THE BIOCHEMICAL AND HISTOLOGICAL EFFECT OF DIAZEPAM ON THE LIVER OF ALBINO MALE RATS

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ABSTRACT

Diazepam is a benzodiazepine derivative that is commonly used for its sedative and anxiolytic activities. The liver plays a major role in the metabolism of this drug. The aim of this study was to investigate the effects of different doses of diazepam on liver enzymes and histology in albino male rats. Forty (40) adult albino male rats were uniformly divided into four groups of ten rats each. Group 1 served as control received distilled water for 8 weeks. While groups 2, 3 and 4 were respectively gavaged with 2mg/kg/day , 5mg/kg/day and 10mg/kg/day of diazepam suspension daily for 8 weeks. At the end of the treatments, animals were sacrificed, liver tissues were obtained for the histopathology analysis and serum for the colorimetric assay of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma- glutamyl transferase (GGT) and albumin concentrations. The results of the present study showed that treatment of rats with the respective doses of diazepam suspension significantly ($P<0.05$) increased the serum enzymatic levels of ALT, AST and ALP and significantly decreased serum albumin level in group3 compared to group 1 and 2 . Also, the serum levels of ALT, AST and ALP enzymes were highly significantly ($P<0.001$) increased and the serum albumin level was highly significantly decreased in group 4 compared to group 1 and 2 . Concerning the GGT serum levels; it was increased significantly in group 2 compared to group 1 and in group 3 compared to group 2 . And, highly significantly in group 3 and 4 compared to group 1 . Also, highly significantly in group 4 compared to both group 2 and 3 . In conclusion; Diazepam when taken in large doses and for long periods might affect the hepatic cell and might leads to liver damage and hepatotoxicity.

Keywords: Diazepam, liver enzymes, liver histology, hepatotoxicity.

INTRODUCTION

Diazepam is a benzodiazepine derivative used to treat anxiety, seizure, muscle spasm, insomnia and restless legs syndrome[1]. It posses sedative, anxiolytic, hypnotic, skeletal muscle relaxant and amnestic properties [2]. Diazepam acts on the γ- aminobutyric acid A receptor complex, found in the central nervous system [3]. In addition to the central receptors described for benzodiazepines, peripheral- type binding sites had been identified in the endocrine steroidogenic tissues, immune cells and liver cells[4] .

The liver plays a central role in the transformation and cleaning of chemical substances and this increased the susceptibility to the toxic effect of these agents. Certain medicines when taken in overdoses and even within therapeutic ranges may injure the organ. The liver plays a

major role in metabolism of drugs and chemicals . Hepatic metabolism is a mechanism in which drugs and other compounds are converted into products that are more easily excreted. A metabolite can be more active and/or more toxic than the original drug[5]. Due to this essential point of view , serum levels of liver enzymes were used as markers in determining drug toxicity [6]. Metabolism of diazepam is carried out in the liver mainly into N-desmethyldiazepam (Nordiazepam) and 3-hydroxy-diazepam (Temazepam) [7]. The mechanism of action of hepatotoxicity is postulated to be unknown but it could be due to the effect of these reactive metabolites on immunity or to variations in the metabolism of diazepam[8] .

Previous studies had documented the role of free radical mediated pro-oxidative processes in rats after the administration of diazepam[9] .

Diazepam as stated previously ; caused oxidative tissue damage, especially after long treatments. Other studies showed that treatment of diazepam increased lipid peroxidation in the cortex and cerebellum of mouse brain along with increasing protein carbonyl formation in the striatum of these animals [10] . Others showed that rats treated with diazepam had significant decreases of glutathione levels and superoxide dismutase activity in their liver[11].

The objective of the present study was to evaluate the effect of the administration of different doses of diazepam suspension by oral gavage on liver enzymes and histology.

MATERIALS AND METHODS

Animals

Forty albino male rats weighing 200-250 gm and 8 weeks old were obtained from the animal house of the College of Pharmacy- University of Baghdad. The animals were maintained on normal conditions of temperature, humidity and light/dark cycles. They were fed standard rodent pellet and they had free access to water.

Preparation of diazepam suspension

Diazepam (Valium®) tablets (5mg) from Roche were powdered and mixed with 5ml of distilled water, 5% carboxymethylcellulose (CMC) was added as a suspending agent to produce a suspension for different doses[12].

The study design

These forty male rats were divided into 4 groups:
First group (control): 10 rats were administered distilled water for 8 weeks by oral gavage.
Second group: 10 rats were used for the study of the hepatotoxic activity of diazepam suspension in which (2mg/kg/day B.W.) of diazepam was given for 8 weeks by oral gavage.
Third group: 10 rats were used for the study of the possible hepatotoxic activity of diazepam in rat model. In this group (5mg/kg/day B.W.) dose of diazepam suspension was used for 8 weeks by oral gavage.

Fourth group: 10 rats were used for the study of the possible hepatotoxic activity of (10mg/kg/day B.W.) diazepam suspension was used for 8 weeks by oral gavage.

Preparation of serum

Rats of all the groups were anaesthetized with diethyl ether and blood samples were collected from the animals through cardiac puncture into centrifuge tubes and allowed to clot for 30

minutes. The clotted blood samples were centrifuged to separate the cells from the serum. Sera were then aspirated into labeled tubes and stored in the freezer at -15^{o}C until use.

Biochemical assays

The alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphate (ALP), gamma- glutamyl transferase (GGT) and albumin levels were evaluated using assay kits (Randox Laboratory Ltd, United Kingdom). The principle was based on the colorimetric measurement.

Histological analysis

The liver tissues isolated from the test animals underwent fixation with formal-saline for 48h. Then, these fixed tissue were processed manually through different grades of ethanol concentrations, cleared with xylene, impregnated and embedded in paraffin wax. Thin sections were obtained after cutting with a rotary microtome . Then, these sections were stained with haematoxylin and eosin (H&E) and examined microscopically for pathological changes[13] .

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-21 (Statistical Packages for Social Sciences version -21). Student t-test was used for testing the significance of difference between two groups and ANOVA between three. Statistical significance was considered whenever the (p value) was equal to or less than 0.05.

RESULTS

Biochemical

Concerning the liver biochemical parameters ; There was no significant difference between Group 1 (control) and group 2 (2mg/kg/day) regarding the serum levels of ALT, AST, and ALP. While the serum levels of these enzymes were increased significantly ($P<0.05$) in group 3 (5mg/kg/day) compared to group 1 (control) and group2 (2mg/kg/day). Also, the serum levels of ALT,AST and ALP enzymes were highly significantly increased ($P<0.001$) in group 4 (10mg/kg/day) compared to group1 (control) and group 2 (2mg/kg/day) and significantly increased ($p<0.05$) compared to group 3 (5mg/kg/day).

Regarding the GGT serum levels; it was significantly increased ($p<0.05$) in group 2 (2mg/kg/day) compared to group 1(control) and in group 3 (5mg/kg/day) compared to group 2 (2mg/kg/day). And , highly significantly increased ($p<0.001$) in group 3 (5mg/kg/day) and group 4 (10mg/kg/day) compared to group 1(control). Also, highly significantly increased ($p<0.001$) in group 4 (10 mg/kg/day) compared to both group 2 (2mg/kg/day) and group 3 (5mg/kg/day). According to the serum albumin level ; There was no significant difference between Group 1 (control) and group 2 (2mg/kg/day). But; it was decreased significantly ($P<0.05$) in group 3 (5mg/kg/day) compared to group 1 (control) and group 2 (2mg/kg/day). Also, the serum albumin level was highly significantly decreased ($P<0.001$) in group 4 (10mg/kg/day) compared to group1 (control) and group 2 (2mg/kg/day) and significantly ($p<0.05$) compared to group 3 (5mg/kg/day). As shown in table 1:

Table 1 comparison of the serum liver enzymes and albumin levels between group1, group2 , group 3 and group 4 in experimental albino rats

Group No.	Group 1 (control)	Group2 (2mg/kg/day)	Group 3 (5mg/kg/day)	Group 4 (10mg/kg/day)
ALT (IU/L)	34.7±1.66	36.9±1.67	40.8± 1.61 b	47.4± 1.38 *ab
AST(IU/L)	88.4±2.08	91.3±2.13	98.8± 2.3 b	106.2± 2.12 *ab
ALP(IU/L)	137.2±5.24	140.1±5.21	163.3± 5.11 b	183.5± 1.8 *ab
GGT(IU/L)	0.54±0.068	0.77± 0.061 b	1.08± 0.061 *ab	1.45± 0.058 *a
Albumin gm/dl	4.02±0.099	3.8±0.089	3.53± 0.091 b	3.21± 0.84 *ab

Data are expressed as mean (±SE); n=10 rats/group. Values with different superscripts are significantly different. P<0.05 significant; p<0.001 highly significant.

Histopathological examination

As per microscopic examination, the liver specimens of rats stained by H and E in group 1 (control) were free of pathological changes; it shows normal structure appearance of hepatocytes arranged as threads around the central vein and near the portal area (Fig. 1). However, group 2 (2mg/kg/day) shows abundant diffusion of inflammatory cells inside the sinusoids (figure 2). While in group 3 (5mg/kg/day); there was a degeneration and decrease of glycoprotein content of hepatocyte cells (Fig. 3). Whereas in group 4, there was an inflammatory cell infilteration with local necrosis of hepatocytic cells and congestion of the central vein (Fig. 4).

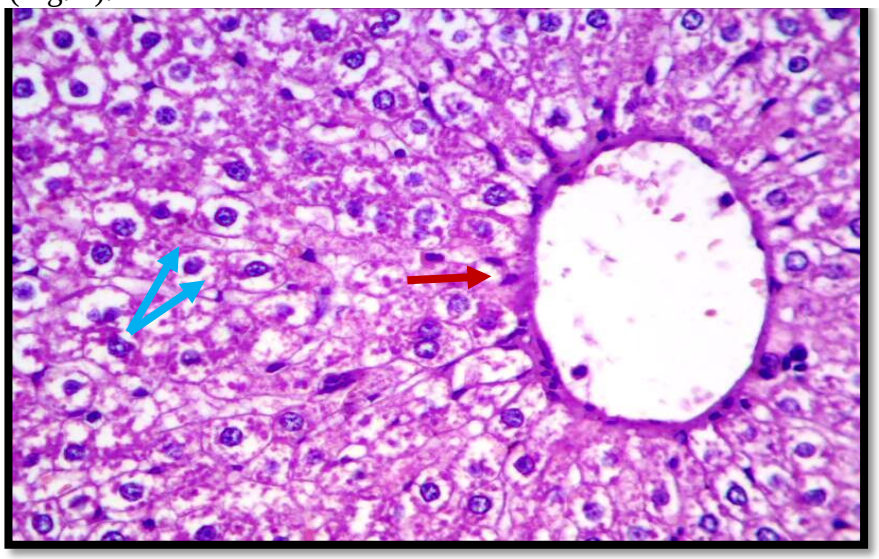

Figure 1: Photomicrograph (x400) of control rat liver stained with H and E; red arrow shows the central vein, blue arrows shownormal hepatocytes

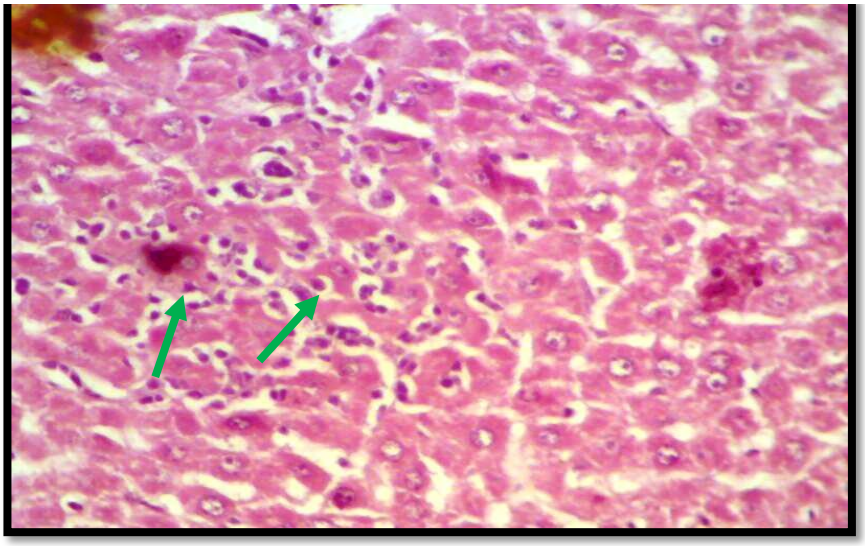

Figure 2: Photomicrograph (x400) of rat liver treated with 2mg/kg body weight of diazepam suspension stained with H and E, green arrows shows diffusion of inflammatory cells inside sinusoids.

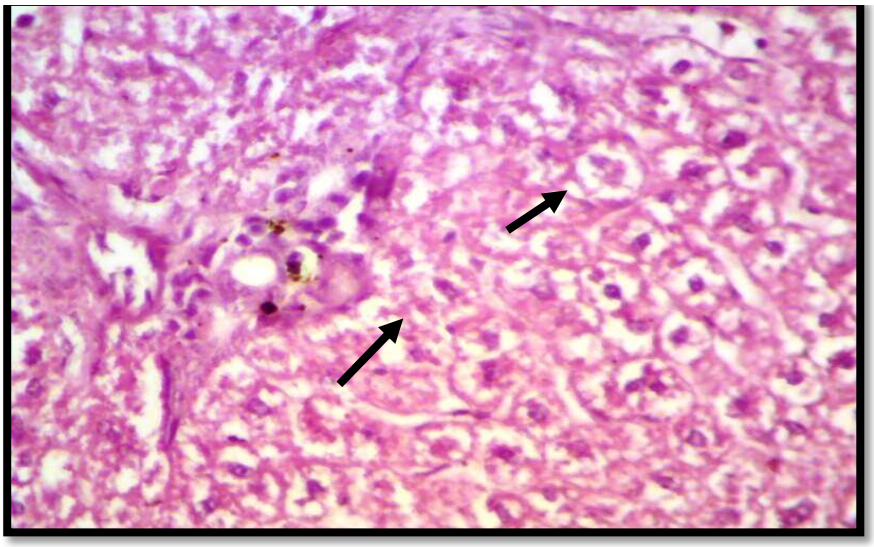

Figure 3: Photomicrograph (x400) of rat liver treated with 5mg/kg body weight of diazepam suspension stained with H and E, black arrows show degeneration and decrease contents of glycoproteins.

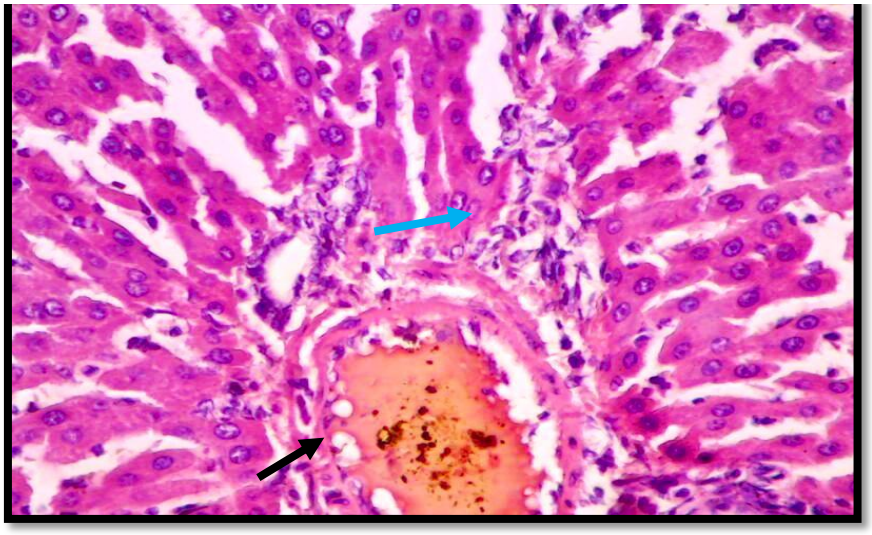

Figure 4: Photomicrograph (x400) of rat liver treated with 10mg/kg body weight of diazepam suspension stained with H and E, black arrow shows congestion of the central vein and the blue arrow shows local necrosis of hepatocytes.

DISCUSSION

The result of the present study showed that the serum levels of the liver enzymes (ALT, AST and ALP) were significantly increased in group3 and highly significantly increased in group 4 ; this might be due to the tissue damage induced in the liver by oxidative stress in response to the repeated ingestion of diazepam . And , also might be due to the adverse effects of its active metabolites (desmethyldiazepam). The increase in the level of these enzymes was mainly due to the leakage of these enzymes from the liver cytosol into the blood stream due to tissue damage[14] .

This result was in agreement with the studies of Jabłońska et al.,(1975) who showed that diazepam caused an increase in liver enzymes activity [15] and also with El- sokkary (2008) who reported that the long- term use of diazepam terminates in a complex liver damage marked by an increase in lipid peroxides activity plus a decrease in glutathione concentration and superoxide dismutaseactivity in livers of diazepam-administered rats[11].
Serum GGT levels was increased highly significantly in group 3 and 4 and this was inconcordance with that stated byKoenig et al.,(2015) who stated that GGT increase together with ALT,AST,ALP increase is associated with liver disease and liver damage and that low antioxidant defenses are also correlated with elevated GGT, particularly reduced levels of glutathione [16] . This was in agreement with Eger et al., (2016) who noted that rats treated with diazepam showed an altered enzymatic activity of oxidative stress enzymes including catalase, glutathione peroxidase , and superoxide dismutase in the brain as well as decreased glutathione levels and superoxide dismutase activity in the liver[17]. Also, with Nunes et al.,(2008) who reported that treatment with diazepam altered glutathione homeostasis and altered the enzymatic activity of glutathione peroxidase, glutathione reductase, superoxide dismutase and glutathione-S-transferases in the liver tissues[18].

The significant decrease in serum albumin level can be explained by the fact that protein synthesis is carried out in the liver. Albumin reflects hepatic function; it is one of the standard liver function tests. The two most influential factors regulating hepatic albumin synthesis are nutritional intake, specifically protein consumption, and illness. Reduced protein consumption slows mRNA synthesis of albumin and results in lower serum levels[19] .

Regarding the histopathological examination of the liver; the present study showed an increase in the infilteration of inflammatory cells inside the sinusoids and with increasing the dose of diazepam; there was a degeneration and necrosis of hepatocytic cells and congestion of the central vein, these results were inagreement with that noticed by Chatterajee et al. (1997) who noticed a prenecrotic and necrotic changes in the liver in the high – dose group of diazepam treatment [20].

The present histopathological results could be attributed to the toxic effect of the drug and its metabolites on the liver. Oxidative stress disrupts lipids, proteins and DNA, induces necrosis and apoptosis of hepatocytes and amplifies the inflammatory response and it stimulate the production of profibrogenic mediators from Kupffer cells and circulating inflammatory cells resulting in the initiation of fibrosis[21] .Also Seqin et al., (2007) stated that chronic administration of diazepam caused an increase in malondialdehyde levels and a decrease in glutathione content and that diazepam markedly lowered Ca^{2+}ATPase activity. Thus, increased lipid peroxidation together with alteration in Ca^{2+} -ATPase activity may play a role in diazepam induced hepatic injury [22] .

CONCLUSION

In agreement with the biochemical findings, which were supported by the histopathological changes in the liver of the treated rat groups, the present results pointed out the risk of hepatic tissue damage due to administration of diazepam in large doses and for long times. Therefore, it is recommended to take the drug only with the prescription of a doctor and the dose should be reduced especially in the presence of liver diseases. Moreover, future studies should be carried out to explore the toxic effect of the long-term use of this drugs on other organs such as the brain, heart, and kidney.
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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