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Immobilization Stress- Related alterations in Ultrastructure of Skeletal Muscle of Albino Rat and the Role of Diazepam.

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ABSTRACT

The current study is planned to study the effect of immobilization stress on the ultrastructure of skeletal muscles of adult male albino rats and the possible curative role of diazepam. The study was carried out on 40 male albino rats; the animals were divided into four groups: group I served as control rats; group II unimmobilized-stressed rats injected intraperitoneally daily with 0.1mg/kg b.w diazepam for 30 days; group III served as immobilized stressed-rats for 30 days (by restricting movement for 2 hrs daily for 30 days); group IV stressed rats treated daily with 0.1mg/kg b.w. diazepam for 30 days**.** By using TEM, rats stressed for 30 days showed marked distortion of the myofibrils of skeletal muscle; the transverse striations of many myofibrils appeared irregular, discontinued and disintegrated. Partial disappearance of light I band, discontinuous Z line and extension of dark A band were also seen. Either shrunken of elongated mitochondria with distorted cristae was seen. Also, dilatation of sarcoplasmic reticulum and reduction of glycogen were demonstrated. Nuclei were appeared swollen with irregular and indented chromatin condensation. After treatment of rat with diazepam at a dose of 0.1mg/kg b.w. for 30 days, the skeletal muscles exhibited an obvious improvement and restoration of their transverse striations of myofibrils almost similar to normal form. The results indicated that diazepam is recommended to be used as a curative drug to improve the disturbances in the skeletal myofibrils assembly caused under the effect of stress.

Keywords: Skeletal muscle – Stress – Diazepam - Rat –Ultrastrucure

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INTRODUCTION

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 [1].

The chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rats [2]. The authers showed that whereas acute stress often results in analgesia, chronic stress can trigger hyperalgesia/allodynia. This influence of long-term stress on nociception is relevant to numerous painful pathologies, such as fibromyalgia characterized by diffuse muscular pain (hyperalgesia) and/or tenderness (allodynia). Stress-induced anxiety and associated oxidative organ injury damage in rats were accompanied by increase of serum cortisol [3].

The histopathological studies of many organs under the influence of stress were demonstrated by many authors. The histological changes occurred in the kidneys during both acute and chronic stress rats subjected to immobilization for 2hrs (4). Also, histological changes in gastric mucosa and hyperactivity of parietal cells were seen post immobilization stress for 2hrs for different durations [5]. Moreover, ultrastructural abnormalities in the adrenal cortical zones appeared after restricted immobilization stress for 15 days [6]. Also, 4-weeks restraint stress down regulated some important calcium transporter mRNA expression in the duodenal epithelial cells of male rats [7].

Diazepam is one of the most representatives of the classical Benzodiazepines (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 [8]. 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 [9]. 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 [10].

The pre-treatment of acute stressed rats with diazepam reduced the enhancement of the corticosterone serum and the level of lipid peroxidation, decreased superoxide dismutase activity and improved the levels of mitochondrial function [11]. Oral administration of diazepam may be applied to help alleviate the stress-induced bone loss and osteoporosis by restoring intestinal calcium absorption to provide calcium for bone formation [7]. Previous studies also suggested a causal role for cortisol in mediating stress effects on risk taking [12,13].

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The aim of this work is to study the impact of immobilization stress on the cortisol level and ultrastrucure of the skeletal myocytes of male albino rats and the possible curative role of diazepam.

MATERIALS AND METHODS

Animals

Forty adult male albino rats, each weighing 100±5 g, were used in the present work. The animals were kept under the same natural environmental condition of temperature and photoperiod with free food and water. The present study was approved by the relevant ethics committee and Guidelines for the care and use of experimental animals.

Immobilization stress

Rats were exposed to stress for 2 hr daily between 10:00 and 12:00 a.m. The animals were individually placed in wire mesh restrainer (5×7×12 cm in dimension) as described by [14]. This procedure effectively restricted movement of the animals.

Treatment

Stressed rats were daily injected intraperitoneally for 30 days with the therapeutic dose of diazepam which is 0.1 mg /kg b.w. according to (15). Diazepam was received from Amoun Pharmaceutical Industries Co. Cairo, Egypt.

Experimental design

The rats were divided into four groups, 10 rats / each. Group I: rats received no treatment and served as control; group II: unstressed rats injected daily intraperitoneally with a dose of diazepam 0.1mg/kg b.w. for 30 days; group III: rats exposed to stress daily for 30 days; group IV: rats injected daily intraperitoneally with a diazepam dose of 0.1 mg/kg b.w. for 30 days after 30 days of applying the stress.

Methods

The blood sera were collected at 9-11 am at the day 5, 15 & 30 to measure the levels of cortisol. Serum cortisol was determined by using a radioimmunoassay kit (Biochemical, Costa Mesa, CA, USA) and the values were expressed as Ug cortisol /dl serum [16]. At the end of the experiment, at the day 30, the rats were sacrificed and small pieces (about 1mm) from the gastrocnemius muscles of all animal groups were dissected out then immediately fixed in 2.5% 0.1 M phosphate- buffered glutaraldehyde at 4C˚ for 2 hours for ultrastructural studies by TEM. The specimens rinsed in 0.1 M phosphate- buffered and post fixed in phosphate- buffered 1% osmium tetroxide in one hour at room temperature then dehydrated in ascending grades of

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ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. Semithin sections (1µm thick) were obtained and stained with toluidine blue, then examined with the light microscope. Ultrathin sections (60-90 nm) were cut and picked up on copper grides; the sections were double stained with uranyl acetate and lead citrate [17] and examined by JEOL transmission electron microscope (TEM) at 80 KV in Faculty of Medicine, Tanta University.

RESULTS

Effect of stress on cortisol hormone

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

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

Group	Control	Stressed rats		
		Stress for 5 days	Stress for 15 days	Stress for 30 days
Cortisol hormone Ug/dl	1.35	1.53	4.5	4.03

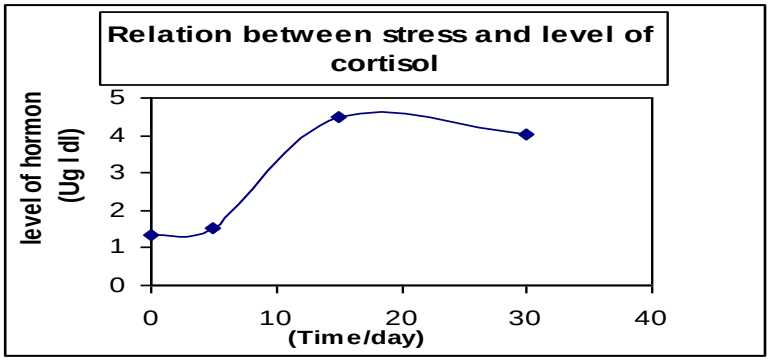

Fig A: The correlation between stress and level of cortisol

Ultrastructure results

The ultrastructure examination of the skeletal muscle of control rats reveals normal structure of skeletal myofibrils, each myofibril contains alternating thin light (I) band (actin filaments) and thicker dark (A) band (actin and myosin filaments). In relaxed fiber, the middle of the dark band is pale and contains only myosin filaments and called H zone. Condensation of myosin filaments in the middle of H zone forms M line. In the middle of I band there is Z line. Myofibrils are separated from each other by space that contained mitochondria and sarcoplasmic reticulum. Myonuclei are peripherally located in the myofibers below the

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sarcolemmae and show a pattern of condensed heterochromatin and euchromatin. The amount of cytoplasm in between myofibrils is minimal, and contains mitochondria or sarcosomes, and glycogen (Figs.1 & 2). Similar results were seen in unstressed-rats treated with diazepam for 30 days and displayed no changeable in the myofibrils (Fig. 3).

The immobilized stress- rats for 30 days showed marked distortion of the myofibrils. The transverse striations of the myofibrils appeared irregular and discontinued. Partial disappearance of light I band, discontinous Z line and extension of dark A band were seen. Myofibril nuclei appeared either swollen, irregular, indented or shrunken with marked chromatin condensation. Mitochondria were shrunken, elongated and irregular with distorted cristae compared to control structure. Glycogen contents were decreased (Figs.4- 6).

Treatment of immobilized stress- rats with diazepam for 30 days at a dose 0.1mg/kg b.w. elucidated partial recovery and improvement of most myofibrils. Normal light I and dark A bands, with irregular aligments of most of Z line, continuous myofibrils with normal spaces between them, normal euochromatic nucleus, recovery in most mitochondria and restoration of glycogen content were seen (Figs.7 & 8).

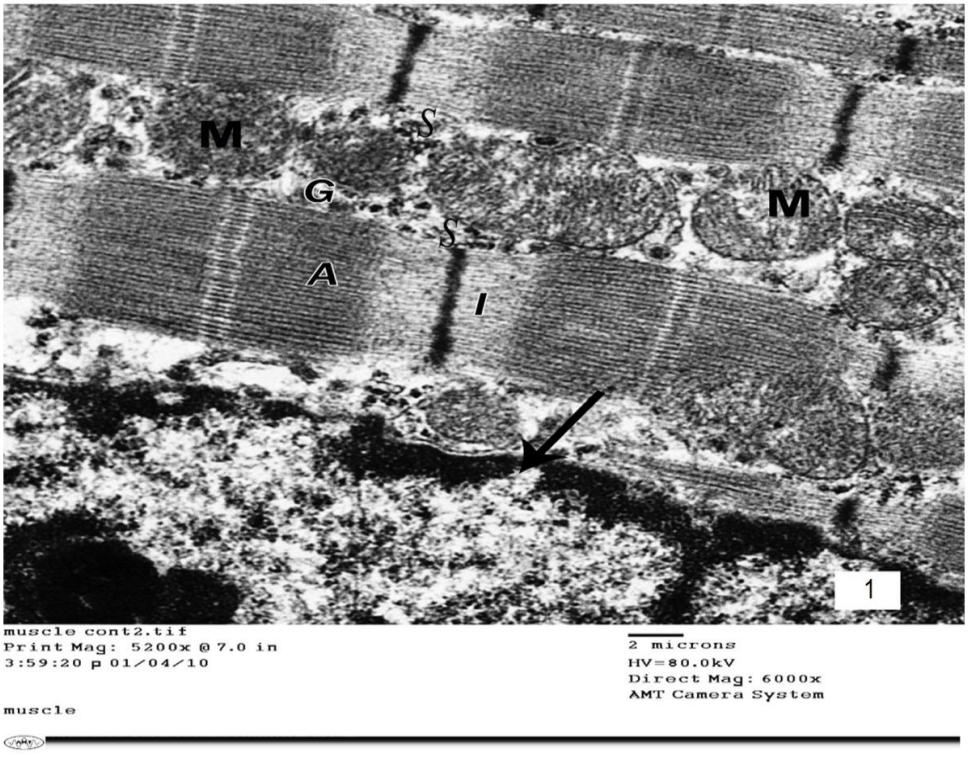

Fig. (1): Electron micrograph of L. S. of skeletal muscle of a control rat showing normal regular arranged of myofibril, with thin light (I) band and thick dark (A) band, each myofibril separated from each other by space that contains mitochondria (M), sarcoplasmic reticulum (S), glycogen (G), normal peripheral nucleus (arrow) with euchromatin and peripherally condensed heterochromatin. Bar = 2 µm

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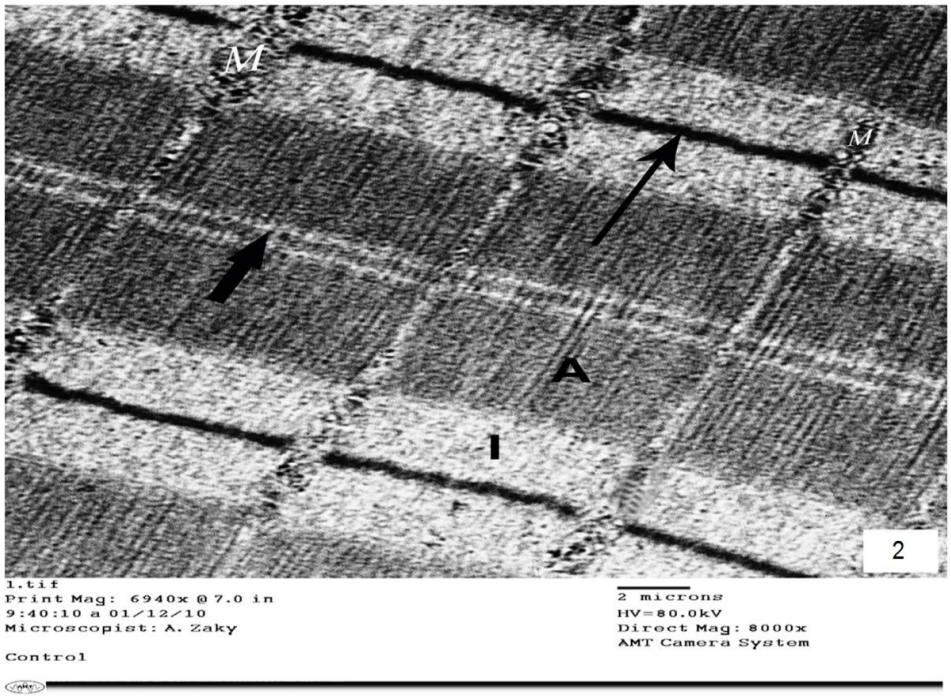

Fig. (2): High magnified part of an electron micrograph of L. S. of the skeletal muscle of a control rat showing normal pattern bands of myofibrils, each revealing alternating thin light (I) band, and thick dark (A) band in the middle of the dark band H zone is seen. Condensation of myosin filaments in the middle of H zone forms M line (thick arrow) and Z line (thin arrow) in the middle of I band are seen. See normal mitochondria (M) inbetween myofibrils. Bar = 2 µm

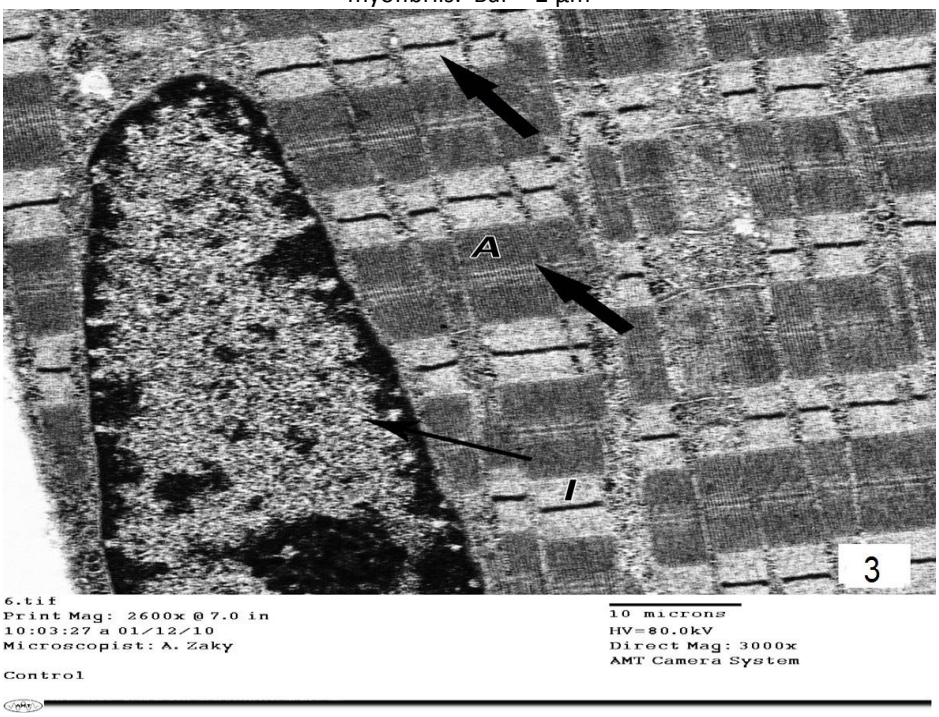

Fig. (3): Electron micrograph of L. S. of skeletal muscle of an unstressed rat treated with diazepam for 30 days showing normal continuous myofibrils (thick arrows) with normal light (I), dark (A) band and normal nucleus (thin arrow) with normal hetero and euchromatin. Bar = 10 µm

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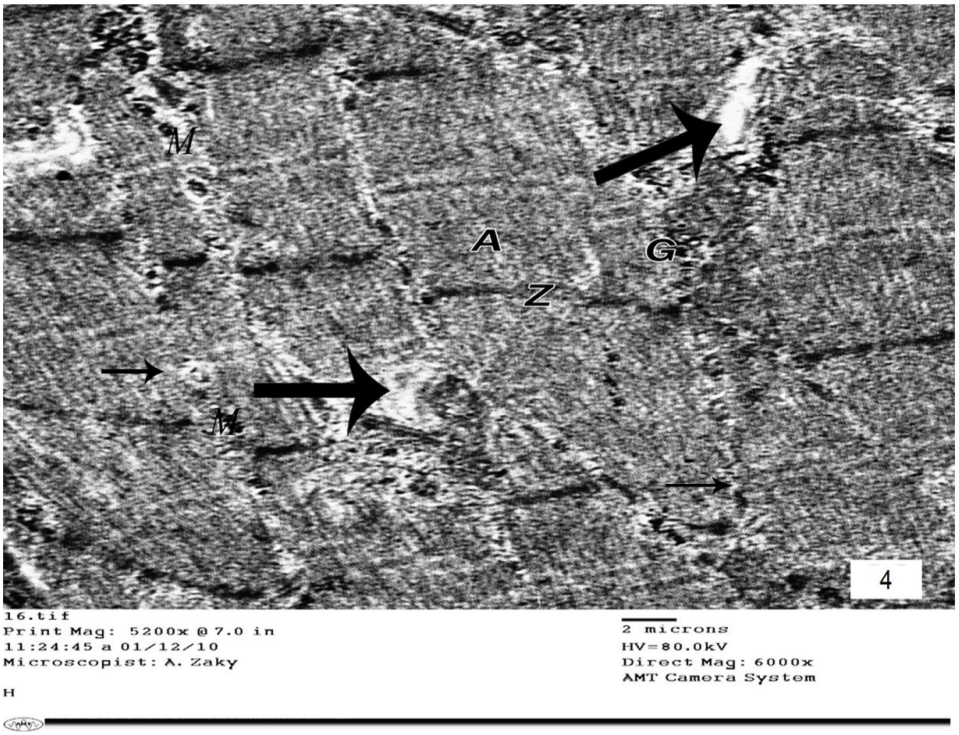

Fig. (4): Electron micrograph of L. S. of skeletal muscle of a rat stressed for 30 days showing distortion and disorganization of the myofibrils, partial reduction or disappearance of light band (actin filament), discontinuations and rupture of Z line and extension of dark A band. See partial disintegration of myofibrils (arrows), focal distortion of myofibrils (thick arrows) with obvious shrinkage of mitochondria (M) and reduction of glycogen (G). Bar = 2 µm

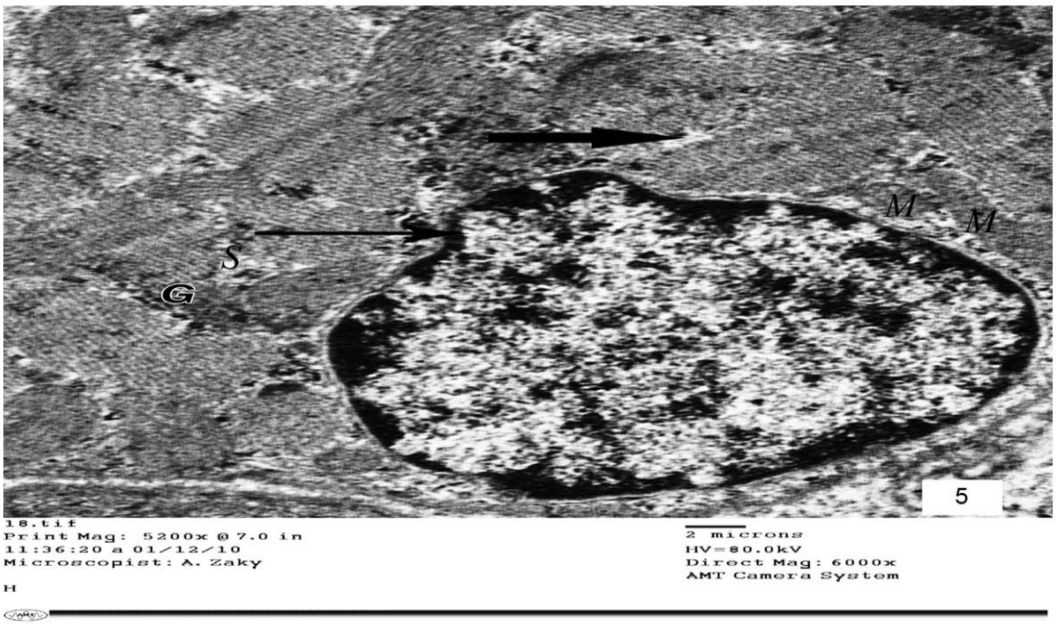

Fig. (5): Electron micrograph of L. S. skeletal muscle of a rat stressed for 30 days showing distortion of the myofibrils with completely disappearance of light I band (thick arrow), shrunken of mitochondria (M), dilatation of sarcoplasmic reticulum (S) and reduction of glycogen contents (G). Myofibril nucleus appears swollen and indented with marked chromatin condensation (thin arrow). Bar = 2 µm

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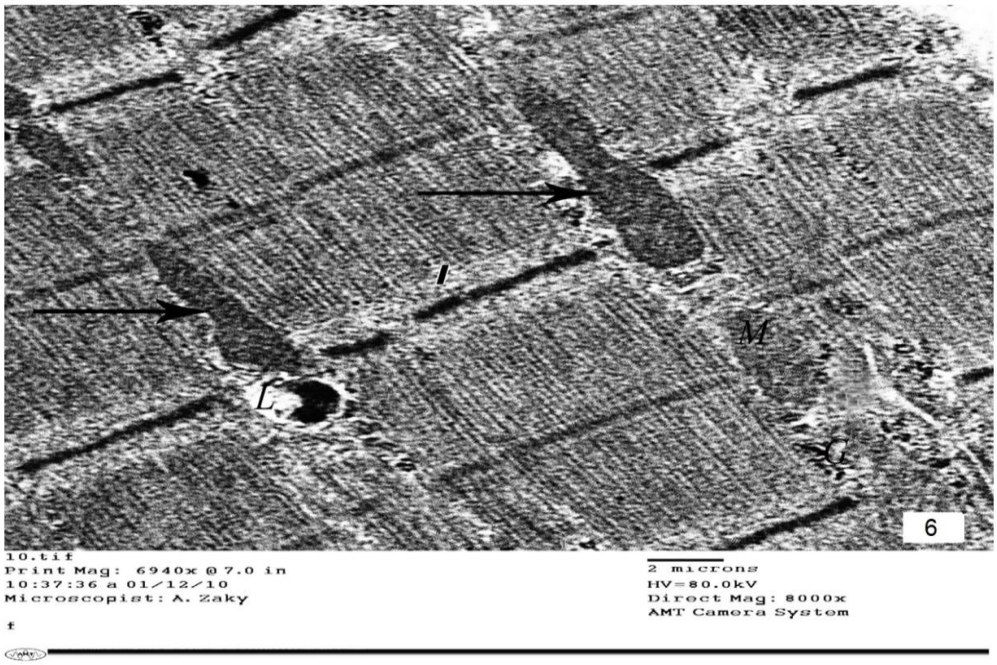

Fig. (6): Electron micrograph of another part of L. S. of skeletal muscle of a rat stressed for 30 days showing reduction of light I band, disintegration of mitochondria (M) and fusion of others (thin arrows), reduction of glycogen contents (G) and appearance of lysosome (L). Bar = 2 µm

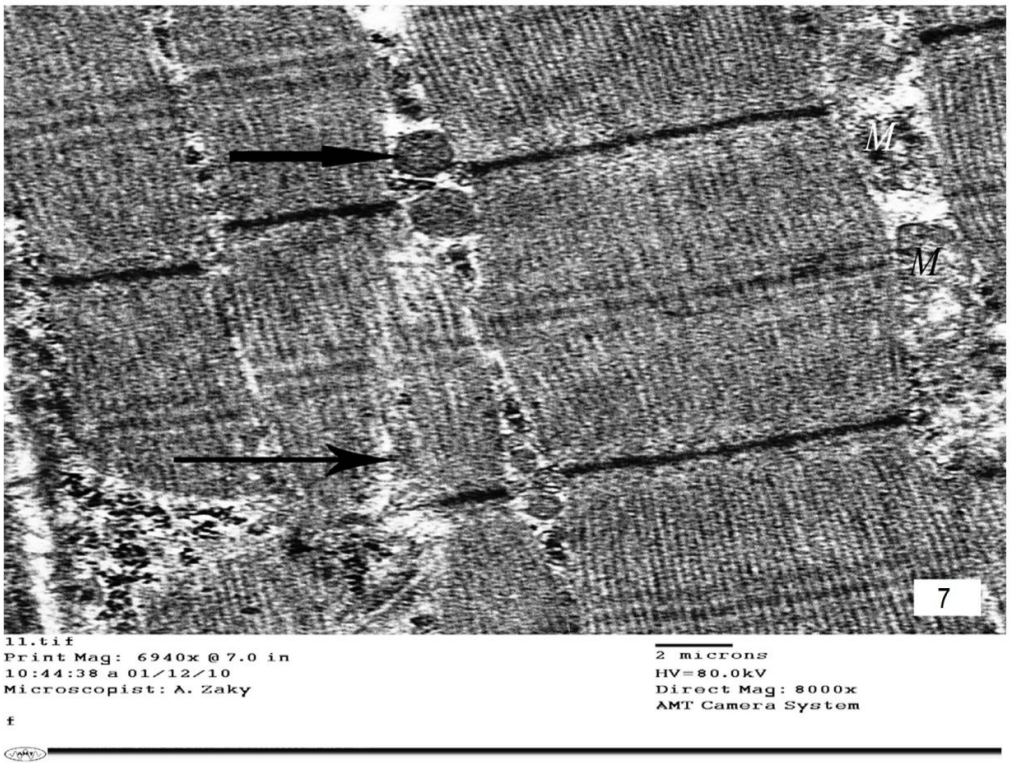

Fig. (7): Electron micrograph of other part of the skeletal muscle of a rat stressed for 30 days showing distortion of some myofibrils (thin arrow), disappearance of light I band, shrunken of mitochondria (thick arrow) and disintegration of others (M). Bar = 2µm

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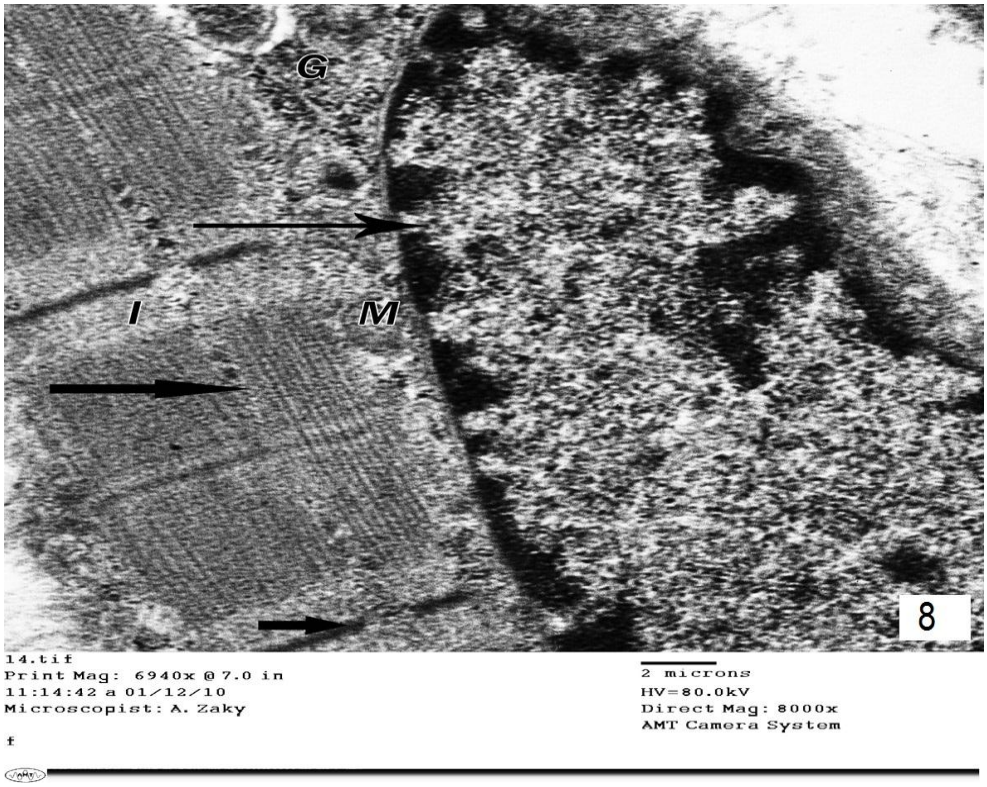

Fig. (8): Electron micrograph the skeletal muscle of a rat stressed for 30 days and treated with diazepam for 30 days showing intact myofibrils with normal I band with recovery of most of Z line (short arrow) and dark A band (thick arrow) and partial restoration of glycogen contents (G). Abnormal mitochondria (M) are still seen. An improvement of nucleus (thin arrow) is seen. Bar = 2 µm

DISCUSSION

Stress has been reported to influence motor activity, caused pain perception and in skeletal muscle [18]; anxiety [19]; depression- like behaviors [20]; effect on immune system [21], evoked histological and ultrastructural changes in the adrenal cortex [6] , gastric mucosa [5] and in cardiac muscle [22].

A familiar stress response in vertebrates is the activation of adrenocortical activity. Acute or chronic stress causes an elevation in corticosterone levels in mammals, hence increased adrenocortical activity is considered as an index of stress response in vertebrates [23]. Cortisol (a natural steroid hormone) is made by the adrenal gland in response to stress such as fasting for prolonged time [24], noise stress [25], high ambient temperature (26) and immobilization stress [6,22].

In the present study, the level of the cortisol hormone increased after exposure to different periods (5, 15 and 30 days) of immobilization stress and was time- dependent. In accordance [27] reported a marked increase cortisol levels in response to noise stressful stimulus that activated the hypothalamic-pituitary-adrenal axis leading to a release of adreno-corticotropic hormone (ACTH). The disturbances may vary by type, intensity, duration of a stressor and the strain / sex differentiation of the experimental subjects [28]. The length of

stress period may alter neurological, behavioral and biochemical parameters, possibly in different ways [29,30].

Moreover, the forced exercise stress induced different levels of plasma corticosterone which were negatively correlated with the amount of functional mitochondria in the plantaris muscle. Therefore, a chronic intermittent stress is able to induce an increase in plasma corticosterone which may be related to deleterious changes in muscle mitochondrial metabolism. Lastly, the acute stress was not associated with a decrease in functional mitochondria but with an increase in cytochrome-C-oxidase activity. This suggests that the relationship between corticosterone and muscle mitochondrial metabolism depends both on the level and duration of endogenous glucocorticoids exposure [31]. Immobilization stress-response related disorder is resulted in the hypersecretion of both ACTH and corticosterone to a subsequent stressor [32]. Also, the increment of cortisol levels in rats after exposure to high ambient temperature was recorded [26].

The present ultrastructural study on skeletal muscles of rat stressed for 30 days showed marked distortion, irregular and discontinued, of the myofibrils. The transverse striations of many myofibrils were disappeared as well as partial disappearance of light I band, discontinous Z line and extension of dark A band. Myofibrils nuclei appeared either swollen, irregular, indented or shrunken with marked chromatin condensation. Mitochondria were shrunken, elongated and irregularly with distorted cristae compared to control ones. Also, reduction of glycogen was seen. In accordance, the immobilization stress lead to abnormal mitochondria and sarcoplasmic changed in rabbit skeletal muscles [33]; and decreased the relaxation of smooth muscles and damaged cardiomyocytes ultrastructure as manifested by caryopyknosis, caryorrhexis, desintegration of myofibrils, vacuolization of mitochondria and endoplasmic reticulum [34].

Additioally, the immobilization stress significantly reduces the glycogen content in skeletal muscles [35]. The reduction of glycogen in muscle may due to the role of reactive oxygen species that causing damage to the mechanisms of carbohydrates synthesis in cells and might be due to inhibition of mitochondrial energy metabolization and the inability of cells to store glycogen and convert lactate and pyruvate to glycogen [36]. Oxidative stress may also induce many damaging processes in stress disorders such as disruption of energy pathways mitochondrial dysfunction and dysregulation of calcium homeostasis [37].

The present results were also in agreement with [38]**,** they demonstrated that exposed to heat stress (39°C for 2 hours daily) provoked structural changes in the myocardium such as loss of the characterstic striation, focal areas of necrotic fibers, vacuolated cytoplasm and mononuclear cell infiltration in the affected areas Similarly, the histopathological examination of the hearts of immobilization stressed rats showed large areas of leucocytic infiltration, marked myocytes vacuolation, undergoing apoptosis with small deeply stained nuclei and widely dilated and engorged blood vessels indicating injury of myocardium [22]. Chronic stress in humans had been correlated with increased risk for ischemic heart disease and those with ischemic heart disease are at higher risk to morbidityand mortality on exposure to stress [39].

Restraint stress produced numerous painful pathologies, such as fibromyalgia, characterized by diffuse skeletal muscular pain and/or tenderness. With following immobilization, the decrease in muscle power, which showed as muscle weakness, might depend not only on a quantitative mechanism (loss of mass), but also on qualitative mechanisms, i.e. loss of the intrinsic capacity of muscle fibres to develope force and, in aged muscle, a slowing of shortening velocity [40]**.** Moreover, psychological stress elevated markers of skeletal muscle atrophy and apoptosis and signaling pathways associated with muscle atrophy. Atrophy may be the result of increased catabolic factors, e.g. glucocorticoids or reduced influence of anabolic factors e.g. insulin [41]. Also, the acute restraint stress for 6 hrs caused severe anxiety like behavior, antinociception and impaired locomotor activity as compared with unstressed mice [42]**.** The histopathological changes were too induced in testis [43], adrenal gland under immobilization stress in male rats [6], in cardiomyocytes [22] and in intermediate filaments of stomach immunohistochemically [44].

The ultrastructure study of rat cardiomyocytes and DNA integrity under the effect of noise exposure was studied [45]**.** The exposure to loud noise for 12 hr caused a significant increase of DNA damage, accompanied by swelling of mitochondria. These alterations were concomitant with increased *in situ* noradrenaline levels and utilization. Genetic and ultrastructural alterations did not decrease 24 hr after the cessation of the stimulus. An elevated oxy-radical generation, possibly related to altered sympathetic innervation, is hypothesized as responsible for the induction and persistence of noise-induced cellular damage [45].

Findings of the present study illustrated that the diazepam could be improved the ultrastructure changes under the effect of immobilization stress in the rats skeletal muscles. The present results were in agreement with the previous studies reporting that benzodiazepines can reduce or suppress the effects induced by various stress stimuli. In fact, diazepam treatment has been found to reduce the ultrastructure alterations in atrial tissue [46], in brain, kidney, adrenals and heart induced by chronic mild stress in rats [47, 48], in ultrastructural changes induced by restraint stressed-rats in adrenal gland [6], and in gastric ultrastructure alteration [49]. Moreover, the stressed-rats treated with diazepam for different durations showed a significant restoration in glycogen and protein contents in cardiac myocytes histochemically [22] and obviously improved the disturbances in the cytoskeletal intermediate protein filaments of the rat stomach by using immunohistochemical markers [44].

Benzodiazepines are among the most commonly used groups of anxiolytic drugs in the world. They are indicated for treatment of generalized anxiety disorders, treatment of panic disorders with or without agoraphobia, sedation, light anesthesia and anterograde amnesia of perioperative events, control of seizures, and skeletal muscle relaxation [50]. 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 [51]. Both central and peripheral BDZ ligands are able to prevent the myocardial damage induced by noise exposure, the extent of this protection depending on the

specific drug used and the duration of stress exposure [52]. Antidepressants drugs have also been reported to elevated antioxidant enzyme defense system particularly superoxide enzyme and catalase activity. These antioxidant enzymes raised the level of oxidative defense against stress [42].

In conclusion, the immobilization stress induced ultrastructure changes in the skeletal muscles of albino rats. Diazepam treatment showed an obvious improvement in these alterations. So, it is recommended that stress should be avoided, and diazepam can be used as a curative drug for fine structure of skeletal muscles alteration induced by stress.

REFERENCES

[1] Gidron Y, Russ K, Tissarchondou H, Warner J. Biol Psychol 2006; 72(3):291-304.
[2] Bardin L, Malfetes N, Tancredi AN, Depoortère R. Behav Brain Res 2009;28: 205(2):360-366.
[3] Çakır B, Kasımay Ö, Kolgazi M, Ersoy Y, Ercan F, Yeğen BC. Cell Biochem Function 2010;28(6): 469–479.
[4] Vesna S, Nada V, Nenad B, Aleksandra D. Stress Health 2011;27(3): e195-e198.
[5] Gabry MS, El-Desouki NI, El-Refaiy AI, Ibrahim MA, Mohamed HN. Egypt J Exp Biol (Zool.) 2011;7(2): 153-161.
[6] El-Desouki NI, El-Refaiy AI, Abdel-Azeem H, El-Baely MA. J Egypt Ger Soc Zool 2011;62: 25-45.
[7] Charoenphandhu N, Teerapornpuntakit J, Lapmanee S, Krishnamra N, Charoenphandhu J Mole Cell Biochem 2012;369(1-2):87-94.
[8] Inada T, Nozki S, Inagaki A, Furukawa T A. Hum J Psychopharmacol Clin Exp 2003;18: 483 – 487.
[9] Engel J, Solomon M, Jean A. 2007. Epilepsy A Comprehensive Textbook. 2nd edn. Vol. 2. Lippincott Williams & Wilkins. Philadelphia, 1433-1466.
[10] Bribes E, Carrière D, Goubet C, Galiègue S, Casellas P, Simony-Lafontaine J. J Histochem Cytochem 2004;52(1): 19-28.
[11] Méndez- Cuesta LA, et al. Basic Clin Pharmacol Toxicol 2011;109 (5): 350-356.
[12] Pabst S, Brand M, Wolf OT. Behav Brain Res 2013;250:39–45.
[13] Buckert M, Schwieren C, Kudielka BM, Fiebach CJ. Front Neurosci 2014;8(82):1-11.
[14] Soliman AA. Egypt J Histol 2006;29(2):259-268.
[15] Paget GE, Barnes, JM. 1964: Evaluation of Drug Activites, 2 nd edn. Laurence and Bacharach, Academic Press, New York.
[16] Yvonne M U, Helmer F F, Michelle M O, Dennis C C, William C E, James P H. Am J Physiol Endocrinol Metab 2006;291:965- 973.
[17] Reynolds ES. J Cell Biol 1963;17: 208-212.
[18] Abdel Baky NA, Ali AA. Inter J Acad Res 2009;1(1): 59.
[19] Metz GA, Jadavji NM, Smith LK. Eur J Neurosci 2005;22:1190-1200.
[20] Sevgi S, Ozek M, Eroglu L. Methods Find Exp Clin Pharmacol 2006;28(2): 95-99
[21] Brehe J, Way AL. Adv Physiol Educ 2008;32: 157-160.

[22] El-Desouki NI, El-Refaiy AI, Afifi DF, Abdel-Kader AA. Egypt J Exp Biol (Zool.) 2012;8(2): 273- 285.
[23] Nirupama R, Devaki M, Yajurvedi HN. J Stress Physiol Biochemi 2010;6(3): 44-55.
[24] Beszczynska B. Acta Biol Cracoviensia Zool 2005; 47: 53-57.
[25] Swami CG, Ramanathan J, Charan- Jeganath C. Malaysian J Med Sci 2007;14(1): 28-35.
[26] Mazroa SA, Asker SA. Egypt J Histol 2010; 33(1): 23-31.
[27] Gesi M, Fornai F, Lenzi P, Natale G, Soldani P, Paparelli A. Eur J Morphol 2001;39:129-135.
[28] Kioukia-Fougia N, Antoniou K Bekrs S, Liapi C, Christofidis I, Papadopoulou- Dafoti Z. Prog Neuropharmacol Biol Psych 2002;26: 823-830.
[29] Rai J, Pandey SN, Srivastava RK. J Anat Soc India 2003;52(1): 55-57.
[30] Weiss SJ. Perspect Psychiatr Care 2007;43(3):114–122.
[31] Duclos M, Martin C, Malgat M, Mazat JPF, Mormède P, Letellier T. Eur J Physiol 2001; 443(2): 218-226.
[32] Armario A, Escorihuela RM, Nadal R. Neurosci. Biobehav Rev 2008;32(6): 1121- 1135.
[33] Leivo I, Kauhanen S, Michelsson JE. APMIS 1998;106: 1113-1123.
[34] Kuoleikaite M, Kirkutis A, Razbadauskas A, Rugevieiene O, Kusleika S. ACTA Med lituanica 2004;11(1): 26–30.
[35] Bosi PL, Borges GD, Durigan JLQ, Cancelliero MK, Da Silva CA. Braz Arch Biol Technol 2008;51(2): 295-301.
[36] Brown R, Mcburney A, Lunec J, Kely F. J Free Rad Biol Med 2005;18(1): 801-806.
[37] Amoroso S, Torrtiglione A, Secondo A, Catalano A, Montagnani S, Di Renzo G, Annunziato L. J Neurochem 2000;74(4):1505-1513.
[38] Mansour AM, Shady MA, Kefafy AM, Hamaad S. Egypt J Histol 2008;31(2): 220 – 232.
[39] Schwartz BG, Mayeda GS, Burstein S, Economides C, Kloner RA. Hosp Pract (Minneap) 2010;38(5):144-152.
[40] Antona GD, Pellegrino MA, Adami R, Rossi R, Carlizzi CN, Canepari M, Saltin B, Bottinelli R. J Physiol 2003;10:1113.
[41] Engelbrecht AM, Smith C, Neethling I, Thomas M, Ellis B, Mattheyse M, Myburgh KH. Stress 2010;13 (2): 132–141.
[42] Kumar A, Garg R, Prakash KA. BMC Complement Altern Med 2010;10-18.
[43] El-Refaiy AI. J Egypt Ger Soc Zool 2010;60(C): 1-22.
[44] El – Desouki NI, El-Refaiy AI, Gabry MS, Ibrahim MA, Nagi HM. Life Sci J 2013;10 (2): 2211-2219.
[45] Lenzi P, Frenzilli G, Gesi M, Ferrucci M, Lazzeri G, Fornai F, Nigro M. Environ Health Perspect 2003;111(4): 467–471.
[46] Pellegrini A, Soldani P, Gesi M, Lenzi P, Paparelli A. J Submicrosc Cytol Pathol 1996;28(4): 507-512.
[47] Nirmal J, Saravana BC, Harisudhan T, Ramanathan M. BMC Complement Altern Med 2008;8: 15.
[48] Abdel Baky NA, Ali AA. Inter J Acad Res 2009;1(1):59.
[49] Nagi H. 2012. Biological studies on the effect of stress on the digestive system of the adult male albino rat and the possible curative role of diazepam. M. Sc. Thesis. Helwan University. Helwan. Egypt.

[50] Iqbal MM, Sobhan T, Ryals T. Psychiatr Serv 2002;53: 39 – 49.
[51] Gehlot A, Godhwani JL, Godhwani S, Aseri ML, Jain P, Vyas MC. Indian J Pharmacol 1997;29(3): 187-189.
[52] Gesi M, Riva A, Soldani P, Fornai F, Natale G, Lenzi P, Pellegrin A, Paparelli A. J Anat Res 1999;255(3): 334-341.