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Effects of chronic unpredictable stress on intestinal morphology in Wistar rats

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Abstract

Objectives: Stressful events might cause immune dysfunction and trigger various disorders. Adverse effects of acute or chronic stress exposure on the gastrointestinal system have been shown previously in several studies. In this experimental study, we used chronic unpredictable stress (CUS) paradigm to better mimic effects of the intermittent exposure to daily life stress and investigated the morphometric alterations occurring in the small intestines of rats.

Methods: Male Wistar rats were randomly divided into stress and control groups (n=8, each). While stress group was subjected to chronic unpredictable stress protocol for 21 days, control group remained undisturbed. Intestinal tissue samples were obtained from two different regions; one was 3-6 cm away from the pylorus and the other one 3-6 cm prior to the ileocaecal valve. Tissue sections were obtained from paraffin blocks at the thickness of 3 micrometers and stained with hematoxylin-eosin (HE) or periodic acid-Shiff (PAS). The lengths of villi were measured from the basal membrane to the top of the villus. The ratio of degranulating and non-degranulating mast cells per unit area were estimated by point counting method.

Results: The mean villi length in the stress group were significantly higher ($p<0.01$) than those of the control group. Degranulation to non-degranulation ratio of the mast cells were 40% and 54% in the control and stress groups, respectively.

Conclusion: Animals exposed to chronic unpredictable stress protocol displayed a significant elongation in the villi of small intestines and an increase in the number of degranulating mast cells in the intestinal mucosa. Since activation of mast cells causes releasing of various chemical mediators and growth factors, it is plausible that stressed animals developed an adaptation mechanism to enhance the capacity for absorption and digestion per unit length of the guts.

Keywords: chronic unpredictable stress; gastrointestinal system; mast cell; villus length

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Introduction

Stress is a usual part of daily living; however, if its level exceeds the adaptive capacity of an individual, it might predispose to illnesses in multiple systems including the gastrointestinal organs.[1,2] It has been shown that diverse stressors have a major influence on the gastrointestinal secretion, motility, epithelial permeability, and inflammation.[3] Also, in different animal models, stress exposure leads to intestinal pathology such as increased ion secretion, macromolecular permeability, microscopic inflammation, visceral hypersensitivity, dysmotility, and even bacterial penetration.[4]

The chronic unpredictable stress (CUS) model is originally used to study mechanisms underlying the stress response.[5] One of the main advantages of this protocol is better imitating the intermittent exposure to daily life stress. It also has a greater face validity than the other animal models of stress exposure.[6] Multiple experimental observations have suggested that endogenous gastrointestinal secretions and structural changes are important mechanisms for adaptation to stress exposure. Among them, mast cells have a special importance since they are associated with diverse modulatory effects in innate and adaptive immunity.[7] The mast cells of rodents and humans are numerous, and if grouped together they would make an organ equal to the size of the spleen.[8] Upon activation, mast cells can release a variety of chemical mediators stored in their secretory granules into the

extracellular environment within minutes, a process known as degranulation.[9] Therefore, in this study, we used CUS model to investigate the morphological changes in the small intestines of rats and specifically aimed to analyze alterations in the mast cells located in the guts.

Materials and Methods

Healthy male Wistar rats were obtained from the breeding colony at the Eskişehir Osmangazi University Animal Care Facility and maintained under constant temperature (21°C) and light (12:12 h light/dark cycle) conditions. Experimental procedures were performed in accordance with protocols approved by the Institutional Animal Usage Committee (Protocol number: 2016/526). Chronic unpredictable stress (CUS) model was applied to the stress group (n=8). During 21 days, rats were randomly exposed to stressors such as change of dark / light cycle, 45 degree tilted cage, wet bedding, crowded conditions, cold environment, predator odor, isolation in steel cage, exposure to bright light, water and food deprivation; two times a day (**Table 1**). Any intervention was not applied to the animals in the control group (n=8) except weekly body weighting. At the end of 21th day, rats were perfused intracardiacally with neutral phosphate buffer saline (pH=7.4) and then with 4% paraformaldehyde solution.

Intestinal tissue samples were obtained from two different regions; 3-6 cm away from pylorus, 3-6 cm prior to the ileocaecal valve. They were dehydrated, cleared, and then embedded in paraffin; 3 µm thick sections were obtained from each sample. The sections were mounted on slides, dried, and subsequently deparaffinized in three changes of xylol and rehydrated in three changes of 95% ethyl alcohol and distilled water. Histomorphological assessment was carried out by staining the sections with hematoxylin-eosin (HE), and periodic acid-Shiff (PAS) staining was used for mast cell counting. The sections were then rinsed and blotted carefully, dehydrated through 95% ethanol, absolute alcohol and xylene. For each animal, 10 sections in which the villi can be identified clearly were selected for the morphometric analyses. Measurements were done in at least 10 different areas from each section. Villi lengths were measured from the basal membrane to the top of the villus in a straight line by the StereoInvestigator software (Version 11; Microbrightfield Inc., Williston, VT, USA). A total of 1600 villi lengths were measured from stress and control groups. The number of non-degranulating and degranulating mast cells were estimated with point counting method using a grid. Systematic random sampling method was applied in the selection of areas under the high power objectives and both degranulating and non-degranulating mast cells per unit area were counted on 10 different sections obtained from each rat (**Figure 1**).

Table 1
The list and the application time of each stressor. Two of the stressors were applied randomly per day to each rat.

Stressor type	Application duration
Change of dark / light cycle	12 /12 h
45 degree tilted cage	8 h
Wet bedding	8 h
Crowded conditions	8 h
Cold environment	15 min
Predator odor	15 min
Isolation in steel cage	8 h
Exposure to bright light	12 h
Water deprivation	4 h
Food deprivation	8 h

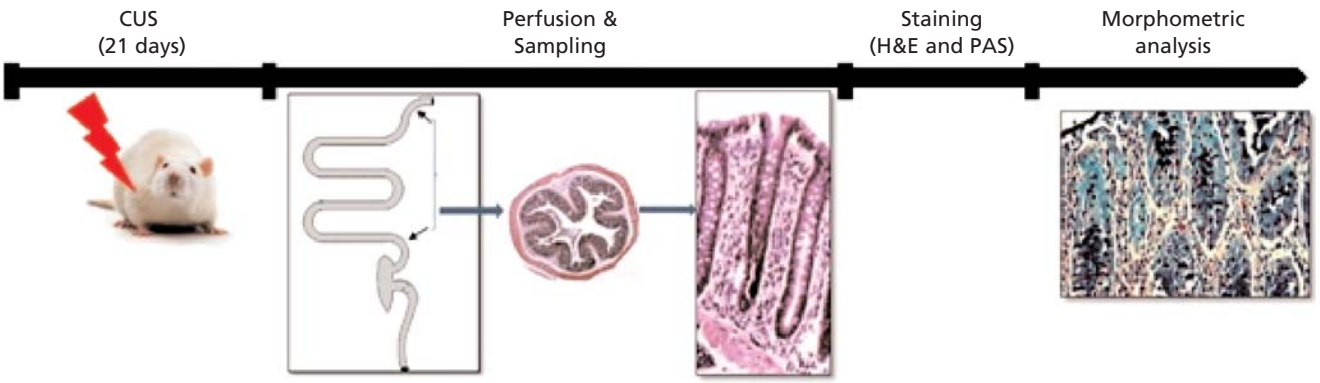

Figure 1. Experimental design. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

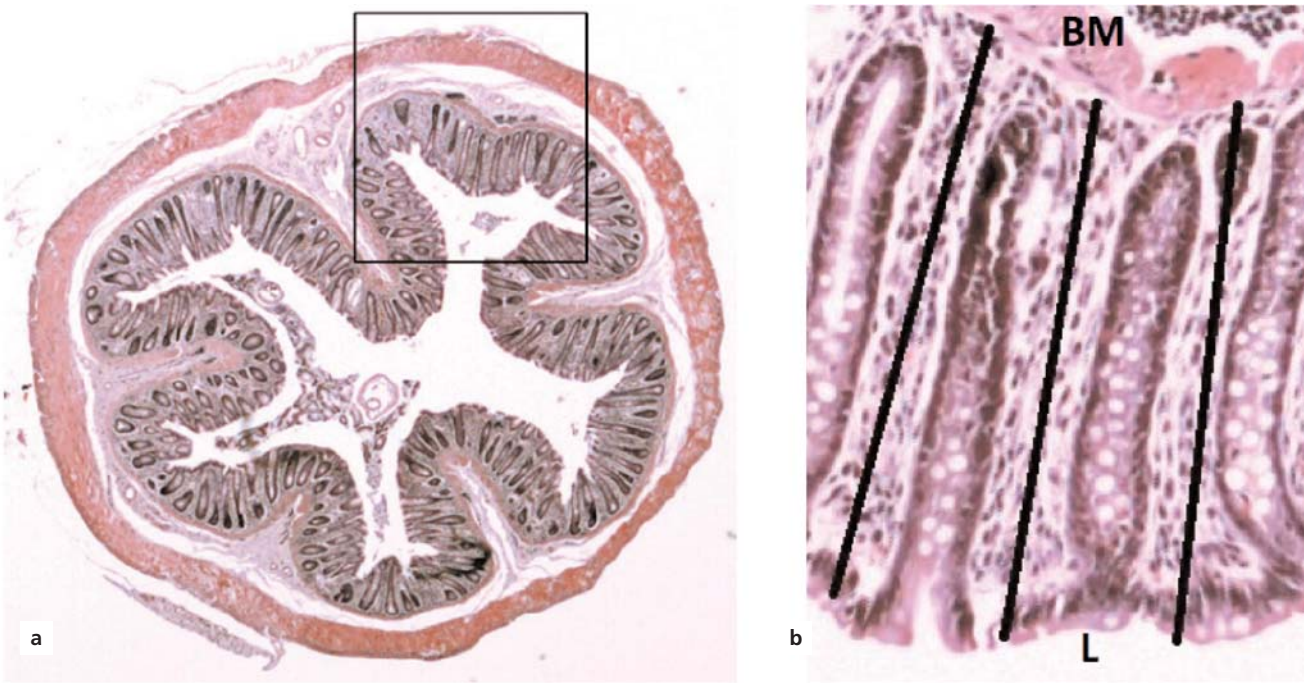

Figure 2. Photomicrographs obtained from the small intestinal tissue. (**a**) Cross sectional view of small intestine (10×) is shown; (**b**) High power view of outlined area (**black box**). Black straight lines in B are used for the villus length measurements. **BM:** basal membrane; **L:** lumen. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

Results

The cross sections obtained from the intestinal tissue of animals were stained and examined under the light microscopy. In both control and stress groups, the integrity of the intestinal mucosa was maintained and there were no irregularities in the glandular structures (**Figure 2a**). No inflammatory infiltration or ulceration was seen in the intestinal wall. Analysis at higher magnifications allowed the measurement of the villus length, from the basal membrane to the tip of the villus (**Figure 2b**). The mean length of the villi was 292.4 µm and 229.1 µm in the stress and control groups, respectively; significantly higher ($p<0.01$) in the stress group compared to those of controls (**Figure 3**). At the same time, a significant increase in the density of mast cells was detected in the stressed group. Degranulating mast cells were detectable in the histological sections stained by PAS method with numerous extracellular meta-chromatic granules. On the other hand, non-degranulating mast cells were characterized by rich intracytoplasmic granule contents. Average density of degranulating and non-degranulating mast cells were estimated by using a counting grid. In the high power view of degranulating mast cells, pink-purple color granules were easily discernable (**Figure 4**). When degranulation to non-degranulation ratio of mast cells were calculated by using the percentages of cells per unit area, the control group had lower (40%) degranulating mast cells than the stress group (54%) in the intestinal mucosa (**Figure 5**).

Discussion

In recent years, increasing number of publications has indicated that stress plays a major role in the gastrointestinal pathophysiology.[10] Stress-related functional

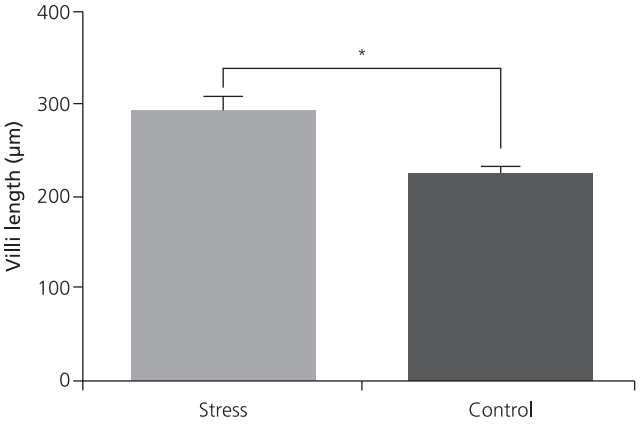

Figure 3. Comparison of villi lengths. The mean lengths of villi are significantly higher in the stressed animals than those of the control (*$p<0.01$).

intestinal diseases are becoming more common in modern human life, almost like the flue infections. Both of these are the most frequently seen causes of the labor loss due to the illness.[11] Experimental studies showed that after repetitive stress exposure, gastrointestinal inflammation is activated.[12] The influence of stress on the clinical course of some intestinal diseases, e.g. irritable bowel syndrome (IBS) a highly prevalent disorder in developed countries, is increasingly being recognized, but the underlying mechanisms are largely unknown.[12–15] Evidence from several studies indicate that mucosal mast cells play an important role in the stress related intestinal diseases, possibly by activating neurons that release corticotropin-releasing hormone and/or acetylcholine.[15] The main function of the intestinal mucosa is to exchange the nutrients with waste products between intestinal lumen and blood.[11] Epithelial cells act as a physical and functional barrier that limits the uptake of luminal antigens and pathogens.[12] Mast cells play an important role in the regulation of epithelial transport in both human and rodent intestine and there is clear evidence that nerve and mast cell interactions are responsible in intestinal epithelial dysfunction.[12]

Mast cells are effector cells of the immune system, found principally in all organs and vascularized tissues which are in contact with the outer environment[16] and regulate adaptive and innate immunity.[16,17] Mast cells could be seen in the skin, the gastrointestinal and the respiratory tracts, and also found in the peritoneum and synovium.[16] They are highly active cells of hypersensitivity reactions and allergic disorders, as seen in the allergic or parasitic inflammations.[13,18] Mast cells produce various inflammatory and immunoregulatory molecules called cytokines and chemokines.[18] While the first identified signaling mediator molecule of mast cell is heparin, more than 200 mediators are produced by mast cells all around the body.[17] In addition, mast cells produce biogenic amines (histamine, serotonin), interleukin (IL-1 to IL-6), leukemia inhibitory factor, tumor necrosis factor, interferon, transforming growth factor, granulocyte-microphage colony-stimulating factor, enzymes (acid hydrolyzes, chymase, phospholipases, rat mast-cell protease I and II, trypase), lipid metabolites (prostaglandins, leukotrienes, platelet-activating factor), ATP, neuropeptides (vasoactive intestinal peptide), growth factors (nerve growth factor), nitric oxide, and heparin.[16] These multifunctional biochemical messengers were collected in the cytoplasmatic granules of mast cells and released via a very rapid process called as degranulation.[10,16] Mast cells are present in all tissue layers of the gastrointestinal tract.[13,19] Human and rodent mast cells derive from

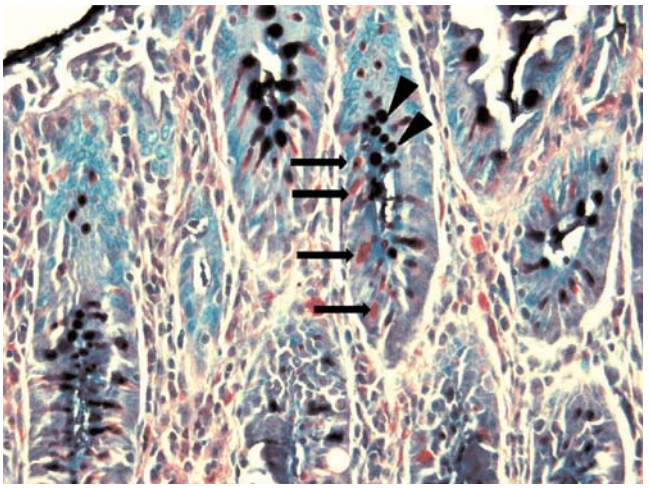

Figure 4. PAS stained histological sections showing degranulated and non-degranulated mast cells in small intestinal villi. The black arrows point the granulated and the black arrowheads indicate the non-degranulated mast cells. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

pluripotent hematopoietic progenitors in the bone marrow and reach to the gastrointestinal system *via* the blood stream during the 16th-22nd week of fetal life.[13,16] After completing their maturation, they are mostly located in the lamina propria of the mucosal layer and in the submucosal layer.[19] It is reported that the intestinal mucosa of irritable bowel syndrome patients contains an increased number of mast cells, also most of these patient have food allergies or adverse reactions to food.[10,18]

The pathophysiological mechanisms of chronic stress exposure on the intestinal tissue remains uncovered.[2] Traumatic experiences during childhood have been shown

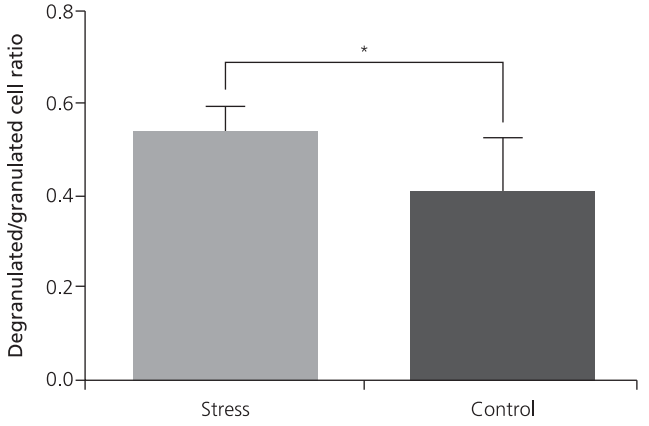

Figure 5. The ratio of degranulated/non-degranulated mast cells in stress and control groups. The stressed animals have significantly more degranulating mast cells than those of control animals (*p<0.01).

to increase the risk of IBS development.[14] Mast cells have been shown to play an important role in the responses of intestinal tissue to acute stressors in humans and rats.[2] During the last decade, studies focused on mast cells and various groups have highlighted the importance of the mast cells in stress-related changes in the intestinal tract.[11,14] The pathological changes caused by mast cell degranulation, goblet cell secretion and endothelial cell membrane alterations in the intestinal tissue are similar to changes produced by oxidative stress.[11] Mast cell activation results in ion transport changes in human and rat intestinal system.[2] Researchers report that all these stress-induced abnormalities seem to rely on biological mediators released by activated mast cells.[4,10] Thus, in adult rats, repeated stress has increased the amount of colonic mucosal mast cells, as demonstrated by using mast cell deficient rats or mast cell stabilizers.[14] Under light microscopy, mast cells were observed in the colon of these rats, but repetitive stress exposure significantly increased the number of mast cells in the mucosa of the colon.[10,12]

Various stress models were used to evaluate the effects of stress on the gastrointestinal tract and gastrointestinal disorders. Crowding, neonatal maternal deprivation, water avoidance stress, immobilization stress, cold pain stress, acquired intestinal infection or intestinal irritation, mild environmental stress such as change of rooms are the most commonly used stress models. Researchers have demonstrated that a mild environmental stress affects the intestinal mucosa by increasing the degranulation of mucosal mast cells, the activation of goblet cells and altering the capillary endothelial ultrastructure.[11,13] It is reported that crowding is reproducing naturalistic psychosocial stress. All these single acute or repetitive homotypic stress models affect the intestinal pathobiology such as increased ion secretion and permeability, inflammation, visceral hypersensitivity and bacterial penetration.[4] Stress has been shown to reactivate colitis in animal models. There are evidences that severe physical stress can cause gastrointestinal dysfunction and pathology. The central nervous system has the ability to modulate intestinal mast cell activity and that mast cells play a role in stress-related gut mucosal dysfunction.[15] The proximity of degranulated mast cells to enteric glia has suggested that stress activates the enteric nervous system and attracting and activating mast cells. In stressed rats gastrointestinal mucosal mast cells were observed as hyperplastic. Stress causes epithelial barrier defects and mucosal mast cell activation in rats.[17]

Conclusion

In this study, animals exposed to chronic unpredictable stress protocol for 21 days displayed a significant increase in the number of degranulating mast cells in the intestinal mucosa. In addition, villus length of stressed animals was significantly higher than the controls. It is known that degranulation of mast cells causes releasing of various chemical mediators, neutral proteases, a group of growth factors and vasoactive intestinal polypeptide. Therefore, structural changes detected in the villi might be associated with these mediators released by activated mast cells. It is also plausible that animals exposed to stress develop an adaptation mechanism characterized by elongation of villus and deepening of crypts, which increases the capacity for absorption and digestion per unit length.

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