

## RAT DUODENAL MOTILITY IN VITRO: PROKINETIC EFFECTS OF DL-HOMOCYSTEINE THIOACTONE AND MODULATION OF NITRIC OXIDE MEDIATED INHIBITION

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**Abstract** - Homocysteine is a significant but modifiable risk factor for vascular diseases. As gastrointestinal smooth musculature is similar to blood vessel muscles, we investigated how elevated homocysteine levels affect nitric oxide-mediated neurotransmission in the gut. There is accumulated evidence that a dysfunction of NO neurons in the myenteric plexus may cause various diseases in the gastrointestinal tract such as achalasia, diabetic gastroparesis and infantile hypertrophic pyloric stenosis. In the present study, we aimed to assess the effects of homocysteine on NO-mediated responses *in vitro*, and to examine the effects of DL-homocysteine thioactone on the spontaneous motility of rat duodenum and nitrergic neurotransmission. DL-homocysteine thioactone concentration of 10  $\mu\text{mol/L}$  leads to the immediate increase in tone, amplitude and frequency of spontaneous movements in isolated rat duodenum. L-NAME (30  $\mu\text{mol/L}$ ) leads to an increase in basal tone, amplitude and frequency of spontaneous contractions. The relaxations induced by EFS were significantly reduced in duodenal segments incubated in DL-homocysteine thioactone compared with the control group. EFS-induced relaxations were inhibited by L-NAME in both experimental and control groups. These results suggest that a high level of homocysteine causes an important impairment of non-adrenergic non-cholinergic innervation of the rat duodenum.

**Key words:** DL-homocysteine thioactone, motility, nitric oxide, electrical field stimulation, duodenum, rat

### INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid that is not used for the synthesis of proteins (de Bree et al., 2002). Hcy is formed when cells metabolize the essential amino acid methionine. An elevated plasma homocysteine level is a risk factor for vascular disease, including coronary, cerebrovascular or peripheral arterial disease, and is a good predictor of atherothrombotic events (Eichinger et al., 1998; Hankey and Eikelboom, 1999; Lentz, 1998). Mecha-

nisms responsible for the association between hyperhomocysteinemia and vascular disease are poorly understood (Lentz et al., 1996). While several pathological processes may be involved, homocysteine can cause significant endothelial impairment and compromise vascular NO bioactivity (Fu et al., 2002).

Gastrointestinal smooth musculature is similar to blood vessel muscles. We investigated how elevated homocysteine levels affect NO-mediated neurotransmission in the gut.

The mammalian gut wall contains a distinct class of intrinsic inhibitory motoneurons, the so-called non-adrenergic non-cholinergic (NANC) neurons (Glaskow et al., 1998). These neurons mediate functional relaxations of the gut. Nitrergic neurotransmitters, acting via the release of NO, are thought to be the responsible transmitters of this NANC inhibitory motor innervation. NO serves as the primary enteric inhibitory neurotransmitter in GI muscles, and nitrergic neurons regulate gut tone, phasic contractile amplitude, frequency, and inhibitory reflexes (Sanders and Ward, 1992). NO also regulates the accommodation reflex of the fundus and the peristaltic reflex of the intestine (Takahashi, 2003). At least a portion of the mechanical effect of NO are a consequence of the hyperpolarization of membrane potential that results in reduced smooth muscle excitability. There is accumulated evidence that a dysfunction in NO neurons in the myenteric plexus may cause various diseases in the gastrointestinal tract such as achalasia, diabetic gastroparesis, infantile hypertrophic pyloric stenosis and Hirschsprung's disease. Reduced NO release and/or nNOS expression are suspicious in a subset of patients with functional dyspepsia. Although the etiology of intestinal pseudo-obstruction remains unknown, it is conceivable that extrinsic denervation may upregulate nNOS expression, resulting in enhanced muscular relaxation and disturbed peristalsis (Takahashi, 2003).

Hyperhomocysteinemia (HHcy) has been implicated in inflammation and remodeling in the intestinal vasculature and it is known to aggravate the pathogenesis of inflammatory bowel disease (IBD). There are results indicating that HHcy decreases intestinal motility due to matrix metallo-proteinase-9 (MMP-9)-induced intestinal remodeling leading to constipation (Givvimani et al., 2012). Oral diet for 12 weeks was able to reduce constipation and laxative use and improve gut health. It is assumed that this will lead to improved plasma levels of homocysteine (Sturtzel et al., 2010). Increased homocysteine levels in the colonic mucosa and plasma of patients with inflammatory bowel disease may play a role in the pathogenesis of Crohn's disease and ulcerative colitis. Patients who have inflammatory bowel disease are

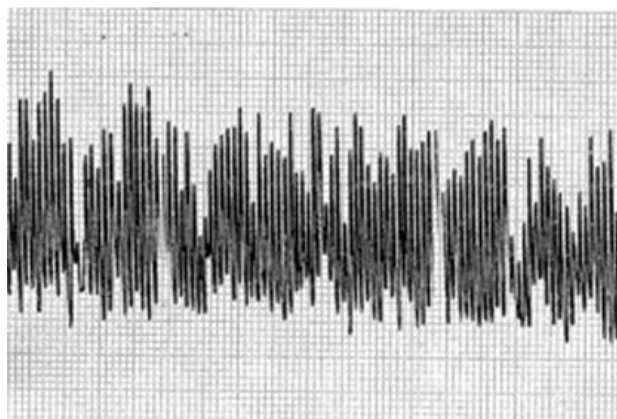
also at higher risk for hyperhomocysteinemia, which increases their susceptibility to thrombotic events. Patients with inflammatory bowel disease (IBD) frequently experience thromboembolic complications (Papa et al., 2001) which represent an important cause of morbidity and mortality. Elevated homocysteine levels increased the risk of Crohn's disease and ulcerative colitis. Vitamin B12 and folate supplementation in patients with IBD significantly reduced homocysteine concentrations (Fernández-Miranda et al., 2005).

The aim of this study was to investigate the effects of DL-homocysteine thiolactone on the spontaneous motility of isolated rat duodenum, and on the effects of NANC relaxation of this part of the small intestine.

## MATERIALS AND METHODS

Experiments were carried out on male Wistar albino rats, weighing from 250-300 g. After the animals were killed by cervical dislocation under urethane anesthesia, a duodenal segment about 2 cm in length was quickly removed just distal to the pylorus and placed in a 50-ml thermostatically controlled (37°C) organ bath containing Tyrode's solution in the following composition (mM): NaCl 136.9, KCl 2.7, CaCl<sub>2</sub> 1.0, NaHCO<sub>3</sub> 11.9, NaH<sub>2</sub>PO<sub>4</sub> 0.4 and glucose 5.6. The pH of the solution was 7.4 during bubbling with 5% CO<sub>2</sub> in O<sub>2</sub>. The preparations were placed under a resting tension of 1 g. Contractile tone and its variations were recorded by means of an isometric transducer connected to a Sensor Medics Dynograph recorder R511A recorder. To produce adrenergic and cholinergic blockade, all experiments were performed in the presence of atropine ( $3 \times 10^{-6}$  M) plus guanethidine ( $3 \times 10^{-6}$  M).

To determine the effect of NO on spontaneous contractions of longitudinal duodenal muscle strips, segments were exposed to L-NAME (NOS inhibitor) and L-arginine (the substrate for NOS). Electrical field stimulation was delivered by platinum electrodes placed parallel to the strip, connected to a Ugo Basile stimulator. Square wave pulses (0.5 ms pulse



**Fig. 1.** Spontaneous activity of longitudinal muscle strips of isolated rat duodenum during a 6 min period.

duration, maximal voltage, 50 V) were delivered for 10 s frequencies of 1 to 5 Hz.

Tissue responses (resting tone, amplitude of contraction) were measured as changes in isometric tension of the duodenum. The responses were then calculated and expressed in milligrams (mg). Frequency of contractions was calculated as the number of contractions per minute.

Before testing, segments were allowed to equilibrate in Tyrodé's solution for 60 min. During this time, the nutrient solution was changed every 20 min.

The drugs used were DL-homocysteine thiolactone, L-N<sup>G</sup>-nitroarginine methyl ester (L-NAME), L-arginine, guanethidine, atropine, and all were obtained from Sigma Chemical Company (St Louis, MO, USA).

#### *Statistical analysis*

All values are expressed as the mean  $\pm$  SEM. Statistical evaluation was done with the software GraphPad Prism InStat 5.0 (GraphPad Software; CA, USA). The data were analyzed by means of two-way analysis of variance (ANOVA). Statistical evaluation of the data

was done following Student's t-test. For all comparisons,  $p < 0.05$  was considered significant.

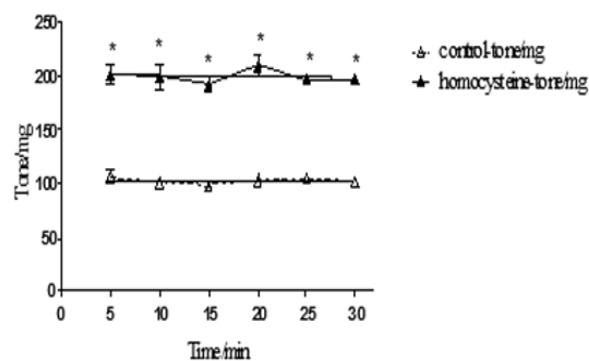
## RESULTS

### *Spontaneous activity of longitudinal muscle strips of isolated rat duodenum*

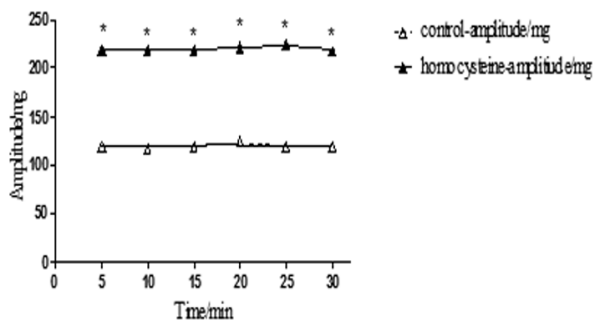
After a one-hour adaptation period, duodenal muscle strips showed spontaneous motility. Amplitude moved from 118.10 to 124.60 mg, with a mean value of  $120.30 \pm 0.93$  mg ( $n=10$ ). Frequency of spontaneous movements was 9.17-10.33 per minute, average  $9.81 \pm 0.17$ . The tonus of spontaneous contractions was 98.33-106.70, with a mean value  $102.90 \pm 1.17$  mg (Fig. 1).

### *Effects of DL-homocysteine thiolactone on the tone, amplitude and frequency of spontaneous contractions of isolated rat duodenum*

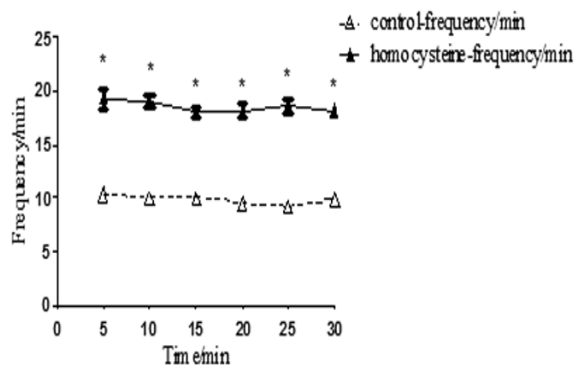
DL-homocysteine thiolactone in a concentration of 10  $\mu\text{mol/L}$  increased the resting tone, amplitude and frequency, and this effect takes place and reaches maximum after 30 min incubation period (Figs. 2, 3, 4). The difference between control values and values in the presence of DL-homocysteine thiolactone in the 30-min period was assessed every 5 min. The mean values were tested by Student t test. The difference was statistically significant ( $*p < 0.05$ ).



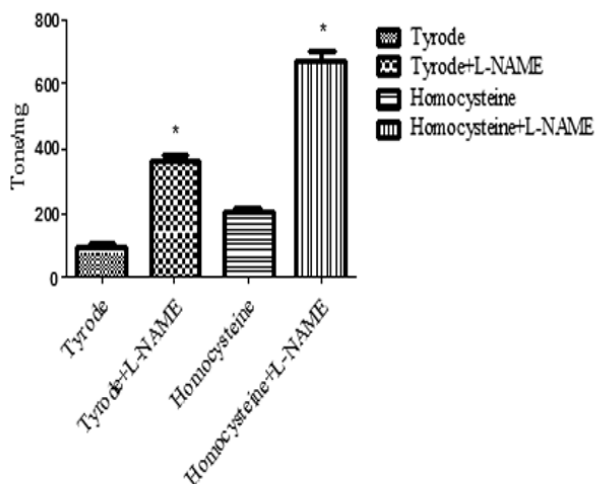
**Fig. 2.** The effects of DL-homocysteine thiolactone on the tone of spontaneous contractions of isolated rat duodenum (\*statistically different from the control group,  $p < 0.05$ ).



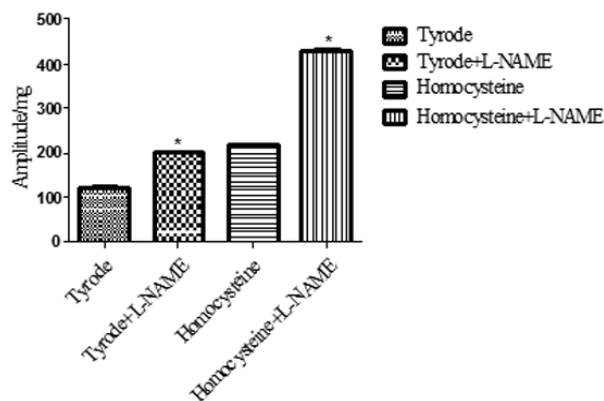
**Fig. 3.** The effects of DL-homocysteine thiolacton on the amplitude of spontaneous contractions of isolated rat duodenum (\*statistically different from the control group,  $p < 0.05$ ).



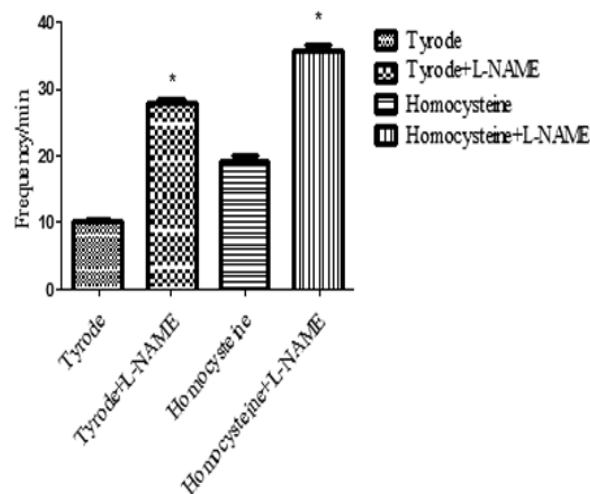
**Fig. 4.** The effects of DL-homocysteine thiolactone on the frequency of spontaneous contractions of isolated rat duodenum (\*statistically different from the control group,  $p < 0.05$ ).



**Fig. 5.** The effects of L-NAME on the tone of spontaneous contractions of isolated rat duodenum (\*statistically different from the control group,  $p < 0.05$ ).



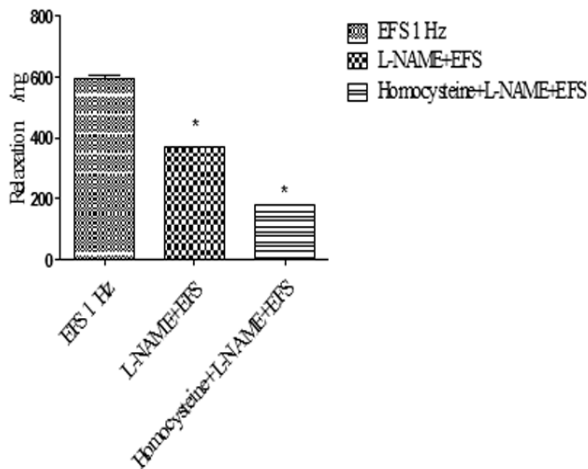
**Fig. 6.** The effects of L-NAME on the amplitude of spontaneous contractions of isolated rat duodenum (\*statistically different from the control group,  $p < 0.05$ ).



**Fig. 7.** The effects of L-NAME on the frequency of spontaneous contractions of isolated rat duodenum (\*statistically different from the control group,  $p < 0.05$ ).

*Effects of L-NAME and L-arginine on the tone, amplitude and frequency of spontaneous contractions of isolated rat duodenum*

L-NAME increases the resting tone, amplitude and frequency of spontaneous contractions of longitudinal muscle layers of isolated rat duodenum compared with the control group (Figs. 5, 6, 7). This effect appears after application and lasts for 5 min. When the compound was applied 5 min before the addition of L-NAME incubated in 10  $\mu\text{mol/L}$  L-arginine, the ef-



**Fig. 8.** L-NAME impact on the effects of electrical field stimulation of isolated duodenal segments in the presence of DL-homocysteine thiolactone (\*statistically different from the control group,  $p < 0.05$ ).

effects of L-NAME were significantly reduced, but not completely blocked.

*Effects of L-NAME on the tone, amplitude and frequency of spontaneous contractions of isolated rat duodenum in the presence of DL-homocysteine thiolactone*

The samples were incubated for 30 min in the presence of DL-homocysteine thiolactone, and then  $30 \mu\text{mol/L}$  L-NAME was added. There was an increase in the resting tone, amplitude and frequency of spontaneous contractions of the longitudinal muscle layers of isolated rat duodenum compared with the control group (Figs. 5, 6, 7). The mean values were tested by Student's *t* test. The difference was statistically significant ( $*p < 0.05$ ).

*Effects of electrical field stimulation on spontaneous activity of isolated duodenal segments*

Isolated longitudinal duodenal segments were stimulated for 10 s. At the beginning of stimulation, there was a relaxation that lasted for 1 s. After stimulation "off" response was made, the contractions lasted 1.5 s in all experiments.

*L-NAME impact on the effects of electrical field stimulation of isolated duodenal segments in the presence of DL-homocysteine thiolactone*

Duodenal preparations were initially exposed to a concentration of DL-homocysteine thiolactone of  $10 \mu\text{mol/L}$ . A current increase of the amplitude of spontaneous contractions was observed. Immediately after the addition of homocysteine in the organ bath, electrical field stimulation (50 V, 1Hz, 0.5 ms, 10 s) was carried out. After 5 min,  $30 \mu\text{mol/L}$  of L-NAME was administered and the effects of L-NAME were recorded at the end of the incubation period (Fig. 8).

## DISCUSSION

Our research was conducted to investigate the effects of DL-homocysteine thiolactone on NANC neurotransmission in isolated rat duodenum. Increasing epidemiological and experimental studies indicate that even a mild elevation of plasma homocysteine (Hcy) concentration (plasma Hcy  $> 16 \mu\text{mol/L}$ ) is an independent risk factor for occlusive diseases, atherosclerosis, diabetes mellitus, hypertension and end-stage renal failure (Stampfer and Malinow, 1995). However, to our knowledge, its effect in human gastrointestinal (GI) smooth muscle has not been studied in detail.

In this paper we wanted to examine the effects of DL-homocysteine thiolactone on duodenal motility, which has proved to have a prokinetic effect. Our results show that DL-homocysteine thiolactone increases the resting tone, amplitude and the frequency of spontaneous contractions. Our results are consistent with those of Park et al., which suggest that sulfur-containing amino acids like DL-homocysteine potentiate the depolarization of murine proximal colon cells. These effects include increasing the amplitude and frequency of spontaneous contractions of murine colonic stripes (Park et al., 2005).

Choe et al. (2012) investigated the effects of methionine on the contractile activity of human colon smooth muscle *in vitro*. Methionine is a sulfur-con-

taining amino acid that is transformed into homocysteine during biometabolism. Their results indicate that methionine increases the amplitude of colonic muscle strips.

It has been widely recognized that stimulation of a certain class of nerves within the wall of the gut elicits relaxation (Allescher et al., 1993; Mourelle et al., 1993; Szilvassy et al., 1996). These nerves are non-adrenergic and non-cholinergic and have been commonly referred to as NANC nerves. These neurons are extremely important because they mediate the majority of inhibitory responses in the GI tract and regulate many important physiological reflexes, such as relaxation of the lower esophageal sphincter after swallowing, receptive relaxation of the proximal stomach during eating, and reducing inhibition in response to distension (Takahashi, 2003). Many reports have identified a role of NO in NANC neuromuscular transmission in many tissues, such as the ileocolonic junction and duodenum of dog, the gastric fundus in rat and the low esophageal sphincter in opossum (Kasakov et al., 1995; Bult et al., 1990; Toda et al., 1991; Boeckxstaens et al., 1991; Tottrup et al., 1991). The most potent inhibitor of NO production is L-NAME (Rees et al., 1990). L-NAME has been used by many investigators to determine the role of endogenous nitric oxide in various physiological and pathophysiological conditions.

In our experiments, L-NAME showed an increase in the resting tone, amplitude and frequency of the contractions of isolated duodenal segments. It has been shown that L-arginine reversed the action of L-NAME. In the presence of L-NAME, treatment with 10  $\mu\text{mol/L}$  DL-homocysteine thiolactone caused a significant increase in resting tone, amplitude and the frequency of contractions. These results suggest that the mechanisms of Hcy action on duodenal segment contractions are based on the modulation of nitrergic neurotransmission in the gut.

Transmural electrical stimulation of all segments induced relaxation that was maximal at frequency of 10 Hz. Relaxation occurred at the beginning of stimulation "on" response. A rebound contraction

was observed after cessation of the stimulus – "off" response.

Karasu et al. (2008) showed that EFS-induced relaxations are mediated mainly by NO in rat duodenum. These findings suggest that the impairment of nitrergic innervation of rat duodenum may contribute to abnormalities of intestinal motility.

In our study, L-NAME, a nitric oxide synthase inhibitor, inhibited low frequency electrical stimulation-induced relaxation. This indicated that endogenous NO is involved in NANC relaxation of rat duodenum. These data confirm the evidence of the participation of the L-arginine-NO pathway in neurally induced isolated rat duodenum. Importantly, NANC relaxations induced by low frequencies of EFS were significantly changed in duodenal preparations obtained from duodenal segments treated with high homocysteine concentration levels. These findings suggest that homocysteine causes an important impairment of NANC innervation of the rat duodenum. Contrary to our results, Karasu et al. (2008) suggest that experimental hyperhomocysteinemia, induced by methionine diet 12 weeks prior to study, does not cause a significant change in NANC-mediated responses of rat duodenum.

DL-homocysteine thiolactone enhances the contractile activity of isolated rat duodenum. DL-homocysteine thiolactone also reverses the effect of nitrergic stimulation in the duodenal muscle strip. We assume that a compound mimicking DL-homocysteine thiolactone may provide prokinetic functions in the human gastrointestinal tract. Motility disorders that seem to benefit from prokinetics are postoperative ileus, acute colonic pseudo-obstruction, chronic intestinal pseudo-obstruction, idiopathic constipation, Hirschsprung's disease and constipation.

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