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Localization of Cholecystokinin and Vasoactive Intestinal Peptide in Lower Biliary Tract in Cats Following Electroacupuncture on Right Qimen (LR14) and Riyue (GB 24): an Immunohistochemistry Study

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ABSTRACT:

Accumulating evidence has shown that control of the motility of the sphincter of Oddi (SO) involves a complex interaction between nerves, neurotransmitters and gastrointestinal hormones such as vasoactive intestinal peptide (VIP) and cholecystokinin (CCK). Our previous studies demonstrated that electroacupuncture (EA) modulated the SO motility in cats and rabbits through activation of non-adrenergic non-cholinergic (NANC) pathway. This study was designed to investigate the changes of neurotransmitters such as CCK and VIP in lower biliary tract in cats receiving EA stimulation. After cats were anesthetized with intramuscular injection of ketamine hydrochloride, they were prepared to conduct EA stimulation on right Qimen (LR14) and Riyue (GB 24). The parameters of EA were 6 pulses/ 3 sec and 45 pulses/ 3 sec alternatively in frequency, 1-2 mA in intensity and 20 min in stimulation duration. After the completeness of EA

stimulation, visceral organs such as gallbladder, duodenum and the sphincter of Oddi were removed and frozen for immunohistochemistry localization of CCK and VIP. The results showed that the distribution of CCK-labeled cells in duodenum, gallbladder and SO were more and distinct after EA than before EA stimulation. Whereas, the VIP-labeled cells were significantly more and distinct in duodenum and SO, but not in gall bladder. We conclude that EA regulates the biliary motility though increasing the distribution of CCK- and VIP-containing cells in duodenum and the sphincter of Oddi.

Key Words: Electroacupuncture; Sphincter of Oddi; Motility; Vasoactive Intestinal Peptide; Immunohistochemisty; Cholecystokinin.

INTRODUCTION

The sphincter of Oddi (SO) plays an important role in regulating the bile flow into the duodenum. Accumulating evidence has shown that control of SO motility involves a complex interaction between nerves, neurotransmitters (nitric oxide) and gastrointestinal hormones such as vasoactive intestinal peptide (VIP) and cholecystokinin (CCK) [1-4]. There is consensus that SO functions differently between herbivorous and carnivorous animals. For example, CCK inhibits the SO in humans, cats, and dogs, but stimulates the SO in herbivorous animals[1,5]. However, in both circumstances, CCK functions to increase the bile flow into the duodenum. VIP is an inhibitory neurotransmitter in gastrointestinal tract, and there are rich VIP nerves found in SO, sphincter of pylorus as well as in lower esophageal sphincter [6,7]. Many reports concerning the interaction of VIP and other hormones onto SO. For example, when VIP is released, peptide histidine isoleucine (PHI) and neuropeptide Y are also released, since they are all the substances released by VIP-ergic nerve, and present inhibitory reactions toward gastrointestinal tract [8,9]. In the experiments of cats, if the anti-serum of VIP is given prior to CCK, SO will contract, which suggests that VIP, joined with CCK, is involved in the relaxation of SO [10]. From clinical human studies, 45 minutes after applying transcutaneous electrical nerve stimulation (TENS) to Hegu (LI 4), VIP in the blood rises, and the pressure of SO falls [11]. However, the exact localization of CCK and VIP in response to electroacupuncture (EA) remains unclear.

Acupuncture and herbal medicine are main frames in traditional Chinese medicine for treatment of many human disorders. Thanks to the advancement of neurophysiology, pharmacology and functional imaging technology, accumulating evidence supports the effectiveness of acupuncture [12-14]. Recently, our works have shown that EA stimulation on acupoint GB 24 (Riyue) regulates the motility of the SO in rabbits and cats through a somatovisceral reflex mediated by the secretion of CCK [15]. The present study was designed to investigate the changes of neurotransmitters such as CCK and VIP in lower biliary tract in cats receiving EA stimulation.

MATERIALS AND METHODS

Animals and experimental setup

Eight cats of either sex, weighting 2.5-3.5 Kg, were studied. They were treated under the regulations of the "Principles of laboratory animal care" (NIH publication No. 86-23, revised,

1985). After fasting for 12 hrs but free access to water, animals were anesthetized with intramuscular injection of ketamine hydrochloride (35 mg/kg body wt) and maintained with small dose of ketamine (5-10 mg/kg) if needed. The depth of anesthesia was kept on steady level without affecting heart rate, arterial blood pressure or inducing pain response to peritoneal traction [5, 15]. They were placed in supine position with a slow infusion of 5% dextrose in normal saline and the respiration was controlled with a respirator (Model 141, New England Medical Instruments Inc. Mass, USA) through a tracheostomy tube. Through an upper midline incision, gall bladder and biliary trees were identified and gall bladder pressure and sphincter of Oddi (SO) pressure were measured as described previously [15-17]. The SO pressure were measured with open-tipped catheters constantly perfused (0.3 ml/min) with physiologic warm saline (38°C) and recorded on a multi-channel polygraphic recorder (Could TA 240, Ohio, USA).

Manometry of the sphincter of Oddi

During manometric tracing, the basal or tonic SO pressure was measured as mean end-expiratory pressure during resting and SO phasic contraction pressure was measured as mean amplitude of the contractions above the tonic pressure as described previously [15-17]. Since the SO in herbivorous and carnivorous animals responded differently to CCK, it was necessary to notice a decrease SO pressure in cats soon after injection of 100 ng/kg cholecystokinin-octapeptide (CCK-8) (Sigma C2175, MO, USA) to validate the manometric method. To avoid confounding effects produced by administration of exogenous CCK in data interpretation, there was no such treatment in the following experiments.

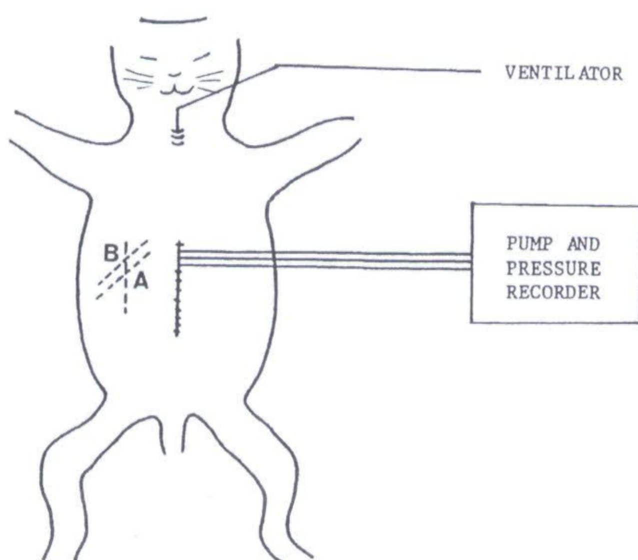

Fig. 1: Experimental setup. Electroacupuncture (EA) was brought about by applying an electric current to two needles (#32) positioned at right Riyue (GB 24, the 7th intercostal space, point A) and Qimen (LR14, the 6th intercostal space, point B).

Electroacupuncture
Electroacupuncture (EA) was brought about by applying an electric current to two needles (#32) positioned at right Qimen (LR14, the 6th intercostal space, point A) and Riyue (GB 24, the 7th intercostal space, point B) as shown in Fig. 1. EA stimulation was conducted by using a pocket nerve stimulator (Han Acuten, WQ1002F, Beijing, China). The stimulation was pulse-waved with frequency 6 pulses/ 3 sec and 45 pulses/ 3 sec alternately and 20 min stimulation duration. The intensity was adjusted until rhythmic contraction of intercostal muscle, usually 1-2 mA. For comparison, acupuncture by needling was applied to acupoints as mentioned above but no electric current was given as sham EA group.

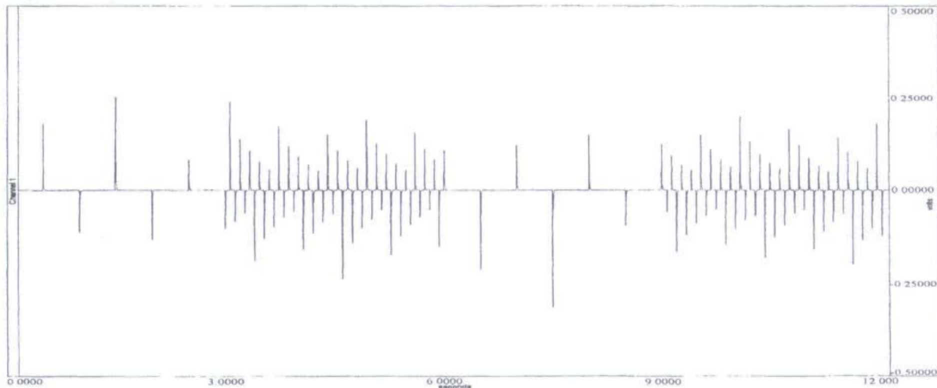

Fig. 2. The wave form of electroacupuncture. The stimulation was pulse-waved with frequency 6 pulses/3 sec and 45 pulses/3 sec alternately (dense and disperse) and 20 min stimulation duration. The intensity was 1-2 mA.

Antibodies and immunohistochemistry stain
The rabbit anti-cholecystokinin octapeptide (CCK 26-33) antibody (H-069-04) and rabbit anti-VIP antibody (H-064-16) were purchased commercially (Phoenix Pharmaceuticals, Inc, CA, USA). After anesthetized cats were EA stimulated on right Qimen (LR14) and Riyue (GB 24) for 20 minutes, they were immediately sacrificed and visceral organs such as gall bladder, duodenum and SO were removed, cut into small pieces, embedded and stored at –20°C until use.

For immunostaining, the frozen tissues were sectioned in 5 μm thickness and stained with ABC kit. In brief, tissue sections were fixed with acetone for 5 min, followed by incubation with 0.3% hydrogen peroxide for 30 minutes. Following washed with phosphate buffered saline (PBS) for 3 times, the samples were incubated with 5% goat serum for 10 min, washed with PBS, then incubated with primary antibody (anti-CCK or anti-VIP or idiotypic antibody) at room temperature (RT) for 30 minutes. The sections, washed with PBS 3 times, were processed with commercially available ABC kit including sequential incubation at RT in the following solutions with PBS washes between them. (1) secondary antibody (dilution 1:500), 30 min. (2) A+B reagents (dilution 1:100), 60 min. (3) 0.05% diaminobenzidine/0.02% hydrogen peroxide in 0.1 mM Tris-Hcl buffer at PH 7.2 for 10 minutes. The sections were then washed, counter-stained, mounted and examined under light microscope. Idiotypic antibody as primary antibody was used as negative control. The positivity of antibody-stained cells was scored semi-quantitatively, namely, (-), no positive stain; (±), < 5% positive stain; (+), 5-25% positive stain; (++), 26-50

% positive stain; (+++), > 50 % positive stain.

Experimental design

To elucidate the specific effect of EA on biliary tract, 3 groups of samples were obtained. They were 1) electroacupuncture (EA) group: animals on stable postoperative status, followed by EA stimulation for 20 minutes; 2) sham EA group: animals on stable postoperative status and kept still without EA for 20 minutes; 3) control group: animals undergoing sham operation without manipulation of biliary tract. The sample taken from visceral organs were obtained immediately after the termination of EA stimulation and so were the other two groups.

RESULTS

Inhibitory effect of EA on the sphincter of Oddi in cats

To validate the manometric tracing in cats, CCK-8 (100 ng/kg) was injected to verify the catheter position, a decrease of resting and phasic contraction pressure of SO was simultaneously recorded (Fig.3, panel 1). When sham EA was applied to the animals, there was no obvious change of SO motility (Fig. 3, panel 2); whereas EA on acupoints right Qimen (LR14) and Riyue (GB 24) for 20 min, there was a gradual decrease of SO activity (Fig. 3, panel 3).

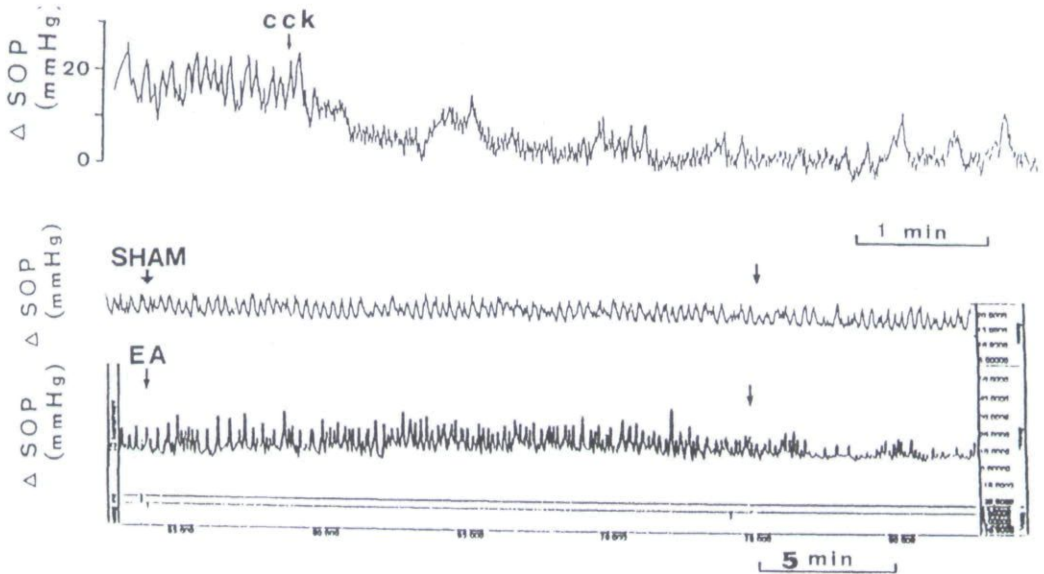

Fig. 3: Manometric tracing illustrating EA-induced response of the sphincter of Oddi in cats. A decrease of SO pressure (SOP) (panel 1) after administration of CCK-8 (100 ng/kg) were necessarily observed. When sham EA was applied for 20 min, there was no obvious change of SO motility (panel 2), whereas EA induced a decrease of SO pressure after EA stimulation (panel 3). Arrows indicate the start and the end of EA stimulation on right Qimen (LR 14) and Riyue (GB 24).

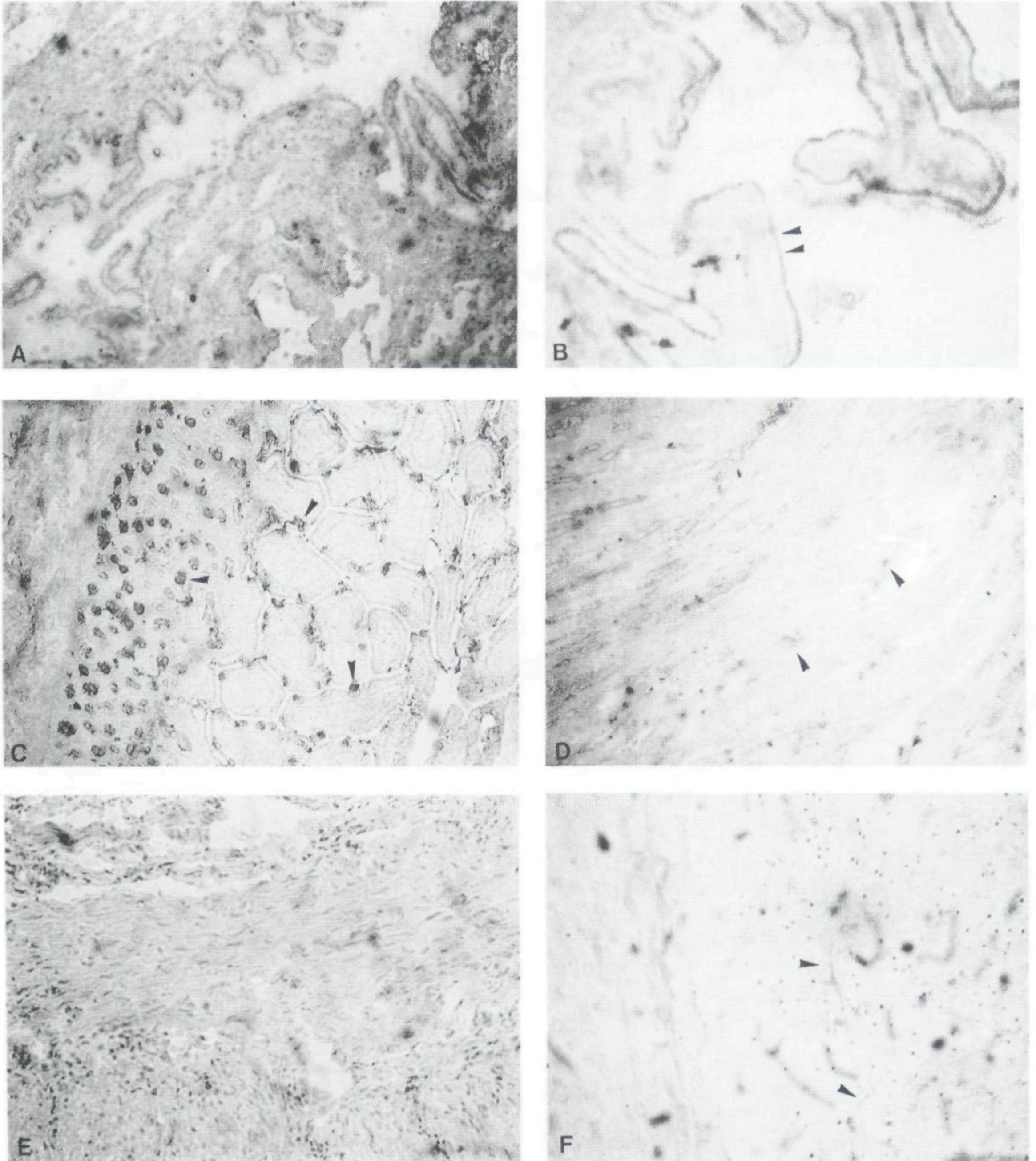

Fig. 4: Immunohistochemical localization of CCK and VIP in cats' biliary tract. When stained with idiotypic antibody (Fig. 3 A), and anti-CCK antibody, the gall bladder (Fig. 3B) and the duodenum (Fig. 3C) showed 3+ CCK-labeled cellular distribution after EA

stimulation on right Qimen (LR14) and Riyue (GB 24). When stained with anti-VIP antibody, the duodenum (Fig. 3D) showed 3+ VIP-labeled cellular distribution after EA stimulation. As compared to control group (Fig. 3E), SO showed 2+ VIP-labeled cellular distribution in cats receiving EA stimulation (Fig. 3F). Arrow heads indicate positive-stained cells.

Immunohistochemical localization of CCK and VIP in cats'biliary tract

When stained with idiotypic antibody (Fig. 3 A), and anti-CCK antibody, the gall bladder (Fig. 3B) and the duodenum (Fig. 3C) showed 3+ CCK-labeled cellular distribution after EA stimulation on right Qimen (LR14) and Riyue (GB 24). When stained with anti-VIP antibody, the duodenum (Fig. 3D) showed 3+ VIP-labeled cellular distribution after EA stimulation. As compared to control group (Fig. 3E), SO showed 2+ VIP-labeled cellular distribution in cats receiving EA stimulation (Fig. 3F).

Scoring for CCK and VIP distribution in cats'biliary tract after EA stimulation

The positivity of antibodies-stained cells was scored semi-quantitatively, namely, (-), no positive stain; (±), < 5% positive stain; (+), 5-25% positive stain; (++), 26-50 % positive stain; (+++), > 50 % positive stain. The results showed that the distribution of CCK-labeled cells in duodenum, gallbladder and SO were more and distinct after EA than before EA stimulation. Whereas, the VIP-labeled cells were significantly more and distinct in duodenum and SO, but not in gall bladder (table 1).

Table 1: Distribution of cholecystokinin and vasoactive intestinal peptide in biliary tract of the cats receiving electroacupuncture.

		CCK (+) cells	VIP (+) nerves
Gall Bladder	sham EA group	-	-
	EA group	+++	-
SO	sham EA group	+	±
	EA group	+++	++
Duodenum	sham EA group	+	±
	EA group	+++	+++

CCK, cholecystokinin; VIP, vasoactive intestinal peptide; SO, sphincter of Oddi.
The degree of positive stained cells were scored semi-quantitatively and described as followings: (-), no positive stain; (±), < 5% positive stain; (+), 5-25% positive stain; (++), 26-50 % positive stain; (+++), > 50 % positive stain.

DISCUSSION

In present studies, we have used lower biliary tract as a model to elucidate the localization of how EA modulates the biliary motility in cats. In combination with our previous report, to our knowledge, this a novel finding that EA modulates the motility of SO through a somatovisceral reflex.

Since the depth of anesthesia might affect the SO activity, namely, hyperactivity on shallow level and hypoactivity on deep level of anesthesia, a well-maintained anesthesia is mandatory to get a steady measurement of gall bladder or SO activity. We have kept animals on the level of surgical anesthesia without affecting the heart rate, arterial blood pressure or inducing pain response to peritoneal traction [18]. Besides, the model used in the present study has been generally accepted as a good system to evaluate the gall bladder and SO motility [15-17]. Fig. 2 showed the reproducibility of our model that supported the biological responses of EA on acupoints right Qimen (LR14) and Riyue (GB 24).

In addition to the well-known peptide CCK, endogenous opioid peptide, VIP, nitric oxide (NO) etc. have been documented to affect SO motility through nonadrenergic noncholinergic (NANC) pathway [1,2,19-21]. It is generally accepted that EA induces mobilization of different opioids in the brain and hence sustains antinoiceptive effect for more than 30 minutes [14,22]. Besides, opioid peptides have been previous recognized as factors sharing the spasmogenic action on SO [23]. Our previous results that EA-induced SO hyperactivity in rabbits was not blocked by pretreatment with naloxon 640ug/kg, a dose previously documented to antagonize three types of opioid peptides [15], lessened the possibility of endogenous opioids playing a role on such SO responses. It was also demonstrated that EA-induced SO hyperactivity in rabbits was blocked by pretreatment with proglumide, a major CCK-A antagonist. Direct evidence by determination of plasma level of CCK suggests the role of CCK in EA-induced SO response in rabbits as well as in cats [15]. It is of note that the initial post-EA SO activity is not obviously changed as compared to the baseline SO activity (Fig.2). We attributed this delayed SO response to a time lag needed for a host to respond to EA. That was why we designed our EA duration up to 20 min.

The functions of CCK as regulatory mediator in physiological phenomena include activation of the release of acetylcholine, induction of VIP secretion, reviving the inhibitory vagus nerve reflex arc and directly enhancing the contraction of gall bladder [24]. VIP is an inhibitory neurotransmitter in gastrointestinal tract. Many reports concerning the interaction of VIP and other hormones onto SO. For example, when VIP is released, peptide histidine isoleucine (PHI) and neuropeptide Y are also released, since they are all the substances released by VIP-ergic nerve [8,9]. From clinical human studies, 45 min after applying transcutaneous electrical nerve stimulation (TENS) to acupoint Hegu (LI 4), VIP in the blood rises, and the pressure of SO falls [11]. Our data show that the distribution of CCK-labeled and VIP-labeled cells in SO and duodenum increase after EA on acupoints right Qimen (LR14) and Riyue (GB 24).

Conventionally, the regulation of SO motility is through a viscero-visceral reflex regulation. For example, when gall bladder pressure is increased, SO was found to produce inhibitory reflex [25]. Recently, accumulating evidence suggests that the motility of SO and lower esophageal sphincter could be modulated by peripheral or somatic stimulation, such as TENS or electroacupuncture [11,26]. Owing to the embryonic development, visceral pain can be perceived as originating from a somatic area, a phenomenon known as ‘referred pain’. Therefore, it is reasonable to speculate that visceral function can be influenced by the stimulation on some somatic area. Cold-induced vasoconstriction may be the best example to explain such somatic visceral reflexes.

Taken together with our previous reports, we conclude that EA regulates the biliary motility though a somato-visceral reflex by increasing the distribution of CCK- and VIP-containing cells in duodenum and the sphincter of Oddi.

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