

Original Article

Traditional Chinese medicine formula Qing Huo Yi Hao as superoxide anion scavenger in high glucose-treated endothelial cells

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Aim: To investigate the effects of a traditional Chinese medicine formula Qing Huo Yi Hao (QHYH) and its components on hydroxyl radical (HO^\bullet) production *in vitro* and the activity of QHYH against free radicals in cultured endothelial cells induced by high glucose.

Methods: Hydroxyl radicals (HO^\bullet) were generated through Fenton reactions *in vitro*, and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was used as a spin trap to form DMPO/ HO^\bullet adducts detected using electron paramagnetic resonance (EPR). Immortalized mouse cerebral microvascular endothelial (bEnd.3) cells were treated with high glucose (35 mmol/L). The free radical scavenging ability of QHYH in the cells was evaluated using EPR. Superoxide dismutase (SOD) was used to identify the free radicals scavenged by QHYH in the cells.

Results: QHYH and its 8 components concentration-dependently reduced DMPO/ HO^\bullet signaling. The DMPO/ HO^\bullet adduct scavenging ability of QHYH was 82.2%, which was higher than each individual component. The free radical scavenging ability of 1% QHYH in high glucose-treated bEnd.3 cells was approximately 70%. In these cells, the free radicals were also specifically reduced by SOD (400 U/mL), implying that the free radicals were primarily superoxide anions.

Conclusion: The results demonstrate that the QHYH formula is potent antioxidant acting as scavenger of superoxide anions in high glucose-treated endothelial cells.

Keywords: TCM; Qing Huo Yi Hao; antioxidant; endothelial cell; high glucose; free radical; superoxide dismutase; electron paramagnetic resonance

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Introduction

Reactive oxygen species (ROS) is a collective term that broadly describes O_2 -derived free radicals, such as superoxide anion ($\text{O}_2^{\bullet-}$), HO^\bullet , peroxy (RO_2^\bullet), and alkoxy (RO^\bullet) radicals, as well as O_2 -derived non-radical species, such as hydrogen peroxide (H_2O_2)^[1]. ROS are formed as by-products of aerobic respiration and various other metabolic processes in cells^[2]. Enzymatic and non-enzymatic antioxidants also exist in cells that degrade and inactivate ROS to maintain them at a low or moderate level^[3, 4]. ROS at physiological concentrations regulate many cellular processes, such as proliferation, differentiation, and cell death^[5, 6]. However, an imbalance between ROS production and intracellular antioxidant defense could lead to ROS accumulation, which causes harmful effects collectively termed oxidative stress^[7]. According to a large body of research, reactive stress is involved in the onset of aging

and many diseases, including diabetes, cardiovascular disease, and neurodegenerative diseases (eg, Alzheimer's disease and Parkinson's disease)^[8–11].

The prevalence of diabetes mellitus (DM) is increasing dramatically, especially in developing countries^[12, 13]. Cardiovascular complications of diabetes are the leading cause of DM-related death^[14]. The earliest pathophysiological stage of DM cardiovascular complications is endothelial dysfunction^[15, 16]. Oxidative stress resulting from hyperglycemia, hyperlipidemia, and insulin resistance is the crucial factor involved in endothelial dysfunction^[17, 18]. Therefore, using an antioxidant to reduce ROS could be effective for the prevention and treatment of cardiovascular complications from diabetes. However, many clinical trials investigating a possible role for antioxidants such as vitamin E, vitamin C and beta-carotene in the prevention of atherosclerosis or clinical events have failed^[19, 20]. One reason for the failure of these trials is that antioxidants do not accumulate in the mitochondria, which is the major intracellular site of ROS production^[21]. Therefore, developing a more potent mitochondria-targeting antioxidant is essential^[22].

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QHYH is a traditional Chinese medicine (TCM) formula, the principles of which are based on “clearing heat and detoxifying” and improving blood circulation and anti-inflammation. In our previous single-blind, randomized, controlled clinical trial, QHYH was demonstrated to reduce urinary albumin excretion ability (UAER), which is an important marker of systemic endothelial function^[23], and to ameliorate micro-circulation nail bed flow in type 2 diabetes^[24]. These results suggested that QHYH could improve endothelial function. In addition, gene chip analysis showed that QHYH acted on high glucose-induced endothelial cells mainly through oxidative phosphorylation and glutathione pathways. Based on the results described above, we presumed that QHYH might be an antioxidant, which would explain its protective effects on high glucose-induced endothelial dysfunction. In this study, we determined the antioxidant effects of QHYH and each of its components *in vitro* by EPR. We further evaluated its antioxidant activity in high glucose-induced endothelial cells and identified the free radicals scavenged by QHYH in these cells.

Materials and methods

Chemicals

For the Fenton reaction, DMPO was purchased from Sigma-Aldrich (St Louis, MO, USA), and ammonium ferrous sulfate, diethylene triamine pentaacetic acid and hydrogen peroxide were obtained from Sangon Biotech Co, Ltd (Shanghai, China). N-Acetyl-L-cysteine (NAC) was used as a positive control and was purchased from Sigma-Aldrich (St Louis, MO, USA). SOD used for free radical identification was purchased from Haixing Co, Ltd (Beijing, China).

Cell cultures

Immortalized mouse cerebral microvascular endothelial (bEnd.3) cells, which were used for the antioxidant study of QHYH, were kindly provided by Hui-ming JIN (Department of Pathophysiology of Shanghai Medical College, Fudan University, Shanghai, China). The cells were cultured in normal glucose (5.6 mmol/L)-Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified 95% air/5% CO₂ incubator at 37 °C.

Preparation of QHYH and its components

Aqueous extracts of QHYH and each of the formula components, named number (Num) 1 to Num 8 below, were prepared and provided by the TCM preparation room of ZhongShan Hospital. The components of QHYH are Ligusticum chuanxiong, Hirudo nipponica, Astragalus membranaceus, Pueraria lobata, Gardenia jasminoides, Artemisia capillaris, Pteris multifida and Pericarpium Citri (ratio 3:1.5:1.5:1.5:1.5:1:0.6:0.6, W/ W). A mixture of these components was boiled in water (ratio 1:5, W/ W) for 50 min to get the crucial solution, which was then extracted by 95% ethanol and left at room temperature for 48 h. After that, supernatant was collected and reserved for detection. A package of crude drug or its corresponding components is equivalent to

15 mL of aqueous extract, with concentrations of 2.0 g/mL, 1.0 g/mL, 1.0 g/mL, 1.0 g/mL, 1.0 g/mL, 0.66 g/mL, 0.4 g/mL, and 0.4 g/mL for each component from Num 1 to Num 8, respectively. Each ingredient was condensed to determine its concentration-dependent effects on free radical scavenging. The concentrations of these condensed components from Num 1 to Num 8 were 8.0 g/mL, 3.0 g/mL, 5.5 g/mL, 3.0 g/mL, 2.5 g/mL, 2.0 g/mL, 2.2 g/mL, and 0.8 g/mL, respectively.

An MTT cell proliferation assay was performed to determine the effects of QHYH, with concentrations ranging from 1/10000 to 1 (*v/v*), on the viability of endothelial cells cultured in 5.6 mmol/L glucose DMEM. The results showed that QHYH with a concentration of 1/100 was the best condition for cell growth. Therefore, a working concentration of 1/100 QHYH aqueous extracts was chosen for the cell studies.

Determining the DMPO/HO[•] adduct scavenging ability of QHYH and each of its components *in vitro*

To study the DMPO/HO[•] adduct scavenging ability of QHYH *in vitro*, the HO[•] free radicals were generated by the Fenton reaction system. This generating system was composed of 100 mmol/L H₂O₂ (5 μL), 0.3 mmol/L Fe²⁺/0.15 mmol/L DTPA (5 μL) and phosphate-buffered saline (5 μL). To form DMPO/HO[•] radicals that were detected by EPR (EMX-8/2.7, Bruker BioSpin GmbH), 100 mmol/L DMPO (5 μL) was used as a spin trap.

To determine the scavenging capacity of DMPO/HO[•] spin adducts, 30-μL aqueous extracts of QHYH or each of its components were directly added to the Fenton reaction system. The mixture was then transferred into a flat quartz cell, and the EPR spectra was measured after Fe²⁺/DTPA had been added for 5 min. The EPR conditions used in this experiment were: microwave frequency=9.87 GHz, microwave power=20 mW, modulating frequency=100 kHz, modulating extent=0.1 mT and measurement time=1 min. Data acquisition and analysis were performed with Win EPR analyzing software (Bruker). The scavenging ability of the samples on the DMPO/HO[•] adducts was calculated using the formula $(h_0-h_1)/h_0 \times 100\%$, where h_1 and h_0 were the peak heights of the second low-field line of the DMPO/HO[•] spin adduct with and without samples, respectively. To study the dose-dependent effects of the components of QHYH on free radical reduction, the following concentrations of the condensed components were used: 3.1%, 6.25%, 12.5%, 25%, 50%, and 100%.

Determining the effects of high glucose on free radical generation in bEnd.3 cells

To evaluate the antioxidant effects of QHYH on high glucose-induced bEnd.3 cells, free radicals generated from high glucose-treated bEnd.3 cells were initially evaluated. The cells were incubated with different glucose concentrations from 25 mmol/L to 40 mmol/L to find an optimum glucose concentration that would induce the strongest free radical signals. Next, bEnd.3 cells were incubated with glucose for 0.5 h to 24 h to obtain an optimal glucose incubation time. After incubation, the cells were digested with 0.25% pancreatin and centrifuged

at 300×g for 5 min. DMEM without fetal bovine serum was added to adjust the cell concentration to 5×10^6 /mL. Forty-five-microliter cell samples were mixed with 100 mmol/L DMPO (5 μ L). The mixture was then transferred to a flat quartz cell, and the EPR spectra were immediately collected using the conditions mentioned above. The EPR signal intensity was quantified by the second peak-to-peak height in the direction of the magnetic field.

To validate specific effects of high glucose on free radical generation in bEnd.3 cells, EPR spectra were collected in the following groups cultured for 1 h, which is the optimum condition for free radical generation: (1) normal glucose-treated cell group: bEnd.3 cells cultured with 5.6 mmol/L glucose-DMEM; (2) high glucose-treated cell group: bEnd.3 cells cultured with 35 mmol/L glucose-DMEM; (3) high-glucose group: 35 mmol/L glucose-DMEM without cells; (4) mannitol group (osmotic control): bEnd.3 cells cultured with normal glucose-DMEM containing 29.4 mmol/L mannitol. After incubation, the free radicals in these cells were determined by the same methods used for the detection of high glucose effects.

Determining the antioxidant effects of QHYH on high glucose-induced bEnd.3 cells

QHYH with a working concentration of 1/100 was incubated with high glucose-induced bEnd.3 cells at the optimum conditions to determine its antioxidant effects. NAC (10 mmol/L) was used as the positive control. After incubating for 1 h, the free radicals were determined by the same methods used for the detection of the high glucose effects.

Identifying the free radicals scavenged by QHYH in high glucose-induced bEnd.3 cells

To identify the free radicals scavenged by QHYH in high glucose-induced bEnd.3 cells, 400 U/mL SOD were added to the cells. After incubation for 1 h, the EPR spectra were collected from this group.

Statistical analysis

Numerical data are expressed as the mean \pm SEM. Statistical evaluation was performed using a Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Results

Hydroxyl radical scavenging activity of QHYH and its components

A strong free radical signal was detected in the DMPO/HO[•] adduct generating system (Figure 1). To evaluate the free radical scavenging ability of QHYH, aqueous extracts of QHYH at various concentrations [12.5%, 25%, 50%, 75%, and 100% (*v/v*)] were added to this DMPO/HO[•] adduct generating system. Compared to the hydroxyl radical model, the DMPO/HO[•] adduct signals decreased significantly in a concentration-dependent manner (Figure 1). Quantitatively, 30 μ L of QHYH (100% QHYH in Figure 1) with the same concentration as that of the crude drug scavenged approximately 82.2% of the DMPO/HO[•] generated in this reaction (Figure 2). The DMPO/HO[•] adduct scavenging ability of each QHYH

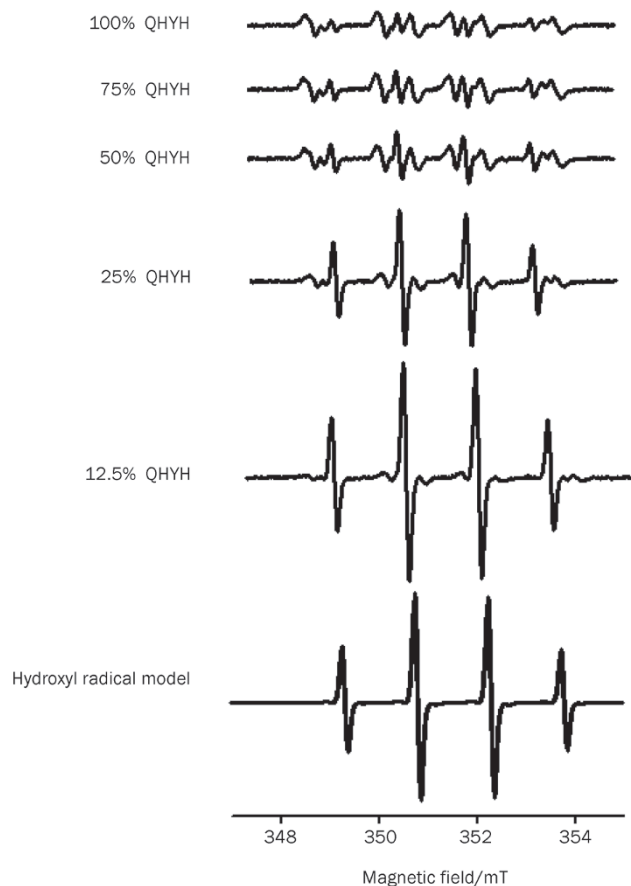


Figure 1. EPR spectra of DMPO/HO[•] adducts before and after QHYH sample.

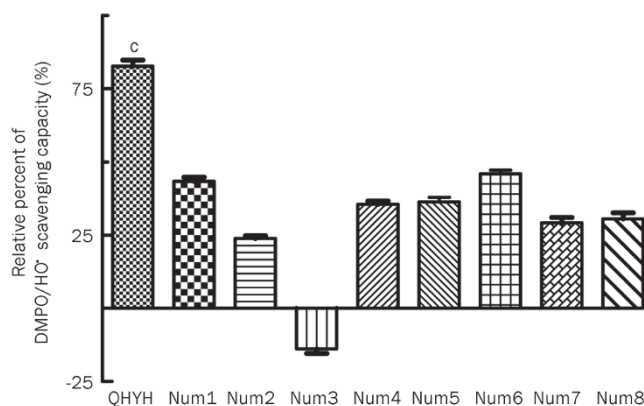


Figure 2. Hydroxyl radical scavenging ability of QHYH and its components. Aqueous extracts of QHYH or its components (30 μ L) were added to the DMPO/HO[•] adduct generating system. The scavenging ability on DMPO/HO[•] adduct was calculated by $(h_0 - h_1)/h_0 \times 100\%$, which was presented as mean \pm SEM ($n=3$). * $P < 0.01$ vs components from Num1 to Num8.

component was also determined. All of the QHYH components except Num 3 reduced the DMPO/HO[•] adducts. The DMPO/HO[•] scavenging abilities of Num 1 to Num 8 ranged from 25% to 46.2%; however, the scavenging ability of Num

3 was -16.7% (Figure 2), suggesting that Num 3 at the dose in the TCM prescription may be a pro-oxidant. After the QHYH components, including Num 3, were condensed and added to the Fenton reaction at increasing concentrations, the DMPO/HO[•] adducts were scavenged in a concentration-dependent manner (Table 1). The results imply that Num 3 may be both an antioxidant and a pro-oxidant depending on its concentration.

The effects of high glucose on free radical generation in bEnd.3 cells

When bEnd.3 cells were induced with glucose for 1 h, the EPR signal intensity was enhanced as the glucose level increased from 25 to 35 mmol/L (Figure 3). However, the EPR signal intensity induced by 40 mmol/L glucose was weaker than that induced by 35 mmol/L. This may be attributed to the alcohol groups in glucose that can decrease the free radical signals. Therefore, 35 mmol/L glucose was chosen as the optimum glucose concentration to induce ROS generation in bEnd.3 cells. Culturing time with high glucose was also various to determine its effects on ROS generation. The results showed that the EPR signal intensity in bEnd.3 cells cultured with 35 mmol/L glucose for 1 h was stronger than that in cells induced by this glucose concentration cultured for 30 min, 2 h,

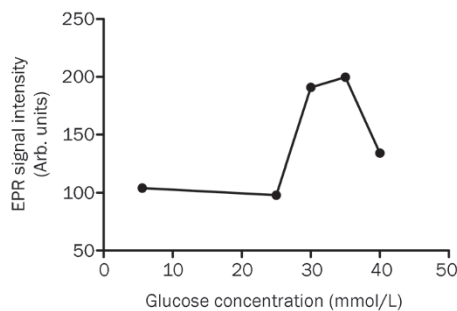


Figure 3. EPR signal intensity of bEnd.3 cells cultured in different glucose concentration. The x-axis is the glucose concentrations that are 5.6 mmol/L, 25 mmol/L, 30 mmol/L, 35 mmol/L, and 40 mmol/L. The y-axis is EPR signal intensity that was quantified by the second peak-peak height in the direction of magnetic field.

6 h, and 24 h (Figure 4). Thus, the optimum incubation condition for ROS generation in bEnd.3 cells was induction with 35 mmol/L glucose for 1 h.

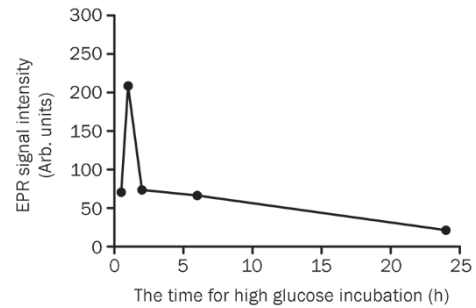


Figure 4. EPR signal intensity of bEnd.3 cells cultured in 35 mmol/L glucose DMEM for 30 min, 1 h, 2 h, 6 h, and 24 h. The x-axis is time of high glucose incubation. The y-axis is EPR signal intensity that was quantified by the second peak-peak height in the direction of magnetic field.

The EPR spectra of the normal glucose-treated cell group, the high glucose-treated cell group, the high-glucose group without cells and the mannitol group were collected to confirm the specific effects of high glucose on free radical generation in bEnd.3 cells. As an osmotic stress control, 29.4 mmol/L mannitol was used. The EPR spectra showed that no obvious signal was detected in the mannitol group compared to the high glucose-treated cell group (Figure 5), indicating that the ROS produced in the high glucose-treated cell group were not generated by osmotic stress. Similarly, there was no obvious signal detected in the normal glucose-treated cell group or the high-glucose group without cells compared to the high glucose-treated cell group (Figure 5).

Antioxidant effects of QHYH on high glucose-treated bEnd.3 cells

To evaluate the antioxidant effects of QHYH on high glucose-treated bEnd.3 cells, 1/100 QHYH was added to the bEnd.3 cells, which were then co-incubated with 35 mmol/L glucose for 1 h. After incubation, DMPO was added and EPR spec-

Table 1. Concentration-dependent property of QHYH's components on HO[•] scavenging. QHYH's components were condensed to evaluate concentration-dependent effects on HO[•] scavenging. The concentrations of the condensed components were 8.0 g/mL, 3.0 g/mL, 5.5 g/mL, 3.0 g/mL, 2.5 g/mL, 2.0 g/mL, 2.2 g/mL, and 0.8 g/mL individually. The scavenging abilities are presented as mean±SEM (n=3).

Content (%)	Scavenging ability of HO [•] (%)							
	Num1 (8.0 g/mL)	Num2 (3.0 g/mL)	Num3 (5.5 g/mL)	Num4 (3.0 g/mL)	Num5 (2.5 g/mL)	Num6 (2.0 g/mL)	Num7 (2.2 g/mL)	Num8 (0.8 g/mL)
3.10	33.52±0.01	29.38±0.23	17.30±7.80	43.11±0.05	67.46±0.08	51.76±0.06	18.62±0.17	12.15±0.72
6.25	53.63±0.91	61.43±0.05	30.57±7.25	62.20±0.10	84.42±0.02	72.67±0.11	43.40±0.13	39.88±0.10
12.50	69.61±0.15	75.70±0.20	55.58±0.01	85.50±1.66	91.77±0.06	86.28±1.44	80.12±0.05	59.47±0.13
25.00	83±0.10	96.36±0.28	82.98±1.78	94.78±0.21	96.87±0.21	94.90±0.20	86.75±0.08	78.69±0.16
50.00	93.46±0.12	97.76±0.23	92.35±0.13	98.42±0.23	98.87±0.18	97.25±0.20	97.78±0.01	94.12±0.17
100.00	98.02±0.40	99.10±0.16	96.74±0.44	99.08±0.32	99.21±0.28	99.30±0.20	98.81±0.04	97.84±0.13

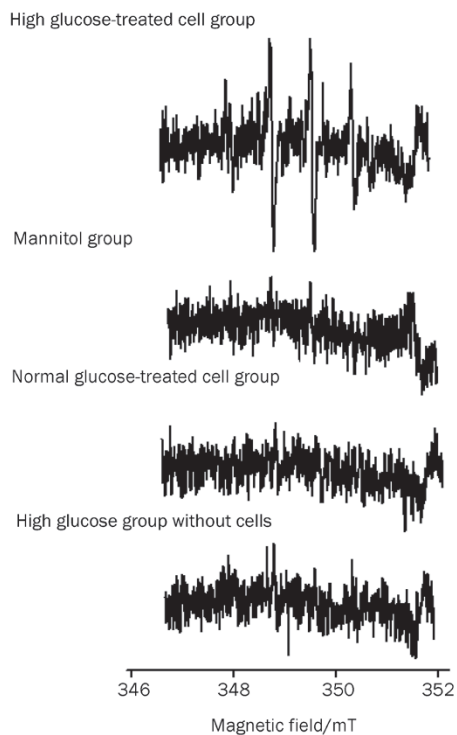


Figure 5. EPR spectra of high glucose-treated cell group (35 mmol/L), mannitol group (29.4 mmol/L), normal glucose-treated cell group (5.6 mmol/L), and high glucose group without cells (35 mmol/L).

tra were collected. Compared to the high glucose-treated cell group, the free radical signals in the QHYH group were remarkably reduced. The free radical scavenging ability of the QHYH group was approximately 70%, which was higher than that of the NAC group (Figure 6).

Identifying the free radicals scavenged by QHYH in high glucose-treated bEnd.3 cells

The free radicals scavenged by QHYH in the high glucose-treated cell group were identified by SOD. bEnd.3 cells treated with high glucose concentrations were co-incubated with 400 U/mL SOD, and the EPR spectra of this group were collected. As shown in Figure 7, the ROS signals were significantly reduced in the SOD group compared with the high glucose-treated cell group, which were decreased by approximately 60%. These results indicated that the ROS generated in bEnd.3 cells induced with 35 mmol/L glucose was $O_2^{\cdot-}$.

Discussion

In this study, we found that QHYH and its components reduced the DMPO/ HO^{\cdot} adduct signal in a concentration-dependent manner, as measured by EPR *in vitro*, and, moreover, that QHYH decreased the free radicals generated in high glucose-induced bEnd.3 cells. The free radicals scavenged by QHYH in these cells were identified as superoxide anions.

EPR is the only approach (other than enzymatic detection) that can provide direct evidence of the presence of a free radi-

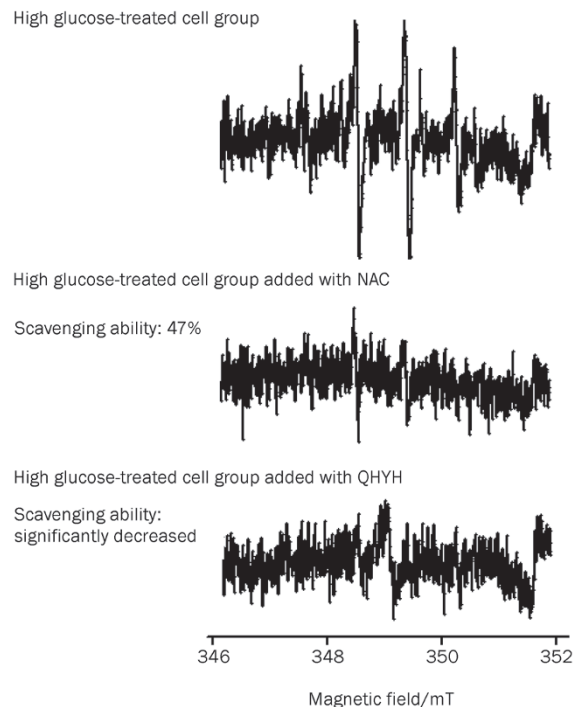


Figure 6. EPR spectra of high glucose-treated bEnd.3 cells before or after addition of QHYH. Cells cultured in high glucose (35 mmol/L) were treated with 1/100 QHYH for 1 h. Free radicals in the groups were then assessed by EPR using DMPO as a spin trap. NAC was used as a positive control.

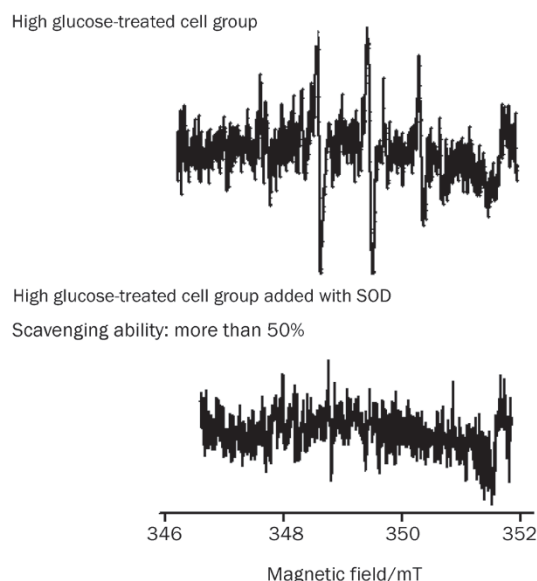


Figure 7. EPR spectra of high glucose-treated cell group (35 mmol/L) and high glucose-treated cell group added with 400 U/mL SOD.

cal^[25]. Using spin traps, EPR allows the detection of free radicals with short life spans, such as $HO^{\cdot}/O_2^{\cdot-}$ ^[26]. In addition, because every free radical has its own typical EPR spectrum, EPR is a unique technique to identify the nature of free radi-

icals. In our study, EPR was applied to evaluate the antioxidant effects of QHYH. The HO[•] free radical was generated through a Fenton reaction (Fe²⁺/H₂O₂). A DMPO spin trap was used to form DMPO/HO[•] adducts with longer half-lives that were detectable with EPR^[26, 27]. The DMPO/HO[•] scavenging ability of QHYH was 82.2%, and it reduced DMPO/HO[•] in a concentration-dependent manner (Figure 1 and 2). Most of the QHYH components also reduced free radicals in a concentration-dependent manner, except for the component Num 3 (Figure 2 and Table 1). However, when Num 3 was condensed, it showed antioxidant effects with concentrations ranging from 3.1% to 100% (Table 1). These results indicate that Num 3 produces pro-oxidant and antioxidant effects at different concentrations, which is similar to the properties of other natural materials, such as curcumin, tea extracts and guava extracts^[28, 29]. Because the mechanism underlying this activity of Num 3 is not clear, more studies are required to determine it. Nonetheless, the scavenging free radical ability of the QHYH formula was higher than each of its individual components (Figure 2). These results demonstrate that the antioxidant effect of QHYH is more potent than that of any of its components, which explains the rationale for using TCM as a prescribed formula but not as a single drug.

It is worth noting that the main peaks in Figure 1 were reduced as the QHYH concentration increased, and a new small radical signal emerged (Figure 1), suggesting that the new signal may be the result of the reaction between DMPO and the radicals produced in the reaction between QHYH and HO[•]. Although the exact nature of the new signal is not known, it does not affect the determination of the antioxidant ability of QHYH.

ROS accumulation induced by hyperglycemia is the main factor contributing to endothelial dysfunction in type 2 diabetes mellitus^[17, 18]. Free radical signals were most prominent in bEnd.3 cells cultured with 35 mmol/L glucose for 1 h compared to bEnd.3 cells in other culturing conditions (Figure 3 and Figure 4). No obvious free radical signal was detected in the high-glucose group without cells, the normal glucose-treated cell group or the osmotic control (mannitol) group (Figure 5). These results demonstrate that the free radicals were generated in the high glucose-treated endothelial cells rather than being affected by osmotic pressure. To determine the antioxidant effects of QHYH, its extracts at a concentration of 1/100 (*v/v*) were added to bEnd.3 cells treated under high-glucose conditions. The results showed that free radicals were reduced, and the scavenging ability of QHYH was higher than that of 10 mmol/L NAC (Figure 6). This indicates that QHYH is able to protect endothelial cells against high glucose-induced oxidative stress.

In this study, we observed that the free radicals generated in bEnd.3 cells treated under high-glucose conditions decreased dramatically when SOD was added (Figure 7). Because SOD catalyzes the reduction of the superoxide anion ($2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$) extremely rapidly^[30, 31], the results demonstrate that the free radical induced by high glucose is O₂^{•-}. Combined with the results described above, these findings show that

QHYH exerts its antioxidant effects by scavenging O₂^{•-}, which is mainly generated during the transfer of electrons through the respiratory chain^[32, 33]. These results indicate that the site of action of QHYH is the mitochondria. In our previous report, we isolated several monomers from QHYH and found that tetramethylpyrazine, one of the strongest antioxidant compounds^[34], can up-regulate mitochondrial biogenesis and reduce mitochondrial ROS production in palmitate-induced muscle cells^[35]. All of these results indicate that QHYH may be a mitochondria-targeted antioxidant.

In this study, we demonstrated that the antioxidant activity of QHYH and its constituents *in vitro* using EPR, and we confirmed the ability of QHYH to specifically scavenge superoxide anions in high glucose-induced bEnd.3 cells. Our results suggest that QHYH is a potent antioxidant that protects endothelial cells against oxidative stress. Therefore, QHYH could be a promising antioxidant drug for the prevention and treatment of diabetic complications.

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Author contribution

Qiong XU analyzed the data and wrote the paper; Bing ZHANG performed the research; Xiao-mu LI helped to design the study and analyze the data. Xin GAO designed the study and analyzed the data.

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