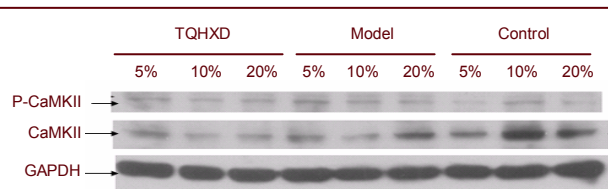


Figure 3 Effect of serum with *Tongqiaohuoxue* decoction (TQHXD) on the expression of calmodulin-dependent protein kinase II (CaMKII) and phosphorylated CaMKII (P-CaMKII) in PC12 cells (western blotting).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. A high gray value (Table 1) represents low protein expression.

## DISCUSSION

Glutamate is an amino acid involved in excitatory

neurotransmission in the central nervous system. Glutamate is present at high levels in the brain of humans and other mammals. Excessive glutamate is harmful to neurons and plays an important role in many pathophysiological states, including anoxia/ischemia, Alzheimer's disease, and neuroendocrine disturbances<sup>[8]</sup>. Oxidative stress triggers apoptosis associated with increased ROS generation, accumulation of free  $[Ca^{2+}]_i$ , and decreased MMP<sup>[9-11]</sup>. It has been hypothesized that three key elements are involved in neuronal death: (1) an increase of intracellular ROS, (2) impaired energy production, and (3) an increase in  $[Ca^{2+}]_i$ <sup>[12-13]</sup>. Cellular energy synthesis takes place primarily within the mitochondria. ATP, a high-energy molecule synthesized through oxidative phosphorylation in the mitochondrial respiratory chain, is the main source of cellular energy<sup>[14]</sup>. ATP is necessary to drive  $Na^+-K^+$ -ATPase activity, which consumes 50 percent of the energy supply in the central nervous system. The onset of cerebral ischemia is accompanied by a reduction of  $Na^+-K^+$ -ATPase activity due to depletion of intracellular ATP<sup>[15-16]</sup>. A previous study has demonstrated that hypoxia can result in an increase in free radicals in mitochondria and a decrease in  $Na^+-K^+$ -ATPase activity, and that these changes are capable of mediating cell degeneration and death<sup>[17]</sup>. SOD, the only known

enzyme that specifically clears superoxide anions, plays an important role in the effectual clearance of harmful free radicals, and also reduces and blocks lipid peroxidation. MDA is one of the end-products of lipid peroxidation, and MDA levels reflect the degree of oxidative injury to the organism. Unsaturated fatty acids of membrane phospholipids are easily attacked by free radicals, causing lipid peroxidation and crosslinking of membrane proteins, enzymes and phosphatides, ultimately leading to membrane damage<sup>[18]</sup>. In this study, TQHXD-containing serum reduced ROS generation, prevented oxidative damage, protected membrane structure and mitochondrial function, increased SOD and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, and reduced MDA levels. These findings suggest that TQHXD protects mitochondria from structural damage by ROS, prevents ROS release into the cytoplasm, and supports the balance between ROS production and clearance.

Mitochondria are also involved in Ca<sup>2+</sup> buffering and ATP generation. Oxidative stress and elevated intramitochondrial Ca<sup>2+</sup> can lead to the opening of the mitochondrial permeability transition pore<sup>[19]</sup>. This results in a loss of ATP synthesis and also allows low molecular weight intramitochondrial components to escape into the cytosol. Using Rh-123 localization as a measure of mitochondrial integrity, we found that glutamate at 12.5 mM led to mitochondrial membrane depolarization. TQHXD-containing serum restored the loss of MMP. This effect may be associated with the inhibition of intracellular ROS accumulation.

Glutamate exposure caused an increase of ROS, as determined by the dramatic increase in the intensity of DCF-labeled cells. The large increase in intracellular Ca<sup>2+</sup> reflects the insult from oxidative stress imposed by ROS on the cells. Sustained elevation of [Ca<sup>2+</sup>]<sub>i</sub> may impair mitochondrial function and lead to irreversible membrane damage and eventually cell death<sup>[20]</sup>.

Therefore, Ca<sup>2+</sup> plays an important role in the development of oxidative injury. Glutamate exposure causes membrane depolarization through to the opening of ion channels and increased Ca<sup>2+</sup> influx. CaM kinase responds to Ca<sup>2+</sup> influx through Ca<sup>2+</sup> channels gated by ATP and acetylcholine, and mediates the actions of many neurotransmitters and Ca<sup>2+</sup> signaling pathways<sup>[21]</sup>. CaMKII, a serine/threonine protein kinase, is the major CaM kinase, and is highly concentrated in postsynaptic densities-exceeding the amounts of other signal transduction molecules<sup>[22-23]</sup>. CaMKII has been implicated in a wide variety of neurobiological processes, including synaptic plasticity<sup>[24-25]</sup>. It is activated by autophosphorylation triggered by binding of Ca<sup>2+</sup>-containing calmodulin. The activity of the phosphorylated form of CaMKII is independent of calmodulin binding<sup>[26-27]</sup>. Phosphorylation of protein

kinases is one of most important means of regulation of protein function in the body, and is the most fundamental and important molecular event in intracellular signal transduction in the nervous system. Nevertheless, hyperphosphorylation will disturb the balance between phosphorylation and dephosphorylation and evoke pathological changes such as apoptosis<sup>[28-29]</sup>. Pretreatment with TQHXD blocked glutamate-induced Ca<sup>2+</sup> influx and significantly decreased the degree of CaMKII phosphorylation.

In summary, TQHXD protected PC12 cells from glutamate-induced toxicity by interrupting the cell death cascade at three distinct steps: (1) blocking ROS production, (2) preventing the decrease of MMP, and (3) inhibiting Ca<sup>2+</sup> influx and regulating intracellular Ca<sup>2+</sup> signaling.

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## MATERIALS AND METHODS

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### Design

A parallel controlled *in vitro* experiment.

### Time and setting

Experiments were performed at the Anhui Key Laboratory of Modernized Chinese Material, Anhui University of Traditional Chinese Medicine, China, from June, 2010 to June, 2011.

### Materials

#### Animals

Twenty clean healthy Sprague-Dawley rats (10 males, 10 females), aged 6–8 weeks, weighing 200 ± 20 g, were provided by the Laboratory Animal Center of Anhui Medical University (license No. SCXK (Wan) 2007-003) and housed in a cage at 23 ± 1°C with a 12-hour light-dark cycle. Food and water were freely available. The protocol was performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China<sup>[30]</sup>.

#### Cells

PC12 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

#### Drugs

The composition of TQHXD was *moschus* (0.15 g), *Carthamus tinctorius* (9 g), *Rhizoma chuanxiong* (3 g), *Semen pruni persicae* (9 g), *Radix Paeoniae Rubra* (9 g) (Hefei He Yi Tang Traditional Chinese Medicines Co., Ltd., Hefei, Anhui Province, China). Ten times of water was added to 10 times of the weight of TQHXD. The mix



was soaked for 2 hours and decocted twice, each time lasting 2 hours, and the solution was filtered and concentrated. The concentrate of the water extract from the prescription was precipitated by 70% ethanol, and centrifuged for 10 minutes at 3 000 r/min. The liquid supernatant was decompressed and condensed to a density of 1.6 g/mL. The extraction from the prescription by water extract-alcohol precipitation was diluted with culture medium and sterilized using a filter membrane. The filtered liquid served as the donor sample of TQHXD<sup>[7]</sup>.

## Methods

### **Preparation of TQHXD according to conventional methods of traditional Chinese drug-containing serum**

The 20 Sprague-Dawley rats were randomly divided into TQHXD ( $n = 10$ ) and blank control ( $n = 10$ ) groups. Rats in the TQHXD group received intragastric administration of TQHXD twice a day for 5 days<sup>[7, 31]</sup>. The dose (4 g/kg/d) was calculated based on the adult human therapeutic dose<sup>[1]</sup>. The control group received intragastric injection of physiological saline twice a day for 5 days. One hour after the last administration, rats were intraperitoneally anesthetized by amobarbital and blood was sampled from the abdominal aorta and centrifuged. The serum was aliquoted into 10 mL ampoules and preserved at  $-70^{\circ}\text{C}$  for future use.

### **PC12 cell culture**

Tubes containing the PC12 cell strain were removed from storage in liquid nitrogen and placed in a water bath at  $37^{\circ}\text{C}$  under constant agitation to allow melting within 30 seconds. The cells were centrifuged at 800 r/min for 5 minutes. The supernatant was discarded and the cells were dispersed with phosphate buffered saline and centrifuged again at 800 r/min for 5 minutes. PC12 cells were routinely cultured in RPM 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% calf serum, 100 units penicillin, and 100 U/mL streptomycin in a humidified atmosphere of 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The medium was changed every 3 days and cells were passaged every 3 days.

### **Establishment of the model of PC12 cell glutamate toxicity**

PC12 cells were divided into control, model, and TQHXD groups. The cell concentration was  $2.0 \times 10^6$  cells/mL. Cells in the normal group were incubated in RPMI 1640 culture medium. Blank rat serum was added in the normal and model groups, and serum containing TQHXD was added in the TQHXD group. PC12 cells in the model and TQHXD groups were cultured in 5%, 10%, or 20% serum for 1 hour, and then treated with 12.5 mM

glutamate (Sigma, St. Louis, MO, USA) to induce cell injury<sup>[7]</sup>. The experiments described below were done after 24 hours of incubation.

### **Detection of SOD, MDA, and ATPase activity**

The PC12 cells were collected in a 96-well plate with  $2 \times 10^6$  cells/well, and then broken by the Ultrasonic Cell Disrupter System (Ningbo XinZhi Biotechnology Co., Ltd., China).  $\text{Na}^+/\text{K}^+$ -ATPase activity, SOD activity, and MDA content were determined using the SOD Assay Kit, MDA Assay Kit, and ATPase Assay Kit (Nanjing Jiancheng Bioengineering Research Institute, China). The measurement of SOD activity was based on the xanthine-xanthine oxidase method<sup>[32]</sup>. The sample supernatant was combined with the xanthine-xanthine oxidase reagent and incubated at  $37^{\circ}\text{C}$  for 40 minutes, and the reaction was terminated with sodium dodecyl sulfate. The absorbance of each sample was recorded at 532 nm. SOD activity was calculated according to the sodium nitrite standard curve:  $y = 0.066\ 83x - 0.009\ 373$ ,  $r = 0.998\ 7$ , where  $x$  represents the concentration of sodium nitrite and  $y$  represents the optical density of sodium nitrite combined with developer. MDA levels were assessed as an index of lipid peroxidation<sup>[33]</sup>. Cells were mixed with 3 mL of  $\text{H}_3\text{PO}_4$  solution (1%, v/v), followed by addition of 1 mL of thiobarbituric acid solution (0.67%, w/v). The mixture was heated in a water bath ( $95^{\circ}\text{C}$ ) for 45 minutes, and the colored complex was extracted in butanol and cooled to room temperature. Absorbance at 532 nm was measured using tetramethoxypropane as the standard.  $\text{Na}^+/\text{K}^+$ -ATPase activity was determined using the ammonium molybdate method<sup>[34]</sup> that measures phosphoric acid formation from ATP. Quantification was performed spectrophotometrically according to the manufacturer's instructions.

### **Detection of cellular ROS levels**

To determine whether TQHXD-containing serum prevented intracellular accumulation of ROS and the resulting oxidative stress, intracellular ROS levels were estimated using 2'-7'-dichlorodihydrofluorescein diacetate (Beyotime Institute of Biotechnology, Nanjing, China). In addition, the fluorescence intensity of cellular oxidized Rh-123 was detected by flow cytometry after incubation with dihydrogenrhodamine 123 for 20 minutes<sup>[35]</sup>. The excitation filter was set at 488 nm and the emission filter at 525 nm.

### **Measurement of MMP**

PC12 cells in a 96-well plate ( $1 \times 10^6$  cells/well) were pretreated with different concentrations of TQHXD-containing serum for 24 hours. Then, Rh-123 (Nanjing KeyGen Biotech Co., Ltd., Nanjing, Jiangsu Province, China) was added to the medium (30  $\mu\text{g}/\text{mL}$ )

and incubated for 30 minutes at 37°C. Culture medium was added to wash unbound dye. A smear of cell suspension was observed and photographed using a fluorescence microscope (Olympus, Tokyo, Japan). Rh-123 fluorescence intensity was measured using flow cytometry (FACScan Becton Dickinson, USA). The excitation filter was set at 505 nm and the emission filter at 534 nm<sup>[36-37]</sup>.

#### Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$  was determined by the intensity of the fluorescent  $Ca^{2+}$ -sensitive dye Fura-2-AM (Dojindo, Japan)<sup>[38-39]</sup>. In a dark room, PC12 cells were loaded with Fura-2-AM (5 mM) in 0.1% dimethyl sulfoxide, and 1% bovine serum albumin for 30 minutes at room temperature, and incubated for 30 minutes at 37°C. Fluorescence measurements were done with a spectrofluorometer (Shimadzu Co., Kyoto, Japan). Fura-2-AM-loaded PC12 cells were exposed sequentially to an excitation wavelength of 340 nm and 380 nm (bandwidth 10 nm), and the emission signal was monitored at a wavelength of 510 nm (bandwidth 10 nm). Fluorescence ratios were converted into calcium concentrations using the following equation:  $[Ca^{2+}]_i = K_d \times [(R - R_{min}) / (R_{max} - R)] \times FD / Fs$ , where  $K_d$  was 224 nM and  $R(F_{340}/F_{380})$  was the fluorescence intensity.  $R_{max}$  was determined by adding Triton X-100 (Sigma; 0.1%) and  $R_{min}$  by adding EGTA (Sigma; 5 mM). FD was the fluorescence of Fura-2-AM at 380 nm under  $Ca^{2+}$ -free conditions and Fs the fluorescence under saturation with  $Ca^{2+}$ .

#### Expression of P-CaMKII determined by western blot analysis

Cells were lysed with buffer containing (in mM) Tris-HCl 50, NaCl 100, MgCl<sub>2</sub> 10, EDTA 2, and 0.1% Triton X-100. The cell lysates were centrifuged at 15 000 r/min and the supernatant collected. The protein concentration was determined using the Bradford assay. The cell lysates (20 mg) were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% goat serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang Province, China), the membranes were incubated in rabbit anti-CaMKII polyclonal antibody (1:250; Abzoom biolabs, Dallas, TX, USA) and rabbit anti-phospho-CaMKII polyclonal antibody (1:300; Abzoom biolabs) at 4°C for one night. This was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3 000, Rockland, Gilbertsville, PA, USA) at 37°C for one hour. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5 000, GAPDH antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the loading control. The gray

levels of electrophoresis strips were analyzed using GeneSnap software (Syngene, Cambridge, UK) and the ratio of P-CaMKII to CaMKII gray levels was calculated to evaluate the protein content<sup>[40]</sup>.

#### Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA), and presented as mean ± SD. One-way analysis of variance was used to evaluate differences between groups. The Fishers least significant difference *post hoc* test was used to analyze differences between groups. A value of  $P < 0.05$  was considered statistically significant.

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**Author contributions:** All authors participated in the study design, conduction, and evaluation. Ning Wang drafted the manuscript. All authors contributed to preparation of the manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Animal Ethics Committee of Anhui Medical University, China.

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