

Effect of Immobilization Stress on the Cytoskeletal Intermediate Filaments of Rat Stomach and the Possible Curative Role of Diazepam

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Abstract: The present study is planned to investigate the effect of immobilization-stress for 30 days on the cytoskeletal intermediate filaments of the rat stomach and the possible curative role of diazepam immunohistochemically. The study was conducted on forty adult male albino rats weighing 110 ± 5 g were used and divided equally into four groups (10 animals each), group (1) served as control rats; group (2) treated unstressed rats with diazepam only for 30 days; group (3) served as immobilized- stressed rats for 2 hrs / day for 30 days and group (4) served as immobilized- stressed rats treated with diazepam (therapeutic dose, 0.1mg /kg b.w.) for 30 days obtained results revealed a significant increase in sera cortisol of the stressed – rats for 30 days. The cytokeratin of cytoskeletal intermediate filaments after stress revealed a marked intense immunoreactivity at the apical part and lateral borders of the surface mucous cells, mucous neck cells, parietal and chief cells. The vimentin demonstrated intensive immunoreactivity in the lamina propria and the blood vessels. Treatment of the stressed-rats with diazepam showed obviously improvements and amelioration in the cytoskeletal intermediate filaments of the stomach. In conclusion; stress should be avoided and supported the using diazepam as a curative drug to improve the disturbances in the stomach intermediate filaments caused under the effect of stress.

[Nabila I. El – Desouki, Amal I. El-Refaiy, Gabry M. Sayed, Mona A.Ibrahim, and Heba N. Mohamed. **The Effect of Immobilization Stress on the Cytoskeletal Intermediate Filaments of Rat Stomach and the Possible Curative Role of Diazepam.** *Life Sci J* 2013; 10 (2): 2211-2219] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 308

Key words: Stress - Diazepam – Rat – Stomach – Intermediate filaments -Immunohistochemistry

1. Introduction

Stress is involved in pathogenesis of variety of diseases. Immobilization stress is a mixture of physical and psychological stressors, restricting movement and isolating the individual from the group (Pacak and Palkovits, 2001). Also, both single and repeated swimming-stress animals had almost used experimentally (Hu *et al.*, 2000). Emotional changes, such as exposure to stress situations can influence feeding behavior; chronic variant stress cause decreased ingestion of sweet food and increased dopaminergic neurotransmission in hypothalamus (Gamaro *et al.*, 2003). Restraint stress is widely used as acute and chronic stress model (Iwa *et al.*, 2006). Additionally, the chronic exposure of rats to noise taped at an industrial cotton room induces alterations in their respiratory epithelia (Oliveira *et al.*, 2005).

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 & 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).

Intermediate filaments (IFs) are one of the three abundant cytoskeletal proteins. IFs include cytokeratin, vimentin, desmin, glial fibrillary acidic proteins, neurofilament proteins, nuclear lamins and nestin (Alberts *et al.*, 1989). They play an important role in the structural integrity, in movement of organelles and their secretory vesicles, and possibly in the transport into and out of the cells. IFs provide flexible intracellular scaffolding whose function is to structure cytoplasm and to resist stresses externally applied to the cell (Djabali, 1999; Omary *et al.*, 2004).

Cytokeratin filamentous proteins used as diagnostic markers in tumor pathology, particularly for the differential diagnosis of carcinomas at the histologic level (Moll *et al.*, 1992). Vimentin is widely distributed in the cells of mesenchymal nature and in stroma (Kameda, 1995). It is used as tumor markers in serum, and as means of detecting micrometastases (Omary *et al.*, 2004).

Benzodiazepines (BDZ) are the most frequently prescribed class of psychotropic drugs. BDZ such as diazepam 'trade 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 symptom (Inada *et al.*, 2003). BDZ reduce anxiety and stress responses by acting on high-affinity receptor sites present in the central nervous system, 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 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).

The reaction of an individual to a given stressor involves the stimulation of pathways within the brain leading to the activation of the hypothalamic-pituitary- adrenal axis and the central sympathetic outflow. The activation of these pathways results in stress response and to the release of the key peripheral mediators of the stress response, namely, glucocorticoids and catecholamines (Swain, 2000).

The diazepam-treatment to immobilize stressed-rats could improve certain histological and histochemical alterations induced in testes of rats (El-Refaiy, 2010). Gabry *et al.* (2011) reported that diazepam suppressed stress-induced histological alterations in stomach of rats. Also, diazepam treatment reduced the ultrastructural alterations induced by stress in rat adrenal cortex (El-Desouki *et al.*, 2011), and improved the cytoskeletal IFs (cytokeratin and vimentin) of the adrenal glands of rats stressed for 30 days, and restored them to normal structural integrity (El-Baely, 2011) as well as desmin of the cardiac myofibrils (El-Desouki *et al.*, 2012).

Aim of the present work is to study the influence of immobilization stress on the cytoskeletal intermediate filaments of rat stomach and the possible curative role of diazepam.

2. Material and Methods

Animals:

forty 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) which is effectively restricted movement of the animals.

Treatment:

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

Experimental design:

The animals were divided into 4 equal groups, 10 animals / each. Group I: served as control; Group II: rats injected with diazepam only for 30 days; Group III: stressed rats for 30 days and 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 levels of cortisol and the rats were sacrificed by decapitation. Serum cortisol was determined by using a radio-immune-assay kit (biochemical, Costa Mesa, CA, USA) according to Ulrich-Lai *et al.* (2006). The stomach tissues were carefully removed and cut into small pieces then processed for immunohistochemical study. Paraffin sections of stomach and monoclonal antibodies against either cytokeratin (anti-CK AE1/AE3) or vimentin (V9) were used. Avidin-biotin immunoperoxidase technique is applied in which a biotinylated secondary antibody reacts with peroxidase conjugated streptavidin molecules. Colour reaction was developed by using diaminobenzidine (DAB) that gives a brown colour. Haematoxylin was used for counterstaining (Hsu *et al.*, 1981).

3. Results

1- Measurement of Cortisol Hormone:-

Serum cortisol hormone level was 1.35Ug/dl in a control rat. After 30 days of stress, the hormone level in the blood sera increased from 1.35Ug/dl to 4.03 Ug/dl. (Fig A).

2- Immunohistochemical studies:-

A- Cytokeratin:-

The cells of the gastric mucosa of both control rats and that treated with diazepam only (0.1mg/kg b.w.) for 30 days expressed normal cytokeratin filaments immunoreactivity as brown colour filaments at the apical part and lateral borders of cells. The surface mucous cells, mucous neck cells,

parietal and chief cells showed the most positive reactivity (Figs. 1- 4). Stressed-rats for 30 days revealed markedly more intense cytokeratin filaments immunoreactivity at the apical part and lateral borders of the surface mucous cells, mucous neck cells, parietal and chief cells (Figs. 5&6). After treatment with diazepam for 30 days to stressed-rats, a marked decrease of cytokeratin filaments immunoreactivity of the surface mucous cells, mucous neck cells, parietal and chief cells was demonstrated. Such reactivity was approximately similar to the control form (Figs. 7&8). The nuclei of such cells exhibited no reaction in all animal groups.

B- Vimentin:

The gastric mucosa of the control rats showed the normal vimentin filaments immunoreactivity in the form of brown colour filaments stained with avidin-biotin immunoperoxidase technique. The vimentin filaments expressed in lamina propria and in the blood vessels (Figs. 9&10). The unstressed-rats treated with diazepam only with a daily dose of 0.1mg/kg b.w. for 30 days expressed vimentin filaments immunoreactivity approximately similar to the control ones (Figs. 11&12). The stressed-rats manifested an obvious intense immunoreactivity to

vimentin filaments in the lamina propria and in the blood vessels (Figs. 13&14). After treatment of stressed-rats with diazepam for 30 days, a noticeable decrease of vimentin filaments immunoreactivity in the gastric mucosa and in the blood vessels was obviously revealed. Such reactivity was almost similar to the control ones (Figs. 15&16). No reaction was seen in the gastric cells nuclei in all animal groups.

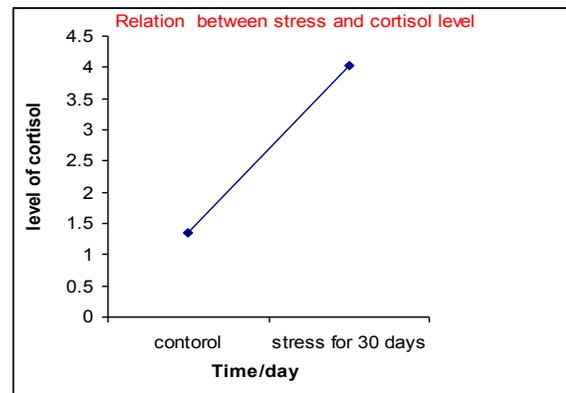


Fig. A: Effect of stress on the levels of cortisol hormone

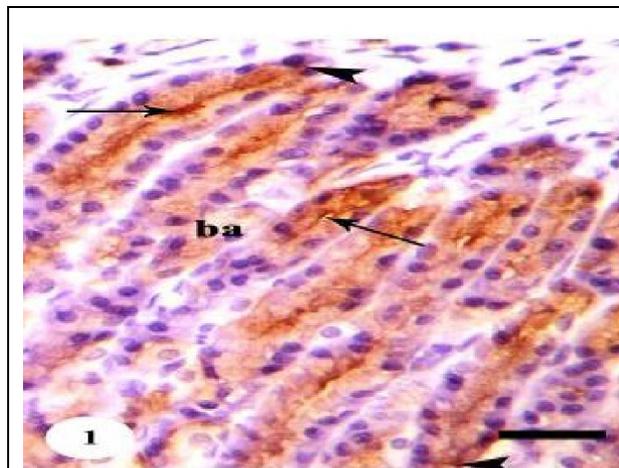


Fig. (1): Cross section of the gastric mucosa of a control rat showing the normal immunoreactivity to cytokeratin at the apical part (arrows) and lateral (arrowhead) borders of cells of base (ba) region. (Immunostain, Bar = 6.25 μ m)

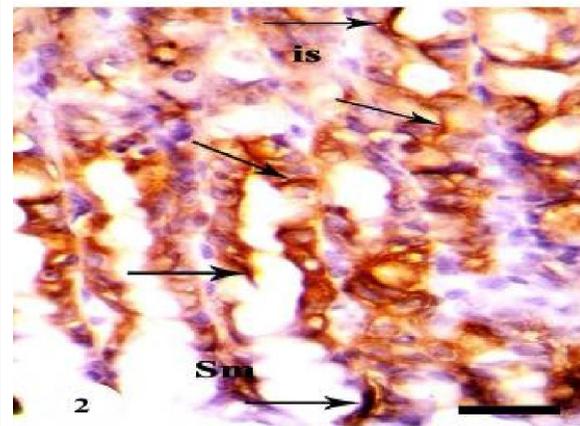


Fig. (2): Cross section of the gastric mucosa of a control rat showing the normal immunoreactivity to cytokeratin in isthmus (is) region and surface mucous (Sm) cells (arrows). (Immunostain, Bar = 6.25 μ m)

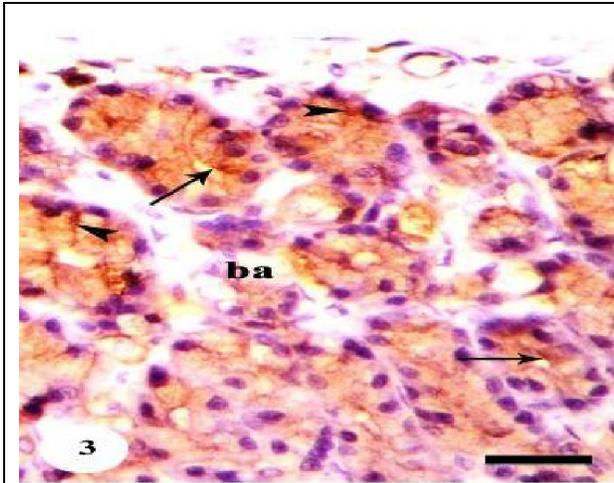


Fig. (3): Cross section of the gastric mucosa of a rat treated with diazepam only showing the normal immunoreactivity to cytokeratin at the apical part (arrows) and lateral (arrowheads) borders of cells of base (ba) region. (Immunostain, Bar = 6.25 μ m)

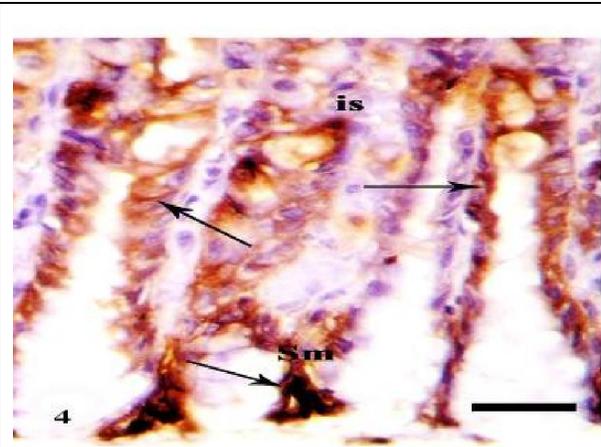


Fig. (4): Cross section of the gastric mucosa of a rat treated with diazepam for 30 days showing approximately normal immunoreactivity to cytokeratin at the apical and lateral borders of the cells of isthmus (is) region and surface mucous (Sm) cells (arrows). (Immunostain, Bar = 6.25 μ m)

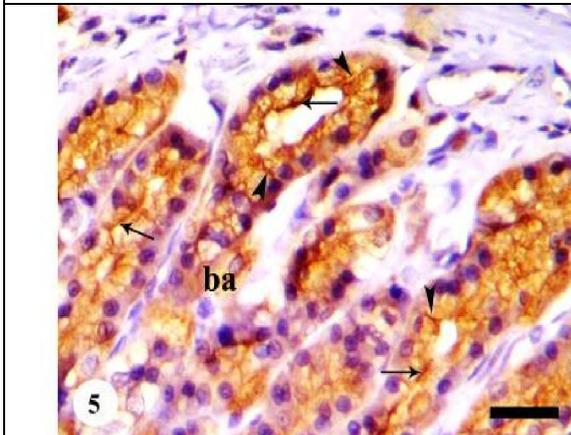


Fig. (5): Cross section of the gastric mucosa of a rat stressed for 30 days showing an obvious intense immunoreactivity to cytokeratin at the apical (arrows) and lateral (arrowheads) borders of cells of base (ba) region. (Immunostain, Bar = 6.25 μ m)

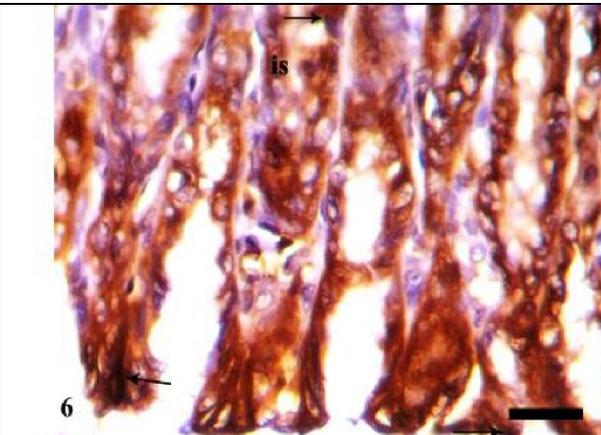


Fig. (6): Cross section of the gastric mucosa of a rat stressed for 30 days showing an obvious intense immunoreactivity to cytokeratin at the apical and lateral borders of the cells of the isthmus (is) region and surface mucous cells (arrows). (Immunostain, Bar = 6.25 μ m)

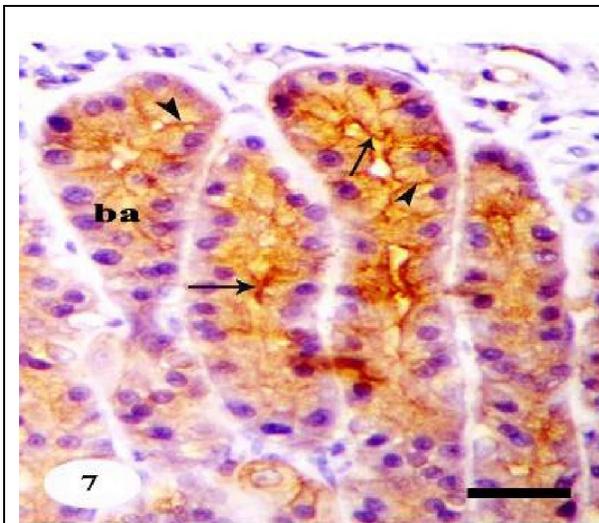


Fig. (7): Cross section of the gastric mucosa of a rat stressed for 30 days treated with diazepam for 30 days showing a marked decrease of immunoreactivity to cytokeratin at the apical (arrows) and lateral (arrow heads) borders of cells of base (ba) region. (Immunostain, Bar = 6.25 μ m)

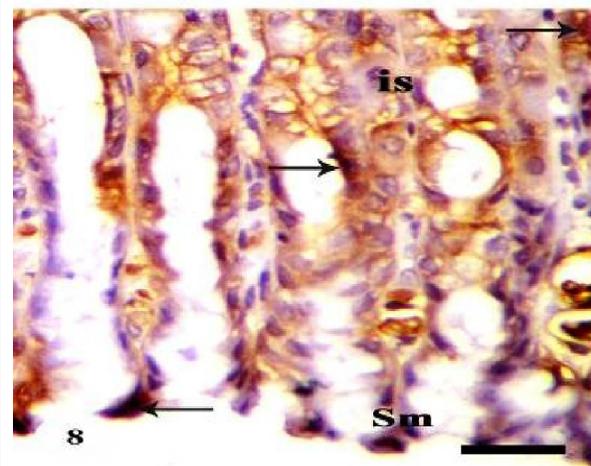


Fig. (8): Cross section of the gastric mucosa of a rat stressed for 30 days treated with diazepam for 30 days showing a marked decrease of immunoreactivity to cytokeratin in isthmus (is) region and surface mucous (Sm) cells (arrows). (Immunostain, Bar = 6.25 μ m)

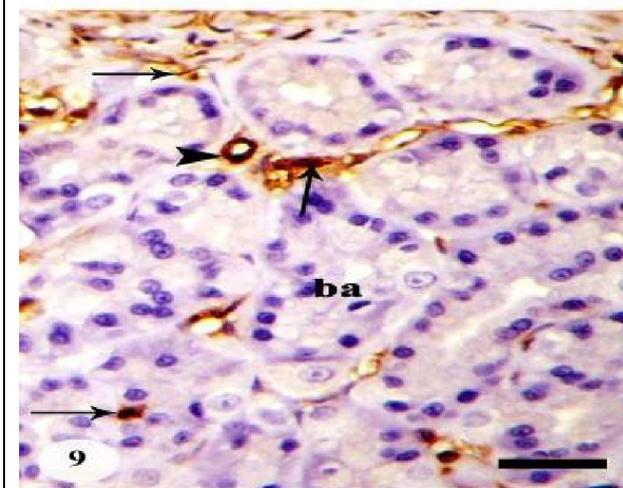


Fig. (9): Cross section of the gastric mucosa of a control rat showing the normal immunoreactivity to vimentin in the lamina propria of base (ba) region (arrows) and the blood vessel (arrowhead). (Immunostain, Bar = 6.25 μ m)

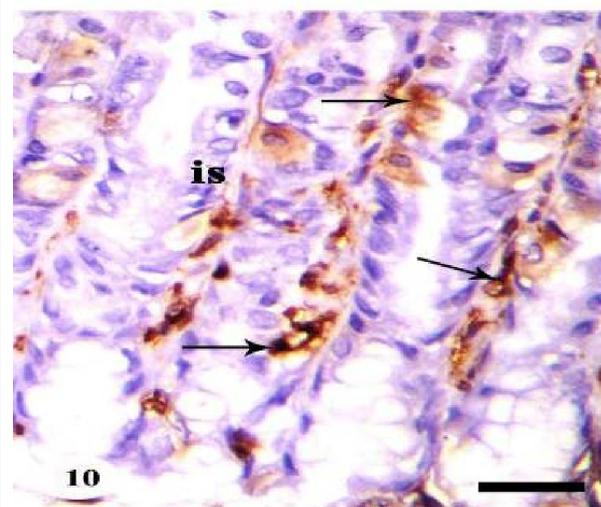


Fig. (10): Cross section of the gastric mucosa of a control rat showing the normal immunoreactivity to vimentin in the lamina propria of isthmus (is) region and in the blood vessels (arrows). (Immunostain, Bar = 6.25 μ m)

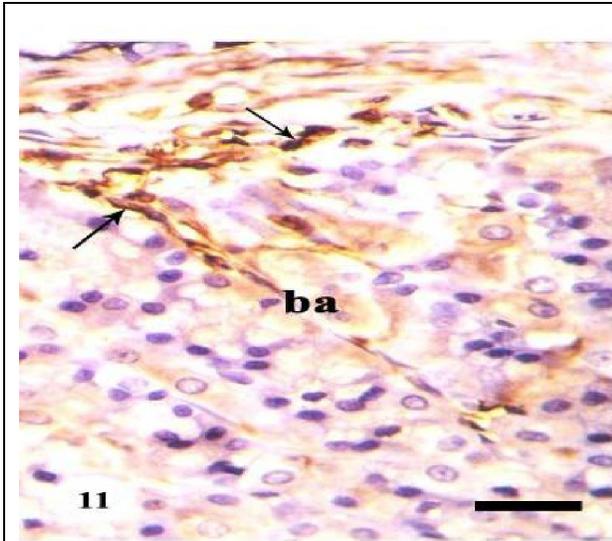


Fig. (11): Cross section of the gastric mucosa a rat treated with diazepam for 30 days showing approximately normal immunoreactivity to vimentin in lamina propria of base (ba) region and the blood vessels (arrows). (Immunostain, Bar = 6.25 μ m)

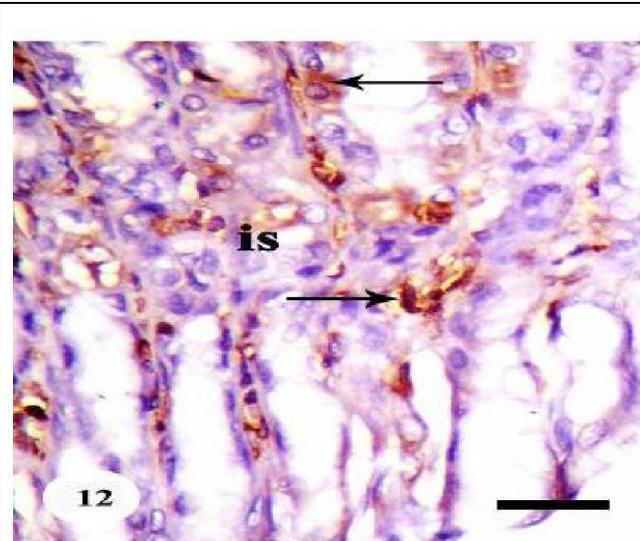


Fig. (12): Cross section of the gastric mucosa a rat treated with diazepam for 30 days showing the normal immunoreactivity to vimentin in isthmus (is) region and in the blood vessels (arrows). (Immunostain, Bar = 6.25 μ m)

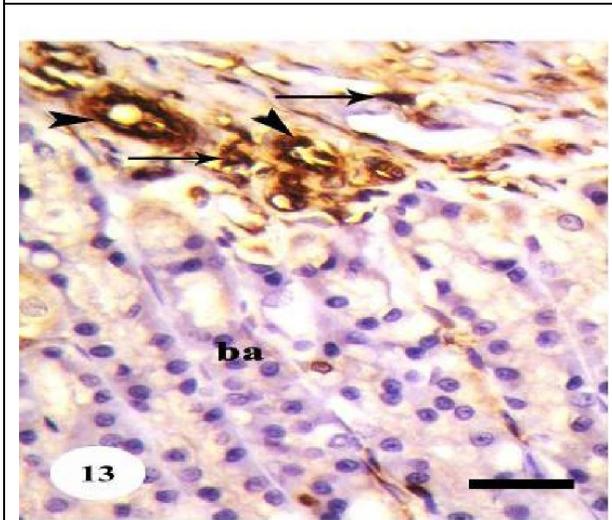


Fig. (13): Cross section of the gastric mucosa a rat stressed for 30 showing intense immunoreactivity to vimentin in the lamina propria of the base (ba) region (arrows) and in the blood vessels (arrowheads). (Immunostain, Bar = 6.25 μ m)

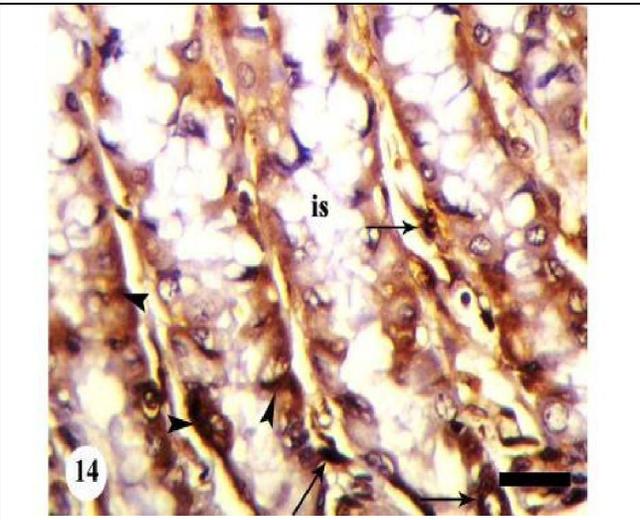


Fig. (14): Cross section of the gastric mucosa of a rat stressed for 30 days showing intense immunoreactivity to vimentin in the lamina propria (arrows) of the neck (ne) and isthmus (is) regions and in the blood vessels (arrowheads). (Immunostain, Bar = 6.25 μ m)

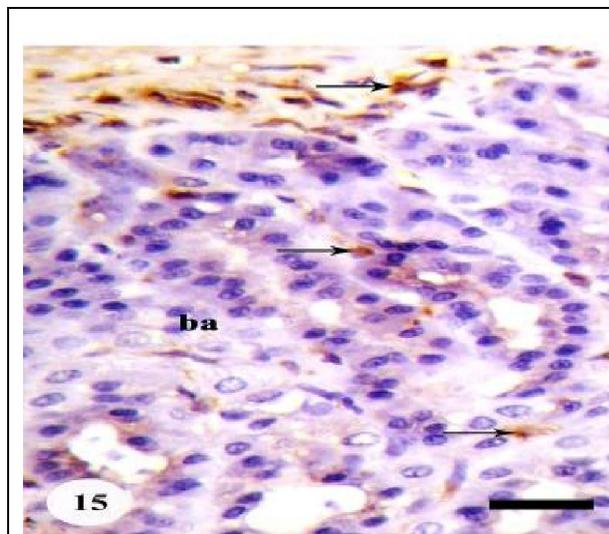


Fig. (15): Cross section of the gastric mucosa of a rat stressed for 30 days treated with diazepam for 30 days showing a marked decrease of immunoreactivity to vimentin in the lamina propria of the base (ba) region (arrows). (Immunostain, Bar = 6.25 μ m)

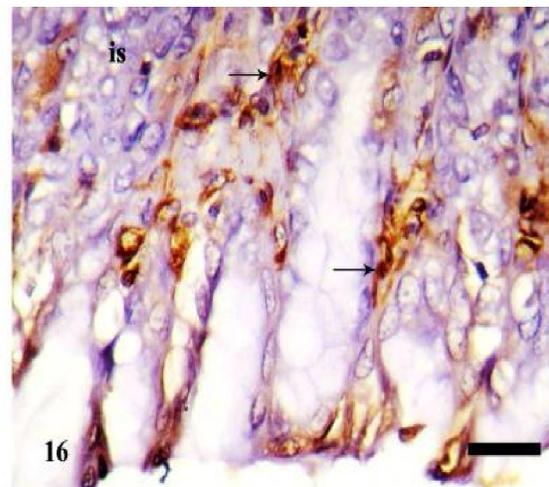


Fig. (16): Cross section of the gastric mucosa of a rat stressed for 30 days treated with diazepam for 30 days showing a marked decrease of immunoreactivity to vimentin in lamina propria of the isthmus (is) region and the blood vessels (arrows). (Immunostain, Bar = 6.25 μ m)

4. 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 is resulted 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, Gesi *et al.* (2001) reported a marked increase of 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-Fougia *et al.*, 2002). The length of stress period may alter neurological, behavioral, and biochemical parameters, possibly in different ways (Rai *et al.*, 2003).

In the current work, the cells of the gastric mucosa of the rats stressed for 30 days revealed

markedly intense cytokeratin filaments immunoreactivity at the apical part and lateral borders of the three types of gastric mucosal cells (mucous, parietal and chief cells) as compared to the control ones. Also, the vimentin filaments manifested an obvious intense immunoreactivity in the lamina propria of gastric mucosa and in the blood vessels during stress as compared to the control ones.

In accordance, Fuchs and Cleveland (1998) illustrated that when the stresses externally applied to the cell, the mutation of IFs were found. These mutations are associated with the weakness of IFs structural framework and increased the risk of cell rupture and cause a variety of human disorders. Moreover, Keratin, desmin and neurofilaments showed morphological changes under the effect of a forced, lateral movement of the atomic force microscopy tip. These changes revealed that the filaments are highly elastic and can be stretched at the expense of reduction in filament diameter (Kreplak *et al.*, 2005).

Also, the cytoskeletal filament protein structures showed alterations as they are actually significant targets in quinone-induced oxidative stress (Bellomo *et al.*, 1990). The increased free radicals in brain of rats affected and degraded the glial filament acidic proteins "GFAP, a type of cytoskeletal IFs" (Baydas *et al.*, 2002). Similarly, the domoic acid-inducer neurotoxicity in the brain of adult rats resulted in the overexpression of GFAP

immunoreactivity in the areas of severe neuronal degeneration (**Ananth et al., 2003**).

Furthermore, the cytokeratin expression pattern of the lung and its morphological alterations after rats exposed to X-irradiation for 6-months were seen, and was a time dependent relationship in terminal bronchial cells. Also, vimentin were focally present in bronchial epithelial cells and a typical type I and II pneumocytes as well as scattered epitheloid cell complexes were seen (**Kasper et al., 1993**). In addition, **Lundkvist et al. (2004)** demonstrated that vimentin plays an important role in maintaining the mechanical integrity of Müller-cell endfeet and the inner retinal layers of mice. Under the mechanical stress, local separation of the inner limiting membrane was apparent. Furthermore, the absence of vimentin in Müller cells leads to an abnormal response to the vascular system to ischemia, specifically decreased ability to newly formed blood vessels.

During compressive stress; the expression of vimentin and actin in the rat mandibular condylar chondrocytes was down regulated at first under stress then increased by feedback. It hinted that there were self-regulate mechanism in the cell response to mechanics stimulate (**Li et al., 2007**).

In pathology, IFs can serve as markers of the tissue origin of poorly differentiated tumors (keratins define epithelial tissues "at least 20 subclasses" (**Miettinen, 1993**). Cytokeratin or/and vimentin coexpression are found in endometrioid adenocarcinoma, ovarian serous adenocarcinoma, salivary gland carcinoma, renal cell carcinoma, spindle cell carcinoma and thyroid follicular carcinoma (**Chen, 2009**).

In the present study, the treatment of stressed-rats with diazepam for 30 days revealed a remarkable improvement and decrease of the cytokeratin and vimentin immunoreactivity in the gastric mucosa to normal appearance. In agreement, **El-Baely (2011)** found that the treatment of rats with diazepam at a therapeutic dose (0.1 mg/ kg b. w.) for 30 days improved the adrenal cytoskeletal IFs of rats stressed for 30 days, and restored the IFs (cytokeratin and vimentin) to normal expression in the adrenal glands (cortex and medulla) of albino rats. Similarly, diazepam administration (0.1 mg/ kg b. w.) for 30 days resulted in the amelioration of a changeable of desmin (IFs proteins) in the cardiac myofibrils of the immobilized-stressed albino rats (**El-Desouki et al., 2012**).

From the present study, diazepam protects the cytoskeletal intermediate filaments (vimentin and cytokeratin) of stomach from the damaging effects of immobilization stress.

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5/17/2013