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Effects of *Ligusticum chuanxiong* and *Gastrodia elata* on blood-brain barrier permeability in migraine rats

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The two herbs *Ligusticum chuanxiong* (LC) Hort. (Umbelliferae) and *Gastrodia elata* (GE) Blume (Orchidaceae), are widely used in the clinic for the treatment of migraine. This article aims to understand the effects of LC and GE on blood-brain barrier (BBB) permeability in migraine rats. Serotonin, excitatory amino acids (EAAs) and matrix metalloproteinase-9 (MMP-9) were determined at different sampling times to assess BBB disruption during a migraine attack. BBB permeability was examined by fluorescence imaging and Evans blue dye (EBD) extravasation. The results showed that the expression of serotonin in migraine rat brain was enhanced from 30 min to 120 min and glutamate (Glu) was suppressed from 30 min to 60 min in LC-GE group compared with the model group ($p < 0.05$ or 0.01), while the MMP-9 levels in migraine rat blood was increased at 30 min as well as decreased at 120 min in LC-GE group compared with the model group ($p < 0.05$ or 0.01). EBD levels in rat brain were significantly lower at 30~60 min and 120~150 min in LC-GE group than that of the model group ($p < 0.05$ or 0.01). Our findings demonstrated that LC and GE might decrease BBB permeability and maintain its integrity through regulating serotonin, EAAs and MMP-9 in migraine rats.

1. Introduction

The blood-brain barrier (BBB) is a dynamically regulated physical barrier between the central nervous system (CNS) and the circulation consisting of endothelial cells (ECs) that line cerebral microvessels (Jiang et al. 2014). Migraine is one of the most common neurological disorders. Abundant experimental evidence indicates that sensitization of peripheral and central brain areas, activation of the trigeminovascular system and cortical spreading depression (CSD) are three important processes in migraine (De-Vries et al. 1999; Jiang et al. 2008). A great many of neurotransmitters such as serotonin (5-hydroxytryptamine [5-HT]), excitatory amino acids (EAAs) and matrix metalloproteinase-9 (MMP-9) are involved in the disruption of the BBB during migraine attacks (Charles 2013). 5-HT could regulate both physiological and pathophysiological conditions in tissues throughout the body, while 5-hydroxytryptophan (5-HTP) and 5-hydroxyindoleacetic acid (5-HIAA) are its direct precursor and metabolite, respectively (Lynn-Bullock et al. 2004; Lorenzi et al. 2009). It has been confirmed that 5-HT and related compounds could inhibit N-methyl D-aspartate (NMDA) receptors and attenuate glutamate-mediated excitatory synaptic responses in a concentration dependent manner, which may play a role in the protection of BBB (Kloda and Adams 2005). EAAs are other typical neurotransmitters including glutamate (Glu) and aspar-

tate (Asp), the increased EAAs in brain may activate NMDA receptors excessively and decrease the phosphorylation of IGF-1 receptors, which may amplify and reinforce pain transmission and brain damage in migraine (Sun et al. 2012). Therefore, taken together, 5-HT is a NMDA receptors antagonist while EAAs are NMDA receptors agonists, both of them affect the same receptor competitively, and affect the integrity of BBB simultaneously. Matrix metalloproteinases (MMPs) are a family of over twenty zinc-dependent endopeptidases that play essential roles in a wide range of proteolytic processes. Their first-recognized and most prominent function is cleaving the extracellular matrix (ECM) (Lakhan and Avramut 2012). Evidence in human and animal models shows that BBB disruption is associated with activation of MMP-9. After cerebral ischemia and inflammation, MMP-9 can degrade the basement membrane and result in structural damage and the increase of BBB permeability. The Evans blue dye (EBD, 961 Da) can bind to serum albumin (69,000 Da) to become a high molecular weight (HMW) protein tracer after injected into the circulation, it is commonly used as an inert tracer in BBB research. Albumin-bound EBD is too large to cross the BBB but can grossly stain tissue blue when the BBB is disrupted (Do et al. 2014).

The two herbs *Ligusticum chuanxiong* (LC) Hort. (Umbelliferae) and *Gastrodia elata* (GE) Blume (Orchidaceae) with a crude weight ratio of 4:1, are commonly used for treating migraine. Preliminary studies show that LC and GE ethanol

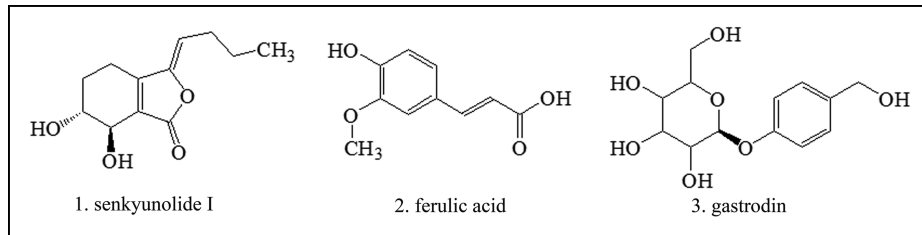


Fig. 1: Chemical structures of senkyunolide I, ferulic acid, and gastrodin.

extracts are the main therapeutically effective parts, on this basis, the extraction and purification are optimized, therefore, active components of LC and GE are obtained and confirmed its weight ratio of 4:3 (Hong et al. 2007). Senkyunolide I, ferulic acid and gastrodin (Fig. 1) are three main active ingredients from LC and GE, an anti-migraine effect of which has been suggested (Wan et al. 2003; Wang et al. 2011; Lin et al. 2013; Xu et al. 2007). However, it is known that BBB permeability is abnormal during migraine attacks and the effects of the two herbs on BBB permeability is still unclear.

In the current study, a dose of LC and GE, as well as their mixture (LC-GE) were given to 6-week old Sprague-Dawley rats orally. The effects of LC and GE on the contents of serotonin, EEAs, MMP-9 and the possible changes in the permeability of the BBB in migraine rats were systematically profiled.

2. Investigations and results

The effects of LC and GE on the contents of serotonin, EEAs, MMP-9 and the possible changes in the permeability of the BBB in experimental migraine rats determined.

2.1. Quantitative analysis of serotonin in rat brain

The rat brain serotonin amounts measured are shown in Tables 1–3. The amount of 5-HTP in LC-GE group was significantly higher than in all the other groups at 60 min ($p < 0.05$ or 0.01). Similarly, the amount of 5-HT in the LC-GE group was significantly higher than that of the model group at all sampling times except 15 min ($p < 0.05$ or 0.01), and was significantly higher than that of the GE group at 30 min ($p < 0.05$). The amount of 5-HIAA in the LC-GE group showed no significant difference compared with the model group at all sampling times, but the amount of 5-HIAA in all groups decreased as time went by.

2.2. Quantitative analysis of EAAs in rat brain

The amounts of the EAAs found in rat brain are presented in Tables 4 and 5, the amount of Glu in the LC-GE group was sig-

nificantly lower than that of the model group at 30 and 60 min ($p < 0.05$ or 0.01), and Glu measured from the LC group at 60 min, GE group at 30 and 60 mins, were significantly lower than that of model group ($p < 0.05$ or 0.01). However, the amount of Asp in the LC-GE group showed no significant difference compared with the model group at all sampling times.

2.3. Quantitative analysis of MMP-9 in rat blood

The amounts of MMP-9 found in rat blood are given in Table 6. The MMP-9 measured from the LC-GE group was significantly higher than that of the model group at 30 min ($p < 0.01$), and the amount of MMP-9 in the LC group was significantly higher than that of the model group at 15 and 30 mins ($p < 0.05$ or 0.01). However, the amount of MMP-9 in the LC-GE group was significantly lower than that of the model group at 120 min ($p < 0.05$).

2.4. Quantitative analysis of EBD in rat brain

The amounts of the EBD found in rat brain are displayed in Table 7, the amount of EBD in LC-GE and GE groups were significantly lower than that of the model group at 0~30 min and 120~150 min ($p < 0.05$ or 0.01), and the LC group was significantly lower than that of model group at 0~30 min, 30~60 min and 120~150 min ($p < 0.05$ or 0.01). As was shown in Fig. 2, strong yellow or red fluorescence (Equivalent to a high content of EBD) was obtained in the model group at 0~30 min, 30~60 min, while that was much weaker in the LC, GE, LC-GE groups. Meanwhile, the fluorescence in the LC group was weaker than in the GE, LC-GE group at all times.

3. Discussion

5-HT is one of the main transmitters in the nervous system. Cerebral vasodilation and low serotonin levels may be involved in the pathogenesis of migraine, as several receptors including 5-HT₁, 5-HT₂ and 5-HT₃ are associated with migraine. The 5-HT₁ receptor is the one mainly expressed in the CNS, therefore 5-HT_{1D} receptor agonists could suppress cerebral

Table 1: Amounts of 5-HTP in rat brain (ng/g) (n = 6)

Group	5-HTP			
	15 min	30 min	60 min	120 min
Normal group	833.78 ± 45.68	805.10 ± 66.34	805.43 ± 30.88 ^{€€}	834.78 ± 51.58
Model group	800.29 ± 51.84	773.48 ± 57.01	799.09 ± 33.81 ^{€€}	870.73 ± 123.13
Positive group	869.80 ± 65.21	832.11 ± 75.56	845.45 ± 51.02 [€]	804.43 ± 65.76
LC group	843.45 ± 44.37	816.77 ± 99.70	808.43 ± 82.72 ^{€€}	838.78 ± 54.05
GE group	843.78 ± 92.79	805.76 ± 89.59	782.08 ± 60.94 ^{€€}	828.77 ± 82.38
LC-GE group	856.79 ± 89.59	779.75 ± 109.57	922.82 ± 41.23 ^{**▲φφ}	856.79 ± 99.10

Note: At one sampling time, $p < 0.05$; $p < 0.01$, Other groups vs Normal group; * $p < 0.05$; ** $p < 0.01$, Other groups vs Model group; ▲ $p < 0.05$; ▲▲ $p < 0.01$, Other groups vs Positive group; φ $p < 0.05$; φφ $p < 0.01$, Other groups vs LC group; § $p < 0.05$; §§ $p < 0.01$, Other groups vs GE group; € $p < 0.05$; €€ $p < 0.01$, Other groups vs LC-GE group.

Table 2: Amounts of 5-HT in rat brain (ng/g) (n = 6)

Group	5-HT			
	15 min	30 min	60 min	120 min
Normal group	393.25 ± 15.67	389.29 ± 23.84	397.20 ± 29.61*	395.00 ± 26.63**
Model group	361.71 ± 13.62	370.67 ± 20.12 [€]	358.02 ± 32.45 ^{ψ€}	356.44 ± 8.21 ^{▲ψ€€}
Positive group	387.10 ± 11.23	372.25 ± 5.71	382.27 ± 6.01	381.39 ± 6.38*
LC group	387.98 ± 51.62	382.71 ± 32.20	401.77 ± 31.54*	385.34 ± 17.75*
GE group	369.09 ± 20.27	372.17 ± 22.57 [€]	386.85 ± 35.66	386.48 ± 25.59*
LC-GE group	386.48 ± 34.94	404.22 ± 34.19*	406.86 ± 29.75*	397.64 ± 19.89**

The note was the same as Table 1.

Table 3: Amounts of 5-HIAA in rat brain (ng/g) (n = 6)

Group	5-HIAA			
	15 min	30 min	60 min	120 min
Normal group	578.33 ± 22.97	560.48 ± 25.25	547.36 ± 19.05	545.26 ± 22.25
Model group	576.44 ± 24.56	558.17 ± 14.01	541.16 ± 11.22	539.91 ± 16.27
Positive group	577.81 ± 23.60	557.86 ± 21.98	544.21 ± 23.86	521.64 ± 16.06 ^{ψψ}
LC group	587.25 ± 45.53	576.23 ± 27.58	557.33 ± 27.36	559.95 ± 22.92 [▲]
GE group	603.53 ± 29.61	573.61 ± 25.72	542.63 ± 28.01	546.83 ± 32.24
LC-GE group	582.53 ± 28.70	566.28 ± 12.66	560.48 ± 40.42	549.46 ± 24.67

The note was the same as Table 1.

Table 4: Amounts of Glu in rat brain (μg/g) (n = 6)

Group	Glu			
	15 min	30 min	60 min	120 min
Normal group	795.90 ± 98.51	810.55 ± 60.62*	793.21 ± 78.35**	799.84 ± 79.11
Model group	871.67 ± 164.76	968.28 ± 50.99 [€]	1045.70 ± 36.32 ^{▲▲ψψ€€}	909.13 ± 30.51
Positive group	825.43 ± 85.25	821.43 ± 68.66*	800.37 ± 98.01**	778.83 ± 110.46
LC group	919.32 ± 93.67	860.06 ± 168.75	883.39 ± 96.19**	859.31 ± 88.68
GE group	831.79 ± 18.41	800.53 ± 109.99*	857.31 ± 66.35**	868.99 ± 127.37
LC-GE group	850.09 ± 135.77	792.97 ± 115.30*	868.58 ± 123.53**	859.07 ± 148.53

The note was the same as Table 1.

Table 5: Amounts of Asp in rat brain (μg/g) (n = 6)

Group	Asp			
	15 min	30 min	60 min	120 min
Normal group	924.11 ± 76.30 ^ψ	905.91 ± 60.15 ^{***€€}	931.48 ± 106.29 ^{**€€€}	950.50 ± 62.44
Model group	1128.06 ± 152.47 ^{▲ψ}	1179.26 ± 62.94 [▲]	1138.03 ± 61.01 [▲]	1162.75 ± 288.18 [▲]
Positive group	840.85 ± 106.57 ^{**€}	924.27 ± 129.76 ^{**€}	880.05 ± 121.79 ^{**€€}	904.22 ± 79.48*
LC group	917.17 ± 145.74*	1040.87 ± 167.80	1005.71 ± 139.46	1112.69 ± 245.20
GE group	990.08 ± 186.15	998.33 ± 146.55*	1030.26 ± 174.51 [▲]	1019.72 ± 209.49
LC-GE group	1066.70 ± 67.43 [▲]	1119.20 ± 124.95 [▲]	1137.33 ± 120.02 [▲]	1097.17 ± 179.69

The note was the same as Table 1.

vasodilation and take an effect for the treatment of migraine (Ahn and Basbaum 2006). In this study, it was found that LC-GE could increase 5-HTP and 5-HT levels. Which play a role in migraine treatment. Additionally, it could relieve brain damage and decrease BBB permeability through inhibition of NMDA receptors. Some drugs have timeliness when modulating the physiological function. It is known that migraine occurred rapidly, and the main active ingredients of LC and GE namely senkyunolide I, ferulic acid and gastrodin were absorbed quickly and eliminated fast, so 120 min was the longest sampling time to research timeliness. The results showed that, compared with

normal rats, the level of 5-HT in migraine rat brain was lower and a downward trend could be seen after 30 min, meanwhile LC-GE could increase 5-HTP levels at 60 min, and increase 5-HT levels in migraine rat brain at all sampling times except 15 min, LC could increase 5-HT levels at 60 and 120 min. However, there was no significant difference between the GE and the model group, suggesting the compatibility of LC and GE might increase 5-HT levels in migraine rat brain from 30 min to 120 min, which resulted in a decrease of BBB permeability and avoidance of brain damage. And between those two herbs, LC played a major role.

Table 6: Amounts of MMP-9 in rat blood (ng/mL) (n = 6)

Group	MMP-9			
	15 min	30 min	60 min	120 min
Normal group	12.90 ± 0.91 ^{φφ}	13.37 ± 1.96 ^{€€}	13.68 ± 1.10	14.29 ± 1.33 ^{φφ€€}
Model group	12.71 ± 1.77 ^{φφ}	13.24 ± 1.88 ^{φφ€€}	14.59 ± 2.60	13.87 ± 1.75 ^{€€}
Positive group	14.31 ± 1.52 ^φ	14.07 ± 1.71 [€]	13.80 ± 1.92	14.16 ± 2.27 ^{€φ}
LC group	17.01 ± 2.28 ^{**▲€}	15.45 ± 1.69 [*]	13.53 ± 2.48	11.93 ± 0.50 [▲]
GE group	14.01 ± 2.18 ^{φφ}	14.89 ± 1.32	14.21 ± 1.62	13.81 ± 1.72 [€]
LC-GE group	14.60 ± 1.82 ^φ	16.60 ± 1.91 ^{**▲}	13.92 ± 2.48	11.63 ± 1.43 ^{*▲}

The note was the same as Table 1.

Table 7: Amounts of EBD in rat brain (μg/g) (n = 6)

Group	EBD			
	0-30 min	30-60 min	60-90 min	120-150 min
Normal group	0.45 ± 0.13 ^{**▲€€}	0.53 ± 0.14 ^{**€}	0.45 ± 0.10 ^{**▲€}	0.58 ± 0.12 ^{**}
Model group	1.61 ± 0.48 ^{φφ€}	1.10 ± 0.28 ^{▲φ}	1.14 ± 0.48	1.14 ± 0.31 ^{▲▲φ}
Positive group	1.49 ± 0.16 ^{φφ€}	0.78 ± 0.23 [*]	0.83 ± 0.21	0.75 ± 0.19 ^{**φ}
LC group	0.57 ± 0.23 ^{**▲€}	0.54 ± 0.15 ^{**€}	0.77 ± 0.27	0.35 ± 0.01 ^{**▲}
GE group	0.98 ± 0.43 ^{*▲}	0.76 ± 0.37	0.79 ± 0.20	0.63 ± 0.28 ^{**}
LC-GE group	1.06 ± 0.27 ^{*▲φ}	0.93 ± 0.24 ^φ	0.91 ± 0.22	0.64 ± 0.19 ^{**}

The note was the same as Table 1.

Glutamate receptors, activated by Glu and Asp, are divided into two types, the ionic and the metabolic type. Glutamate ionotropic receptors of the NMDA and AMPA /kainate type are abundantly expressed on neurons and participate to the regulation of both motor and secretory functions of the whole body (Szegedi et al. 2005), while the metabotropic glutamate receptors (mGluR) are involved in many developmental processes and immune and synaptic functions, but the mode of their signal transduction is unclear (Tambuyzer et al. 2013). When the EAAs activate NMDA receptors, brain damage can be caused. In this

study, it was found that GE and LC-GE could decrease Glu levels in migraine rat brain, which resulted in weakening the effect of the NMDA receptors and maintaining the integrity of the BBB. It was shown that the Glu levels in migraine rats were higher than that of normal rats and an increasing trend was seen up to 60 min GE and LC-GE could decrease Glu levels in migraine rat brain at 30 and 60 min. However, there was no significant difference among all groups at 120 min, probably because astrocytes could respond to synaptic activation and react in a way that sustained or enhanced further communication (Uwechue et al. 2012). It could

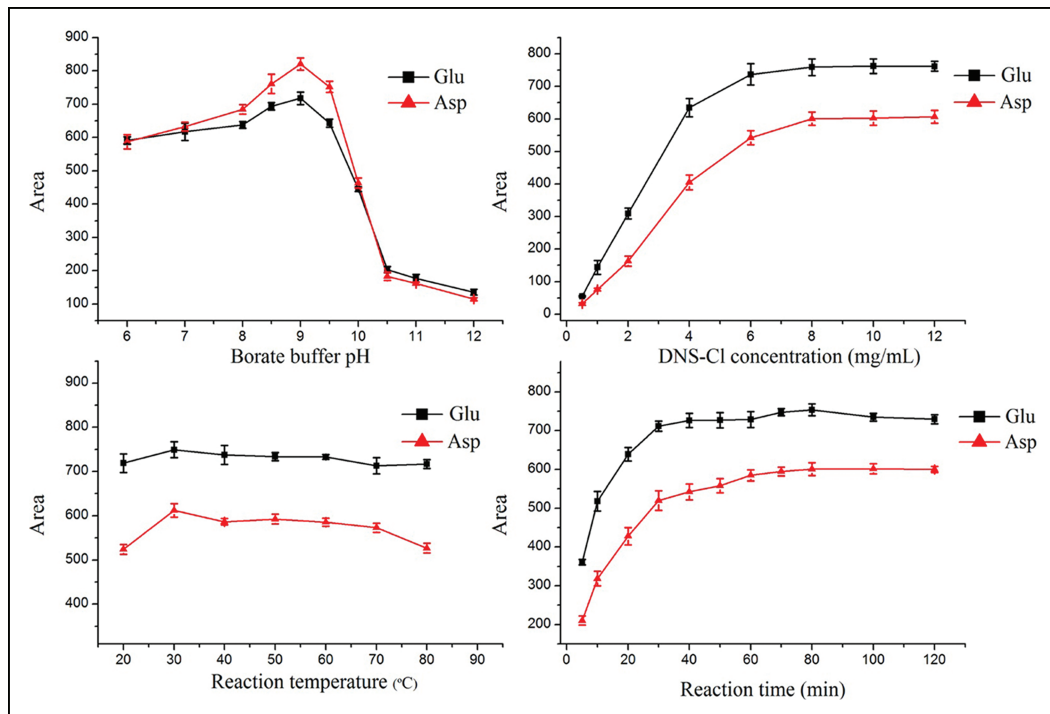


Fig. 2: Fluorescence intensity of EBD in rat brain from different groups in different periods of Migraine attack.

be concluded that LC and GE compatibility might decrease Glu levels in migraine rats so as to maintain the integrity of the BBB from 30 min to 60 min with GE playing a major role.

It is possible that MMP-9 levels might be up-regulated during a migraine attack, and that increased MMP-9 could degrade the basement membrane and change BBB permeability. In the present study, it was found that LC could increase MMP-9 levels in migraine rat blood at 15 and 30 mins, LC-GE could increase MMP-9 levels at 30 min, indicating that LC and GE compatibility might increase the BBB permeability at 30 min and possibly promote CNS drug delivery, during which, LC played a major role. However, LC-GE could decrease MMP-9 levels at 120 min, indicating that LC and GE compatibility might decrease the BBB permeability at 120 min after administration.

All of the 5-HT, Glu and MMP-9 levels could impact the integrity of the BBB, which had synergistic or antagonistic effects at different sampling times. Therefore, EBD was used as a direct tracer to investigate BBB disruption. In the present study, it was found that the EBD extravasation was lower in LC, GE and LC-GE groups than in the model group at 0~30 min, 30~60 min and 120~150 min, illustrating LC and GE compatibility might indeed reduce the BBB permeability through regulating neurotransmitters during a migraine attack. EBD extravasation in the LC-GE group was higher than in the LC group at all sampling times, indicating LC and GE compatibility might promote drug absorption in the BBB to some extent by changing the BBB permeability. Therefore, LC and GE compatibility might have these possible pathways to protect brain damage for treating migraine. In summary, LC and GE compatibility could up-regulate 5-HT levels from 30 min to 120 min, down-regulate Glu levels from 30 min to 60 min in migraine rat brain. They could first increase MMP-9 levels at 30 min in migraine rat serum, then decrease MMP-9 levels at 120 min. Practically, the results demonstrated that LC and GE compatibility could regulate the BBB permeability and relieve brain damage through these three approaches. These findings provided further evidence for traditional Chinese medicine to influence the integrity of the BBB.

4. Experimental

4.1. Chemicals and reagents

Co-dergocrine mesylate tablets and nitroglycerin injection were purchased from Tianjin Huajin Pharmaceutical Co. Ltd. (Tianjin, China) and Beijing Yimin Pharmaceutical Co. Ltd. (Peking, China), respectively. 5-HTP, 5-HIAA and 5-HT were obtained from Sigma-Aldrich. L-Glutamate, L-aspartate were from Sinopharm Chemical Reagent (Shanghai, China). Dansyl chloride was from TCI (Tokyo, Japan). Rat Total MMP-9 Quantikine ELISA Kit was from R&D Systems (Minneapolis, USA). Evans blue dye (EBD) was from Sinopharm Chemical Reagent (Shanghai, China). HPLC grade acetonitrile and methanol were obtained from Merck, Inc. (Merck, Darmstadt, Germany). All other chemical solvents were of analytical grade from Sinopharm Chemical Reagent (Shanghai, China).

4.2. Plant extraction

The standards of ferulic acid and gastrodin were purchased from National Institute for food and drug Control (Peking, China). The standard of senkyunolide I was separated from LC with a purity not less than 98%. LC and GE were from Shanghai Cambridge Chinese Herbal Medicine Co. Ltd., which were identified as *Ligusticum chuanxiong* Hort. (Umbelliferae) and *Gastrodia elata* Blume (Orchidaceae) by Professor Bao-Kang Huang from Second Military Medical University, respectively (Shanghai, China). 1 kg of the dry powder from the LC was loaded in 12 L of 70% ethanol for 2 h and 1.5 h at 80 °C, respectively. The mixed extraction solutions were concentrated to 2 L and centrifuged at 5000 rpm for 15 min. The supernatant was added in HPD-100 macroporous resin with a flow rate of 1 BV/h, firstly the ultrapure water was added until the eluent remained nearly colorless, then 50% ethanol was added to 4 BV, the ethanol eluent was collected, evaporated and dried. The LC extract contained 6.20% of ferulic acid and 10.74% of senkyunolide I after HPLC analysis.

Similarly, 1 kg of plant powder from GE were extracted using 10 L of 70% ethanol for 1 h for two times at 80 °C. The mixed extraction solutions were concentrated to 2 L and centrifuged at 5000 rpm for 15 min. The supernatant was evaporated to 250 mL, 95% ethanol was added until its contents of 70%, after laying 24 h, filtered. 40% sodium hydroxide was added until its pH 8.0, after laying 24 h again, filtered and the filtrate was evaporated and dried. The GE extract contained 14.85% of gastrodin after HPLC analysis.

4.3. Preparation of sample solutions

Co-dergocrine mesylate tablets (each tablet contains 1 mg co-dergocrine mesylate) were prepared in physiological saline and diluted to 0.1 mg/mL co-dergocrine mesylate (CDM) solution, which was used as positive drug. The extracts of LC and GE were dissolved in physiological saline and diluted to 65.20 mg/mL (equivalent to 4.04 mg/mL ferulic acid, 7.00 mg/mL senkyunolide I) and 48.40 mg/mL (equivalent to 7.19 mg/mL gastrodin) LC and GE solution, respectively. The extracts of LC and GE were mixed in a weight ratio of 4:3 and the mixture was dissolved in physiological saline and diluted to form the solution of the same concentration as in LC and GE solution. All solutions were stored at 4 °C and shaken well before using. The dansyl chloride (DNS-Cl) solution was prepared just before derivatization by dissolving 80.00 mg of DNS-Cl in 10 mL of acetonitrile. The borate buffer (pH 9.0; 0.2 M) was prepared by dissolving 1.25 g of boric acid and 1.50 g NaCl in 100 mL of ultrapure water, and 0.2 M NaOH was added to adjust the pH to 9.0.

4.4. Animals, drug administration and sampling

4.4.1. Quantification of serotonin, EAAs and MMP-9

144 male Sprague-Dawley rats (180-220 g) were supplied by the Lab Animal Center of Shanghai University of Traditional Chinese Medicine (TCM), and were randomly divided into six groups, named normal group, model group, positive group, LC group, GE group and LC-GE group. They were kept on a 12-hour light/ dark cycle with access to food and water ad libitum. The animal facilities and protocols were approved by the Institutional Animal Care and Use Committee, Shanghai University of TCM. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animal (The National Academies Press, revised edition 2010). Except the normal group, as reported by Tassorelli et al. (2003), nitroglycerin was s.c. injected in a dose of 10 mg/kg, then the physiological saline, CDM, LC, GE and LC-GE solutions (10 mL/Kg, p.o.) were administered to rats in each group after 30 min. The rats in each group were anesthetized at 15 min, 30 min, 60 min, 120 min, respectively. Blood was collected for analysis of MMP-9 by ELISA, brains were rapidly removed after collecting blood, the blood and brain samples were stored at -80 °C.

The brains were homogenized in one volume of cold physiological saline. An equal volume of 0.4 M perchloric acid was added to an appropriate amount of homogenate, followed by centrifugation (10000 rpm for 10 min at 4 °C) to remove the protein precipitate. The supernatant (20 µL) was injected into the HPLC system for determination of serotonin, another 50 µL of the supernatant were for the determination of EAAs after derivative reaction. The supernatant 50 µL was sucked precisely and put into a centrifuge tube, 1.0 M sodium hydroxide (10 µL), 0.2 M borate buffer (50 µL) and 8.00 mg/mL DNS-Cl solution (50 µL) were added. After mixing vigorously for 10 s, the mixture was reacted in the dark at 30 °C in a water bath for 80 min. Then, an aliquot (40 µL) of acetic acid was added into the tube to stop the reaction. The mixture was centrifuged at 4000 rpm for 5 min. The supernatant (10 µL) of the reaction mixture was injected into the HPLC system for determination of EAAs.

4.4.2. Quantification of EBD extravasation

The six groups of rats were i.v. injected with 3 mL/kg of 2% EBD solution in physiological saline at 0, 30, 60 and 120 min following the drug administration, 30 minutes later, the rats were perfused with physiological saline to wash out any remaining dye in the blood vessels and then the brains were removed. The brains were firstly inspected by using the Lumina XR Series III imaging system (PerkinElmer, USA) (The fluorescence at an emission wavelength of 680 nm and excitation at 620 nm), then 3 mL of dimethylformamide were added to each brain and incubated at 37 °C for 72 h, the supernatant was analyzed spectrophotometrically at 620 nm. The EBD contents were expressed as µg/g of tissue weight and calculated from a standard curve from known amounts of the dye.

4.5. HPLC analysis

Aqueous stock solutions of 5-HTP (124.00 µg/mL), 5-HT (40.00 µg/mL), 5-HIAA (110.00 µg/mL), Glu (182.40 µg/mL) and Asp (102.20 µg/mL) were prepared in 0.1 M HCl and aliquoted to brown volumetric flask. These solu-

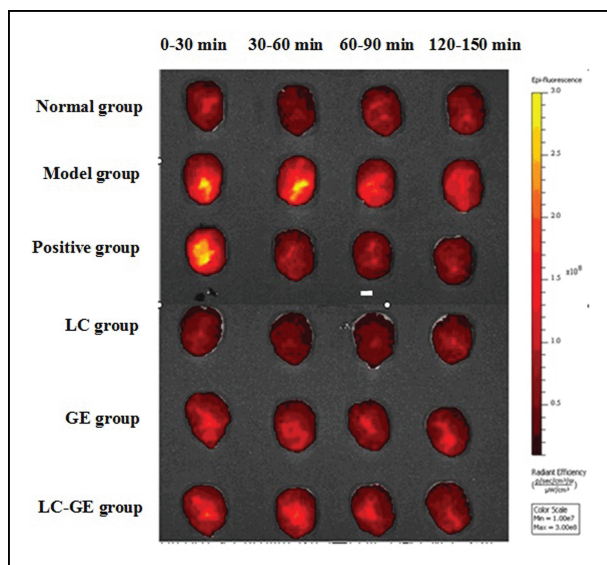


Fig. 3: Effect of pH value of borate buffer solution, DNS-Cl concentration, reaction temperature and reaction time on derivatization reaction.

tions were diluted daily to working concentrations with 0.1 M HCl. All solutions were stored at 4 °C in the dark.

HPLC analysis for 5-HTP, 5-HT and 5-HIAA were performed on an Agilent 1200 HPLC, equipped with fluorescence detector (Agilent technology, USA). The column used was ZORBAX SB-C₁₈, 5 μm, 4.6 × 250 mm (Agilent technology, USA). The mobile phase was composed of methanol: sodium acetate buffer (pH 5.1; 0.1 M) (8:92%, v/v) at flow rate 1 mL/min. Acetate buffer was prepared by dissolving 8.20 g sodium acetate, 33.62 mg sodium-EDTA in triple-distilled water and its pH 5.1 was adjusted with acetic acid. The column was heated to 25 °C and the serotonin was detected by its fluorescence at an emission wavelength of 330 nm and excitation at 290 nm. The retention times for 5-HTP, 5-HIAA, 5-HT were approximately 6.82 min, 11.18 min, 11.86 min. The limit of detection (LOD) and limit of quantitation (LOQ), defined as a signal/noise ratio ≥ 3 and ≥ 10 , were 0.93-8.53 ng/mL and 2.84-25.94 ng/mL, respectively. The linear regression equation for 5-HTP was $Y = 0.1999X - 7.4347$ with R of 0.9998, for 5-HT was $Y = 0.1518X - 11.707$ with R of 0.9996 and for 5-HIAA was $Y = 0.127X - 12.662$ with R of 0.9992, where Y was the peak area of 5-HTP, 5-HT and 5-HIAA, and X was the concentration of the corresponding substance. The accuracy and precision RSD were 95.12%-104.76% and 0.52%-4.88% across 0.10-1.50 μg/mL. The stability RSD and recoveries were 2.47-7.50 and 93.41%-107.12%.

The chromatographic system and the column for Glu and Asp were of the same composition as described above. The flow rate was 0.8 mL/min and the temperature of the column oven was maintained at 30 °C. The derivatized DNS-amino acids were detected by their fluorescence at an emission wavelength of 515 nm and excitation at 340 nm. (A) Acetonitrile and (B) ammonium acetate buffer (pH 3.5; 5 mM) were used for gradient elution, the ammonium acetate buffer was prepared by dissolving 0.39 g ammonium acetate, 0.96 g citric acid, 64.47 mg tetrabutyl ammonium bromide and 16.81 mg sodium-EDTA in 1L triple-distilled water and its pH 3.5 was adjusted with formic acid. The initial elution condition was A-B (20:80, v/v), then linearly changed to A-B (25:75, v/v) at 3 min, A-B (30:70, v/v) at 10 min, A-B (32:68, v/v) at 16 min, A-B (33:67, v/v) at 25 min, and A-B (20:80, v/v) at 28 min.

This reaction of EAAs with DNS-Cl was optimized by assaying the borate buffer pH (pH 6.0-12.0), the DNS-Cl concentration (0.5-12.0 mg/mL); temperature (20-80 °C); and reaction time (5-120 min) (Fig. 3), the optimal conditions for the derivatization reaction were: borate buffer (pH 9.0; 0.2 M), 8.0 mg/mL DNS-Cl, reaction temperature 30 °C and reaction time 80 min. The retention times for DNS-Glu, DNS-Asp were approximately 20.06 min, and 21.45 min. The LOD and LOQ were 103.50-119.07 ng/mL and 314.61-361.90 ng/mL, respectively. The linear regression equation for DNS-Glu was $Y = 3.9094X + 27.106$ with R of 0.9998 and for DNS-Asp was $Y = 3.5222X - 2.6123$ with R of 0.9999, where Y was the peak area of DNS-Glu and DNS-Asp, and X was the concentration of the corresponding substance. The accuracy and precision RSD were 96.19%-103.42% and 0.37%-3.31% across 1.25-480.00 μg/mL. The stability RSD and recoveries were 0.37%-2.27% and 97.85%-107.63%.

4.6. Data analysis

Data are expressed as means \pm SD. For the comparisons of different groups sampled at the same timeline, one-way analysis of variance (ANOVA) and further *LSD-t* multiple comparisons were employed via SPSS 15.0. A *p*-value below 0.05 or 0.01 was taken to indicate significant difference between data means.

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