Original Article
Therapeutic effects of acupuncture treatment on experimental rats with intracerebral hemorrhage via promoting neovascularization

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Abstract: This research was aimed to investigate the therapeutic effects of acupuncture treatment (APT) on experimental rats with intracerebral hemorrhage (ICH) and its potential mechanisms. An ICH animal model was established by infusion of autologous blood. Then, the local temperature, blood flow volume, nitric oxide (NO) and carbon monoxide (CO) contents in tissues surrounding hematoma were determined at 3 and 7 days after ICH model prepared. Furthermore, the mRNA and proteins expressions of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), angiogenin (ANG) I, tissue type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI) were determined by using real-time polymerase chain reaction analysis (RT-PCR) and immunohistochemical assays. Our results showed that APT increased the local temperature ($P<0.05$), blood flow volume ($P<0.05$), and contents of NO ($P<0.05$) and CO ($P<0.05$) at both day 3 and 7, compared with the control rats. In addition, from the RT-PCR and immunohistochemical assays, APT also up-regulated the mRNA and proteins expressions of VEGF ($P<0.05$), b-FGF ($P<0.05$), ANG I ($P<0.05$) and t-PA ($P<0.05$) in brain tissues surrounding hematoma whereas the PAI ($P<0.05$) down-regulated compared with normal rats at both 3 and 7 days. Collectively, our present results indicated that APT possesses notable therapeutic effects on experimental ICH rats via promoting neovascularization.

Keywords: Acupuncture treatment, intracerebral hemorrhage, neovascularization, molecular mechanism

Introduction

Intracerebral hemorrhage (ICH), the spontaneous non-traumatic bleeding induced by angiorrhexis in brain parenchyma, accounts for over 10-20% of all strokes with high rates of morbidity and mortality as well as poor prognosis [1, 2]. In recent years, although the mortality of ICH has significantly decreased with the great improvements of diagnosis and emergency treatments of acute phase of ICH, it has been reported that only 20% of ICH patients could be functionally independent due to hemiparesis, dementia and cognitive impairment caused by severe hemorrhagic brain injury [3-5]. It is therefore urgent to find out novel strategy for the long-term functional recover of ICH patients.

Acupuncture treatment (APT), also called *Zhenjiu* in Chinese, is an ancient and effective non-

pharmacological therapy, which is commonly used in conjunction with herbal remedies for treating various diseases and conditions [6, 7]. Increasing investigations have demonstrated that APT could be beneficial for the cure and functional recover of some chronic diseases, such as cervical spondylosis, hemiparesis, arthritis, tinnitus and chronic pains, *etc* [8-12]. In our hospital and other hospital in China, APT is commonly used for the long-term functional recover of ICH patients, and achieves good effects [13-16]. However, the experimental evidences of therapeutic effects of APT and its potential mechanisms are lacking. In our present study, we designed an experimental ICH rat model to evaluate the therapeutic effects of APT and explore its possible molecular mechanism, which have significant reference value for using APT to treat ICH related diseases in clinical.
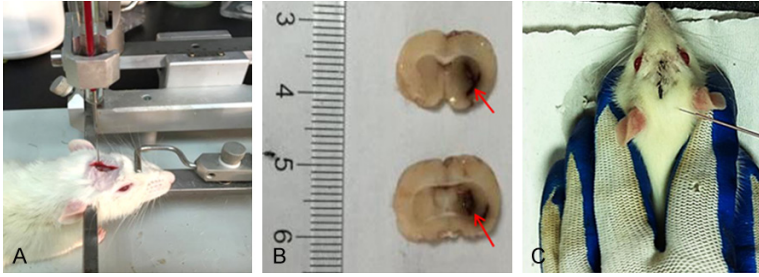
Figure 1. Preparation of the ICH rats and acupuncture treatment. A. Preparation of ICH rats; B. Hematoma in rats' brain (red arrow); C. Acupuncture treatment on ICH rats.

Materials and methods

Animals

Male Sprague Dawley (SD) rats (6-8 weeks old, 280±10 g) were purchased from the Animal Center of the Shanghai University of Traditional Chinese Medicine (Shanghai, China). All the animals were housed in humidity/temperature controlled house with 12 h light/dark cycle. All animal experimental protocols were prepared according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of the Shanghai University of Traditional Chinese Medicine.

Chemicals and reagents

Nitric oxide (NO) assay kit and carbon monoxide (CO) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); Trizol reagent was purchased from Gibco Biotech Co. (Grand Island, NY, USA); formaldehyde, hematoxylin and eosin (H&E), horseradish-peroxidase-conjugated (HRP) secondary antibody and chloral hydrate were purchased from the Shanghai Long Island Biotech (Shanghai, China); primary antibodies for vascular endothelial growth factor (VEGF) (cat. No. ab46154; dilution: 1:1000), tissue type plasminogen activator (t-PA) (Cat. No. ab157469; dilution: 1:2000) and plasminogen activator inhibitor (PAI) (Cat. No. ab66705; dilution: 1:400) were purchased from Abcam (Cambridge, MA, USA). Basic fibroblast growth factor (b-FGF) (Cat. No. 3196; dilution: 1:1000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Angiogenin (ANG) I (Cat. No. sc-6320; dilution: 1:200) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). RNA reverse transcription kit

and Real-time polymerase chain reaction analysis (PCR) SYBR green kit were purchased from Thermo Scientific (Waltham, MA, USA). Acupuncture needles were purchased from Suzhou Hwato Medical Instruments Co. (Suzhou, China). All other chemicals used in this study were of analytical reagent grade.

Preparation of ICH rats

ICH rats were prepared according to the reported method with minor modifications [17, 18]. Rats were anesthetized by intraperitoneal injection of 10% chloralhydrate (4 ml/kg, ip) and fixed in a stereotaxic frame (ALCOTT BIO-TECH Co., Shanghai, China). After sterilization, the calvaria skin was cut and skull was exposed, and subsequently a burr hole (1 mm diameter) was made at the leftward top of caudatum by using a skull drill (ALCOTT BIO-TECH Co., Shanghai, China). Then, a volume of 100 µl of autologous blood obtained from the arteria cruralis was infused into the leftward caudatum (coordinates relative to bregma: 0.1 mm posterior, 3.5 mm lateral to the left) at the speed of 10 µl/min by using a 100 µl microsyringe (ALCOTT BIOTECH Co., Shanghai, China). After the blood infusion, the microsyringe was held for another 9 min to prevent blood leakage. Finally, the opened burr hole was closed with bone wax and the skin incision closed by suture. For the rats in normal rats (sham ICH rats), a same surgery protocol was carried out with infusion of saline instead of autologous blood (**Figure 1A**).

After the surgery, an obvious hematoma could be observed in the rats' brain (**Figure 1B**); in addition, some indicates of neurological disorders among the prepared ICH rats were also found according to the previous reference [19], such as: right-fore limb extension disorder, right ring-shaped movement and even movement disorder, etc.

Experimental protocol

After the prepared ICH rats' awaking, a total of 48 ICH rats were selected and divided randomly into two groups (n=24): control group (ICH model control) and acupuncture treatment

Table 1. Primers used in the Real-time PCR assays

Genes	Primer sequences (5'-3')
VEGF	F: 5'-GAGTCTGTGCTCTGGGATTTG-3'
	R: 5'-TCCTGCTACCTCTTTCCTCTG-3'
b-FGF	F: 5'-TCTGTCTCCCGCACCCTATC-3'
	R: 5'-ACCAGCTTCCACCCAAAGC-3'
ANG I	F: 5'-CCATAACCTTGTCAGCCTTTG-3'
	R: 5'-TGTCCATCCTCCTTCATTCAC-3'
t-PA	F: 5'-GCCCTGACGGATTTGTTG-3'
	R: 5'-TCTCGGTCTGGGTTTCTG-3'
PAI	F: 5'-GCTTCTCATCCCACTCTC-3'
	R: 5'-GCTTCTCATCCCACTCTC-3'
GAPDH	F: 5'-GTCGGTGTGAACGGATTTG-3'
	R: 5'-TCCCATTCTCAGCCTTGAC-3'

F: Forward; R: Reverse.

(APT) group. In addition, another 12 sham ICH rats were used as the normal group. In APT group, "point-through-point method" was performed once a day (**Figure 1C**). Briefly, an acupuncture needle (0.25 mm, id) acupunctured from "*Baihui*acupoint" to the left "temple" with a needle retaining time of 30 min. During the needle retaining period, twirling needle manipulation technique was carried out one minute every 10 min with the frequency of 200 times/min.

Local temperature and blood flow volume in tissues surrounding hematoma of ICH rats were determined by using FLIR Therma CAM-TM P30 thermal infrared imager (FLIR Systems Inc, Wilsonville, AL, USA) and moorPLPI-2™ laser speckle detecting system (Moor Instruments, MillweyAxminster, Devon, UK) at the 3 and 7 days after surgery, respectively. In addition, 8 rats were selected and sacrificed by cervical dislocation under anesthesia by 10% chloralhydrate (4 ml/kg, ip) at the 3 and 7 days after surgery respectively, and the brains were harvested for the following immunohistochemical and biochemical assays.

Determination of the NO and CO contents

The brain tissues surrounding hematoma (0.1 g) was weighed and homogenized at 4°C, 2000 rpm for 20 min. Subsequently, the contents of NO and CO were determined by using commercial kits according to the manufactures' instructions with colorimetric method.

Determination of quantitative mRNA expressions by real-time PCR

Total RNA of the brain tissues surrounding hematoma was extracted by Trizol reagents. Then, RNA (2 µg) of each sample was used to synthesize the cDNA by using a RNA reverse transcription kit. All primers were designed by Primer 5.0 software and described in **Table 1**, and cDNAs were amplified using PCR SYBR green kit by using an ABI-7500 Real time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA) and the amplification condition was conducted as following: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, and then 72°C for 5 min. Amplification products were analyzed and quantitatively determined by ABI Prism 7300 SDS Software (Applied Biosystems, Inc., Carlsbad, CA, USA).

Immunohistochemical assays

The brain tissues were fixed by formaldehyde and the sections (4 µm) were successively prepared according to a normal and standard protocol [20]. Then, the tissues sections were respectively incubated with primary antibodies of VEGF, b-FGF, ANG I, t-PA and PAI, and subsequently incubated with HPR secondary antibody for immunohistochemical examinations.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The statistical significances of differences between groups were evaluated by using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance (ANOVA) followed by LSD-t test. P value less than 0.05 was recognized as statistically significant.
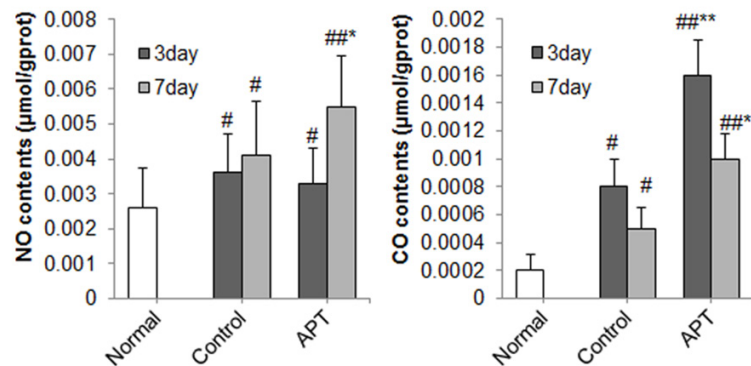
Results

APT increased the local temperature and blood flow volume in brain tissues surrounding hematoma

After infusion of autologous blood, the local temperature ($P<0.05$, $P<0.05$) and blood flow volume ($P<0.05$, $P<0.05$) in brain tissues surrounding hematoma significantly decreased at both day 3 and 7, compared with the normal rats (**Figure 2**). Furthermore, APT could obviously increase the local temperature ($P<0.05$,
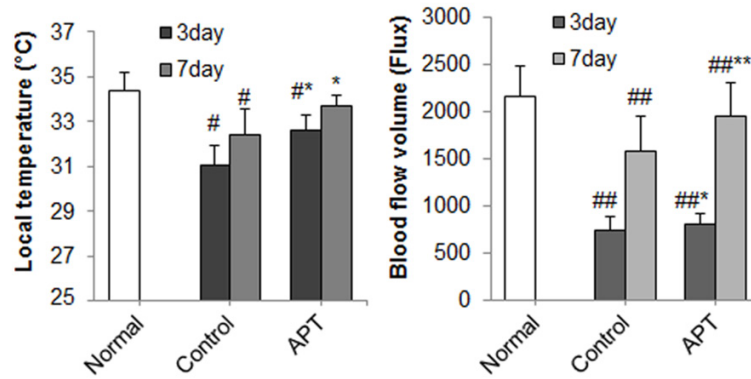
Figure 2. Effects of APT on the local temperature and blood flow volume in brain tissues surrounding hematoma of ICH rats. APT: acupuncture treatment; Data were expressed as Mean ± SD (n=8), #$P<0.05$, ##$P<0.01$, compared with normal rats; *$P<0.05$, **$P<0.01$, compared with control rats.

Figure 3. Effects of APT on the endogenous gaseous signal molecules in brain tissues surrounding hematoma of ICH rats. APT: acupuncture treatment; Data were expressed as Mean ± SD (n=8), #$P<0.05$, ##$P<0.01$, compared with normal rats; *$P<0.05$, **$P<0.01$, compared with control rats.

$P<0.05$) and blood flow volume ($P<0.05$, $P<0.01$) at both day 3 and 7, compared with the control rats. Importantly, for the local temperature and blood flow volume, no obvious difference was observed between the normal group and APT group at the 7 days after surgery ($P>0.05$).

APT increased endogenous gaseous signal molecules in brain tissues surrounding hematoma

Compared with normal rats, the control rats possessed higher levels of NO contents at both the 3 ($P<0.05$) and 7 days ($P<0.05$) after surgery. In addition, APT could further increase the NO contents compared with the control rats at 7 days ($P<0.05$) after surgery. Similarly, the CO contents were also increased in control rats compared with normal rats, and

the APT could significantly increase the CO contents compared with both the normal and control rats at the 3 ($P<0.01$, both) and 7 days ($P<0.01$, both) after surgery (**Figure 3**).
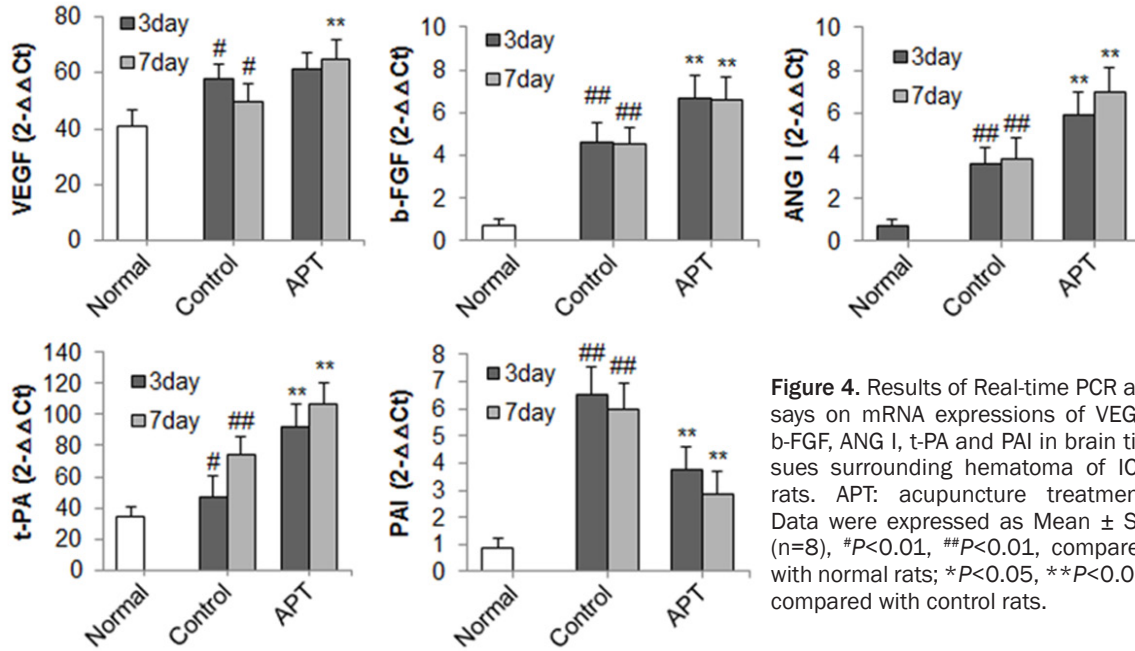
APT increased mRAN expressions of VEGF, b-FGF, ANG I and t-PA, and whereas decreased PAI in brain tissues surrounding hematoma

The mRNA expressions of VEGF, b-FGF, ANG I and t-PA, PAI in brain tissues surrounding hematoma of ICH rats were showed in **Figure 4**. After surgery, the mRNA expressions of VEGF ($P<0.05$, both), b-FGF ($P<0.01$, both), ANG I ($P<0.01$, both) and t-PA ($P<0.05$, $P<0.01$) were significantly up-regulated whereas the PAI ($P<0.01$, both) obviously down-regulated compared with normal rats at both 3 and 7 days. Interestingly, by treatment with APT, mRNA expressions of VEGF ($P<0.01$ at 7 day), b-FGF ($P<0.01$, both), ANG I ($P<0.01$, both) and t-PA ($P<0.01$, both) could be further up-regulated as well as the PAI ($P<0.01$, both) could be further down-regulated compared with normal rats at both 3 and 7 days.

APT increased proteins expressions of VEGF, b-FGF, ANG I and t-PA, and whereas decreased PAI in brain tissues surrounding hematoma

The immunohistochemical assays results were described in **Figure 5** and **Table 2**. Similar to the mRNA expressions assay results, after the ICH rats model was prepared, expressions of VEGF ($P<0.05$, $P<0.01$), b-FGF ($P<0.05$, $P<0.05$), ANG I ($P<0.05$, $P<0.05$) and t-PA ($P<0.05$, $P<0.01$) significantly increased whereas the PAI ($P<0.01$, $P<0.05$) decreased compared with normal rats at both 3 and 7 days. Interestingly, by APT treatment, VEGF ($P<0.05$, both), b-FGF ($P<0.05$, both), ANG I ($P<0.05$, both) and t-PA ($P<0.05$, $P<0.01$) could be further up-regulated as well as the PAI ($P<0.05$,

Figure 4. Results of Real-time PCR assays on mRNA expressions of VEGF, b-FGF, ANG I, t-PA and PAI in brain tissues surrounding hematoma of ICH rats. APT: acupuncture treatment; Data were expressed as Mean ± SD (n=8), #P<0.01, ##P<0.01, compared with normal rats; *P<0.05, **P<0.01, compared with control rats.
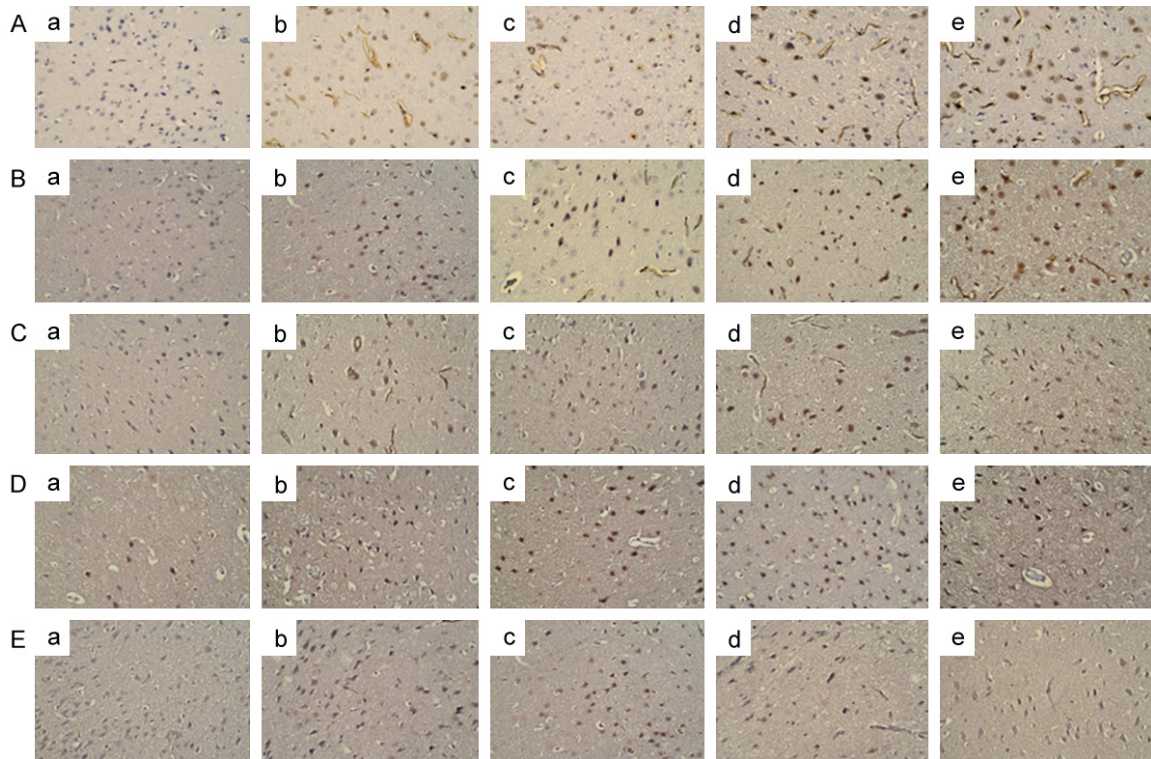
Figure 5. Results of immunohistochemical assays on expressions of VEGF, b-FGF, ANG I, t-PA and PAI in brain tissues surrounding hematoma of ICH rats. (×200, brown means the protein expressions). APT: acupuncture treatment; A-E. Represented the rats in groups of normal, model (3 days and 7 days) and APT (3 days and 7 days), respectively.

both) could be further down-regulated compared with normal rats at both 3 and 7 days. Importantly, for the PAI, no obvious difference was observed between the normal group and APT group at the 3 and 7 days after surgery (P>0.05, P>0.05).

Table 2. Effects of APT on angiogenic proteins in brain tissues surrounding hematoma of ICH rats

	VEGF (µm²)		b-FGF (µm²)	
	3 d	7 d	3 d	7 d
Normal	657.88 ± 57.23		711.00 ± 16.16	
Control	710.00 ± 52.54[#]	756.13 ± 27.79[##]	732.25 ± 16.71[#]	745.75 ± 17.85[#]
APT	772.50 ± 33.62[##,*]	816.00 ± 51.32[##,*]	757.75 ± 19.47[##,*]	773.63 ± 17.10[##,*]
	ANG I (µm²)		t-PA (µm²)	
	3 d	7 d	3 d	7 d
Normal	751.88 ± 19.25		710.25 ± 18.23	
Control	789.88 ± 16.06[#]	823.13 ± 18.62[#]	740.25 ± 18.23[#]	766.00 ± 16.48[#]
APT	817.63 ± 17.52[#,*]	846.88 ± 12.84[#,*]	772.25 ± 18.55[##,*]	804.63 ± 15.25[##,**]
	PAI (µm²)			
	3 d	7 d		
Normal	753.25 ± 17.16			
Control	832.25 ± 18.82[##]	803.63 ± 11.49[#]		
745.25	780.62 ± 15.26[*]	767.38 ± 16.04[*]		

APT: Acupuncture treatment; Data were expressed as Mean ± SD (n=8), [#]$P<0.01$, [##]$P<0.01$, compared with normal rats; [*]$P<0.05$, [**]$P<0.01$, compared with control rats.

Discussion

Currently, intracerebral hemorrhage (ICH), could result in devastating damage to brain, remains one of the leading causes of death and disability worldwide for which there is still no effective therapeutic strategy [21-23]. Complementary and alternative medicine, such as acupuncture treatment, is widely applied for treating chronic cerebro-/cardiovascular diseases [12, 24]. In our present investigation, we investigated the therapeutic activity of acupuncture on experimental ICH rats, and explored its potential molecular mechanisms for the first time.

Previous researches demonstrated that ICH commonly causes primary and secondary brain injury [24]. The primary brain injury is mainly about hematoma expansion and the consequent increase in intracranial pressure after ICH which could be alleviated by early emergency treatments, including surgical hematoma treatment, reduction of hypertension, hemostatic treatment and reversal of coagulopathies, etc [25, 26]. However, the subsequent secondary injuries, induced by tissue ischemia, disturbance of blood circulation, cell apoptosis and inflammatory responses, could result in severe neurologic deterioration and cognitive impairments, leading to functional disorders and disability [25-28]. In our represent results, acupuncture treatment could enhance the local temperature and blood flow volume in brain tissues surrounding hematoma, suggesting that acupuncture treatment could be beneficial for alleviating hematoma edema and increasing blood circulation.

Moderate endogenous gaseous signal molecules such as NO and CO could increase the expressions of VEGF, relaxation of blood vessel, inhibition of platelet aggregation [27, 29]. The genes of VEGF, b-FGF and ANG I are three crucial factors for the neo-vascularization. VEGF gene is reported to be the most powerful vascular growth factor so far, and could promote the proliferation of endothelial cells, accelerating the neovascularization [30, 31]. Similar to the VEGF, ANG I is another promoting factor for neo-vascularization, sustaining the vascular integrity [32]. The b-FGF, a neurotrophic factor possesses various pharmacological effects including promoting neovascularization, regulating local blood flow volume, etc [33]. In our results, the acupuncture treatment up-regulated all these 3 genes mentioned above, suggesting that acupuncture treatment could promoting the neovascularization and local blood flow volume in brain tissues surrounding the hematoma of ICH rats. All these results suggested that acupuncture treatment could increase blood circulation and promote the growth and development of new vessels. Fibrinolysis mediated by vascular endothelial cells mainly relate to the release and synthetize of t-PA and PAI. The t-PA could active the profibri-

nolysin to fibrinolysin and could dissociate fibrous proteins, clearing the hematoma. In contrary, PAI could specific inhibition of the t-PA, resulting in the inactivation of t-PA [34, 35]. Our results revealed that acupuncture treatment could up-regulate the t-PA whereas down-regulate the PAI, suggesting that acupuncture treatment could active the fibrinolysis process and inhibit blood coagulation.

In conclusion, our present results indicated that acupuncture treatment possesses notable therapeutic effects on experimental rats with intracerebral hemorrhage via promoting neovascularization.

Acknowledgements

Disclosure of conflict of interest

None.

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