Impairment of CRH in the intestinal mucosal epithelial barrier of pregnant Bama miniature pig induced by restraint stress

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Abstract. Female, especially for pregnant female, are vulnerable to psychological stress. The morphology and metabolism of the maternal intestine are both obviously changed during pregnancy, thus making intestinal health status more fragile under psychological stress. The aim of the present study was to investigate the role of CRH and CRHR1 in the pregnant maternal intestine under psychological stress, thus exploring the mechanism of psychological stress in the pregnant maternal intestine. Bama miniature pigs were divided into the control and restraint stress groups from the first day of pregnancy. After restraint stress treatment for 18 consecutive days (D18), the plasma, duodenum, jejunum, ileum and colon were collected for study. Pregnant Bama miniature pigs subjected to restraint stress had significantly elevated CRH, adrenocorticotropic hormone (ACTH) and cortisol (COR) levels in plasma. Consistent with the increase in CRH levels, we observed enhanced oxidative stress levels in the intestine, which resulted in intestinal mucosal injury, including impaired intestinal morphology, a reduced number of goblet cells and proliferating cell nuclear antigen-positive cells, decreased expression of MUC2 and tight junctions, and elevated expression of CRHR1 and caspase-3. Moreover, exogenous CRH could directly promote IPEC-J2 cell apoptosis and influence its cell cycle (S and G2 phase) through CRHR1, and antalarmin could alleviate this phenomenon. Therefore, our results illustrated that the intestinal dysfunction of pregnant Bama miniature pigs was caused by restraint stress, and these changes were associated with the enhanced expression of CRH and CRHR1 in the intestine.

Key words: Psychological stress, Intestine, Pregnancy, CRH, CRHR1

WITH the accelerating of pace of life, the pressure people experience in their jobs, lives and education is constantly increasing. Notably, professional women need to maintain the balance between career and family, which inevitably brings more pressure to them. A study demonstrated that 78% of women from different urban areas experienced low-to-moderate antenatal psychosocial stress and 6% suffered high levels [1], so the anxiety is more serious among pregnant women [2]. Relevant studies have found increased corticosterone levels [3] and impaired intestinal barrier [4] of non-pregnant female animals under the stress, but there is no related study about pregnant female. Pregnancy is a time of marked neural, physiological and behavioral plasticity in the female and is often a time when women are more vulnerable to stress-related intestinal diseases. Numerous epidemiological and case-control studies have shown that chronic stress in mothers during pregnancy brought the negative affectivity to offspring [5, 6]. However, studies on the impact of psychological stress on pregnant women themselves are limited.

Intestine is the primary target altered by a variety of stressful stimuli, and the morphology and metabolism of the maternal intestine are both obviously changed during pregnancy. Since feed intake of pregnant rats increased by 40 to 50%, hypertrophy of all layers of the small intestinal wall is observed in the late stages of pregnancy [7], as well as the composition of the gut microbiota in mammals changed dramatically during pregnancy and was in parallel with the alteration of whole-body metabolic status [8]. These results mean that pregnant female need to absorb more nutrients through intestine and the whole-body healthy status will be influenced if intestine is impaired during pregnancy. Therefore, intestinal health is critical during pregnancy, people are supposed to pay more attention to that. Moreover, intestinal status during...
pregnancy is susceptible, so changes in the intestine caused by psychological stress are imperative.

Many studies by different animal stress models have demonstrated that psychological stress caused increased intestinal permeability [9] and altered gastrointestinal motility as well as ion secretion [10], and these consequences were also investigated in humans under acute stress [11]. There have been reports claimed that intestinal barrier of non-pregnant female was impaired under stress [4]. This may be more serious for pregnant women’s intestinal health, but there is no relevant study. A search for ‘stress and pregnancy’ in NCBI PubMed yielded 29,762 related entries (up to September 30, 2020), many of which are about descendants’ health, and different keywords account for diverse proportions: fetal (8,822 results), infants (6,309 results), newborns (6,214 results), children (6,165 results) and offspring (3,681 results). However, in a search for ‘intestine’ and ‘stress and pregnancy’, yielded were 326 entries, and only 11 of them were related to maternal intestinal health. Therefore, we choose the intestine as well as pregnant animals for this study to explore the influence of psychological stress on pregnant animals’ intestinal health.

Corticotropin-releasing hormone (CRH) and its receptor CRHR1 may play an important role in intestinal health of pregnant female under psychological stress. As for the adaptive response to stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated, releasing the hypothalamic hormone CRH [12]. Exogenous CRH administration can also simulate the stress response [13, 14], so CRH is a key hormone in stress response. For intestinal health, the systemic application of CRH induces a colonic motility response, which is more prominent in patients with intestinal diseases than in healthy controls [15]. At the same time, the peripheral CRH signaling pathways in the intestine may be also associated with a series of intestinal diseases. The expression of CRHR1 is tissue specific [16] and it has been detected both at the gene level and by immunohistochemistry in goblet and stem cells of crypts and on surface intestinal epithelial cells, lamina propria [17], and the myenteric nervous plexus [18]. In addition, some studies have reported that peripheral CRHR1 exerts proinflammatory effects on the intestine [19] and increases colonic secretion and permeability [20], which was alleviated by selective CRHR1 antagonists [21]. Peripherally acting CRHR1 antagonists might directly improve IBS symptoms related to motility, secretion and immune response [22]. Intestinal epithelial cells and the tight junctions between them are the first physical defense of the intestine; however, the direct effect of CRH and CRHR1 on these components has not been determined. Intestinal dysbiosis may induce pregnancy complications via affecting maternal adaptation [23], so it’s necessary to explore the influence of stress-induced CRH on pregnant female intestine.

Since a review [24] noted that the restraint technique has evolved as a standard model for studying stress effects, restraint stress has continued to be used in a variety of physiological and behavioral studies. Restraint stress is a well-known and ethologically acceptable model of chronic psychosocial stress, and Glavin noted an important role of the central nervous system in peripheral disease during restraint stress, especially gastrointestinal disease [25]. The restraint stress model is predominantly used in rodents. Nevertheless, pigs offer several advantages as preclinical models for humans because pigs are more similar to humans than rodents in many anatomical and physiological characteristics. Unlike in rodents, the main stress-induced glucocorticoid in humans and pigs is cortisol with a similar circadian rhythm [26]. Therefore, we aimed to investigate the involvement of peripheral CRH and CRHR1 in the intestinal barrier function caused by restraint stress using the pregnant Bama miniature pig model, which closely resembles the psychological stress of pregnant women in modern society, thus helping to refine rational strategies to maintain and repair stress-associated intestinal barrier function in pregnant women.

Materials and Methods

Animal treatment

A total of 6 healthy Bama miniature pigs (female; 8–9 months of age; Beijing Strong Century Min Sows Breeding Base, Beijing, China) weighing 20 to 30 kg were used in the study. The animals were raised under controlled conditions (temperature 22°C; 12-hour light/dark cycle; lights on at 8 AM and off at 8 PM). After successful natural mating, the pregnant Bama miniature pigs were randomly divided into two groups: (1) the control group, in which animals were raised in a wide environment and were allowed to move freely; (2) the restraint stress group, in which animals were raised (1 pig per cage) in a metal cage (50 cm × 60 cm × 100 cm) and were only allowed to move backwards and forwards but not turn around. Each group included 3 pregnant Bama miniature pigs, and all of them were given free access to food and tap water. After the restraint stress treatment for 18 days (D18), the pigs were sacrificed by cardiac bleeding and anesthetized using Zoletil 50 at a dose of 0.1 mL/kg body weight. The experimental procedures used for animal care and handling were approved by the China Agricultural University Institutional Animal Care and Use Committee (AW12119102-2).
**Blood samples and tissue preparation**

Blood samples obtained from jugular vein puncture of pigs were used for ELISA and radioimmunoassay detection. The duodenum (posterior to stomach), middle jejunum, ileum (anterior to ileocecal junction) and ascending colon were excised, and segments were fixed in 4% paraformaldehyde and stored at −80°C for subsequent experiments.

**Enzyme-linked immunosorbent assay**

Plasma samples were collected for the detection of CRH by competitive ELISA kits (CEA835Po, Wuhan USCN Business Co., Ltd., Wuhan, China). The experimental procedures were carried out according to the manufacturer’s instructions. The optical density was measured at 450 nm using an enzyme-linked immune detector (550, BIO-RAD, USA) immediately after the end of the reaction. The concentrations of CRH in plasma were calculated according to standard curves. The detection range was from 12.35 pg/mL to 1,000 pg/mL, and the lowest detection limit was 5.53 pg/mL. The intra- and inter-assay variations were lower than 10% and 12%, respectively.

**Radioimmunoassay**

Radioimmunoassay is a labeled immunoassay with radionuclide as the marker that is used to quantitatively measure an antigen in samples. Serum was separated from blood samples, and the ACTH (D14PDA, Beijing North Institute of Biotechnology Co., Ltd., Beijing, China) and COR (D10PZA, Beijing North Institute of Biotechnology Co., Ltd.) levels in serum samples were determined with commercially available kits. Standards, serum samples, 125I-ACTH or 125I-COR, and antibodies were added into test tubes successively according to the manufacturer’s instructions, mixed well and left at 4°C overnight. The next day, the mixture was mixed again and set aside for 15 min at room temperature. The supernatant was removed by centrifugation (3,500 × g for 10 min) at 4°C, and the radioactivity counts were determined with a multichannel radioimmunoassay counter (Zhongcheng Electromechanical Technology Co., Ltd., Hefei, China). The intra- and inter-assay variations were lower than 10% and 15.2%, respectively.

**Hematoxylin and eosin staining**

Tissue sections were mounted on gelatinized glass slides, deparaffinized with xylene, and then rehydrated with serial ethanol dilution. For histological studies, the sections were stained with hematoxylin and eosin (HE). Cell nuclei are shown in blue, and the cytoplasm is shown in soft red. In different intestinal segments, at least 3 random fields in 5 sections of each sample stained with HE were photographed with a microscope (BX51; Olympus). Villus heights (VHs) and crypt depth (CDs) were determined in the 5 longest and well-oriented villi and crypts in each image using Image-Pro Plus software (IPP 6.0, Media Cybernetics, Silver Spring, MD, USA). VH was measured from the opening of the intestinal gland to the top of the villus, and CD was measured from the opening of the intestinal gland to the muscularis mucosa.

**PAS staining**

The tissue sections were stained with periodic acid-Schiff (PAS). First, the polysaccharides in goblet cells were oxidized by periodic acid, exposing the aldehyde group, and reacted with colorless basic fuchsia. Finally, the polysaccharide in goblet cells appeared purple red. In different intestinal segments, at least 3 random fields in 5 sections of each sample stained with PAS were photographed with a microscope (BX51; Olympus). The number of columnar epithelial cells and goblet cells was counted for the 5 longest villi in each image, and the number of goblet cells per 100 intestinal columnar epithelial cells was calculated. The number of goblet cells in the colon was not counted because the colon had no intestinal villi.

**Immunohistochemical staining**

For the immunohistochemical studies, the sections were incubated with primary PCNA antibody (1:500, AV03018, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. Then, the sections were incubated with goat anti-rabbit IgG and biotin-conjugated antibody (1:300, CW0107, CWBioTech, Beijing, China) for 2 h 30 min at room temperature. Horseradish peroxidase streptavidin (SA-5004, Vector, Germany) and a diaminobenzidine (DAB) kit (ZLI-9017, ZsBio, Beijing, China) were used for staining and imaging. The mean density (IOD/area) of positive cells was measured from 3 random fields of 5 sections in each sample using Image-Pro Plus 6.0.

**Transmission electron microscopy**

Intestinal segments were immediately fixed in 2.5% glutaraldehyde and 1% osmium tetroxide. After dehydration by different concentrations of ethanol and acetone, the tissues were processed and embedded in Epon Araldite. Semithin sections (2 μm) were stained with toluidine blue to determine the location. Afterwards, ultrathin sections (50 nm) were made and stained with uranyl acetate and lead citrate and then examined on a transmission electron microscope (H-7500, HITACHI, Japan).
Measurements of antioxidant enzyme levels and lipid peroxidation

Portions of the various intestinal segments were rapidly homogenized, and clarified lysates were isolated by centrifugation (200 g for 10 min) at 4°C. The tissue extracts were stored at –80°C for antioxidant activity analysis. Five commercial kits (Beyotime Biotechnology Co. Ltd., Shanghai, China) were used to assay glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) activities, total antioxidant capability (T-AOC) and malondialdehyde (MDA) content using an enzyme-linked immune detector (550, BIO-RAD, USA).

Real-time quantitative PCR (qPCR) analysis

Total RNA was extracted and purified using TRIzol Reagent (CW0580A, CWBioTech, Beijing, China) following the protocol provided by the manufacturer. cDNA was reverse-transcribed and amplified using the Reverse Transcription System (A3500, Promega, USA). Real-time polymerase chain reaction (PCR) was performed with AceQ® qPCR SYBR® Green Master Mix (Q111-02, Vazyme, Nanjing, China). The primers of genes (Sangon Biotech, Shanghai, China) are shown in Table 1. β-actin was used as a housekeeping gene to normalize target gene transcript levels. Relative expression was normalized and expressed as a ratio to the expression in the control group.

Western blot analysis

The concentration of protein samples was determined by a bicinchoninic acid (BCA) protein assay kit (CW0014, CWBioTech, Beijing, China). Equal amounts of protein samples (30 μg) were separated and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, CA). After blocking with 5% skim milk, the membranes were incubated overnight with primary antibodies at 4°C, including claudin-1 (1:1,000, ab15098, Abcam, USA), occludin (1:1,000, CSB-PA190654, Cusabio, Wuhan, China), CRHR1 (1:500, NHA16988, Novogene, Tianjin, China), caspase-3 (1:1,000, Asp175, CST, USA) and β-actin (1:4,000, CW0096, CWBioTech, Beijing, China). The membranes were subsequently washed with TBST and incubated with secondary horse-radish peroxidase-conjugated IgG for 2 h at room temperature. An enhanced chemiluminescence western blot kit (CW0049A, CWBioTech, Beijing, China) was used according to the manufacturer’s instructions to visualize the bands. The intensities of the bands were analyzed using a Gel-Pro Analyzer 4.5 (Media Cybernetics, USA). The relative protein expression of claudin-1, occludin, CRHR1 and caspase-3 was expressed relative to β-actin protein.

Cell culture

Porcine intestinal epithelial cells (the IPEC-J2 cell line) were cultured in DMEM/F12 medium (SH30023.01B, HyClone, Beijing, China) containing 10% FBS, 1% antibiotics (penicillin-streptomycin), and grown in a humidified incubator at 37°C with 5% CO₂ and 95% air.

MTT assay

Cell viability was evaluated using an MTT assay. Then, 200 μL of IPEC-J2 cells (5 × 10⁴/mL) was added to each well of 96-well plates and cultured in DMEM/F12 with 10% FBS (complete medium) for 24 h. Then, the cells were cultured in DMEM/F12 without FBS (basal medium) for 12 h. After that, the cells were treated with H₂O₂ at the concentrations of 0, 100, 200, 400, 600, 800, 1,000, 1,200, 1,400, and 1,600 μM or CRH (C3042, Sigma-Aldrich, St. Louis, Missouri, USA) at the concentrations of 0, 0.0001, 0.001, 0.01, 0.1, and 1 nM for 24 h, or the cells were treated with 0.01 nM CRH for 6, 12, 24, 36, or 48 h or 0.01 nM CRH and antalarmin (A8727, Sigma-Aldrich, St. Louis, Missouri, USA) at the concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 μM for 36 h. Finally, the medium was discarded, and 10 μL MTT reagent and 90 μL basal medium were added. The cells were further cultured for 4 h, and the optical density (OD) was measured at a wavelength of 570 nm using an enzyme-linked immune detector (550, BIO-RAD, USA). The number of cells is presented as the percent of control cells.

Table 1 Primer sequences used for Quantitative real-time RT-PCR

| Gene       | Forward (5’-3’)            | Reverse (5’-3’)          |
|------------|-----------------------------|--------------------------|
| Claudiin-1 | ACCACTTTTGCAGAACACCCG       | CATTGACTGGGTCATGGGGTC    |
| Occludin   | CAGTGTTAACCCTGGAGGCGGT      | CCCTGCTGAGTCTGGTCTCG     |
| MUC2       | CTGGCTCCGGGGTCCCTGAGGGA     | CCCGCTGGCTGGGTCGATAC     |
| β-actin    | TCTGGCACCCACACCTCTAC        | TTCTCACGTTGGCCTTTGGG     |
Flow cytometry

A total of 1 mL of IPEC-J2 cells (1 × 10^5/mL) was added to each well of 12-well plates and cultured in complete medium for 24 h, and the cells were cultured in basal medium for 12 h. After that, the cells were treated for 36 h with (1) basal medium; (2) 1% DMSO; (3) H₂O₂ (1,000 μM); (4) CRH (0.01 nM); (5) CRH (0.01 nM) + antalarmin (1 μM); and (6) antalarmin (1 μM). After 36 h of incubation, some collected cells were treated with 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI) by Annexin V-FITC/PI kits (C1062M, Beyotime Biotechnology Co. Ltd., Shanghai, China) for 15 min at room temperature in the dark to measure cell apoptosis, and some cells were treated with 0.5 mL of propidium iodide (PI) by Cell Cycle and Apoptosis Analysis Kit (C1052, Beyotime Biotechnology Co. Ltd., Shanghai, China) for 30 min at 37°C in the dark to assess the cell cycle. The Apoptotic cells and cell cycle phases were finally identified using a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). The data were analyzed by using the CellQuest program (BD Biosciences). Experiments were performed in triplicate.

Statistical analysis

The SPSS 23.0 (SPSS Sciences, Chicago, USA) statistical package was used for statistical analyses. The results are presented as the mean ± SEM. The difference between the two different treatment groups (control and stress group) was assessed by independent-samples t test, considering p ≤ 0.05 to be significant; the effects of different treatments on IPEC-J2 cells were analyzed using one-way ANOVA and a post hoc least significant difference (LSD) test, considering p ≤ 0.05 to be significant.

Results

Restraint stress increased plasma hormone levels

To determine whether a stress response was induced by restricted space, we examined the changes in stress hormone levels in plasma. As shown in Fig. 1A–C, the plasma concentrations of CRH, ACTH and COR of pregnant pigs subjected to the restraint stress protocol were 335.58% (t = –5.531, p = 0.030), 62.98% (t = –4.549, p = 0.010) and 109.86% (t = –5.602, p = 0.005), respectively, higher than the control group on the 18th day of treatment (D18), while there was no difference before the restraint stress treatment (D0). The enhanced level of stress hormones illustrated that a restraint stress model in pregnant pigs that simulated chronic psychological stress was successfully established.

Increased oxidative stress in the intestine

Under restraint stress, we determined five antioxidant parameters, including antioxidant enzymes (GSH-Px, CAT and SOD), T-AOC and MDA, to examine whether restraint stress caused oxidative stress in various intestinal segments. The GSH-Px level (Fig. 2A) was reduced by 44.34% (jejenum, t = 8.386, p = 0.001), 76.58% (ileum, t = 10.165, p = 0.001) and 49.79% (colon, t = 9.693, p = 0.001) in the restraint stress group compared to the control group. The CAT level (Fig. 2B) was reduced by 26.27% (duodenum, t = 3.905, p = 0.017), 26.88% (jejenum, t = 3.248, p = 0.031), and 7.71% (ileum, t = 6.431, p = 0.003). The SOD level (Fig. 2C) was reduced by 27.92% (duodenum, t = 5.405, p = 0.027), 31.24% (jejenum, t = 4.955, p = 0.008), 26.52% (ileum, t = 4.325, p = 0.012) and 20.98% (colon, t = 3.162, p = 0.034). The T-AOC level (Fig. 2D) was reduced by 51.30% (duodenum, t = 7.763, p = 0.001), 51.19% (jejenum, t = 12.902, p = 0.000), 25.08% (ileum, t = 4.537, p = 0.044) and 37.58% (colon, t = 6.967, p = 0.002). In contrast, we observed a significant increase in MDA levels (Fig. 2E), an end-product of lipid peroxidation. MDA content was significantly elevated by 72.61% (duodenum, t = –4.732, p = 0.009), 14.22% (jejenum, t = –2.938, p = 0.042), and 87.07% (colon, t = 4.131, p = 0.014) in the restraint stress group vs. the control group. These results indicated that the oxidative stress level was significantly increased in the intestine of pregnant pigs after restraint stress.

Impairment of intestinal mucosa

To determine the influence of oxidative stress on the intestinal mucosa, we examined pathological changes after restraint stress. Compared to the control group (Fig. 3A), the restraint stress group showed obvious pathological changes in the duodenum and ileum (Fig. 3B): hyperemia appeared in the intestinal villus, and the intestinal epithelium cells had fallen away and exposed the lamina propria (black arrows). In addition, the villus height (VH) and crypt depth (CD), which represent the absorptive and secretive function of the intestinal villus, respectively, were measured. The VH (Fig. 3C) was significantly decreased in the stressed animals compared with the control animals, reaching 15.37% in the duodenum (t = 23.15, p = 0.000), 21.73% in the jejunum (t = 7.896, p = 0.000) and 18.36% in the ileum (t = 7.393, p = 0.000) of the control animals. In contrast to the VH, the CD (Fig. 3D) of the stress group, at 23.15% in the duodenum (t = –6.547, p = 0.000), and 12.80% in the jejunum (t = –3.566, p = 0.000), was considerably increased compared to that of the control group. Therefore, the villus height/crypt depth (V/C) ratio (Fig. 3E), which comprehensively reflects the digestion and absorption capacity of the small intestine, was tremendously decreased by 31.96% in the duodenum.
Fig. 1  Restraint stress increased plasma hormone levels (CRH, ACTH and COR) in pregnant pigs. (A) Plasma CRH concentrations were measured by ELISA. (B) Plasma ACTH and (C) COR concentrations were measured by radioimmunoassay. The lowercase letters represent the control group comparisons, and the uppercase letters represent the stress group comparisons. Differences were assessed by independent-samples t test, and the values are represented as the mean ± SEM. Different letters indicate a significant difference between the same treated groups on the different days ($p \leq 0.05$). * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ represent the comparison between the stress and control groups. D0, the day before restraint stress treatment; D18, the 18th day of restraint stress treatment.

Fig. 2  The levels of five antioxidant parameters were increased in the intestine of restraint-stressed pregnant pigs. (A) GSH-Px (B) CAT (C) SOD (D) T-AOC and (E) MDA concentrations were measured by an oxidative stress-related enzyme test. Differences were assessed by independent-samples t test, and values are presented as the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the control group.
(t = 5.738, p = 0.000), 30.56% in the jejunum (t = 8.033, p = 0.000) and 24.08% in the ileum (t = 7.857, p = 0.000) in the restraint stress group compared to the control group. These results indicated that restraint stress impaired intestinal morphology, thus influencing several functions of the intestine.
**Impaired secretion of intestinal goblet cells**

To determine whether the secretion of intestinal goblet cells was changed after restraint stress, we measured the number of goblet cells in the intestine by PAS staining and the changes in the ultrastructure of goblet cells through transmission electron microscopy. In Fig. 4, the black arrow indicates the goblet cell (Fig. 4A), and the PAS staining results showed that the number of goblet cells was dramatically decreased by 28.02% (duodenum, $t = 10.476, p = 0.000$) in the duodenum, 22.08% (jejenum, $t = 8.258, p = 0.000$) in the jejunum, and 17.65% (ileum, $t = 7.218, p = 0.000$) in the ileum of the restraint stress group (Fig. 4E). Moreover, the transmission electron microscope results (Fig. 4C–D) showed that the secretory granules in the goblet cells of the control group had well-defined boundaries and homogeneous densities. However, rarefaction of the goblet cells’ secretory granules and abnormal boundaries were observed in the restraint stress group. A similar effect was also observed in the expression level of MUC2, which was significantly decreased by 20.05% (duodenum, $t = 4.454, p = 0.011$), 35.84% (jejenum, $t = 3.120, p = 0.036$), 42.16% (ileum, $t = 3.579, p = 0.023$), and 37.02% (colon, $t = 7.358, p = 0.002$) in the restraint stress group compared with the control group (Fig. 4F). These results illustrated that the secretion of intestinal goblet cells was impaired because of the number of goblet cells was decreased, and the structure of goblet cells was damaged.

**Imbalance of proliferation and apoptosis in the intestine**

To confirm the proliferative and apoptotic state of the intestine, we performed the following experiments. Intestinal cell proliferation was measured by determining the number of PCNA-positive cells using immunohistochemistry staining, and the reaction-positive cells appeared in brown color and were mainly distributed in lamina propria and crypts (Fig. 5A–B). A significant decrease in the PCNA-positive density (Fig. 5C) was observed in animals in the stress group compared with those in the control group, including a 18.34% decrease in the duodenum ($t = 2.373, p = 0.025$), a 33.30% decrease in the jejunum ($t = 5.962, p = 0.000$), a 29.16% decrease in the ileum ($t = 4.157, p = 0.001$), and a 20.44% decrease in the colon ($t = 2.849, p = 0.008$). In addition, intestinal cell apoptosis was measured by western blotting analysis of cleaved caspase-3 using a 15% polyacrylamide gel for electrophoresis. Two cleaved caspase-3-specific bands (Fig. 5D) of approximately 17 kDa and 19 kDa were observed, and a significant increase in cleaved caspase-3 levels in the intestine was observed in stress-induced pregnant pigs compared to the control pigs. For the 17 kDa protein (Fig. 5E), the expression of cleaved caspase-3 was increased by 345.02% (duodenum, $t = –5.010, p = 0.007$) and 119.22% (colon, $t = –3.849, p = 0.018$). For the 19 kDa protein (Fig. 5F), the expression of cleaved caspase-3 was increased by 219.13% (duodenum, $t = –5.215, p = 0.006$), 397.46% (jejenum, $t = –3.113, p = 0.036$), 177.63% (ileum, $t = –3.698, p = 0.021$) and 92.91% (colon, $t = –4.834, p = 0.008$). These results suggested that the balance of proliferation and apoptosis in the intestine of pregnant pigs was disrupted after restraint stress.

**Decreased tight junction expression in the intestine**

The intestinal epithelial barrier can become leaky as a result of tight junction (TJ) impairment, which results in many diseases, such as IBD and systemic inflammation. To ascertain the changes in tight junction expression due to restraint stress, we also observed the ultrastructure of tight junctions by transmission electron microscopy. Through transmission electron microscopy observations (Fig. 6A), we found that the tight junctions between epithelial cells were extremely tight in the control group. However, gap expansion and tight junction damage between intestinal epithelial cells were visible in the restraint stress group (Fig. 6B).

To further investigate whether restraint stress could affect pregnant maternal intestinal mucosal integrity, we detected changes in the expression of tight junction genes and proteins in the mucosal epithelium. The mRNA expression of the tight junction proteins (Fig. 6C–D) claudin-1 and occludin was decreased by 55.95% ($t = 3.430, p = 0.027$) and 25.19% ($t = 5.479, p = 0.005$) in the duodenum, 50.56% ($t = 3.586, p = 0.023$) and 54.93% ($t = 11.620, p = 0.000$) in the jejunum, 52.73% ($t = 4.196, p = 0.014$) and 69.50% ($t = 32.838, p = 0.000$) in the ileum, and 53.34% ($t = 6.673, p = 0.003$) and 73.86% ($t = 14.655, p = 0.000$) in the colon, respectively. In addition, the protein expression of claudin-1 and occludin (Fig. 6E–G) was also reduced by 67.80% ($t = 3.366, p = 0.028$) and 60.37% ($t = 2.928, p = 0.043$) in the duodenum, 61.43% ($t = 4.449, p = 0.011$) and 45.82% ($t = 4.092, p = 0.015$) in the jejunum, 58.93% ($t = 5.189, p = 0.007$) and 67.91% ($t = 5.017, p = 0.007$) in the ileum, and 60.72% ($t = 3.690, p = 0.021$) and 53.19% ($t = 3.284, p = 0.030$) in the colon, respectively. These results suggested that the intestinal epithelial barrier of pregnant pigs became leaky due to restraint stress.

**Increased CRHR1 expression in the intestine**

To further explore the link between the above changes in the maternal intestine, we determined the expression of CRHR1 in various intestinal segments of pregnant pigs. The results showed that CRHR1 protein expression
Fig. 4 Impairment of the secretion of intestinal goblet cells in restraint-stressed pregnant pigs. 
(A–B) PAS staining of different intestinal segments in the control (A) and stress (B) groups (black arrows: goblet cell; scale bar: 20 μm). (C–D) Transmission electron microscope observation of colonic goblet cells in the control (C) and stress (D) groups (scale bar: 2 μm). (E) The number of goblet cells per 100 intestinal columnar epithelial cells in the control and stress groups. (F) MUC2 gene expression of different intestinal segments in control and stress groups and relative gene levels were normalized to β-actin. Differences were assessed by independent-samples t test, and values are represented as the means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared with the control group.
(Fig. 6G–H) was evidently increased by 86.13% in the duodenum ($t = -3.213, p = 0.032$), 214.76% in the jejunum ($t = -9.997, p = 0.001$), 79.33% in the ileum ($t = -2.844, p = 0.047$), and 62.31% in the colon ($t = -3.204, p = 0.033$). Therefore, psychological stress can strengthen the expression of CRHR1, which was positively correlated with the expression of CRH in plasma, indicating the involvement of CRH and CRHR1 in intestinal barrier function.

**CRH suppressed the growth of IPEC-J2 cells**

To explore how CRH and CRHR1 influence the intes-
Fig. 6 Decreased tight junction and increased CRHR1 expression in the intestine of restraint-stressed pregnant pigs.
(A–B) Transmission electron microscope observation of tight junctions in the control (A) and stress (B) groups. (C–D) claudin-1, occludin and β-actin gene expression in different intestinal segments in the control and stress groups. Relative gene levels were normalized to β-actin. (E–H) claudin-1, occludin, CRHR1 and β-actin protein expression in different intestinal segments in the control and stress groups. Relative protein levels were normalized to β-actin. Differences were assessed by independent-samples t test, and the values are presented as the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared with the control group. CD, duodenum of the control group; SD, duodenum of the stress group; CJ, jejunum of the control group; SJ, jejunum of the stress group; CI, ileum of the control group; SI, ileum of the stress group; CC, colon of the control group; SC, colon of the stress group.

Impairment of CRH in maternity gut

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tinal epithelial barrier, we added CRH to the medium of intestinal epithelial cells (IPEC-J2 cells). First, we observed the morphological characteristics of IPEC-J2 cells. As shown by the HE staining in Fig. 7A–B, IPEC-J2 cells are polygonal in shape, with a round or elliptical shape. Then, we indicated that CRHR1 was located at the cytomembrane of IPEC-J2 cells (Fig. 7C) compared to the negative control group with immunohistochemistry (Fig. 7D). Based on these results, we added H2O2 and CRH to the medium, and the cytotoxic effects of H2O2 and CRH on IPEC-J2 cells were evaluated by MTT assay. The growth of IPEC-J2 cells is shown in Fig. 7. The results showed that the number of cells was significantly reduced in the 1,000 μM H2O2 group compared with the 0, 100, 200, 400, 600, and 800 μM H2O2 groups ($F_{(9, 70)} = 79.616$, $p = 0.000$), and no difference was found among the 1,200, 1,400, and 1,600 μM H2O2 groups (Fig. 7E); 0.01 nM CRH caused a significant reduction ($F_{(8, 42)} = 9.487$, $p = 0.000$) in the number of IPEC-J2 cells (Fig. 7F). The number of IPEC-J2 cells was significantly reduced by treatment with 0.01 nM CRH for 36 or 48 h ($F_{(4, 35)} = 114.277$, $p = 0.000$) (Fig. 7G). Moreover, 1 μM antalarmin could obviously alleviate the inhibitory effect of CRH, and no difference was found between the 10 and 100 μM antalarmin groups ($F_{(6, 49)} = 41.835$, $p = 0.000$) (Fig. 7H). Therefore, these results showed that CRH suppressed the growth of IPEC-J2 cells through CRHR1. Moreover, 1,000 μM H2O2, 0.01 nM CRH, 36 h of cell culture, and 1 μM antalarmin were chosen for subsequent experiments.

**CRH promoted IPEC-J2 cell apoptosis and influenced the cell cycle**

To further verify the impact of CRH and CRHR1 on IPEC-J2 cells, we detected the apoptosis rate and cell cycle of IPEC-J2 cells with flow cytometry. According to the flow cytometry results (Fig. 8), the percentage of apoptotic cells in the CRH-treated group was significantly ($F_{(5, 12)} = 205.193$, $p = 0.000$) higher than that in the control group. In contrast, treatment of IPEC-J2 cells with CRH + antalarmin significantly inhibited the cell apoptosis induced by CRH. This result indicated that CRH had a proapoptotic effect on IPEC-J2 cells through CRHR1.

In addition, as shown in Fig. 9, we found that the number of IPEC-J2 cells in S phase was decreased ($F_{(5, 12)} = 365.801$, $p = 0.000$) and that the number of cells in G2 phase ($F_{(5, 12)} = 52.210$, $p = 0.000$) was increased significantly in the CRH-treated group, which indicates that CRH mainly impacted the DNA synthesis phase (S phase) and mitotic preparation phase (G2 phase) of IPEC-J2 cells, and antalarmin could reverse this effect.

**Discussion**

In the current study, we demonstrated that psychological stress impaired pregnant maternal intestinal barrier function, and this effect was accompanied by increased CRH levels in the plasma and CRHR1 expression in the intestine. Therefore, the impaired intestinal function of pregnant pigs may be dependent on the activation of CRH and CRHR1 in the intestine after restraint stress, which was supported by an in vitro experiment in which exogenous CRH could directly influence the cell cycle of intestinal epithelial cells and promote apoptosis through CRHR1.

Psychological stress is often suspected to adversely affect intestinal health, including maternal intestinal function during pregnancy. To prevent the adverse outcomes of maternal stress associated with negative health consequences, the activity of corticotrophin-releasing hormone (CRH) neurons is attenuated during pregnancy [27]. Regarding the role of CRH in the periphery under stress, it has been reported that the direct peripheral administration of CRH in animals could mimic the visceral responses to stress [28]. For instance, CRH strongly alters gut motility and transit in several mammalian species, including rodents, dogs, and humans [15, 29, 30]. Therefore, CRH is an important mediator in the induction of psychological stress-related disorders. In our study, the level of CRH in the plasma of pregnant pigs was prominently enhanced, which was consistent with the results of many studies [31, 32], in which these changes were observed in mice after restraint stress and in pregnant women under chronic stress. At the same time, ACTH and COR levels were also significantly enhanced in the plasma of pregnant pigs in the restraint stress group, which was consistent with the finding that chronic exposure to stress results in long-term cortisol exposure, with maladaptive effects [33]. Therefore, a restraint stress model of pregnant pigs could help illustrate the link between psychological stress and maternal intestinal health during pregnancy.

In addition to increased stress hormone levels, the present findings also showed that restraint stress could induce oxidative stress by decreasing the activities of antioxidant enzymes and T-AOC and increasing the formation of MDA. This is because these antioxidant enzymes, namely, GSH-Px, SOD and CAT, are the most potent scavengers of harmful reactive oxygen species (ROS), which result in lipid peroxidation (formation of MDA) and oxidative stress. Under this adverse status, the body will become impaired. The intestinal mucosal barrier is the first line of defense against a hostile environment [34]. The morphology of the mucous membrane, such as the depth of crypts, architecture of villi...
Fig. 7 CRH suppressed the growth of IPEC-J2 cells.

(A–B) HE staining of IPEC-J2 cells (scale bars: (A) 100 μm and (B) 50 μm). (C–D) Immunohistochemical staining of CRHR1 in IPEC-J2 cells (scale bar: 50 μm), (C) CRHR1-positive cells, and (D) negative control. (E–H) MTT assay. (E) Effect of different concentrations of H$_2$O$_2$ on the growth of IPEC-J2 cells. (F) Effect of different concentrations of CRH on the growth of IPEC-J2 cells. (G) Effect of CRH on the growth of IPEC-J2 cells at different times. (H) Effect of different concentrations of antalarmin on the CRHR1 expression in IPEC-J2 cell membranes. Differences were assessed by one-way ANOVA, and the values are presented as the mean ± SEM. The different letters indicate a significant difference among the different treatment groups ($p \leq 0.05$).
and number of goblet cells, are often used to evaluate gut barrier function [35]. For instance, villus height influences the absorption of nutrients, and crypt depth represents the production rate of crypt cells. During pregnancy, food intake usually increases; therefore, the absorption and digestion of the intestine should increase. Nevertheless, similar to previous experiments [36], our current findings showed that intestinal villus height was significantly decreased and that crypt depth was increased in stress-induced animals compared to control animals. Accordingly, the V/C ratio was tremendously decreased, illustrating the reduction in intestinal maturation and nutritional effects.

Under severe oxidative stress, or decreased protective enzymes, cells may “sacrifice themselves” through apoptosis, which protects surrounding healthy tissue from further damage [37]. Interestingly, in our study, a pronounced decrease in the PCNA-positive rate and a corresponding increase in caspase-3 expression in the intestine of restraint stress-induced pregnant pigs, suggesting that the increased cell apoptosis impaired the intestinal mucosal barrier of pregnant pigs. In addition, there are tight junctions between adjacent intestinal epithelial cells, and their expression influences the integrity and permeability of the intestinal mucosa. If tight junctions are impaired, foreign antigens may penetrate across the defective intestinal barrier. Moreover, claudin-1 and occludin are members of the critical components in the structural and functional organization of tight junctions [38, 39], thus closely influencing intestinal permeability.

Fig. 8  CRH promoted IPEC-J2 cell apoptosis.
(A) Flow cytometry analyses of apoptosis using FITC-labeled Annexin V/PI staining. IPECs situated in the M1 area were regarded as apoptotic cells. (B) Statistical analysis of the percentage of apoptotic cells in the IPECs under different treatments. Differences were assessed by one-way ANOVA, and values are presented as the mean ± SEM. The different letters indicate a significant difference among the different treated groups (p ≤ 0.05).
CRH influenced the cell cycle of IPEC-J2 cells.

(A) Flow cytometry analyses of the cell cycle of IPECs using PI staining. (B–D) Statistical analysis of the cell cycle of IPECs under different treatments. (B) Percentage of IPECs in the gap 1 (G1) phase of the cell cycle under different treatments. (C) Percentage of IPECs in the synthesis (S) phase of the cell cycle under different treatments. (D) Percentage of IPECs in the mitotic (M) phase of the cell cycle under different treatments. Differences were assessed by one-way ANOVA, and values are presented as the mean ± SEM. The different letters indicate a significant difference among the different treatment groups (p ≤ 0.05).
Meanwhile, mucin (MUC2), which is secreted by goblet cells, also plays an important role in maintaining the normal permeability of the intestine [40]. In agreement with many reports [41-43], our observations showed that restraint stress resulted in a decrease in the number of goblet cells and the downregulation of occludin, claudin-1 and MUC2. These results indicated that restraint stress could damage the intestinal mucosa, thus disrupting the mechanical barrier.

The mechanism through which restraint stress impairs the intestinal epithelial barrier of the pregnant matrix remains elusive. A relevant study has reported that the stress-induced CRHR1-dependent stimulation of colonic motor function is not altered by blocking the activation of the HPA axis [44]. This is because CRHR1 is also widely expressed in the gastrointestinal tract in addition to being located in the brain and pituitary, and this expression of CRH ligands and receptors in the gastrointestinal tract gives rise to a local CRH signaling pathway that can act directly on the gut in either a paracrine or an autocrine manner [28, 45]. In our study, we observed the obvious improvement of CRHR1 in the intestine of stress-induced pregnant pigs, and these alterations were similar to the report by Huo [46], in which mice were treated with chronic restraint stress. CRHR1–/– mice displayed reduced activation of NF-kB with decreased proliferating cells and enhanced apoptotic cells in the colon [47], which underlines the role of CRHR1 in cell apoptosis of the intestine. To further explore the role of CRH and CRHR1 in intestinal epithelial cells, we cultured IPEC-J2 cells in vitro. The results showed that CRH could promote the apoptosis of IPEC-J2 cells and impact the S phase and G2 phase of the cell cycle, which was completely overcome by the addition of the CRHR1 antagonist antalarmin. A similar analysis showed that the addition of CRH induced ovarian cell apoptosis by interacting with elevated CRHR1 [31]. We speculated that CRH may impair the intestinal mucosal epithelial barrier through its proapoptotic action on intestinal epithelial cells by interacting with CRHR1.

In summary, we demonstrated that elevated CRH may influence the intestinal mucosal epithelial barrier of pregnant pigs by increasing the oxidative stress level and damaging intestinal morphology. Furthermore, the participation of CRH in the stress-related intestinal responses of pregnant pigs and its proapoptotic action on IPEC-J2 cells suggests the potential regulatory role of CRH and CRHR1 in the intestinal health of pregnant women. Therefore, our findings could drive the development of maternal intestinal health research and may provide a tractable target in the treatment and prevention of the intestinal diseases of pregnant women under many kinds of social and psychological stress.

Acknowledgements

The present study work was supported by the National Natural Science Foundation of China (grant nos. 31972633, 31572476, 31272483) and National Natural Science Foundation of Beijing (grant nos. 6172022).

Disclosure

The authors declare that they have no competing interests.

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