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EXPERIMENTAL STUDY

Effect of Qilongtoutong granule on calcitonin gene-related peptide, beta-endorphin, serotonin, dopamine, and noradrenalin in migraine model rats and mice

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Abstract

OBJECTIVE: To study the effect of Qilongtoutong granule (QLTT) on plasma calcitonin gene-related peptide (CGRP), beta-endorphin (β-EP), 5-HT, dopamine (DA), noradrenalin (NE), and blood viscosity in migraine model rats and mice.

METHODS: Both the acute blood stasis model group and nitroglycerin-induced migraine model group included 60 Sprague-Dawley rats. The reserpine-reduced model group had 60 Kunming mice. Rats from each test were grouped into normal control group, model group, Zhengtian pill (ZTP) group, and high, moderate, or low-dose QLTT groups. In the acute blood stasis model test, after gavage for 7 days, rats were given 0.8 mL/kg adrenaline hydrochloride subcutaneously twice, and kept in ice water for 5 min. After fasting for 12 h, rats were anesthetized and blood samples were collected for detection of blood viscosity. In the nitroglycerin-induced migraine group, after gavage for 7 days, rats were intraperitoneally injected nitroglycerin (10 mg/kg), and 4 h later, blood samples were collected from postcava for measuring the plasma CGRP and β-EP levels. In the reserpine-reduced model test, except the normal control group, mice were administered reserpine (0.25 mg/kg, i.h.) for 9 days. Mice received intragastric administration from the third day to the ninth day. One hour after the last gavage, blood and brain tissue samples were obtained. Then, blood clotting time and the contents of neurotransmitters were determined.

RESULTS: QLTT- (3.6, 1.8, and 0.9 g/kg) and ZTP-treated rats had lower blood viscosity than that in model rats under different shear rates ($P<0.01$). QLTT- (3.6, 1.8 g/kg) and ZTP-treated rats had significantly lower plasma CGRP levels and higher plasma β-EP levels than those in model rats ($P<0.01$). QLTT treatment at dose of 0.9 g/kg had lower plasma CGRP levels as well ($P<0.05$). QLTT- (5.2, 2.6 g/kg) and ZTP-treated mice had longer blood clotting time than that in model mice ($P<0.01$). QLTT- (2.6 g/kg) and ZTP-treated mice had higher plasma serotonin (5-HT) levels than those in model mice ($P<0.05$).

CONCLUSION: QLTT-treated animals had lower plasma CGRP level, higher plasma β-EP, 5-HT, higher brain tissue 5-HT, NE, DA levels, and lower blood viscosity than those in the migraine model animals.

Key words: Migraine disorders; Calcitonin gene-related peptide; Beta-endorphin; Medicine, Chinese traditional; Qilongtoutong granule

INTRODUCTION

Migraine is a common disease that adversely affects quality of life.[1] However, few migraine medications achieve an effective rate over 50% and side effects limit their use.[2] Therefore, it is clinically significant to seek new drugs for migraine. A Chinese medicine formula for the treatment of migraine, Qilongtoutong granule (QLTT), has been used in clinic over 50 years.

According to the theory of Traditional Chinese Medicine (TCM), the stagnation of *Qi* and blood may bring about pain. Therefore, activating blood, expelling blood stasis, and dredging collaterals are the principles for migraine management. Research indicates that calcitonin gene-related peptide (CGRP) is responsible for neurogenic inflammation and vasodilatation of the cranial vessels in migraine pathophysiology.[3] Some scholars found that beta-endorphin (β-EP), by modulating the release of sympathetic transmitters, played a role in the pathogenesis of migraine.[4] Research also showed that a fall of serotonin (5-HT) in plasma and brain tissue was a specific feature of migraine attacks.[5] Additionally, a study showed that anomalies in the metabolism of glutamate, γ-aminobutyric acid, and those that govern pain and vegetative functions, such as 5-HT, dopamine (DA), and noradrenalin (NE), constituted the phenotypical biochemical cause of the migraine.[6] Based on these studies, an acute blood stasis model was used to assess the effect of QLTT on activating blood and expelling blood stasis. A nitroglycerin-induced migraine model was used to investigate the efficacy of QLTT on plasma CGRP and β-EP, and a reserpine-induced low 5-HT model was employed to explore the effect of QLTT on plasma 5-HT concentrations and the contents of 5-HT, DA, and NE in brain tissue.

MATERIALS AND METHODS

Preparation of QLTT

QLTT was manufactured in the pharmacy department of Chinese People's Liberation Army General Hospital (Beijing, China). It was prepared with 12 medicinal herbs: 2 parts Huangqi (*Radix Astragali Mongolici*) and Baishao (*Radix Paeoniae Alba*); 1.5 parts Danggui (*Radix Angelicae Sinensis*), Gegen (*Radix Puerariae Lobatae*) and Jili (*Fructus Tribuli*); 1.2 parts Chuanxiong (*Rhizoma Chuanxiong*), Juhua (*Flos Chrysanthemi*) and Dilong (*Pheretima Aspergillum*); and 1 part stir-frying Gancao (*Radix Glycyrrhizae*), Ruxiang (*Olibanum*), Moyao (*Myrrh*), and Tianma (*Rhizoma Gastrodiae*). Herbs were purchased from Anguoshi Changda Chinese Herbal Pieces Co., Ltd. (Hebei, China). All herbs met the standards of the Chinese Pharmacopoeia (2010 Edition, Volume I).[7] Dilong (*Pheretima Aspergillum*) and Moyao (*Myrrh*) were ground into a fine powder and filtered through a 100 mesh. Jili (*Fructus Tribuli*) and Tianma (*Rhizoma Gastrodiae*) were pulverized into a coarse powder and decocted with the other eight herbs three times. The herbs were decocted with an eight-fold volume of water for 1.5 h, then six-fold and four-fold volumes of water for 1 h. The three decoctions were filtered, mixed together and concentrated under reduced pressure to a thick paste with relative density 1.13 to 1.15 (50℃). The thick paste was dried at 80℃ and ground into a fine powder with 100 mesh. This powder was then mixed with the Dilong (*Pheretima Aspergillum*) and Moyao (*Myrrh*) powder. Sucrose and dextrin in the ratio of 2∶1 were also added. After mixing evenly, all herbs were dried and packed. The wet granulation technique was employed to prepare QLTT and the yield of the extract was about 20%.

Reagents and apparatus

The following reagents and apparatus were used in the experiment: Zhengtian pill (ZTP, China Resources Sanjiu Medical & Pharmaceutical Co., Ltd., Shenzhen, China), adrenaline hydrochloride injection (Tianjin Pharmaceutical Group Xinzheng Co., Ltd., Tianjin, China), nitroglycerin injection (Beijing Yimin Pharmaceutical Co., Ltd., Beijing, China), trichloroacetaldehyde hydrate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, analytical reagent), reserpine injection (Guangdong Bangmin Pharmaceutical Co., Ltd., Jiangmen, China), 5-Hydroxytryptamine hydrochloride (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), noradrenaline bitartrate (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), dopamine hydrochloride (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), evacuated blood collection system (Greiner Bio-One GmbH, Krems munster, Austria), Rheogeniometer SA-7000 (Beijing Sicceeder Technology Development Co., Ltd., Beijing, China), CGRP RIA kit (Beijing Huaying Biotechnology Research Institute, Beijing, China), β-EP RIA kit (Beijing Huaying Biotechnology Research Institute, Beijing, China), phosphoric acid (Sinopharm Chemical Reagent Co., Ltd., analytical reagent, Shanghai, China), sodium heptanesulfonate (Sinopharm Chemical Reagent Co., Ltd., analytical reagent, Shanghai, China), acetonitrile (Fisher Scientific, Langenselbold, Germany, spectrographic grade), Agilent Technologies Series 1200 system (Agilent, Santa Clara, CA, USA), mouse 5-Hydroxytryptamine ELISA Kit (Beijing Aike Boya Biotechnology Co., Ltd., Beijing, China), and ELISA plate reader (Multiskan Ex Primary EIA V.2.3, Thermo, Vantaa, Finland).

Animals

A total of 120 Sprague-Dawley rats (60 males and 60 females, 6-8 weeks old, 180-220 g, SPF) and 60 Kun-

ming mice (30 males and 30 females, 2 months old, 23-27 g, SPF) were provided by the experimental animal center, Academy of Military Medical Sciences of Chinese People's Liberation Army (Beijing, China, Certificate of quality No: SCXK- (jun) 2007-004). The rats were housed four per cage (545 mm × 359 mm × 200 mm) and mice were housed eight per cage. The rats were kept at 22℃±2℃ with a 12-h light/dark cycle (lights on at 8 a.m.) and had access to food and water ad libitum. Animal welfare and experimental procedures were carried out in accordance with international ethical guidelines, and the trial was approved by the ethics committee of Academy of Military Medical Sciences of Chinese People's Liberation Army.

Acute blood stasis model test

Sixty Sprague-Dawley rats were randomly divided into six groups by random number table method: (a) normal control group; (b) model group; (c) ZTP (1.62 g/kg) group; (d) QLTT high-dose (3.6 g/kg) group; (e) QLTT moderate-dose (1.8 g/kg) group; and (f) QLTT low-dose (0.9 g/kg) group. Rats were administered drugs or tap water (normal and model groups) orally once a day for 7 days. After the last gavage, rats in the model group and medicine treatment groups were injected with 0.1% adrenaline hydrochloride (0.8 mL/kg, i.h.). After 2 h, the rats received a cold stimulation in ice water for 5 min, and then an injection of an equal amount of adrenaline hydrochloride was given. After fasting for 12 h, rats were anesthetized with 10% chloral hydrate (4 mL/kg, i.p.). Then, 3 mL blood per rat was drawn from the postcava and collected in heparin anticoagulation tubes. The whole blood viscosity of rats was detected by the Department of Laboratory Medicine, Chinese People's Liberation Army General Hospital.

Determination of plasma CGRP and β-EP in rats with nitroglycerin-induced migraine

Sixty Sprague-Dawley rats were grouped with the same method used for the acute blood stasis model rats. Rats received the last gavage 1 h before injection of nitroglycerin (NTG, 10 mg/kg, i.p.) and were given prophylactic treatment with corresponding medicine for the previous 7 days. After 4 h, rats were anesthetized with 10% chloral hydrate (4 mL/kg, i.p.). Then, 2 mL blood per rat was drawn from the postcava and collected in centrifuge tubes containing 7.5% ethylene diamine tetraacetic acid (EDTA, 30 μL) and aprotinin (40 μL). Samples were separated by centrifugation (4377 g) at 4℃ within 10 min and supernatants were immediately stored at - 20℃. The concentrations of CGRP and β-EP were determined using respective ^{125}I-CGRP and ^{125}I-β-EP Radioimmunoassay Kits.

Reserpine-induced low 5-HT model test

Sixty Kunming mice were randomly divided into the following six groups by random number table method: (a) normal control group; (b) mode group; (c) ZTP (2.34 g/kg) group; (d) QLTT (5.2 g/kg) group; (e) QLTT moderate-dose (2.6 g/kg) group; and (f) QLTT low-dose (1.3 g/kg) group. Apart from the mice in the normal control group, mice were administered reserpine (0.25 mg/kg, i.h.) once a day for 9 days. From the third day of reserpine injection, mice in the normal control group and model group were given tap water orally, and medicine treatment groups were given corresponding medications.

Blood clotting time

The assay of blood clotting time was carried out as Chen *et al.* [8] After 1 h from the last reserpine injection and gavage, blood samples were taken with glass capillary tubes from the posterior orbital venous plexus of mice, and a stopwatch was started immediately. A small piece of capillary was broken from one end every 30 s until fibrin threads of blood appeared between the broken ends of the glass capillary. Then the stopwatch was stopped and the blood clotting time was recorded.

Plasma samples and cerebral homogenate samples for 5-HT, DA, and NE determination

After completion of the blood clotting test, the eyeballs of mice were taken out for the collection of blood samples. Then, brains were rapidly removed and cerebellums were discarded. The remaining tissue samples were weighed and stored at - 80℃ until homogenization. Blood samples (0.5 mL) were collected in centrifuge tubes containing 7.5% EDTA (7.5 μL) and separated by centrifugation (4377 ×*g*) at 4℃ within 10 min. Supernatants were immediately stored at - 20℃ and the concentrations of 5-HT were determined using ELISA.

Per mouse, 0.1 g brain tissue was homogenized in 0.2 mL perchloric acid (0.1 M), which included 0.01% cysteine as an antioxidant. The homogenate was centrifuged at 20 428 × *g* at 4℃ for 15 min, and 20 μL supernatant was directly injected into an Agilent 1200 series HPLC equipped with a phenomenex luna C18 column (250 mm × 4.6 mm × 5 μm). A VWD detector (Agilent G1314B) was set at a wavelength of 280 nm. The mobile phase consisted of 15 volumes of acetonitrile and 85 volumes of 0.14% sodium heptanesulfonate (adjusted to pH 3.0 with phosphoric acid). The column temperature was 40℃ and the flow rate was 1.0 mL/min.

Statistical analyses

Data are expressed as mean±standard deviation ($\bar{x} \pm s$). All data analysis was tested by one-way analysis of variance (ANOVA). The least-significant difference (LSD) test was used for the Post hoc multiple comparisons in the data with homogeneity of variance and Dunnett's T3 testing in the case of variance. The significance level was $P<0.05$. All data were analyzed with IBM SPSS (version 19.0, SPSS Inc, Chicago, IL, USA).

RESULTS

Whole blood viscosity of acute blood stasis model rats

Compared with that of the normal control group, the blood viscosity of the model group was significantly higher. However, rats treated with ZTP and the high (3.6 g/kg), moderate (1.8 g/kg), and low (0.9 g/kg) doses of QLTT had significantly lower blood viscosity than those of the control rats (Table 1).

Determination of the levels of CGRP and β-EP

As shown in Table 2, NTG stimulated the release of CGRP and induced a decrease in plasma β-EP. QLTT-treated rats at dosages of 3.6, 1.8 and 0.9 g/kg as well as those treated with ZTP had lower NTG-induced plasma CGRP levels. Apart from rats in the QLTT (0.9 g/kg) group, the plasma β-EP levels in rats of the other treatment groups were significantly higher than those in the model rats.

Blood clotting time of reserpine-induce model mice

The time of blood coagulation was greatly shortened by the administration of reserpine (Table 3). In this experiment, QLTT at dosages of 5.2 and 2.6 g/kg and ZTP significantly prolonged the clotting time compared with the model group.

Table 1 Differences in whole blood viscosity of rats under different shear rates ($\bar{x} \pm s$)

Group	*n*	Whole blood viscosity (mPa·S)			
		1/s	5/s	30/s	200/s
Normal control	9	33.81±3.47[a]	13.22±1.11[a]	6.72±0.44[a]	4.63±0.25[a]
Model	10	46.40±4.91[b]	17.65±1.49[b]	8.65±0.61[b]	5.89±0.35[b]
ZTP (1.62 g/kg)	9	34.61±5.23[a]	13.60±1.79[a]	6.96±0.78[a]	4.81±0.48[a]
QLTT (3.6 g/kg)	10	35.34±3.57[a]	13.84±1.18[a]	7.06±0.50[a]	4.87±0.29[a]
QLTT (1.8 g/kg)	8	34.82±4.14[a]	13.77±1.35[a]	7.10±0.55[a]	4.95±0.34[a]
QLTT (0.9 g/kg)	8	36.31±5.74[a]	14.29±1.80[a]	7.33±0.69[a]	5.08±0.39[a]

Notes: normal control group treated with saline only; model group treated with saline and epinephrine; ZTP group treated with ZTP (1.62 g/kg) and epinephrine; QLTT groups treated with epinephrine and high-dose QLTT (3.6 g/kg), moderate-dose QLTT (1.8 g/kg), and low-dose QLTT (0.9 g/kg). ZTP: Zhengtian pill; QLTT: Qilongtoutong granule. Compared with model group, [a]P<0.01; compared with the normal control group, [b]P<0.01.

Table 2 Comparison of plasma CGRP and β-EP levels of rats ($\bar{x} \pm s$)

Group	*n*	CGRP (pg/mL)	β-EP (pg/mL)
Normal control	10	73±11[a]	177±20[a]
Model	10	92±5[b]	149±14[b]
ZTP (1.62 g/kg)	10	76±11[a]	176±20[a]
QLTT (3.6 g/kg)	10	77±10[a]	182±22[a]
QLTT (1.8 g/kg)	10	76±11[a]	183±26[a]
QLTT (0.9 g/kg)	10	82±8[c]	165±21

Notes: normal control group treated with saline only; model group treated with saline and nitroglycerin; ZTP group treated with ZTP (1.62 g/kg) and nitroglycerin; QLTT groups treated with nitroglycerin and high-dose QLTT (3.6 g/kg), moderate-dose QLTT (1.8 g/kg), and low-dose QLTT (0.9 g/kg). ZTP: Zhengtian pill; QLTT: Qilongtoutong granule; CGRP: calcitonin gene-related peptide; β-EP: beta-endorphin. Compared with model group, [a]P<0.01, [c]P<0.05; compared with normal control group, [b]P<0.01.

Table 3 Differences in blood clotting time of mice ($\bar{x} \pm s$)

Group	*n*	Clotting time (s)	*P* value (*vs* normal control)	*P* value (*vs* model)
Normal control	10	93±22	-	0.001
Model	9	50±15	0.001	-
ZTP (2.34 g/kg)	9	90±26	0.797	0.001
QLTT (5.2 g/kg)	10	96±24	0.791	0.000
QLTT (2.6 g/kg)	9	93±32	0.977	0.001
QLTT (1.3 g/kg)	9	70±30	0.053	0.099

Notes: normal control group treated with saline only; model group treated with saline and reserpine; ZTP group treated with ZTP (2.34 g/kg) and reserpine; QLTT groups treated with reserpine and high-dose QLTT (5.2 g/kg), moderate-dose QLTT (2.6 g/kg), and low-dose QLTT (1.3 g/kg). ZTP: Zhengtian pill; QLTT: Qilongtoutong granule.

Contents of 5-HT, NE, and DA in plasma and brain tissue of mice

Compared with the model group, QLTT- (2.6 g/kg) and ZTP-treated mice had significantly higher plasma 5-HT levels (P<0.05). Meanwhile, QLTT- (5.2, 2.6 and 1.3 g/kg) and ZTP-treated mice had significantly higher 5-HT, NE, and DA contents in the brain tissue (P<0.01) than those in model mice, although they were not as high as those in the normal mice. Typical high-performance liquid chromatography chromatograms of brain homogenate for detecting the 5-HT, NE, and DA concentrations are shown in Figure 1 and Table 4.

DISCUSSION

Our studies demonstrated that rats treated with QLTT at the high, moderate, and low dosages, and ZTP, had lower whole blood viscosity than those in blood stasis model rats under different shear rates. Treated rats also had longer clotting times than those in reserpine-induced model mice. CGRP is considered an important mediator in migraine and has been found at increased concentrations in peripheral blood plasma during attacks of migraine.[9] Meanwhile, some studies indicated that migraine is closely related to the impairment of the endogenous opioid peptide system, and especially

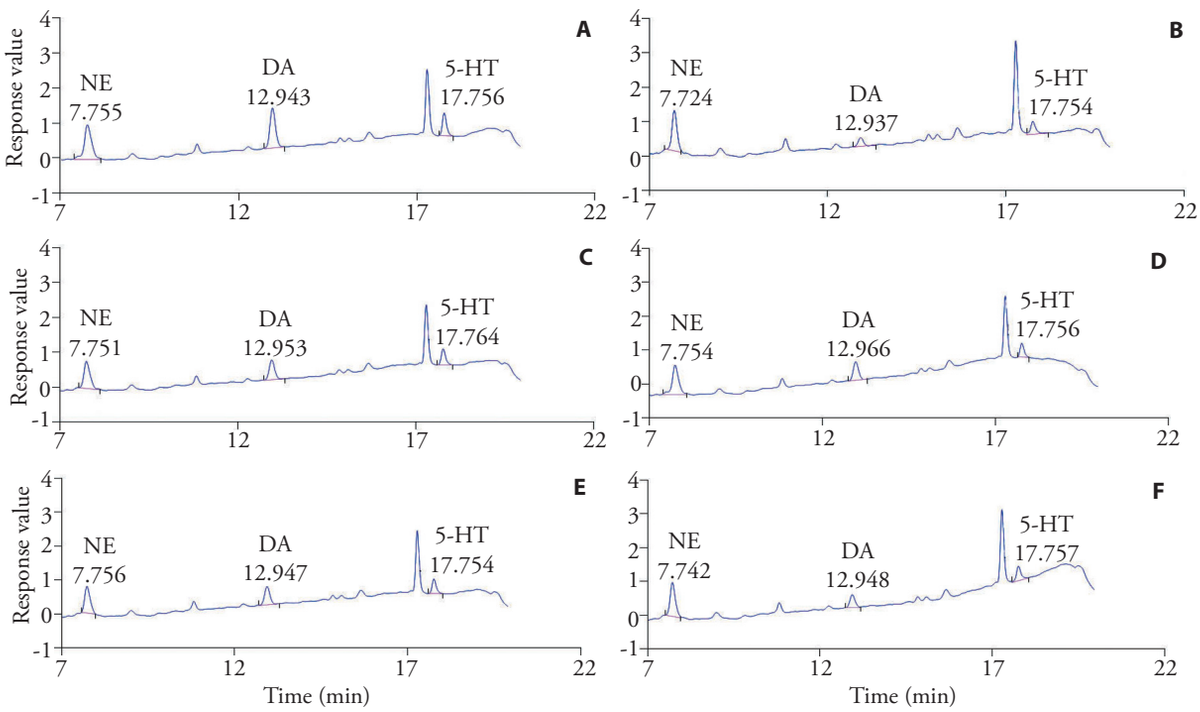

Figure 1 High-performance liquid chromatography chromatograms of brain homogenate

A: normal control group, treated with saline only; B: model group, treated with saline and reserpine; C: Zhengtian pill group, treated with ZTP (2.34 g/kg) and reserpine; D: Qilongtoutong high-dose (5.2 g/kg) group, treated with QLTT (5.2 g/kg) and reserpine; E: Qilongtoutong moderate-dose (2.6 g/kg) group, treated with QLTT (2.6 g/kg) and reserpine; F: Qilongtoutong low-dose (1.3 g/kg) group, treated with QLTT (1.3 g/kg) and reserpine.

Table 4 Differences in serotonin, noradrenalin, and dopamine contents in plasma and brain tissue in mice (n=10, $\bar{x} \pm s$)

Group	Dosage (g/kg)	Plasma serotonin (ng/mL)	Brain tissue (ng/g)		
			Serotonin	Noradrenalin	Dopamine
Normal control	-	259±98[a]	556±44[a]	675±47[a]	2042±250[a]
Model	-	107±56[b]	268±34[b]	279±34[b]	510±45[b]
ZTP	2.34	191±90[c]	358±45[ab]	358±42[ab]	978±130[ab]
QLTT	5.20	180±75[d]	353±33[ab]	367±31[ab]	985±158[ab]
QLTT	2.60	194±84[c]	353±32[ab]	367±30[ab]	988±141[ab]
QLTT	1.30	145±66[b]	305±36[bc]	315±36[bc]	675±47[ba]

Notes: normal control group treated with saline only; model group treated with saline and reserpine; ZTP group treated with ZTP (2.34 g/kg) and reserpine; QLTT groups treated with reserpine and high-dose QLTT (5.2 g/kg), moderate-dose QLTT (2.6 g/kg), and low-dose QLTT (1.3 g/kg). ZTP: Zhengtian pill; QLTT: Qilongtoutong granule. Compared with model group, [a]P<0.01, [c]P<0.05; compared with normal control group, [b]P<0.01, [d]P<0.05.

that the disturbance of beta-endorphin may give rise to regulation disorders of afferent nociception.[10] Plasma β-EP levels are remarkably low in migraine patients and there is an inverse relationship between endorphin levels and headache severity scores.[11] To investigate the influence of QLTT on plasma CGRP and β-EP, a reliable animal experimental migraine model induced by NTG was chosen.[12] The rats showed a series of symptoms after being injected with NTG, which are similar to the symptoms of human migraine onset. The symptoms include frequent scratching of the head, climbing cages and flushing. These typical manifestations of migraine were used as indicators of successful models establishment.[13] We found that QLTT-treated rats had significantly lower plasma CGRP levels and higher plasma β-EP levels than those in model rats.

Among the many neurotransmitters in the brain, 5-HT from the brainstem raphe nucleus has been most convincingly implicated in migraine.[14] Some researchers also hypothesize that 5-HT dysfunction in the circulatory system is involved in the pathogenesis of migraine.[15] Recent evidence suggests that a low level of 5-HT facilitated activation of the trigeminovascular nociceptive pathway.[14] One study also showed that norepinephrine inhibited the migration of a cortical spreading depression wave in the adult gray matter.[16] Additionally, dopamine appears to have a beneficial effect on pain by inhibiting nociceptive transmission from the trigeminocervical complex up to the thalamus.[17] Many studies indicate that 5-HT, NE, and DA play an important role in migraine onset. However, reserpine can deplete monoamine neurotransmitters and precipitate migraine onset.[18] In our experiments, mice treated with QLTT at high, moderate, and low doses, and ZTP, had higher 5-HT levels than those in model mice with migraine induced by reserpine. This suggests that QLTT and ZTP rely on the serotonergic system and tyrosine metabolism pathway to relieve the pain caused by migraine. To ease the pain, QLTT might act through multiple pathways including activating blood, expelling blood stasis, and regulating the levels of CGRP, β-EP, 5-HT, NE, and DA. However, further study is needed to elucidate the QLTT mechanism underpinning its pain-relieving action.

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