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Microscopic Studies on the Effect of Alprazolam (Xanax) on the Liver of Mice

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Abstract: Effects of the administration of Alprazolam (Xanax) were studied on the liver of the mice. Animals were treated orally with different daily doses of 0.01, 0.1 and 0.2 mg of alprazolam/kg body wt. for 21 days. In the animals treated with the two high therapeutic doses (0.1 and 0.2 mg), light microscopic examination showed enlargement of the nuclei, vacuolation of the cytoplasm, proliferation (hyperplasia) and hypertrophied of Kupffer cells. Extensive morphological changes in the liver cells were revealed by electron microscope including decrease in heterochromatin, extensive enlargement and fragmentation of the nucleoli, decrease of mitochondria, rough endoplasmic reticulum and glycogen contents, hypertrophied golgi apparatus, increase and dilation of smooth endoplasmic reticulum, increase of lipid droplets, vacuolation of cytoplasm.

Key words: Alprazolam, mice, liver, electron microscopy, nucleus

Introduction

The distinction between 'pathological' and 'normal' state of anxiety is hard to draw. Because of this uncertainly, anxiolytic drugs are among the most frequently prescribed substances, used regularly by upwards of 10% of the population in developing countries (Salzman and Sheikh, 1998). Drugs that relieve anxiety generally cause a degree of sedation and drowsiness, which is one of the main drawbacks in the clinical use of anxiolytic drugs. In high doses all these drugs cause unconsciousness and eventually death from respiratory and cardiovascular depression. Benzodiazepines from the most important group, though anxiolytic and hypnotic drugs from an era are still in use (Harro *et al*., 1993). The use of benzodiazepines as anxiolytic agents is reviewed by Shader and Greenblatt (1993). Alprazolam, triazolobenzodiazepine, is a benzodiazepine derivative, is a sedative hypnotic, anxiolytic, muscle relaxant and antipanic medication that may have some antidepressant properties. Alprazolam was found to be effective for premenstrual symptoms in several (Harrison *et al*., 1990; Freeman *et al*., 1995) placebo-controlled double-blind studies.

The most important effects of the benzodiazepines are on the central nervous system and consist of: reduction of anxiety and aggression; sedation and induction of sleep; reduction of muscle tone and coordination; anticonvulsant effect. Benzodiazepines exert significant anxiolytic effect by enhancing neuronal-inhibiting properties of a central nervous system neurotransmitter, gamma-amino butyric acid (GABA). They act by binding to the specific regulatory site on GABA-receptor, thus enhancing the inhibitory effect of GABA (Salzman, 1997). They are usually classified by their elimination half-life rather than by differences in efficacy or adverse effect profile. Long-half-life benzodiazepines, such as diazepam, clonazepam and chlordiazepoxide, accumulate with repeated doses and clearance is significantly increased with age. For this reason, short-half-life benzodiazepines, such as lorazepan and oxazepan, are usually recommended for the elderly because they do not accumulate and present greater flexibility dosage (Salzman, 1998; Aparasu *et al*., 1998). Alprazolam is intermediate-half-life compound (approximately 14 h) whose clearance is somewhat delayed by the aging process (Lucki *et al*., 1986). The usual starting dose of alprazolam varies between 0.5 and 1 mg per day. The average dose reported in an extensive, multi-clinical study amounted to 6± 2 mg. In exceptional cases, a peak dose of 10 mg/day was necessary (Salzman, 1999).

Benzodiazepines produce dose-dependent adverse effects typical of sedative-hypnotics, although they possess a higher therapeutic-toxic ratio (Salzman, 1999; Shader and Greenblatt, 1993) than many other drugs. Sedation and unsteadiness, often worse with advancing age, are readily apparent when benzodiazepines are first taken or when dosages are raised, these effects can interfere with daily functioning and are potentially dangerous. Long-term benzodiazepines use may induce physiological dependence. For high doses, such as those used for panic disorder, dependence may develop sooner. When benzodiazepines are discontinued a well-described discontinuation syndrome may appear (Anonymous, 1990), typically include restlessness, agitation, anxiety, poor sleep- the opposite of therapeutic benzodiazepines effects. It is likely that many patients continue benzodiazepine use to avoid the appearance of these discontinuation symptoms.

Many researches have described the adverse effects of benzodiazepines and alprazolam in human. There is little information about the possible influence of hypnotic and anxiolytic drugs on different tissue organs. No histological and ultrastructural studies were conducted about the influence of alprazolam on the body tissues including liver. It was thought to be of particular interest to determine the effect of this commonly used drug on this organ at low and high therapeutic doses. In the view of the above mentioned notes the present work was planned to illustrate and assess the possible ultrastructural impacts of alprazolam on the liver.

Materials and Methods

Female Swiss mice (*Mus musculus*) aged 2-3 months and weighing 22-25 g were used in this study. Food and water were provided *ad libitum*. For comparison, three different doses of alprazolam were used in present study. These doses represent the low and two high recommended therapeutic doses. Xanax tablets containing 0.5 mg of alprazolam were uniformly suspended in distilled water. The animals were divided into 4 experimental groups each of 5 animals. The first group served as a control and received no treatment, while animals of the other three groups were orally treated via a bent stainless steel feeding tube with daily doses of 0.01, 0.1 and 0.2 mg kg^{-1} body weight for 21 days.

Small blocks of liver of control and the experimental groups treated with the high therapeutic doses were fixed for one hour in 2.5% glutaraldehyde, phosphate buffer pH 7.4 at 4°C, post fixed in 2% OsO_4 for one hour, dehydrated through graded alcohol and kept them embedded in Epon. Thin (50 nm) sections were cut on LKB ultramicrotome. After double staining with uranyl acetate and lead citrate, the sections were examined in Jeol CX electron microscope. For light microscopic studies semithin sections of liver of the control and all treated groups were stained with toluidine blue stain.

Results

Control liver: The ultrastructural examination of control liver cells is demonstrated (Fig. 1). Hepatocyte are exposed on each side to the sinusoids (filled with erythrocytes), which are lined by a discontinuous layer of fenestrated endothelial cells. The endothelial cells are separated from the underlying hepatocyte surfaces by space of Disse. Via the gaps in the sinusoidal lining, the space of Disse is continuous with the sinusoid lumen, thus bathing the hepatocyte surface with plasma. The surface of the hepatocyte that faces the space of Disse bears many irregular microvilli protruding in that space, but there is always a space between

them and the cells of sinusoidal wall. Reflecting the extraordinary range of biosynthestic and regenerative activities, the hepatocytes cytoplasm is crowded with organelles particularly mitochondria, rough endoplasmic reticulum, smooth endoplasmic reticulum, golgi stacks, lipid droplets, free ribosomes and lysosomes. The nuclei are large, spherical or sometimes irregular, central and contain heterochromatin clumps of varying shapes and sizes, dispersed chromatin particles (euchromatin) and one or two nucleoli. The nucleus is bounded by distinct nuclear envelope. Mitochondria are abundant and randomly scattered. Of particular interest in the present context are the appearance and distribution of the rough endoplasmic reticulum. Rough endoplasmic reticulum stacked in close parallel arrays is abundant in most hepatocytes observed in control animals. The rough endoplasmic reticulum forms aggregates dispersed in the cytoplasm. A close association between mitochondria and rough endoplasmic reticulum has been noticed (Fig. 1). Most of the mitochondria appeared almost completely encircled with heavily stained parts of rough endoplasmic reticulum. Golgi apparatus is numerous, consists of flattened cisternae, small vesicles and vacuoles and is mainly seen near the nucleus and rough endoplasmic reticulum aggregations, with an extension lying close to canalicular surface. There are numerous free ribsomes as well as large glycogen deposits in the cytoplasm. The smooth endoplasmic reticulum usually appear in the form of tubules and associated with glycogen areas in the cytoplasm, i.e. the glycogen often being closely related to the smooth endoplasmic reticulum. Another common cellular component is the lipid droplets. The lipid droplets of variable sizes are present in variable amount in different hepatocytes depending on nutritional status. The lysosomes appear in the control hepatocytes as dense membrane-bound bodies and vary in size, number and position from cell to cell.

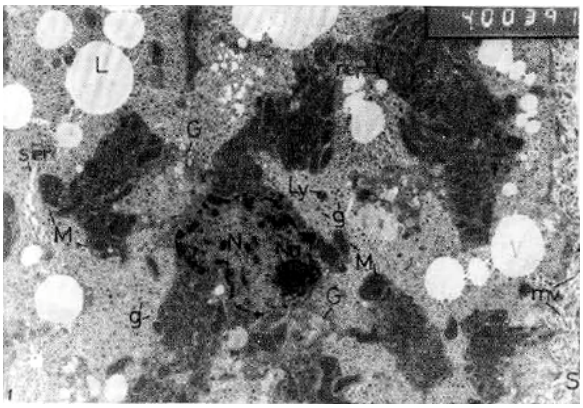

Fig. 1: Electron micrograph of control liver. Note: hepatocyte nucleus (N) with one nucleolus (Nu), heterochromatin (He), mitochondria (M) and rough endoplasmic reticulum (rer) alternate with glycogen (g) areas, smooth endoplasmic reticulum (ser), lipid droplets (L), golgi apparatus (G), lysosome (Ly), sinusoid (S), microvilli (mv). (x 4.000).

Alprasolam-treated liver: No obvious structural changes were observed in the light microscopy of the liver of the animals treated with low therapeutic dose (0.01 mg kg^{-1} b.wt.).
Light microscopic examination of the liver of animals treated with the high therapeutic doses (0.1 and 0.2 mg kg^{-1} b.wt.) of alprazolam revealed hypertrophied of the nuclei which appeared pale, nucleolar fragmentation (multiple nucleoli), cytoplasmic vacuolation and dilation of sinusoids. Kupffer cells were increased and hypertrophied (Fig. 2).
Electron microscopy showed that inspection of hepatocytes, 21 days post 0.1 and 0.2 mg kg^{-1} body weight alprazolam treatment revealed the same pathological alterations. These alterations were more severe and extensive in the higher dose (0.2 mg kg^{-1} b. wt.). The treatment with these doses of the drug affected all constituents of the liver, hepatocytes, sinusoids and blood vessels. The nuclei appeared hypertrophied in the hepatocytes. The most striking nuclear changes of the hepatocytes was the nucleolar fragmentation. Nucleoli were enlarged and increased in number. Most nuclei contained 5 to 6 nucleoli, several of which were adherent to the nuclear membrane. Some nuclei contained 2 very enlarged nucleoli. Extensive changes were observed in the chromatin. Heterochromatin clumps appeared obviously decreased. Chromatin particles of different sizes dispersed in the nucleoplasm. Nuclear envelope was clearly visible (Figs. 3, 4). Other changes observed in hepatocytes were a decrease in density of mitochondria. The number of mitochondria was low in all hepatocytes. Some of mitochondria were highly elongated, or curved. Some mitochondria had ring shaped appearance (Fig. 5); others appeared to divide. Many small rounded mitochondria were also observed (Figs. 3-5). The golgi apparatus appeared obviously hypertrophied (Fig. 3). A striking alteration in the rough endoplasmic reticulum of treated hepatocytes was the decreased frequency of the cisternal units of it associated in the typical parallel stacks (Figs. 3, 4). In some areas large segments of rough endoplasmic reticulum were divided into smaller ones (Fig. 4). Smooth endoplasmic reticulum occasionally showed numerous dilated elements in some cells and in other cells severe dilation in

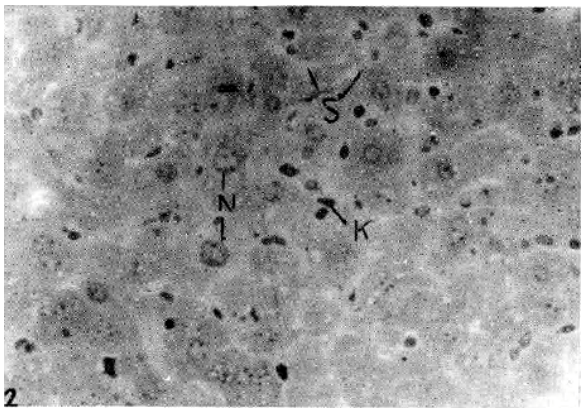

Fig. 2: Semithin section of treated liver (0.2 mg kg^{-1}). Note: hepatocytes with enlarged nuclei (N), fragmented nucleoli, vacuolation in cytoplasm, dilated sinusoids (S), hyperplasia of Kupffer cells (K). Toludin blue (x 400).

smooth endoplasmic reticulum was clearly noticed (Figs. 3, 4). Ultrastructural examination of the hepatocytes treated with alprazolam revealed massive cytoplasmic lipid inclusions in most hepatocytes. As in the control a moderate amount of the well defined cytoplasmic pale membrane-bound vacuoles of variable sizes, which represent the lipid droplets appeared in some cells (Fig. 3). In other cells a very large number of lipid droplets of varying sizes had developed and this was more frequently observed in hepatocyte of the animals treated with 0.2 mg kg^{-1} alprazolam (Fig. 4). These lipid droplets were distributed irregularly. Electron microscope showed loss of glycogen areas in all hepatocytes only a few scattered glycogen granules were observed among dilated smooth endoplasmic reticulum (Fig. 3). The amount of glycogen in highly vacuolated cells of treated

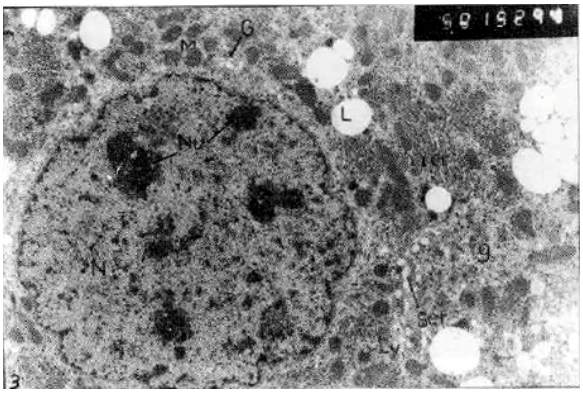

Fig. 3: Electron micrograph of treated liver (0.1 mg kg^{-1}). Note: enlarged nuclei with multiple nucleoli; heterochromatin highly decreased; decreased mitochondria (M) and glycogen (g), a dark particle (arrow) besides the lipid vacuoles (L), smooth endoplasmic reticulum (ser), lysosomes (Ly). (x 5.000).

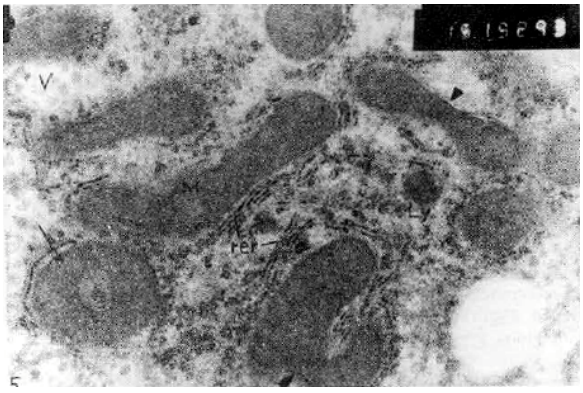

Fig. 5: Electron micrograph of treated liver (0.2 mg kg^{-1}). Note: mitochondria (M) highly elongated, ring shaped mitochondria (arrow), others appeared to divide (head arrow), ribosomes (r), rough endoplasmic reticulum (rer), vacuoles (V), glycogen (g), lysosome(Ly). (x 10.000).

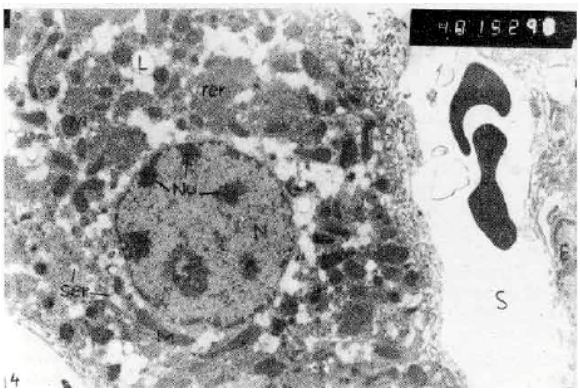

Fig. 4: Electron micrograph of treated liver (0.2 mg kg^{-1}). Note: enlarged nucleus (N), fragmented nucleoli, heterochromatin obviously decreased, few and elongated mitochondria (M), fragmented rough endoplasmic reticulum (rer) obvious reduction of glycogen areas, dilated smooth endoplasmic reticulum (ser), lipid vacuoles (L), dilated blood sinusoids (S), endothelial cell (E). (x 4.000).

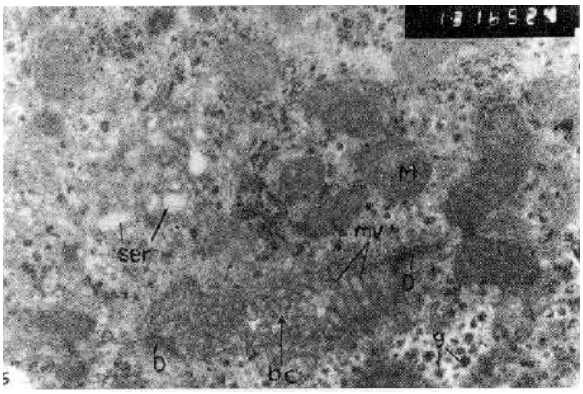

Fig. 6: Electron micrograph of treated liver (0.1 mg kg^{-1}). Note: bile canaliculi (bc), microvilli (mv) slightly swollen, glycogen (g), dilated smooth endoplasmic reticulum (ser), desmosomes (D). (x 13.000).

animals was considerably reduced compared with control (Fig. 1 with 4). The most striking feature in the alprazolam treated liver cells was the presence of dark particles besides the lipid vacuoles (Fig. 3). The number of lysosomes slightly increased. The cytoplasm contained small empty-appeared parts, i.e. vacuolation in the cytoplasm was noticed. Microvilli of bile canaliculi were slightly swollen (Fig. 6). The cell membrane of the adjacent hepatocytes showed desmosomal junctions. The microvilli of intercellular bile canaliculi obscured its lumen (Fig. 6).

Electron microscopic examination showed marked dilatation of sinusoids (Fig. 4); Kupffer cells appeared hypertrophied and protrude in the sinusoidal lumen (Fig. 7). Enlarged Kupffer cell had large lobulated nucleus with marginated large masses of heterochromatin. The cytoplasm contained small rounded mitochondria a few individual profiles of rough endoplasmic reticulum and small residual bodies or phagolysosomes (Fig. 7).

Discussion

In present study, obvious alterations in the ultrastructure of liver cells of the mice treated with daily high therapeutic doses (0.1 and 0.2 mg kg^{-1} b.wt.) of Alprazolam for 21 days were noticed. Sinusoidal dilated and prominent hyperplasia of enlarged Kupffer cells were detected by light microscopy.

Massoud *et al.* (1991) studied the effect of tempazepam on the rat and found that benzodiazepines derivatives was shown to cause

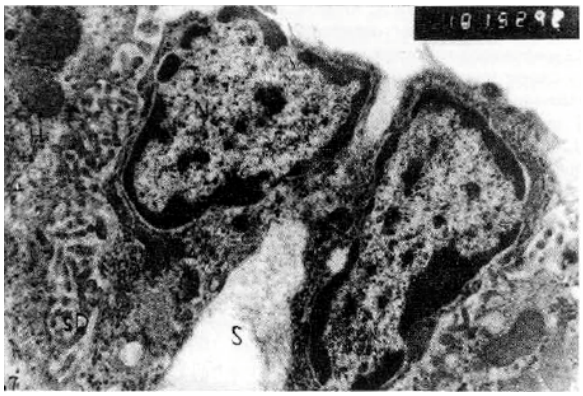

Fig. 7: Electron micrograph of treated liver (0.2 mg kg^{-1}). Note: enlarged Kupffer cells with enlarged nucleus, protrude into sinusoidal lumin, space of Disse (SD), hepatocyte (H). (x 10.000).

significant decrease in the amount of DNA, RNA and total proteins in the liver and they cause significant chromosomal aberrations in rat bone marrow. Similarly, Stenghever *et al.* (1970) reported that diazepam has proved to cause chromosomal breakage *in vitro.* Pisciotta and Kaldahl (1962) reported that chlorpromazine inhibited DNA synthesis through inhibition of a wide variety of the DNA synthesizing enzymes. Pawlikowaski *et al.* (1988) found that diazepam suppressed H^3-thymidine incorporation into DNA of human glioma cells *in vitro.* Additionally, diazepam exert a potent antiproliferative action on some types of neurons and anterior pituitary; on the other hand, it caused a significant increase in mitotic activity of the thymus gland (Pawlikowaski *et al.*, 1987). Moris (1991) found that administration of a daily dose of 0.1 mg diazepam/rat for 4 weeks suppressed lymphocytes proliferation in both red and white pulps of the spleen. In agreement with the above observations, the nuclei of hepatocytes showed obvious and extensive ultrastructural changes including enlargement of the nucleus, nucleolar enlargement and fragmentation, decreased of heterochromatin clumps. Nucleoli fragmentation patterns, similar to those observed in present study, have been found in liver cells, cardiac muscle fibers and other cells following exposure to drugs that inhibit RNA synthesis such as actinomycin D, adriamycin and erythromycin (Merski *et* al., 1976; Yousif and El Rawi, 2000). Schoefl (1964) stated that if any nucleolar synthetic processes are blocked, the structure undergoes marked alteration. It is generally agreed that the primary function of the nucleolus is related to ribosomal and protein synthesis. Interference with an adequate supply of ribosomes, as a result of decreased rRNA synthesis, would severely decrease the protein synthetic activity of the cells (Hjalmarson *et al.*, 1975). Chipchase and Birnsiel (1963) concluded that most, if not all, of the ribosomal RNA is synthesized by non-nucleolar portions of chromatin and then transferred to the nucleolus for assembly into ribosomes. Autoradiographic studies (Karasaki, 1965) have also shown that nuclear RNA synthesis occurs in both the nucleolus and the chromatin with the former consistently containing more label than the later. Accordingly, it is possible that the nucleolar changes may be a nonspecific morphologic representation of interference with the chromatin-nucleolar axis and that reflect variations in fundamental cell activity, particularly protein synthesis. The nucleolar zonal rearrangement could have been a result of increased nuclear RNase activity. Chevemont *et al.* (1956) reported, by light microscopy of cultured cells, that RNase caused shrinkage and fragmentation of nucleoli. Ultrastructural changes in the nucleus may represent functional changes and an important aspect of toxicity due to the effect of alprazolam on the liver cells at the high therapeutic doses. Ferguson-Chanowitz *et al.* (1990) studied the effect of alprazolam (platelets-activating factor antagonist, PAF) on Tumor Necrosis Factors-α (TNFα) in plasma of mice treated with endotoxin and found that administration at dose of 5-20 mg kg^{-1} body weight caused a dose dependent reduction in plasma TNFα levels of LPS-treated mice. Alprazolam or PAF is known to induce synthesis of prostaglandin E2 (Levine, 1988). Ferguson-Chanowitz *et al.* (1990) also concluded that the amounts of TNFα mRNA present in kidneys and the livers of LPS-treated mice, were increased. There is an increase in ribosomes of the cytoplasm in the liver cells.

In the present study, there was more or less moderate decrease in the mitochondrial amount. Klatskin (1961) stated that although alcohol has been shown to affect mitochondrial enzymes, no abnormality of mitochondrial structure was demonstrable in rats given large doses of alcohol for 6 weeks. Greenblatt *et al.* (1998) stated that alprazolam plasma level may be increased by inhibitors of the cytochrome P450 3A/4 hepatic isozyme. The golgi apparatus showed marked hypertrophy and was dispersed over a large area in hepatocytes after alprazolam treatment. Ramadan *et al.* (1997) stated that the alteration of the Golgi apparatus consisted of dilatation of the cisternae and vacuolation of its membranes in the hepatic cells after insulin administration.

In present study, other cytoplasmic changes in the alprazolam-treated animals included a marked reduction in glycogen and dilatation and an apparent increase in the amount of smooth endoplasmic reticulum. Depletion and loss of glycogen in mammalian liver cell was also induced by other different agents such as caffeine treatment (Tupikava, 1957), morphinism (Paroli and Caprino, 1959) and chronic alcoholism (Moustafa *et al.*, 1983). Cardell (1971) suggested that the development of smooth endoplasmic reticulum was dependent upon the glycogen content of the cells, it was abundant in cells with little glycogen and sparse in those with large quantities of glycogen. The principal function of smooth endoplasmic reticulum are lipid biosynthesis; in the liver cells, smooth endoplasmic reticulum also play a major role in detoxification of various noxious metabolite by-products and drugs (Junqueira *et al.*, 1995). The dilation and the apparent increase in the amount of smooth endoplasmic reticulum was observed in experimental animals following the administration of a variety of chemical agents (Gustafsson and Afzelius, 1963). Such a change would be found if liver biopsies were made in patients under intensive therapy with drugs such as phenobarbital and corticosteroids which are known to induce hypertrophy of smooth endoplasmic reticulum in animals (Biava *et al.*, 1965). Similarly, Hewers (1973) stated that large increases in the amount of smooth endoplasmic reticulum occur in the hepatocytes after the administration of drugs such as barbiturates and liver poisons e.g., carbon tetrachloride. This is apparently a response to the need to breakdown the foreign substance by enzymic action.

In present study, majority of liver cells exhibited a considerable increase in lipid droplets. The fatty changes in pathological conditions take place in the liver, according to Glaser and Magner (1972) due to imbalance between the normal rates of lipid synthesis, utilization and secretion. It must be taken into consideration that, the presence of very large number of mitochondria in the hepatocytes enables the liver to play a major role in the oxidation of fatty acids. The neutral fat is first absorbed by the hepatocytes and split into glycerol and fatty acids (Snell, 1984).

In the present study alprazolam caused dilation of the sinusoids and an increase and enlargement of the Kupffer cells. Kupffer cell proliferation occures in viral diseases of the liver and during hepatocellular necrosis in man and in laboratory animals (Ruebner and Miyai, 1962). Vascular altrations have been reported following intra-arterial (Knill and Evans, 1975) and intravenous (Graham *et al.*, 1977) injection of diazepam including; veinous dilation, stasis, intrstitial infiltration and thrombosis as early as 48 h after injection. Similarly Labib *et al.* (2000) found that the therapeutic dose of flunitrazepam showed vascular changes in the lung and

cerebral tissues including marked congestion and dilation of blood vessels.

Essential features of premenstrual dysphoric disorder (PDD) are symptoms such as profoundly depressed mood, anxiety, lability of effect and decreased interest in activities. Freeman *et al.* (1995) studied a double-blind trial of progesterone, alprazolam and placebo in treatment of (PDD) and found that, after 3 months, alprazolam group showing significantly more improvement compared with progesterone but not with placebo.

Many of the common adverse effects of drugs in the class of sedative-hypnotics are those resulting from dose-related depression of central nervous system functions (Trevor and Way, 1998). The action of peripheral type benzodiazepine receptor ligands seems to be connected with the blockage of voltage-dependent calcium channels (Cantor *et al.*, 1984). Furthermore, calcium influxes producing factors such as ionophore A 2318-7, which increase intracellular C^{2+} (Lichtman *et al.*, 1983).

From the previous observations, we can conclude that alprazolam (Xanax) commonly prescribed by physicians for anxiety, sedation, premenstrual dysphoric disorder etc. and recently may be used as platelets-activating factors antagonist (PAF) to reduce TNFα level. This paper critically assessed the effect of alprazolam on liver and found that the high therapeutic doses for 21 days may cause histological and ultrastructural changes in the liver, which lead to disturbance in the function of the organ.

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