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Electron Microscopic Studies on the Effect of the Diazepam on Mouse Sartorius Muscle

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Abstract: The effect of the high therapeutic doses of diazepam on the histology and ultrastructure of the mice muscular tissue was investigated. Diazepam administration caused obvious histological and ultrastructural alterations in sartorius muscle fibers; distortion of their normal architecture was clearly observed. The sartorius muscle showed obvious enlargement of the nucleus, small and degenerated myofibrils, small, few and disorganized mitochondria, decrease of glycogen granules increase of fat vacuoles and destruction and dilation of SR, large interfibrillar spaces. Moreover, sartorius muscle revealed increase and vacuolation of connective tissue between the muscle fibers. Connective tissue contained dilated and congested blood capillaries, macrophages and many fibroblasts. In addition, disorganized myoneural junctions were noticed. Empty and dilated axons were noticed at the myoneural junction.

Key words: Diazepam, muscle, electron microscopy, myoneural junction, mice

INTRODUCTION

Benzodiazepines are medications that are frequently prescribed for the symptomatic treatment of anxiety and insomnia (sleep disorders). Benzodiazepines are also widely prescribed for other reasons such as muscle spasticity, convulsive, disorders presurgical sedation, involuntary movement disorders, detoxification of alcohol and other substances and anxiety associated with cardiovascular or gastrointestinal conditions. Diazepam dosage are, anxiety states: 3 mg, muscle spams and insomnia: 15 mg, epilepsy: 60 mg daily. Women are more likely than men to take sleeping pills (benzodiazepines), their use increases with age (Salzman, 1997). According to the some reports on benzodiazepines, 11 to 15% of the adult population has taken a benzodiazepine one or more times during the preceding years (Curran, 1992; Buston *et al*., 1998). Benzodiazepines receptors are ubiquitous throughout the CNS and are linked predominantly to gamma-aminobutyric acid (GABA) receptors, the neurotransmitter GABA the most prominent inhibitory neurotransmitter in the CNS. Diazepam and its active metabolites interact with the benzodiazepine receptors and subsequently increase the frequency of GABA-gated chloride ion channel openings. This facilitates the pre- and post-synaptic inhibitory action of GABA resulting in CNS depression (Barbone *et al*., 1998). Many of the common adverse effects of drugs in the class of sedative-hypnotics are those resulting from dose-related depression of the CNS functions (Trevor and Way, 1995). Use in elderly is associated with an increased rate of falling that cause hip and femur fractures (Herings *et al*., 1995; Hemmelgam *et al*., 1997). These medications may be fatal when used in combination with alcohol and other drugs that depress the CNS (Ciraulo *et al*., 1988; Ashton, 1995). Stopping abruptly can bring on such symptoms as trouble sleeping gastrointestinal upset, feeling unwell, loss of appetite, sweating, trembling, weakness, anxiety changes in perception (Borbone *et al*., 1998). The onset and severity of withdrawal are often more marked from benzodiazepine that are rapidly eliminated from the body (e.g., alprazolam) than from those that are slowly eliminated (e.g., diazepam). High doses lead to heavier sedation and can impair both mental sharpness and physical coordination (Curran, 1992). Insomnia is a common sequela of numerous medical and psychiatric conditions and is often associated with substance use disorder. If benzodiazepine used regularly (for example-every day) for insomnia they usually are not effective for more than a few weeks. Long-term use can be proplematic due to the development of tolerance and dependency (Ciraulo *et al*., 1988; Buston *et al*., 1998). Diazepam are metabolized and eliminated from the body quite slowly, the medication can accumulate in body tissue with long-term use (Mellinger *et al*., 1984; Mamiott and Tyrer, 1993). Data suggest that highly lipophilic benzodiazepines (for example, those that cross the blood-brain barrier more rapidly) such as diazepam are the most reinforcing benzodiazepines and therefore the ones most likely to be associated with abuse (Roache and

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Meisch, 1995). Although benzodiazepines are effective in a wide range of medical and psychiatric condition, caution must be exercised with their use.

Skeletal muscle have a wide variety of morphological forms and mode of action, nevertheless all have the same basic structure. Skeletal muscle undergoes profound rapid changes in the composition of contractile, regulatory and energy yield systems during growth, malnutrition and starvation (Goldspink and Ward, 1979; Yousif and Sorour, 1992), in pathological conditions (Stevens and Lowe, 1993) and under the effect of some agents (Muoio *et al*., 1999). The masses of fibers that make up the different types of muscle are not grouped in random fashion but are arranged in regular bundles surrounded by the epimysium, an external sheath of dense connective tissue. The connective tissue around each bundle of muscle fibers is the perimysium. Each muscle fiber is itself surrounded by a delicate layer of connective tissue, the endomysium composed mainly of a basal lamina, fibroblast and collagen fibers. One of the most important roles of the connective tissue is that of mechanical transmission of the forces generated by contracting muscle cells, since in most instances, individual muscle cells do not extend from one end of a muscle to the other, but are arranged in overlapping bundles, the force of contraction being transmitted through the arrangement of the support tissue (Junqueira *et al*., 1995; Stevens and Lowe, 1993). Connective tissue has been noted to increase in dystrophic muscle (Comfort, 1979), denervated muscle (Tomanck and Lund, 1973) and immobilized muscle (Williams and Goldspink, 1981). Skeletal muscle fibers are syncytial cells, nuclei, mitochondria and glycogen granules are displaced to the cell periphery by myofibrils (Johnson, 1992). Innervation and control by specialized neurons (motor neuron), which terminate on muscle cells at specialized nerve endings, myoneural junctions (motor end plate): muscle cells have scattered surface invaginations called primary synaptic cleft; myocyte cytoplasm near the myoneural junction contains an abundance of mitochondria, SR. Muscle characterized by rich blood supply reflect their high metabolic demands (Stevens and Lowe, 1993; Johnson, 1992). Generally speaking, histopathology findings in muscle can be divided into two broad categories myopathy or neurogenic. Indulging in a useful oversimplification, diseases detectable by microscopic examination can be due to interruption of signal from the CNS, as when a motor nerve is severed by trauma, or to problem intrinsic to muscle fibers as when there loss of the normal myofibrillar protein dystrophin, while other patients are recognized, it is helpful to first attempt to categorize muscle findings as myopathic or neurogenic (Barbona, 1996).

In the recent years, many drugs (e.g., benzodiazepine) prescribed in low and high therapeutic doses for treatment of different pathologic conditions. The present work, an attempt was made to get some information about the muscle ultrastructural changes when the animal treated with high therapeutic doses of one of the common anxiolytic drug, diazepam and a possible dangerous of the drug on the function of the muscle. Moreover, the available literature indicated that very rare non specific studies have been done concerning such subject.

MATERIALS AND METHODS

Female mice aged 2-3 months and weighing 20-25 mg were used in this study. The high therapeutic dose of diazepam were used in the present study. Farcozepam tablets containing 2 mg of diazepam were uniformly suspended in distilled water. The animals were divided into two groups. The first group served as a control group and received no treatment, while animals of the other group were orally given via a bent stainless steel feeding tube daily high therapeutic doses of 0.02 mg kg^{-1} body weight for a month. The daily dose was in equally divided and spaced amounts, given every 12 h.

Small slices of the sartorius muscles of control and the experimental groups immediately fixed in 2% glutraldehyde buffered with phosphate buffer (pH 7.6) for 1 h and post-fixed in 2% osmium tetraoxide (in the same buffer) for 1-2 h at 4°C, dehydrated through graded series of ethyl alcohol and embedded in Araldite-Epon mixture. Semithin (1 um) and ultrathin (50 nm) sections for selected areas were cut on LKB ultramicrotome. Semithin sections were stained with toluidine blue (TB) and examined with light microscopy, while ultrathin sections were double stained with uranyl acetate and lead citrate and examined by Jeol 100 CX Electron Microscope.

RESULTS

The treated animals become less active and drowsy. There is inhibition in the locomotory activities of the animals. An increase in food intake is noticed. Large amount of fatty tissue is observed in the viscera.

Control sartorius muscle fibers: Sartorius muscle is composed of elongated muscle fibers. Individual muscle fibers are large and vary slightly in diameter and grouped together into elongated bundles (fasciculi) with delicate supporting tissue (endomysium) occupy the spaces between individual muscle fibers (Fig. 1 and 2). Endomysium is composed of sheets of external lamina identical to the basement membrane (Fig. 3). Endomysium anchors the muscle fibers to each other and contains both

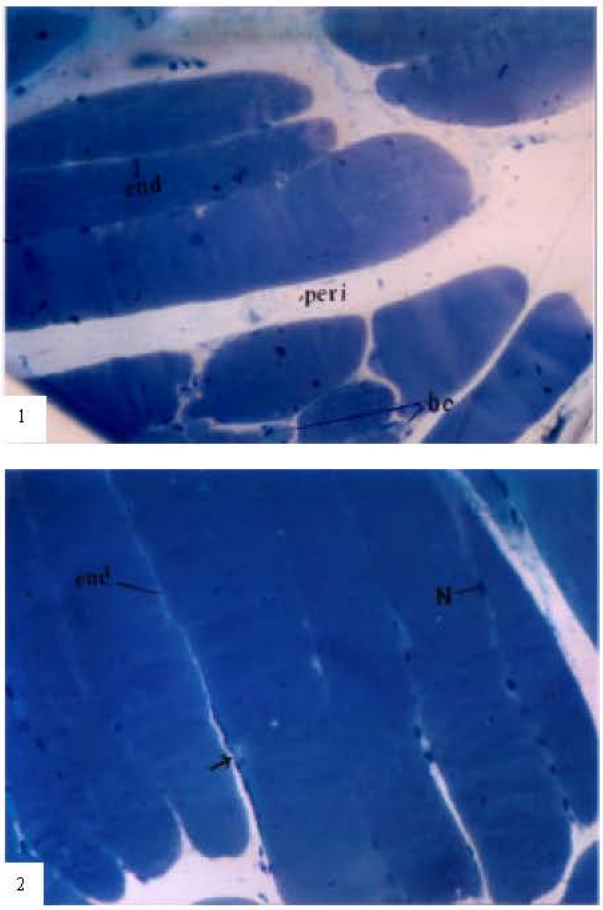

Fig. 1, 2: Photomicrograph of control mouse sartorius muscle fibers showing large muscle fibers, nucleus (N), endmysium (end), perimysium (peri) and myoneural junction (arrow). (x 400)

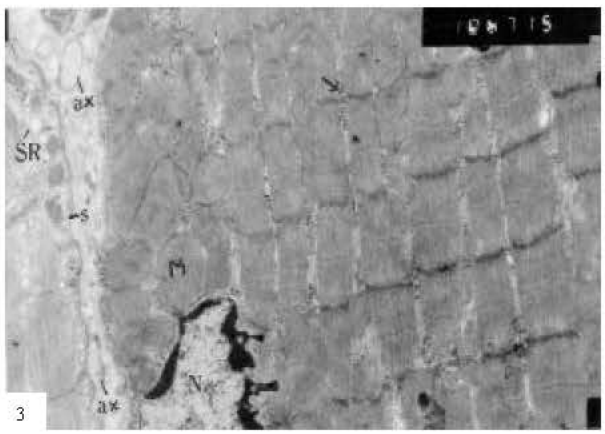

Fig. 3: Electron micrograph of control mouse sartorius muscle fibers showing nucleus (N) with distinct irregular nuclear membrane, nuclear pores (head arrow), peripheral heterochromatin, mitochondria (M), glycogen (arrow), SR (numerous at the myoneural junctions), well developed sarcolemma (s), few sarcoplasm inbetween regular myofbrils, individual nerve axons (ax) at the level of the Z lines. (x 10.000)

Fig. 4: Electron micrograph of control mouse sartorius muscle fibers showing SR, A,I bands, Z lines, t-tubules & trials (head arrow), myoneural junction at the Z line (arrow), mitochondria (M). (x 13.000)

capillaries and individual nerve axons (Fig. 2-4). The sarcolemma of control muscle fiber is well defined and is not straight throughout its length but with slight indentation in the region of the Z line (Fig. 3 and 4). In electron micrograph, the muscle fibers showed different degree of contraction, i.e., the length of the A and I bands differ in different muscle fibers (Fig. 3 and 4). Individual axons containing synaptic vesicles are clearly noticed in the endomysium and appeared innervated the muscle fibers at the myoneural junction at the region of the Z line (Fig. 3 and 4). In the sarcoplasm surrounding the synapsing axon however the tubules of the Sarcoplasmic Reticulum (SR) ramify profusely thus providing a great membranous surface than in non synaptic region (Fig. 3 and 4). In general, the nucleus in the control muscle fibers appeared large, ovoid but often with an irregular outline, nuclear envelope and nuclear pores, nucleolus are seen (Fig. 3). The peripheral large aggregates of the heterochromatin surrounding the electron light euchromatin are present, small particles of various sizes and density are scattered throughout the nuclear interior (Fig. 3). The sarcoplasm is distributed in a small quantities between the fibrils and in greater amounts in peripheral and perinuclear regions of the muscle fibers (Fig. 3 and 4). The myofilaments are very distinct in the A band, but thinner filaments in the I band are resolved with difficulty (Fig. 4). The sarcoplasmic reticulum appears as an elaborate network of tubular and vesicular elements existion as a structural component of the interfibrillar sarcoplasm. Usually the tubules are oriented along the longitudinal axis of the fiber. However, transverse orientation of tubules (t-tubules) can be seen and trials are clearly noticed at junction between the A and I bands (Fig. 3 and 4). Sartorius muscle fibers contain numerous mitochondria of different sizes shapes and quantities are interposed between the myofibrils. Although the mitochondria commonly aligned in rows in interfibrillar clefts their arrangement in some muscle fibers take the form of frequent pairs symmetrically disposed in relation to the Z line, these organelles are concentrated in groups immediately beneath the sarcolemma and often aggregated about the nucleus (Fig. 3 and 4), mitochondria contain slightly to moderately electron dense matrix. In the control muscle fibers, abundant glycogen granules are found restricted to the thin layer of sarcoplasm that separate the myofibrils and are more concentrated at the I band. The region of the sarcoplasm at the pole of the nucleus and beneath the sarcolemam are crowded with glycogen (Fig. 3). Fat vacuoles are few in the sarcoplasm of the control muscle fibers. They are rounded and oval vacuoles limited with an indistinct membrane.

Treated sartorius muscle fibers: As demonstrated by light microscopy, muscle fibers of treated animals appeared small, relaxed, vary in diameter, have obviously enlarged nucleus, degenerated muscle fibers and an increase in the connective tissue (Fig. 5-7). Many muscle fibers ended (terminated) by a pointed end (Fig. 5-7). Sartorius muscle fibers of treated animals could be distinguished by wide extracellular space (Fig. 5-10) occupied by connective tissue contain fibroblast macrophages, dilated and congested blood capillaries. Vacuolation in the connective tissue has been observed (Fig. 5-7, 9 and 10). Nuclei increase greatly in size and contain more peripheral heterochromatin aggregations (Fig. 8 and 9). The nucleoli increase in size and become highly electron dense. Chromatin particles of various densities are scattered throughout the nuclear interior

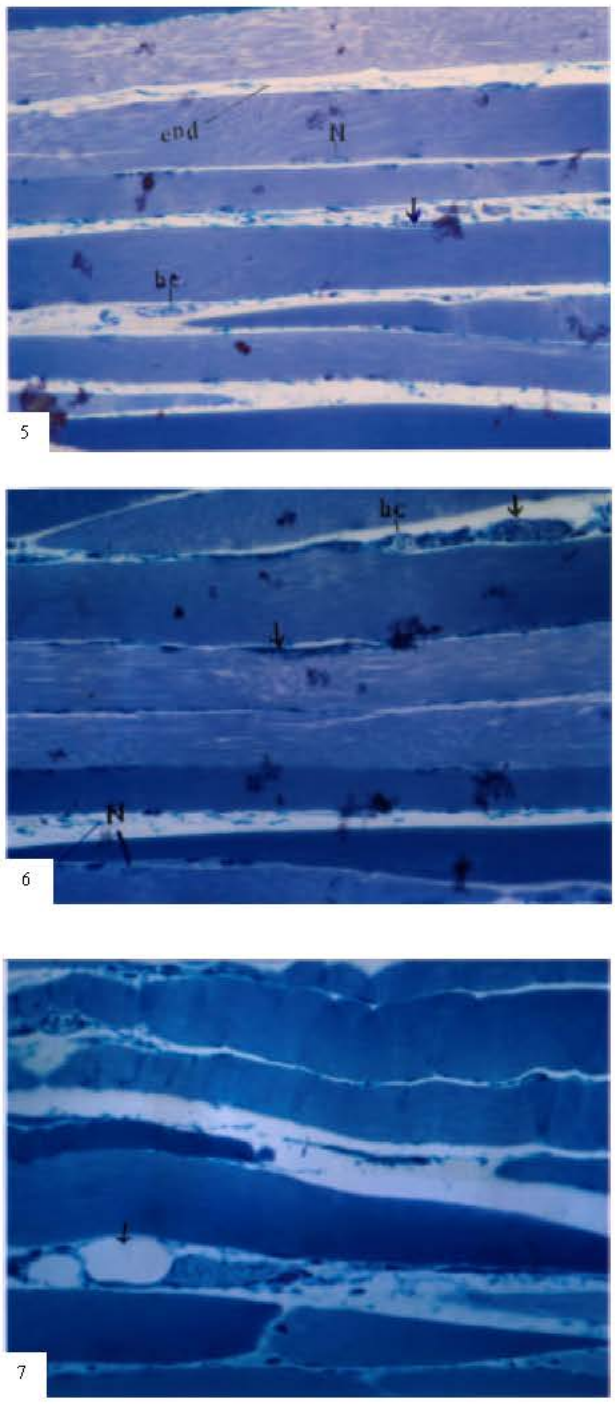

Fig. 5-7: Photomicrograph of treated mouse sartorius muscle fibers showing small, relaxed muscle fibers, some are degenerated (head arrow), others with pointed end nucleus (N), increased and vacuolated endmysium (end), congested blood capillaries (bc), dilated nerve axons (arrow). (x 400)

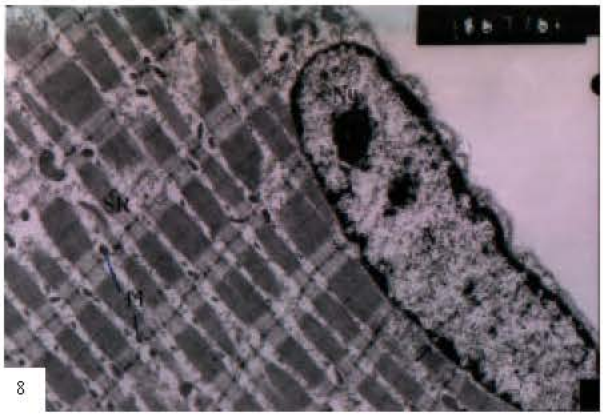

Fig. 8: Electron micrograph of treated mouse sartorius muscle fibers showing large nucleus (N) with distinct nuclear membrane, nuclear pores (arrow), peripheral heterochromatin, dense nucleolus (Nu), few mitochondria (M), dilated SR, relaxed A and I bands, degenerated Z lines, indented sarcolemma small and splitting myofibrils. (x 7.500)

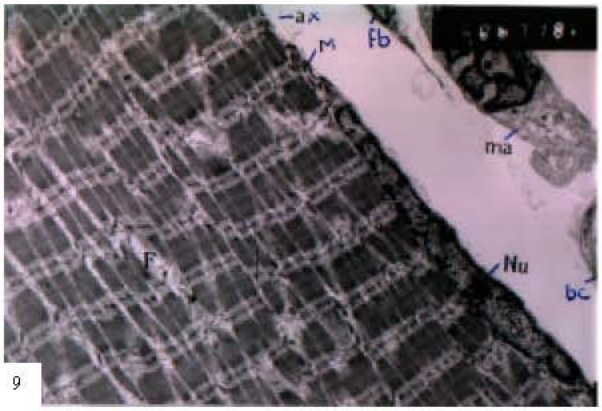

Fig. 9: Electron micrograph of treated mouse sartorius muscle fibers showing large (long) nucleus (N) with peripheral heterochromatin, dense nucleolus (Nu), few small mitochondria (M), small and disorganized myofibril, relaxed A and I bands, degenerated Z lines, empty and dilated nerve axon of myoneural junction (ax), fat vacuoles (F), fibroblast (fb), macrophage (ma), blood capillary (bc). (x 5.000)

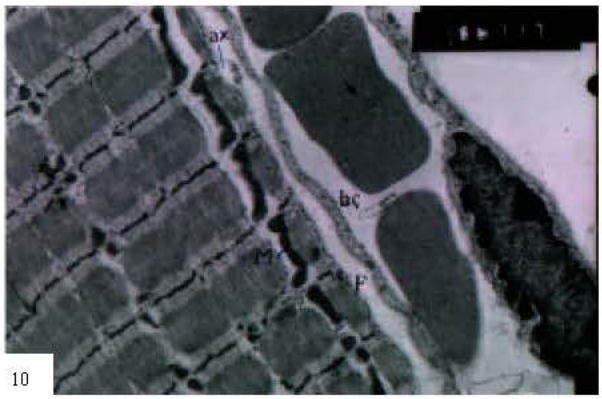

Fig. 10: Electron micrograph of treated mouse sartorius muscle fibers showing relaxed myofibrils with thick Z lines few small mitochondria (M) with dense matrix, empty axon (ax) at the myoneural junction, congested blood capillary (bc), fat vacuoles (F) in the endmysium. (x 7.500)

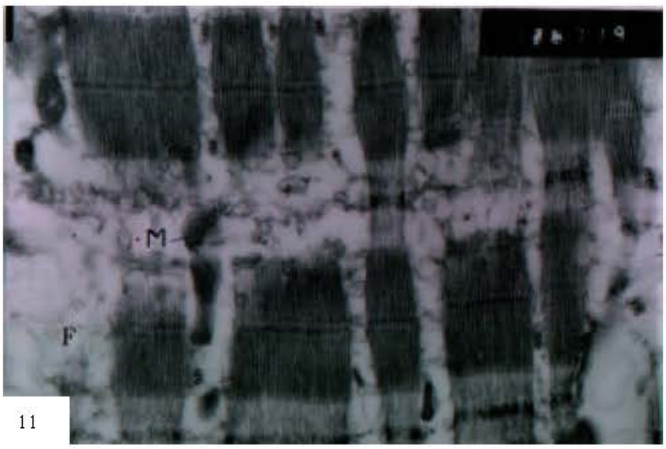

Fig. 11: Electron micrograph of treated mouse sartorius muscle fibers showing relaxed and degenerated myofibrils with degenerated Z lines, sarcoplasm with degenerated organelles, small mitochondria (M) with dense matrix, fat vacuoles (F). (x 13.000)

(Fig. 8 and 9). In some muscle fibers distinct myofibrils are not distinguished in longitudinal sections (Fig. 9 and 10), i.e., the myofibrils in the muscle fibers are not arranged in regular parallel pattern (Fig. 9). In some regions in the muscle fibers discrete and incomplete myofibrils are observed. In all muscle fibers myofibrils are appeared relaxed and the I bands are wide (Fig. 8-11). The Z lines are thicker than in the normal and sometimes followed a less regular coarse across the width of the myofibrils (Fig. 8-10), disintegrated Z line is observed in some myofibrils (Fig. 8, 9 and 11). The myofibrils seem to be separated by interfibrillar area which is clearly wider than in the control muscle fibers, therefore the myofibrils components appeared reduced, i.e., thin myofibrils are clearly noticed (Fig. 8). The interfibrillar sarcoplasm is relatively abundant (Fig. 8 and 11). The sarcoplasm between the myofibrils contain degenerated sarcoplasmic organelles (Fig. 8 and 9). In these muscle fibers degenerated myofibrils (atrophied) are observed (Fig. 9 and 11). In the interfibrillar sarcoplasm numerous profiles of the SR of varying size, shapes and densities are not noticed as in the control (Compare Fig. 4 of control muscle and Fig. 10 of treated muscle). Dilatation of SR are noticed (Fig. 8). Whereas mitochondrial profiles are abundant in sections from the muscle of control animals, mitochondria are few in the muscle of treated animals (Fig. 8-11), have irregular outline (Fig. 8 and 10), their matrix is filled with numerous electron dense granules (Fig. 10), i.e., treated muscle fibers are recognized by sparseness of mitochondria. Fat which is represented by vacuoles is normally present in few amount or rarely present in the sarcoplasm of the control muscle fibers are more numerous in the treated muscle fibers. In the interfibrillar sarcoplasm numerous fat vacuoles are present, these fat vacuoles are also present around the nucleus and subsarcolemmar regions (Fig. 9 and 11). Also numerous fat vacuoles are present in the connective tissue between the muscle fibers (Fig. 10). The appearance of well developed individual small axons near the sarcolemma or forming myoneural junctions are not clearly observed in the treated muscle fibers, empty and dilated axons (no synaptic vesicles in the cytoplasm of the axon) are noticed at the myoneural junction on the sarcolemma (Fig. 9 and 10).

DISCUSSION

No work has been reported on possible histopathological effects of diazepam on the muscular tissue. The histological and ultrastructural changes included alterations of the general architecture of the sartorius muscle which showed variable degrees of degeneration. Many of the common adverse effect of drugs in the class of sedated hypnotics are those resulting from dose-related depression of the central nervous system functions (Trevor and Way, 1995). Most studies of the effect of benzodiazepines and other hypnotic agents are carried out on the nervous tissue (Stenchever *et al*., 1970; Pawlikowaski *et al*., 1987; Andrada-Martinez *et al*., 1993; Labib *et al*., 2000). Necrosis was reported in histopathological studies on the rat brain under the effect of diazepam (Andrada-Martinez *et al*., 1993; Labib *et al*., 2000). On the other hand diazepam cause chromosomal breakage *in vitro* (Stenchever *et al*., 1970), suppression of DNA in the human glioma cells *in vitro* (Pawlikowaski *et al*., 1988) and

exert potent antiproliferative action on some types of neurons, also cause a significant increase in mitotic activity of the thymus gland (Pawlikowaski *et al*., 1987), suppression of lymphocyte proliferation in the spleen (Morsi, 1991). Moreover, Ingum *et al*. (1994) stated that flunitrazepam and diazepam have a fast penetration into the brain tissue from the plasma. Electron microscopic studies of the hippocams and cerebral cortex of the gasoline-treated rats revealed dilatation and fragmentation of rER, degeneration of mitochondria as well as increase of ribosomes, lysosomes; gasoline vapours induced a significant decrease in locomotor activity of rat (Faris *et al*., 2001). Yousif (2002) studied the ultrastructure of the liver of alprazolam-treated mice and found extensive morphological changes including, increase in euchromatin enlargement and fragmentation of nucleoli, decrease of mitochondria, rER and glycogen content, hypertrophied Golgi apparatus, increase and dilatation of sER increase of lipid droplets. On the other hand, Greenblatt *et al*. (1977) suggested that the administration of diazepam caused a significant increase in p CO_2 and fall in pH of blood of pregnant women.

Electron microscopic studies of the sartorius muscle of the mice treated with high therapeutic doses of diazepam showed extensive and obvious changes in connective tissue, myofibrils, sarcoplasm, neuromuscular junctions and blood vessels. Chaikules and Pauly (1965) and Alnaqeeb *et al*. (1984) found that as the animal mature there is a decrease in the connective tissue in the skeletal muscles throughout the entire growth period. On the other hand, biochemical analysis indicated that the concentration of connective tissue within the muscle belly increase in initial stages and then again in the senility. The size of the fasciculi reflects the function of the particular muscle concerned. Muscles responsible for fine, highly controlled movements, e.g., the external muscles of the eye, have small fasciculi and a relatively greater proportion of perimysial supporting tissue. In contrast muscle of the buttocks, have large fasciculi and relatively little perimysial tissue (Burkitt *et al*., 1996). The loss of sarcomeres and reduction in muscle fiber length occur when muscles are working at a shortened length (Williams and Goldspink, 1973). In the present study the muscle fibers which have pointed end in the treated animals may result from the loss of sarcomeres. At the same time the muscle of the diazepam treated mice showed an obvious increase in the connective tissue.

Fast muscle fibers have fibrils of regular size which are separate evenly from each other. They are also characterized by abundant amounts of SR and a straight Z line (Page, 1965), Also, the myofibrils in the flight muscles of insects are large and exceptionally uniform in diameter (Hodge, 1955). Similar observation are noticed in the control muscle fibers. Slow muscle fibers have fibrils of irregular size which are unevenly separated from each other. They have relatively small amount of SR and a zig-zag Z line (Page, 1965), similar observations are noticed in the treated muscle fibers. The variation in diameter of skeletal muscle fibers depends on such factors as the specific muscle, the age and gender, state of nutrition and physiological training. It is a common observation that exercise enlarges the musculature (Goldspink and Ward, 1979). Salmons *et al*. (1978) found that the Z discs of the slow muscle were thicker than that of the fast muscle and that they different in their mitochondrial content, similar results are noticed in treated muscle fibers. In the present study the initial histological and ultrastructural changes started in the cytoplasm, earlier than in the nucleus. Similar observations are illustrated by Moussa and El Beih (1972) and Labib *et al*. (2000) in the nerve cells. The extent of the SR can usually be correlated with a muscle fiber's speed of contraction, the SR is prominent in fast-acting muscle cells (Peachy and Porter, 1959). On the other hand, dilated SR cisternae are observed in the muscle fibers of the new born (Yousif and Sorour, 1992). In the treated muscle fibers dilated SR are noticed.

A special characteristics of muscle is the possession of a rich sensory nerve supply which detection position and velocity of movement. The integration of sensory information by the CNS is vital for the muscle to function normally (Stevens and Lowe, 1993). The vitality of the skeletal muscle fibers is depend on their nerve supply which if damaged results in atrophy of the fibers (Burkitt *et al*., 1995). At the myoneural junction small unmyelinated axons enter depressions in the surface of the muscle fibers, a connection between the Z bands of the myofibrils and the axon endings of motor end plates (Robertson, 1956). Huxley (1956) showing that local response to small stimuli occurs only at those muscle regions where the SR is in contact with the plasma membrane is necessary. All the above observations are clearly noticed in the control muscle fibers but not observed in the treated muscle fibers. It has been observed that, there is inhibition in the locomotory activities of the animals of the treated animals. Thus, It is possible to suggest that the inhibition of the locomotory activities, reported in the present study, may be related to the histological damage of the brain tissues of these areas and consequently lack of proper control of locomotion due to the injury of motor center in the brain cortex.

George and Naik (1985) have shown that a considerable amount of fat is reduced in the breast muscle of the bird and bat during exercise and thus fat is the chief

fuel during sustained muscular activity. According to Yousif and Sorour (1992) the fat concentration decreased during development of the muscle fibers. Skeletal muscle lipid metabolism controls by lepin. Lepin is the first identified adipocyte-derived hormone that directly regulate adiposity and energy homeostasis by decreasing food intake (Pellymounter *et al*., 1995). Homozygous ob/ob mice, which lake functional lepins, are characterized by severe adiposity and hyperlipidemia (Zhang *et al*., 1994). Lepin receptor are expressed primarily in the hypothalamus but are also present in several peripheral tissues including skeletal muscles (Ghilurdi *et al*., 1996). In the present study, in the treated muscle, it has been noticed that large amount of fatty tissue is noticed in the viscera, at the same time, the sarcoplasm of the muscle fibers and also the connective tissue contain large amount of fat vacuoles. We believed that there is no muscular exercise and the animals did not utilize the fat for energy, therefore large numbers of fat vacuoles are present in the muscle, similar observation are noticed in the tortoise skeletal muscle (Yousif, 1991). Diazepam may also induce a certain effect on the lepin, causing a disturbance in its function.

Vascular alterations have been reported following intra-arterial (Knil and Evans, 1975) and intra-venous (Graham *et al*., 1977) injection of diazepam including venous dilation and stasis as early as 48 h after injection. Similarly Labib *et al.* (2000) found that therapeutic dose of flunitrazepam showed vascular changes in the lung and cerebral tissues including marked congestion and dilatation of blood vessels, also in the present study diazepam induced the same vascular changes in the treated muscle.

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