HISTOLOGICAL STUDY ON THE STOMACH OF IMMOBILIZED - STRESSED ALBINO RAT AND THE CURATIVE ROLE OF DIAZEPAM

ABSTRACT:
The present work is planned to study the histopathological changes that may occur in the stomach of the immobilized-stressed rats and the possible curative role of diazepam injected intraperitoneally with therapeutic dose (0.1 mg / kg BW) daily for 30 days. Eighty adult male albino rats weighing 110 ± 5 g were used and divided equally into eight groups, group (I) served as control rats; group (II) rats treated with diazepam; group (III), (IV), and (V) served as stressed rats (10 animals each) in which the rats were immobilized individually for 2 hrs / day for different durations (5, 15, and 30 days, respectively); groups (VI), (VII), and (VIII) served as immobilized-stressed rats (10 animals each) for 2 hrs / day for 5, 15, 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, 15, and 30 days. Histological results of stomach demonstrated hypertrophy of parietal cells, detachment of gastric cells from the basement membrane, degeneration of the gastric glandular cells and irregular topography of the gastric gland. Appearance of karyolytic and pyknotic nuclei was also seen. Infiltration of inflammatory cells in lamina propria of mucosa was elucidated as well as congestion and dilatation of blood vessels. Moreover, the increment of collagen fibres in the lamina propria of mucosa and submucosa of the stressed rats was obviously demonstrated. These alterations were time-dependent. Treatment with diazepam resulted in 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 stomach caused under the effect of stress.

KEY WORDS:
Histology, Stomach, Rat, Stress, Benzodiazepines

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INTRODUCTION:
Stress and anxiety were thought to have an etiological role in chronic inflammatory diseases and gastrointestinal pathophysiology. Stress is widely believed to play a major role in developing functional gastrointestinal disorders. Patients with serious stress frequently complain of gastrointestinal symptoms. Common upper gastrointestinal symptoms include fullness and bloating after small meals, abdominal distention, nausea and loss of appetite (Tack and Lee, 2005). These symptoms are, at least in part, likely to be due to gastrointestinal motility disorders such as functional dyspepsia. Functional gastrointestinal disorders are multifactorial in which the pathophysiological mechanisms are variably combined in different patients (Zheng et al., 2009).

Acute restraint stress inhibits solid gastric emptying via the central corticotropin-releasing factor (CRF) and peripheral parasympathetic / sympathetic neural pathways in conscious rats (Taché and Bonaz, 2005). CRF, a stress-related neuropeptide, is...
known to act in the brain to influence the gastrointestinal tract. Gastric emptying and acid secretion are greatly attenuated when CRF is exogenously applied to the central nervous system (Lenz et al., 1988) and solid meal gastric emptying was delayed by acute stress such as in rats (Nakade et al., 2005). Moreover, Erosy et al. (2008) documented that the stomach epithelium of Wister albino rat showed ulceration in some areas, dilatation of gastric glands, degeneration of gastric glandular cells, and prominent congestion of the blood capillaries. In a similar fashion, degenerated epithelium and severe vascular congestion were observed in the ileum and colon after the exposure to acute water-avoidance stress for two hrs daily and chronic stress for 5 days. El-Refaiy (2010) documented the immobilization stress of rats for 2hrs daily for 5, 15 and 30 days caused marked reduction of spermatogenesis, degeneration of germinal epithelium, thickening of basement membrane, haemorrhage and marked increment of collagen fibres.

Benzodiazepines (BDZ) are the most frequently prescribed class of psychotropic drugs. BDZ such as diazepam ʽtrade name is Valium, chemically it is phenyl benzodiazepine containing 7- chloro 1,3 di hydro -1- methyl -5-phenyl -2H- 1,4 benzodiazepine” (Abdelmajeed, 2009). Diazepam is one of the most representatives of the classical BDZ, and is widely used as an anti-anxiety agent. It has a basic clinical profile that is typical of BDZ, exhibiting muscle relaxant, anticonvulsant, sedative / hypnotic and anxiolytic activity. Its clinical indications cover a wide range of anxiety states, seizures and other symptoms (Inada et al., 2003).

BDZ reduce anxiety and stress responses by acting on high-affinity receptor sites present in the central nervous system (CNS), these specific binding sites on γ-aminobutyric acid (GABA)-gated chloride channels called GABA- receptor-chloride-complex (Engel et al., 2007). Nevertheless, besides the central receptors described for BDZ, peripheral-type binding sites (PBR) have also been identified for them in human stomach, small intestine, colon, liver, lung, thyroid gland, pancreas, breast, prostate, ovary and in mitochondrial membrane (Bribes et al., 2004).

Gabry et al. (2002) reported that rats exposed to 4 hrs. immobilization and treated with diazepam (5 mg/kg) received half the dose of the drug intraperitoneally twice before and after 2 hrs of starting stress, diazepam suppressed stress-induced gastric ulceration, inhibited colon motility, mucin depletion and decreased struggling behaviour. The administration of diazepam reversed the stress-induced alterations, and these effects were dose dependent. Also, diazepam increased food-intake, water intake and growth rate (Reshama, 2008). Moreover, the diazepam-treatment to immobilize stressed-rats could improve certain histological and histochemical alterations induced in testes of rats (El-Refaiy, 2010).

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 stomach of albino rats and the probable ameliorative effect of diazepam.

**MATERIAL AND METHODS:**

**Animals:**

Eighty adult male albino rats, each weighing 110 ± 5g, were used in present experiment. The animals were housed in environmentally controlled optimal conditions for two weeks before the beginning of the experiment. Diet and water were allowed ad libitum.

**Immobilization stress:**

Rats were exposed to stress for 2hrs daily between 10:00 and 12:00 a.m. The animals were individually placed in wire mesh restrainers (5×7×12 cm in dimension) as described by Soliman (2006). This procedure effectively restricted movement of the animals.

**Treatment:**

Stressed-rats were injected intraperitoneally with the therapeutic dose of diazepam (0.1 mg /kg BW according to Paget and Barnes, 1964), diluted in distilled water, daily for 30 days. Diazepam was supplied from Amoun Pharmaceutical Industries Co. Cairo, Egypt.

**Experimental design:**

The animals were divided into 8 groups, 10 animals / each. Group I: served as control; Group II: rats injected with diazepam only for 30 days; Groups III, IV and V: stressed rats for 5, 15 and 30 days, respectively; Groups: VI, VII and VIII: stressed-rats for 5, 15 and 30 days and treated with diazepam for 30 days. At the end of the experimental period, the blood sera were collected to measure the levels of cortisol and the rats were sacrificed by decapitation. Serum cortisol was determined using a radio-immune-assay kit (biochemical, Costa Mesa, CA, USA) and the values were expressed as Ug cortisol / dl serum (Ulrich-Lai et al., 2006).

The stomach samples were carefully removed, cut into small pieces, fixed in 10% buffered formalin and processed to get sections of 5 μm thick. Sections were stained with Harris haematoxylin and eosin (H&E) for histological study (Delafield, 1984) and other sections were stained with azan to demonstrate the collagenous fibres (Humason, 1972).
RESULTS:
Effect of stress on Cortisol:

The cortisol hormone values measured in the blood sera of control rats recorded 1.35Ug/dl. After 5 days of stress, the hormone levels in the blood sera were increased from 1.35Ug/dl to 1.53Ug/dl. The increment of the hormone levels continued after 15 days of stress where it reached 4.535Ug/dl then, the cortisol levels post 30 days of stress reached 4.03 35 Ug/dl (Table 1 & Fig. 1).

Fig. 1. The correlation between stress and level of cortisol hormone

Table 1. Effect of stress on the level of cortisol hormone

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Stressed rats</th>
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<tbody>
<tr>
<td>Stress for 5 days</td>
<td>Stress for 15 days</td>
<td>Stress for 30 days</td>
</tr>
<tr>
<td>Cortisol hormone Ug/dl</td>
<td>1.35</td>
<td>1.53</td>
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Histological results:

Sections of stomach of control rats stained with H&E showed normal histological architectures. The gastric gland is divided into three regions: isthmus, neck and base. Three types of cells line the pit and gland regions; a) surface mucous cells line the gastric pits, they are columnar acidophilic with oval basal nuclei. B) parietal cells are dispersed throughout the gland characterized by their acidophilic cytoplasm and central rounded nuclei. C) chief cells line the gastric base region having basal basophilia, apical acidophilic cytoplasm and basal nuclei. The lamina propria is composed of loose connective tissue interspersed between gastric glands (Fig. 2a&b). Treatment of rats with diazepam for 30 days revealed no changes in gastric glandular cells except slightly mononuclear cellular infiltration in lamina propria of mucosa layer was seen (Fig. 3 a&b).

Fig. 2. Cross section of stomach of a control rat showing: a) the gastric base region; chief cells (cc) and parietal cells (Pc). Also, a part of muscularis mucosa (mm) is noticed. b) a part of neck region and isthmus region including parietal cells (Pc), surface mucous cells (sm) and gastric pits (arrows). (H&E, Bar = 6.25 µm)

Fig. 3. Cross section of stomach of a rat treated with diazepam only (0.1 mg/kg) for 30 days showing: a) normal appearance of gastric base region; chief cells (cc), parietal cells (Pc) and slightly mononuclear cellular infiltration (arrows). b) a part of neck region (ne) with parietal cells (Pc), mucous neck cells (mn) and a part of isthmus region (is) with surface mucous cells (sm) and gastric pits (arrows). (H&E, Bar = 6.25 µm)

The stressed-rats for 5 days showed many histopathological changes in the stomach as: irregular architecture of chief cells with degeneration of cytoplasm and appearance of pyknotic nuclei. In addition, parietal cells exhibited vacuolated cytoplasm, karyolytic or pyknotic nuclei. Additionally,
fibrosis and congestion of blood vessels were also seen (Fig. 4 a&b).

Fig. 4. Cross section of stomach of a rat stressed for 5 days showing: a) degeneration of the cytoplasm of chief cells (arrows) and pyknotic nuclei (double arrows). b) Vacuolated cytoplasm of parietal cells (V), karyolysis of some nuclei (arrowheads) and pyknosis in others (thin arrows). In addition, dilatation and congestion of blood vessels are observed (thick arrow). (H&E, Bar = 6.25 µm)

Treatment with diazepam at a dose of 0.1 mg /kg BW for 30 days showed an improvement in the gastric glandular cells. However, slight cytoplasmic degeneration of chief cells in gastric base region and mild mononuclear cellular infiltration in lamina propria of mucosa were still seen (Fig. 5 a&b).

Fig. 5. Cross section of stomach of a rat stressed for 5 days and treated with diazepam for 30 days showing: a) slight degeneration of chief cells and slightly mononuclear cellular infiltration (arrows). b) an obvious improvement of neck cells and isthmus regions (arrows). (H&E, Bar=12.5µm)

The stressed-rats for 15 days demonstrated remarkable disarrangement of chief cells at the gastric base region with increased degeneration of cytoplasm and more illustration of pyknotic nuclei, hypertrophy of parietal cells accompanied with vacuolated cytoplasm, detachment of gastric basal cells from their basement membrane, infiltration of mononuclear cells in isthmus region. In addition, fibrosis, dilatation and congestion of blood vessels were noticed (Figs 6 a&b and 7 a&b). These alterations became more prominent in the stomach cross sections of rats stressed for 30 days (Fig. 9 a&b, Fig. 10).

Fig. 6. Cross section of stomach of a rat stressed for 15 days showing: a) degeneration of gastric base chief cells (thin arrows), vacuolation of the cytoplasm of parietal cells (V), pyknotic (double arrows) and karyolytic (arrowhead) nuclei. In addition, dilatation and congestion of blood vessels could be noticed (asterisk). b) Disarrangement of chief cells of gastric base region, degeneration of cytoplasm (thin arrows), pyknotic nuclei (thick arrows) and vacuolated cytoplasm of parietal cells (V). (H&E, Bar= 6.25 µm)

Fig. 7. Cross section of stomach of a rat stressed for 15 days showing: a) hypertrophy of parietal cells with vacuolated cytoplasm (thin arrows), mononuclear cellular infiltration (thick arrows) and congestion of blood vessels (double arrows). b) Other part showing the previous alterations and dilatation with congestion of blood vessels (double arrow). (H&E, Bar = 6.25 µm)
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Treating rats with diazepam for 30 days after immobilization stress for 15 days, exhibited an obvious improvement of the gastric cells except few cells with slight vacuolation were still noticed (Fig. 8 a&b). Moreover, the stressed-rats for 30 days treated with diazepam for 30 days, restored the normal architecture of the stomach and exhibited normal appearance of gastric glandular cells; chief, parietal and surface mucous cells (Fig. 11 a&b).

Sections of the stomach of control rats stained with azan revealed normal distribution of collagen fibres that demonstrated as a blue colour in muscularis mucosa, lamina propria of mucosa and the gastric glandular cells (Fig. 12 a&b). Rats treated with diazepam only (0.1 mg/kg) for 30 days, exhibited normal distribution of the collagen fibres as in control ones (Fig. 13 a&b). In stressed-rats for 5, 15, and 30 days,
the collagen fibres were increased and the increment was time-dependent (Figs 14 a&amp;b, 16 a&amp;b, and 18 a&amp;b). After treatment with diazepam, a marked reduction in the distribution of collagen fibres was observed to reach the normal ones indicating the curative role of diazepam (Figs 15 a&amp;b, 17 a&amp;b, and 19 a&amp;b).

Fig. 12. Cross section of stomach of a control rat showing moderate normal distribution of collagen fibres in: a) muscularis mucosa and lamina propria (arrows). b) Lamina propria and isthmus region (arrows). (Azan, Bar=12.5 µm)

Fig. 13. Cross section of stomach of a rat treated with diazepam (0.1 mg/kg) for 30 days showing moderate distribution of collagen fibres in: a) muscularis mucosa and lamina propria (arrows). b) lamina propria and surface mucous cells (arrows). (Azan, Bar=12.5 µm)

Fig. 14. Cross section of stomach of a rat stressed for 5 days showing marked increase of collagen fibres in: a) muscularis mucosa and lamina propria between the gastric base cells (arrows). b) lamina propria of neck and isthmus regions (arrows). (Azan, Bar=6.25 µm)

Fig. 15. Cross section of stomach of a rat stressed for 5 days and treated with diazepam for 30 days showing marked decrease of collagen fibres in: a) muscularis mucosa and lamina propria (arrows). b) lamina propria of neck and isthmus regions (arrows). (Azan, Bar=6.25 µm)

Fig. 16. Cross section of stomach of a rat stressed for 15 days showing an obvious increase of collagen fibres in: a) muscularis mucosa and lamina propria (arrows). b) Lamina propria of neck and isthmus regions (arrows), note, dilatation and congestion of blood vessels. (Azan, Bar=6.25 µm)
DISCUSSION:
Stress has been postulated to be involved in the etiopathogenesis of a variety of disease states, including anxiety, hypertension, peptic ulcers, diabetes, depression of immune system and reproductive dysfunctions because of an involvement of the central nervous system and the endocrine system (Gidron et al., 2006). Hyperactivity of the pituitary-adrenocortical axis is the most prominent neuroendocrine abnormality in major stress. Cortisol secretion in individuals who have been exposed to severe stress or suffer from immobilization stress-response-related disorders results in the hypersecretion of both ACTH and corticosterone to a subsequent stressor (Armario et al., 2008).

In the present study, the level of the cortisol hormone increased after exposure to different periods of stress. In accordance with this result, Gesi et al. (2001) reported a marked increase cortisol levels in response to noise stressful stimulus. Mazroa and Asker (2010) recorded the increment of cortisol levels in rats after exposure to high ambient temperature. The disturbances may vary by type, intensity, and duration of a stressor, and the strain / sex differentiation of the experimental subjects (Kioukia-Fuougia et al., 2002). Restraint stress is widely used as acute and chronic stress model (Iwa et al., 2006). The length of stress period may alter neurological, behavioral, and biochemical parameters, possibly in different ways (Rai et al., 2003).

In the present study, a daily two hrs immobilized - stress of albino rats for different periods 5, 15 and 30 days resulted in the degeneration of the chief cells, appearance of karyolytic and pyknotic nuclei, hypertrophy of parietal cells accompanied with vacuolated cytoplasm, detachment of gastric cells from the basement membrane and irregular topography of the gastric gland. Infiltration of inflammatory cells in lamina propria of mucosa was elucidated. The congestion and dilatation of blood vessels were also seen. These results agreed with the previous study that illustrated by Erosy et al. (2008) who investigated the effect of water avoidance stress on stomach and proposed that in both acute and chronic stress, the stomach epithelium showed ulceration in some areas, dilation and degeneration of gastric glandular cells as well as prominent congestion of the blood vessels. Moreover, cold-restraint stress caused morphological changes of the mucosa, increased ulcer indices, altered gastric muscular contractility and reduced gastric mucosal blood flow (Erin et al., 2000).

Exposure to stress can stimulate numerous pathways leading to an increased production of free radicals (Rai et al., 2003). It is well known that free radicals activate a cascade, producing lipid peroxidation, protein oxidation, DNA damage, and cell death, and
that they contribute to the occurrence of pathological conditions (Bagchi et al., 1999). Restraint stress has been shown to induce the production of oxygen-derived free radicals in the stomach, which leads to mucosal ulceration, even in the absence of luminal acid (Izgut-Uysal et al., 2001). Stress may also impair the antioxidant defense system, leading to oxidative damage, by changing the balance between oxidant and antioxidant factors (Fontella et al., 2005). Moreover, water-avoidance stress resulted in dense inflammatory cells infiltration and mast cells degranulation in mucosa was observed in stomach, ileum and colon. Malondialdehyde levels (a biomarker of lipid peroxidation) were also significantly increased while glutathione levels (a biomarker of protective oxidative injury) were significantly decreased in all tissues after exposure to stress (Erosy et al., 2008).

Findings of the present study showed that treatment with diazepam stimulated an obvious amelioration of the histopathological changes caused under the effect of immobilization stress. These results agreed with many authors who reported that diazepam had an effective role in improving the various changes induced in stomach, intestine, testis and adrenal gland by immobilization stress in male rats (Gabry et al., 2002; El-Refaiy, 2010; El-Desouki et al., 2011). Moreover, diazepam treatment reduced changes in brain, kidney, adrenals and heart induced by chronic mild stress in rats (Nirmal et al., 2008; Abdel Baky and Ali (2009). The preventive effect of diazepam is suggested to be attributed to its ability to inhibit the stress-induced activation of HPA-axis and sympathetic stimulation and functional alteration of cell membranes due to steroids (Gehlot et al., 1997).

In the current study, collagen fibres were increased after exposure to restraint stress. The increment was time-dependent. In agreement, El-Drieny and Mousa (2006) reported that the colonic mucosa of rats exposed to stress showed features indicating fibrosis as a result of increased collagen synthesis by fibroblasts. Moreover, El-Desouki et al. (2011) reported that the immobilized stressed rats showed increment of collagen fibres in adrenal cortex, and the diazepam administration improved the histological and ultrastructural alterations.

In conclusion, the immobilization stress induced histopathological changes in the stomach of albino rats. These alterations were time-dependent. Diazepam treatment showed an obvious improvement in the stomach alterations. Therefore, it is recommended that stress should be avoided, and diazepam can be used as a curative drug for stomach histopathological alterations induced by stress.

REFERENCES:


