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Involvement of ghrelin in nucleus tractus solitaries on gastric signal afferent and gastric motility in cisplatin-treated rats

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Abstract. – OBJECTIVE: Ghrelin had been known to promote gastric motility in human and animals previously. We aim to investigate the role of ghrelin in chemotherapy-induced nausea and vomiting.

MATERIALS AND METHODS: In the present study, we observed the changes in food intake, kaolin consumption, body weight, plasma ghrelin concentration and expression of ghrelin and its receptor GHS-R1a in the stomach and nucleus tractus solitaries (NTS) in cisplatin-treated rats, and the effects of ghrelin microinjected into NTS on the discharge activity of gastric distension (GD) responsive neurons and gastric motility were also observed.

RESULTS: Cisplatin induced the decrease in food intake and the increase in kaolin consumption of rats. In addition, mRNA expression of GHS-R1a in the stomach and NTS increased significantly after cisplatin treatment. The discharge activity of GD excited (GD-E) and GD inhibited (GD-I) neurons in cisplatin-treated rats was weaker than that of saline treatment, while ghrelin administration into NTS excited most of GD-E and GD-I neurons. Cisplatin induced the decrease in gastric contraction while ghrelin administrated into NTS promoted the gastric motility significantly. However, the amplitude and frequency of gastric contraction promoted by ghrelin in NTS of cisplatin-treated rats were lower than that of saline treated rats. The effects of ghrelin could be completely blocked by its receptor antagonist BIM28163.

CONCLUSIONS: These results indicated that ghrelin in the NTS might participate in the regulation of GD-neurons and gastric motility via its receptor in cisplatin-treated rats.

Words: Ghrelin, Nucleus tractus solitaries, Gastric motility, Cisplatin, Gastric distension responsive neurons.

Introduction

Chemotherapy-induced nausea and vomiting (CINV) is one of the most dreaded side effects associated with chemotherapy, playing a significant role in cancer patients' morbidity and quality of life, rendering difficulty of continuation of chemotherapy[1-3]. According to time of onset, CINV is classified into acute CINV (occurs during the first 24 h after chemotherapy administration), delayed CINV (occurs from at least 24 h, or later, after chemotherapy administration) and anticipatory CINV (occurs before drug administration), which involves both central and peripheral mechanisms[4-7]. Although antiemetic drugs are available for management of acute CINV[8], few treatments are effective for delayed CINV. Thus, it is critical to reveal the pathophysiological mechanisms of CINV and develop new therapeutic strategies.

It is well known that the emetic process is mainly controlled by the medullary dorsal vagal complex (DVC) in the brain stem, which includes the area postrema (AP), the dorsal motor nucleus of the vagus (DMNX), and the nucleus tractus solitarius (NTS). These areas receive and process various emetic stimuli and also generate efferent signals to the vasomotor, respiratory and salivary centers, as well as to the cranial nerves VIII and X, which results in nausea and vomiting[9]. NTS is one of the most important parts which integrate signals from vomiting related central nucleus and vagus afferent information[4]. Meanwhile, the emetic circuits are not yet fully defined anatomically and physiologically, which has hampered the development of treatments for the distress produced by CINV.

Ghrelin is a 28-amino acid peptide produced by the endocrine cells of the upper GI tract and recognized as a potent orexigenic hormone[10]. Ghrelin stimulates growth hormone (GH) release, regulates appetite and nutrient ingestion, and promotes digestive motility and secretion[11-14]. Ghrelin distributes widely in the peripheral tissues and

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central nervous system[15,16]. Findings have indicated that ghrelin acts as an anabolic hormone, targeting the brain to stimulate food intake and fat storage[17,18]. In addition to the regulation of normal physiological function, ghrelin is also involved in many pathological processes such as carcinogenesis, gastrointestinal diseases, obesity and diabetes mellitus, etc., implying it has a potential clinical application[19-21]. Recent studies have reported the involvement of ghrelin in CINV. Circulating ghrelin level decreased in the early stage of CINV, and administration of exogenous ghrelin reduced these symptoms in rodents[22]. However, in the delayed CINV, plasma ghrelin showed an adaptive up-regulation, despite reduced food intake, which might be due to the decreased synthesis and/or transduction of ghrelin outside the gastrointestinal tract[23]. As ghrelin distributes widely in the central nervous system including brain and hypothalamus areas[24] and regulates gastric motility, the ghrelin neural pathway might be involved in the CINV. However, an involvement of the NTS ghrelin in the regulation of gastric functions in the CINV state has yet not been investigated. In the present work, we observed the effects of ghrelin on the electrical activity of GD sensitive neurons in the NTS and gastric motility in cisplatin-treated rats. The changes in plasma ghrelin and expression of ghrelin and its receptors in the stomach and NTS were also observed.

Materials and Methods

Animals

Male adult Wistar rats, weighing 280-320 g, were obtained from Institute of Pharmaceutical Research of Qingdao, China. They were housed in a temperature controlled room (25 ± 2°C) with 12 h light:12 h light-dark cycle (light on at 8:00 am). The rats were caged individually with standard laboratory chow pellets, pelleted kaolin clay and tap water available ad libitum throughout experiments. The study was approved and all procedures were complied with institutional guidelines of the Animal Care and Use Committee at Qingdao University of Science and Technology.

Drug Administration

After adaptive feeding in the cages for 3 days, the rats were given the intra-peritoneal injection (i.p.) either saline (0.15 M NaCl) or cisplatin (6 mg/kg, Qilu Pharmaceutical Co. Ltd, Hainan, China) and dose volume were 0.4 ml/100 g. The cisplatin injection was prepared by dissolving 15 mg cisplatinum (II) diamine dichloride in 10 ml saline with 60 s of sonication and the concentration was 1.5 mg/ml. The experiments were conducted 2 days after administration of either cisplatin or saline.

Measurement of Body Weight, Food Intake and Kaolin Consumption

Body weight, food and kaolin intake were measured on each day of the experiments between 10:00 am and 11:00 am starting from the day cisplatin or saline administration. Pica in rats was assessed by measurement of kaolin consumption.

Analysis of Plasma Ghrelin Concentrations

At 2 days after drug administration, blood samples were collected by decapitation and delivered into tubes containing potassium-EDTA on the ice. Plasma was separated by centrifugation, frozen and stored at -20°C until analysis. Plasma ghrelin concentration was measured by enzyme-linked immunosorbent assay using commercially available kits (Phoenix Pharmaceuticals, CA, USA).

Measurement of mRNA Expression of Ghrelin and Ghrelin Receptor GHS-R1a

After blood colleting, rats were placed on a bed of dry ice for tissue removal. The stomach was removed from each rat, opened, contents removed and the tissue washed in chilled sterile saline to remove adherent particulate matter. The same part of the distal stomach was cut and then snap-frozen in liquid nitrogen before storage at -70°C. The LHA and the NTS were removed immediately from the same animal respectively and snap-frozen in liquid nitrogen prior to storage at -70°C. Homogenization and total RNA extraction were conducted using the RNase universal tissue kit according to the manufacturer's instruction (Qiagen, Valencia, CA, USA). 1 μg of total RNA was reverse transcribed using first-strand cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time quantitative PCR was performed on a GeneAmp 5700 Sequence detection system (Applied Biosystems, Warrington, UK) using SYBR Green I as a double-stranded DNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out in a total volume of 25 μL containing 2×PCR Master Mix (Ap-

plied Biosystems, Warrington, UK), 2 μL of 1:4 diluted cDNA and 5 μmol L^{-1} of each specific primer. PCR primers for rat GHS-R1a (312 bp) were 5' -GAGATCGCTCAGATCAGCCAGTAC -3' (sense), 5' -TAATCCCCAAACTGAG-GTTCTGC -3'(antisense), for ghrelin (347 bp) were 5'-TTG AGC CCA GAG CAC CAG AAA-3' (sense), 5'-AGT TGC AGA GGA GGC AGA AGC-T-3' (antisense), and for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 134 bp) were 5′- CG-GCAAGTTCAACGGCACAG -3′ (sense), 5′-ACTCCACGACATACTCAGCAC-3′ (antisense). The PCR reaction conditions were as follows: 40 cycles of 95°C for 15 s and 64°C for 60 s (GH-SR-1a) or 60°C for 60 s (GAPDH). Data was analyzed by Gene Amp 5700 SDS software (Applied Biosystems, Warrington, UK). Relative quantification was performed by calculating the difference of the threshold cycle ($\Delta C_t = C_{tGHS-R1a/ghrelin} - C_{tGAPDH}$) of GHS-R1a or ghrelin and GAPDH.

Abdominal Surgery and Electrophysiology

After fasted for 18 h, rats were anesthetized with thiobutabarbitol (100 mg/kg, i.p., Sigma-Aldrich, CA, USA) and the maintenance anesthetic was given whenever necessary. The trachea was cannulated to maintain an open airway and the hearts were continuously monitored. The rectal temperature was kept at 36-37°C by a heating pad.

Gastric distension (GD) was produced by air inflation of a latex balloon surgically placed in the stomach as described in literature[25]. When necessary, GD was performed by inflating the gastric balloon with 3-5 mL 37°C water at a rate of 0.5 mL/s for 20 s. After the abdominal surgery, the rat was anesthetized and positioned on a stereotaxic frame (Narashige SN-3, Tokyo, Japan), and a cranial surgery was performed for neuronal recording. With the aid of a hydraulic micropositioner, a four-barrel glass microelectrode (total tip diameter 3-10 μm, resistance 5-15 MΩ) was inserted and then advanced in 10 μm steps into the area of NTS (bregma: P: 12.84-14.04 mm, L (R): 0.5-1.5 mm, H: 7.6-8.0 mm)[26], which was used for extracellular potential recording and micro-pressure injection. The recording glass microelectrode was filled with 0.5 M sodium acetate and 2% Pontamine sky blue. The other three barrels connected with a 3-channel pressure injector were filled with 15 nmol L^{-1} solution of ghrelin (Sigma-Aldrich, St. Louis, MO, USA), 45 nmol L^{-1} solution of BIM28163 (Anaspec, Inc. Fremont, CA, USA) and normal saline, respectively.

Once the microelectrode was advanced into the NTS, the extracellular action potentials of single neurons were recorded by the glass microelectrode (another electrode was placed on the epicranium of the rat), amplified using a high input impedance amplifier (MEZ8201, Nihon Kohden, Tokyo, Japan), and displayed on an oscilloscope (VC-11, Nihon Kohden, Tokyo, Japan). All signals were recorded and stored in a computer for further analyses with the SUMP-PC biological signal processing system.

The unit discharge was tested with a GD stimulus to determine whether there was input from gastric mechanoreceptors. A neuron was identified as a GD sensitive neuron if its mean firing frequency changed via GD by at least 20% from the mean basal firing level. The GD sensitive neurons were further sorted into GD-excitatory (GD-E) neurons and GD-inhibitory (GD-I) neurons according to the spontaneous discharge increased or decreased with GD, respectively.

Histochemistry Verification

At the end of each experiment, a direct current (10 μA, 20 min) was passed through the electrode to form an iron deposit of Pontamine sky blue to check the position of the recording electrode. After perfusion and fixation of the brain, 50 μm frozen coronal sections were cut through the regions of the NTS, stained with Neutral Red, cleared with xylene, coverslipped, and observed under a light microscope. Samples with incorrect locations were eliminated in the analysis.

Gastric Motility Recording in Conscious Rats

After fasted for 18 h, the rats were anesthetized and placed on a stereotaxic frame. A stainless steel guide cannula was implanted unilaterally into the NTS to deliver drug and the stylet protruded 0.5 mm beyond the tip of the guide cannula[26]]. The injection cannula (29 gauge) was connected to a syringe by a 10 cm piece of polyethylene tubing. A midline laparotomy was performed after the cannula was implanted. The force transducer was implanted in the stomach[27], and the animals were allowed to recover for 3 days before chemicals were administered.

Thirty-two normal rats and 32 cisplatin-treated rats were used in this experiment. After fasted overnight, the rats were acclimatized for 30 min

at the recording area. Gastric motility was recorded on a polygraph (3066-23, Chengdu Precision Instruments, Sichuan, China). After recording baseline motility for 30 min, 1.0 μL of 250 nmol L^{-1} ghrelin, 1.0 μL of 1250 nmol L^{-1} BIM28163, 1.0 μL mixed solution of 250 nmol L^{-1} ghrelin and 1250 nmol L^{-1} BIM28163 were slowly injected via the cranial cannula respectively (n=8, respectively). Equal volumes of 0.9% saline were injected as control (n=8). The rats were gently held during the injection, but during the experiment, they could move around freely in their cages. The amplitude and frequency of gastric motility were measured.

Statistical Analysis

Data were presented as mean ± SD. Paired and non-paired Student's *t*-test were used to check the difference in treatment or two groups. Data of multi-group were analyzed by one-way ANOVA and differences among means were analyzed using Dunnett's test or Fisher's protected LSD multiple comparison tests. Significant differences were considered at $p<0.05$.

Results

Changes of Body Weight, Food Intake and Kaolin Consumption in Cisplatin-Treated Rats

Sixteen rats were used in this experiment and divided into saline treated and cisplatin-treated group randomly. Body weight, food intake and kaolin consumption were recorded over 1 and 2 days after drug administration. Cisplatin treatment led to a significant decrease in the total food intake 2 days after dosing compared with saline treatment group (n=8, $p<0.05$, Figure 1A). Kaolin consumption over 1 day and 2 days increased significantly in rats treated with cisplatin in comparison to those treated with saline (n=8, $p<0.01$, Figure 1B). However, body weight had a decreased trend after cisplatin administration but had no statistical significance (n=8, $p>0.05$, Figure 1C).

Changes of Ghrelin and its Receptor GHSR1a in Cisplatin-Treated Rats

Data obtained from ELISA assay showed that the mean concentration of plasma ghrelin decreased after 2 days of cisplatin treatment compared with saline treatment but had no statistical significance (25.64 ± 6.47 fmol/ml versus 33.22 ± 9.86 fmol/mol, n=8, $p>0.05$, Figure 2A). Ghrelin and ghrelin receptor GHS-R1a mRNA expression were detected in both stomach and NTS in rats treated with saline. Two days after cisplatin dosing, there were no changes in the level of ghrelin mRNA expression in stomach and NTS (n=8, $p>0.05$, Figure 2B), while GHS-R1a mRNA expression increased significantly (n=8, $p<0.05$, Figure 2C).

Effects of Ghrelin in NTS on the Discharges of GD Neurons in Cisplatin-Treated Rats

One hundred and twenty-five rats were used to observe the effects of ghrelin in NTS on the discharges of GD neurons in this experiment. At 2 days after drug administration, abdominal surgery and electrophysiology were conducted to record the discharges of GD neurons. One hundred and fifty-two spontaneously discharging neurons in the NTS in 68 cisplatin-treated rats and 136 neurons in 57 rats treated with saline were recorded respectively. Of 152 neurons, 96 responded to GD stimulus defined as GD-neurons. After gastric distension, the firing frequency increased in 61 neurons (GD-E, 63.54%) and

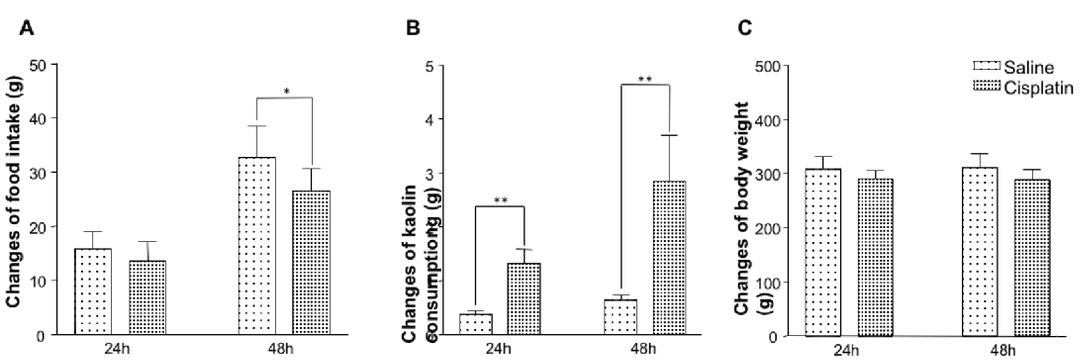

Figure 1. Changes of food intake ***(A)***, kaolin consumption ***(B)*** and body weight ***(C)*** in cisplatin-treated rats. n=8, compared with saline treated group, $^{*}p<0.05$, $^{**}p<0.01$.

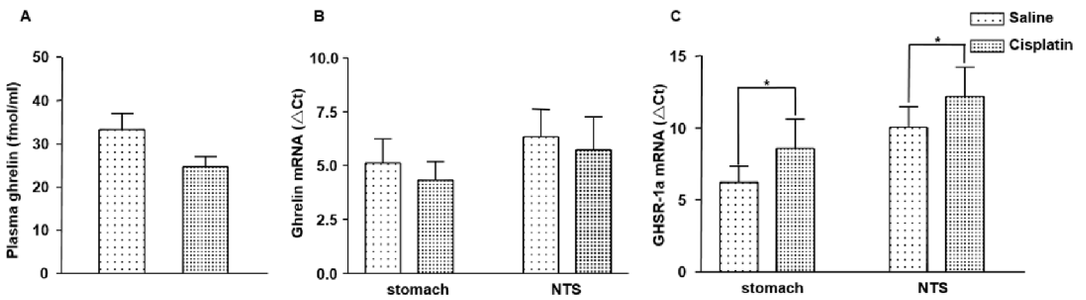

Figure 2. Changes of plasma ghrelin levels ***(A)***, expression of ghrelin mRNA ***(B)*** and GHS-R1a mRNA ***(B)*** in stomach and NTS in cisplatin-treated rats. n=8, compared with saline treated group, $^{*}p<0.05$, $^{**}p<0.01$.

decreased in 35 neurons (GD-I, 36.46%). There were no statistical differences in the ratio of GD-E and GD-I neurons and the changes of firing frequency between NS and cisplatin-treated rats ($p>0.05$).

After ghrelin administration into the NTS in cisplatin-treated rats, 23 (23/61, 37.70%) GD-E neurons and 14 (14/35, 40.00%) GD-I neurons were activated with the firing frequency increased from 2.32 ± 0.97 Hz to 3.46 ± 1.02 Hz and 2.58 ± 1.22 Hz to 3.56 ± 1.15 Hz, respectively ($p<0.01$, Figure 3C and 3D). Compared with the saline-treated rats, the ratio of GD neurons excited by ghrelin significantly increased in cis-

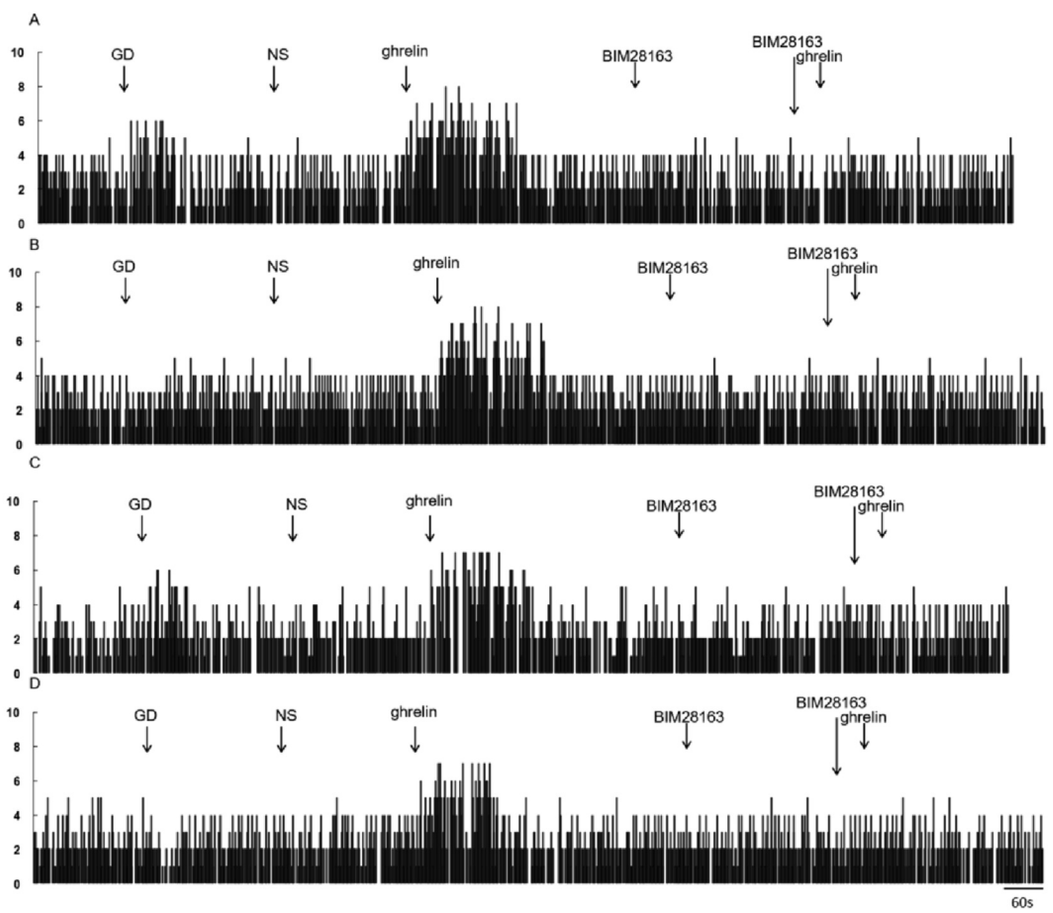

Figure 3. Effects of ghrelin on firing activity of GD neurons in the NTS. ***(A-B)*** Effects of ghrelin on firing frequency of GDE ***(A)*** and GDI ***(B)*** neurons in the NTS in saline treated rats. ***(C-D)*** Effects of ghrelin on firing frequency of GDE ***(C)*** and GDI ***(D)*** neurons in the NTS in cisplatin-treated rats.

platin-treated rats (37.70% *vs.* 62.75% in GD-E neurons and 40.00% *vs.* 66.67% in GD-I neurons, $p<0.01$, Figure 3A and 3C, Figure 3B and 3D, respectively). The average increase in firing frequency also decreased in GD-E neurons (49.14 ± 9.27% *vs.* 78.25 ± 23.67%, $p<0.01$, Figure 3A and 3C) and GD-I neurons (36.55 ± 11.42% *vs.* 60.32 ± 17.17%, $p<0.01$, Figure 3B and 3D). The excitatory responses induced by ghrelin could be completely abolished by pretreatment with the ghrelin receptor antagonist BIM28163 (Figure 3). BIM28163 or saline alone had no effect on GD neuron discharge (Figure 3).

Effects of Ghrelin in NTS on Gastric Motility in Cisplatin-Treated Rats

Gastric motility assay revealed that the gastric contraction decreased in cisplatin-treated rats with the reduced contraction amplitude ($p<0.01$, Figure 4A) and frequency ($p<0.01$, Figure 4B) in the recording period compared to saline treated rats. Administration of ghrelin in the NTS could significantly promote the gastric motility in cisplatin-treated rats, which was similar to the saline treated rats (Figure 4A and 4B). The increase of contraction amplitude and frequency had a latency of 10 min around, reached a peak at around 15 min and lasted for 10 min (Figure 4A and 4B). Otherwise, the increased gastric contraction induced by ghrelin in cisplatin-treated rats was significantly lower than that of saline treated rats ($p<0.05$ in amplitude and $p<0.01$ in frequency, Figure 4A and 4B). The excitatory effects of ghrelin on the gastric motility could be completely blocked by BIM28163 both in cisplatin and NS-treated rats ($p>0.05$, Figure 4A and 4B). Administration of BIM28163 or saline singly had no effect on the gastric motility (data not shown).

Discussion

Effects of ghrelin in the NTS on the discharges of GD neurons and the regulation of gastric motility in cisplatin-treated rats were investigated in this preliminary experiment. The results revealed that the cisplatin treatment induced the decrease of total food intake and increase of kaolin consumption while had no significant effect on the body weight in rats over 2 days of treatment. Worthy to be mentioned, the effects of cisplatin on body weight over longer times of treatment should be investigated in later experiments. Expression of ghrelin receptor GHS-R1a mRNA in the stomach and NTS was increased by cisplatin treatment. Ghrelin administration into the NTS excited most of the GD-E and GD-I neurons. Cisplatin treatment induced the decrease of gastric contraction which indicated the gastric function disorders. Ghrelin in the NTS promoted the gastric motility both in saline and cisplatin-treated rats, which could be completely blocked by the ghrelin receptor antagonist. However, the amplitude and frequency of gastric contraction promoted by ghrelin administrated into the NTS in the cisplatin-treated rats were lower than the saline treated rats. In all, these results obtained in this study indicated that ghrelin in the NTS might participate in the regulation of GD-neurons and gastric motility via its receptor.

Cisplatin, an agent for cancer chemotherapy, generates nausea, vomiting, anorexia and other behaviors indicative of malaise[9]. Clinical data revealed that almost all patients had nausea and vomiting during the first 2 h after cisplatin administration. A reduction in the severity of emesis is observed during a period of 18 to 24 h and reappeared in the second and third day after cisplatin administration[28]. Pica, an alteration of feeding behavior consisting of the intake of non-nutritive substances, such as kaolin, has been used as a proxy for emesis in rodents since they lack a vomiting response[29]. In our experiment, kaolin consumption increased at 24 h and 48 h after cisplatin dosing while the food intake decreased on 48 h in rats. These results indicated that cisplatin induced pica and decreased appetite in rats. The mechanism of CINV has been explored recently. Excessive serotonin release and abnormal ghrelin dynamics might contribute to the production of CINV. It is reported[30] that 2 mg/kg of cisplatin administration to rats intraperitoneally decreased plasma ghrelin levels after 2 h. Yakabi et al[31] detected a markable decrease in plasma ghrelin concentration several hours after 4 mg/kg cisplatin administration, which subsequently recovered within 24 h. Malik et al[32] reported an increased trend of plasma ghrelin levels over 48 h after intraperitoneal of 6 mg/kg cisplatin compared to that of administration of saline. We measured the changes of ghrelin and its receptor over 2 days after cisplatin dosing in this experiment. Although plasma ghrelin concentration, gastric and NTS

ghrelin mRNA expression decreased in cisplatin-treated rats, but had no statistical significance compared with the saline-treated group. Ghrelin receptor GHSR-1a mRNA expression in the stomach and NTS increased significantly in cisplatin group. It revealed that ghrelin might contribute to cisplatin induced nausea and vomiting.

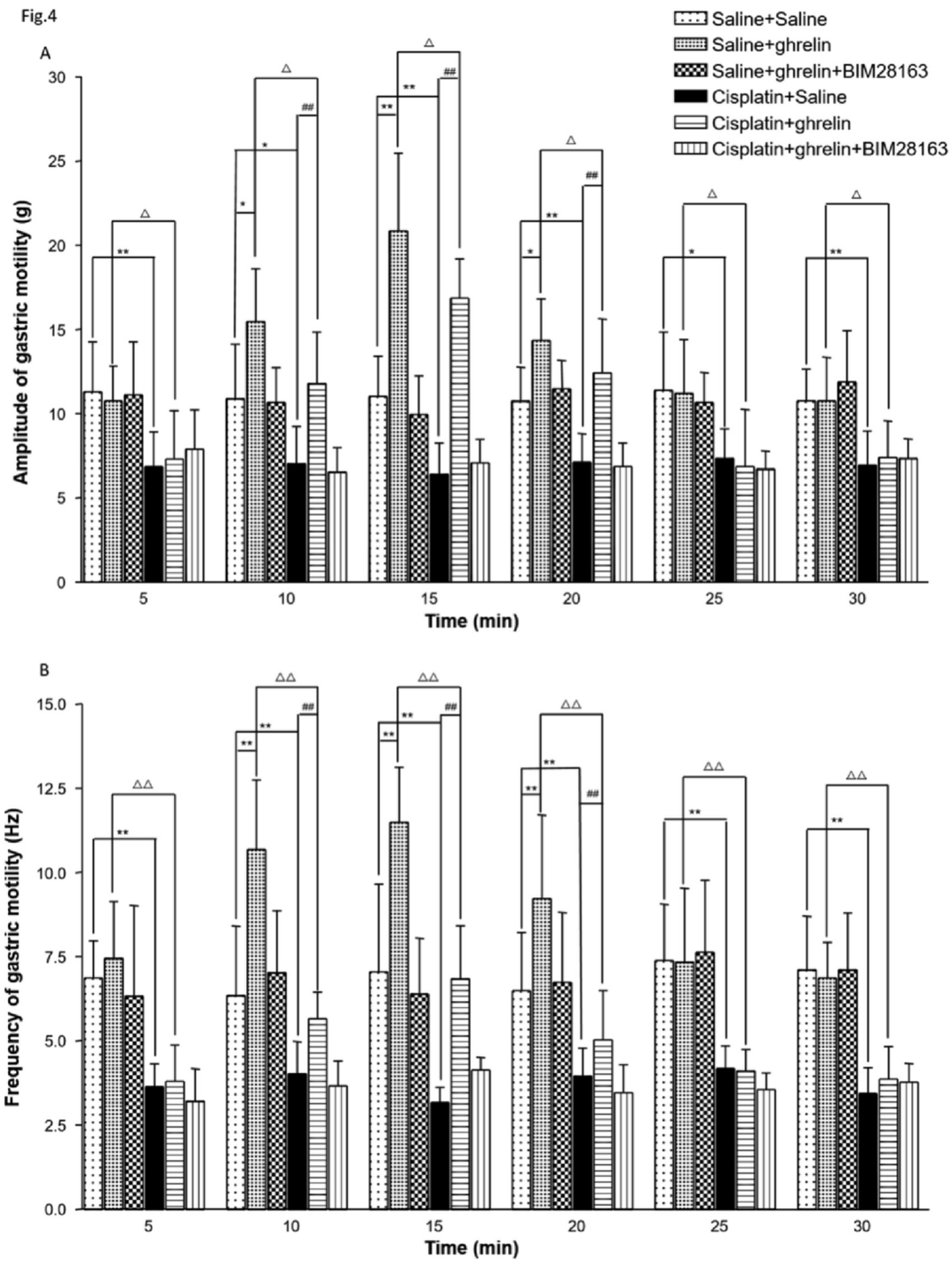

Figure 4. Effects of ghrelin administrated into the NTS on the amplitude ***(A)*** and frequency ***(B)*** of gastric contraction. n=8, compared with saline+saline group, $^{*}p<0.05$, $^{**}p<0.01$; compared with cisplatin+saline group, $^{\#}p<0.05$, $^{\#\#}p<0.01$; compared with saline+ghrelin group, $\triangle p<0.05$, $\triangle\triangle\ p<0.01$.

Chemotherapy administration causes the release of several mediators by stimulating the enterochromaffin cells in the intestinal mucosa. These mediators bind to the specific receptors in vagal primary afferent neurons. The projection of vagal stimulus in the central nervous system triggers the emesis reflex. The NTS is the original site through which peripheral afferent information from the gastrointestinal tract enters the brain[33]. In the present study, we found that ghrelin receptor GHSR-1a existed in the NTS and its expression level adaptively upregulated in cisplatin-treated rats. GD neurons were detected in the NTS and ghrelin could excite most of GDE and GDI neurons when microinjected into the NTS both in saline and cisplatin-treated rats. The ratio of GD neurons excited by ghrelin, as well as the average increase in firing frequency, decreased significantly in cisplatin-treated rats compared with saline group. The excitatory effect induced by ghrelin could be completely blocked by ghrelin receptor antagonist BIM28163, indicating that ghrelin regulated the firing activity of the GD neurons in the NTS via its receptor in normal and cisplatin-treated rats.

Emetogenic stimuli might trigger the alterations in gastric rhythm in human nausea and animal models of nausea and vomiting[34,35]. Delayed gastric emptying is the main response to substances that might induce vomiting in other species such as ferret[9]. Cisplatin delayed gastric emptying in a dose-dependent manner, which showed a strict temporal relationship with the induction of both acute and delayed pica[36]. Chronic administration of cisplatin exacerbated this effect and also induced gastric distension, with only minor alterations in intestinal motility[37]. Although pica and gastric emptying are an index for assessment of different effects of cisplatin, both they have traditionally been used as markers of nausea in nonvomiting species[38]. These findings might have important implications in the search for new antiemetics. In agreement with these studies, amplitude and frequency of gastric contraction decreased significantly in cisplatin-treated rats in our experiment. When ghrelin microinjected into the NTS, the gastric motility increased both in saline and cisplatin-treated rats, which could be completely blocked by ghrelin receptor antagonist BIM28163. Furthermore, the improvement of the gastric motility in the cisplatin-treated rats induced by ghrelin was much less than that of saline.

Conclusions

Cisplatin induced pica and upregulation of GHS-R1a mRNA expression in the stomach and NTS, as well as decreases of gastric motility. Ghrelin in the NTS excited the GD neurons and promoted the gastric motility via its receptor GHS-R1a, indicating that ghrelin might be a new potential target for therapy of CINV.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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