CD4+/IL-4+ lymphocytes of the lamina propria and substance P promote colonic protection during acute stress

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Abstract. Life stress may influence symptom onset and severity in certain gastrointestinal disorders in association with a dysregulated intestinal barrier. It has been widely accepted that stress triggers the hypothalamus-pituitary-adrenal (HPA) axis, releasing corticosterone, which promotes intestinal permeability. In response, colonic inflammation alters mucosal immune homeostasis and destroys the colonic architecture, leading to severe intestinal diseases. Endogenous substance P (SP) does not inhibit the initial extent of the HPA axis response to restraint stress, but it reduces the duration of the stress, suggesting that SP plays an important role in the transition between acute and chronic stress. The present study aimed to investigate the effect of two groups of mice exposed to stress, including acute and chronic stress. The corticosterone was evaluated by ELISA, colon samples were obtained to detected polymorphonuclear cells by hematoxylin and eosin staining, goblet and mast cells were identified by immunocytochemistry and cytokine-producing $CD4^+$ T cells were analyzed by flow cytometry assays, adhesion proteins in the colon epithelium by western blotting and serum SP levels by ELISA. The results demonstrated an increase in the number of polymorphonuclear, goblet and mast cells, a decrease in claudin-1 expression and an elevation in E-cadherin expression during acute stress. Increased E-cadherin expression was also detected during chronic stress. Moreover, it was found that acute stress caused a shift towards a predominantly anti-inflammatory immune response (T helper 2 cells), as shown by the increase in the percentage of $CD4^+$/IL-6^+ and $CD4^+$/$IL4^+$ lymphocytes in the lamina propria and the increase in serum SP. In conclusion, this response promoted colonic protection during acute stress.

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Introduction

It is well established that cortisol is the main hormone that regulates stress in humans and it plays an equivalent role in rodents (1). Stress begins when the hypothalamus releases corticotropin-releasing factor (CRF), thereby activating the hypothalamic-pituitary-adrenal (HPA) axis (2). In animal models, stress is associated with abnormalities in processes that maintain gut homeostasis, such as in visceral perception (3), the integrity of the intestinal-epithelial barrier (4), ion transport (5) and host defense mechanisms. Increasing gut permeability promotes an inflammatory environment, such as that observed in chronic gastrointestinal diseases (6). Accumulating evidence has shown that, in adult rats, acute or chronic stress involves the release of CRF (7); furthermore, other models of stress, such as neonatal maternal deprivation stress, restraint and immobilization stress (8,9), and chronic unpredictable stress (10), mimic the epithelial response to stress, stimulating ion secretion and enhancing permeability. Data from chronic stress models have revealed that a region-specific reduction in epithelial tight junction (TJ) protein expression in the colon is induced by corticosterone (11). Recently, the same authors proposed that chronic elevation of glucocorticoid (GC) levels may impair the epithelial barrier function of human/rat claudin 1 promoters in the colon via the transcription repressor HES family bHLH transcription factor 1 and GCs natural cytotoxicity triggering receptor 3 axis (12). Therefore, intestinal permeability during stress is a process that is highly regulated and is crucial for maintaining interactions between microbiota, colonic epithelial cells, immune system cells and nervous system cells. Although it has been hypothesized that acute or

short-term stress can be an adaptive mechanism that confers greater immune protection after injury or infection (13,14), to date, little is known regarding epithelial cells (goblet, mast and immune cells) and other effector mechanisms involved in conferring this protection.

While observations have documented that mast cells are recruited by high levels of corticosterone (15), it has been proposed that these cells are sentinels that are responsible for supporting stress-induced colonic mucosal pathophysiology, and they also communicate with immune cells (16) and the central nervous system via substance P (SP) (17). SP is a neuropeptide that is released throughout the body by the neuro-endocrine system in all organs and inflammatory cells, such as lymphocytes (18). SP acts by binding to the neurokinin-1 receptor (NK1R), and it exerts proinflammatory effects on immune and epithelial cells. In addition, SP regulates smooth muscle contractility, hyperalgesia, epithelial ion transport, vascular permeability and immune function in the gastrointestinal tract (18). At present, the two types of stress imposed on intestinal epithelial components (goblet and mast cells, TJ proteins) and non-epithelial components (inflammatory infiltrate and lymphocyte cytokines of the lamina propria) in the colon and serum SP were evaluated. These changes could help elucidate the initiation and perpetuation mechanisms of gut inflammation by stress.

The current study used a stress model in which mice were subjected to repeated movement restriction, including one session (acute) or three repeated sessions over 2 h (chronic). The objective of the present study was to evaluate the effects of these two stress protocols on mucosal components and immune response parameters in the colon, as well as on serum SP levels. The results of the present study could provide a useful reference for the management of therapies that regulate the effects of stress on intestinal bowel disease dysfunction.

Materials and methods

Experimental animals. A total of 45 male BALB/c mice (weight, 20-30 g; age, 10-12 weeks) were used in this study. The mice were allowed free access to food and water (Labdiet 5013; LabDiet). All mice were kept on a 12-h light/dark cycle (lights on at 6:00 a.m.) at room temperature (RT) at 20˚C, with a relative humidity of 55%. The current protocol was developed based on the ARRIVE guidelines for reporting animal research (19) and was approved by the Ethics in Research Committee of the Escuela Superior de Medicina (IPN). The animals were handled following the Mexican Federal Regulations for animal experimentation and care (Regulation-062-ZOO-1999, Ministry of Agriculture, México City, México).

Stress protocol. The animals were randomly divided into three groups (n=15): i) Control group without stress; ii) acute stress group (2 h of movement restriction repeated once); and iii) chronic stress group (2 h of movement restriction repeated for 3 days). Mice were placed in cylindrical plexiglass containers according to a previously described method (20). The animals from the control group remained in their home cages without water and food for the same duration as that for which the animals in both stressed groups were exposed to stress.

Tissue collection. Male BALB/c mice were anaesthetized by an intraperitoneal injection of a lethal dose of 100 mg/kg body weight pentobarbital sodium salt (cat. no. P3761; Sigma-Aldrich; Merck KGaA). Blood was obtained via cardiac puncture (0.7-1 ml), and serum was obtained via centrifugation at 1,660 x g for 7 min at 4˚C. The serum samples were stored at -20˚C until use. After exsanguination the large intestines were dissected, a colon segments were cut (1 cm), fixed with 4% paraformaldehyde at RT for 24 h and processed for paraffin embedding. Sections (7-μm thick) were generated with a microtome (Rotatory Microtome; Leica Microsystems GmbH), placed on coverslips and stained with H&E or Alcian Blue (AB) or toluidine blue at RT.

Corticosterone assay. Plasma corticosterone concentrations were determined using a commercially available ELISA kit for corticosterone analysis according to the manufacturer's instructions (cat. no. ADI-901-097; Enzo Life Sciences, Inc.). The corticosterone concentrations in the plasma samples were calculated based on a standard curve and are expressed in ng/ml.

Quantitative analysis of the polymorphonuclear cells (PMN) in the colon. For the quantification of leukocyte infiltration, colon sections were stained with H&E. After deparaffinization with xylol and rehydration in a descending alcohol gradient, the samples were immersed in Harris hematoxylin solution (cat. no. H3136; Sigma-Aldrich; Merck KGaA) and incubated for 20 min at RT. After incubation in an eosin solution for 2 min at RT (cat. no. E4009; Sigma-Aldrich; Merck KGaA), the samples were then washed with distilled water. Finally, the sections were dehydrated and mounted with Entellan® (cat. no. 1079610500; Merck KGaA). The samples were analyzed via optical microscopy, and the PMN numbers were determined. The PMNs in the inflammatory infiltrates were counted (12 per slide/3 slides per animal). The percentage of PMNs in each sample was calculated. The number of cells counted was evaluated using Image-Pro Plus version 5.1 software (Media Cybernetics, Inc.) and an E600 microscope (Eclipse E-600; Nikon Corporation) at a x40 magnification, and the total number of PMNs was quantified (21).

Acid mucin staining and goblet cell quantification. Colon samples were fixed and stained for acidic mucins as previously described (22). Acid mucins were stained with AB (cat. no. C.I.74240; Sigma-Aldrich; Merck KGaA). After deparaffinization with xylene for 30 min at 60˚C, the samples were rehydrated in a descending alcohol gradient. The samples were incubated in 3% acetic acid for 3 min at RT and then incubated with 1% AB solution in 3% acetic acid pH 2.5 for 25 min at RT. Then, the samples were washed with warm water until the color changed. Finally, the sections were dehydrated and mounted with Entellan. The samples were observed via light microscopy, and the positive staining of five randomly selected Lieberkühn crypts in each colon sample was observed (n=5). The number of goblet cells per group was determined (magnification, x40; Eclipse E-600; Nikon Corporation) and analyzed

using Image-Pro Plus 5.1 software (Media Cybernetics, Inc.) and an E600 microscope; the average numbers of goblet cells per crypt and per group were determined (22).

Mast cell staining and quantification. Mast cells were observed using toluidine blue staining (cat. no. 198161; Sigma-Aldrich; Merck KGaA). After deparaffinization with xylol and rehydration in a descending alcohol gradient, the samples were immersed in a 0.5% toluidine blue solution and incubated for 30 min at RT. Then, the samples were washed with distilled water, dehydrated and mounted with Entellan. The numbers of mast cells in the intestinal lamina propria of each group were determined. The mast cells were identified and counted randomly via light microscopy at a x40 magnification with an E600 microscope (Eclipse E-600; Nikon Corporation). The average numbers of mast cells per section per group were determined (23).

Epithelial cell isolation. Claudin-1 and E-cadherin expression in isolated epithelial cells from the large intestine was determined. Briefly, fragments of the large intestine were incubated in RPMI-1640 medium (cat. no. R7388; Sigma-Aldrich; Merck KGaA) with 1 mM dithiothreitol (cat. no. 20290; Thermo Fisher Scientific, Inc.) and 1.5 mM EDTA (cat. no. E6511; Sigma-Aldrich; Merck KGaA), with continuous shaking at 415 x g for 30 min at 37˚C. The cell suspension was passed through organza to remove the mucus and centrifuged at 415 x g for 10 min at 4˚C. The pellet was suspended in 15 ml RPMI-1640 medium, passed through an organza filter and washed twice with 15 ml RPMI-1640 medium followed by centrifugation at 415 x g for 10 min at 4˚C. The washed pellet was suspended in 20% Percoll® (cat. no. P1644; Sigma-Aldrich; Merck KGaA) and centrifuged over a discontinuous Percoll gradient at 1,160 x g for 30 min at 25˚C. Epithelial cells were recovered from the interphase between 20 and 40%. The cells were washed with PBS and centrifuged as aforementioned. The cells were resuspended in RPMI-1640 medium. The purity of the samples was analyzed via light microscopy based on the normal morphology of epithelial cells. Cell viability was determined using a Neubauer chamber and an optical microscope (magnification, x20). Then 10 μl of cell suspension were added to an equal volume of 0.4% trypan blue. The number of cells were counted and their viability (viable cells excluding trypan blue; cat. no. T8154; Sigma-Aldrich; Merck KGaA) was determined to be 90%. The samples contained up to 85% epithelial cells (24).

Western blot analysis of claudin-1 and E-cadherin expression. The protein expression levels of claudin-1 and E-cadherin were determined via western blotting. Samples were homogenized in 100 μl lysis buffer [10 mmol Tris pH 7.4, containing 1% SDS (cat. no. 1610301; Bio-Rad Laboratories, Inc.), 2 mmol/l sodium orthovanadate (cat. no. S6508; Sigma-Aldrich; Merck KGaA) and 12.55 μg/ml phenylmethylsulphonyl fluoride (cat. no. P7626; Sigma-Aldrich; Merck KGaA)]. The samples were sonicated four times for 15 sec with 30 sec of rest at 4˚C (22). Proteins were quantified by Nanodrop Lite (Thermo Fisher Scientific, Inc.). Proteins were equally loaded (20 μg per well), separated via 12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). After blocking with 3% powdered skimmed milk, 0.1% Tween-20 PBS for 2 h at RT, the membranes were incubated for 1 h at RT with polyclonal antibodies against claudin-1 (cat. no. GTX134842; polyclonal; 23 kDa; GeneTex, Inc.) and E-cadherin (cat. no. GTX100443; polyclonal; 97 kDa; GeneTex, Inc.), followed by incubation for 1 h at RT with HRP-conjugated secondary antibodies (cat. no. A27036; Oligoclonal; Thermo Fisher Scientific, Inc.). The immunoreactive bands were detected using an ECL detection kit (Amersham; Cytiva) and semi-quantified via densitometry using ImageJ 1.49 software (National Institutes of Health). The membranes were stripped and reprobed with an anti-β-actin primary antibody (cat. no. GTX109639; polyclonal; GeneTex, Inc.) to ensure equal loading (25).

Lamina propria lymphocytes. After treatment with EDTA 30 min at 37˚C, the large intestines were washed twice with 25 ml RPMI-1640 medium and then transferred to 50-ml tubes containing 25 ml RPMI-1640 medium with 60 U/ml type IV collagenase (cat. no. C5138; Sigma-Aldrich; Merck KGaA), 1% fetal calf serum and 50 g/ml gentamicin (cat. no. G1397; Sigma-Aldrich; Merck KGaA). The tubes were incubated horizontally for 30 min at 37˚C in a shaking water bath. The contents of each tube were then transferred to Petri dishes. The intestinal mucosa samples were filtered through an organza filter with a syringe plunger; then, the single cell suspensions containing lamina propria cells were filtered and centrifuged at 415 x g for 10 min at 4˚C. The cells were resuspended, collected and centrifuged in a discontinuous 40/75% Percoll gradient at 1,160 x g for 30 min at 4˚C. The cells were recovered from the interphase and then washed with RPMI-1640 medium. Viability was determined using trypan blue exclusion, and it was found to be >90% (24).

Flow cytometry assays. The isolation of lymphocytes from the lamina propria of the large intestine was carried out as previously described with some modifications (24,26). Cells from the lamina propria of the large intestine were resuspended, and the concentration was adjusted to $1x10^6$ cells/ml in PBS for cytofluorometric analysis (26). Anti-$CD4^+$/PerCP (cat. no. GTX79970; MEM-241; GeneTex Inc.) was used to determine the predominant cytokines produced by the $CD4^+$ T cell population. The $CD4^+$ cells were fixed and permeabilized with 200 μl Cytofix/Cytoperm Fixation/Permeabilization Solution kit (cat. no. BD 554714; Thermo Fisher Scientific, Inc.) and then incubated for 20 min at RT in the dark. Subsequently, the cells were centrifuged at 415 x g for 5 min at 4˚C, the excess solution was removed and the obtained pellet was resuspended again. In total, 500 μl 1X Perm/Wash solution was added, and the samples were centrifuged again at 415 x g for 5 min at 4˚C. Then, the supernatants were decanted and the cells were resuspended again. Antibody cocktails (10 μl) were added and incubated for 20 min in the dark at RT. Markers of the T helper (Th)1 profile were detected with anti-IL-12/APC (cat. no. 554480; C15-6; BD Pharmingen; BD Biosciences), anti-IFN-γ/FITC (cat. no. 554411; XMG1.2; BD Pharmingen; BD Biosciences), anti-TNF-α/PE (cat. no. 554419; MX6-XT22; BD Pharmingen; BD Biosciences) and anti-IL-1β/FITC (cat. no. IC4013F; NJTEN3; R&D Systems, Inc.). Then, two more washes and centrifugation cycles were performed with 300 μl 1X Perm/Wash solution at 415 x g for

5 min at 4˚C. Markers of the Th2/T regulatory (Treg) profiles were detected with anti-IL-4/PE (cat. no. 554435; 11B11; BD Pharmingen; BD Biosciences), anti-IL-6/APC (cat. no. 561367; MP5-20F3; BD Pharmingen; BD Biosciences), anti-IL-10/FITC (cat. no. 554466; JE55-E3; BD Pharmingen; BD Biosciences), anti-CD25/FITC (cat. no. 553072; BD Pharmingen; BD Biosciences) and anti-FoxP3/PE (cat. no. 50-5773-U100; G3 Tonbo Biosciences) antibodies. Then, two more washes and spin cycles were performed with 300 μl 1X Perm/Wash solution at 415 x g for 5 min at 4˚C. Finally, the samples were stored at 4˚C in the dark until analysis. The fluorescent signal intensities were recorded and analyzed using a FACSArial flow cytometer (Becton, Dickinson and Company). Events were collected from the lymphocyte gate on the FSC/SSC dot plot. Overall, 20,000 gated events were acquired from each sample using BD FACSDIVA™ software 6.1 (Becton, Dickinson and Company). The data were analyzed using Summit software v4.3 (Dako; Agilent Technologies, Inc.) and are reported as percentages. The data from five mice per group are reported as the mean ± SD.

Serum SP ELISA. Serum SP concentrations were measured using a competitive ELISA kit according to the recommendations of the manufacturer (cat. no. 583751; Cayman Chemical Company). A total of 50 μl per well of serum samples from the acute, chronic and control groups were added, and the assay was performed in triplicate. The plate was read with a microplate reader (BioTek Instruments, Inc.) at 420 nm. The SP concentrations were calculated based on a standard curve and are expressed in pg/ml.

Statistical analysis. The experimental assays were repeated for ≥3 independent assays (n=5 mice per group). The data are expressed as the mean ± SD, and multiple comparisons between groups were analyzed using one-way ANOVA, and the means of the respective groups were compared using the post hoc Tukey's multiple comparison test. Statistical analysis was performed using GraphPad Prism Version 9 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Serum corticosterone concentrations are increased in the stressed animal models. The serum corticosterone concentration is an indicator of stress, and different stressors are associated with increases in serum corticosterone concentrations (27). The results demonstrated that the serum corticosterone hormone concentrations were increased in the acute (P<0.05) and chronic (P<0.001) stress groups compared with the control group (Fig. 1). Additionally, the concentration of corticosterone was increased in the chronic stress group compared with the acute group (P<0.001). These results indicated that serum corticosterone concentrations are modified with respect to the type of stress, acute or chronic.

Acute stress induces changes in inflammatory cell infiltration. The acute stress group (Fig. 2B) exhibited an accumulation and increase in crypt inflammatory cell infiltration compared with the control and chronic stress groups (Fig. 2A and C). The quantification of the infiltrating

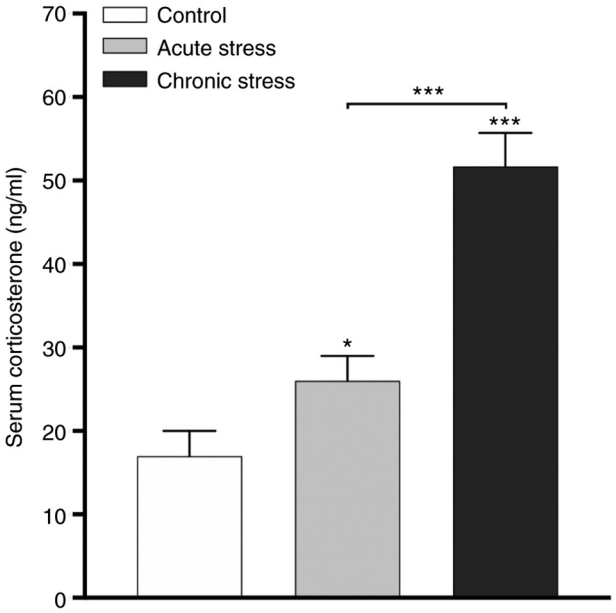

Figure 1. Corticosterone serum concentrations. Mice were subjected to different experimental conditions, and then, peripheral blood was collected and analyzed using a colorimetric-enzymatic assay. The data are expressed as the mean ± SD (n=5). *P<0.05, ***P<0.001 vs. control group; ***P<0.001 (umbrella line comparison).

PMN showed a significant increase in the acute stress group compared with the control and chronic stress groups (P<0.05; Fig. 2J). However, no significant differences were observed between the chronic stress and control groups. The infiltrating PMN was significantly higher in the acute stress group compared with in the chronic stress group (P<0.01; Fig. 2J). These results indicated that acute stress induced differential modifications of the inflammatory infiltrate in mice under stress-induced conditions.

Acute stress increases the number of goblet cells. Acute stress altered goblet cell numbers, and an increase was observed in the acute stress group compared with the control group (Fig. 2D and E). The chronic stress group showed a similar goblet cell number as the control group (Fig. 2F). Furthermore, goblet cell quantification revealed a statistically significant increase in the acute stress group compared with the control group (P<0.05; Fig. 2K). Additionally, the goblet cell number was increased in the acute stress group compared with the chronic group (P<0.01). The results demonstrated that acute stress stimulates an increase in the number of goblet cells.

Mast cell numbers increase during acute stress. The presence of metachromatic mast cells in colon was detected via toluidine blue staining. The number of mast cells was markedly increased in the acute stress group (Fig. 2H) compared with the chronic stress (Fig. 2I) and control groups (Fig. 2G). The quantification of mast cells showed a significant increase in the acute stress group compared with the control and chronic stress groups (P<0.05 and P<0.001; Fig. 2L). No significant differences were found between the chronic stress and control groups. These results showed a differential number of mast cells in acute or chronic stress.

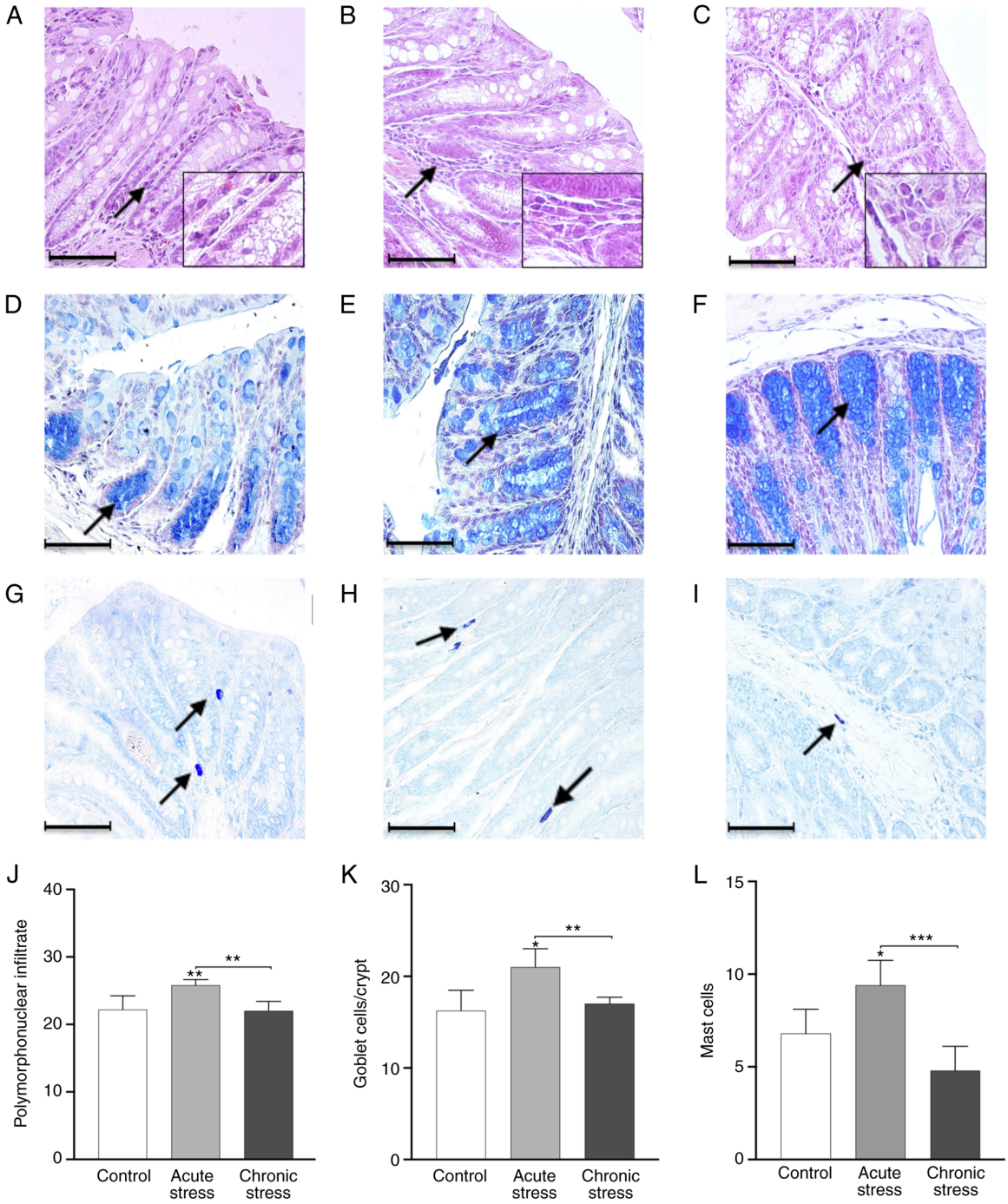

Figure 2. Representative photomicrographs obtained via light microscopy. (A) control, (B) acute stress and chronic stress (C) Colon samples stained with H&E to detect infiltrating inflammatory cells (arrows). (D) control, (E) acute stress and chronic stress (F) Colon samples stained with Alcian blue to visualize goblet cells (arrows). (G) control, (H) acute stress and chronic stress. (I) Colon samples stained with toluidine blue for the detection of mast cells (arrows). Scale bar, 25 μm. (J) Quantification of the polymorphonuclear infiltrate in mouse colon. (K) Goblet cell quantification in mouse colon. (L) Mast cell quantification in mouse colon. Data are expressed as the mean ± SD (n=5). *P<0.05, **P<0.01 vs. control group; **P<0.01, ***P<0.001 (umbrella line comparison).

Claudin-1 and E-cadherin expression is altered under stress. Western blot analysis demonstrated different protein expression patterns under stress conditions. Densitometric analysis revealed lower levels of claudin-1 expression in the acute and chronic stress groups compared with those in the control group (P<0.001). Claudin-1 expression was also significantly higher in the acute stress group compared with in the chronic stress group (P<0.05; Fig. 3A and C). By contrast, densitometric analysis showed higher expression levels of E-cadherin in the acute (P<0.01) and chronic (P<0.001) stress groups compared with in the control group. E-cadherin expression was also significantly higher in the chronic stress group than in the acute stress group (P<0.001; Fig. 3B and C). These data indicated that stress conditions generated changes in claudin-1 and E-cadherin protein expression.

Stress induces changes in the percentage of cytokine/IL CD4^{+} T cells and CD4^{+} Treg cells from the lamina propria.

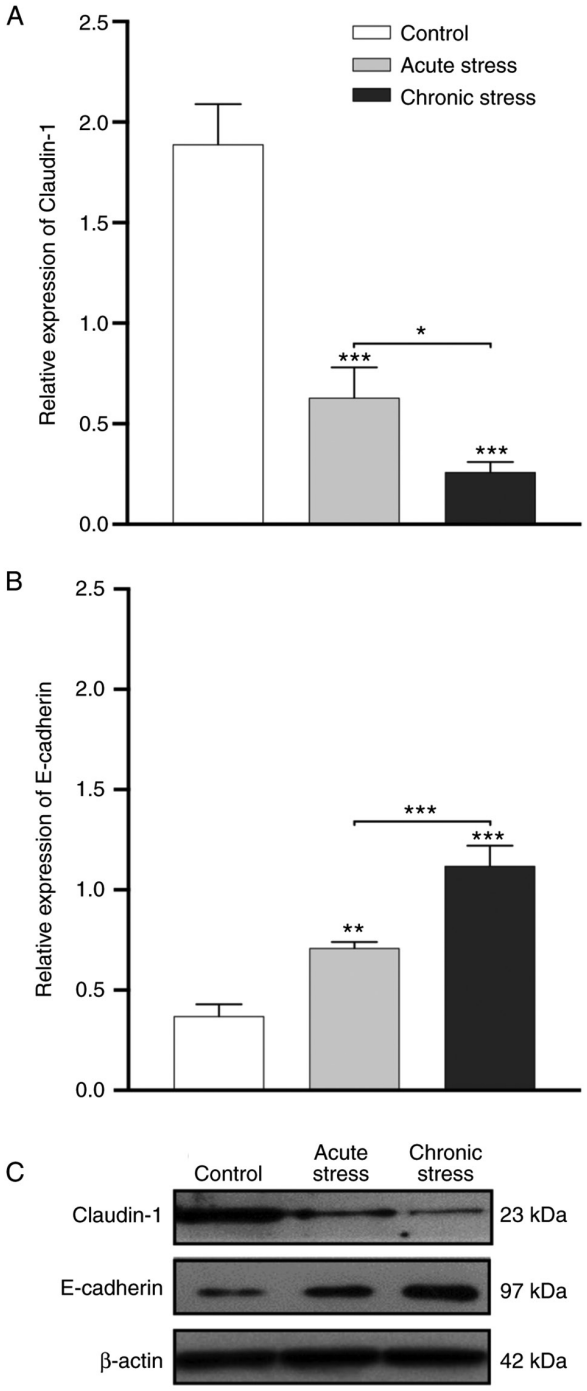

Figure 3. Relative protein expression levels of claudin-1 and E-cadherin, as analyzed via western blotting. (A) Claudin-1 expression. (B) E-cadherin expression. Data are expressed as the mean ± SD for each group (n=5). (C) Intensities of the bands were measured via densitometric analysis and normalized to the intensities of the corresponding β-actin bands. **P<0.01, ***P<0.001 vs. control group; *P<0.05, ***P<0.001 (umbrella line comparison).

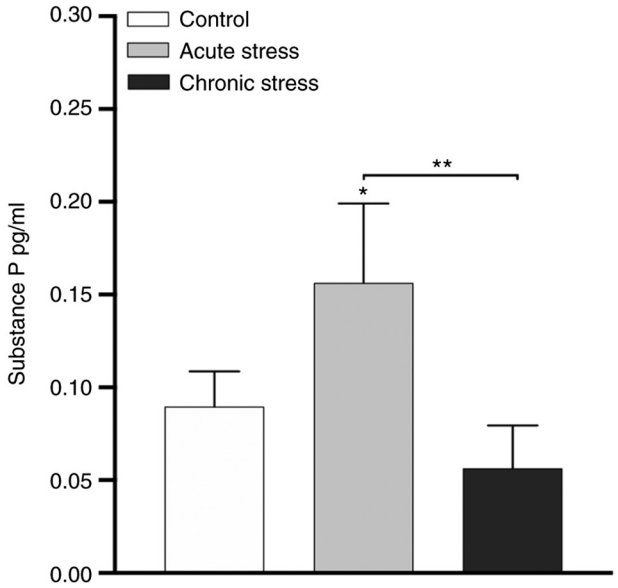

Figure 4. Serum substance P concentration. Data are expressed as the mean ± SD (n=5). *P<0.05 vs. control group; **P<0.01 (umbrella line comparison).

Compared with the control, acute and chronic stress diminished the percentages of CD4+ T cells and CD4+ Treg cells in the lamina propria (Table I). Acute stress significantly increased IL-6 and IL-4 cytokine CD4+ lymphocyte percentages in the lamina propria compared with the control (P<0.05; Table I). Compared with the control group, the acute and chronic stress groups had significantly decreased percentages of CD4+ lymphocytes/IFN-γ, TNF-α, IL-1β and IL-10 in the lamina propria (P<0.05; Table I). A significant reduction in the IL-6 and IL-4 cytokine CD4+ lymphocyte and IL-12 cytokine CD4+ lymphocyte percentages in the lamina propria of the chronic stress group compared with those in the lamina propria of the acute and control groups was observed (P<0.05; Table I). Comparison of the acute stress and control groups did not reveal differences with respect to the IL-12 cytokine CD4+ lymphocytes. Both groups of stressed animals showed a significant reduction in FoxP3/CD4+ Treg cell expression compared with the control group without stress (P<0.05). Based on these results, stress induced a differential percentage of cytokine/IL CD4+ T cells and CD4+ Treg cells. The gating strategy and a representative dot-plots from lamina propria lymphocytes from large intestine are shown in the supplementary material (Fig. S1).

Influence of acute stress on serum SP concentration. Stimulation of the sensory nerve causes an axonal reflex, producing neuropeptides, especially SP, that activate inflammatory cells. Next, the effect of stress on serum SP concentration was examined. As shown in Fig. 4, acute stress significantly increased the serum SP concentration compared with the chronic stress and control groups (P<0.05 and P<0.01). No significant difference was observed between the chronic stress and control groups. These data indicate that the release of serum SP was stimulated during acute stress.

Discussion

The present study identified an association between plasma corticosterone and the stress type in the mouse model, and similar results have been reported previously under different stress conditions (27-30). The current study observed significantly increased inflammatory infiltration during acute stress, and this increase has been observed since pioneering studies (13,14); such studies propose that the increases in the corticosterone

Table I. Cytokine/IL $CD4^+$ T and Treg cell responses in the lamina propria of the large intestine under acute stress and chronic stress.

Cytokine/IL	Control	Acute stress	Chronic stress
IFN-γ	2.77±0.15	0.50±0.10[a]	0.23±0.06[a]
TNF-α	1.93±0.15	0.73±0.12[a]	0.70±0.10[a]
IL-1β	1.47±0.15	0.43±0.15[a]	0.30±0.10[a]
IL-12	4.20±0.20	4.10±0.20	0.57±0.12[a]
IL-6	2.60±0.20	11.60±1.18[a]	0.70±0.10[a]
IL-4	2.03±0.25	8.03±0.15[a]	0.23±0.12[a]
IL-10	2.70±0.20	0.63±0.15[a]	0.13±0.06[a]
FoxP3	5.17±0.21	1.07±0.31[a]	0.93±0.15[a]

Percentage of cytokine/IL $CD3^+/CD4^+$ T and $FoxP3^+/CD3^+/CD4^+$ Treg cell from lamina propria is expressed as mean ± SD of 5 mice per group from three independent experiments. [a]$P<0.05$ vs. control group. Treg, regulatory T cells.

levels in the plasma and intestinal lamina propria are associated with immune cell trafficking, which is a crucial event for the surveillance and effector functions of the immune system (13). Furthermore, the influence of GCs on leukocyte redistribution is likely the most important factor in supporting the immune response (31,32). Conversely, the present study also demonstrated an increase in the number of goblet cells in the lamina propria; these cells are responsible for synthesizing mucus (22), and an increase in their numbers can enhance the thickness of the mucosal layer to prevent contact with luminal bacteria. By contrast, another report has shown that chronic stress reduced the number of goblet cells, and neutrophil cellularity was unaltered (22). Moreover, chronic stress studies (33) have shown that this type of stress promotes an increase in the immunopathology, long term goblet cell depletion and elevations in local and/or systemic inflammatory mediators (33). Unlike other studies, the number of mast cells was increased during chronic stress, but the current study did not observe significant changes during chronic stress. This result could be consistent with those from an overcrowding stress model, in which the increase in plasma corticosterone levels was associated with the recruitment of mast cells over time (28). In models of acute immobilization stress, it has been widely accepted that mast cells are activated by corticotropin releasing factor (34,35) and that they play a role in mediating colonic goblet cell secretion (35).

Despite the decreased claudin-1 protein expression in the TJ region during acute and chronic stress, the current study observed that the mucosa in those experimental groups was similar to that in the control group. A previous study (11) showed that stress mainly promotes functional and morphological changes in the colonic epithelium, and increases colonic permeability due to a decrease in claudin-1 expression in the TJ region (11). The decrease in claudin-1 expression in the present acute stress model was consistent with these studies. The current study also identified an increase in E-cadherin expression. E-cadherin is the core component of epithelial adherent junctions and is essential for tissue development, differentiation and maintenance of tissue barrier formation, a critical function of epithelial tissues (36-38). A previous study investigated the viability and cell-cell adhesion in sodium lauryl sulphate (SLS)-treated human keratinocytes pretreated, co-treated and post-treated with SP, and proposed that SP-treated cells had increased E-cadherin expression. These author suggested that E-cadherins on the membrane of keratinocytes are shifted to desmosomes under physiological conditions and therein may mediate an adhesion function in association with other desmosomal cadherins. SP could provide protection against SLS-induced toxicity by maintaining E-cadherin expression, as well as by exerting anti-inflammatory effects. Therefore, with a low dose of SP may protect against this condition (39).

The present results revealed an increase in the $CD4^+/IL\text{-}4^+$ lymphocyte percentage under acute stress. Although it is well established that stress in the colon increases the number of CD4 lymphocytes (40), to the best of our knowledge, there is no evidence of the effects of repeated restriction stress on the cytokine lymphocytes profiles in the lamina propria of mouse colon. The current data demonstrated that the predominant phenotype in lymphocytes during acute restriction stress consisted of anti-inflammatory Th2 cells, and increase in $CD4^+/IL\text{-}6^+$ and $CD4^+/IL4^+$ lymphocytes in the lamina propria was observed. Additionally, decreases in the numbers of γδ T lymphocytes and $CD4^+$ and $CD8^+$ T lymphocytes in the epithelium of the small intestine of mice have been reported due to the combined action of higher concentrations of catecholamines and GCs (41). During stress, GCs, such as corticosterone, bind to GC response elements and suppress the expression of proinflammatory genes or induce the expression of suppressive factors, such as NFκB inhibitor α, dual specificity phosphatase 1 (MKP-1), glucocorticoid-induced leucine zipper and ZFP36 ring finger protein (TTP). For example, TTP binds to AU-rich elements in the 3'untranslatef region of the mRNA of several inflammatory cytokines to destabilize these mRNAs. GC also reduces the stability of mRNAs encoding IL-1β, IL-2, IL-6 and TNF-α (42). However, data on the effects of different types of stress on T lymphocyte/cytokine profiles in the lamina propria of the mouse colon are lacking.

The delicate balance between pro- and anti-inflammatory mechanisms, essential for intestinal immune homeostasis, is regulated by Treg cells that express the transcription factor FoxP3 and play role in limiting inflammatory responses in the intestine (43). Moreover, evidence has shown that butyrate, produced by commensal microorganisms during starch fermentation, facilitates extrathymic generation of Treg cells (44). The

present study demonstrated a decrease in the percentage of $FoxP3^+$/$CD25^+$/$CD4^+$ Treg lymphocytes in the lamina propria of the large intestine under acute and chronic stress vs. the control group.

Additionally, in the current study, the serum SP concentration after repeated exposure to restriction stress increased during acute stress, and this observation differed from the results after exposure to chronic stress. This phenomenon could be consistent with a study showing that SP reduces the duration of acute stress, and therefore, SP plays an essential role in the transition between acute and chronic stress (45). Conversely, SP exerts antiapoptotic effects on colon epithelial cells, thereby promoting accelerated intestinal healing (46,47). Serum SP levels have been associated with injury severity and mortality in patients with severe traumatic brain injury (TBI), indicating that serum SP levels could be used as a biomarker to predict mortality in patients with severe TBI and may be of great pathophysiological significance in these patients (48). During inflammation and injury in the colon, sensory nerves release SP locally within tissues to promote 'neurogenic inflammation', and it is well established that SP and the NK1R may initiate this inflammation. The cell-surface enzyme neutral endopeptidase (NEP) degrades SP in the extracellular fluid and may terminate its proinflammatory effects. Evidence has shown that NEP in the colon may contribute to uncontrolled intestinal inflammation (49). Thus, it was suggested that the increase in the serum SP concentration during acute stress could be a mechanism via which HPA activation is regulated.

The increase in the serum SP concentration during acute stress may be associated with two factors that are not necessarily contradictory. First, a previous study has proposed that SP does not inhibit the initial activation of the HPA axis in response to restraint stress, but does act via NK1R at a central level (50) to reduce the duration of the stress response; conversely, SP can promote epithelial cell proliferation (antiapoptotic effect) at the site of injury in the large intestine, which promotes intestinal healing (47).

An important limitation of the present study was to analyze substance P in serum and not in colonic tissue, this would have allowed the present study to relate *in situ* possible effects of substance P in the cells studied. In the future experimental and therapeutic studies will be performed that will allow us to relate stress and the immune system, with molecules such as neurotransmitters or neuropeptides.

In conclusion, the parameters evaluated in the current study suggest that acute stress may facilitate stress management therapy to benefit the resolution of intestinal diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JPY and EAR conceptualized the study. IMAM, AARA, LMCJ, BMA, RFV, JMGM, MYO, JPY and EAR designed the study. IMAM, AARA, LMCJ, JPY and EAR utilised the software. IMAM, AARA, LMCJ, JPY and EAR performed formal analysis. JPY, AARA and EAR provided the resources. JPY and EAR contributed to data curation. IMAM, AARA, JPY and EAR wrote initial draft of the manuscript. IMAM, AARA, LMCJ, BMA, RFV, JMGM, MYO, JPY and EAR wrote, reviewed and edited the manuscript. JPY and EAR supervised the study. JPY and EAR performed project administration and acquired the funding. IMAM and EAR confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines the Mexican Federal Regulations for animal experimentation and care (Regulation-062-ZOO-1999; Ministry of Agriculture, Mexico City, México) and was approved by the Superior School of Medicine, National Polytechnic Institute.

Patient consent for publication

Not applicable.

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Competing interests

The authors declare that they have no competing interests.

References

1. McEwen BS: Physiology and neurobiology of stress and adaptation: Central role of the brain. Physiol Rev 87: 873-904, 2007.
2. Chrousos GP: The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 332: 1351-1362, 1995.
3. Grundy D, Al-Chaer ED, Aziz Q, Collins SM, Ke M, Taché Y and Wood JD: Fundamentals of neurogastroenterology: Basic science. Gastroenterology 130: 1391-1411, 2006.
4. Barreau F, Cartier C, Leveque M, Ferrier L, Moriez R, Laroute V, Rosztoczy A, Fioramonti J and Bueno L: Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: Corticotrophin-releasing factor and nerve growth factor interplay. J Physiol 580: 347-356, 2007.

5. Barclay GR and Turnberg LA: Effect of psychological stress on salt and water transport in the human jejunum. Gastroenterology 93: 91-97, 1987.
6. Camilleri M, Madsen K, Spiller R, Greenwood-Van Meerveld B and Verne GN: Intestinal barrier function in health and gastrointestinal disease. Neurogastroenterol Motil 24: 503-512, 2012.
7. Santos J, Saunders PR, Hanssen NP, Yang PC, Yates D, Groot JA and Perdue MH: Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. Am J Physiol 277: G391-G399, 1999.
8. Campos AC, Ferreira FR, Guimaraes FS and Lemos JI: Facilitation of endocannabinoid effects in the ventral hippocampus modulates anxiety-like behaviors depending on previous stress experience. Neuroscience 167: 238-246, 2010.
9. Padovan CM and Guimaraes FS: Restraint-induced hypoactivity in an elevated plus-maze. Braz J Med Biol Res 33: 79-83, 2000.
10. Willner P: Validity, reliability and utility of the chronic mild stress model of depression: A 10-year review and evaluation. Psychopharmacology (Berl) 134: 319-329, 1997.
11. Zheng G, Wu SP, Hu Y, Smith DE, Wiley JW and Hong S: Corticosterone mediates stress-related increased intestinal permeability in a region-specific manner. Neurogastroenterol Motil 25: e127-e139, 2013.
12. Zheng G, Victor Fon G, Meixner W, Creekmore A, Zong Y, K Dame M, Colacino J, Dedhia PH, Hong S and Wiley JW: Chronic stress and intestinal barrier dysfunction: Glucocorticoid receptor and transcription repressor HES1 regulate tight junction protein Claudin-1 promoter. Sci Rep 7: 4502, 2017.
13. Dhabhar FS and McEwen BS: Stress-induced enhancement of antigen-specific cell-mediated immunity. J Immunol 156: 2608-2615, 1996.
14. Dhabhar FS, Miller AH, McEwen BS and Spencer RL: Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. J Immunol 154: 5511-5527, 1995.
15. Keita AV, Soderholm JD and Ericson AC: Stress-induced barrier disruption of rat follicle-associated epithelium involves Corticotropin-releasing hormone, acetylcholine, substance P, and mast cells. Neurogastroenterol Motil 22: 770-778, e221-e222, 2010.
16. Santos J, Yates D, Guilarte M, Vicario M, Alonso C and Perdue MH: Stress neuropeptides evoke epithelial responses via mast cell activation in the rat colon. Psychoneuroendocrinology 33: 1248-1256, 2008.
17. Vergnolle N: The enteric nervous system in inflammation and pain: The role of proteinase-activated receptors. Can J Gastroenterol 17: 589-592, 2003.
18. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP and Shanahan F: The role of substance P in inflammatory disease. J Cell Physiol 201: 167-180, 2004.
19. Kilkenny C, Browne WJ, Cuthill IC, Emerson M and Altman DG: Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. PLoS Biol 8: e1000412, 2010.
20. Martinez-Carrillo BE, Godinez-Victoria M, Jarillo-Luna A, Oros-Pantoja R, Abarca-Rojano E, Rivera-Aguilar V, Yépez JP, Sánchez-Torres LE and Campos-Rodríguez R: Repeated restraint stress reduces the number of IgA-producing cells in Peyer's patches. Neuroimmunomodulation 18: 131-141, 2011.
21. Cruz-Baquero A, Cardenas Jaramillo LM, Gutierrez-Meza M, Jarillo-Luna RA, Campos-Rodríguez R, Rivera-Aguilar V, Miliar-García A and Pacheco-Yepez J: Different behavior of myeloperoxidase in two rodent amoebic liver abscess models. PLoS One 12: e0182480, 2017.
22. Machorro-Rojas N, Sainz-Espuñes T, Godínez-Victoria M, Castañeda-Sánchez JI, Campos-Rodríguez R, Pacheco-Yepez J and Drago-Serrano ME: Impact of chronic immobilization stress on parameters of colonic homeostasis in BALB/c mice. Mol Med Rep 20: 2083-2090, 2019.
23. Santana T, Nagata G, Saturno JL and Trierveiler M: Histopathological features of photodamage and mast cell infiltrate in actinic Cheilitis with different grades of epithelial dysplasia. J Cutan Pathol 47: 592-600, 2020.
24. Resendiz-Albor AA, Reina-Garfias H, Rojas-Hernández S, Jarillo-Luna A, Rivera-Aguilar V, Miliar-García A and Campos-Rodríguez R: Regionalization of pIgR expression in the mucosa of mouse small intestine. Immunol Lett 128: 59-67, 2010.
25. Lafuente N, Matesanz N, Azcutia V, Romacho T, Nevado J, Rodríguez-Mañas L, Moncada S, Peiró C and Sánchez-Ferrer CF: The deleterious effect of high concentrations of D-glucose requires pro-inflammatory preconditioning. J Hypertens 26: 478-485, 2008.
26. Resendiz-Albor AA, Esquivel R, Lopez-Revilla R, Verdin L and Moreno-Fierros L: Striking phenotypic and functional differences in lamina Propria lymphocytes from the large and small intestine of mice. Life Sci 76: 2783-2803, 2005.
27. Gong S, Miao YL, Jiao GZ, Sun MJ, Li H, Lin J, Luo MJ and Tan JH: Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. PLoS One 10: e0117503, 2015.
28. Vicario M, Guilarte M, Alonso C, Yang P, Martínez C, Ramos L, Lobo B, González A, Guilà M, Pigrau M, *et al*: Chronological assessment of mast cell-mediated gut dysfunction and mucosal inflammation in a rat model of chronic psychosocial stress. Brain Behav Immun 24: 1166-1175, 2010.
29. Flores CM, Hernandez MC, Hargreaves KM and Bayer BM: Restraint stress-induced elevations in plasma corticosterone and beta-endorphin are not accompanied by alterations in immune function. J Neuroimmunol 28: 219-225, 1990.
30. Kvetnansky R, Fukuhara K, Pacak K, Cizza G, Goldstein DS and Kopin IJ: Endogenous glucocorticoids restrain catecholamine synthesis and release at rest and during immobilization stress in rats. Endocrinology 133: 1411-1419, 1993.
31. Dhabhar FS and McEwen BS: Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci USA 96: 1059-1064, 1999.
32. Straub RH, Dhabhar FS, Bijlsma JW and Cutolo M: How psychological stress via hormones and nerve fibers may exacerbate rheumatoid arthritis. Arthritis Rheum 52: 16-26, 2005.
33. Dhabhar FS: Enhancing versus suppressive effects of stress on immune function: Implications for Immunoprotection versus immunopathology. Allergy Asthma Clin Immunol 4: 2-11, 2008.
34. Castagliuolo I, Lamont JT, Qiu B, Fleming SM, Bhaskar KR, Nikulasson ST, Kornetsky C and Pothoulakis C: Acute stress causes mucin release from rat colon: Role of corticotropin releasing factor and mast cells. Am J Physiol 271: G884-G892, 1996.
35. Castagliuolo I, Wershil BK, Karalis K, Pasha A, Nikulasson ST and Pothoulakis C: Colonic mucin release in response to immobilization stress is mast cell dependent. Am J Physiol 274: G1094-G1100, 1998.
36. Daulagala AC, Bridges MC and Kourtidis A: E-cadherin beyond structure: A signaling hub in colon homeostasis and disease. Int J Mol Sci 20: 2756, 2019.
37. Hwang DY, Kim S and Hong HS: Substance-P ameliorates dextran sodium sulfate-induced intestinal damage by preserving tissue barrier function. Tissue Eng Regen Med 15: 63-73, 2018.
38. Sayer B, Lu J, Green C, Soderholm JD, Akhtar M and McKay DM: Dextran sodium Sulphate-induced colitis perturbs muscarinic cholinergic control of colonic epithelial ion transport. Br J Pharmacol 135: 1794-1800, 2002.
39. Choi HW, Ahn HJ, Shin MK, Son YS and Kim KS: Pretreatment with substance P alleviates irritation due to sodium lauryl sulphate exposure by maintaining E-cadherin expression on human keratinocytes. Clin Exp Dermatol 43: 291-295, 2018.
40. Qiu BS, Vallance BA, Blennerhassett PA and Collins SM: The role of CD4+ lymphocytes in the susceptibility of mice to stress-induced reactivation of experimental colitis. Nat Med 5: 1178-1182, 1999.
41. Jarillo-Luna A, Rivera-Aguilar V, Martinez-Carrillo BE, Barbosa-Cabrera E, Garfias HR and Campos-Rodriguez R: Effect of restraint stress on the population of intestinal intraepithelial lymphocytes in mice. Brain Behav Immun 22: 265-275, 2008.
42. Shimba A and Ikuta K: Glucocorticoids regulate circadian rhythm of innate and adaptive immunity. Front Immunol 11: 2143, 2020.
43. Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, Umetsu DT and Rudensky AY: Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature 482: 395-399, 2012.
44. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ and Rudensky AY: Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504: 451-455, 2013.
45. Mello DM, Marcinichen DR, Madruga D, Branco R, Paschoalini MA and De Lima TC: Involvement of NK1 receptors in metabolic stress markers after the central administration of substance P. Behav Brain Res 181: 232-238, 2007.
46. Kang MH, Kim DY, Yi JY and Son Y: Substance P accelerates intestinal tissue regeneration after gamma-irradiation-induced damage. Wound Repair Regen 17: 216-223, 2009.

47. Koon HW, Zhao D, Zhan Y, Moyer MP and Pothoulakis C: Substance P mediates antiapoptotic responses in human colonocytes by Akt activation. Proc Natl Acad Sci USA 104: 2013-2018, 2007.
48. Lorente L: New prognostic biomarkers in patients with traumatic brain injury. Arch Trauma Res 4: e30165, 2015.
49. Sturiale S, Barbara G, Qiu B, Figini M, Geppetti P, Gerard N, Gerard C, Grady EF, Bunnett NW and Collins SM: Neutral endopeptidase (EC 3.4.24.11) terminates colitis by degrading substance P. Proc Natl Acad Sci USA 96: 11653-11658, 1999.
50. Jessop DS, Renshaw D, Larsen PJ, Chowdrey HS and Harbuz MS: Substance P is involved in terminating the hypothalamo-pituitary-adrenal axis response to acute stress through centrally located neurokinin-1 receptors. Stress 3: 209-220, 2000.

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