

PAPER DETAILS

TITLE: HAYATIN ERKEN DÖNEMİNDE KARSILASILAN SOSYAL IZOLASYON STRESİ, KOLON INFLAMASYONUNUN SİDDETİNİ AZALTIR

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ORIGINAL RESEARCH

SOCIAL ISOLATION STRESS IN THE EARLY LIFE REDUCES THE SEVERITY OF COLONIC INFLAMMATION

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ABSTRACT

Objective: To investigate whether the early-life stress interact with a brief stress exposure and acute colonic inflammation in adulthood.

Methods: Female Sprague–Dawley rats on the postnatal 21st day were exposed to isolation stress until they reach 250 g. On the last 3 days with or without isolation, water-avoidance-stress (WAS) for 30 min/day or acetic acid(5 %) colitis were performed. After the last WAS or on the 4th day of colitis, rats were decapitated to collect serum for TNF-alpha levels, colonic tissues for histological analysis, myeloperoxidase activity (MPO), evidence of neutrophil infiltration, and glutathione (GSH) levels, a key antioxidant.

Results: In the non-isolated and isolated groups, WAS and colitis both elevated the TNF-alpha, MDA levels and MPO activity compared to control ($p<0.05$ - $p<0.001$). GSH levels were reduced in the non-isolated - WAS or -colitis groups and in the isolated-WAS group ($p<0.01$ - 0.001), while GSH level of the isolated-colitis group was not different than control. In the isolated-colitis group, MPO activity and microscopic scores were lower compared to non-isolated group ($p<0.05$ - 0.001). TNF-alpha levels were not different between non-isolated and isolated colitis groups.

Conclusion: Post-weaning isolation stress has a protective effect on colonic inflammation and does not exacerbate the colonic response to acute stressors.

Keywords: myeloperoxidase, colitis, psychological stress, water avoidance stress (WAS), social isolation

HAYATIN ERKEN DÖNEMİNDE KARŞILAŞILAN SOSYAL İZOLASYON STRESİ, KOLON İNFLAMASYONUNUN ŞİDDETİNİ AZALTIR

ÖZET

Amaç: Hayatın erken dönemindeki stresin, ileri yaştaki akut barsak inflamasyonu ve strese maruz kalma üzerine etkilerini araştırmak.

Material Metod: Dişi Sprague–Dawley sıçanlara, doğum sonrası 21. günde, sosyal izolasyon stresi uygulandı. 250 g. ağırlığa ulaşılana dek beklendi. 60 günlük protokolün son 3 gününde, izole edilen, edilmeyen gruplara sudan kaçınma stresi (WAS) 30 dak./gün uygulandı. Sıçanların kalan yarısında ise izole olan, olmayan gruplara, kolona %5'lik asetik asit verilerek kolit oluşturuldu. Son WAS uygulamasından hemen sonra ya da kolit indüksiyonunun 4. gününde sıçanlar dekapite edilerek, TNF-alpha seviyelerinin ölçümü için kan, histolojik değerlendirme, doku myeloperoksidaz (MPO) aktivitesi, nötrofil infiltrasyonunun göstergesi, ölçümü ve glutatyon (GSH) seviyelerinin, antioksidan, ölçümü için kolon dokusu alındı.

Bulgular: İzole olan, olmayan gruplarda, WAS ve kolit indüksiyonu serum TNF-alpha, doku MDA seviyelerini, MPO aktivitelerini kontrole göre anlamlı arttırmıştır ($p<0.05$ - $p<0.001$). GSH seviyeleri izole olmayan grupta WAS ya da kolit indüksiyonu ile azalmış, fakat GSH izole grupta WAS sonrası azalırken ($p<0.01$ - 0.001), kolit sonrası kontrole göre değişiklik olmamıştır. Kolitli izole grupta, MPO aktivitesi ve mikroskopik hasar skorları, izole olmayan grupla karşılaştırıldığında anlamlı azalmıştır ($p<0.05$ - 0.001). TNF-alpha seviyeleri izole olan veya olmayan kolit gruplarında farklılık göstermemiştir.

Sonuç: Sütten kesilme sonrasındaki sosyal izolasyon stresi kolit hasarından koruyucu etkili ve kolon inflamasyonunun stresle artışına yol açmaz.

Anahtar Kelimeler: myeloperoksidaz, kolit, psikolojik stres, sudan kaçınma stresi, sosyal izolasyon

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INTRODUCTION

Stress of various types during daily life, and adequate responses to these stressors are necessary for survival. If the severity or the chronicity of the stressful experience exceeds the adaptive capacity, the individual will be predisposed to illness and disease in multiple organ systems¹. Stress can induce inflammatory responses in various organs and when stress is chronic it may induce or aggravate chronic inflammatory diseases. Although the influence of psychological stress on the symptoms and clinical course of intestinal inflammatory processes has long been recognized, it has recently received the attention of the researchers². Recent data suggest that stress induced alterations in gastrointestinal inflammation may be mediated through changes in hypothalamic-pituitary-adrenal (HPA) axis function and alterations in bacterial-mucosal interactions, and via mucosal mast cells and mediators such as corticotrophin releasing factor (CRF)³.

Psychological stress in adulthood has long been reported to increase disease activity in inflammatory bowel disease (IBD), and recent well-designed studies have confirmed that chronic stress and depression increase the likelihood of relapse in patients with quiescent IBD³. Although long-term perceived stress in patients with ulcerative colitis increases the risk of exacerbation over a period of months to years, it was shown that short-term stress does not trigger exacerbation⁴. Similarly, it has been shown that different stress models in animals may have varying effects on colonic inflammation induced by different methods. Partial restraint stress applied during 4 consecutive days causes exacerbation of 2,4,6-trinitrobenzenesulfonic acid (TNB)-induced colitis in adult rats⁵. In contrary, we have previously shown that acute stress exposure applied as water avoidance stress (WAS)⁶ or electric shock⁷ reduces the severity of colitis induced by acetic acid or TNBS, respectively.

A large number of studies have also shown that early-life trauma can affect the development and clinical course of intestinal disorders and reactivate inflammation in experimental colitis^{2,5}. The neonatal period, roughly extending in rats from birth to day 14, is often referred to as a stress hyporesponsive period characterized by a diminished adrenocorticotropin and corticosterone response to most stressors⁸.

Repeated maternal deprivation during this neonatal period yields to a more tentative behavior and decreased gain of body weight⁹, exacerbates the severity of trinitrobenzene sulfonic acid (TNBS)-induced colitis¹⁰ and increases gastric ulcer susceptibility in the adult¹¹. On the other hand, brief maternal separation has a protective effect on adult stress exposure, protecting the animals from dextran sodium sulphate (DSS)- induced colitis, while the non-handling condition sensitizes mucosa to DSS exposure¹². Social isolation stress during the post-weaning period also leads to behavioural and neurochemical sequelae in rats¹³. However, the effect of post-weaning social isolation stress on secondary stress exposures during the later phases of life, whether it will alleviate or exacerbate the inflammatory responses, is not clarified yet.

Based on the current knowledge about the impact of stress on the course of inflammatory bowel diseases, the present study was designed to investigate whether the early-life stress at the post-weaning period might interact with a brief stress exposure and acute colonic inflammation in adulthood.

METHODS

Animals

Adult female Sprague-Dawley rats (250–300 g) were kept in a light- and temperature-controlled room with 12:12 h- light-dark cycle, where the temperature (22 ± 0.5 °C) and relative humidity (65–70 %) were kept constant. The animals were fed a standard pellet and food was withdrawn overnight before colitis induction. Access to water was allowed ad libitum. Experiments were approved by the Marmara University School of Medicine Animal Care and Use Committee.

Social isolation stress

Dams and their litters were assigned to the non-isolation (n=18) protocol or to social isolation stress (n=18) protocol. To induce post-weaning social isolation stress, rats were removed from their cages and dams on the postnatal 21st day and were placed in individual plastic cages to be kept in another room. Before the experiments, pups were allowed to reach at least to 250 g body weight for 60 days. The growth of the animals was followed only by inspection and the rats were not handled following the separation. The animals were transferred to clean cages with new chip bedding at weekly intervals without handling.



Water avoidance stress (WAS)

At the end of the 60-days protocol with or without isolation, when the required weight was reached, water avoidance stress (WAS) was performed as previously described¹⁴. Rats were individually placed onto a plastic platform (6 cm × 6 cm × 8 cm) located in the middle of a plastic cylinder container (50 cm × 56 cm) filled with warm water (25 °C) up to 1 cm below the height of the platform. Stress sessions, which lasted for 30 min, were performed for 3 consecutive days between 16:00 and 16:30 p.m. to minimize any diurnal variation in the responses. Rats were then put back to their home cages with free access to water and food.

Induction of colitis

At the end of 60-days follow-up period, another group of rats (n=12; isolated and non-isolated) were fasted for 18 h before the induction of colitis. Colitis was induced by a modification of the method introduced by MacPherson and Pfeiffer¹⁵. Under light ether anesthesia, a polyethylene catheter (PE-60) was inserted into the colon with its tip positioned 8 cm from the anus. To induce colitis, a single solution of 1 ml of 5 % (v/v) acetic acid diluted in saline (pH 2.3) was instilled.

Experimental protocol

On the 4th day, immediately after the last WAS exposure or on the 4th day of colitis induction, rats were decapitated. The rats in the control group (n=12) were handled at the same time points as in the WAS-applied group, but were not placed on the platform and isotonic saline was instilled intracolonic instead of acetic acid. Control rats were decapitated on the 4th day of intracolonic saline application. Trunk blood was collected for the assessment of TNF- α levels. The distal 8 cm of the colon were opened down their mesenteric borders and cleansed of luminal contents. Colonic tissue was obtained from each animal and stored at -80 °C until the determination of tissue myeloperoxidase activity (MPO), as an indirect evidence of neutrophil infiltration, and the level of glutathione (GSH), a key antioxidant. For the histological analysis, a 1 square cm sample at 8 cm from anus was obtained from each animal to be fixed in formaldehyde.

Measurement of serum TNF- α level

Serum TNF- α level was evaluated by a RIA-IRMA (radioimmunoassay-immunoradiometric assay) method. All samples were assayed in

duplicates using the commercial kit (Biosource Europe S.A., Nivelles, Belgium). The activity of radioactive assays was measured by a gamma counter (LKB WALLAC 1270 RACK, Canada) and the values were expressed as ng/ml.

Myeloperoxidase (MPO) activity

Tissue-associated myeloperoxidase (MPO) activity was determined in the colonic samples as an indication of accumulation of neutrophils. All reagents for MPO assay were obtained from Sigma. The tissue samples (0.2–0.3 g) were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mM K₂HPO₄, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mM K₂HPO₄ containing 0.5% (w/v) hexadecyltrimethylammonium bromide and 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma). MPO activity was assessed by measuring the H₂O₂-dependent oxidation of o-dianizidine·2HCl. One unit (U) of enzyme activity was defined as the amount of the MPO present per gram of tissue weight that caused a change in absorbance of 1.0 min⁻¹ at 460 nm and 37 °C¹⁶.

Determination of glutathione level

Tissue samples were homogenized in 10 ml vol. of ice-cold 10% trichloroacetic acid, in an Ultra Turrax tissue homogenizer. Homogenized tissue samples were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was removed and recentrifuged at 15,000 rpm for 8 min. Glutathione measurements were performed using a modification of the Ellman procedure¹⁷.

Histological assessment of colonic injury

For light microscopic investigations samples from the colon were fixed with 10% formaldehyde, dehydrated in graded alcohol series, cleared with toluen and embeded in paraffin. Tissue sections (5 μ m) were stained with hematoxylin and eosin (H&E) for general morphology and examined under a Olympus BX51 photomicroscope (Tokyo, Japan). All tissue sections were examined microscopically for characterization of histopathological changes by an experienced histologist (F.E.) who was unaware of the treatment conditions. Assessment of the colonic injury was performed using the previously described criteria: Damage/necrosis (0: None, 1: Localised, 2: Moderate, 3: Severe); Submucosal edema (0:None, 1: Mild, 2: Moderate, 3: Severe);



Inflammatory cell infiltration (0: None, 1: Mild, 2: Moderate, 3: Severe); Vasculitis (0: None, 1: Mild, 2: Moderate, 3: Severe); Perforation (0: Absent, 1: Present); with a maximum score of 13³⁹

Statistics

The results are expressed as means \pm SEM. Following the assurance of normal distribution of data, one-way analysis of variance (ANOVA) was used for multiple comparisons and Student's t-test was used to evaluate the level of statistical significance between pairs (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant if $P < 0.05$.

RESULTS

In the non-isolated groups, exposure to WAS and induction of colitis both elevated the serum levels of TNF- α significantly, as compared to the control group that had neither colitis nor stress exposure ($p < 0.001$; Fig. 1). However, isolation for two months per se in the control group, resulted in elevated serum TNF- α ($p < 0.01$). WAS enhanced the isolation-induced increase in TNF- α level when compared to animals without acute stress. On the other hand, colitis induction had no further effect on the cytokine level that was already elevated by chronic isolation stress.

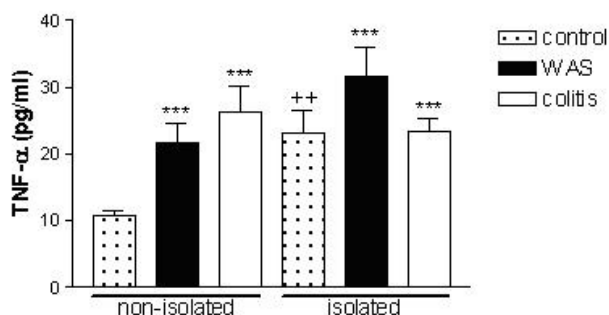


Figure 1: TNF- α levels, in colonic tissues of non-isolated and isolated groups of control, WAS-exposure and colitis-induced groups. *** $p < 0.001$ compared to control group, ++ $p < 0.01$ compared to corresponding group in non-isolated group.

The microscopic damage scores of the two control groups with or without isolation stress were found to be similar (Fig. 2a), indicating that chronic isolation stress alone did not cause any significant changes in the histological appearance of the colon. Exposure to acute water avoidance stress and acetic acid instillation resulted in significant microscopic damage in the non-isolated groups ($p < 0.05$ and $p < 0.001$). The score given in the chemically-induced colitis was nearly 3-folds higher than the acute stressed group. In the post-weaning isolated group, the microscopic damage scores in both WAS and colitis groups were found to be less with respect to corresponding non-isolated groups ($p < 0.05$ and $p < 0.001$). As seen in the non-isolated groups, the damage score reached by acetic acid in the group with a previous isolation stress was clearly higher than the group exposed to the secondary stress.

The histological analysis in the non-isolated control group showed regular colonic morphology (Fig. 3A). Colonic tissues of both non-isolated and isolated WAS groups (Fig. 3B and E) and isolated control group (Fig. 3D) demonstrated only mild inflammatory cell infiltration. In the non-isolated colitis group (Fig. 3C), severe damage of mucosa with epithelial and glandular degeneration, severe inflammatory cell infiltration, vasculitis and submucosal edema were evident. However, in the isolated colitis group (Fig. 3F), mild mucosal degeneration with localized epithelial and glandular degeneration, moderate submucosal edema, vasculitis and inflammatory cell infiltration were observed.

Social isolation for two months did not alter the colonic GSH content (Fig. 2b). However, exposure to acute WAS depleted the GSH content in both the non-isolated and isolated groups ($p < 0.001$). Similarly, colonic GSH levels of the rats with acetic acid colitis were also reduced in the non-isolated group ($p < 0.01$), but the reduction was relatively less than the WAS group.

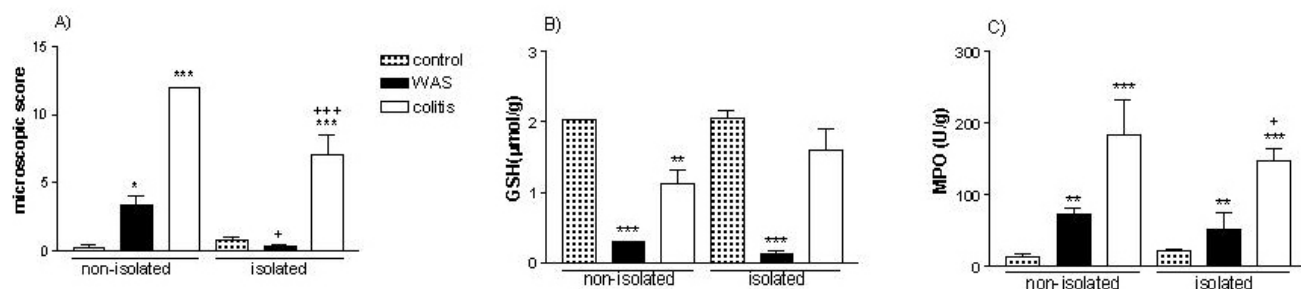


Figure 2: A) Microscopic score B) Glutathione (GSH) levels C) Myeloperoxidase (MPO) activity in colonic tissues of non-isolated and isolated groups of control, WAS-exposure and colitis-induced groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control group, + $p < 0.05$, +++ $p < 0.001$ compared to corresponding group in non-isolated group.

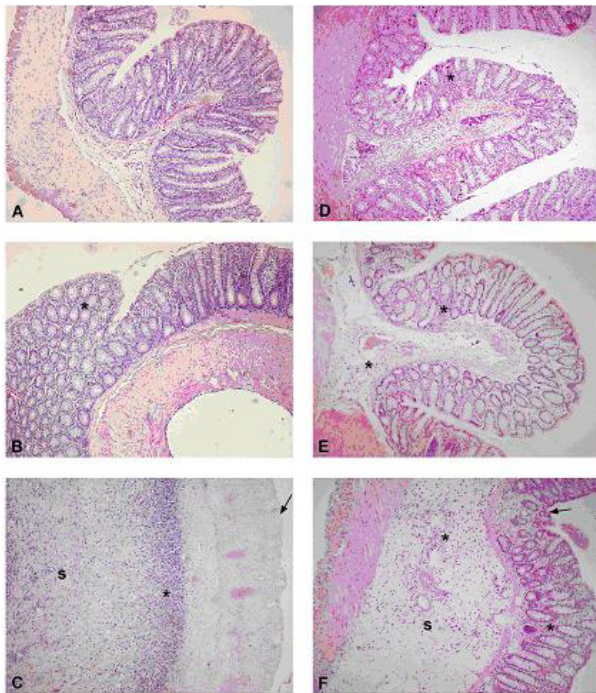


Figure 3: Micrographs illustrating the histological appearances of colonic tissues in different experimental groups. Non-isolated control group (A), regular colon morphology; non-isolated WAS group (B), mild inflammatory cell infiltration; non-isolated colitis group (C), severe damage of mucosa with epithelial degeneration, severe submucosal edema, vasculitis and inflammatory cell infiltration; isolated control group (D), mild inflammatory cell infiltration; isolated WAS group (E) mild inflammatory cell infiltration; isolated colitis group (F) mild damage of mucosa with localized epithelial degeneration, moderate submucosal edema, vasculitis and inflammatory cell infiltration. H&E staining, original magnifications, $\times 100$. Epithelial degeneration (\rightarrow), submucosal edema (s) and inflammatory cell infiltration (*).

On the other hand, colonic GSH in the rats with colitis that were previously isolated was found to be similar to that of the control levels, showing that the depletion of colonic GSH by colitis does not occur in chronically stressed animals. In contrary, the pre-existing isolation stress did not alter the reduction in the colonic GSH induced by the acute WAS.

MPO activity, indicating tissue neutrophil infiltration, was elevated in the colonic tissues of both the isolated and non-isolated rats that were exposed to acute WAS ($p < 0.01$; Fig. 2c). A similar elevation in the colonic MPO activity was observed in the colitis groups with or without isolation stress ($p < 0.001$). However, colitis-induced increase in colonic MPO activity was found to be less in the rats that had isolation stress before, as compared to non-isolated colitis group ($p < 0.05$).

DISCUSSION

In the present study, the data show that rats exposed to prolonged isolation stress in the early life immediately after the weaning period, develop a resistance to colonic inflammation occurring later in life. Social isolation that was experienced before the adult life reduced the colonic microscopic damage, prevented the depletion of colonic glutathione and reduced the neutrophil accumulation to colonic tissue, making the rats less vulnerable to experimentally induced colonic inflammation. Interestingly, having a history of early-life stress does not change the colonic response to acute psychological stress introduced in the adult life, but still alleviates the severity of histological damage.

Physical and emotional stress-related disturbances of homeostasis and induction of the pathogenesis of various diseases are well established. Maternal separation of pups from dams is often used as an early life stressor that causes profound neurochemical and behavioral changes in the pups that persist into adulthood. The neonatal period, roughly extending in rats from birth to day 14, is often referred to as a stress hypo-responsive period characterized by a blunted activation of HPA responses to a variety of environmental stressor, and circulating levels of adrenocorticotropin and corticosterone are low¹⁸. If newborn rats are subjected to chronic or prolonged maternal separation, a hyperresponsiveness to stress is observed along with increased release of corticotropin-releasing hormone (CRH) and altered expression of glucocorticoid receptors,^{19,20,21}. Early life events in humans were also found to be associated with a long-term enhancement in stress-responsiveness and CRH secretion in adults^{22,23,24}. Alterations in the HPA axis are related with numerous long-term disorders, such as anxiety, depression, feeding behaviour abnormalities and increased gastric ulcer susceptibility in the adult^{11,25}. It has been shown that prolonged neonatal maternal deprivation may alter the mucosal immunity and predispose adult animals to increased vulnerability to pathogenic bacteria and to colonic barrier dysfunction in response to mild stress^{26,27}. On the other hand, brief maternal separation during the critical phase of development has been shown to lower the CRH and ACTH response¹² and decrease the acute stress-induced anxious behavior and HPA activation during adulthood^{28,29}. It is postulated that these brief separations are normal for development³⁰. Our results show that isolation-induced stress in the



early life, but clearly beyond the vulnerable period, yields to a reduction in the inflammatory response to colitis induction, which may suggest a decrease in the HPA activation. As the other mild stressors, post-weaning isolation may induce an adaptive response to protect the individual to subsequent pathological conditions. Thus, adult rats exposed to isolation stress during their post-weaning period are protected from chemically induced colitis.

Experimental and clinical studies have shown that any harmful tissue event is perceived by macrophages and monocytes, which in turn secrete cytokines. Cytokines then activate inflammatory cells (neutrophils, macrophages/monocytes, platelets, mastocytes) releasing large amounts of toxic oxygen and nitrogen species, which cause cellular injury via several mechanisms. Glutathione is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress. However, reduced glutathione as the main component of endogenous non-protein sulfhydryl pool, is known to be a major low molecular weight scavenger of free radicals in the cytoplasm^{31,32}. In accordance with the previous reports, our results support the notion that depletion of tissue GSH, as observed in the acetic acid- and WAS-induced colonic injury, is one of the major factors that permit tissue damage. Since exposure to isolation stress previously has reduced the colonic GSH depletion, it appears that the protective effect of the early life stressor may involve the maintenance of antioxidant capacity in protecting the colonic tissue against oxidative stress. However, the presence of isolation stress in the early life period has not altered the WAS-induced GSH depletion. Interestingly, the previous stress has not exacerbated the destruction of antioxidant capacity induced by an acute stressor.

The tissue-associated MPO, which is known as the index of neutrophil infiltration, plays a fundamental role in oxidant production by neutrophils³³. In our observation, elevated MPO levels in the colonic tissues indicate that neutrophil accumulation contributes to the colitis- and WAS-induced oxidative injury. As shared by other inflammatory disorders in the gut, active lesions in the ulcerative colitis involve the migration of activated neutrophils and macrophages³⁴. A growing body of evidence suggests that neutrophils release chemotactic substances, which further promote neutrophil migration to the tissue, activate neutrophils, and

increase the damage³⁵. In the present study, colitis-induced increase in MPO activity was relatively less when the rats were exposed to isolation stress previously, suggesting that the protective effect of the early stressor on colonic inflammation may involve the inhibition of neutrophil recruitment, which then inhibits the adhesion and aggregation of neutrophil leukocytes³⁶. On the other hand, it seems that isolation stress during post-weaning period protected the colonic tissue against WAS exposure by a mechanism independent of neutrophil accumulation. However, the TNF- α level was already increased in the rats that were exposed to isolation stress, suggesting that the cytokine response was enhanced by the early life stressor. Nevertheless, the important function of antibodies in recognizing and neutralizing some of the damaging cytokines like TNF- α may be changed by alterations in the HPA axis¹². The present data demonstrates that hypersensitivity to inflammation, via increased synthesis or release of proinflammatory mediators, is not further enhanced by new stressors of chemical or psychological origin.

The newborn is totally dependent on maternal care. Feeding and maintaining optimal body temperature depend on parental sensitivity to the offspring's signals, and tactile stimulation appears to be crucial for the infant's biological and behavioral responses³⁷. Therefore, repeated maternal separation delays the gain in body weight²⁸. Since neonatal stress consisted of early weaning, milk deprivation has been proposed as a major factor involved in numerous long-term disorders, such as increased ulcer susceptibility^{11,38}. However, in our study, the rats were isolated after the weaning period and the weight gain was found to be similar in both the isolated and non-isolated rats. Since the separated animals were capable of self-care, isolation did not directly affect the feeding behavior. Along with the behavioral symptoms, the current data show that post-weaning isolation has a protective effect on chemically induced colonic inflammation. Furthermore, isolation following weaning does not exacerbate colonic response to subsequent acute stressors. In conclusion, our results show that stress exposure does not always contribute to the exacerbation of the inflammatory bowel disease. Stress, depending on intensity, duration and the time of exposure, may elicit adaptive or maladaptive physiological changes, providing advantageous or deleterious effects on psychosomatic vulnerability.



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