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Original Article

Qi Shen Yi Qi dropping pill prevents nitroglycerin-induced tolerance in rats

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Abstract: A useful method for the progressive attenuation of the effects of organic nitrates during long-term therapy (nitroglycerin tolerance) remains lacking. Qi Shen Yi Qi Dropping Pill (QSYQ) is a commercial herbal medicinal compound that has been widely used in therapy for cardiovascular diseases. The aim of this study was to investigate the potential effect of QSYQ to prevent nitroglycerin (GTN) tolerance in vivo and in vitro. In the present study, we used a nitroglycerin-induced tolerance model in rats and investigated the effect of QSYQ using the evaluation of cardiac function by echocardiography, vascular function by isolated aortic rings and anti-oxidative action. Pretreatment of QSYQ for 4 days showed improved cardiac function and vascular function and significantly decreased the ejection fraction (EF) and fractional shortening (FS) and increased the peak Vel of the left ventricular outflow tract (LVOT) in nitroglycerin-induced tolerance rats (with an intervention of 50 mg/kg GTN for 3 days). QSYQ also attenuated oxidative stress, such as NO and MDA activities. The results provide experimental evidence for treating cardiovascular disease through the use of QSYQ, particularly in nitroglycerin-induced tolerance. The mechanism is related to the use of anti-oxidants after the excessive intake of nitrates.

Keywords: Qi Shen Yi Qi, nitroglycerin, tolerance, echocardiograph, aortic ring, antioxidant

Introduction

Nitroglycerin (glyceryl trinitrate, GTN) has been used for the treatment of congestive heart failure and ischemic heart disease for more than a century [1]. However, it has been found that prolonged and prior nitroglycerin therapy may cause desensitization, a phenomenon that is known as “nitroglycerin tolerance” [2]. Because GTN is a commonly used life-saving chemical, researchers have tried to find the mechanism leading to the appearance of nitroglycerin tolerance. It was previously reported that reactive oxygen species and oxidative stress play a major role in nitroglycerin tolerance; GTN treatment was accompanied by the increased formation of superoxide [3]. In clinical practice, carvedilol can act as an antioxidant to prevent or attenuate the development of tolerance [4]. However, an effective medicine to inhibit nitroglycerin tolerance in the clinic is still lacking.

Qi Shen Yi Qi Dropping Pill (QSYQ) is a commercial herbal medicine compound, which was approved by China State Food and Drug Administration (CFDA) in 2003 for the treatment of cardiac dysfunction [5]. It contains four herbal medicines, *Astragalus membranaceus* (Leguminosae, Astragalus Radix), *Salvia miltiorrhiza Bunge* (Labiatae, Salviae miltiorrhizae Radix et Rhizoma), *Panax notoginseng* (Araliaceae, Panax Notoginseng Radix et Rhizoma), and *Dalbergia odorifera* (Leguminosae, Dalbergia odorifera T. Chen Heartwood). Modern studies have indicated that QSYQ improves heart function [6] and anti-oxidative stress [7].

This study aimed to investigate the potential effects of QSYQ on nitroglycerin-induced tolerance in rats. We used a nitroglycerin-induced tolerance model in rats and investigated the potential effects of QSYQ through the evaluation of cardiac function by echocardiography,

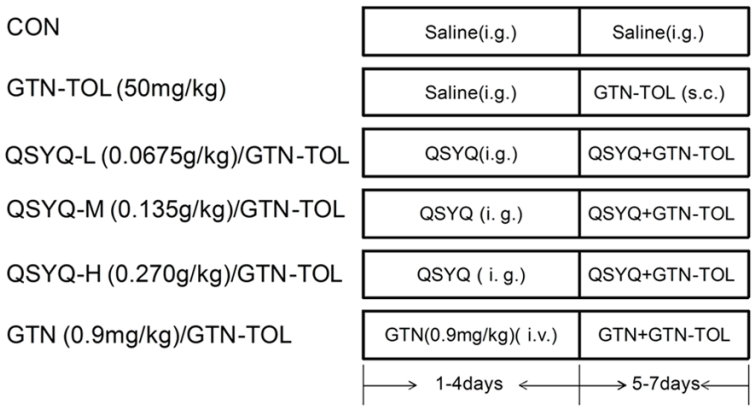

Figure 1. Experiment protocol. Rats of three QSYQ/GTN-TOL groups were pretreated with QSYQ via intragastric administration for four days. Rats in the control, GTN-TOL and GTN/GTN-TOL groups were treated with saline. After the fifth day, rats in all of the groups except the control were administered 50 mg/kg GTN via subcutaneous injection for three days. GTN: glyceryl trinitrate.

vascular function by isolated aortic rings and anti-oxidative action.

Materials and methods

Animals

Adult male Sprague Dawley (SD) rats, weighing 230-250 g, were purchased from Vital River Company (Beijing, China). The animals were housed in cages at a temperature of 22 ± 2°C, humidity 40 ± 5%, a 12-hour light/dark cycle, and received a standard diet and water ad libitum. The animals fasted for 12 hours before the experiment but were allowed free access to water. All of the experiments were conducted under the Guidelines for Animal Experiments of the Tianjin University of Traditional Chinese Medicine.

Drug and reagents

QSYQ (batch number: 20091005) was purchased from Tasly Pharmaceutical Group Co. Ltd. (Tianjin, China) according to the guidelines of Good Manufacturing Practice and Good Laboratory Practice, and the contents of its major components were determined by high performance liquid chromatography finger print [8]. Nitroglycerin Injection was obtained from Beijing Yimin Pharmaceutical Co. Ltd. (Beijing, China); Isoflurane was obtained from YEERAN Technology Co. Ltd. (Beijing, China); Chloral hydrate was obtained from the Tianjin Kermel Chemical Reagent Co. Ltd., (Tianjin, China); Acetylcholine (Ach) was purchased from Sigma-Aldrich (St Louis, MO, U.S.A.); Norepinephrine (NE) was obtained from Tianjin Pharmaceutical Group Xinzheng Co, Ltd. (Tianjin, China). NaCl, KCl, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $NaHCO_3$ and Glucose were obtained from the Tianjin Chemical Reagent Factory (Tianjin, China).

Nitroglycerin-tolerance model and drug administration

The rats were randomly divided into six groups: control groups, GTN-TOL (50 mg/kg, GTN-tolerance), QSYQ-L (0.0675 g/kg)/GTN-TOL, QSYQ-M (0.135 g/kg, clinical equivalent dose)/GTN-TOL, QSYQ-H (0.270 g/kg)/GTN-TOL and GTN (0.9 mg/kg, clinical equivalent dose)/GTN-TOL groups. The rats of three QSYQ/GTN-TOL groups were pretreated with QSYQ via intragastrical administration for four days. The rats in the control and GTN-TOL groups were treated with saline via intragastrical administration. After the fifth day, the rats in all of the groups except the control were administered 50 mg/kg GTN via subcutaneous injection for three days. Rats in the GTN/GTN-TOL group were co-treated with an intravenous tail injection of 0.9 mg/kg GTN from the 5^{th} to 7^{th} days. The other groups were treated with relevant reagents similarly to the first 4 days. Each experimental group included 10 animals (a total of 60 rats) (**Figure 1**).

Ultrasound echocardiography evaluation in vivo

The cardiac function was evaluated with a Vevo 2100 ultra-high resolution small ultrasound imaging system in real time (Visual Sonics Vevo 2100, Canada) with a MS-250 ultrasound scanning transducer to evaluate the left ventricular function [9]. The animals were anesthetized with 3% isoflurane to induce anesthesia and maintained with 1.5% isoflurane during the echocardiogram. Two-dimensional cine loops and guided M-mode frames were recorded from the parasternal long axis [8]. The following parameters were measured as indicators of the

left ventricular function: left ventricular ejection fraction percentage (EF %), left ventricular fractional shortening percentage (FS %) and left ventricular outflow tract (Peak Vel, mm/s).

Aortic ring assay

After the last time of drug administration, the rats were anesthetized with 5% chloral hydrate (0.6 mL/100 g) by intraperitoneal injection. The thoracic aorta was immediately isolated and placed in Krebs Henseleit (NaCl 118 mM, KCl 4.75 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM and glucose 11 mM). Two parallel stainless steel hooks were introduced through the lumen of the aorta rings; one fixed to the bottom of the organ bath (Radnoti, AD Instruments Pty Ltd., Australia) while the other was connected to a force displacement transducer (AD Instruments Pty Ltd., Australia), which recorded the changes during the experiments [10, 11]. The organ bath included 10 mL of K-H solution with a pH of 7.3-7.4 at 37°C and was bubbled with 95% O_2 and 5% CO_2 to keep blood vessels alive. The aorta rings were stretched to a based tension of 2.0 g and allowed to equilibrate for 60 minutes, the K-H solution was changed every 15 minutes. Every experiment started with a repeated KCl treatment to test the contractility, and after which, the rings were rinsed with a pre-warmed and oxygenated K-H solution until the muscle tension returned to the basal level [12]. The endothelial was evaluated by NE and Ach, 1 μM norepinephrine (NE) to achieve a plateau phase, and then 10 μM of Acetylcholine (Ach) was added to induce vasoconstriction. The functions of the drugs were compared with intact aortic rings and denuded aorta rings, and denuded aorta rings were prepared by the mechanical method with cotton pipe-cleaners.

Oxidative stress

At the end of the experiments (drawing serum from the abdominal aorta), the serum was collected and centrifuged at 3000 rpm for 10 minutes. The serum was used to detect nitric oxide (NO) and the lipid peroxidation products (MDA) kit. The activity of NO was investigated with the Nitric Oxide Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China), and the light absorbance was detected at 540 nm by an Enspire multimode plate reader (Perkin Elmer, America). The content of MDA was measured by the Lipid Peroxidation MDA Assay Kit (Beyotime) and the light absorbance was detected at 532 nm. All of the procedures were performed according to the manufacturer's instructions. An Enspire multimode plate reader was used to read the absorbance.

Histopathological examination of the myocardial tissues

On the seventh day after the nitroglycerin-injection, the rats were anesthetized with 5% chloral hydrate (0.6 mL/100 g) by intraperitoneal injection. The heart was removed from the chest, fixed in 10% formaldehyde solution for greater than 48 hours and embedded in paraffin. Serial sections (4 μm) were cut and the toasted slices, after standing at room temperature overnight, were stained with hematoxylin-eosin. Hematoxylin staining lasted three minutes, whereas eosin staining occurred for ninety seconds.

Statistical analysis

All of the data are presented as the mean ± standard deviation (S.D.). A statistical analysis was performed using SPSS 19.0 statistical software. A one-way analysis of variance followed by the Tukey's test for multiple comparisons was used between the groups. A value of $P<0.05$ was considered statistically significant.

Results

Echocardiography evaluation

The effect of QSYQ pretreatment on nitroglycerin-induced tolerance was confirmed by quantitative analysis of the echocardiograms. The representative echocardiograms in the different groups are presented in **Figure 2**. The rats were treated with GTN (50 mg/kg) or a combination of GTN and QSYQ (0.0675, 0.135, and 0.270 g/kg) for seven days and examined by M-mode and LVOT echocardiography (**Figure 2A** and **2B**). Compared with the control groups, the ejection fraction (EF) and fractional shortening (FS) were significantly increased in the rats given GTN treatment alone. However, the peak Vel of LVOT was significantly decreased in the rats given the GTN treatment alone. Co-treatment with QSYQ significantly decreased the ejection fraction (EF) of 72.17 ± 7.91% by 0.0675 g/kg QSYQ ($P<0.05$), significantly decreased the fractional shortening (FS) by 0.0675 and 0.135 g/kg QSYQ ($P<0.05$) and sig-

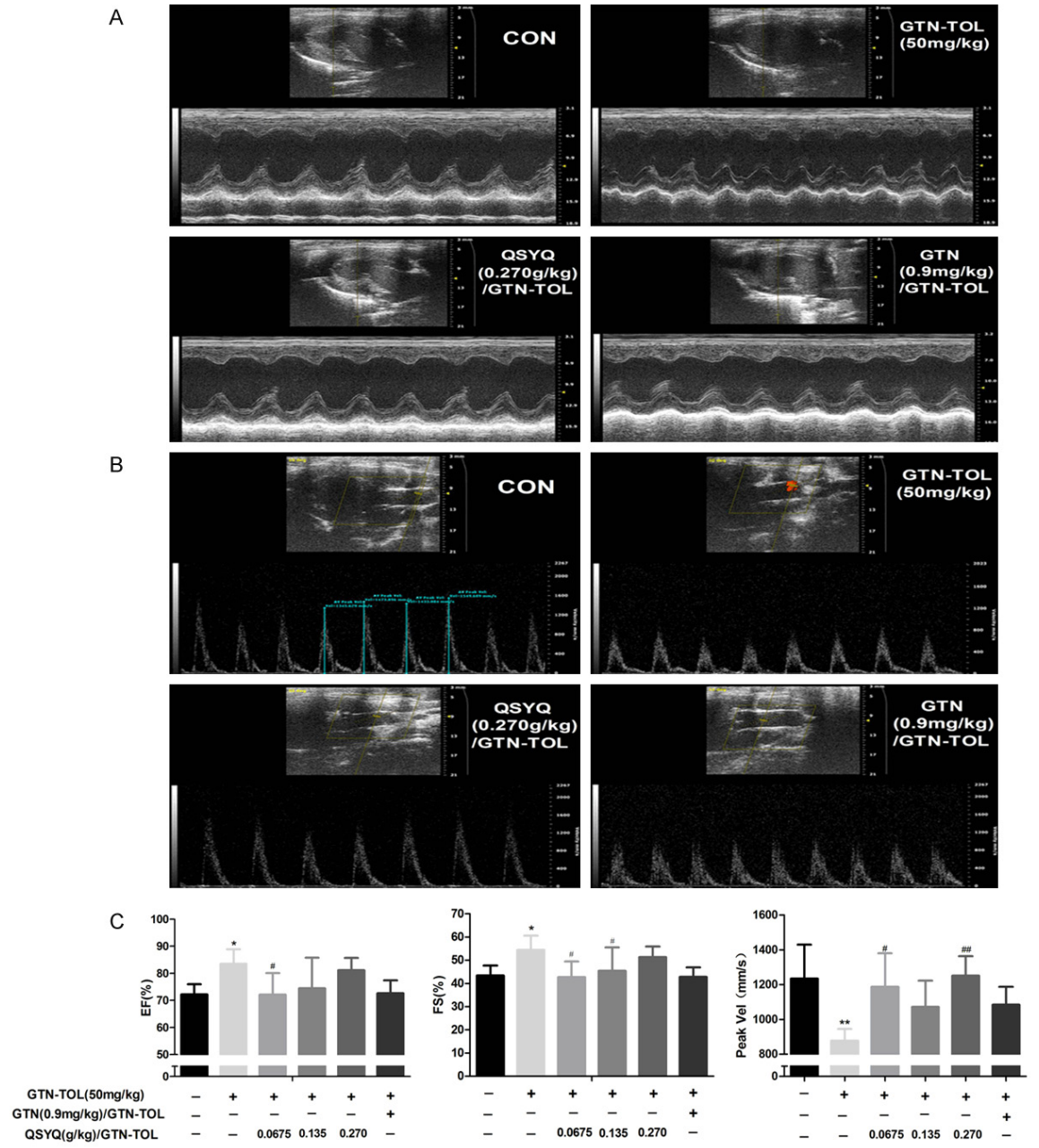

Figure 2. Echocardiography evaluation. GTN and QSYQ combination treatment protected the cardiac function in vivo. A. Representative M-mode echocardiograms of control and GTN-TOL treated and combination-treated groups showed the wall motion. B. Peak Vel echocardiograms on Doppler of the left ventricular outflow tract. C. Ejection fraction (EF), fractional shortening (FS) and peak Vel of LVOT in the groups.

nificantly increased the peak Vel to maximums of 1251.28 ± 112.26 mm/s by 0.270 g/kg QSYQ (P<0.01).

Endothelium function in response to nitroglycerin-induced tolerance

The thoracic aortas were immediately isolated and immersed in Krebs-Henseleit (K-H) solution on the seventh day. The pictures of this assay for each of group showed in **Figure 3A**. The aorta rings were pre-treated with 1 μM NE to achieve a plateau phase. The vascular tone of endothelium-intact groups reached 4.25 ± 0.20 g, and the endothelium-denuded groups reached 5.43 ± 0.21 g. Next, 10 μM of Ach was added to induce vasodilation. Endothelium was considered intact when the relaxation response

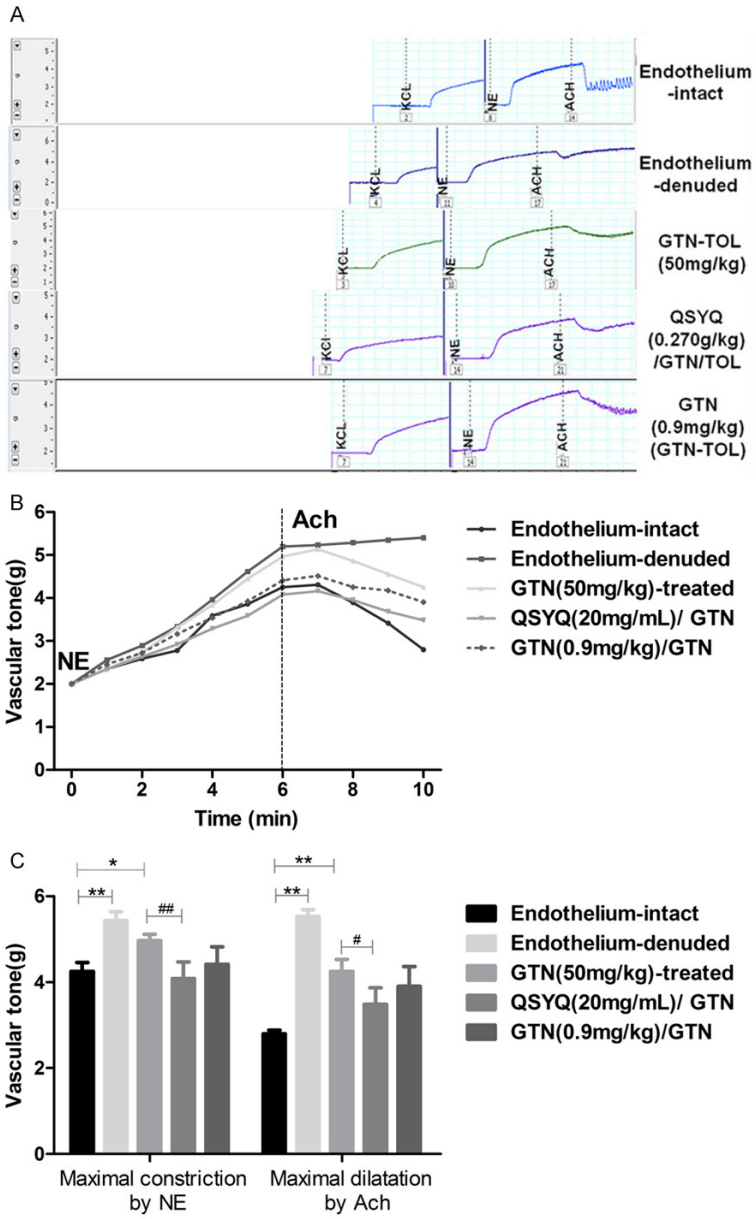

Figure 3. Endothelium function in response to nitroglycerin-induced tolerance. Testing of the endothelium function through an aortic ring assay. The control (endothelium-intact and endothelium-denuded) group, the GTN-TOL (50 mg/kg), QSYQ (0.270 g/kg)/GTN-TOL and GTN (0.9 mg/kg)/GTN-TOL groups treated S.D. rats were killed on the seventh day after the GTN-injection. The aortic rings were pre-treated with 1 μm NE to reach a plateau phase, and 10 μm Ach was then added to induce vasodilation. A. The picture of this assay for each of group. B. Vascular tone of NE and Ach treatment. The maximal constriction tone appeared six minutes after the NE incubation, and Ach was then added to reach vasodilation. C. Maximal constriction by NE and maximal dilatation by Ach.

was more than 70% and less than 30% in the endothelium-denuded aortas [13]. In the GTN-TOL (50 mg/kg) groups, the maximal constriction by NE was 4.96 ± 0.15 g after treatment with Ach, the vascular tone was decreased to 4.25 ± 0.28 g, and the maximal vasodilation was 23%, which showed a significant difference with the endothelium-intact groups and significant endothelium dysfunction. Compared with the GTN-TOL (50 mg/kg) groups, the QSYQ (0.270 g/kg)/GTN-TOL groups and GTN (0.9 mg/kg)/GTN-TOL groups showed less NE-induced constriction and more Ach-induced vasodilation (**Figure 3B** and **3C**).

Effects of QSYQ on serum MDA and NO

The level of serum MDA decreased in the GTN-TOL group, compared with the control group ($P<0.01$). Pretreatment with QSYQ (0.0675 g/kg, 0.135 g/kg, and 0.270 g/kg) attenuated the GTN-induced tolerance decrease in MDA ($P<0.01$) (**Figure 4A**). The serum NO in the GTN-TOL group was higher than the control group ($P<0.01$). In the QSYQ (0.0675 g/kg, 0.135 g/kg, and 0.270 g/kg) group, the content of NO increased to 9.68 ± 1.71, 11.70 ± 0.89 and 12.45 ± 0.74 umol/L ($P<0.01$), respectively (**Figure 4B**).

Histopathological examination of myocardial tissues

To directly observe the effect of the QSYQ pretreatment on nitroglycerin-induced tolerance in the myocardium structure, we examined the histology of the myocardial tissues (**Figure 5**). Compared with the control group, the GTN-TOL group showed intracellular edema and the muscle fibers were arranged irregularly. Co-treatment with QSYQ significantly alleviated the degree of irregularly arranged muscle fibers and did not show expanded myocardial fibers. Application software Image Pro-plus 6.0 was used to measure the myocardial fiber

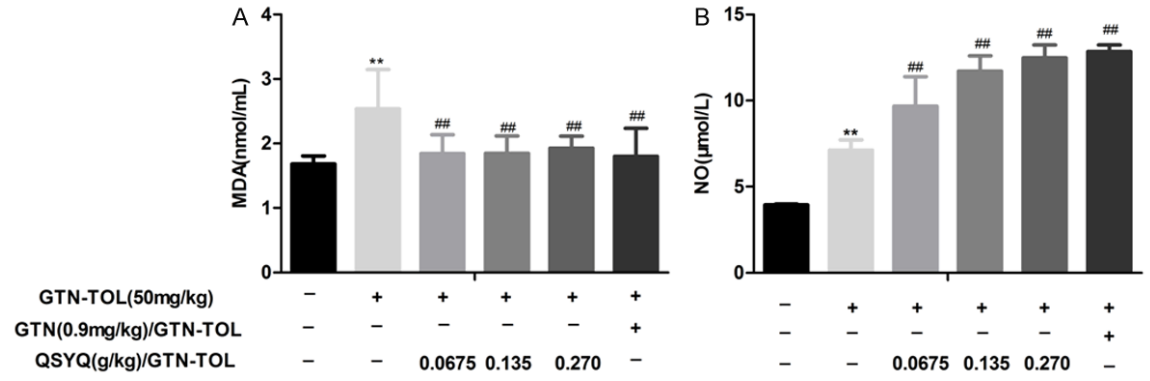

Figure 4. Effects of QSYQ on serum MDA and NO. A. Lipid peroxidation product malondlaldehyde (MDA) was determined to evaluate the antioxidant activity of QSYQ. $^{**}P<0.01$ versus CON, $^{\#\#}P<0.01$ versus GTN-TOL (50 mg/kg). The values are the mean ± S.D., n=10. B. Nitric oxide (NO). $^{**}P<0.01$ versus CON, $^{\#\#}P<0.01$ versus GTN-TOL (50 mg/kg). The values are the mean ± S.D., n=10.

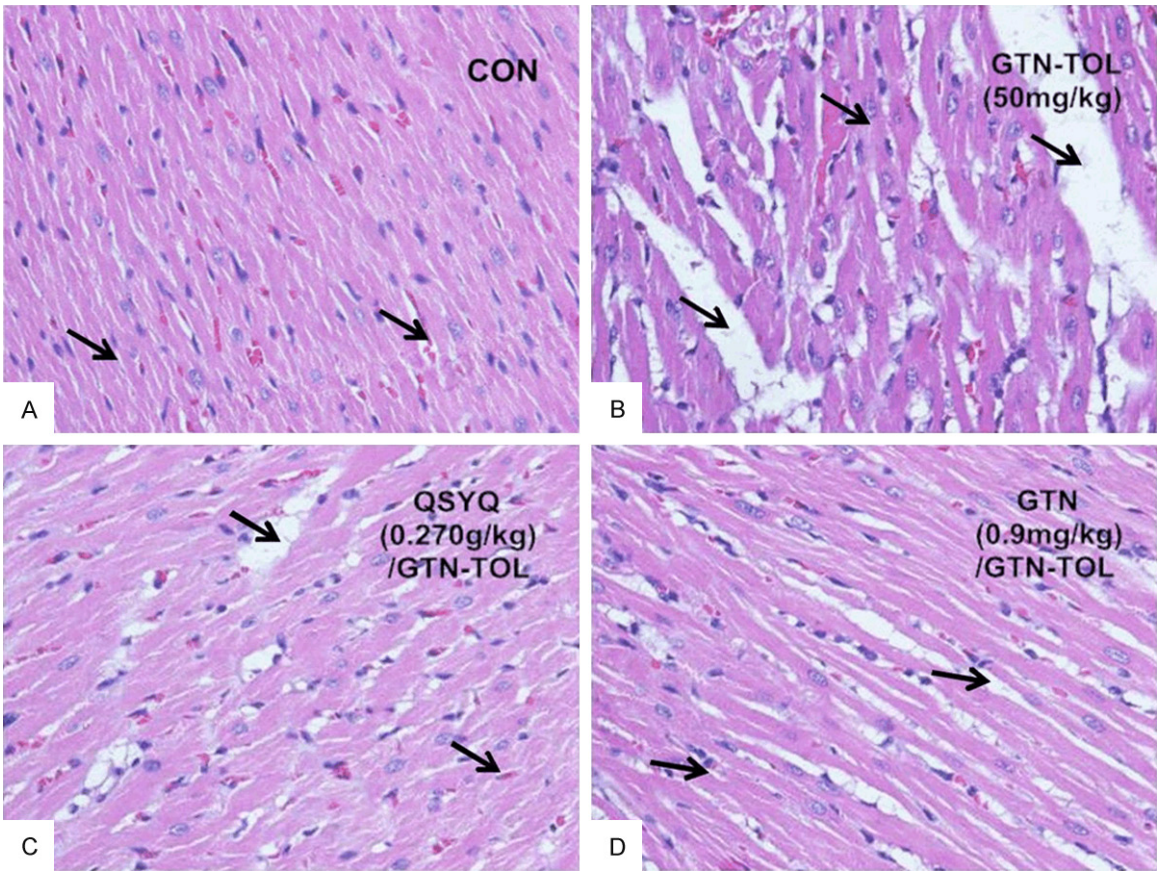

Figure 5. Histopathological examination of myocardial tissues. The histology effect of QSYQ on the myocardial tissues. (A) Control group, (B) GTN-TOL (50 mg/kg) group, (C) QSYQ-H (0.270 g/kg)/GTN-TOL group and (D) GTN (0.9 mg/kg)/GTN-TOL group.

gap distance; the control group was 0.0035 mm; the GTN-TOL group was 0.0355 mm; the QSYQ (0.270 g/kg)/GTN-TOL group was 0.0152 mm and the GTN (0.9 mg/kg)/GTN-TOL group was 0.0087 mm.

Discussion

Nitroglycerin and other organic nitrates are widely used in the management of cardiovascular disease. However, sustained administration

of nitroglycerin can cause tolerance to develop rapidly. This will undermine the treatment of the patients, which may bring difficulties to the therapeutic treatment. The research for the tolerance to nitroglycerin still has some problems, i.e., there is no special clinical medicine.

The ability of QSYQ to improve cardiac function has been documented in rats with acute myocardial infarction [14]. A recently published study showed that ASIV has a protective effect on cardiac hypertrophy induced by isoprenaline through attenuating inflammatory cytokines [15]. DIA can prevent isoproterenol-induced myocardial hypertrophy and improve cardiac function by acting as an antioxidant [16]. In the present study, our results demonstrated that GTN-TOL (50 mg/kg) significantly increased the EF and FS while reducing the peak Vel of LVOT compared with controls; co-treatment with QSYQ and GTN effectively prevented nitroglycerin-induced cardiac dysfunction.

Recent experimental and clinical studies have revealed that nitroglycerin-induced tolerance may cause endothelium dysfunction or an increase in the sensitivity of the vasculature to vasoconstrictors [4, 17]. Endothelium-derived mediators are essential in vascular homeostasis. Nitric oxide (NO) is the active metabolite of GTN. NO contributes to the regulation of the vascular tone by relaxing the smooth muscle. Once nitric oxide is formed, it is instantly spread to the vascular smooth muscle, which activates guanylate cyclase (GC) so that the vascular smooth muscle relaxes the diastolic blood vessels. Norepinephrine effects on the α receptors of vascular smooth muscle activates the receptor-operated calcium channel (ROC) and increases the internal calcium ions, which leads to vasoconstriction. Our results are in agreement with prior reports in animal models that QSYQ prevents the development of endothelium dysfunction from continuous nitroglycerin-induced tolerance. In our study, treatment with the QSYQ partially prevented the development of GTN-induced endothelium dysfunction.

Chronic nitroglycerin anti-ischemic therapy induces side effects such as nitrate tolerance and dysfunction. Both phenomena could be based on oxidation [18]. Oxidative stress generated by overproduction of free radical species, mostly superoxide anions and NO-derived peroxynitrite, has been suggested to play a pivotal role in the development of nitroglycerin tolerance [19]. The effect of QSYQ in antioxidant production has been reported [7]; QSYQ can exert cardioprotective effects on myocardial ischemia/reperfusion (I/R) injury through antioxidative stress. This indicates that an important cause of nitrate tolerance is an increase in the vascular bioavailability of reactive oxygen species [20]. From this study, QSYQ can significantly increase the content of NO and decrease the content of MDA, which showed a potential antioxidant effect.

Traditional Chinese medicine can interact with multiple targets and regulate multiple biological pathways simultaneously, which has been certified to be especially effective in treating complex diseases [21]. The active components of QSYQ are astragaloside IV (ASIV, from *Astragalus membranaceus*), 3,4-dihydroxyphenyl lactic acid (DLA, from *Salvia miltiorrhiza*), and notoginsenoside R1 (from *Panax notoginseng*) [5]. QSYQ has shown its unique advantage in terms of coronary artery disease (CAD). In this study, we found that pretreatment with QSYQ was effective against nitroglycerin-induced tolerance including improvement of cardiac function and antioxidative stress and amelioration of endothelium dysfunction.

Additionally, the fact that QSYQ can protect cardiac function from nitroglycerin-induced tolerance needs further clarification; the effective components may be astragaloside IV, which is the monarch drug in Qi Shen Yi Qi Dropping Pill. Several studies have shown that astragaloside IV has oxidative stress against various heart diseases [22, 23]. We will perform more experiments to verify the feasibility of QSYQ application in the clinical setting.

Conclusion

In summary, Qi Shen Yi Qi Dropping Pill provides prominent pre-protection against nitroglycerin-induced tolerance. The clinical equivalent dose can improve cardiac function, as seen by the ejection fraction (EF) and fractional shortening (FS). It can also improve vascular function, and its mechanism might be related to the enhancement of antioxidants.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

Ach, Acetylcholine; CAD, coronary artery disease; EF, ejection fraction; FS, fractional shortening; GC, guanylate cyclase; GTN, glyceryl trinitrate; LVOT, left ventricular outflow tract; MDA, malondialdehyde; NE, norepinephrine; NO, nitric oxide; ROC, receptor-operated calcium channel; QSYQ, Qi Shen Yi Qi.

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