

## THE EFFECTS AND POTENTIAL MOLECULAR MECHANISMS OF ACUPUNCTURE COMBINED WITH HYPERBARIC OXYGEN IN THE TREATMENT OF DYSPHAGIA AFTER STROKE

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### ABSTRACT

**Objective:** Dysphagia is common after stroke and in relation to increased risk of prolonged hospital stay, poor clinical prognosis, and increased mortality. This study aimed to observe the therapeutic effects and molecular mechanisms of acupuncture combined with hyperbaric oxygen on dysphagia after stroke.

**Methods:** Dysphagia patients after stroke were randomly divided into control, acupuncture and acupuncture combined with hyperbaric oxygen groups (n=30 each group). After 4 weeks of treatment, swallowing function was assessed by video fluorography (VFG), swallowing-related quality of life (SWAL-QOL) scale and Watian drinking water tests. The rat models of dysphagia after stroke were constructed. Thirty rats were separated into sham operation, model, acupuncture, hyperbaric oxygen, acupuncture combined with hyperbaric oxygen groups (n=6 each group). After treatment for 8 and 16 days, the number of swallows and swallowing response time were examined. Tryptophan hydroxylase (TPH) in brain homogenate and serum substance P (SP) levels were detected via ELISA. After 16 days, cerebral infarct volume was detected via TTC staining and the expression of 5-HT and its subtypes 5-HT1A and 5-HT3 was detected by immunohistochemistry and western blot.

**Results:** Acupuncture combined with hyperbaric oxygen treatment significantly increased VFG scores and decreased SWAL-QOL scores for dysphagia patients. Furthermore, total effective rate was up to 93.3% in combination treatment group. This combination treatment distinctly increased the number of swallows and lowered swallowing response time for model rats on the 8th and 16th day. The lower infarct volume and higher TPH and SP levels were found in combination group. Also, the combination treatment elevated the expression of 5-HT, 5-HT1A and 5-HT3 in the nucleus of the solitary tract of model rats.

**Conclusion:** Our data demonstrated that acupuncture combined with hyperbaric oxygen exhibited a good therapeutic effect on dysphagia after stroke, which may be related to the activation of 5-HT pathway.

**Keywords:** stroke, dysphagia, acupuncture, hyperbaric oxygen, 5-HT.

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### Introduction

Dysphagia is a common comorbidity of stroke<sup>(1-3)</sup>, which can cause severe complications such as malnutrition, skin pressure sores, aspiration pneumonia, and disturbance of consciousness, thereby seriously affecting and damaging patients' lives and quality of life, even life-threatening<sup>(4-6)</sup>. The incidence of dysphagia exceeds 50%<sup>(7)</sup>. It seriously affects the nutritional intake, and disease recovery of stroke patients<sup>(8)</sup>. Therefore, it is very important to find new treatment methods.

At present, there are three main types of treatment methods for dysphagia, as follows: (1) compensatory strategy may change the way or direction of food passing through the mouth and pharynx, thereby reducing the symptoms of dysphagia<sup>(9)</sup>; (2) for indirect strategy, the patient does not really eat during training, but through similar exercise training to improve the control ability of the neuromuscular related to swallowing; (3) for direct strategy, during training, the patient is guided to directly achieve swallowing actions to improve the pathophysiological condition of

swallowing<sup>(10-12)</sup>. With the continuous exploration of treatment methods, acupuncture has been used in clinical treatment because of its high therapeutic effect, safe operation, and economical simplicity<sup>(13-15)</sup>. A meta-analysis study found that acupuncture combined with other intervention strategies exhibited higher therapeutic efficacy<sup>(16)</sup>. However, stricter evaluation standards should be required for the treatment strategies. Furthermore, its mechanism of action is still unclear, which may limit the clinical application<sup>(17)</sup>. This study found that acupuncture combined with hyperbaric oxygen could efficiently improve swallowing function of dysphagia patients. Our further analysis indicated that the well clinical efficacy was related to the activation of 5-HT pathway. Therefore, acupuncture combined with hyperbaric oxygen strategy was simple, safe and practical, which might be worthy of clinical promotion.

## Materials and methods

### *Study populations*

Patients with dysphagia after stroke were recruited from Jinhua Central Hospital, Zhejiang Province between January 2020 to January 2021 as subjects.

*The inclusion criteria were as follows:* (1) age  $\geq 18$  years; (2) stable vital signs, Abbreviated Mental Test (AMT)  $\geq 8$  points; (3) acute ischemic stroke confirmed by CT or MRI; (4) within 1 week of onset.

*The exclusion criteria were as follows:* (1) deaf-mute or sensory aphasia; (2) coexisting severe heart, lung, kidney insufficiency or cancer; (3) other diseases leading to impaired swallowing function. The swallowing function assessment was completed by doctors, rehabilitation technicians, and stroke rehabilitation specialist nurses within 48 hours of the patient's admission. Preliminary functions of patients including oral organs (lips, cheeks, jaws, tongue, soft palate, and throat) were assessed. Thereafter, through repeated saliva swallowing tests, the basic situation of swallowing function was evaluated to confirm whether the patient could safely proceed to the next assessment. Those who had completed the above assessment would be further assessed by rehabilitation technicians and stroke rehabilitation specialist nurses for dysphagia. Finally, a total of 90 patients were included in this study. This study was approved by the Ethics Committee of Jinhua Central Hospital, Zhejiang Province (2020011). Each patient provided written informed consent.

### *Procedures*

With the random number table method, the patients were divided into three groups, including control group, acupuncture group and acupuncture combined with hyperbaric oxygen group, 30 cases per group. Patients in the control group received conventional drug treatment, physical exercise training and swallowing training. For acupuncture group, the acupuncture points were firstly determined, including Fengchi (double), Lianquan, Jialianquan (double), Bailao (double), Jinjin, and Yuye. The specific acupuncture method was as follows: at the Lianquan and Jialianquan points, a 3-inch needle was used to pierce the tongue root direction, 1-1.5 inch deep, and lightly twisted 3-5 times; at Bailao point, 2-inch needle was used to pierce 1 inch deep with acupuncture needles to fill and relieve 3-5 times. The above steps of acupuncture needed to be connected to the electricity for 25 minutes, and the 6805A pulse electro-acupuncture treatment instrument was applied. At Fengchi acupoint, a 2-inch needle with the tip of the needle was used to point to the direction of the nose to pierce 1 inch, so that the needle feels sore and swollen. It last for 25 minutes with acupuncture needles to fill and relieve 1 times / 10 minutes. At the Jinjin and Yuye points, a 1.5-inch needle was utilized to pierce 0.5 inch into the meridian of the tongue frenulum. After getting the qi, it twisted 3-5 times to remove it. Above procedures were performed for 1 time / day, 6 times / week, for a total of 4 weeks. For the acupuncture combined with hyperbaric oxygen group, after acupuncture, a multi-person hyperbaric oxygen chamber was selected with the pressure as 0.22 MPa and the treatment time as 120 minutes. The specific time allocation was as follows: the patients wore a mask and inhaled pure oxygen for 60 minutes, inhaling 3 times, 20 minutes per time. Then, the patients inhaled mixed oxygen at intervals of 10 minutes, 20 minutes in total between the two sessions, followed by pressurization for 20 minutes and depressurization for 20 minutes. Above procedures were performed for 1 time / day, 1 course of treatment for 10 days, 3 courses in total.

### *Outcome measures*

Video fluorography (VFG) method was used to evaluate the swallowing function of patients before and after treatment. According to the patients' swallowing contrast agent, 1-10 points were given according to the relevant standards. The swallowing-related quality of life (SWAL-QOL) scale was used to compare the quality-of-life scores of patients

before and after treatment. The scale had a total of 44 items, composed of 11 dimensions. Each dimension was divided into 5 levels, with a score of 0-100. The quality of life was inversely proportional to the score. The scores of patients in each group were compared before and after treatment. Watian drinking water test was applied for assessing clinical curative effect. The patients took the seated position, and were asked to drink 30 mL of warm water. Then, the time required and the situation of coughing were observed.

*The classification was as follows:* level 1: swallow smoothly once; level 2: swallow more than 2 times; level 3: swallow 1 time but cough; level 4: swallow more than 2 times but cough; level 5: uncomfortably swallow the whole amount, and frequently cough.

*After the dysphagia disappeared, the drinking test was evaluated as follows:* grade 1: cured; grade 2: dysphagia improved significantly and effective; grade 3: dysphagia improved, effective; grade 4: dysphagia improved insignificantly, invalid. Total effective rate = (cure + significantly effective + effective) × 100% / total number of cases.

#### ***Construction of an animal model of dysphagia after stroke***

This animal experiment was approved by the Ethics Committee of Jinhua Central Hospital, Zhejiang Province (2020011). Thirty Sprague Dawley (SD) male rats were randomly divided into five groups: sham operation group, model group, acupuncture group, hyperbaric oxygen group, acupuncture combined with hyperbaric oxygen group, 6 rats in each group. SD male rats were adaptively fed for one week. The day before the model-making operation, the rats were fasted with water for 12 hours. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital. After anesthesia, the vascular clamp was used to clamp the rat's paw. When the rat's paw did not respond, it was fixed on the rat board. The rat's neck was prepared and disinfected by iodophor. A longitudinal incision with approximately 1.5 cm along the midline of the neck was made. By bluntly separating the intermuscular space between the sternocleidomastoid and sternohyoid muscles, the common carotid artery (CCA) was exposed. The common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were separated in sequence, and the proximal ends of ECA and CCA were ligated.

Furthermore, in the CCA close to the ICA, the slip knot was reserved at the bifurcation of the ECA and was clamped by the ECA. The 2 mL syringe needle was used to assist the MCAO thread plug, which was inserted into the CCA. After the needle was pulled out, the middle cerebral artery occlusion (MCAO) thread plug was continued to be inserted. The ICA arterial clamp was loosened and the MCAO thread was tethered at the bifurcation and entered the ICA about 18-20 mm. The CCA was ligated at the reserved slipknot at the distal end to fix the wire bolt. The incision was sutured layer by layer. The end of the MCAO thread plug left outside was blackened with an oil-based pen. Each rat was given intraperitoneal injection of penicillin. The rat was placed in a lateral decubitus position and placed on an insulation blanket. After 90 minutes, the MCAO thread bolt was pulled out for about 1 cm, and the pulled part of the thread bolt was cut off. In the sham operation group, the operation process was the same as that of the model group except that MCAO thread plug was not inserted. The acupuncture group, hyperoxia group, and combination group were given treatment on the second day after modeling lasting 16 days (one time / each day).

#### ***Detection of swallowing times and swallowing response time***

On the 8th and 16th day of treatment, the swallowing times and swallowing response time were measured for rats in each group. After intraperitoneal injection of sodium pentobarbital for anesthesia, an incision of about 1 cm was cut along the midline of the neck. The anterior abdomen of the digastric muscle was exposed. One end of the tension converter was connected to the anterior abdomen of the digastric muscle, and the other end was connected to the biosignal converter. A 0.5mm diameter hose was inserted through the mouth of the rat to reach the base of the tongue. A micro syringe pump was used to inject distilled water at a rate of 3 nL/s for 30 seconds. This operation was repeated 3 times after stopping for 1 minutes. The number of swallows and the time from the distilled water stimulation to the first swallowing were recorded by the biological signal acquisition system.

#### ***TTC staining***

The brain sections were incubated with 3% TTC solution at 37 °C in the dark for 15 minutes. Subsequently, the sections were immersed in a 4% paraformaldehyde solution for 24 hours.

The infarct volume of each brain slice was counted with Image-Pro Plus 6.01 image analysis software.

#### **Enzyme-linked immunosorbent assay (ELISA)**

On the 8th and 16th day after treatment, serum samples were prepared from each rat. Following the instructions of ELISA kit (CSB-E08358r; CUSABIO, Wuhan, China), five standards with concentrations of 100 pg/ml, 50 pg/ml, 25 pg/ml, 12.5 pg/ml and 6.25 pg/ml were prepared. The sample wells, standard wells and blank wells were set on the microtiter plate. 50  $\mu$ L different concentrations of standards were added to the standard wells. 10  $\mu$ L samples to be tested were added to the sample wells, followed by 40  $\mu$ L sample diluent. No liquid was added to the blank hole. ELISA was presented to detect serum substance P (SP) levels. Tryptophan hydroxylase (TPH; CSB-E13984r; CUSABIO, Wuhan, China) was detected in brain homogenate samples on the 8th and 16th day after treatment by corresponding ELISA kits.

#### **Western blot**

Brain homogenate samples were lysed by RIPA lysis buffer (P0013B; Beyotime, Shanghai, China). Protein concentration was assessed via BCA kit (P0009; Beyotime, Shanghai, China). Then, protein samples were separated via SDS-PAGE electrophoresis and transferred onto PVDF membrane. The membrane was blocked by 5% milk/TBST for 1 hour and incubated with primary antibodies against 5-HT1A (1:1000; AF0482; AFFINITY, Shanghai, China), 5-HT3 (1:1000; DF3502; AFFINITY, Shanghai, China) and GAPDH (1:5000; ATA00013Rb; ATAGENIX, Wuhan, China) at 4 °C overnight. Afterwards, the membrane was incubated with secondary antibody (1:5000; SA00001-2; Proteintech, Wuhan, China). ECL reagent was applied to develop. Protein bands were observed by gel imaging system.

#### **Immunohistochemistry**

On the 8th and 16th day after treatment, paraffin sections of the medulla oblongata were prepared. Immunohistochemistry assay was performed according to the operating method of the kit (KIHC-5; Proteintech, Wuhan, China). The sections were incubated with 5-HT antibodies (1:100; bs-1126R; Bioss, Beijing, China) at 4 °C overnight, followed by secondary antibody incubation.

#### **Statistical analysis**

Statistical analysis was presented via SPSS and Graphpad Prism software. The data of normal distribution were expressed as mean  $\pm$  standard deviation. Student's t-test and one-way analysis of variance were used to compare the groups. The count data were tested with  $\chi^2$  test.  $P < 0.05$  indicated that the difference was statistically significant.

#### **Results**

##### **Acupuncture combined with hyperbaric oxygen ameliorates dysphagia of stroke patients**

In this study, 90 patients with dysphagia after stroke were randomly divided into control group, acupuncture group and acupuncture combined with hyperbaric oxygen group ( $n=30$  each group). Before and after treatment for 4 weeks, we compared the therapeutic effects on swallowing functions for patients in each group. Compared with before treatment, VFG scores were all significantly increased after treatment with conventional treatment ( $3.13 \pm 1.07$  vs  $4.93 \pm 1.26$ ;  $p < 0.01$ ), acupuncture ( $3.17 \pm 1.15$  vs  $5.47 \pm 1.50$ ;  $p < 0.01$ ) or acupuncture combined with hyperbaric oxygen ( $3.20 \pm 1.13$  vs  $6.83 \pm 1.72$ ;  $p < 0.01$ ) for 4 weeks, demonstrating that all three treatments can improve dysphagia of patients (Table 1). Among the three groups, patients in acupuncture combined with hyperbaric oxygen group had the highest VFG scores after treatment for 4 weeks ( $p < 0.01$ ). Above data indicated that acupuncture combined with hyperbaric oxygen could significantly improve the swallowing function of patients.

Groups	n	VFG scores		t	P
		Before treatment	After treatment		
Control group	30	3.13 $\pm$ 1.07	4.93 $\pm$ 1.26	-8.115	<0.01
Acupuncture group	30	3.17 $\pm$ 1.15	5.47 $\pm$ 1.50	-15.315	<0.01
Acupuncture combined with hyperbaric oxygen group	30	3.20 $\pm$ 1.13	6.83 $\pm$ 1.72	-13.762	<0.01
F		0.027	12.691		
P		0.974	<0.01		

**Table 1:** VFG scores of patients with dysphagia after stroke before and after treatment with routine, acupuncture or acupuncture combined with hyperbaric oxygen.

##### **Acupuncture combined with hyperbaric oxygen improves swallowing quality of life for patients with dysphagia**

Swallowing quality of life was evaluated for patients in each group by applying SWAL-QOL scales. In comparison to before treatment, SWAL-QOL scores were all significantly decreased after routine treatment ( $859.17 \pm 47.56$  vs

738.33±56.76,  $p < 0.01$ ), acupuncture (858.33±48.84 vs 699.17±53.53,  $p < 0.01$ ) and acupuncture combined with hyperbaric oxygen (862.50±50.32 vs 640.00±54.77,  $p < 0.01$ ; Table 2). Thus, above treatments all improved swallowing quality of life for patients. But acupuncture combined with hyperbaric oxygen group had the lowest SWAL-QOL scores after treatment, indicating that this treatment strategy had the best performance on improving patients' swallowing quality of life.

Groups	n	SWAL-QOL scores		t	P
		Before treatment	After treatment		
Control group	30	859.17±47.56	738.33±56.76	13.005	<0.01
Acupuncture group	30	858.33±48.84	699.17±53.53	21.132	<0.01
Acupuncture combined with hyperbaric oxygen group	30	862.50±50.32	640.00±54.77	17.283	<0.01
F		0.061	24.273		
P		0.941	<0.01		

**Table 2:** SWAL-QOL scores of patients with dysphagia after stroke before and after treatment with routine, acupuncture or acupuncture combined with hyperbaric oxygen.

**Acupuncture combined with hyperbaric oxygen effectively cures dysphagia of stroke patients**

After treatment for 4 weeks, we evaluated the clinical efficacy of patients through the Watian drinking water test. Total effective rates were separately 60%, 83.3% and 93.3% for control group, acupuncture group and acupuncture combined with hyperbaric oxygen group (Table 3). Acupuncture combined with hyperbaric oxygen treatment exhibited the highest total effective rate among them ( $p < 0.01$ ).

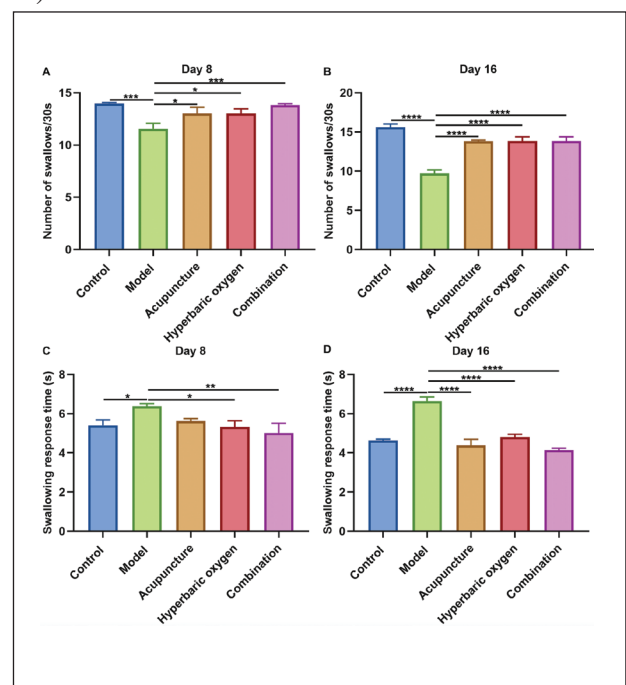
Groups	n	Recovery/cases	Significantly effective/cases	Effective/cases	Ineffective/cases	Total effective rate/%
Control group	30	0	9	9	12	60
Acupuncture group	30	6	10	9	5	83.3
Acupuncture combined with hyperbaric oxygen group	30	13	12	3	2	93.3
$\chi^2$						25.564
P						<0.01

**Table 3:** Assessment of clinical efficacy for patients with dysphagia after stroke after treatment with routine treatment, acupuncture or acupuncture combined with hyperbaric oxygen.

**Acupuncture combined with hyperbaric oxygen ameliorates dysphagia of model rats**

Post-stroke dysphagia models were established in this study. Following treatment with acupuncture,

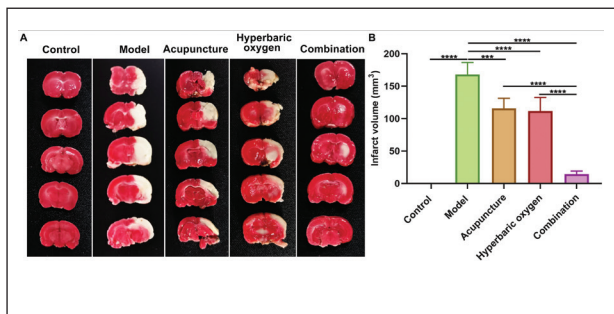
hyperbaric oxygen or acupuncture combined with hyperbaric oxygen for 8 and 16 days, the number of swallows and swallowing response time were examined in each group, respectively. Compared to sham operation control group, the number of swallows was significantly lowered in the model group ( $p < 0.001$  and  $p < 0.0001$ ; Fig. 1A, B). The number of swallows of model rats was significantly increased after treatment with acupuncture ( $p < 0.05$  and  $p < 0.0001$ ), hyperbaric oxygen ( $p < 0.05$  and  $p < 0.0001$ ) or acupuncture combined with hyperbaric oxygen ( $p < 0.001$  and  $p < 0.0001$ ) for 8 and 16 days (Fig. 1A, B). The swallowing response time of model rats was significantly longer than controls ( $p < 0.05$  and  $p < 0.0001$ ; Fig. 1C, D). Hyperbaric oxygen ( $p < 0.05$ ) and acupuncture combined with hyperbaric oxygen ( $p < 0.01$ ) significantly shortened the swallowing response time of model rats on the 8th day (Fig. 1C, D). On the 16th day of treatment, the swallowing response time was significantly cut down by above three treatment strategies (Fig. 1C, D).



**Figure 1:** The effects of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen treatment on the number of swallows and swallowing response time in dysphagia model rats after stroke. (A, B) Assessment of the number of swallows in dysphagia model rats with acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen treatment on the 8th and 16th day. (C, D) evaluation of the swallowing response time in dysphagia model rats with acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen treatment on the 8<sup>th</sup> and 16<sup>th</sup> day. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .

### Acupuncture combined with hyperbaric oxygen weakens cerebral infarction volume of model rats

After treatment for 16 days, cerebral infarction volume was assessed by TTC staining. As a result, acupuncture ( $p < 0.001$ ), hyperbaric oxygen ( $p < 0.0001$ ) and acupuncture combined with hyperbaric oxygen ( $p < 0.0001$ ) treatment all significantly decreased cerebral infarction volume of model rats (Fig. 2A, B). Furthermore, the lowest infarction volume was found in acupuncture combined with hyperbaric oxygen group.

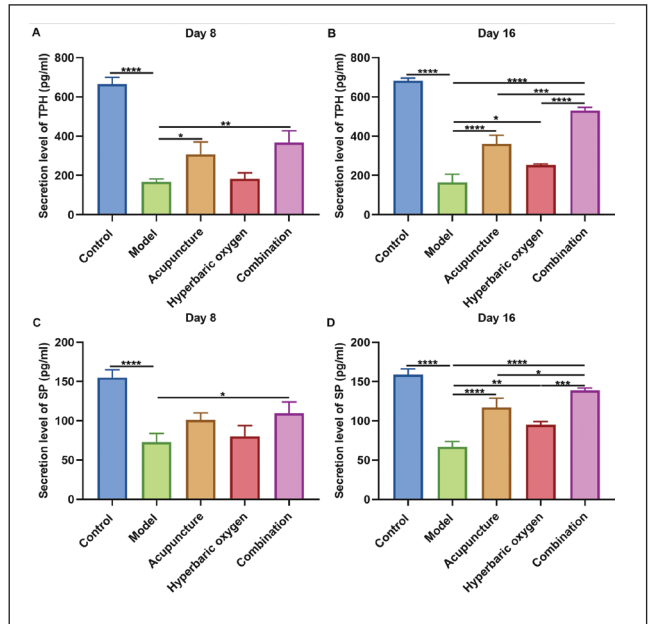


**Figure 2:** The effects of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen treatment on cerebral infarction volume in dysphagia model rats after stroke. (A, B) TTC staining for quantifying the infarction volume for each group. \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .

### Acupuncture combined with hyperbaric oxygen increases TPH and SP levels in model rats

Following treatment for 8 and 16 days, TPH levels in brain homogenate were detected in each group by ELISA. Compared to the control group, there were lower TPH levels in brain homogenate of model rats ( $p < 0.0001$ ; Fig. 3A, B). On the 8<sup>th</sup> day of treatment, acupuncture ( $p < 0.05$ ) and acupuncture combined with hyperbaric oxygen ( $p < 0.01$ ) significantly increased TPH levels of model rats. After 16 days, TPH levels of model rats were all significantly increased by the three treatment methods (all  $p < 0.0001$ ). Among them, acupuncture combined with hyperbaric oxygen group had the highest TPH levels in brain homogenate. Furthermore, we found that serum SP levels of model rats were distinctly decreased than controls ( $p < 0.0001$ ; Fig. 3C, D). On the 8<sup>th</sup> day of treatment, only acupuncture combined with hyperbaric oxygen increased serum SP levels of model rats ( $p < 0.05$ ). After treatment for 16 days, compared to the model group, higher SP levels were detected in the acupuncture ( $p < 0.0001$ ), hyperbaric oxygen ( $p < 0.01$ ) and acupuncture combined with hyperbaric oxygen groups ( $p < 0.0001$ ; Fig. 3C, D).

Among the three groups, acupuncture combined with hyperbaric oxygen group had the highest serum SP levels.

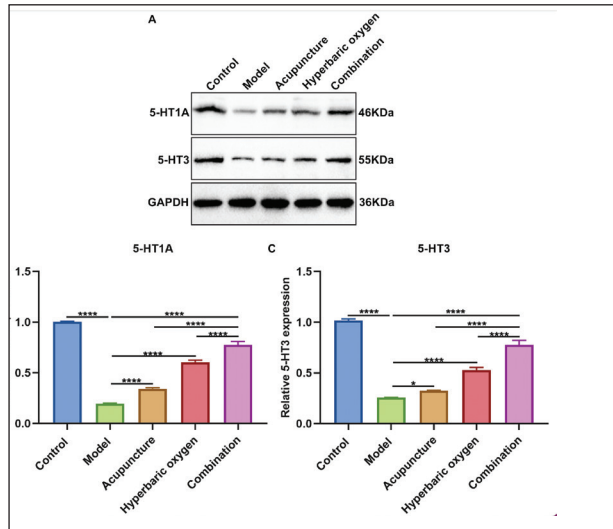


**Figure 3:** The effects of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen on the levels of TPH and SP of dysphagia model rats after stroke. (A, B) ELISA for assessing the levels of TPH in brain homogenate of dysphagia model rats after treatment of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen for 8 and 16 days. (C, D) ELISA for examining the serum SP levels in dysphagia model rats after treatment of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen for 8 and 16 days. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

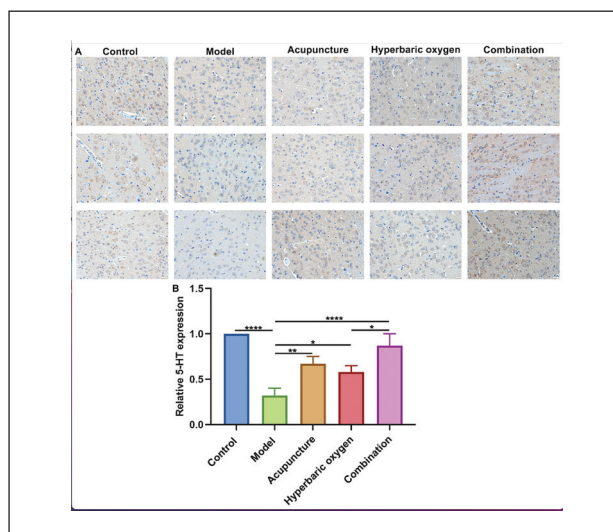
### Acupuncture combined with hyperbaric oxygen increases the expression of 5-HT and its subtypes 5-HT1A and 5-HT3 in model rats

Western blot was applied to examine the expression of 5-HT subtypes including 5-HT1A and 5-HT3 in brain homogenate from each group (Fig. 4A). Compared to the control group, lower 5-HT1A and 5-HT3 expression was found in the model group (both  $p < 0.0001$ ; Fig. 4B, C). Acupuncture, hyperbaric oxygen and acupuncture combined with hyperbaric oxygen treatment all significantly increased 5-HT1A and 5-HT3 expression in the model group. Among them, there were the highest 5-HT1A and 5-HT3 expression levels in the acupuncture combined with hyperbaric oxygen group. Immunohistochemistry results showed that 5-HT had significantly lower expression in the nucleus of the solitary tract of model rats than controls ( $p < 0.0001$ ; Fig. 5A, B).

Acupuncture, hyperbaric oxygen and acupuncture combined with hyperbaric oxygen treatment all distinctly increased the expression of 5-HT in the model rats. Among the three treatments, 5-HT had the highest expression of 5-HT in the nucleus of the solitary tract.



**Figure 4:** The effects of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen on the expression of 5-HT1A and 5-HT3 in dysphagia model rats after stroke. (A) Representative images of western blot results. (B, C) Quantification of 5-HT1A and 5-HT3 expression levels in brain homogenate of dysphagia model rats after treatment of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen for 16 days. \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ .



**Figure 5:** Immunohistochemistry for detecting the expression of 5-HT in the nucleus of the solitary tract of dysphagia model rats after treatment of acupuncture, hyperbaric oxygen or acupuncture combined with hyperbaric oxygen for 16 days. (A) Representative images of immunohistochemistry assay. Bar value = 20  $\mu\text{m}$ . (B) Quantification results of 5-HT expression. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ .

## Discussion

In this study, we observed that acupuncture combined with hyperbaric oxygen efficiently improved the swallowing function of dysphagia patients after stroke. The therapeutic effects of acupuncture combined with hyperbaric oxygen were also confirmed in the model rats. In-depth analysis demonstrated that this combination treatment could be related to the activation of 5-HT pathway. More research should be presented to explore its molecular mechanisms in treating dysphagia.

In our prospective cohort study, we applied acupuncture combined with hyperbaric oxygen therapy to treat post-stroke dysphagia. The clinical efficacy was evaluated by VFG score, SWAL-QOL score, and Watian drinking water test. A previous meta-analysis demonstrated that acupuncture exhibited the well efficacy and safety in treatment of post-stroke dysphagia<sup>(18)</sup>. Following treatment for 4 weeks, our data demonstrated that acupuncture combined with hyperbaric oxygen therapy had the higher clinical effects compared to routine treatment or acupuncture. Acupuncture combined with hyperbaric oxygen to treat post-stroke dysphagia can reduce the pain caused by nasal feeding and gastric fistula to patients with post-stroke dysphagia<sup>(19)</sup>. Our research indicated that this combination treatment might be used in clinical practice for post-stroke dysphagia patients.

This study constructed a rat model of post-stroke dysphagia. Swallowing function was assessed by examining the number of swallows and swallowing response time. Our results demonstrated that acupuncture combined with hyperbaric oxygen had the better treatment effects on treating dysphagia and cerebral infarction compared to acupuncture or hyperbaric oxygen. Studies have shown that 5-HT molecules in the nucleus tractus solitarius and their signaling pathways play an important role in improving dysphagia<sup>(20,21)</sup>. 5-HT is widely present in the peripheral tissues and central nervous system of animals and plants in nature<sup>(22-24)</sup>.

Studies have shown that stimulants and antagonists of 5-HT and receptor subtypes play an important excitatory effect in reflex and spontaneous swallowing<sup>(25)</sup>. The nucleus tractus solitarius is the main place where 5-HT promotes swallowing<sup>(26)</sup>. Therefore, 5-HT may be a potential target for improving dysphagia<sup>(27)</sup>. Our clinical trial and animal experiment results showed that acupuncture combined with hyperoxia had a good effect on the

treatment of dysphagia after stroke. At the same time, we detected that acupuncture combined with hyperoxia had the ability to up-regulate the expression of 5-HT. At present, 5-HT in the nucleus tractus solitarius generally originates from the raphe nucleus group<sup>(28-30)</sup>, and the regulation of pharyngeal muscle movement by 5-HTergic neurons in the raphe nucleus group of the brainstem may be mediated by 5-HT<sub>3</sub> and 5-HT<sub>1A</sub> receptors<sup>(31)</sup>. Our data showed that acupuncture combined with hyperoxia can also elevate the expression of 5-HT<sub>3</sub> and 5-HT<sub>1A</sub> in the model rats. These findings indicated that this combination treatment can improve post-stroke dysphagia, which may be related to the 5-HT pathway activation.

## Conclusion

In this study, our clinical trial and rat model experiment demonstrated that acupuncture combined with hyperbaric oxygen had a significant effect on the treatment of post-stroke dysphagia. Furthermore, this study preliminarily clarified that the 5-HT pathway was activated by this combination treatment, which provided a new insight into the treatment of dysphagia. Collectively, our research provided a reliable scientific experimental basis for the principle of acupuncture combined with hyperbaric oxygen in the treatment of dysphagia, which was conducive to its clinical promotion.

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#### *Authors' contributions*

*Weixin Zhu conceived and designed the study. Hehe Chen, Meifei Xu and Feihong Zhu conducted most of the experiments and data analysis, and wrote the manuscript. Yali Pan, Jing Yu and Xiajun Zhang participated in collecting data and helped to draft the manuscript. All authors reviewed and approved the manuscript.*

#### *Ethics approval and consent to participate*

*The study was approved by the Ethics Committee of Jinhua Central Hospital, Zhejiang Province (2020011).*

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