Research Journal of Medicine and Medical Sciences, 4(2): 295-302, 2009

Diazepam-induced Oxidative Stress Inrat Different Organs

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Abstract: The objective of the present study was to demonstrate the role of diazepam in inducing oxidative stress and tissue damage on rat different organs. Animals were divided into two groups; G1: normal control (not received any drug), G2: diazepam drug treated group. diazepam administered orally using a single dose of 1 mg/ 100gm body weight. The effect of this drug toxicity on different tissue organs (liver, kidney and heart) was studied after three different experimental periods (after 10, 20 and 30 days) commenced just after of drug ingestion. The results revealed that administration of this toxic drug induces oxidative tissue damage indicated by marked elevated activity of xanthin oxidase (XO) accompanied by increased nitric oxide (NO) level in liver, kidney and heart in diazepam-treated rats compared with normal animals. The increment of such oxidative stress markers was accompanied by increased malondialdehyde (MDA, index of lipid peroxidation) in kidney ,and decrease in adenosine triphosphatase (ATPase) and lactate dehdrogenase (LDH) activities in heart tissue indicating tissue oxidative damage. The oxidative tissue damage induced in liver in response to diazepam ingestion was ensured by a decrease in the activity of hepatic sorbitol dehydrogenase (SD) with concomitant increase in liver serum marker enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT). Also the marked increment of serum markers of kidney, creatinine and uric acid levels and increase in serum marker enzyme of heart, creatine phosphokinase (CPK) in diazepam treated animals versus normal ones ,indicating kidney and heart disfunction. The current study also revealed that ingestion of diazepam to rats led to decreased level of hemoglobin (Hb) compared with normal animals The alteration in the biochemical results were severe 20 and 30 days after the drug administration and documented by the histopathological pictures of the studied organs.

Key words: diazepam, oxidative stress, organs, enzymes.

INTRODUCTION

Benzodiazepines are the most frequently prescribed class of psychotropic drugs, possibly worldwide[37]. Benzodiazepines, such as diazepam (trade name is Valium, chemically it is phenyl benzodiazepine containing 7- chloro 1,3 di hydro -1- methyl -5- phenyl -2H- 1,4 benzodiazepin), are commonly used for their anxiolytic and sedative effects, that is, by their action on high-affinity receptor sites coupled to the γ-aminobutyric acid A receptor complex, present in the central nervous system[9]. Nevertheless, in addition to the central receptors described for benzodiazepine, peripheral-type binding sites have been identified for them in liver cells[45], endocrine steroidogenic tissues [47] and immune cells, such as macrophages and lymphocytes[46], and also in tumour cells[16]. Peripheral-type benzodiazepine receptor expression has been shown to increase in some neoplastic tissues and tumour cells, particularly in the liver[40], ovary[20], colon [21], breast[17] and in the brain[25]. Peripheral-type benzodiazepine receptor expression has also been associated with both tumour progression and aggressiveness, because higher levels of its expression were found in tumour cells that display increased rates of proliferation, such as some breast cancer cells hepatic cancer cells[8] and glioma cells[4]. Many studies have been conducted to ascertain any role and/or involvement of free radical mediated pro-oxidative processes in the brain following diazepam administration[28]. This team has reported that a single dose of diazepam caused stimulation of free radical-mediated changes.

The objective of the present study was to demonstrate the role of diazepam in inducing oxidative stress and tissue damage on rat different organs (liver, kidney and heart).

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MATERIALS AND METHODS

Chemicals: All chemical reagents were of analytical grades purchased from Sigma Chemical Co. (St. Louis, Mo, USA), Merk (Germany) and BDH (England). Diazepam drug was obtained from Swiss Hofman Laroch Limited Company.

Animals: 60 adult male albino rats (50-70 gm) were obtained from animal house, King Fahed Center for Medicinal Research, King Abdul-Aziz University, Jeddah. The animals were housed in cages under standard hygienic condition and were fed with rat chow and water *ad libitum*. In order to optimize drug absorption, all animals were fasted for 1 hour prior to drug administration.

Experimental Design: Rats were divided into Two groups normal healthy group (group 1) and diazepam drug treated group (group 2), each of 30 rats. Diazepam drug induced tissue damage was administered orally using a single dose of 1 mg/ 100gm body weight. The effect of this drug toxicity on different tissue organs (liver, kidney and heart) was studied after three different experimental periods (after 10, 20 and 30 days) commenced just after of drug ingestion. After each studied period the blood samples were collected from some animals into sterilized tubes for serum separation and into tubes containing heparin for hemoglobin determination. Serum was separated by centrifugation at 3000× g for 10 minutes and used for biochemical serum analysis. After blood collection, rats of each experimental peroid were sacrificed under ether anesthesia and the liver, kidney and heart samples were collected, minced and homogenized in either ice cold bidistilled water or 10 % to yield 10% homogenates using a glass homogenizer. The homogenates were centrifuged for 15 minutes at 10000g. at 4°C and the supernatants were used for different biochemical tissue analysis.

Biochemical Analysis: All the following biochemical parameters were measured spectrophotometrically .

Tissue Analysis: Nitrite concentration (an indirect measurement of NO synthesis) was assayed using Griess reagent (sulfanilamide and N-1-naphthylethylenediamine dihydrochloride) in acidic medium[27]. Lipid peroxidation was determined by measuring the formed MDA (an end product of fatty acid peroxidation) by using thiobarbituric acid reactive substances (TBARS) method[5]. This assay is based on the formation of red adduct in acidic medium between thiobarbituric acid and MDA, the product of lipid peroxidation was measured at 532 nm. MDA concentration was calculated using extinction coefficient value(ε) of MDA-thiobarbituric acid complex (1.56 $\times 10^5$ /M/cm). XO (EC 1.1.3.22) activity was determined by the reduction of nitroblue tetrazolium (NBT) in the presence of xanthine , forming formazan. The enzyme activity was calculated using the extinction coefficient of reduced NBT (7.5 cm2/µmol at 540nm) [14]. The activity of the enzyme is expressed as nmol uric acid /min/mg protein. ATPase was determined using the method of Tsakiris and Deliconstantinos [39]. LDH activity was evaluated in a reaction mixture containing tris buffer (50Mm, pH,7.5), sodium pyruvate (0.6mM) and NADH (0.18 mM). The rate of NADH consumption is determined at 340nm and is directly proportional to the LDH activity (Bergmeyer, 1975). Sorbitol dehydrogenase (SD, EC 1×1×1×14) was measured by the method of Bergmeyer (1974). The reaction mixture contained the following in the final concentration of: 0×107 M Triethanolamine buffer (pH 7×4), 300 Mm and 0.4Mm NADH. The decrease in the optical density was recorded at 340nm.

Serum Analysis: ALT and AST activities were determined according to the method described by Bergmeyer *et al.,*[3]. Gamma Glutamyl Transferees (GGT) was measured by the method described by Shaw *et al.,*[35], uric Acid (UA) by the method described by Bulgar and Johns[6], creatinine (Crea) by the method of Larsen[24] and creatine phosphokinase (CPK) by the method described by Rosalki[32]. GGT was assayed by the method of Schmidt and Schmidt[34].

Blood Analysis: Hb was estimated in the whole heparinized blood by cyanmethaemoglobin method [11].

Histological Evaluation: Representative slices from liver, kidney and heart tissues were taken from the eviscerated animals and fixed in 10% formalin. For light microscopy examination, the tissues were embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (H&E).

Statistical Analyses: Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as mean ± S.D. The significant differences among values were analyzed using analysis of variance (one-way ANOVA) coupled with post-hoc (LSD) and followed by Bonferoni as a post ANOVA test.

RESULTS AND DISCUSSION

The levels of oxidative stress markers in different tissue organs in normal and diazepam treated groups

after the three stdied different periods are shown in table 1. From the table it can be observed that administration of diazepam to rats led to marked elevated activity of xanthin oxidase (XO) accompanied by increased nitric oxide (NO) level in liver, kidney and heart in diazepam-treated rats compared with normal animals. The increment of such oxidative stress markers was accompanied by decreased level of sorbitol dehydrogenase (SD) in liver, increased malondialdehyde (MDA, index of lipid peroxidation) in kidney ,and decrease in adenosine triphosphatase (ATPase) and lactate dehdrogenase (LDH) activities in heart tissue. Table 2 shows the levels of blood functional markers in normal and diazepam treated rats after three different periods. The results revealed increased in liver serum marker enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT). Marked increment of serum markers of kidney, creatinine and uric acid levels and increase in serum marker enzyme of heart, creatine phosphokinase (CPK) in diazepam treated animals versus normal ones were also observed. The table also shows that ingestion of diazepam to rats led to decreased level of hemoglobin (Hb) in diazepam treated animals compared with normal animals. The histopathological changes in in livers, kidneys and hearts of diazepam treated animals were observed in figures 1,2 and 3 respectively.The pictures showed necrotic degenerative changes in the three studied organs in rats under the effect of the used drug. These changes were severe in 20 and 30 days after the drug ingestion.

Discussion: The results of this study showed that, administration of diazepam drug to rats induces oxidative stress in different organs of rats which is indicated by marked elevated activity of XO accompanied by increased NO level in liver, kidney and heart in compared with normal animals. The increment of such oxidative stress markers was accompanied by increased MDA (index of lipid peroxidation) in kidney ,and decrease in ATPase and LDH activities in heart tissue indicating tissue oxidative damage. These results are in coped with previous study revealed an involvement of free radical-mediated pro-oxidative processes in brain and liver following diazepam administration[28,10]. suggesting that diazepam has a portant role in development of oxidative stress in brains and livers of diazepam-treated rats.

XO is an enzyme catalyzes the oxidation of hypoxanthine to xanthine and the later to uric acid. It was reported that XO is an endogenous source of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can produce oxidative stress which inflects tissue injury[44,18,12]. It catalyzes the reduction of nitrite to NO which exerts various influences on the pathogenesis of tissues[26]. NO has a double- edged knife in pathophysiology, since both the abundance and paucity of NO causes diseases[22]. The action of NO depends on the relative degree of its expression by nitric oxide synthase isomers,endothelial ,neuronal and inducible (eNOS, nNOS & iNOS respectively).The eNOS expresses the eNO that causes vasodilatation[29], inhibition of eNOS leads to vasoconstriction and atherosclerosis. NO produced by nNOS is resistant to brain damage caused by vascular strokes[30], while iNO plays an important role in vascular smooth muscle dilation[42] and its over production has linked to a variety of clinical inflammatory diseases[22]. The direct toxicity of NO is enhanced by reacting with superoxide radical to give powerful secondary toxic oxidizing species,such as peroxynitrite (ONOO̅) which is capable of oxidizing cellular structure and causes lipid peroxidation[43,33], which is coupled with many deleterious effects, both on cell membrane and cytosol. The effects on cell membrane may include an increase in osmotic fragility[19], an increase in permeability, inactivation of membrane-bound enzymes such as ATPases, and cross-linking of membrane constituents [41]. Transformation of eNO by superoxide radical to peroxynitrite diminshes the capacity of endothelial cells to generate bioactive useful NO ,which is important in maintenance of normal blood pressure ,thereby decreases in NO bioavailability, causing endothelial sclerosis and subsequently hypertension[37]. Thus from our result it can be stated that increased of XO activity which plays a crucial role in reducing the level of bioactive NO may be used as important marker for vascular disorder.

On the other hand, ATPases is essential for the regulation of ionic content and membrane excitability of myocardial cells, impaired of its function would lead to the coronary artery vasospasm, arrhythmias, ischemic damage and cardiac failure. This contention is in concert with the some findings that certain cardiovascular diseases, such as cardiomyopathy, and hypertension, associate with decreased ATPase activity [31,7].

The current study showed that the oxidative tissue damage induced in liver in response to diazepam ingestion was ensured by a decrease in the activity of hepatic SD with concomitant increase in liver serum marker enzymes, AST, ALT and GGT. The alteration in such marker enzymes activities is mainly due to the leakage of these enzymes from liver cytosol into the blood stream as a result of tissue damage. This result was also documented by liver histopathological hepatocyte severe damage which indicated by

Table 1: Levels of oxidative stress markers in different organs of rats in normal and diazepam treated groups after three different peroids.

Parameters	Normal	10 days	20 days	30 days
Liver				
XO	3.05 ± 0.194	16.46 ± 1.50*	19.98 ± 1.5*	25.78 ± 1.5*
NO	15.83 ± 1.18	31.13 ± 2.2**	35.38 ± 2.5**	34.05 ± 2.50**
SD	36.07 ± 2.01	2.95 ± 0. 52*	1.77 ± 0.16*	1.69 ± 0.10*
Kidney				
XO	1.87 ± 0.38	13.27± 1.54*	22.04 ± 2.65*	30.02 ± 2.66*
NO	5.51 ± 0.86	24.26 ± 2.59*	22.93 ± 1.89*	23.10 ± 2.26*
MDA	11.18 ± 1.62	25.54 ± 2.58**	33.96 ± 2.03**	42.82± 2.12*
Heart				
XO	1.98 ± 0.18	9.19 ± 1.05*	13.37 ± 0.96*	19.46 ± 0.5*
NO	12.40 ± 2.17	37.49 ± 3.88**	51.67 ± 3.94*	68.94 ± 0.5*
LDH	5.01 ± 0.15	2.40 ± 0.12**	1.76 ± 0.13*	1.54 ± 0.11*
ATPase	5.08 ± 0.35	1.65 ± 0.138*	0.85 ± 0.07*	0.45 ± 0.006*

Data are expressed as mean± SD of 6 rats in each group, XO and SD are expressed in n mol/ min/mg protrein, NO is expressed in umol/g tissue, LDH and ATPase are expressed in u mol/ min/mg, MDA is expressed in nmol/g tissue. *P> 0.0001, **P > 0.01, ***P > 0.05 when compared with normal group.

Table 2: Levels of blood functional markers in normal and diazepam treated rats after three different periods.

Parameters	Normal	10 days	20 days	30 days
AST (u/l)	15.12± 2.6	126.16 ±14.3*	135.83 ± 13.79*	142.50 ± 14.4*
ALT(u/l)	26.50 ± 0.41	50.00 ± 5.89**	62.83 ± 7.22**	64.66 ± 7.14**
GGT (u/l)	12.00 ± 0.89	35. 28 ± 1.4**	37.63 ± 0.90**	36.03 ± 3.67**
UA (mg/dl	2.2 0 ± 0.065	2.86 ± 0.37***	3.93 ± 0.42***	5.13 ± 0.26**
Crea (mg/dl)	2.2± 0.31	2.86± 0.67	3.93 ± 0.43***	6.76± 0.67**
CPK (u/l)	30.33 ± 8.59	92.11 ± 11.36*	103.75 ± 2.94*	113.66 ± 10.93*
Hb (g/dl)	17.68 ± 0.69	12.15 ± 0.7***	11.90 ± 0.5***	10.6 0 ± 0.5***

Data are expressed as mean± SD of 6 rats in each group . $^{*}P > 0.0001$, $^{**}P > 0.01$, $^{***}P > 0.05$ when compared with normal group.

degeneration of hepatocyte in some area and shrinkage of other in another area and lose of tissue normal architecture Also the marked increment of serum creatinine and uric acid levels in animals with concomitant necrosis in kidney tissue observed in histopathological pictures of animals receiving diazepam presented in this study during the three studied different periods are well indicators of renopathy. the oxidative cardiac tissue damage induced by toxic effect of diazepam in rats was ensured by pronounced increased in the activities of diagnostic serum marker enzymes, CPK and a decrease LDH in heart tissue in diazepam treated rats compared to normal rats and confirmed by the hitopathological picture which demonstrated focal myonecrotic lesion. These findings confirm the onset of myocardial lesion and leaking out of the marker enzymes from heart to blood[38,15].

The current study also revealed that ingestion of diazepam to rats led to decreased level of Hb in diazepam treated animals compared with normal animals. Decreased Hb level induce a state of anemia which may lead to a number of biochemical abnormalities and impaired cell-mediated immunity with increased susceptibility to infection[13].

The changes in both biochemical analysis as well as the in histological pictures in liver kidney and heart were sever in animals left for 30 days after diazepam ingestion. In the light of these results, it can be suggested that diazepam is a hazards drug and an oxidative stress inducer.

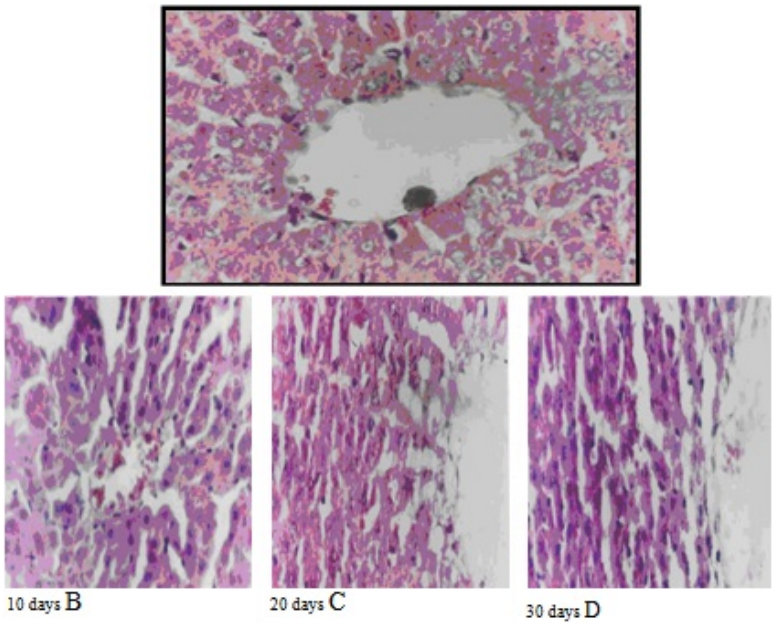

Fig. 1: Effect of Diazepam on Liver Tissues. A-light microscopic picture of normal liver showing normal hepatocytes B - liver of rats treated with diazepam after 10 days showing focal lesion and inflammation (C and D) -Liver of rats received diazepam after 20 days and 30 days respectively showing severe degenerative change in hepatocytes (H&E ×400).

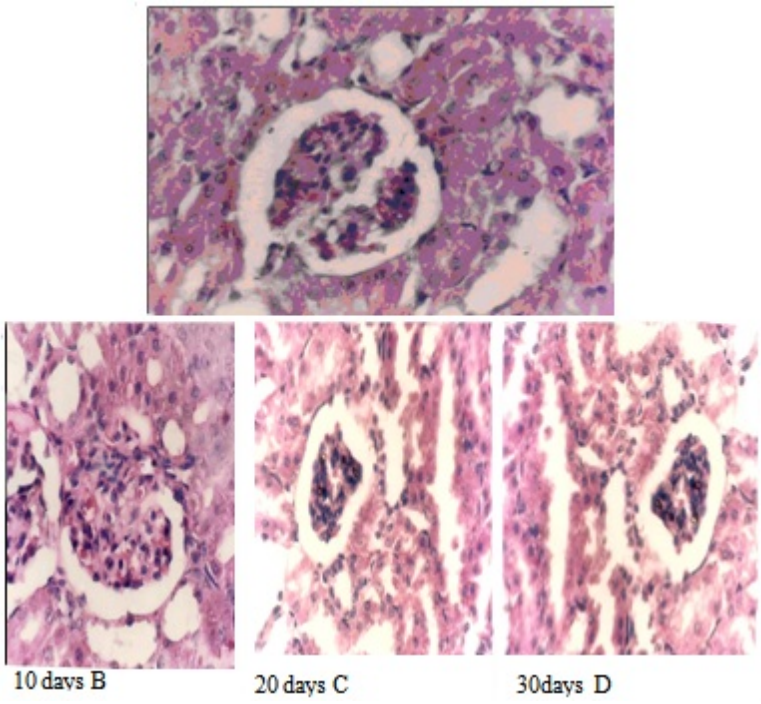

Fig. 2: Effect of Diazepam on Kidney Tissues. A-light microscopic picture of normal kidney showing normal tubules and glomeruli B - kidney of rats treated with diazepam after 10 days showing distended hypercellular glomeruli and degeneration of epithelial lining of tubules (C and D) -kidney of rat received diazepam after 20 days and 30 days respectively showing severe degenerative change in glomeruli and tubules (H&E ×400).

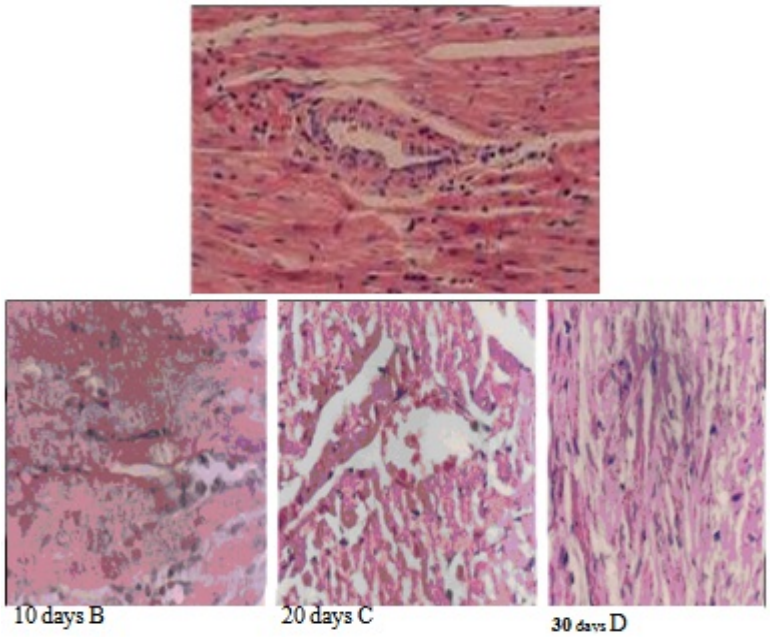

Fig. 3: Effect of Diazepam on heart Tissues. A-light microscopic picture of normal heart showing normal muscle fibers B - Heart of rat treated with diazepam after 10 days showing congestion and inflammation (C and D) -Hearts of rats received diazepam after 20 days and 30 days respectively showing severe necrotic changes in heart muscle fibers (H&E ×400).

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