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Research article

DIFFERENT PATHWAYS INVOLVED IN THE STIMULATORY EFFECTS OF HOMOCYSTEINE ON RAT DUODENAL SMOOTH MUSCLE

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Recent studies have confirmed that hyperhomocysteinemia is associated with gastrointestinal diseases; however, the direct effect of homocysteine on gastrointestinal reactivity still remains unknown. The aim of this study was to demonstrate how homocysteine may affect nitric oxide mediated duodenal relaxation and whether cholinergic receptors and K^+ channels take part in stimulating motility, as well as to explore whether oxidative stress is associated with homocysteine-mediated effects. Experiments were carried out on male rats, body mass 250-300 g. Two groups of animals were treated by i.p. application of saline and D,L-Hcy (0.6 μmol/g bm). After 2h of incubation, the duodenal segments were prepared for biochemical analysis and contractile response measurements in an organ bath with Tyrode's solution. Effects of TEA (10 mmol/L) and L-NAME (30 μmol/L) on duodenal contractility in the presence of D,L-Hcy (0.6 μmol/g bm) were investigated. Elevated homocysteine levels seem to be of crucial importance for the deterioration of contractility through nitric oxide mediated relaxation, and, in part, by activation of K^+ channels. Hcy showed direct promuscarinic effects, since 30 min pretreatment of rat duodenum significantly enhanced the contractile effect of increasing concentrations of ACh (10^{-9}-10^{-2} mol/L). Catalase activity, superoxide dismutase, glutathione peroxidase and the total antioxidant system were reduced while the thiobarbituric acid-reactive substances level was elevated. Our data showed a consistent profile of gastrointestinal injury elicited by sulfur-containing amino acid-homocysteine. This could contribute to explain, at least in part, the mechanisms involved in human gastrointestinal diseases associated to hyperhomocysteinemia.

Keywords Contractility; Duodenum; Homocysteine; Oxidative stress; Rat

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INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid synthesized during transformation of methionine to cysteine in methionine metabolism [1]. Several observational studies have shown that a raised blood Hcy level – hyperhomocysteinemia (HHC) is a risk factor for cardiovascular events, including ischemic heart disease, chronic kidney disease and cerebrovascular disease [2,3].

Numerous clinical studies have shown that an elevated plasma homocysteine level might be an independent risk factor for gastrointestinal diseases. Recent data have explored the relation between HHC and inflammatory bowel disease (IBD) condition, and some authors have suggested an involvement of Hcy in the pathogenesis of these diseases [4,5]. Inflammatory bowel disease, including Crohn's disease (CD) and ulcerative colitis (UC), are a group of chronic intestinal disorders with a multifactorial etiology [6]. A study by Jiang et al. [7] showed that Hcy-related gene and metabolites are involved in the pathogenesis of UC. Increased homocysteine levels in the colonic mucosa and plasma of patients with IBD may play a role in the pathogenesis of CD and UC [8]. Evolution of CD can be riddled with intestinal and extra-intestinal complications, particularly atherothrombotic events [9]. However, the increased Hcy concentrations in patients with IBD may also be a consequence of the disease itself because the gastrointestinal tract is responsible for much of the metabolism of sulfur amino acids.

A proinflammatory role of Hcy in IBD was also suggested [10]. Hyperhomocysteinemia is relatively frequent in patients with celiac disease [11], and may be a risk marker for colorectal cancer, gastric cancer and increased colorectal carcinogenesis in IBD patients [12-14]. These findings lead us to investigate Hcy effects on the gastrointestinal system under laboratory conditions.

Literature data are controversial about the effects of HHC on gastrointestinal contractility. Intestinal contractility is decreased in HHC due to matrix metalloproteinase-9 (MMP-9)-induced intestinal remodeling in mice colon [15]. Increased plasma Hcy concentration has been implicated in constipation (lowered fecal output), indicating that HHC itself may be causing gastrointestinal distress. Constipation as a motility disorder is common in the elderly. Lowering plasma levels of homocysteine in nursing home residents improved constipation [16].

It is well known that NO is released by non-adrenergic, non-cholinergic (NANC) inhibitory neurons in a variety of tissues, including gastrointestinal (GI) smooth muscles [17]. Nitric oxide synthesizing neurons are distributed extensively in the mysenteric and submucosal nerve networks of rats and humans [18-21]. Functional *in vitro* and *in vivo* studies provide convincing evidence in support of the notion that NO is released by NANC inhibitory motoneurons mediating relaxation of the mammalian gut [22-24].

Recent studies have suggested that part of the hyperpolarizing effects of NO may be mediated by stretch-dependent Kþ (SDK) channels that are expressed in GI smooth muscles [25]. Previously was demonstrated that the sulfur-containing amino acid methionine acted as a specific blocker of stretch dependent potassium channels and nitrergic responses in the murine colon [26].

Hyperhomocysteinaemia has been suggested to be implicated in the imbalance between pro-oxidants and antioxidants linked to its pro-oxidant properties or in the impairment of antioxidant systems. Antioxidant defense is composed of enzymatic (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx), and non-enzymatic (vitamin A, vitamin E, vitamin C, thiol antioxidants, albumin, bilirubin, uric acid) antioxidants [27]. Total antioxidant status (TAS) consists of all antioxidants present in body fluids. Lipid peroxidation, which is mediated by free radicals, is considered to be the major mechanism of cell membrane destruction and cell damage. Alteration in the oxidant - antioxidant profile is known to occur in IBD. Growing evidence suggests that HHC may promote chronic intestinal mucosa inflammation, mainly through oxidative stress [28-30]. Oxidative stress induces not only gastric mucosal injury, but also gastric motility dysfunction, such as diabetic gastroparesis. Gastroparesis is thought to be caused by ROS-induced damage of the networks of the interstitial cells of Cajal [31].

The aim of the current study was to test the hypotheses that a high level of homocysteine was associated with the digestive motility disorders by impaired NO-dependent relaxation of the gastrointestinal smooth muscle by activating SDK channels and by increasing oxidative stress. We also tested cholinergic receptors involvement in homocysteine effects.

MATERIAL AND METHODS

Acute D,L-homocysteine administration

Sixteen male adult albino rats (Rattus norvegicus) of the Wistar strain (3 months old) weighing 220 ± 20 g were used. Four animals were housed per cage. Animals were acclimatized to standard animal laboratory conditions for five days (12:12-h light–dark cycle, temperature 22 ± 2 °C and relative humidity 50 ± 5%). Rats were randomly divided into two groups of eight rats each for the control group (C) and experimental group (EG). They received a single intraperitoneal (i.p.) injection of 1 ml of saline (C) or 0.6 μmol/g body mass of D,L-homocysteine (EG). After incubation of 2 hours in standard laboratory conditions, the animals were sacrificed by decapitation and dissection of the duodenum was performed. Organ samples of both groups were immediately removed and prepared for biochemical assays and contractile response measurements. After the weight was measured, 0.5 g segments of tissue were homogenized in 5ml of phosphate buffer. The homogenates were centrifuged (10 min, 10000 rpm) and the clear supernatant was kept at -20C° for biochemical analysis (index of lipid peroxidation, total antioxidant status- TAS, CAT, SOD, GPx activity

and acetylcholinesterase activity). The contractile response was obtained in the isolated organ bath.

Isolated duodenum preparation

As previously described, after 2 h incubation in the D,L-Hcy, duodenal segments of about 2 cm in length were quickly removed just distal to the pylorus and vertically suspended in a 50 ml isolated organ bath filled with Tyrode's solution of the following composition (mM) : NaCl 136.9, KCl 2.7, $CaCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.6. The solution was maintained at 36±1ºC and gassed with a mixture of O_2 (95%) and CO_2 (5%) at atmospheric pressure. After an equilibration period of 30 min, contractile responses were recorded with an isometric transducer connected to a Sensor Medics Dynograph R511A recorder. Preparations were placed under a resting tension of 1 g. Tissue responses (resting tone, amplitude of contractions) were measured as changes in isometric tension of the duodenum. The responses were than calculated and expressed in milligrams (mg). Frequency of contractions was calculated as the number of contractions per minute.

Cumulative concentration-contractile response curves for acetylcholine in the absence and presence of D,L-homocysteine were determined at 15 min intervals. Each dose of acetylcholine was allowed to produce its full effect (15 ± 3 s contact) before the concentration of the drug in the bath was increased in geometric progression by ratio two. Each experiment was separated from the other by at least two washes, in order to obtain the same baseline. Responses from each experiment were demonstrated as amplitude of the contraction shown as mg of tension. Subsequent concentration-response curves were drawn and the EC_{50} values (the concentration producing a 50% maximal contractile effect) were determined for both groups by the Hill transformation: log E/log (Emax - E) versus log concentration.

To determine the involvement of NO and potassium channels on D,L-homocysteine effects on duodenal contractions, segments were exposed to NOS inhibitor L-NAME (N-nitro-L-arginine methyl ester) 10μmol/L and K-channels blocker TEA (Tetraethylammonium chloride) 10 mmol/L.

Measurement of acetylcholinesterase activity

Acetylcholinesterase activity was determined by Ellman's method. The incubation mixture contained duodenal homogenate in phosphate buffer (pH 8.0). The mixture was incubated at 37 ºC for 10 minutes. Acetylcholine iodide and 5,5'-dithionitrobenzoic acid (DTNB), used as substrates, were added, and the reaction was started. The reaction was monitored spectrophotometrically (Gilford Instrument, Model 250) by an increase in the absorbance (ΔA) at 412nm. An assay, without the tissue homogenate, was used as a blank probe. The measurements were assessed with double probes, and the specific AChE activity was presented as U/mg protein.

Index of lipid peroxidation measurement

Lipid peroxidation level was estimated by measurement of thiobarbituric acid reactive substances (TBARS) in the tissue according to the fluorimetric method by Yagi [32]. The pink chromogen produced by the reaction of thiobarbituric acid with lipid peroxidation products such as malondialdehyde (MDA) was estimated using 1,1,3,3-tetraethoxypropane as standard MDA. The absorbance of clear supernatant was measured against reference blank at 535 nm. Results were expressed as μmol/mg proteins.

Tissue total antioxidant status (TAS)

TAS was measured on Ultrospect 2000 Analyzer with Randox reagent set (Randox). The determination was based on the reaction of 2,2'-azino-di-(3-ethylbenythiazoline sulfonate) ($ABTS^+$) with peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$ [33]. The ABTS radical cation ($ABTS^+$) was produced by reacting ABTS stock solution with a 2.45 mM potassium persulfate (final concentration) and incubating the solution in the dark at room temperature for 12–16 h before use. Trolox (a Vitamin E analogue) standards (Calbiochem) were prepared in PBS over the range 0–15 μM. For the standardization of the starting point of all assays and standards, a 10 μl sample/Trolox standard was added to 1 ml ABTS ± solution (A_{734} nm = 0.700 ± 0.020) and an absorbance reading taken exactly 1 min after initial mixing until the decrease in absorbance ceased. Antioxidant activity was expressed as mmol Trolox/ mg protein).

Catalase assay

Catalase (CAT) activity was determined spectrophotometrically according to Aebi et al. [34]. This method was based on the disappearance of H_2O_2 at 240 nm in the reaction medium containing 30 mM H_2O_2, 50 mmol/L potassium phosphate buffer pH 7.0 and 0.1 ml sample. Catalase activity was expressed as U/mg protein.

Determination of superoxide dismutase (SOD) activity

The activity of SOD was measured as the percent of inhibition of epinephrine autooxidation under base conditions by tissue sampling. The activity of total SOD was measured kinetically, as the change of extinction in time (10 minutes) at 480 nm wavelength [35]. The reaction mixture contained Na-bicarbonate buffer (50 mmol/L, pH 10.2) and epinephrine (0.5mmol/L) and 0.1 ml of the sample; the reaction started by adding 0.1 ml of epinephrine solution (0.01 M into 0.01 M HCl). Change of extinction of the same reaction mixture into which 0.1 ml 0.01 M HCl was added was used as a blind trial relative to which the inhibition was calculated. Activity of SOD was presented as U/mg of protein.

Determination of GPx activity

Glutathione peroxidase (GPx) determination was based on oxidation of reduced GSH with GPx using NADPH in reaction catalyzed by enzyme glutathione reductase (GR). Decrease of absorbance at 340nm as a result of used NADPH+H^+ represents the measure of GPx activity in coupled reaction with GR [36].

Protein content

The protein content of the samples was determined by the method of Lowry et al. [37] using bovine serum albumin as standard.

All chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Statistical Analysis

Results were analyzed by standard statistical methods, expressed as mean ± standard error of the mean (SEM), and graphically presented (Statistical program GraphPad Prism 6). Significance of the differences between the experimental and control groups was determined by Student's t-test and Two way ANOVA. P values below 0.05 were considered statistically significant.

Ethical Considerations

The ethics protocol was approved by The Laboratory Animals Maintenance and Usage Committee of the Faculty of Medicine, Belgrade University.

RESULTS

Effects of D,L-homocysteine on isolated rat duodenal motility

The values of tone, amplitude and frequency of spontaneous contractions in longitudinal smooth muscle segments from the rat duodenum in Tyrode solution are shown in Table 1. Two hours incubation in D,L-homocysteine significantly enhanced the tone, amplitude and frequency of spontaneous contractions (Table 1.). The mean values were tested by Student's t- test. The difference was statistically significant (***$p<0.001$).

Table 1. Effects of D,L-homocysteine on the tone, amplitude and frequency of isolated rat duodenum- longitudinal muscle layer. Values are expressed as mean ± SE. ***$p<0.001$, statistically different from the control group.

	Tone (mg) (mean±SD)	Amplitude (mg) (mean±SD)	Frequency (min) (mean±SD)
Control	102.9±2.88	120.3±2.27	9.81±0.41
Hcy (0.6 μmol/g bm)	199.9±5.88***	219.3±2.29***	18.53±0.53***

Effect of D,L-homocysteine on the contractile response of rat duodenal smooth muscle

Cumulative concentration-response relationships for acetylcholine-induced contraction in duodenal smooth muscle were determined by addition of acetylcholine (ACh) in successive concentrations increments (10^{-9}-10^{-2} M). To determine whether the contractile response of ACh is modified by D,L-homocysteine (D,L-Hcy), cumulative additions of acetylcholine was also performed in the presence of D,L-homocysteine (0.6 μmol/g bm). D,L-homocysteine has been added 30 min prior to acetylcholine. Addition of the sulfur amino acid to the incubation buffer increased the baseline tone. The effects of homocysteine on the cumulative contractile responses of acetylcholine are shown in Figure 1. D,L-homocysteine modified the contractile effect of acetylcholine (Fig. 1.). The concentrations of acetylcholine that produced a half-maximal contraction were significantly different in the presence of homocysteine (*p<0.05). The EC50 values in the control (ACh) and experimental group (ACh plus D,L-Hcy) were also calculated and presented in Table 2. The dose response curve was shifted to the right in the presence of D,L- homocysteine.

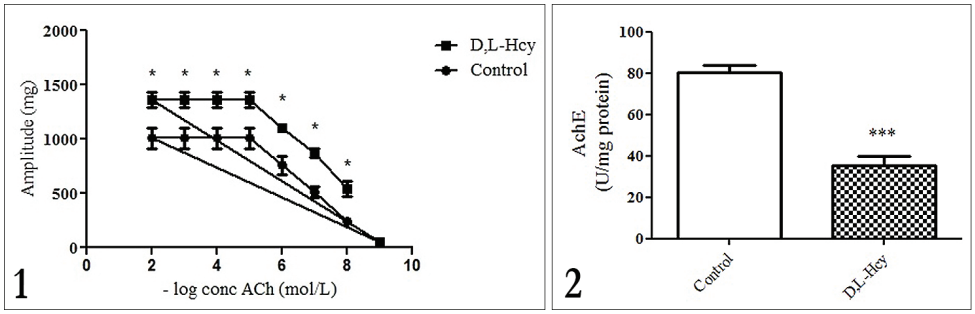

Figure 1. Dose-dependent response of rat duodenal segments to ACh alone (control) and ACh plus D,L-homocysteine (treated with 0.6 μmol/g D,L-Hcy). Each curve was drawn from the results obtained in six experiments. Ordinate scales show contractions expressed as milligrams of the maximal contractile effect. Vertical lines shows mean ± SE. (*p<0.05, statistically different from Tyrode; #p<0.05, statistically different from Homocysteine)

Figure 2. Acetylcholinesterase activity (means ± SE) in duodenal homogenates in the control group and group treated with D,L-Homocysteine (D,L-Hcy); ***p<0.001, statistically different from the control

Table 2. Values of EC50 in the control (only ACh) and experimental group (ACh plus D,L-Hcy)

EC50 value	Control	D,L-Hcy
LogEC50	-6.624	-6.827
EC50	4.207 μM	6.712μM

Acetylcholinesterase activity in homogenized duodenal segments

The AchE activity determined in homogenized duodenum in the control and experimental group (treated with D,L-Hcy) is presented in Figure 3. Enzyme activity from homogenized tissue of rats treated with D,L-homocysteine was decreased compared to the control group. Moreover, this activity was significantly lower compared to control values for the duodenum (by 55.90%) (Fig 3.).

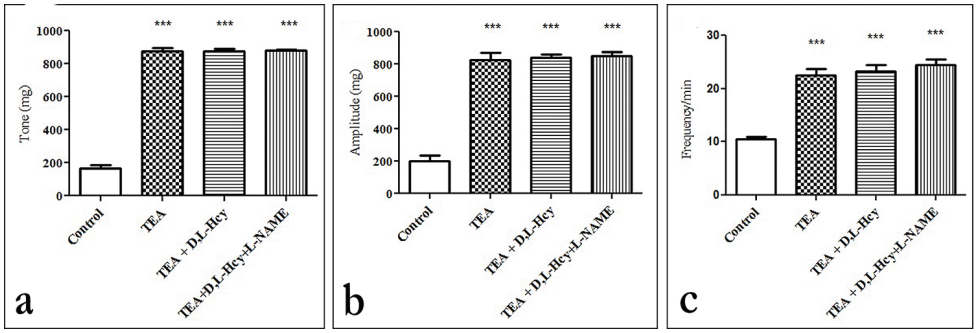

Figure 3. Effects of TEA on tone **(A)**, amplitude **(B)** and frequency **(C)** of spontaneous contractions of isolated rat duodenum in Tyrode solution (TEA) and in presence of D,L-homocysteine (TEA+D,L-Hcy). Values are expressed as mean ± SE (statistically different from the control group, ***$p<0.001$)

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

Involvement of SDK channels in nitrergic responses was tested by SDK channels nonselective blocker teatraethylamonium chloride (TEA). After 30 min incubation in the presence of 10 μmol/L TEA, L-NAME (30 μmol/L) was added (Fig. 3.). Effects of L-NAME tone ($p<0.05$), amplitude ($p<0.05$) and frequency ($p<0.05$) of spontaneous contractions of isolated duodenal muscle strips were potentiated in the presence of TEA and D,L-Hcy (Table 3.)

Table 3a. Effects of L-NAME on isolated duodenal contractions in the presence of D,L-Hcy

	Control	Tyrode+L-NAME	D,L-Hcy	D,L-Hcy+L-NAME
Tone	100%	376.47%	202.63%	665.46%
Amplitude	100%	160.02%	180.59%	355.94%
Frequency	100%	290.55%	193.02%	366.78%

Table 3b. Effects of L-NAME on isolated duodenal contractions in the presence of D,L-Hcy and TEA

	Control	TEA	TEA+D,L-Hcy	TEA+D,L-Hcy+L-NAME
Tone	100%	535.92%	578.95%	526.66%***
Amplitude	100%	401.03%	411.97%	445.53%***
Frequency	100%	200.11%	219.29%	253.46%***

Data are presented as % of changes compared to control value; ***p<0.001).

Effects of D,L-homocysteine on the biomarkers of oxidative stress in isolated rat duodenum

The acute administration of D,L-Hcy induced statistically significant changes in the values of the biomarkers of oxidative stress in the duodenum of rats sacrificed 2 h after the i.p. injection. Importantly, the activity of the anti-oxidative enzymes (CAT, SOD, GPx) was reduced when compared to the controls (Fig. 4.). Furthermore, lipid peroxidation, as assessed by TBARS production, was increased compared to the control group (Fig. 4). Our results showed a statistically significant decrease of TAS, measured as mmol Trolox/mg protein (Fig. 4).

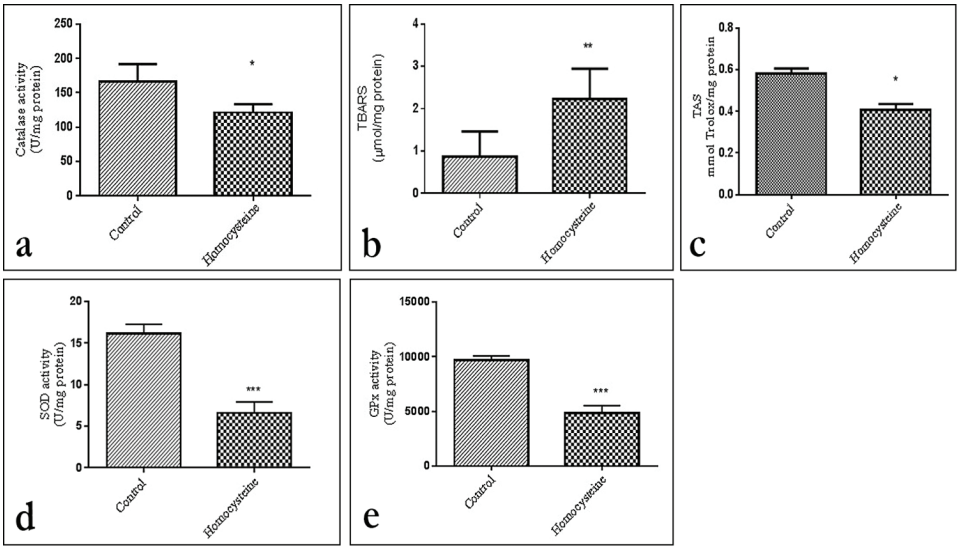

Figure 4. Effects of D,L-homocysteine on activity of catalase **(A),** TBARS concentration **(B)**, TAS concentration **(C),** SOD activity **(D)** and GPx activity (E) (statistically different from the control group; *p<0.05,**p<0.01, ***p<0.001)

DISCUSSION

The gastrointestinal tract is a significant site of sulfur amino acid metabolism [38]. The gastrointestinal tract accounts for 25% of whole body transmethylation and

transsulfuration pathways and is a prominent site of net Hcy release [15]. As reported previously, Hcy has been shown to change intestinal motility, in both a procinetic and inhibitory pattern, acting by modulation of nitrergic innervation or by potassium channels [26,39]. The present study was undertaken to expand upon those initial findings and to assess the effect of D,L-homocysteine on the duodenal motility, especially mechanisms involving nitrergic and cholinergic pathways.

In the experiments reported here, the results indicate an increase in the tone, amplitude and frequency of spontaneous contractions of isolated rat duodenum in D,L-Hcy-treated animals versus the control (Tyrode solution). The largest increase was observed in the amplitude of contraction. It has been shown previously that Hcy potentiates the depolarization of murine proximal colon cells, including the increase of the amplitude and frequency of spontaneous contractions of murine colonic stripes [26]. Our results supported this finding. Our previous work [40] showed that D,L-homocysteine thiolactone (D,L-HCT) stimulated duodenal contractility. Choe et al. [41] demonstrated that methionine, the Hcy precursor, enhanced the contractile activity of human colon smooth muscle *in vitro*. In contrast, HHC mice model showed delayed intestinal transit due to elevated levels of inflammatory cytokines and increased expression and activity of MMP-9 in colonic mucosa [15]. Although there is proven higher contractility during D,L-Hcy treatment, the mechanism by which D,L-Hcy affects duodenal contractility has not been clearly elucidated. Some of the effects could be achieved by modulating cholinergic or nitrergic innervation. Homocysteine can cause significant endothelial impairment of NO bioactivity [42]. Gastrointestinal smooth musculature is similar to blood vessel muscles, so we investigated how elevated homocysteine levels affect NO-mediated and Ach-mediated neurotransmission in the gut.

The present data demonstrated that an acute application of D,L-Hcy intensify acetylcholine-induced contraction of the isolated rat duodenum. The potentiation of ACh-induced contraction by D,L-Hcy in our preparation seems not to be related to a direct agonistic effect of D,L-Hcy on muscarinic receptors, since this sulfur amino acid does not alter the contractile effect of acetylcholine on intestinal smooth muscle, and the effect is mediated through the activation of M2 or M3 receptors [43,44].

The mammalian gut wall contains a distinct class of intrinsic inhibitory motoneurons, the so-called non-adrenergic non-cholinergic (NANC) neurons [45]. These neurons mediate functional relaxations of the gut [46]. NO also regulates the peristaltic reflex of the intestine [47]. At least a portion of the mechanical effect of NO are a consequence of the hyperpolarization of the membrane potential that results in reduced smooth muscle excitability. The most potent inhibitor of NO production is L-NAME [48] which has been used by many investigators to determine the role of nitric oxide in gastrointestinal physiological and pathophysiological conditions.

In our experiments, L-NAME enhanced the tone, amplitude and frequency of the contractions of isolated duodenal muscle strips compared to the control, but after adding D,L-Hcy in the presence of L-NAME, the higher increase appeared. Our

results suggest that the mechanisms of Hcy action on duodenal contractions are based on the modulation of nitrergic neurotransmission.

Mechanosensitive neural reflexes modulate the contractile behavior and movement of luminal contents in the GI tract [49,50]. Mechanosensitive neurons exist in both the small and large intestines and neural reflexes have been studied in vivo and in vitro [51,52]. Neurotoxins, receptor antagonists, block post-junctional ion channels mediating inhibitory responses depolarize GI muscles, increase action potentials and augment contractions [53,54]. Recent data showed that responses of colonic muscle strips to stretch are superimposed upon ionic mechanisms activated by inhibitory neurotransmitters. Stretch responses require tonic release of NO, but replacement of NO synthesized by neurons is capable of supporting stretch responses. They interpret these findings by considering that mechanosensitive ion channels in post-junctional cells modulate the gain on enteric neural inhibitory input to the colon [55].

Previous studies have shown that SDK channels are blocked by sulfur-containing amino acids, such as L-methionine, and this compound has also been shown to inhibit the SDK channels activated during nitrergic stimulation in murine colonic muscles. According to our results, TEA (10 mmol/L), a nonselective blocker of K channels, stimulates spontaneous contractility of longitudinal duodenal muscle layer. Data showed that effects of addition of L-NAME to tissues pre-incubated with D,L-Hcy and TEA (tone, amplitude, frequency) were blocked compared to the effects of L-NAME on segments preincubated in D,L homocysteine alone. We thought that K+ ion channels may modulate the enteric neural inhibitory input to the duodenum. Park et al [26] suggested that spontaneous neural activity and release of NO tonically activated SDK channels. They showed that nitrergic responses to nerve stimulation were reduced by sulfur-containing amino acids. Our data suggest that nitrergic inhibition is mediated, in part, by activation of SDK channels in rat duodenal muscles. The pharmacology of SDK channels is ambiguous at the present time, but new blockers of these channels may be potentially useful in controlling GI motility, particularly in disorders involving organ distention.

In the current study, we tested the hypotheses that homocysteine impairs oxidative stress parameters of the duodenal smooth muscle tissue. In order to verify whether high Hcy levels could alter oxidative status, we evaluated the effect of acute Hcy administration on antioxidant activity in rats.

Our results showed that acute Hcy administration decreased CAT, SOD, GPx activity, suggesting that this amino acid causes a reduction on enzymatic antioxidants in the duodenum. In agreement with our data, other studies suggest a negative correlation between plasma Hcy levels and CAT activity in the liver of rats, pointing a significant reduction of hepatic antioxidant defenses [56,57].

We also investigated the effect of Hcy on the parameters of lipid damage. Lipid peroxidation was assessed by TBARS, which identify malondialdehyde, a final product of peroxidation [32]. Lipid peroxidation serves as a marker of cellular oxidative stress

and it is recognized as a major causative factor of oxidative damage in gastrointestinal diseases. Results showed that Hcy increased lipid peroxidation in duodenal tissue homogenates. In agreement, data from literature showed that HHC increased superoxide anion production, by NAD(P)H oxidase activation, and peroxynitrite formation, resulting in lipid peroxidation in the liver of rats, which could explain many processes associated with Hcy-induced cell damage including inflammation and apoptosis in liver diseases [56,58]. Furthermore, in our previuos study, it has been demonstrated that TBARS concentrations increased after 3h incubation in D,L-homocysteine thiolactone. We may conclude that duodenal lipid peroxidation appears to be strongly associated to Hcy levels.

Our results showed that Hcy decreased the total antioxidant status of homogenates from the duodenum suggesting that this amino acid causes the acute decrease of non-enzymatic antioxidants in the duodenum. According to literature, patients with diabetes mellitus, cardiovascular disease and gastrointestinal disease, obesity and metabolic syndrome showed decreased level of TAS during chronic exposition to high levels of Hcy [59]. Hyperhomocysteinemia decreased antioxidant defenses, and increased lipid peroxidation in the duodenum of rats, characterizing a reliable oxidative stress.

Taken together, our results presented above qualified the occurrence of excitatory effects of D,L-Hcy on duodenal motility. The present results provide an additional insight into the stimulatory mechanisms of Hcy, and may contribute to explain the complex factors involved in injury exhibited in hyperhomocysteinemic patients: cholinergic stimulation, nitrergic inhibition, potassium channel inhibition and oxidative stress augmentation. So, the therapy of these patients should be complex and include corrections in all potential pathways involved in homocysteine effects on the gastrointestinal tract: antioxidative therapy, new selective blockers of potassium channels and stimulation of production of NO in nitrergic neurons.

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Author's contributions
DDj, LjŠ, DM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. MS performed research, drafted the manuscript. MS,MDj carried out biochemical analyses. VŠ, TŠ contributed new analytic tools. RŠ, SI analyzed data. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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MNOGOBROJNI MEHANIZMI UKLJUČENI SU U STIMULATORNE EFEKTE HOMOCISTEINA NA GLATKU MUSKULATURU DUODENUMA PACOVA

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Novije studije potvrdile su povezanost hiperhomocisteinemije i gastrointestinalnih oboljenja, mada su i dalje nepoznati direktni efekti homocisteina na gastrointestinalnu reaktivnost. Cilj ovog rada bio je da demonstrira kako homocistein moduliše azot monoksid zavisnu duodenalnu relaksaciju i da li holinergički receptori i K+ kanali imaju uticaja na stimulaciju motiliteta, kao i da ispita da li je oksidativni stres udružen sa efektima homocisteina. Eksperimenti su izvedeni na mužjacima pacova, telesne mase 250-300g. Dve grupe životinja tretirane su i.p. aplikacijom fiziološkog rastvora i D,L-Hcy (0,6 μmol/g tm). Posle 2h inkubacije, duodenalni segmenti su pripremljeni za biohemijske analize i praćenje kontraktilnog odgovora u izolovanom kupatilu sa Tirodovim rastvorom. Praćeni su efekti TEA (10 mmol/L) i L-NAME (30 μmol/L) na kontraktilnost duodenuma u prisustvu D,L-Hcy (0,6 μmol/g tm). Povišen nivo homocisteina bio je od ključne važnosti za pogoršanje kontraktilne funkcije, kroz relaksaciju posredovanu azot monoksidom, kao i, delom kroz aktivaciju K+ kanala. Hcy je pokazao direktno promuskarinsko dejstvo, s obzirom na to da je tridesetominutni pretretman duodenuma značajno povećao kontraktilne efekte rastućih koncentracija Ach (10-9-10-2 mol/L). Aktivnost katalaze, superoksid dismutaze, glutation peroksidaze i totalni antioksidativni status bili su sniženi, dok su vrednosti TBARS-a bile povišene.

Naši rezultati ukazali su na konzistentno oštećenje gastrointestinalnog trakta izazvano sumporovitom aminokiselinom-homocisteinom. Ovo može doprineti, bar delom, objašnjenju mehanizama uključenim u povezanost hiperhomocisteinemije sa gastrointestinalnim oboljenjima u humanoj populaciji.