Beneficial Role of Diazepam in the Histological Alterations of Colon Post Immobilization Stress-Induced in Adult Albino Rats

Nabila I. El–Desouki1*, Mohamed S. Gabry2, Heba M. Nagi2

Abstract— The present work was planned to study the histological changes that may occur in the colon of the immobilized-stressed albino rats and the ameliorative role of diazepam injected intraperitoneally with therapeutic dose (0.1 mg/ kg b.w.). Sixty adult male albino rats weighing 110 ± 5g were used and divided equally into 6 groups, group(I) served as control rats; group(II) rats treated with diazepam; group (III) and (IV) served as stressed rats (10 animals / each); in which the rats were immobilized individually for 2 hrs. daily for different durations (5 and 30 days, respectively); groups (V) and (VI) served as immobilized-stressed rats (10 animals /each) for 2 hrs daily for 5 and 30 days and treated with diazepam for 30 days, respectively. The results recorded a significant increase in sera cortisol of the stressed-rats for 5 and 30 days. Histological results of colon demonstrated epithelial damage in the form of cytoplasmatic vacuolation, pyknotic nuclei and some others were karyolytic. Desquamation of the absorptive columnar cells, detachment of cells from the basement membrane, reduction in number of superficial goblet cells containing mucus and distortion of the crypt architecture were observed. Moreover, fibrosis between crypts and marked leucocytic infiltration in lamina propria were elucidated as well as congestion and dilatation of the blood vessels. Additionally, the increment of collagen fibres in the lamina propria of mucosa and muscularis mucosa of the stressed rats was obviously demonstrated. These alterations were time-depended. Treatment with diazepam resulted in decreased cortisol levels, marked improvement and restoration of the histological changes. The results indicated that diazepam is recommended to be used as a curative drug to improve the disturbances in the colon caused under the effect of stress.

Keywords— Colon, Histology, Stress, Benzodiazepines, Rat

1 Introduction

Stress is an aversive stimulus which disturbs physiological homeostasis, and it is reported to play an important role in the genesis and pathophysiology of different psychological disorders. Stress can be measured as the actual exposure to events assumed to be stressful or as individual’s interpretation and perception of stressors and can be assessed across all context of life [1]. Stress induces adreno-medullary response in man to release adrenaline which in turn stimulates receptors on the pituitary gland, and it leads to a greater release of adrenocorticotropic hormone (ACTH) that stimulates the adrenal cortex to release cortisol resulting in further release of adrenaline [2]. Stressors can have multiple effects on individuals depending on the nature of the stressors and susceptibility of the individual [3].

Acute restraint stress produces several emotional and autonomic responses. The autonomic responses include increased mean arterial pressure and heart rate, skeletal muscle vasodilatation and cutaneous vaso-contraction which are accompanied by a rapid skin temperature drop and followed by body temperature increment [4], [5],[6]. Chronic stress is most prevalent in human beings and has been associated with the development of different pathologies, including cardiovascular, immunological and neurodegenerative diseases and even psychiatric disorders [7]. Stress can modulate intestinal inflammatory response through multiple routes, stress has various effects on the gastrointestinal functions, including intestinal barrier function, luminal bacteria adherence, mucosal immune function and stress may directly or indirectly influence the balance of pro-inflammatory and anti-inflammatory cytokines in the intestine [8],[9].

Stress promotes long term alterations in the colonic epithelial barrier associated translocation. These alterations associated with macroscopic damage and an increase in mucosal mast cell density and cytokine mRNA expression. This suggests the role of early psychological factors in the regulation of colonic mucosal barrier in later life [10]. Previous study revealed that wrap-restraint stress (4 hrs daily for 15 days) resulted in increase and activation of mast cells in colonic mucosa [11]. Additionally, water avoidance stress (2 hrs daily for 5 days) resulted in severe histological changes in gastrointestinal tract and liver of albino rats [12]. Gaby et al. [13] reported that immobilization stress for different periods resulted in many histological alterations in rat stomach such as hypertrophy of parietal cells, detachment of gastric cells from their basement membrane and irregular topography of the gastric glands. Hunger stress caused an obvious reduction of the mucus content in mucosal epithelial cells of stomach [14].

Benzodiazepines (BDz) such as diazepam are well known to induce antistress properties. BDz have many clinical and therapeutic applications as a preoperative anesthetic medication and have anxiolytic, sedative, hypnotics, anticonvulsants, skeletal muscle relaxant and amnestic properties [15]. Diazepam is known as one of the widely prescribed therapeutic

1 Department of Zoology, Faculty of Science, Tanta University, Tanta 31527, Egypt. E-mail: nabiladesoky@yahoo.com
2 Department of Zoology, Faculty of Science, Helwan University, Egypt
*Corresponding author: Nabila Ibrahim El–Desouki, Department of Zoology, Faculty of Science, Tanta University, Egypt. E-mail: nabiladesoky@yahoo.com

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agents, these drugs affect the central nervous system through specific binding sites on γ-aminobutyricacid (GABA)-gated chloride channels called GABA-receptor-Chloride-complex [16]. In addition to the aforementioned receptors, peripheral binding receptors are widely distributed in stomach, small intestine, colon, liver, pancreas, lung, testis, breast, ovary and on the inner and outer mitochondrial membranes [17],[18].

The administration of diazepam produced a significant anxiolytic effect in mice [19] and decreased the plasma corticosterone and adrenocorticotropic hormone levels in adult female rats [20]. Diazepam reversed the immobilized-stress induced histopathological alterations in testis of adult male albino rats [21], in ultrastructure of adrenal cortex [22], in stomach [13], in the cytoskeletal intermediate filaments impairments in rat stomach [23], in cardiac muscles [24] and in ultrastructure of skeletal muscle [25]. As stress is increasing in our life day by day, the present study was planned to investigate the effects of immobilization stress on the histology of the colon of albino rats and the ameliorative role of diazepam.

2 MATERIALS AND METHODS

2.1 Animals:- Sixty adult male albino rats weighing 110±5g were used in the present experiment. The animals were housed in environmentally controlled optimal conditions for one week in accordance with the Ethics Committee of Accommodation and Care for Animals. Diet and water were allowed ad- libitum. Rats were exposed to stress for 2 hrs daily between 9:00 and 11:00 a.m. The animals were placed individually in wire mesh restrainers (5×7×12 cm in dimension) as described by Soliman [26]. This procedure effectively restricted movement of the animal.

2.2 Treatment:- Stressed-rat were injected intraperitoneally (i.p.) with the therapeautic dose of diazepam (0.1 mg/kg b.w. according to Paget & Barenns [27], diluted in distilled water, daily for 30 days. Diazepam was received from Amoun Pharmaceutical Industries Co. Cairo, Egypt.

2.3 Experimental design:- The rats were divided into 6 equal groups, 10 animals/each. Group I: served as control; Group II: rats injected daily with diazepam only for 30 days; Group III: stressed- rats for 5 days; Group IV: stressed -rats for 30 days; Group V: stressed-rats for 5 days and treated with diazepam for 30 days; Group VI: stressed-rats for 30 days and treated with diazepam for 30 days. At the end of each experimental period, the blood sera were collected to measure the level of cortisol and rats were sacrificed by decapitation. Serum cortisol was determined by using a radio-immunoassay kit (RIA) (biochemical, Costa Mesa, CA, USA) and the values were expressed as Ug cortisol/dl serum [28], [29]. The colon specimens were carefully removed, cut into small pieces then fixed in 10 % neutral formalin for 24 hrs and processed to get sections of 5μ thickness. Sections were stained with Harris haematoxylin and eosin (H&E) for histopathology [30] and azan stain to demonstrate the collagenous fibers and mucous cells [31].

2.4 Statistical analysis:- All values were expressed as Mean ±SD. Statistical significance was determined using one way ANOVA followed by Dunnett’s comparison test.

3 RESULTS

3.1 Effect of Immobilization-stress and diazepam on cortisol levels:
The cortisol hormone values were measured in the blood sera of rats. The value was 1.35Ug/dl in a control rat and cortisol hormone of diazepam treated rats was 1.38 Ug/dl. After 5 days of stress, the hormone levels in the blood sera were increased to 1.53Ug/dl. The increment of the hormone levels continued after 30 days of stress where it reached 4.03Ug/dl. Then, the cortisol levels in stressed rats for 5 days and treated with diazepam for 30 days decreased to reach 1.4Ug/dl, while those stressed for 30 days and treated with diazepam for 30 days decreased to 2.09Ug/dl (see Table 1 and Fig. A).

Table 1: Effect of stress and diazepam treatment on the levels of cortisol hormone

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cortisol hormone level (ug/dL)</th>
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<tbody>
<tr>
<td>G1 Control gp</td>
<td>1.35</td>
</tr>
<tr>
<td>G2 Diazepam alone gp</td>
<td>1.38</td>
</tr>
<tr>
<td>G3 stress 5 days gp</td>
<td>1.53</td>
</tr>
<tr>
<td>G5 stress 30 days gp</td>
<td>4.03</td>
</tr>
<tr>
<td>G5 stress 5 days + Dz gp</td>
<td>1.40</td>
</tr>
<tr>
<td>G6 stress 30 days + Dz gp</td>
<td>2.09</td>
</tr>
</tbody>
</table>

P-value: G1&G2: 0.796, G1&G3: 0.005*, G1&G4: <0.001*, G3&G5: <0.001**, G4&G6: <0.001**

All values are mean ± SD, n-10 animals in each group; P*<0.01 significant as compared to control ; P**< 0.001 significant as compared to stress.

3.2- Histological observations:-

A. H&E:-

![Fig. A. The correlation between stress, diazepam treatment and the levels of cortisol hormone](http://www.ijser.org)
A1. Control group:
Sections of colon of control rats stained with H&E revealed the normal histological structure; the colonic mucosa showed simple columnar epithelial cells, appeared as tall cells with basally located large oval nuclei with many goblet cells, in-between which appeared with vacuolated cytoplasm. The underlying lamina propria is apparently normal formed of loose connective tissue (Fig. 1). The treatment of rats with diazepam only for 30 days revealed no changes in histological pattern (Fig. 2).

A2. Stressed rats groups:
The stressed-rats for 5 days showed many histopathological changes in the colonic epithelial cells in the form of vacuolation of the cytoplasm, desquamation of the absorptive columnar cells, reduction in number of superficial goblet cells containing mucus, distortion of the crypt architecture and pyknotic or karyolitic nuclei were observed. Moreover, fibrosis between crypts and marked leucocytic infiltration in lamina propria were noticed. Also, congestion of the blood vessels was seen (Fig.3 a&b). The previous alterations became more prominent in the colon sections obtained from stressed-rats for 30 days. Moreover, detachment of epithelial cells from the basement membrane, severe dilatation and congestion of blood vessels were observed (Fig. 4 a&b).

A3. Treated-rats groups:
Treatment of stressed-rats for 5 days with diazepam daily at a dose of 0.1 mg/kg b.w for 30 days showed an improvement and restoration of the colonic tissue; appearance of normal absorptive columnar cells, normal goblet cells and the contact nuclei. The crypt architecture and blood vessels exhibited normal appearance as more or less similar to the control ones (Fig. 5). Immobilized-stressed rats for 30 days treated with diazepam for 30 days also exhibited an obvious improvement in the structure of colon (Fig. 6).
the crypts of Liberkuhn (CL) with their absorptive (Ab) and mucous goblet cells (G). (H&E, Bar = 12.5 µm).

**Fig. (2):** Cross section of the colonic mucosa of a rat treated with diazepam only for 30 days showing approximately normal appearance of the colonic mucosa; muscularis mucosa (mm), crypts of Liberkuhn (CL), absorptive columnar epithelial cells (Ab) and goblet cells (G). (H&E, Bar = 12.5 µm).

**Fig. (3):** Cross section of the colonic mucosa of a rat-stressed for 5 days showing:

**a)** vacuolated cytoplasm (thin arrows), depletion of superficial goblet cells, pyknotic (thick arrows) and karyolitic (double arrows) nuclei. Also, leucocytic infiltration (arrowhead) and congestion of the blood vessels (*) are noticed.

**b)** necrosis of many cells (double arrow), desquamation of surface absorptive columnar cells (arrow), distortion of crypt architecture (double thick arrows) and leucocytic infiltration (arrowhead). (H&E, Bar = 12.5 µm).

**Fig. (4):** Cross section of the colonic mucosa of a rat-stressed for 30 days showing:

**a)** vacuolated cytoplasm (thin arrows), pyknotic (thick arrows) and karyolitic (double arrow) nuclei, depletion of goblet cells, distortion of crypt architecture (D), fibers extending inbetween crypts (curved arrows) and leucocytic infiltration in lamina propria (arrowhead).

**b)** necrotic cells (arrow), depletion of goblet cells, distortion of crypt architecture (D) and distortion of absorptive columnar cells (double arrows). Also, dilatation and congestion of the blood vessels in lamina propria are observed (*). (H&E, Bar = 12.5 µm).

**Fig. (5):** Cross section of the colonic mucosa of a rat-stressed for 5 days and treated with diazepam for 30 days showing approximately normal appearance of the colonic mucosa (arrows). (H&E, Bar=12.5µm).

**Fig. (6):** Cross section of the colonic mucosa of a rat-stressed for 30 days and treated with diazepam for 30 days showing nearly normal appearance of colonic mucosa; crypts (thick arrows) and contact absorptive columnar cells (thin arrow). (H&E, Bar=12.5µm).

A. **Azan stain:**
Sections of the colon of control normal rats stained with azan revealed normal distribution of collagen fibers that demonstrated as a blue color in muscularis mucosa and lamina propria (Fig. 7). Rats treated with diazepam only for 30 days, exhibited similar normal distribution of the collagen fibers as in control ones (Fig. 8). In stressed-rats for 5 and 30 days, the collagen fibers were increased and the increment was time-dependent (Figs. 9 & 10). After treatment with diazepam, a marked reduction of collagen fibers was observed to reach the normal ones indicating the curative role of diazepam (Figs. 11 & 12).

Fig. (7): Cross section of the colon of a control rat showing moderate normal distribution of collagen fibers in submucosa (arrowhead), muscularis mucosa (thick arrow) and lamina propria inbetween the crypts (thin arrow), and the mucoid secretion of goblet cells (double arrows). (Azan, Bar = 12.5 µm).

Fig. (8): Cross section of colonic mucosa of a rat treated with diazepam alone for 30 days showing approximately moderate normal distribution of collagen fibers in muscularis mucosa (thick arrow) and lamina propria inbetween the crypts (thin arrows) and in the mucoid secretion of goblet cells (double arrow). (Azan, Bar = 12.5 µm).

Fig. (9): Cross section of the colonic mucosa of a rat-stressed for 5 days showing the increment of collagen fibers in muscularis mucosa (thick arrow) and lamina propria inbetween the crypts (thin arrows). (Azan, Bar = 6.25 µm).

Fig. (10): Cross section of the colonic mucosa of a rat-stressed for 30 days showing marked increment of collagen fibers in muscularis mucosa (thick arrow) and lamina propria inbetween the crypts (thin arrows). (Azan, Bar = 6.25 µm).
Fig. (11): Cross section of the colonic mucosa of a rat-stressed for 5
days and treated with diazepam for 30 days showing decrease of
collagen fibers distributed in muscularis mucosa (thick arrow) and
lamina propria inbetween the crypts (thin arrows). (Azan, Bar = 6.25
µm).

Fig. (12): Cross section of the colonic mucosa of a rat-stressed for 30
days and treated with diazepam for 30 days showing an obvious
reduction of collagen fibers distributed in muscularis mucosa (thick
arrow) and lamina propria inbetween the crypts (thin arrows). (Azan,
Bar = 6.25 µm).

4. Discussion
The exposure to stress induces a cluster of physiological and
behavioral changes in an effort to maintain the homeostasis of
the organism [32]. Stress and anxiety are believed to play a
major role in developing functional gastrointestinal disorders.
Patients with serious stress frequently complain of gastrointestinal
symptoms include fullness and bloating after small meals, abdominal distention, nausea and loss of appetite [33]. Psychological stress aggravated colitis with enhanced expres-
sion of pro-inflammatory cytokines (Tumor necrosis factor-α).

Stress also induced shortening of colonic length, colonic in-
flammation and body weight loss. Both the epithelial cells and
macrophages expressed interleukin-18 in the colonic mucosa of
murine colitis [34],[35]. Chronic immobilization stress during
pregnancy produced a decrease in cellular proliferation index at 12 and 17 gestation days which may be related to
changes in plasmatic concentrations of corticosterone and pro-
lactin, and to the reduction of specific growth factors [36].
In the present study, the cortisol hormone levels were in-
creased after exposure to immobilization stress. In accordance,
Gabry et al. [13] declared that immobilization stress for 2 hrs
daily for different periods resulted in increment of cortisol level and many histological alteration in stomach in male albi-
no rats. Chronic immobilization stress increased the plasma
corticosterone level and neuroinflammation in mice brain [37].
Also, El-Desouki et al. [24][23] reported the increment of cor-
tisol hormone in immobilized-stressed rats. Previously,
Mazroa and Asker [38] recorded high cortisol levels in rats
after exposure to high ambient temperature. Stress induces
adreno-medullary response to release adrenaline which in turn stimu-
lates β-receptors on the pituitary gland. It leads to
greater release of adrenocorticotropic hormone that stimu-
lates adrenal glands (Cortex and medulla) resulting in the ex-
cess release of corticosterone and adrenaline from the cortical
cells to combat stress [39].
The present study revealed that immobilization stress pro-
voked many histopathological alterations in rat colonic mucosal cells such vacuolation of the cytoplasm, pyknotic and kar-
yolitic nuclei. Desquamation of the absorptive columnar cells,
reduction in number of superficial goblet cells containing mus-
cus and distortion of the crypt architecture were observed.
Moreover, enhancement of fibers between crypts, marked leukocytic infiltration and increment of collagen fibers in lamina
propria were noticed. Also, dilatation and congestion of the
blood vessels of stressed animals were seen.
In agreement, exposure to the social interaction stress dis-
played behavioral and physiological changes, which correla-
ted with increase in corticosterone and pro-inflammatory cyto-
kines, and mild damage of the colon. These behavioral and
physiological impairments similar to that observed in patients
with dysregulated brain-gut axis function [40]. Restraint stress
significantly increased malondialdehyde levels and reduced
levels of antioxidants in the normal colon. ATP and the muco-
sal energy charge decreased substantially with chronic stress.
Chronic stress worsened the extent of inflammation in 2, 4, 6-
trinitrobenzene sulfonic acid-induced colitis; mucosal sub-
stance p content was increased after induction of colitis and exposure to chronic stress [41]. Also, de Souza et al. [42] pro-
posed that the morphological alterations induced by immobi-
lization stress in the kidneys of rats may have serious implica-
tions, predisposing individuals to renal diseases and hyper-
tension in adult life. Physiological stress induces gastrointesti-
nal symptoms (including dyspepsia, abdominal pain and in-
creased colonic motility). Stress too has an important role in
the pathogenesis of irritable bowel syndrome as risk, trigger
and perpetuating factor [43].
Additionally, El Drieny and Mousa [11] reported wrap re-
straint stress for 4hrs/day for 15 days resulted in epithelial
separation, cell loss, ulceration, depletion of goblet cells as
well as mononuclear cellular infiltration in lamina propria. Moreover, increase in hyperplasia and activation of mast cells in colonic mucosa after exposure to stress. Similar results were
documented by Zebek et al. [12], [44] who demonstrated the water avoidance-stress resulted in severe vascular congestion with degeneration of ileal and colonic epithelium of albino rats. Also, Rober et al. [45] documented the exposure to stress led to macroscopic damage of the mucosal layers of colon and increased secretion of pro-and anti-inflammatory cytokines by mesenteric lymph node cells in male mice.

Benzodiazepines are used for their anxiolytic and sedative properties in the treatment of a variety of neuropsychiatric disorders including anxiety and depression which are often related to disturbances in the activity of the hypothalamic-pituitary-adrenal axis. These drugs exert their pharmacological effects via GABA-receptors [20]. Diazepam is a classical benzodiazepine derivative. In the present study, diazepam administration for 30 days decreased the high levels of cortisol hormone induced by immobilization stress and revealed a remarkable improvement of alterations in the rat colon. In agreement, Zhao et al. [46] diazepam slowed the progress of chronic stress induced-impairment of hippocampal structural plasticity and depression like behavior in mice by normalizing glucocorticoids.

Moreover, Nagi [47] found that diazepam administration for 30 days decreased cortisol level and ameliorated the histological alterations induced by immobilization stress in rat gastric mucosa. Similarly, diazepam improved the changeable of the cytoskeletal intermediate filaments impairments in rat stomach [23], cytoskeletal intermediate filaments (cytokeratin and vimentin) of both cortex and medulla of adrenal glands of rats stressed for 30 days [48], the ultrastructure of adenral cortex [22] and in desmin (intermediate filament protein) in the cardiomyo-fibirls of the immobilized-stressed albino rats [24]. Subsequently, El-Desouki et al. [25] illustrated the immobilized stress- rats for 30 days showed the ultrastructure of the skeletal muscle with a marked distortion in the myofibirls. The transverse striations of the myofibrils appeared irregular and discontinued. Partial disappearance of light I band and extension of dark A band with discontinuous Z line, and after treatment with diazepam for 30 days at a dose 0.1mg/kg bw, the skeletal muscles elucidated partial recovery and improvement of most myofibrils with the appearance of normal light I and dark A bands accompanied with irregular alignments of most of Z line.

Use of several antistress agents such benzodiazepines (diazepam), certain central nervous system stimulants such as amphetamine and caffeine as well as some anabolic steroids, showing significant antistress activity against various models of stress [49]. Also, diazepam with metformin caused a remarkable reduction of the anxiety-like symptoms in the experimental type 2-diabetic animals exposed to stress [1]. So the diazepam-induced antioxidant effects was due to the modulation of the GABA receptors through different benzodiazepine receptor agonists can reduce the oxidative damage produced by acute immobilization and psychological stress [50], [51]. The mechanisms producing the protective effects suggested that at least 2 important mechanisms could be take place: (1) the increased GABA receptor activation through diazepam would decrease the excitatory glutamateric transmission, thereby decreasing the excitotoxicity and (2) the activation of peripheral benzodiazepine receptors or translocator protein located at the mitochondrial inner membrane, these receptors are involved in micro- and astrogial activation and in many other mitochondrial functions like apoptotic pathways and also participates in regulating cell sensitivity to oxidative stress [52].

From the present study, diazepam is recommended to be used as a curative drug to ameliorate the histopathological alterations in the colon caused under the damaging effects of acute and chronic immobilization stress.

5. Conclusion
The present study revealed that diazepam is recommended to be used as a curative drug to improve the histological alterations in the colon induced by immobilization stress in adult rats

References


