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Research Article

Effects of 5-hydroxytryptamine Receptor on the Reaction of the Lower Esophageal Sphincter Under the Electrical Field Stimulation

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Fund: Effect of 5-hydroxytryptamine Receptor in the Lower Esophageal Sphincter Regulation Mechanism(Number: 18ZF23)

Abstract: In the first and second parts of this study, 5-hydroxytryptamine (5HT) receptors, including 5-HT3 and 5-HT4 with the highest expression level, were found in clasp and sling fibres of the lower esophageal sphincter (LES). Specific 5-HT3 and 5-HT4 receptor agonists can induce the contraction effect of clasp and sling fibres of the LES while specific 5-HT7 receptor agonists showed no effects. In the study of this part, the in-vitro muscle tension measurement technology and EFS methods were used to detect the effect of the selective 5-HT receptor antagonist on the clasp and sling fibres of the in-vitro LES under the electrical field stimulation (EFS), and further to ensure the effect of 5-HT receptor in the LES neuroregulatory pathway, and deeply explore the effect of 5-HT receptor in the systolic and diastolic function regulation of the LES.

Key words: 5-hydroxytryptamine (5HT) receptors; Lower Esophageal Sphincter (LES); In-vitro muscle tension

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1 Introduction

As the major structure of esophagogastric junction, the LES usually controls its opening and closing, and prevents the reflux of gastric contents[1]. The LES usually has a high resting tension but relaxes at the control of strong inhabitation neurons at the moment of swallowing or hiccupping[2,3], which is mainly mediated by the neurogenic and myogenic mechanisms.

The EFS activates and depolarizes the nerve fibres so that the nerve reflex can happen after the ions in the nerve fibres move along the axon[4]. Due to the similar effect of the electric field to nerve reflex, the EFS if often used for many gastrointestinal motility functions, including the esophagus. Studies have found that the stimulation to the Enteric Motor Neurons (EMN) between the muscular layers of the digestive tract can activate the neural conduction pathway; and the electrical stimulation on the in-vitro smooth muscles of esophagus out of the innervation can cause the esophageal peristalsis[5]. Many studies have verified that the EFS activates the nervous signal conduction pathway via stimulating the intestinal mobility neuron. During the study on the EFS on the esophageal muscle strip, Christensen J found the EFS activated the nervous signal conduction pathway and caused obvious peristalsis[5]. Therefore, the EFS can be used to simulate the nervous reflex within the human environment and also serves as the major research method in the study on the lower esophageal sphincter.

Previous studies have confirmed that the normal physiological functions of the LES are regulated by different aspects of the excitatory and inhibitory neural factors, as well as muscle-derived and body fluid factors under the central nervous system. By connecting the EMN synapse, the LES intermuscular vagus nerve endings can constitute the neural conduction pathway, including the excitatory and inhibitory conduction pathway. After being activated, the excitatory nervous conduction pathway composed by preganglionic and

postganglionic cholinergic neurons can induce the release of acetyl choline (ACH) and substance P (SP), etc.[6] by the postganglionic cholinergic neurons, and further cause the contraction of the LES; while the preganglionic cholinergic neurons constitute the inhibitory conduction pathway with the postganglionic Nonadrenergic Noncholinergic (NANC) neurons[7]. Then, the activated inhibitory conduction pathway can cause the release of vasoactive intestinal peptide[8] (VIP) and nitric oxide (NO)[9] by the postganglionic NANC neurons, and further lead to the relaxation of the LES. When the LES nervous conduction pathway is activated, the release of the excitatory and inhibitory neurotransmitters mentioned above, as well as some other body fluids and muscle sources also influence the excitatory and inhibitory mobility conduction pathways of the esophagus. All these factors combine to achieve control of the LES vasomotor function and resting pressures[10].

2 Research Objective

24 patients, including 14 male patients and female patients, averagely aged 61 with subtotal gastrectomy and digestive tract reconstruction surgery for thoracic middle segment esophageal carcinoma in the Thoracic Surgery Department, the Affiliated Hospital of Hebei University during the period from May 2018 to August 2019 were chosen as the research objective. No patients showed the symptoms of sour regurgitation, hiccup, heartburn, emesis, etc., the medical history of esophageal motility disorder disease, like achalasia and gastroesophageal reflux disease and relevant medication history. This research has been approved by the Ethics Committee of the Affiliated Hospital of Hebei University, and all patients have the right to informed consent and have signed it.

3 Experimental Apparatus and Reagents

Experimental Apparatus and Reagents see Table 1 and 2.

Table 1. Experimental Apparatus

Medlab Signal Acquisition System	Nanjing Medease Science & Technology Co., Ltd.
Medlab Isolated Organ Detector	Nanjing Medease Science & Technology Co., Ltd.
JZ101 Muscle Tension Transducer	Beijing Xinhang Electromechanical Equipment Co. Ltd.
pHS-3 Acidometer	Shanghai Hongyi Instrument Factory
Eppendorf Micro-Adjustable Pipette	Eppendorf-Netheler-Hinz, Germany
Electronic Analytical Balance	Shanghai JingKe Balance Co., Ltd.
SC-15 Numerical Control Super Thermostatic Bath	Ningbo Tianheng Instrument Plant
Isolated Organ Perfusion Chamber	Nanjing Medease Science & Technology Co., Ltd.
Platinum Electrode	Beijing Liuyi Instrument Factory
YSD-4G Pharmacological & Physiological Laboratory Multifunctional Apparatus	Huaibei Zhenghua Biologic Apparatus Facilities Co., Ltd.

Table 2. Laboratory Reagents

HCI	Tianjin Tianyi Chemical Reagents Factory
KCl	Tianjin Tianyi Chemical Reagents Factory
Glucose	Tianjin Tianyi Chemical Reagents Factory
NaCl	Tianjin Tianyi Chemical Reagents Factory
$CaCl_2$	Tianjin Tianyi Chemical Reagents Factory
$NaHCO_3$	Tianjin Tianyi Chemical Reagents Factory
$MgSO_4$	Tianjin Tianyi Chemical Reagents Factory
Gas mixture of 95%O_2 and 5%CO_2	Shijiazhuang Xisanjiao Oxygen Manufacturing Station
$NaH_2PO_4 \cdot 2H_2O$	Tianjin Tianyi Chemical Reagents Factory
Granisetron	Abcam, Britain
GR113808	Abcam, Britain
SB 269970	Abcam, Britain
TTX	BELLANCOM, America

4 Experimental Methods

4.1 Specimen collections and Muscle strips preparation

The specimen of the fresh gastroesophageal junction with the upper and lower parts of 3 cm taken from the operating room was placed in the Krebs at 4℃ immediately. After being washed, the specimen was fixed in the Krebs cake wax with the mucosa upward and the mixed gas of 5% CO_2 and 95% O_2 was continuously injected. A scalpel was used to dissect the specimen from the greater curvature and sharply separate the mucosa layer and the lower layer. Then, the sling fibres diagonally distributed on the greater curvature and the clasp fibres on the lesser curvature were found respectively after figuring out the locally thickened muscle layer as the lower esophageal sphincter. The sling and clasp fibres were separated along with the texture and made into muscle strips of 2mm*10mm. However, during the preparation, attention should be paid to carefully ensure the parallel of the long shaft of the muscle strips and the muscle bundle and prevent the muscle strips from being broken. In addition, the mucosa, its submucous membranes as well as longitudinal esophageal muscles had better be fully used. However, it should be abandoned if the specimen invaded by tumour is seen in the surgery or shown by the postoperative pathology or the resection range of the specimen is too small[11-13].

The muscle strips were tied firmly with threads and vertically hang in the 10 ml Krebs liquid in the constant temperature bath at 37℃ and the mixed gas of 5%CO_2 and 95% O_2 was continuously injected. The lower end of the strip was fixed with the L-frame with the platinum electrode, while the upper end was connected to the JZ101 Muscle Tension Transducer fixed on a precisely adjustable lifting frame, and the changing situation of all muscle strip tensions was gathered by the Medlab signal collector. The muscle strips placed in two annular and paralleled platinum electrodes with the distance of 3 mm at least, and all experimental muscle strips were treated with electric field stimulation (EFS) with the Pharmacological and Physiological Laboratory Multifunctional Apparatus on one end of the platinum electrode. The lifting frame was precisely adjusted to ensure the tension of 200mg, at which the muscle strip length was the original one L0. Then, the muscle strip at L0 was bathed warmly at 37℃ for 60 minutes and the Krebs liquid was updated every 20 minutes, after which the muscle strip was pulled slowly and gently once per 15 minutes at nearly 25%[14] to twice the muscle strip length, that is, the most appropriate original length[15].

When reaching the optimum length, the muscle strip still needed to bath in the Krebs liquid for 1 hour and then was treated with the EFS. Parameters for the EFS include: the monopulse square wave, the voltage of 50V, the wave width of 5ms, and the frequency of 1~512Hz in multiples (1Hz, 2Hz, 4Hz, 8Hz, 16Hz, 32Hz, 64Hz, 128Hz, 256Hz and 512Hz). The EFS was conducted according to the frequency from small to large, and then the maximum effect (Emax) for each EFS was calculated. When the EFS was stopped and the muscle strip regained balance, the tetrodotoxin (TTX) was added for 20 minutes before the next EFS. The reaction of the muscle strip to the EFS was compared. Then, when the EFS was stopped and the muscle strip regained balance again, the selective 5-HT3 receptor antagonist Granisetron, 5-HT 4 receptor antagonist GR113808 as well as 5-HT7 receptor antagonist SB 269970 was added for 20 minutes before the next EFS. Then, the reaction of the muscle strip to the EFS was compared.

4.2 Statistical Analysis

Statistical software SPSS19.0 and GraphPad Prism 6.0 were used to conduct statistical analysis. The muscle strip reaction induced by the EFS was expressed in the Average Contraction Percent of Muscle Strips ± Standard Error ($\bar{x}$ ± SE). The two-factor analysis of variance was used for the comparison between the muscle strip drug concentration after administration and the dose-effect curve. If P<0.05, it is considered statistically significant.

5 Results

5.1 Effects of the EFS on the clasp and sling fibres of the lower esophageal sphincter

The EFS can induce the frequency-dependent relaxation of the clasp and sling fibres, in which the EFS frequency at the biggest relaxation of 64Hz, and the biggest relaxation percent of the clasp and sling fibres was (19.1±2.3)% and (21.3±2.7)% respectively. The relaxation of two fibres induced by the EFS showed no difference and statistical significance (F=0.21 and P=0.66)(Shown in Figure 1 and Table 3).

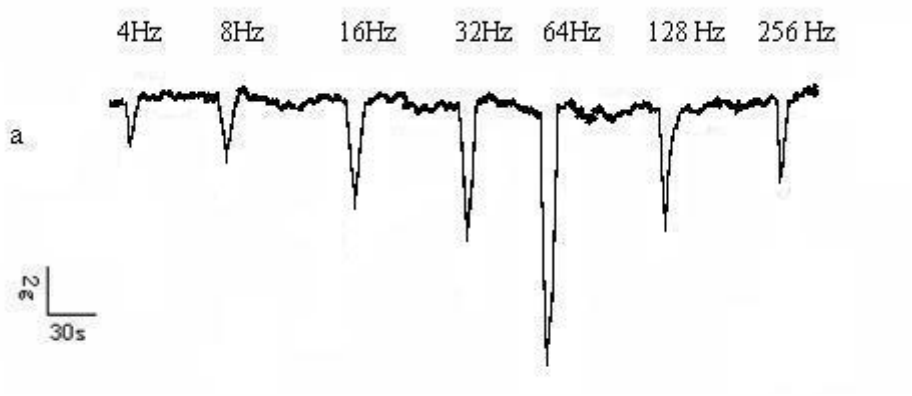

Figure 1. Effect of EFS in LES clasp and sling fibres. The EFS induced a frequency-dependent relaxation in clasp and sling fibres. The optimal frequency leading to maximum relaxation was all 64 Hz.

Table 3. Relaxation responses of clasp and sling fibres induced by EFS

Grouping	Relaxation responses (%)				
	8Hz	16Hz	32Hz	64Hz	128Hz
Clasp fiber	7.6±0.8	11.3±2.3	12.7±1.5	19.1±2.3	13.1±1.4
Sling fiber	8.3±1.2	12.6±1.7	13.6±1.9	21.3±2.7	14.2±2.1

5.2 Effects of the TTX in the relaxation of the lower esophageal sphincter induced by the EFS

The EEX can significantly reduce the relaxation of the lower esophageal sphincter induced by the EFS. After the addition of the TTX, the relaxation of the muscle strip decreased and the comparison between relaxation before and after the addition of the TTX showed statically difference(Shown in Figure 2 and Table 4).

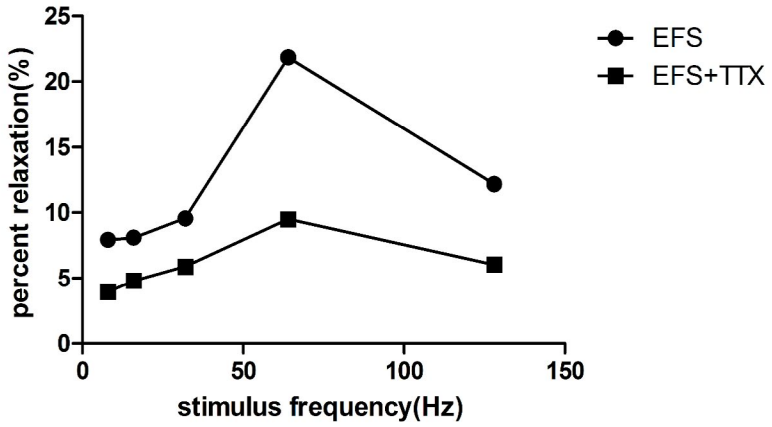

Figure 2. Effect of TTX on the EFS-induced relaxation of LES fibres. TTX produced a significant change in the responses. (F=135, P<0.01)

Table 4. Relaxation of LES induced by EFS, before and after the administration of TTX

Grouping	Relaxation responses (%)				
	8Hz	16Hz	32Hz	64Hz	128Hz
EFS	7.9±1.3	8.1±2.4	9.6±2.2	21.9±3.3	12.2±2.9
EFS+TTX	4.0±0.2	4.8±0.9	5.7±1.2	9.2±1.8	6.1±2.4

5.3 Effects of the selective 5-HT3 and 5HT7 receptor antagonists granisetron and SB 269970 in the EFS-induced relaxation of the LES fibres

For the EFS-induced frequency-dependent relaxation of the LES fibres, the selective 5-HT3 and 5HT7 Receptor Antagonists Granisetron and SB 269970 showed little significant influence, and the difference of relaxation before or after administration showed no statistical significance (Shown in Figure 4 and Table 5).

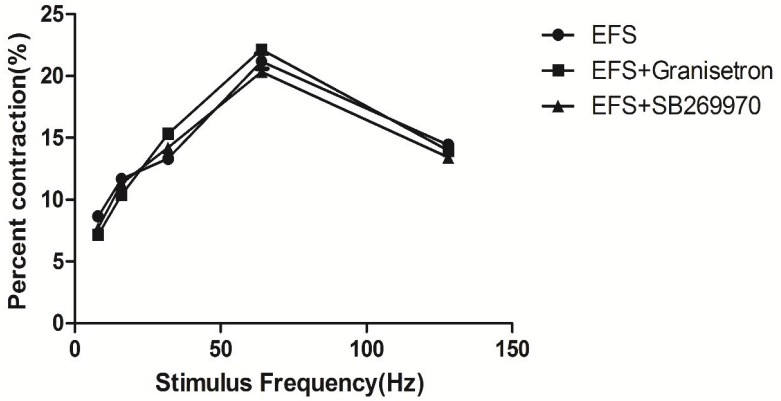

Figure 3. Effect of the selective 5-HT3R antagonist (Granisetron) and selective 5-HT7R antagonist (SB269970) on the EFS-induced relaxation of the LES. The selective 5-HT3R and 5-HT7R antagonist produced no significant change in the responses (F=0.82, P>0.05)

Table 5. The relaxation of LES produced by EFS, before and after the administration of Granisetron and SB269970

Groups	Percent contraction (%)				
	8Hz	16Hz	32Hz	64Hz	128Hz
EFS	8.6±1.5	11.7±1.8	13.3±2.3	21.2±3.6	14.4±2.5
EFS+ Granisetron	7.2±1.4	10.4±2.1	15.3±0.6	22.1±2.8	14.0±1.6
EFS+ SB269970	7.7±1.2	11.3±2.5	14.2±1.2	20.3±3.7	13.4±1.3

5.4 Effects of the selective 5-HT4 receptor antagonist GR113808 in the EFS-induced relaxation of the LES fibres

For the EFS-induced frequency-dependent relaxation of the LES fibres, the selective 5-HT4 Receptor Antagonist GR113808 showed little significant influence, and the difference of relaxation before or after administration showed no statistical significance. (Shown in Figure 4 and Table 6)

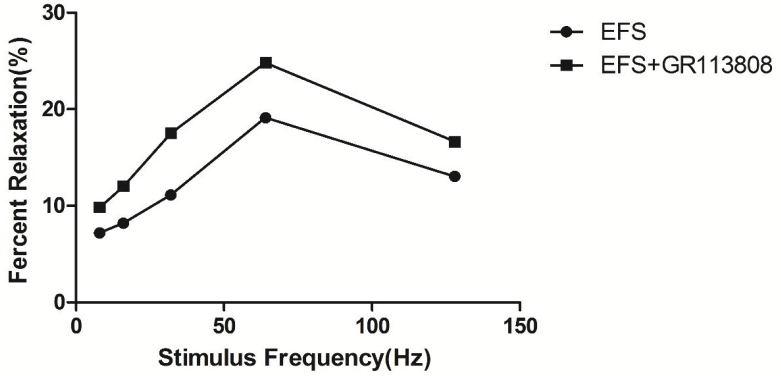

Figure 4. Influence of the selective 5-HT4R antagonist (GR113808) on the EFS-induced relaxation responses of LES. The selective 5-HTR antagonist produced a significant change in the responses. (F=7.28, P<0.05)

Table 6. Relaxation of LES induced by EFS, before and after the administration of GR113808

Grouping	Relaxation responses (%)				
	8Hz	16Hz	32Hz	64Hz	128Hz
EFS	7.2±1.3	8.2±2.0	11.1±1.2	19.1±1.7	13.0±1.7
EFS+GR113808	9.8±1.4	12.0±2.2	17.5±1.3	24.8±2.3	16.6±2.4

6 Discussions

As the important physiological structure of esophagogastric junction, the LES usually regulates its contraction and relaxation function with the central nervous system, which not only involves some neurotransmitters and hormones, as well as its myogenic factors. The LES can cause its relaxation under the control of the inhibitory nerve, and contract under the control of the excitatory nerve. These two always maintain a dynamic balance and each nerve may dominate at a certain moment. When not eating, resting or fasting, the LES is physiologically nervous and under a certain pressure to prevent from reflux esophagitis and bucking caused by the reflux of gastric contents. When eating, the LES can temporarily relax[16] and foods are permitted into the stomach. In addition, when hiccupping or vomiting, the LES can also temporarily relax, and permit the gastric contents and gases to be removed outside, which is also an important protection mechanism.

The excitatory nervous conduction pathway is composed of preganglionic and postganglionic cholinergic neurons, and the inhibitory nervous conduction pathway consists of preganglionic cholinergic neurons and postganglionic non-adrenergic non-cholinergic neurons. The activation of these two pathways can induce the release of various neurotransmitters, such as ganglionic cholinergic neurons and gastrointestinal neurons, which plays a significant role in regulating the contraction and relaxation of the LES. Specifically, the LES can be regulated by the NANC and vagus nerves[17-20]. The major neurotransmitters of the NANC nerves, VIP and NO, can relax the LES, and the major neurotransmitters of the vagus nerve, acetyl choline and substance P[21,22], can induce the contraction and relaxation of the LES. The vagus nerve endings contact the intestinal mobility neurons of the LES to form a synaptic junction[23,24], which play a significant role in the neuroregulation of the LES. Moreover, many different kinds of hormones and neurotransmitters also play a significant role in the inherent neurons of the LES, the contraction and relaxation regulation of the LES through various signal conduction pathway and the maintenance of the resting pressure of the LES. Due to its effect in activating the motor neurons and simulating the nervous reflex inside the body, the ELS can be widely used in the functional research of the smooth muscle of digestive tract, and serve as the important methods in the functional research of the LES[25-30].

As a neurotransmitter with important physiological function, the 5-HT is widely distributed in the central nervous system and closely related to the peristalsis and secretion functions of the gastrointestinal tract[31,32]. Nowadays, it has confirmed that 16 5-HT receptor subtypes are related with human functions, including 5-HT1 to 5-HT7; and except for 5-HT5 and 5-HT6 receptors, other five receptor subtypes exist in the gastrointestinal tract, in which 5-HT1 and 5-HT7 receptors are related to the contraction and relaxation functions of the gastrointestinal tract[33,34], and 5-HT2, 5-HT3 and 5-HT4 receptors are also related to that of the gastric and intestinal smooth muscle[35-37]. In all the 5-HT receptors, the 5-HT7 receptor was confirmed recently; While the 5-HT3 and 5-HT4 receptors have the closest relationship with gastroenteric function, in which the 5-HT3 receptor antagonist has been widely used in clinical practice as the antiemetic, and the good effect of the 5-HT4 receptor antagonist has been verified as the gastroenteric power medicine.

In the first experiment, we have confirmed that mRNA of 7 kinds of the 5-HT receptors existed in the LES, and other 5 receptor proteins except for the 5-HT5 and 5-HT6 receptors also existed in the LES. In the second one, we chose the 5-HT3, 5-HT4 and 5-HT7 receptors for research, further confirming that the 5-HT3 and 5-HT4 receptor agonists had induced the concentration-independent concentration and relaxation, and the 5-HT7 receptor agonist had no obvious influence. The second research revealed that the 5-HT3 and 5-HT4 receptors had been involved in the regulation of the LES' contraction and relaxation. This experiment aims to further confirm whether three 5-HR receptors play a role in the LES' contraction and relaxation and explore the experimental basis for its role

in the regulation of the LES' contraction and relaxation.

In this part, the EFS was first used to activate the clasp and sling fibres of the isolated LES respectively. The result showed that the EFS could induce the frequency-independent contraction and relaxation of the clasp and sling fibres, and the largest EFS frequency of 64Hz. The reaction degree of the clasp and sling fibres to the EFS has no statistical significance. After the application of the neuroleptics TTX, the relaxation of the EFS-induced LES was restricted, indicating this reaction is neurogenic. Later, the selective 5-HT3, 5-HT4 and 5-HT7 receptor antagonists were used respectively to observe its effects in the EFS-induced LES relaxation and contraction, and to further ensure the effects of these three 5-HT receptors under the EFS. The result showed that the comparison between the EFS-induced contraction and relaxation before and after the selective 5-HT3 and 5-HT7 receptor antagonists in the LES had no statistical significance. Then, the comparison between the EFS-induced contraction and relaxation before and after the application of the 5-HT4 receptor antagonist in the LES had statistical significance, specifically, the EFS can be conducted after the application of the 5-HT4 receptor antagonist and the percentage of the contraction and relaxation produced by the muscle strips was bigger than that without the application of antagonists. Through the experiment of this part, we have identified that the neurogenic EFS can activate the contraction and relaxation of the LES. The 5-HT4 receptor got involved and played a significant role in contraction and relaxation regulated by the LES

This study found that the 5-HT4 receptor was involved in the EFS-induced contraction and relaxation of the LES, and the 5-HT can produce the physiological effects after combining with its corresponding receptor. Nowadays, the studies on the LES don't only exist in the isolated muscle strip and cell level, but further focus on specific signal transduction research. As the study on the effect of the 5-HT receptor in the LES signal transduction research is furthered, whether and how different 5-HT receptors play a role in regulating the LES functions have been better explored.

7 Conclusions

Through the isolated muscle tension measurement technology, this experiment further explored the reaction of the LES to the EFS and whether there was the 5-HT receptor involved. The conclusions are as follows:

(1)The EFS can induce the frequency-dependent relaxation of the LES. When the EFS reaches 64Hz, the biggest degree of relaxation occurs in clasp and sling fibres;

(2)The TXX can significantly reduce the EFS-induced frequency-dependent relaxation of the LES sourced from the nerve.

(3)The EFS-induced frequency-dependent relaxation of the LES might have involved the 5-HT4 receptor, but not the 5-HT3 and 5-HT7 receptors.

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