Upregulation of gastric norepinephrine with β-adrenoceptors and gastric dysmotility in a rat model of functional dyspepsia

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Short title: Role of noradrenergic system in gastric dysmotility of FD

Summary

Disordered motility is one of the most important pathogenic characteristics of functional dyspepsia (FD), although the underlying mechanisms remain unclear. Since the sympathetic system is important to the regulation of gastrointestinal motility, the present study aimed to investigate the role of norepinephrine (NE) and adrenoceptors in disordered gastric motility in a rat model with FD. The effect of exogenous NE on gastric motility in control and FD rats was measured through an organ bath study. The expression and distribution of β-adrenoceptors were examined by real-time PCR, Western blotting and immunofluorescence. The results showed that endogenous gastric NE was elevated in FD rats, and hyperreactivity of gastric smooth muscle to NE and delayed gastric emptying were observed in the rat model of FD. The mRNA levels of β_1-adrenoceptor and norepinephrine transporter (NET) and the protein levels of β_2-adrenoceptor and NET were increased significantly in the gastric corpus of FD rats. All three subtypes of β-adrenoceptors were abundantly distributed in the gastric corpus of rats. In conclusion, the enhanced NE and β-adrenoceptors and NETs may be contributed to the disordered gastric motility in FD rats.

Key words: Norepinephrine, β -adrenoceptors, Disordered Gastric Motility, Functional dyspepsia

Introduction

Dyspepsia comprises a constellation of symptoms referable to the gastroduodenal region of the upper gastrointestinal tract (Talley et al., 2015). According to a recent internet-based cross-sectional health survey of adults in the USA, Canada and the UK, approximately 10% of the adult population fulfills symptom-based criteria for Rome IV functional dyspepsia (FD) (Aziz et al., 2018). FD is not a life-threatening disease but often reduces patients' quality of life and is associated with high societal costs (Lacy et al., 2013; Talley et al., 1995). Elucidating the pathogenesis of FD would be conducive to searching for new therapeutic targets and reducing the burden on society. Although not fully understood, several mechanisms have been considered to be involved in the pathogenesis of FD: disordered motility, visceral hypersensitivity and mucosal alterations (Tack et al., 2018). Disordered motility is one of the most important mechanisms because delayed gastric emptying occurs in up to one-third of FD patients (Carbone et al., 2014; Stanghellini et al., 2014). However, the underlying mechanism of disordered motility in FD patients remains debatable.

The enteric nervous system (ENS), which is linked to the central nervous system (CNS) mediated by the autonomic nervous system (ANS), regulates the motility and secretory functions of the GI tract. FD has been described as a multifactorial disease that involves the ENS and the CNS (Sahan et al., 2018). It has been reported that FD patients manifest an imbalance of ANS function and vulnerability to recovery from external stimuli (Tominaga et al., 2016). Automatic dysfunction, including that of the sympathetic and parasympathetic system, has been observed in patients with FD (Park et al., 2001). Norepinephrine (NE) is the main neurotransmitter in the sympathetic nervous system and is widely involved in many physiological and pathological functions, including gastrointestinal motility. Enhanced sympathetic nerve activity and elevated plasma NE have been reported to be related to gastric hypersensitivity in a rat model of FD (Winston et al., 2016). However, there are no published studies describing the association between the sympathetic nervous system and disordered motility in FD.

The aim of the present study was to identify the role of the noradrenergic system (including neurotransmitters and receptors) in disordered gastric motility in a rat model of FD by means of gastric emptying study, *ex vivo* motility recording, enzyme-linked immunosorbent assay (ELISA), real-time reverse transcription-polymerase chain reaction, Western blotting and immunofluorescence. This study helps elucidate the mechanism underlying FD-associated gastric dysmotility.

Materials and methods

Animals

Adult male Sprague−Dawley rats weighing 200−220 g were used in all experiments. The rats were housed in an animal facility with 12 h light and dark cycles and free access to food and water. All procedures were carried out according to the ethics and animal welfare regulation requirements approved by the Institutional Animal Care and Use Committee (Beijing Institute of Traditional Chinese Medicine).

The procedure for creating FD model rats has been described previously (Chang et al., 2017). A sponge clamp was used to clinch the distal end of the rats' tail, and the strength was enough to induce pain without skin damage. FD model rats received 7 days of clamp stimulation 3 times a day for 30 minutes each time. To avoid infection due to a scuffle injury, the injured area was rubbed with 0.5% iodine at the end of stimulation. After one week, the FD model rats showed symptoms such as irritability, reduced consumption and weight growth, and loose stools.

Gastric emptying

Before the experiment, each rat in the control and FD groups was kept separately in a single cage and fasted for 24 hours while having free access to water. Then, each rat was free to eat preweighed pellets for 1 hour until the food was removed again. Two hours later, each rat was sacrificed by an overdose of anesthetics, the pylorus and cardia were ligated, and then the stomach was carefully excised. The contents of the stomach were removed, dried and weighed. The percentage of gastric emptying was calculated according to the following formula:

Gastric emptying%=(1- weight of residue in the stomach/weight of food intake)× 100%

Recording of contractile activity through an organ bath

Rats in the control and FD groups were sacrificed by an overdose of anesthetics, and the stomach was removed and placed in Krebs-Henseleit solution (K-HS, composition: NaCl, 118.4 mM; KCl, 4.7 mM; $MgCl_2$ $7H_2O$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 11 mM; and $CaCl_2$, 2.5 mM, pH 7.4, all chemicals were purchased from Beijing Chemical Works) maintained at 37°C and bubbled with 95% O_2/5% CO_2. The luminal contents were gently flushed out, and smooth muscle strips (approximately 9–11 mm in length and 1–2 mm in width) were cut along the direction of the longitudinal smooth muscle. The strips were mounted vertically under an initial tension of 1 mN in an organ bath containing 20 mL of oxygenated (95% O_2 and 5% CO_2) K-HS at 37°C and equilibrated for 1 hour. Tension changes in the muscle preparation were recorded isometrically through a force transducer (MLT0201/RAD; AD Instruments, Barcelona, Spain). The mechanical activity was digitized using a bridge amplifier (ML228; AD Instruments, Bella Vista, Australia), and tracings were visualized and analyzed by using LabChart 7 software (AD Instruments). The motility index (MI) was calculated based on the area under the curve (gS^{-3}) before and after NE administration.

Measurement of NE by ELISA

Rats in the control and FD groups were sacrificed by an overdose of anesthetics. The method of tissue preparation has been described previously (Zhang et al., 2015a; Zhang et al., 2015b). The stomachs were quickly removed, cleaned and placed in Krebs-Hensleit solution (K-HS). The antrum and fundus of stomachs were quickly removed and the gastric corpus was pinned flat with the muscle side down in a petri dish containing ice-cold oxygenated K-HS. The mucosa/submucosa layers were carefully removed under a dissecting microscope to obtain the serosa and the muscle preparations. The separated muscularis of gastric corpus was flash-frozen in liquid nitrogen and then homogenated. A commercial ELISA kit (E02N0013, Blue Gene) was used to detect the NE content in the muscular layer of the gastric corpus. All

reagents were used at room temperature, and the prepared standard and sample were added to the appropriate wells in an antibody precoated microtiter plate. Then, 10 μL of balance solution and 50 μL of conjugate were added separately to each well. After 1 hour of incubation at 37 °C, the incubation mixture was removed, and the plate was washed manually and automatically (five times each). Two types of substrate (10 μL of substrate A and 50 μL of substrate B) were added to each well of the plate and incubated for 15 minutes at 25°C (avoid sunlight). After 50 μL of stop solution was added to each well, the optical density (O.D.) at 450 nm was immediately determined using a microplate reader. A standard curve was constructed based on the O.D. value of each standard, and the concentration of samples corresponding to the mean absorbance was calculated from the standard curve.

Real-time polymerase chain reaction

Control and FD model rats were sacrificed by an overdose of anesthetics, and the lamina muscularis of the gastric corpus was obtained. Total RNA was extracted by homogenization in an extraction kit (Servicebio, Wuhan, China), and complementary DNAs were synthesized by using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, Waltham, USA). The specific primers are listed in Table 1. As mentioned in our previous study, the β_1-, β_2-, β_3-adrenoceptors and NET transcript levels were measured with the FastStart Universal SYBR Green Master kit (Roche, Basel, Switzerland) using a light cycler instrument (ABI, Waltham, USA). Data were analyzed using StepOne^{TM} Software (Version 2.3, ThermoFisher, Waltham, USA)

Western blotting analysis

As described in our previous study (Song et al., 2014), the frozen muscular layer of the gastric corpus (30 mg) was homogenized in 300 μL of ice cold RIPA lysis buffer (R0010, Solarbio) containing fresh protease inhibitor, namely, 1% phenylmethanesulfonyl fluoride (PMSF, Solarbio). The total tissue lysates were sonicated and centrifuged at 12,000 rpm for 30 minutes at 4°C, and the cellular debris was removed. After the total protein concentration was determined by a bicinchoninic acid assay (BCA), protein samples (100 μg, dissolved in loading buffer with 20% bromophenol blue) were separated by 10% sodium dodecyl sulfate polyacrylamide

gel electrophoresis (120 V, 80 minutes) and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica) at 0°C (295 mA, 90 minutes). Nonspecific binding sites were blocked in a blocking buffer containing 10% nonfat milk in Tris-buffered saline (TBST, 20 mM Tris-Cl, pH 7.5, containing 0.15 M NaCl, 2.7 mM KCl and 0.05% Tween 20) for 1 hour at room temperature. The blocked membrane was incubated with the primary antibodies (see Table 2) at 4°C overnight and then incubated with the appropriate secondary antibodies (see Table 2) for 1 hour at room temperature. The membranes were washed 3 times (each for 5 minutes) in TBST, reacted with ECL solution for 1-2 min and then exposed to a film. After the film was developed, the integrated intensity of the bands was analyzed by ImageJ, and the expression levels of β-actin were used as an internal reference.

Statistics and data analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using a paired or unpaired t-test. “n” refers to the number of rats or the number of pairs. A *p* value less than 0.05 was considered statistically significant. Statistics and graphs were performed by Prism, version 6.01 (GraphPad Software, Inc., San Diego, USA).

Results

NE content and gastric motility in control and FD rats

As shown in **Figure 1A**, the GE rate of solid food in FD model rats was significantly reduced compared with that in controls (56.25 ± 4.16% vs 69.75 ± 2.86%, n = 6, $P < 0.05$). Because norepinephrine (NE) is the main neurotransmitter of the sympathetic nervous system (SNS), we measured its content in the gastric corpus by ELISA. The results showed a significantly elevated NE content in the muscular layer of the gastric corpus in FD model rats compared with that in control rats (38.63 ±4.97 ng/mg vs 18.26±2.12 ng/mg, n = 7, $P < 0.01$, **Figure 1B**). Representative contractile tracings of longitudinal strips of the gastric corpus are shown in **Figure 1C**. Exogenous NE (1 μM) inhibited the contractile activity of the longitudinal strip of gastric corpus in both control and FD model rats. Interestingly, the reduction rate of the motility index induced by NE was higher in the FD model than in control rats

(84.23 ± 1.95% vs 69.68 ± 3.39%, n = 8, $P < 0.01$, **Figure 1D**), reflecting enhanced adrenergic reactivity in the gastric motility of FD rats.

Expression of β-adrenoceptors and NET in gastric corpus

To further investigate the mechanism of hyperreactivity of gastric smooth muscle to NE in FD rats, the expression of β-adrenoceptors in the muscular layer of gastric corpus was measured in control and FD rats. Compared with that in the control group, the mRNA expression level of the β_1-adrenoceptor (n = 9, $P < 0.05$) was increased by 34.46% in FD rats (**Figure 2A**), and the protein level showed an upregulated tendency (**Figure 2B, C**). The protein level of the β_2-adrenoceptor increased by 54.52% in FD rats, and the mRNA level showed an upregulated tendency. The β_3-adrenoceptor mRNA level was decreased while the protein level was increased (**Figure 2B, C**).

The norepinephrine transporter (NET) is mainly responsible for the reuptake of NE, which is important for maintaining synaptic homeostasis. Because NET is a hallmark protein of noradrenergic neurons (Fan et al., 2009), we examined its expression in the gastric corpus of control and FD rats. The NET mRNA level was increased by 96.87% (n = 9, $P < 0.05$), and the protein level was increased by 89.23% in FD rats compared with that in control rats (**Figure 2B, C**). Our present results indicated that sympathetic regulation was enhanced in the gastric corpus of FD rats.

Distribution of β-adrenoceptors in the gastric corpus in rats

All three subtypes were observed in the gastric corpus of rats by immunofluorescence. As shown in **Figure 3**, β_1-IR was mainly distributed in the mucous layer (**Figure 3A**), while β_2 was abundantly distributed in the muscularis layer. In addition, β_2-IR was highly expressed in the myenteric plexus (**Figure 3B**). β_3-IR were distributed in both mucous and muscularis layer (**Figure 3C**). The present data provided morphological evidence for the above data.

Discussion

Disturbed gastric motility has been reported in most FD patients. However, it is poorly managed in the clinic, and the underlying mechanisms are unclear. FD rats manifest delayed emptying. The present study demonstrated that enhanced adrenergic reactivity, elevated NE content, and upregulated expression of NET and of β_1- and β_2-

adrenoceptors were observed in the muscularis layer of the gastric corpus in FD rats. Our present results indicated that enhanced sympathetic regulation was involved in disordered gastric motility in FD rats.

Life stress contributes to symptom onset and exacerbation in the majority of patients with FD (Bennett et al., 1998). In our present study, tail-pinch stress was used to create a rat model of FD, which has been used in our previous study (Chang et al., 2017) as well as other studies (Wei et al., 2011). This method simulates the main etiology and symptoms of FD, as well as the pathological state of anxiety and stress in FD patients. It has been reported that tail-pinch stress enhances the release of catecholamines (NE and dopamine) in the locus coeruleus and NE release in the hippocampus (Kaehler et al., 2000; Rosario et al., 1999). The sympathetic division of the ANS is important in the control of GI function under both basal and stress conditions (Mcintyre et al., 1992). However, no published paper has established the role of the sympathetic system in the regulation of GI motility in FD patients or animal models of FD. For the first time, our present study demonstrated that enhanced sympathetic regulation of gastric motility may be partly involved in the pathogenesis of FD. Our present results were, to some extent, consistent with the results of the abovementioned studies in brain (Kaehler et al., 2000; Rosario et al., 1999).

The sympathetic nervous system predominantly inhibits GI motility and plays a tonic inhibitory role on mucosal secretion (Browning et al., 2014). NE is a primary neurotransmitter of the sympathetic nervous system and can exert an inhibitory effect on gastric motility via β-adrenoceptors located on the smooth muscle (Song et al., 2014). It has been reported that selective agonists of α_2-adrenoceptors can exert inhibitory effects on gastric motor activity in rats (Zadori et al., 2007). However, in our previous study, α-adrenoceptors were not involved in the inhibitory effect of NE on gastric motility, as the specific antagonists did not block the effect induced by NE (Song et al., 2014). These inconsistent results might be related to the different experimental designs and techniques. Canciani et al. investigated the intragastric pressure by *in vivo* balloon measurement, whereas our studies were carried out on freshly isolated gastric muscle strips via an *ex vivo* force transducer. Moreover, the

expression of α_2-adrenoceptors has been reported to be widely distributed in the brain and myenteric plexus (Canciani et al., 2006; Sjoholm et al., 1999), indicating that α_2-adrenoceptors are mainly involved in the regulation of gastric motility via the neural pathway.

The endogenous NE in the gastric corpus was elevated in FD rats in our present study, which might be the consequence of stress conditions. Furthermore, exogenous NE induced more inhibition of gastric motility in FD rats, indicating hyperreactivity to NE in FD. Our present results might partly explain the mechanism of decreased gastric motility in patients with FD. The upregulated β-adrenoceptors might be an adapted response to the elevated endogenous NE, which is also related to the disordered gastric motility in FD. NET is a 12-transmembrane protein that is localized presynaptically on noradrenergic nerve terminals. The reuptake of NE is accomplished by the NETs located on the synapse, which is the main mechanism for the inactivation of released NE (Aggarwal et al., 2017). Sympathetic neurons are a predominant source of NETs, and their axons are projected into the gut; therefore, NETs play a strong role in determining the distribution of sympathetic nerves (Mayer et al., 2006). In our present study, the mRNA and protein levels of NETs in the gastric corpus were consistently upregulated in FD rats. We hypothesized that the elevated endogenous NE may lead to increased NE reuptake, which might be an adaptive alteration to maintain physiological NE concentrations in the stomach.

In conclusion, our present study showed that elevated gastric NE, hyperreactivity of gastric smooth muscle to NE and upregulated β-adrenoceptors might contribute to delayed gastric emptying observed in the rat model of FD. The present study provides new clues for the pathogenesis and therapeutic target of disordered gastric motility in FD.

Conflict of interest: We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (Grant No.81774215).

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Table 1. Sequences of primers

Primers	GenBank accession number	Primer sequence	Primer location in the sequence
GAPDH	NM_017008.4	S: CTGGAGAAACCTGCCAAGTATG A: GGTGGAAGAATGGGAGTTGCT	814-835 931-951
Adr-β_1	NM_012701.1	S: CTCGTCCGTCGTCTCCTTCTA A: CCATGATGATGCCCAGTGTCTT	681-701 937-958
Adr-β_2	NM_012492.2	S: CGACTACAAACCGTCACCAACT A: GAAGGGCGATGTGATAGCAAC	386-407 596-616
Adr-β_3	NM_013108.2	S: TTCAACCCGCTCATCTACTGC A: CACCTTCATAGCCATCAAACCT	1232-1252 1382-1403
NET	NM_031343.1	S: ACTTTGTCCTCTTTGTGCTCCTG A: GATCCATACCGTGGCCTCCT	854-876 950-969

Table 2. Antibodies used in this study

Antigen	Immunizing antigen or conjugation	Host species	Dilution	Source/Catalog No.
Adr-β_1	Synthetic peptide corresponding to Mouse β_1-AR aa 394-408	Rabbit	1: 1000	Abcam/ab3442
Adr-β_2	Synthetic within Human β_2-AR aa 350 to the C-terminus	Rabbit	1: 1000	Abcam/ab182136
Adr-β_3	Synthetic peptide corresponding to Mouse β_3-AR aa 350 to the conjugated to keyhole limpet haemocyanin	Rabbit	1: 1000	Abcam/ab94506
NET	Generated against unique N-terminal peptides that are unique to the noradrenaline transporter protein	Rabbit	1: 1000	Abcam/ab41559
Actin	KLH conjugated Synthetic peptide corresponding to Mouse β-Actin	Mouse	1: 1000	Servicebio/GB12001
Goat anti-rabbit IgG	horseradish peroxidase	Goat	1: 3000	KPL/5220-0336
Goat anti-mouse IgG	horseradish peroxidase	Goat	1: 3000	KPL/5220-0341

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Adr, Adrenoceptor; NET, norepinephrine transporter.

Figures and legends:

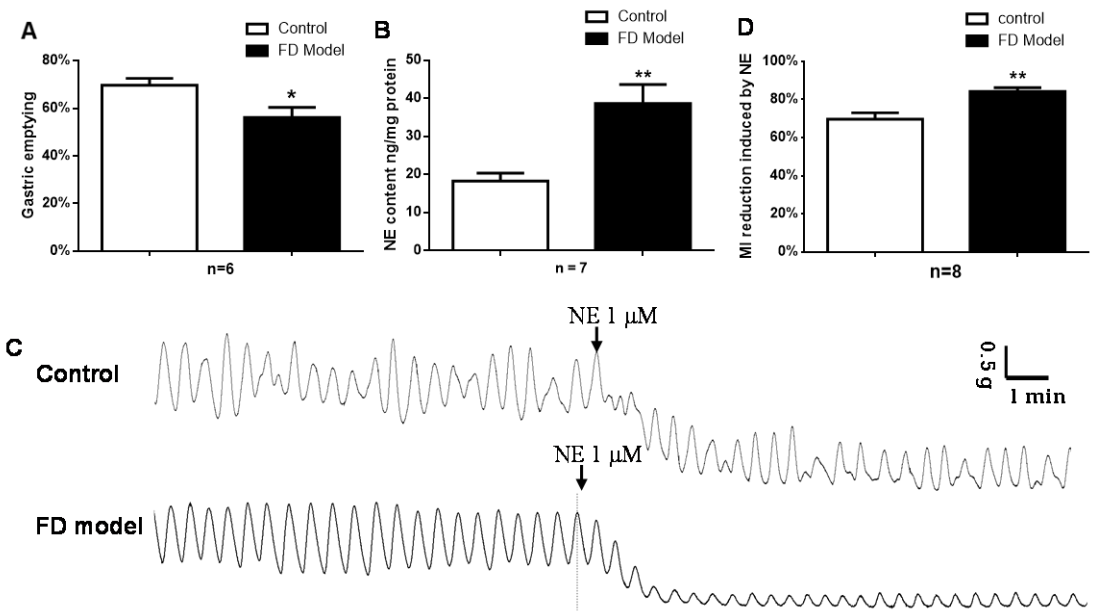

Figure 1. NE-induced reduction of gastric motility in control and FD model rats. (A) Gastric emptying of solid food in FD model rats was reduced significantly (n=6, p=0.036). (B) The NE content in the lamina muscularis of the stomach was increased in the FD model rats (n=7, p=0.007). (C) Representative tracing of a strip in the gastric corpus of control and FD model rats. (D) The reduction rate of the motility index after NE (10^{-6} M) treatment compared with the basal condition was lower in FD model rats (n=7, p=0.0221).

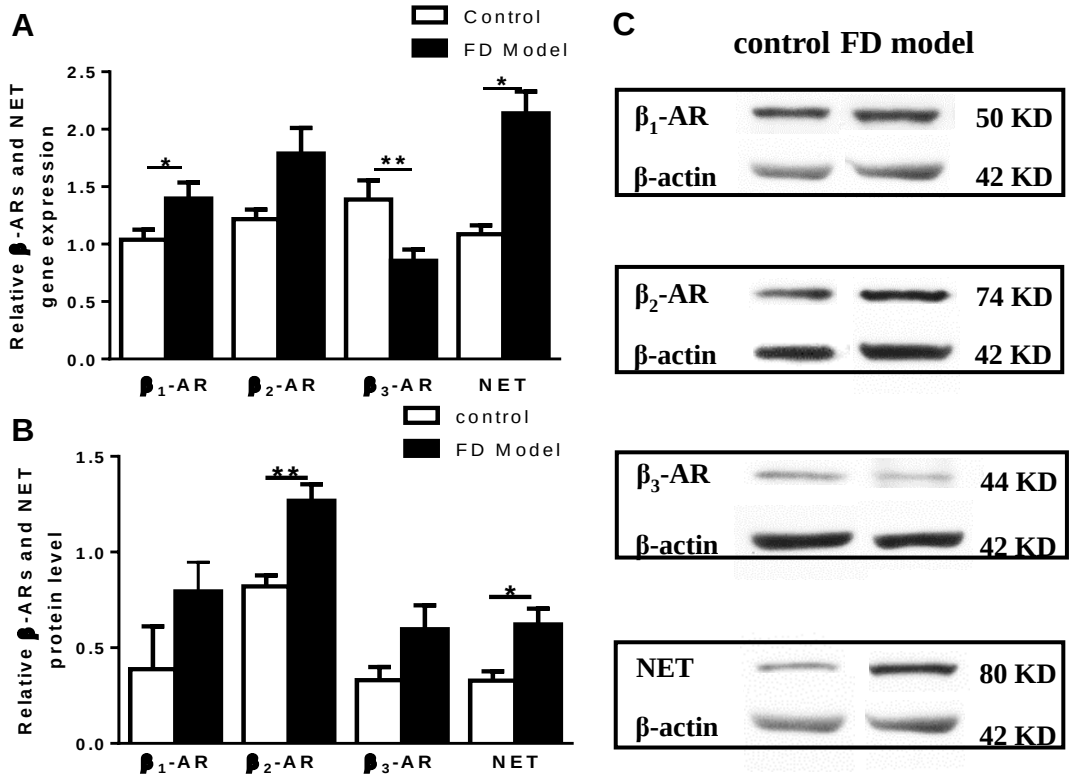

Figure 2. Expression of the β-adrenoceptor in the muscular layer of the gastric corpus in control and FD rats. (A) The mRNA levels of β-adrenoceptors and NETs in the lamina muscularis of control and FD rats. (B, C) The protein levels of β-adrenoceptors and NETs in the muscular layer of control and FD rats. β-actin was the internal control for normalization. The data are expressed as the mean ± SEM; * $P < 0.05$; ** $P < 0.01$.

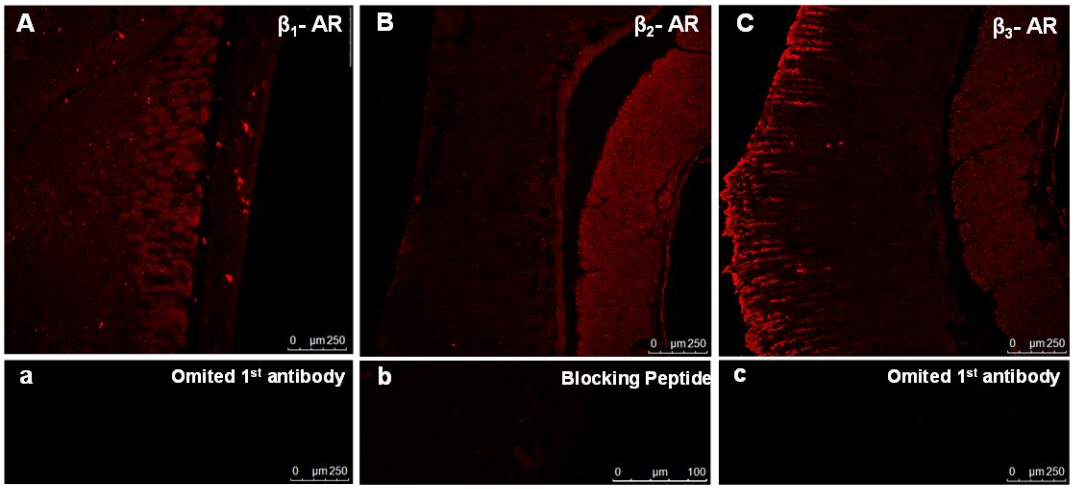

Figure 3. Distribution of β-adrenoceptors (β-ARs) in the full thickness of normal rats' gastric corpus. The distribution of β_1-AR (A), β_2-AR (B) and β_3-AR (C) in the gastric corpus of normal rats. Control without primary antibodies of β_1-AR(a), β_3-AR(c) and pretreatment with a blocking peptide of β_2-AR (b). Scale bar: 250 μm.