THE THERAPEUTIC EFFECT OF TONGYAN SPRAY ON RATS WITH DYSPHAGIA AND THE INFLUENCE OF HYPOGLOSSAL NUCLEUS MOTION-RELATED TRANSMITTER

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ABSTRACT

Objective: *The present stud was conducted to investigate the therapeutic effects of Tongyan Spray on different models of rats with dysphagia and the effects of sublingual nuclear motion-related transmitters.*

Methods: *SD rats were divided into control group, dysphagia group and drug treatment group. The control group was not treated, and the dysphagia group and drug treatment group were modeled for dysphagia. The control group and the dysphagia group were treated with normal saline in the same way. The changes of body weight, water intake, swallowing times and swallowing latency of rats were recorded in each group. Nissl bodies, 5-HT, AChE, P38 and neuronal nitric oxide synthase (nNOS) in sublingual nucleus of rats were detected by Nissl bodies staining and immunohistochemistry.*

Results: *In the drug treatment group, swallowing times, swallowing latency, food intake, water intake, and body weight increased significantly, compared to the dysphagia group (P<0.05). In addition, the levels of Nissl bodies, 5-HT and AChE increased in the drug treatment group (P<0.05), while p38 and nNOS contents decreased (P<0.05).*

Conclusion: *Tongyan Spray could inhibit the activation of the p38MAPK pathway, reduce the expression of nNOS and the production of NO, increase the content of 5-HT and ACh, protect the sublingual neurons, and effectively improve the swallowing function of rats.*

Keywords: *Tongyan spray, dysphagia, rate.*

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Introduction

Dysphagia refers to the damage of the jaw, tongue, soft palate, throat, esophageal sphincter or esophageal function, which leads to difficulty in eating[(1)]. The main clinical symptoms are often accompanied by complications such as aspiration, pneumonia, malnutrition, psychological and social communication disorders, etc. Therefore, dysphagia is a serious topic in clinical research[(2-3)]. At present, the incidence of swallowing dysfunction in stroke is as high as 50%[(4)]. Especially in the later stage, it can reach more than 78%[(5)]. In addition, like superior laryngeal nerve injury, many diseases such as amyotrophic lateral sclerosis, Parkinson's disease, and oropharyngeal neuromuscular diseases can also cause dysphagia. Tongyan Spray is made of ginger, cinnamon, clematis Chinensis, etc. It can be sprayed directly on the tongue. At present, its early clinical trials have achieved remarkable results. As Feng et al.[(6)] reported, they treated pseudobulbar paralysis caused by stroke with Tongyan Spray. Yuan et al.[(7)] studied the effect of Tongyan Spray on swallowing function of rats with transient middle cerebral

artery embolism, and found that the swallowing frequency of rats with dysphagia increased after drug treatment. Jian Guo et al.[8] studied the effect of Tongyan Spray Recipe on the contraction threshold intensity of tongue muscle after sublingual nerve injury in rats, and found that this medicine can reduce the contraction threshold intensity of tongue muscle. To further study the mechanism of action, they also studied the relationship between the movement-related transmitters (Nissl body number, 5-HT, AChE, p38, nNOS, etc.) of sublingual nerve nucleus in rats with dysphagia and the treatment with Tongyan Spray[9].

On this basis, this study attempts to further explore the therapeutic effect of Tongyan Spray on rats with dysphagia and its influence on hypoglossal nucleus movement related transmitters.

Materials and methods

Experimental animals

As many as 65 SD male rats (average weight 190.56±3.24g) purchased from Beijing Spafford Experimental Animal Center were bred for 1 week, and 60 rats were selected for this study.

Reagents and instruments

The main reagents used in this study are as follows:

• Tongyan Spray was prepared according to the method reported in the literature[8];

• 5-HT antibody, nNOS, p38 antibody and AChE antibody (Proteintech company); Nissl staining kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.);

• Chloral hydrate (Baiao Baile Biotechnology Co., Ltd.);

• Isoflurane (Lunanbert Pharmaceutical Co., Ltd.).

The main instruments used in this study are:

Automatic intelligent frontal microscope (Olympus BX53), small animal anesthesia machine (MatrxVIP3000, MIDMARK Company, USA), and Powerlab physiological experiment system (Power Lab/8sp, Instruments Company).

Animal grouping and administration

Grouping and model establishment

60 SD rats were divided into control group, dysphagia group and drug treatment group; subsequently, the model of dysphagia was established for rats of dysphagia group and drug treatment group[9]. The modeling process of dysphagia was as follows: firstly, rats in dysphagia group and drug treatment group were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.04 mL/kg); then, after cutting off the hair on the mandible and neck of anesthetized rats fixed on the operating plate on their back, an incision (1.5 cm) was made in the middle of the neck of the rats, and then the sublingual nerve (the posterior abdominal space between masseter muscle and digastric muscle) was passively separated by glass needle; then, after squeezing the hypoglossal nerve 2 mm before bifurcation with hemostatic forceps for 10s, it can be seen that the squeezing area is flat and intact after loosening; finally, the neck incision of rats was cleaned, sutured and disinfected after modeling.

Administration mode

From the 2nd day of modeling, micro-syringe was used to drip Tongyan Spray on the sublingual and tongue of rats in the drug treatment group (note: the liquid medicine should completely infiltrate the tongue and throat of rats to avoid the accidental inhalation of the liquid medicine through the wrong airway), with 0.1mL each time and an interval of 1min one at a time.

Rats in control group and dysphagia group were given the same dose of normal saline in the same way. The administration should continue for 14 days.

Monitoring indicators and methods

Detection of neurotransmitters

The detection of Nissl body and 5-HT, AChE, p38 and nNOS protein in hypoglossal nucleus was reported in literature[9].

Monitoring of swallowing function

In this study, the monitoring of the swallowing function of rats was reported in literature[7].

The specific process is as follows

On the 14th day, the rats anesthetized with 1.5% ~2.0% isoflurane were fixed on the operation plate in supine position, and then a 1cm incision was made in the middle of the neck of the rats, and the front abdomen of their abdominal muscles was exposed to the operator's vision; then, the front abdomen of the rat's abdominal muscles was connected with one end of the high-precision tension transducer, and

the Power lab physiological experiment system was connected with the other end of the high-precision tension transducer. Then, a hose (diameter=0.5 mm) was inserted into the tongue base of the rat, and distilled water was orally injected at a rate of 3 μL/s for 50s. After stopping for 1min, the above steps were repeated three more times.

Swallowing times of rats

Lab Chart software was used to monitor the swallowing times of rats within 50 seconds during the above experiment; during swallowing latency: the time from the beginning of stimulation to the first swallowing of rats in the above experiment. The statistical data are obtained the average of the three measurements.

Statistical methods

In this study, SPSS17.0 software was used for statistical analysis, and the statistical data were expressed as mean ± standard deviation ($\bar{x}\pm s$), and variance analysis or nonparametric test was used for comparison among multiple groups. P<0.05 indicated a significant difference.

Results

Survival of rats in different groups

As shown in Table 1, the rats in the control group did not die within 14 days, but all rats in the dysphagia group and all rats in the drug treatment group died. In addition, the mortality of rats in the drug treatment group (25.0%) was significantly lower than that of dysphagia group (65.0%), and the difference was statistically significant (P<0.05).

Group	Monitoring time	Death (rat)	Survive (rat)	Mortality rate (%)
Control group	1d	0	20	0.0
	7d	0	20	0.0
	14d	0	20	0.0
Dysphagia group	1d	0	20	0.0
	7d	7	13	35.0
	14d	13	7	65.0
Drug treatment group	1d	0	20	0.0
	7d	3	17	15.0
	14d	5	15	25.0*

Table 1: Survival of rats in different groups at different time.
*Note: *Compared with dysphagia group at the same time, P<0.05.*

Comparison of body weight, food intake and water intake of rats in different groups

The comparison of weight, food intake and water intake of rats in each group is shown in Table 2. In terms of body weight, there was no significant difference in baseline body weight among the three groups (P>0.05). On the 7th day, the weight of dysphagia group and drug treatment group was significantly lower than that of the control group, and the difference was statistically significant (P<0.05).

Monitoring index	Monitoring time	Group			Z	*P*
		Control group	Dysphagia group	Drug treatment group		
Weight/g	1d (n=20)	215.51±5.12	213.45±4.47	217.87±4.17	59.567	
	7d (n=20)	274.23±7.37	198.32±6.76	206.37±4.38	16.232	*
	14d (n=20)	359.72±8.28	175.92±4.09	235.57±8.28	102.326	**
Food intake/g	1d (n=20)	25.66±1.35	24.99±0.98	25.81±1.03	39.944	
	7d (n=13)	35.57±1.45	18.16±1.96	28.45±1.84	26.437	**
	14d (n=7)	39.48±2.45	26.58±1.78	37.75±2.02	12.853	**
Water inflow/g	1d (n=20)	33.35±1.56	34.12±1.28	32.98±1.87	15.327	
	7d (n=17)	38.56±2.45	20.44±2.89	23.45±3.19	9.326	*
	14d (n=15)	44.28±3.53	22.48±3.01	34.29±3.95	146.316	**

Table 2: Comparison of body weight, food intake and water inflow of rats in each group.
*Note: *means P<0.05; **means p<0.01.*

However, there was no significant difference in body weight between dysphagia group and drug treatment group. When it came to the 14th day, the weight difference among control group, dysphagia group and drug treatment group was even more significant (P<0.01). In addition, compared with the control group, the weight of rats in dysphagia group and drug treatment group decreased significantly. In terms of food intake, there was no significant difference in the initial food intake among the three groups (P>0.05). On the 7th day, there was a significant difference in terms of food intake among control group, dysphagia group and drug treatment group (P<0.01). Compared with tats in dysphagia group, the food intake of rats in the drug treatment group increased significantly. In addition, on the 14th day, there were significant differences in food intake among the control group, dysphagia group and drug treatment group (P<0.01). At the same time, the food intake of rats in the drug treatment group increased significantly and was faster than

that of the dysphagia group. In terms of water inflow, there was no significant difference in the initial water inflow among the three groups (P>0.05). However, after feeding to the 7th day, the water intake of rats in dysphagia group and drug treatment group was significantly lower than that in the control group, and the difference was statistically significant (P<0.05). Furthermore, after feeding on the 14th day, the water intake of rats in three groups was significantly different in different groups (P<0.01), and the water intake of rats in the drug treatment group was much higher than that of the dysphagia group.

Comparison of swallowing function in different groups of rats

As shown in Figure 1, on the 14th day, there was a significant difference in terms of swallowing times among the three groups (p<0.05), and the swallowing times in the drug treatment group were significantly higher than those of the dysphagia group (p<0.01).

In addition, on the 14th day, there was a significant difference in swallowing times among the three groups (P<0.05). The swallowing times in drug treatment group were significantly lower than those of the dysphagia group (P<0.05), but higher than those of the control group (P<0.01).

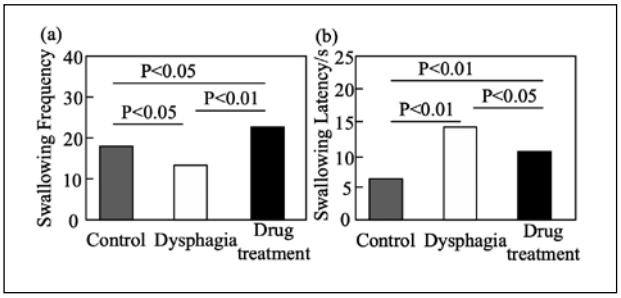

Figure 1: Comparison of swallowing function of different groups of rats (a: swallowing times; B: swallowing latency).

Comparison of Nissl bodies in hypoglossal nucleus of rats in different groups

The results shown in Figure 2 were obtained by staining Nissl bodies in the hypoglossal nucleus of rats that survived 14 days later. The number of Nissl bodies (dark blue) in the drug treatment group was higher than those of the control and dysphagia groups; five rats randomly selected from each group were stained, and the statistical results shown in Figure 3 were obtained. The number of Nissl bodies detected in the hypoglossal nucleus in the drug treatment group was higher than those of the dysphagia and control groups, and there were significant differences among the three groups (P<0.05).

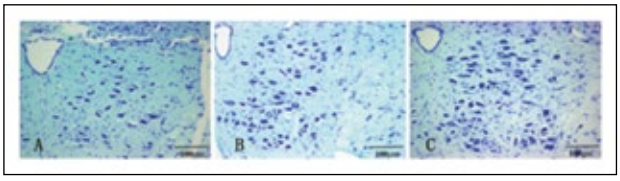

Figure 2: Nissl staining results in sublingual nucleus of rats in different groups (A: control group, B: dysphagia group, C: drug treatment group).

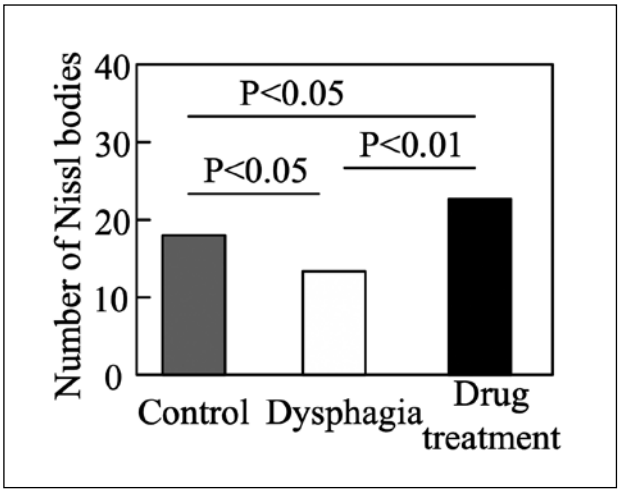

Figure 3: Comparison of Nissl bodies in sublingual nucleus of different groups of rats (n=5).

Comparison of neurotransmitters in sublingual nucleus of different groups of rats

Immunohistochemical staining of 5-HT, AChE, p38, and nNOS expressed in the sublingual nucleus of rats in the control group, dysphagia group and drug treatment group was carried out to obtain the sublingual neurotransmitter (brown-yellow) of rats in each group under inverted microscope, as shown in Figure 4.

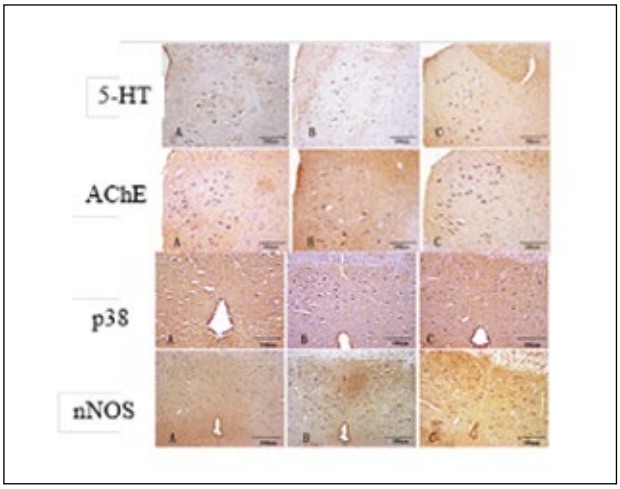

Figure 4: Expression levels of 5-HT, AChE, p38 and nNOS in sublingual nucleus of rats in different groups (a: control group, b: dysphagia group, c: drug treatment group).

Then, the results shown in Table 3 were obtained by counting the number of sublingual neurotransmitters detected in each group of rats (5 rats). Compared with the control group, the number of 5-HT positive cells in the drug treatment group and dysphagia group decreased, but the number of positive cells in dysphagia group decreased even more, and the difference among the three groups was statistically significant (P<0.01).

Similarly, when comparing the number of AChE in the three groups, the trend was the same as that of 5-HT, and there were significant differences among the control group, dysphagia group and drug treatment group (P<0.01). However, in terms of p38 and nNOS neurotransmitters, the number of positive cells detected in dysphagia group and drug treatment group was higher than that of control group, and the number of positive cells of p38 and nNOS neurotransmitters in the drug treatment group was lower than that of dysphagia group; the three groups were significantly different (P<0.01).

Group	Types of neurotransmitters			
	5-HT	AChE	p38	nNOS
Control group (n=5)	18.57±2.78	21.92±1.46	5.27±1.85	3.48±0.58
Dysphagia group (n=5)	6.67±1.59	7.56±1.38	15.13±2.04	13.09±1.78
Drug treatment group (n=5)	16.26±2.05	14.76±1.17	11.03±2.33	8.53±1.54
Z	39.763	120.782	62.831	14.897
P	<0.01	<0.01	<0.05	<0.01

Table 3: The positive cells of sublingual neurotransmitters (5-HT, AChE, p38 and nNOS) in rats of each group (individual; $\bar{x}\pm s$).

Discussion

Swallowing is a series of complex sequential activities of nerves and muscles to complete the task of moving food from mouth to stomach and protecting the airway. Dysphagia can be caused by a variety of diseases, such as stroke, amyotrophic lateral sclerosis, Parkinson's disease, oropharyngeal neuromuscular diseases as well as superior laryngeal nerve injury, etc.[10]. However, there are no specific drugs to treat dysphagia at present. Therefore, the pathogenesis and treatment of dysphagia is one of the research hotspots. In this study, the swallowing frequency of rats in dysphagia group was significantly less than that of the control group, but the swallowing latency was prolonged, indicating that the model of rats in dysphagia group had good feasibility and value in the related research of neurogenic dysphagia. In addition, the rats in the dysphagia group also showed weight loss and reduced food intake and water inflow, reflecting can well indicate dysphagia. That was the same for the clinical dysphagia patients. Subsequently, the rats with dysphagia were treated with Tongyan Spray. Although the rats showed a decrease in swallowing times on the 7th day, the decreased swallowing times gradually increased to the swallowing times of the control group after 14 days of continuous intervention, and even exceeded that. In addition, the body weight, food intake and water inflow of rats in the drug treatment group significantly improved, especially the data monitored on the 14th day.

Based on the above results, it can be seen that Tongyan Spray can effectively improve the swallowing function of rats, and gradually recover the swallowing reflex of rats. Similarly, Tongyan Spray can also improve the food intake and water intake of rats with dysphagia. Obviously, the recovery of swallowing-related functions is closely related to swallowing reflex. However, the mechanism of Tongyan Spray in recovering swallowing reflex is not clear. Therefore, the effects of Tongyan Sprayon motor-related neurotransmitters (Nissl body number, 5-HT, AChE, p38, nNOS) in hypoglossal nucleus of rats with dysphagia were studied. Nissl is an essential protein for nerve cell synthesis. The metabolic function of neurons can be detected by monitoring the number and morphology of Nissl bodies[11].

After feeding rats for 14 days, the number of Nissl bodies in the drug treatment group was significantly higher than that in the dysphagia group, which indicated that Tongyan Spray played an active role in improving neuron metabolism and protein synthesis, and it could repair injured neurons. In addition, for sublingual neurotransmitters 5-HT and AChE, the experimental results show that Tongyan Spray can also increase the contents of 5-HT and AChE neurotransmitters in rats with dysphagia. The reasons are that: on the one hand, 5-HT can excite sublingual nerve; on the other hand, AChE can decompose the neurotransmitter ACh from motor endplate. That is, when ACh neurotransmitter increases, the synthesized and expressed AChE will also increase. Therefore, Tongyan Spray has a positive effect on improving excitatory transmitters 5-HT and ACh, and reducing excitability caused by damaged sublingual nerve. Finally, p38 is an important member of the MAPK family, which

can control inflammatory reaction[12]. When the p38-MAPK pathway is activated, it will induce up-regulation of nNOS and increase NO content[13]. In this study, the contents of p38 and nNOS in rats with dysphagia increased, but the contents of p38 and nNOS in sublingual nerve of rats decreased obviously when Tongyan Spray was used for prognosis ($P<0.05$), which indicated that Tongyan Spray reversed the up-regulation of p38 and nNOS.

To sum up, this study first evaluated the therapeutic effect of Tongyan Spray on rats with dysphagia, and then discussed its internal possible mechanism. It was found that Tongyan Spray could inhibit the activation of the p38MAPK pathway, reduce the expression of nNOS and the production of NO, and increase the content of 5-HT and ACh finally play the role of protecting hypoglossal neurons.

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