Bratisl Lek Listy 2011; 112 (1)
13 – 17

EXPERIMENTAL STUDY

Spectral changes in electrical activity of pylorus due to L-NAME induced hypertrophic pyloric stenosis

Aslan M[1], Celik O[2], Dogan DG[1], Tagluk ME[3], Ulas M[4], Aydin E[5]

Department of Pediatrics, Inonu University School of Medicine, Malatya, Turkey. **mirzaaslan@hotmail.com**

Abstract: *Objectives*: To investigate the relation between hypertrophic pyloric stenosis (HPS) and the changes in the myoelectrical activity of the pyloric and gastric areas.
Methods: Three pregnant females, at 14 days of gestation two of which were named as D14n (NOS inhibitor group) and one was named as D14c (control). From the beginning of the study until the end of gestation, rats in D14n group received nitric oxide synthase inhibitor L-NAME for administrating their pups, and the rat in D14c group was drinking water for 21 days. The pups of each group underwent laparotomy at 42 days of their life and myoelectrical signals of their pyloric and gastric regions were recorded via bipolar electrodes and then evaluated through signal processing.
Results: Signal analysis showed that HPS induced pyloric segment reveals a suppressed spectral component that was detected in normal pyloric segment. The HPS induced pyloric segment also revealed higher power/min and ±SD compared to that of normal and gastric areas. In the pyloric segment, while the number of interstitial cells of Cajal (ICC) was lesser, the number of smooth muscle cells was higher than in the pyloric segment of controls.
Conclusions: The spectral differentials depend on the type, population and condition of locally specialized muscular mechanism which can be affected from HPS. The HPS also has a relation to specific cells, such as ICC that generates NO, provoke the spontaneous pacemakers and biological slow waves *(Tab. 1, Fig. 1, Ref. 19).*
Full Text in free PDF *www.bmj.sk.*
Key words: pyloric stenosis, L-NAME, Cajall cells, myoelectrical activity.

Even in the absence of stimulation, most regions of the gastrointestinal tract can generate some spontaneous electrical activity and consequently mechanical motoric function of the stomach. The recordings made from isolated muscle cells in the gastrointestinal tract show a regular discharge and recharge phenomenon characterized as a plateau followed by slow waves (1–3). These pacemaking potentials are generated by a specialized population of cells, known as interstitial cells of Cajal (ICC) (1). Together with the enteric nerve system, composed of both the myenteric (intermuscular) plexus and the submucosal plexus, the ICC play a major role in gastrointestinal motility (2). Although a developmental process of gastric myoelectrical activity can be observed during the first 6 months of life (3), the dominant 2–4 cycles per minute (CPM) so called slow waves is the characteristic function of healthy gastric system in infants.

[1]Department of Pediatrics, Inonu University School of Medicine, Malatya, Turkey, [2]Department of Obstetrics and Gynecology, Inonu University School of Medicine, Malatya, Turkey, [3]Department of Electrical and Electronics, Inonu University Engineering Faculty, Malatya, Turkey, [4]Department of Physiology, Firat University School of Medicine, Elazig, Turkey, and [5]Department of Pathology, Inonu University School of Medicine, Malatya, Turkey

Address for correspondence: M. Aslan, MD, Dept of Pediatrics, Inonu University School of Medicine, Malatya, Turkey
Phone: +90.422.4441996, Fax: +90.422.3410728

Infantile hypertrophic pyloric stenosis (HPS), characterized by the hypertrophy of the circular muscles of the pylorus, is one of the most common congenital disorder requiring surgery. The occurrence of infantile HPS has been associated with genetic (4), environmental (5) mechanical and hormonal factors (6). The gradual development of gastric outlet obstruction is associated with marked hyperperistalsis and repeated vomiting. Dysfunction of pyloric inhibition has been implicated in the pathophysiology of HPS. Normal inhibition process is mediated by peptidergic and Nitric Oxide (NO) enteric nerves. NO is a gaseous free radical synthesised from L-arginine in a reaction catalysed by Neuronal Nitric Oxide Synthesis (nNOS) that is associated with the population of ICC (7). The role of NO as a major non-adrenergic non-cholinergic inhibitory neurotransmitter that mediates pyloric relaxation in the enteric nervous system has been well-described (7).

In children with infantile HPS, a significant decrease in the number of ICC occurs (8, 9). Biopsy from pyloric muscle sections from children with infantile HPS presented decreased NO in neural fibers of the circular muscle layer of the pylorum (10). Although myenteric neurons can appear normal, innervation the circular layer of the pyloric sphincter lack NO synthesis (10). Abel reported diminished nNOS expression in the muscle layers as well as the myenteric plexus in HPS (11). Barbosa et al (12) administered Nitro-L-Arginine Methyl Ester (L-NAME), a known

nitric oxide synthase inhibitor, to pregnant rats and their newborns and noted that the L-NAME rats had larger stomachs and pyloric hypertrophy. These findings suggest that the function of stomach and pylorus is possibly dependent on NO, and its absence prone to dysfunction of stomach.

We, therefore, designed our experiment to investigate the effects of L-NAME on the myoelectrical activity of pylorus in rat. Our investigation includes 1- the possibility of experimental reproduction of the histopathological case corresponding to infantile HPS through inhibition of NO, and 2- the relation of changes in the myoelectrical activity of pyloric and gastric areas in relation to the development of induced pyloric stenosis using a computerized recording system and dedicated signal processing methods.

Materials and methods

This study was carried out in the Experimental Research Laboratory of the Inonu University Faculty of Medicine, complying with the approval of the ethic committee and the guidelines for care and use of experimental animals. Three mature, healthy, 4-month-old pregnant female rats of Wistar strain, weighing between 200 and 250 g and having a 4–5 day regular estrous cycle, were used in the study.

At 14 days of gestation two of the rats were named as D14n (NOS inhibitor group) and one was named as D14c (control). Until the end of gestation the animals were kept under standard conditions: 12-h light and 12-h dark periods, 20 °C constant temperature, and a humidity range between 40 and 60 %. The rats had free access to standard dry pellets ad libitum and tap water until the end of the study.

From the beginning of the study until the end of gestation, rats in D14n group received nitric oxide synthase inhibitor L-NAME (N (G)-nitro-L-arginine methyl ester hydrochloride; Sigma Chemical Co.; St. Louis; 98H1427) at 50 mg/kg/day. The inhibitor diluted in drinking water was administered at sufficient minimal daily amounts. There was no significant difference in the volume intake of animals. The rat assigned as D14c was not administered with L-NAME (control). The litter sizes of D14n and D14c were, six, eight, and seven pups respectively. On the 21st day of life, pups were removed from their mothers, weighed and followed-up for another 21 days. After weaning, all pups were fed with the same diet. That is: L-NAME was administered to D14n pups, but not D14c, with drinking water for another 21 days and at 50 mg/kg/day (12).

Measurement of myoelectrical activity

The signal recording was conducted with a BIOPAC MP100 A-CE data acquisition system (model MP100; version 3.7.2; Goleta, CA, USA) with a sampling frequency of 500 Hz. At 42 days of life, each rat in D14n group underwent laparotomy. Bipolar electrodes were subserously implanted into their pyloric and gastric region with 1-cm inter-electrode spacing as has been suggested (13), and the reference electrode was placed on the left leg which was considered as far enough from the interested region. The myoelectrical activities of pyloric and gastric regions were recorded for at least 3 min under anesthesia. Then the signals were analyzed in Matlab (version 6; The Math-Works, Natick, MA, USA) environment.

Histological evaluation

In order to understand the level of mitotic indices of the pylorus the stomach including the pylorus and upper part of the duodenum were excised and processed for histological and immunohistochemical studies. Next, fragments from the whole extension of the wall of the pyloric region were fixed in buffered formalin at 10 % at room temperature for 48 hours. Subsequently, fragments were embedded in paraffin wax and divided into sections. One section was sliced at 5-ěm thickness, and stained with hematoxylin and eosin (H&E), Masson's trichrome stain, and the other section was sliced and stained for Ki67 and c-Kit immunoperoxidase. Monoclonal mouse anti-human c-Kit (CD117), clone 595 (BioGenex, San Ramon, CA, USA) ready to use were applied to 5 ě paraffin sections deparaffinized in xylene and for antigen retrival incubated in 10 mmol/L citrate buffer (pH 6.0) at 98 °C for 20 min. Primary antibody was applied to the samples and incubated at room temperature for 20 minutes. Immunodetection was performed by using Ultra Tek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories, Utah, USA). The sections, for Ki67 detection, were incubated in 10 mmol/L citrate buffer (pH 6.0) at 98 °C for 20 min, and then quenched in Super Block (ScyTek Laboratories, Utah, USA) for 5 min at room temperature. Primary antibodies for Ki67 (rabbit polyclonal, ready to use ScyTek Laboratories, Utah, USA) were applied to the samples and incubated at room temperature for 30 minutes. Immunodetection was performed using Ultra Tek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories, Utah, USA). Ki67 was expressed in the cell during M, G1, S and G2 phases of cell cycle and was absent in resting cells (Go). In each case the final products were visualized by aminoethylcarbazole chromogen and counterstaining was performed with hematoxylin. For c-Kit, as positive controls, sections from tissues with known occurrence of the antigens were investigated. Negative controls (primary antibody was omitted) were routinely performed on adjacent serial sections.

A semiquantitative grading system was used to score the degree of histologic change of pylorus. The scaling was conducted for the degree of c-kit immunoreactivity of pyloric segment cells (0 = no c-kit positive cell per high power field (x 40); 1 = between 1 and 10 c-kit positive cells; 2 = between 10 and 20 c-kit positive cells; and 3 = greater than 20 c-kit positive cells), the degree of Ki67 immunoreactivity of pyloric segment cells (0 = no Ki67 positive cells per high power field (x 40); 1 = between 1 and 10 Ki67 positive cells; 2 = between 10 and 20 Ki67 positive cells; and 3 = greater than 20 Ki67 positive cells) and the degree of smooth muscle cells of pyloric segment (0 = no Masson's trichrome positive cells per high power field (x 40); 1 = between 1 and 10 Masson's trichrome positive cells; 2 = be-

tween 10 and 20 Masson's trichrome positive cells; and 3 = greater than 20 Masson's trichrome positive cells).

Signal preprocessing

Since the recordings were not based on excite and then record strategies, the temporal characteristics of the recorded signals were, naturally, not expected to be time-coherent. The assessment of such time signals having improper and unpredictable phase differences is too difficult. However at least a mutual spectrum common to all subjects within a population can be investigated and evaluated for partly determining the correlation of the neuronal signal to the common symptom in the group. Therefore, the myoelectrical signals of pyloric and gastric segments were firstly transferred into frequency domain via fast Fourier transform and two stop-band notch filters, one at zero frequency with ±0.5 CPM bandwidth to remove the DC level and the very low frequency components and one at 50 Hz with a bandwidth of ±1 Hz to remove the line effect from the signal, were applied to the signal's spectrum. The signals were then processed in frequency domain independently of any temporal references. Similar to the probability of occurrence of a particular case over a collection of independent events it was considered that the square root of the multiplication of spectrums of two signals should resolve the common harmonics contributed to the signals. In a similar fashion for N signals the common or spectrally coherent signal may be obtained as:

$$\overline{U}_i(f) = \{\overline{U}_{i-1}(f)\, U_{i+1}(f)\}^{1/2}; \quad i = 2,3,.....,(N-1) \tag{1}$$

where $\overline{U}_1(f) = \{U_1(f)\, U_2(f)\}^{1/2}$, and the inverse Fourier transform of the $\overline{U}_{N-1}(f)$ gives the spectrally coherent spectrum that is common over the population. Taking the square root of the function obtained from the multiplication of the spectrum is for somehow controlling the mutual spectral power not to exceed the maximum expected value of spectral power of one signal. The inverse Fourier transform of this common spectrum gives a common time signal which we consider to hold the information relative to both the control and function of the biological tissue. That is:

$$s_c(t) = \frac{1}{2\pi}\int \overline{U}_{N-1}(f)\exp(j\omega t)\, d\omega \tag{2}$$

This mutual/common time signal can also be further processed with time-frequency techniques, but we do not expect any significant information going beyond that of revealed by spectral content of the signal.

Statistical analysis

The Statistical Package for Social Sciences, version 11.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Individual group parameters were assessed with one sample Kolmogorov-Smirnov Z-test and were found to be abnormally distributed. Mann-Whitney U-test was used to detect statistically significant differences between the animals given L-NAME and controls. $p<0.05$ was considered statistically significant, and the results are given as the mean value ± standard deviation (SD).

Results

In this study, using the described signal processing procedure, for each group the time signals and power spectrums common for all subjects involved in the experiment (five rats in each group) were obtained. As respectively shown in Figure 1–A, B and C, the common time signals cannot be interpreted visually. However, the common spectral content of the signals logically changed and showed the effect of the received oxide inhibitor L-NAME on D14n group. While the myoelectrical activity of pyloric segment of control group, D14c, showed two valuable spectral components (around 1.5 and 5 CPM), the pyloric segment of DI4n group showed only one component (around 1 CPM) with a higher power spectral density. The 5 CPM component in the overall spectrum of D14n has been suppressed which could be due to the received L-NAME inhibitor. The myoelectrical activity of

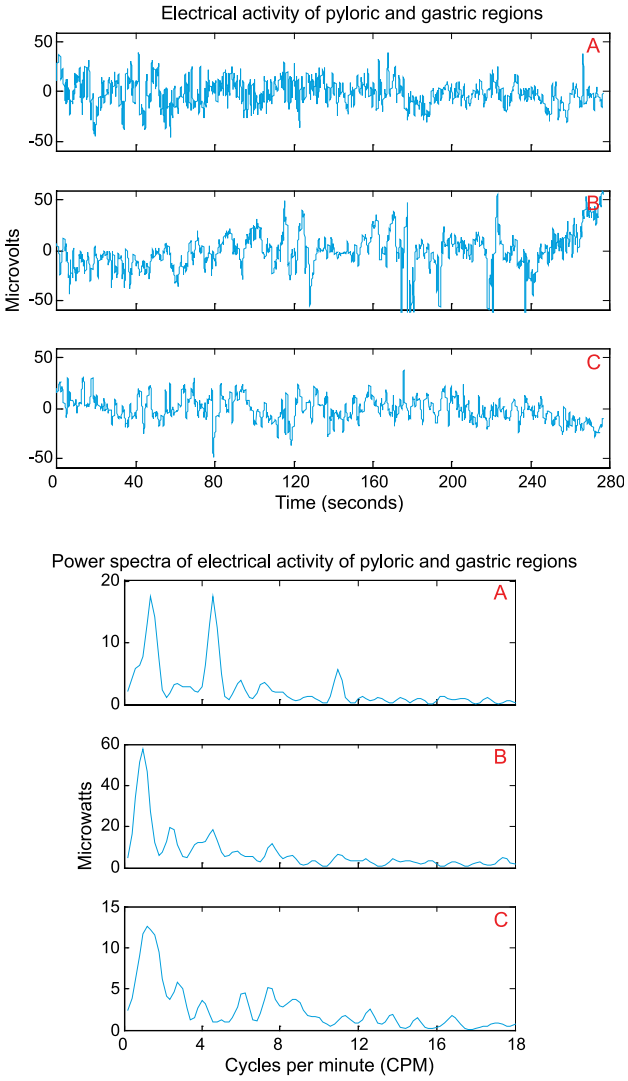

Fig. 1. Pyloric and gastric segments myoelectrical spectrally coherent signals derived from L-NAME and control groups, and their respective power spectra. A – normal pyloric (control); B – HPS pyloric; and C – gastric.

Tab. 1. A subjective grading system for scoring the degree of pyloric segment histologic change and power emitted per second in each group.

	Mean-power ±STD (μW)	c-kit	Ki67	Masson's trichrome
I – Control	45±0.22	0.6±0.5	1.4±0.5	1.2±0.5
II – L-NAME (pyloric)	74.5±0.33	2.0±0.0	2.2±0.0	2.0±0.0
III – L-NAME (gastric)	44.9 ±0.2	2.2±0.0	1.6±0.5	1.4±0.5
p value				
I vs II	0.005	0.001	0.001	0.001
I vs III	0.533	0.001	0.260	0.454
II vs III	0.001	0.454	0.001	0.005

gastric segment of D14n group (local control) demonstrated only one dominant component around 1.5 CPM but with a wider band. Concerning these findings one may speculate that the lower component may be common for gastric and pyloric but the one around 5 CPM may be specific for pyloric segment that probably suppressed by the given L-NAME inhibitor. In the literature the low frequency components lying within 1–4 CPM band had been correlated to the functional impact of the HPS myoelectrical activity which controls or modulates the muscular contractions or movements needed for digestion. Moreover, the statistical quantities such as mean power/min and standard deviation (SD) of the signal might provide gross information relative to the function of the mechanism. Therefore these quantities were also provided and given in Table 1. This information showed that both signal power and SD of the HPS pyloric segment were greater than that of gastric and normal pyloric segments. The differences detected between normal gastric and pyloric electrical activities perhaps depend on the type of muscular tissue that needs on appropriate signal level for activation.

The groups were also assessed according to the weight of pups. Comparison of weight on the 21st day of life of L-NAME groups and control was not significant (55 vs 57 g). On the 42nd day, however, it was found that the weights of the subjects in the pyloric stenosis group were smaller compared to controls (160 vs 190 g). From the histological study it was observed that the c-kit stained cajal cells in the pyloric segment of the HPS animals were lesser then in the pyloric region of the controls ($p<0.001$). As shown in Table 1, in the HPS group the number of smooth muscle cells was significantly higher than in the pylorus of control animals ($p<0.005$). Similarly, in the HPS group with respect to mitotic index the number of Ki67 stained pyloric segment cells was significantly higher than in the pylorus of control animals ($p<0.001$).

Discussion

Pyloric sphincter function and motility is organized via a complex controlling system which involves enteric nervous system, gastrointestinal hormones and ICC (1–3). Estimation and evaluation of changes in the myoelectrical activity of the stomach and intestine in relation to the development of infantile HPS are important in the diagnosis of such cases. The dominant components of mutual spectral content of the pyloric signals investigated here were considered as modulators of the muscular dynamism needed for digestion. Those spectral components emerging about 1–5 CPM can correlate with the functionality or controlling mechanism behind the HPS (14). The systematic action of the distinct frequency components which were detected in both control and pyloric segment could be similar to a frequency division multiple access; each of which is controlling a relatively specialized muscular system. The type and population of particular cells, such as ICC, that locally regulate the biological slow waves could also be actively involved in the regulation of such parametric dynamism of digestion system. As has been suggested ICC produce NO and accordingly amplify nitrergic signaling (15). Supporting this suggestion, experimental studies on mutant animal models lacking intramuscular ICC have shown that intramuscular ICC are essential for nitrergic neurotransmission (16). In particular, myenteric ICC trigger the generation of spontaneous pacemaker currents which are essential for effective peristalsis, while intramuscular ICC mediate excitatory and inhibitory neurotransmission (17). That is, the loss of population of ICC results in loss of NO-dependent neurotransmission (16). The role of NO in the enteric nervous system mediating pyloric relaxation has been well-described in the literature (7).

Any pathologic condition could cause the rhythmic events to change toward bradygastria or tachygastria according to the involved pathogenesis as has been mentioned earlier (14). For example, in the present experiment it was found that the number of immunopositive cells for c-kit has significantly decreased in the pyloric area of the L-NAME received animals compared with pyloric region of the controls. The Cajal cells in the pyloric region of the HPS animals were lesser then in the control groups. In contrast, the number of smooth muscle cells was significantly higher in the pyloric segment of the HPS group. The reduction of intra muscular ICC and associated nerves in the pyloric area could explain the emergence of high pyloric signal's tone or spectrum and decreased compliance of the stomach. Previously, in this direction, Barajas-Lopez et al (4) have demonstrated that ICC, as the pacemaker cells, are regulating electrical waves in intestinal smooth muscles. Langer et al. (18) reported a decrease in ICC count in the HPS patients. This finding was confirmed by immunohistochemistry using a specific antiserum raised against c-kit, a tyrosine kinase receptor expressed by interstitial cells (19).

The association between ICC and NO in the HPS patients has recently been explored using immunohistochemical staining and semi-quantitative analysis. Through such analysis both ICC and NO expressions were found to be either absent or reduced in the HPS (3, 11). Therefore, the hypomotility of the postpyloric segment can also be explained by the absence of slow wave generation by the ICC. On the other hand, the hypertrophy of smooth muscle cells in the HPS group may contribute to the strength of the signal. Nonetheless, to go further than speculations the reality behind the digestive mechanism and the assumption made for the explanation of pyloric myoelectrical signals need further investigation.

This particular study, to our knowledge, is the first trial toward demonstrating the direct influence of L-NAME on the myoelectrical activity of the pyloric muscle cells in vivo. Our results give rise to questions about the possible role of NO in human gastrointestinal system. The findings suggest that the functional regulation of the pylorus may be particularly dependent on NO and especially prone to dysfunction in its absence.

References

1. Langer J, Berezin I, Daniel EE. Hypertrophic pyloric stenosis: ultrastructural abnormalities of enteric nerves and the interstitial cells of Cajal. J Pediatr Surg 1995; 30: 1535–1543.

2. Takaki M. Gut pacemaker cells: the interstitial cells of Cajal (ICC). J Smooth Muscle Res 2003; 39: 137–161.

3. Liang J, Co E, Zhang M, Pineda J, Chen JD. Development of gastric slow waves in preterm infants measured by electrogastrography. Am J Physiol 1998; 274: G503–508.

4. Barajas-Lopez C, Berezin I, Daniel EE, Huizinga JD. Pacemaker activity recorded in interstitial cells of Cajal of the gastrointestinal tract. Am J Physiol 1989; 257: C830–835.

5. Cooper WO, Griffin MR Arbogast P, Hickson GB, Gautam S, Ray WA. Very early exposure to erythromycin and infantile hypertrophic pyloric stenosis. Arch Pediatr Adolesc Med 2002; 156 (7): 647–650.

6. Rogers IM, Macgillion F, Drainer IK. Congenital hypertrophic pyloric stenosis: a gastrin hypothesis pursued. J Pediatr Surg 1976; 11 (2): 173–176.

7. Wiley JW. The many faces of nitric oxide: cytotoxic, cytoprotective or both. Neurogastroenterol Motil 2007; 19: 541–544.

8. Ordog T, Redelman D, Horvath VJ, Miller LJ, Horowitz B, Sanders KM. Quantitative analysis by flow cytometry of interstitial cells of Cajal, pacemakers, and mediators of neurotransmission in the gastrointestinal tract. Cytometry A 2004; 62: 139–149.

9. Vanderwinden JM, Rumessen JJ. Interstitial cells of Cajal in human gut and gastrointestinal disease. Microsc Res Tech 1999; 47: 344–360.

10. Vanderwinden JM, Mailleux P, Schiffmann SN, Vanderhaeghen JJ, Laet MHD. Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. N Engl J Med 1992; 327: 511–515.

11. Abel RM. The ontogeny of the peptide innervation of the human pylorus, with special reference to understanding the aetiology and pathogenesis of infantile hypertrophic pyloric stenosis. J Pediatr Surg 1996; 31 (4): 490–497.

12. Barbosa IM, Ferrante SM, Mandarim-De-Lacerda CA. Role of nitric oxide synthase in the etiopathogenesis of hypertrophic pyloric stenosis in infants. J Pediatr (Rio J) 2001; 77 (4): 307–312.

13. Celik O, Tagluk ME, Hascalik S,. Spectrotemporal changes in electrical activity of myometrium due to recombinant follicle-stimulating hormone preparations follitropin alfa and beta. Fertil Steril 2008; 90 (Suppl 4): 1348–1356.

14. Bókay J, Kis E, Verebély T. Changes in gastric myoelectrical activity in hypertrophic pyloric stenosis and after surgical correction. Pediatr Surg Int 2004; 20 (5): 343–347.

15. Publicover NG, Hammond EM, Sanders KM. Amplification of nitric oxide signaling by interstitial cells isolated from canine colon. Proc Natl Acad Sci USA 1993; 90: 2087–2091.

16. Ward SM. Interstitial cells of Cajal in enteric neurotransmission. Gut 2000; 47: iv40–43.

17. Huizinga JD. Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside II. Gastric motility: lessons from mutant mice on slow waves and innervation. Am J Physiol Gastrointest Liver Physiol 200; 281: 1129–1134.

18. Langer JC, Berezin I, Daniel EE. Hypertrophic pyloric stenosis: ultrastructural abnormalities of enteric nerves and the interstitial cells of Cajal. J Pediatr Surg 1995; 30 (11): 1535–1543.

19. Vanderwinden JM, Liu H, De Laet MH, Vanderhaeghen JJ. Study of interstitial cells of Cajal in infantile hypertrophic pyloric stenosis. Gastroenterol 1996; 111: 279–288.

Received October 7, 2010.
Accepted November 8, 2010.