CLONAZEPAM INDUCED SUB CHRONIC HEPATORENAL TOXICITY IN RATS AND THE PROTECTIVE ROLE OF ALPHA-TOCOPHEROL

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ABSTRACT

Background: Clonazepam (CZP) is an antiepileptic drug approved in 1976 by Food and Drug Administration (FDA) for treatment of various types of seizures, Clonazepam has a high potential for abuse, as well as tolerance, physical dependence, and ultimately addiction. Although many studies confirmed the deleterious effects of other antiepileptic like valproic acid and carbamazepine, those of clonazepam are minimal. **Objectives:** This research aimed to study and evaluate the hepatotoxic and nephrotoxic effects of sub chronic high dose administration of clonazepam and the protective effect of alpha-tocopherol "vitamin E" (V.E). **Methods:** Forty (40) healthy male albino rats were included. They were randomly divided into four equal groups (10 rats each): group I (normal saline), group II (CZP misuse), and group III (V.E) and group IV (CZP + V.E). All rats received the commenced drugs for 50 days. Serum levels of AST, ALT, ALP, urea, creatinine, and uric acid were measured. Liver and kidney tissues were taken for histopathology. **Results:** clonazepam in high doses increased hepatic and renal biomarkers levels, disrupted hepatic and renal tissues, and increased the number of degenerated cells. V.E treatment significantly attenuated the deleterious effects induced by clonazepam.

Keywords: Clonazepam, Misuse, Hepatotoxicity, Nephrotoxicity, alpha-tocopherol, Rats.

INTRODUCTION

Clonazepam is considered a high-potency and long-acting benzodiazepine. It is approved in 1976 as an antiepileptic drug by the United States Food and Drug Administration (FDA) for seizure and panic disorders treatment. Clonazepam has anticonvulsant and anxiolytic effects. Clonazepam inhibits seizure activity in paroxysmal types of activity on the electroencephalogram of neurological patients and many animal models of epilepsy **(Teschke et al., 2017).**

Clonazepam is structurally like diazepam, nitrazepam and chlordiazepoxide. Clonazepam behaves as a positive allosteric facilitator on GABA-A receptors. It acts by facilitation of GABA-A action by increasing the opening frequency of chloride channel leading to neuronal hyperpolarization and decreased firing, that result in calming effects. GABA is an inhibitory neurotransmitter that reduces activity in CNS and therefore incites feelings of relaxation and euphoria **(Sakata et al., 2008).**

Clonazepam is used for treatment of epilepsy, non-convulsive status epilepticus and panic disorder. It also has many off-label indications for its use insomnia, acute mania, tardive dyskinesia, like restless leg syndrome and sleep behavior disorder **(Stroh et al., 2014).**

Clonazepam has a high potential for abuse, as well as for forming a tolerance and physical dependence, and ultimately addiction. The problem of abusing clonazepam becomes devastating especially it is purchased in some developing countries without prescription. The development of euphoria as well as mood changes as adverse effects of clonazepam use had been suggested as the enforcing factors to take more frequent doses **(Larrey and Ripault, 2013).**

Sudden clonazepam withdrawal should be avoided, especially in persons on long-term

therapy and high doses for panic disorder or seizures as it may result in withdrawal manifestations and status epilepticus. The listed withdrawal manifestations are irritability, tremors, anxiety, insomnia, headache, sweating, depression, confusion, seizures, and hallucinations **(Riss et al., 2008).**

Clonazepam is metabolized in the liver and excreted by the kidney; patients with hepatic or renal impairment requires monitoring for clonazepam level as it may lead to accumulation of the drug in the body especially in extreme of age "elderly and children". Clonazepam is contraindicated in severe liver and kidney disease **(Dash et al., 2017).**

Clonazepam initiation in patients with dyslipidemia has led to exacerbation of dyslipidemia and development of remarkable fatty liver. The author considered this as Clonazepam-induced steatohepatitis or steatosis. Patient hepatic conditions may render the hepatocytes more vulnerable to drug effects, change clonazepam hepatic metabolism, and can exacerbate clonazepam induced hepatic necrosis **(Kishimoto et al., 2019).**

Vitamin E (Alpha-tocopherol, "VE") is known to decrease lipid peroxidation, toxic effects and oxidative damage caused by reactive oxygen species (ROS). Alpha-tocopherol is the most active form of vitamin E and considered as a strong antioxidant that acts by many mechanisms for example it acts as a scavenger for free radicals, inhibiting peroxidation of lipids and suppressing apoptosis caused by ROS **(Rezq, 2014).**

In addition, Alpha-tocopherol is considered important factor in peroxidase-dependent antioxidant defense complex system, such as glutathione peroxidase (GPx) and catalase enzymes. Furthermore, Alpha-tocopherol, such as a powerful biological antioxidant, acts by synergistic mechanisms with other antioxidants to decrease free radicals **(Duan et al., 2018).**

Clonazepam misuse and abuse is along lasting health problem. Although several research had proved the deleterious hepatorenal effects of other antiepileptics like valproic acid and carbamazepine, those of clonazepam are understudied as well as understood. Few studies in the literature have discussed the hepatotoxic and nephrotoxic effects of clonazepam and the potential protective effect of Alpha-tocopherol against clonazepam induced chronic toxicity **(Björnsson, 2008).**

Both clonazepam and vitamin E are lipophilic and fat-soluble drugs. This combination hasn't been studied previously in literature. This work is the first, to our knowledge, to study the potential hepatotoxic and nephrotoxic deleterious effects of sub chronic misuse of high dose-clonazepam in an addiction module in albino rats to simulate what occur in human addicts and to illustrate the Alpha-tocopherol protective role. So, it can be considered as an adjuvant treatment for clonazepam dependence or for patients on prolonged clonazepam treatment as those complaining of recurrent manic episodes or prolonged seizures.

MATERIAL AND METHODS:

Study locality and ethical approval:

The current prospective randomized controlled experimental study was performed at Animal house of Research Institute of Ophthalmology (RIO) – in accordance with the institutional Animal Care and Use Committee (CU-IACUC), Faculty of Medicine, Cairo University. Approval of (CU-IACUC) was obtained (code number is CU-III-F-78-22). The care and use of Laboratory Animals in the present experiment had followed the National Research Council's Guide.

Animals

Forty (40) healthy adult albino male rats with average weight of (200-250) gram were included in the present experiment. Rats were kept under a standard laboratory condition of12-h light and 12-h dark cycles at a fixed room temperature average (23- 25°C) in plastic cages. Libitum, pellet and water were given as a standard laboratory feeding diet. Animals were divided into four groups randomly (10 rats in each group).

Drugs and chemicals

- Clonazepam (Rivotril 2 MG 30 tablets F. HOFFMAN LA ROCHE, Egyptian Pharmaceutical Trading Company).
- Vitamin E (Vitamin E cap 1000 mg cap Pharco Pharmaceuticals Company, Cairo, Egypt).

Study design:

1. Group I: Each rat received one ml 0.9% normal saline / day oral by gavage for 50 days and used as negative control group.
2. Group II (clonazepam treated group): a starting dose of (0.18 mg/kg/day) of

clonazepam was given to each rat orally by gavage for three days. The daily doses were gradually increased by addition of the starting dose every three days to the end of the first month in order to reach the maximum therapeutic or dependent dose of (0.36 mg/day) which was reported to produce the desired euphoria and dissociative effects in human addicts at the end of the 30 days. Furthermore, this last dependent dose (0.36 mg/day) was given every day for the next 10 days. After that, 5 rats of the ten were sacrificed and the remaining five rats were kept for another 10 days to evaluate clonazepam induced histopathological changes during withdrawal period after sudden stoppage of clonazepam (Schifano et al., 2011).

3. Group III (vitamin E): Each rat received alpha tocopherol (100 mg/kg/day) orally by gavage for 50 days and used as positive control group (Taha et al., 2020).

4. Group IV (clonazepam + Vitamin E): Each rat received clonazepam (0.18 mg/kg/day) plus alpha tocopherol (100 mg/kg/day) orally by gavage for three days. After that each rat received the same daily dose of alpha tocopherol while the dose of clonazepam was increased by addition of the starting dose every three days to the end of the 30 days. Further, the dependent dose of clonazepam (0.36 mg/day) + alpha tocopherol (100 mg/kg/day) were given daily for another 10 days. Then alpha tocopherol (100 mg/kg/day) were continued alone during the period of withdrawal of ten days after sudden stoppage of clonazepam (Schifano et al., 2011; Taha et al., 2020).

Clonazepam starting dose was equal to the daily therapeutic dose of 2 mg/day while the dependent dose is equal to the dose which leads to the dissociative effects and desired euphoria in human addicts (20 mg/day) with conversion to the rat dose using Paget calculation. The dose of vitamin E (100 mg/kg/day) or (20 mg/day) is equal to the upper tolerable intake levels of vitamin E in human adult according to Paget equation (Taha et al., 2020). The equivalent dose for a 200 g rat is = 18/1000 x average adult human therapeutic daily dose (Paget and Barnes, 1964; Badawi et al., 2016; Nair and Jacob, 2016).

Sampling:

Cervical decapitation was used as a method of euthanasia of the animals for avoiding any contamination or chemical injury to rat tissues (Nakai et al., 2005; Underwood and Anthony, 2020). At end of the study, the weight of the rat of all groups was measured and compared with their weight at the start of the study. Samples of the blood were taken from abdominal aorta. Centrifugation at 5000 rpm for 15 min was performed to obtain the serum and then was kept frozen for biochemical testing at - 80 ◦C. The liver and kidney tissues were excised, the liver and kidney weight were measured and compared between different groups and then were held and washed using cold saline solution then were divided and prepared for histopathological examination.

METHODS

- Evaluation of hepatic functions:

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) serum levels were measured by colorimetric kinetic assays using commercially available kits from (Diamond diagnostic, Egypt) following the manufacturer's instructions.

- Evaluation of renal functions:

Serum creatinine was measured by standard Jaffe method (colorimetric kinetic method) using commercial kits (Diamond diagnostic, Egypt). Serum urea was measured by Brethelot enzymatic colorimetric assay (Diamond diagnostic, Egypt). Serum uric acid was measured by uricase method, colorimetric assay (Diamond diagnostic, Egypt). The concentration was measured against known standard concentrations according to the manufacturer.

- Histopathological study:

Paraffin blocks were made by putting liver and kidney specimens in 10% formol-saline then sectioned at 5-µm-thickness and stained with hematoxylin and eosin (H & E) (Carleton and Drury, 1980).

- Histopathological score:

Hepatic histopathological score was determined in 10 non-overlapping fields chosen randomly. The score showed histopathological changes in venous congestion, necrosis, hydropic degeneration of hepatocytes and lymphatic infiltration. The following score grades were used +++ (high damage), ++ (moderate damage), + (mild damage) and – (no lesion).

Renal histopathological score was determined in 10 non-overlapping fields

chosen randomly. The score showed some renal corpuscle histopathological changes, renal tubules degeneration, and the interstitial mononuclear cell infiltration and hemorrhage. The following score grades were used +++ (high damage), ++ (moderate damage), + (mild damage) and – (no lesion).

Statistical analysis:

The collected data were processed, coded, and analyzed using SPSS version 27 for Windows ® (IBM SPSS Inc, Chicago, IL, USA). The normality of distribution for the analyzed variables was tested using Kolmogorov-Smirnov test assuming normality at $p > 0.05$. Parametric data were expressed as mean ± standard deviation, while the non-parametric data were expressed as median (range). The one-way analysis of the variance (one-way ANOVA) was used to compare the normally distributed quantitative variables and Krauskal Wallis test was used as test of significance to compare independent non-parametric quantitative data. The highly statistically significant (HS) was ($p \leq 0.001$), while the accepted significance level was ($p < 0.05$) and non-statistically significant (NS) was ($p > 0.05$).

RESULTS

1) Effect of long-term clonazepam administration on weight gain:

Initial body weight was statistically comparable between the four groups. Table 1 shows initial and final body weights as well as weight gain percentage of all groups. There was significant increased body weight in clonazepam misuse groups (G II, IV) (p 0.001) at the end of the study. The body weight change percent was significantly high in clonazepam misuse groups (G II, IV) as compared to group (G I) (p 0.001, 0.003) respectively and as compared to group (G III) (p 0.001, 0.021) respectively (table 1, Fig 1).

Table (1): Comparison of body weight among the studied groups.

Variables	Group I Control (n= 10)	Group II Clonazepam (n= 10)	Group III Vit E (n= 10)	Group IV Clonazepam + Vit E (n= 10)	Significance test
Initial (g) Mean ± SD	187.60 ± 4.06	189.30 ± 4.16	187.90 ± 6.26	188.80 ± 6.46	**F= 0.216 P= 0.885**
At 6 weeks (g) Mean ± SD	237.70 ± 5.64	262.50 ± 8.58	241.10 ± 5.67	256.40 ± 8.88	**F= 26.268 P < 0.001***
P1		**< 0.001***	0.731	**< 0.001***	
P2			**< 0.001***	0.266	
P3				**< 0.001***	
Percent of change (%) Mean ± SD	26.80 ± 5.19	38.73 ± 5.34	28.46 ± 5.76	35.90 ± 5.44	**F= 11.183 P < 0.001***
P1		**< 0.001***	0.902	**0.003***	
P2			**0.001***	0.654	
P3				**0.021***	

F: One-Way ANOVA
*: Statistically significant ($p \leq 0.05$)
P1: Significance in relation to G1
P2: Significance in relation to G2
P3: Significance in relation to G3

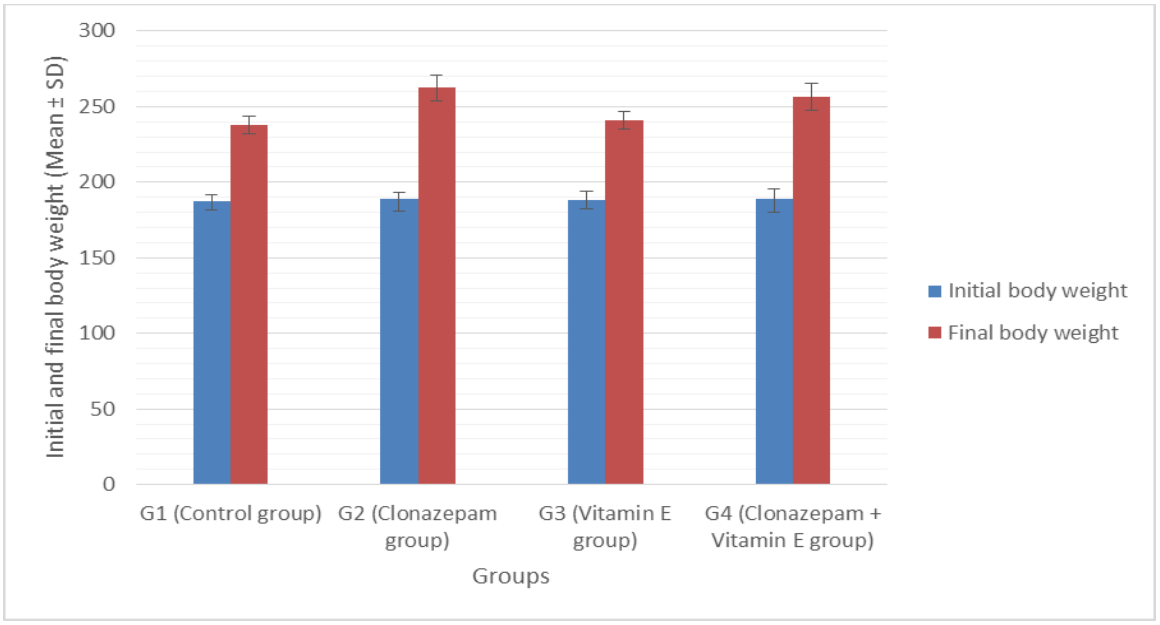

Figure (1): initial and final body weights between different groups.

2) Effect of long-term clonazepam administration on liver & kidney weight: No significant results were detected as regarding liver and kidney weight changes in clonazepam groups in comparison to control groups (table 2, Fig 2).

Table (2): Comparison of liver and kidney weight within the study groups.

Variables	Group I Control (n= 10)	Group II Clonazepam (n= 10)	Group III Vit E (n= 10)	Group IV Clonazepam + Vit E (n= 10)	Significance test
Liver weight (g) Mean ± SD	7.31 ± 0.72	7.40 ± 0.72	7.60 ± 0.57	7.27 ± 0.48	**F= 0.546 P = 0.654**
Kidney weight (g) Mean ± SD	1.34 ± 0.04	1.37 ± 0.15	1.37 ± 0.05	1.42 ± 0.05	**F= 1.384 P = 0.263**

F: One-Way ANOVA

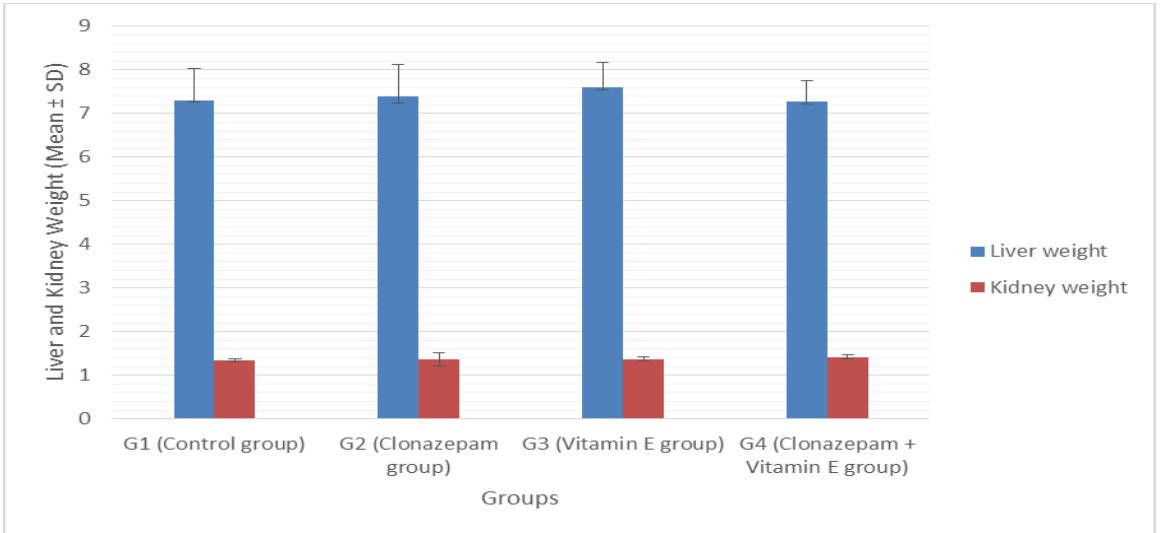

Figure (2): Effect of clonazepam and vitamin E administration on liver and kidney weights.

3) Evaluation of biochemical markers:

Clonazepam treatment (G II, IV) showed significant increased ALT, AST and ALP serum levels, as compared to control groups (G I, III) ($p < 0.001$). Co-administration of Vitamin E with Clonazepam (G IV) showed significant decreased ALT and AST levels as compared to clonazepam group (G II) ($p < 0.001$) that indicates its protective role (table 3, Fig 3, 4).

Similarly, Clonazepam treatment (G II, IV) significant increased urea, creatinine and uric acid serum levels as compared to the control group (G I, III) ($p < 0.001$). Co-administration of Vitamin E with Clonazepam (G IV) showed significant decreased serum levels of urea, creatinine and uric acid as compared to clonazepam group (G II) ($p < 0.001$). This indicates its renal ameliorative role (table 4, Fig 5, 6).

Table (3): Liver function tests within the study groups.

Variables	Group I Control (n= 10)	Group II Clonazepam (n= 10)	Group III Vit E (n= 10)	Group IV Clonazepam + Vit E (n= 10)	Significance test
AST (IU/L) Mean ± SD	20.80 ± 1.81	57.20 ± 4.47	22.40 ± 2.32	39.10 ± 2.51	**F= 331.549 P < 0.001***
P1		**< 0.001***	0.624	**< 0.001***	
P2			**< 0.001***	**< 0.001***	
P3				**< 0.001***	
ALT (IU/L) Mean ± SD	20.10 ± 2.51	46.50 ± 3.27	17.10 ± 2.56	32.40 ± 3.20	**F= 212.128 P < 0.001***
P1		**< 0.001***	0.116	**< 0.001***	
P2			**< 0.001***	**< 0.001***	
P3				**< 0.001***	
ALP (IU/L) Mean ± SD	516.90 ± 21.97	656.90 ± 77.36	500.90 ± 31.91	616.70 ± 73.87	**F= 17.817 P < 0.001***
P1		**< 0.001***	0.922	**0.002***	
P2			**< 0.001***	0.402	
P3				**< 0.001***	

F: One-Way ANOVA
*: Statistically significant ($p \leq 0.05$)
P1: Significance in relation to G1
P2: Significance in relation to G2
P3: Significance in relation to G3

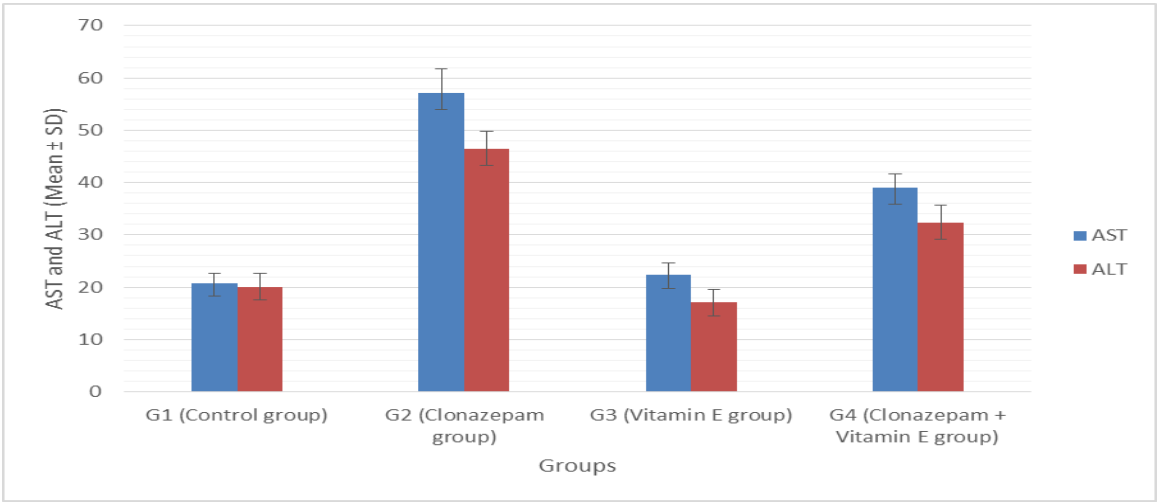

Figure (3): Effect of clonazepam and vitamin E on AST and ALT levels.

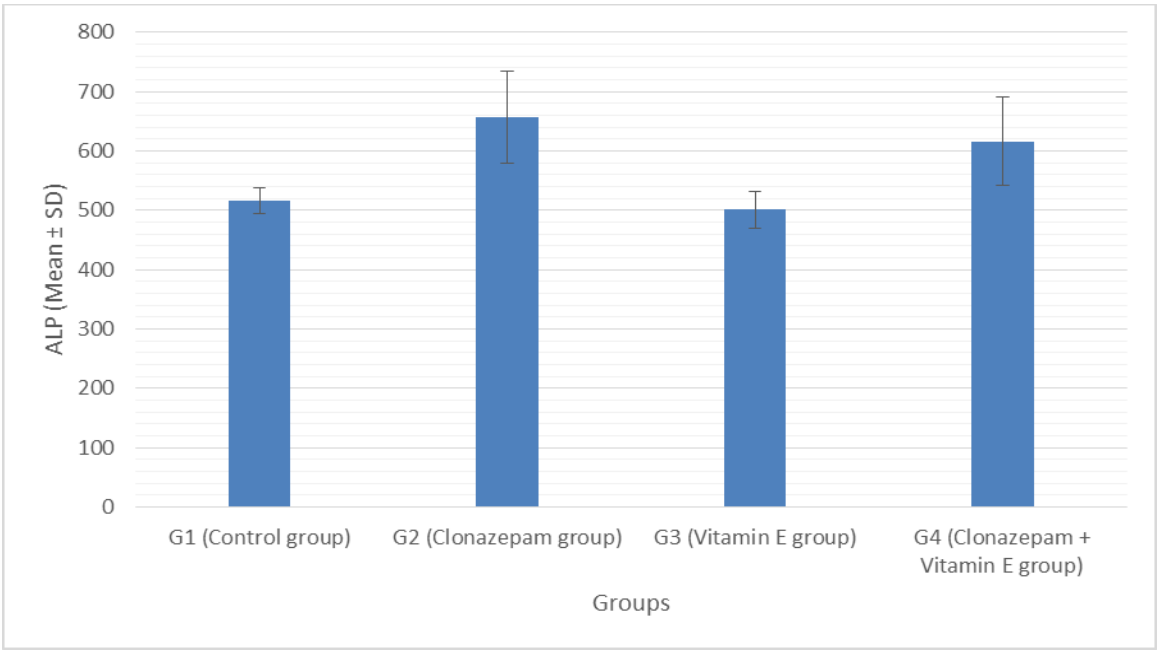

Figure (4): Effect of clonazepam and vitamin E on ALP level.

Table 3 shows that AST, ALT & ALP levels were elevated (P value = 0.001) among clonazepam treated groups (G II, IV) than other groups (G I, III). Also more significant elevations in AST and ALT levels were showed in group II than group IV (P value = 0.001*).

Table (4): Kidney function tests within the study groups.

Variables	Group I Control (n= 10)	Group II Clonazepam (n= 10)	Group III Vit E (n= 10)	Group IV Clonazepam + Vit E (n= 10)	Significance test
Urea mg/dl Mean ± SD	20.80 ± 2.44	34.10 ± 2.64	23.20 ± 3.91	28.50 ± 2.68	**F= 69.567 P < 0.001***
P1		**< 0.001***	0.288	**< 0.001***	
P2			**< 0.001***	**0.001***	
P3				**0.002***	
Creatinine mg/dl Mean ± SD	0.60 ± 0.03	0.76 ± 0.05	0.61 ± 0.03	0.68 ± 0.02	**F= 54.764 P < 0.001***
P1		**< 0.001***	0.963	**< 0.001***	
P2			**< 0.001***	**< 0.001***	
P3				**< 0.001***	
Uric acid mg/dl Mean ± SD	3.16 ± 0.18	5.52 ± 0.54	3.23 ± 0.18	4.31 ± 0.56	**F= 73.735 P < 0.001***
P1		**< 0.001***	0.981	**< 0.001***	
P2			**< 0.001***	**< 0.001***	
P3				**< 0.001***	

F: One-Way ANOVA
*: Statistically significant ($p \leq 0.05$)
P1: Significance in relation to G1
P2: Significance in relation to G2
P3: Significance in relation to G3

Table 4 shows that urea, creatinine & uric acid levels were elevated (P value = 0.001) among clonazepam treated groups (G II, IV) than other groups (G I, III). Also more significant elevation was showed in group II than group IV (P value = 0.001*).

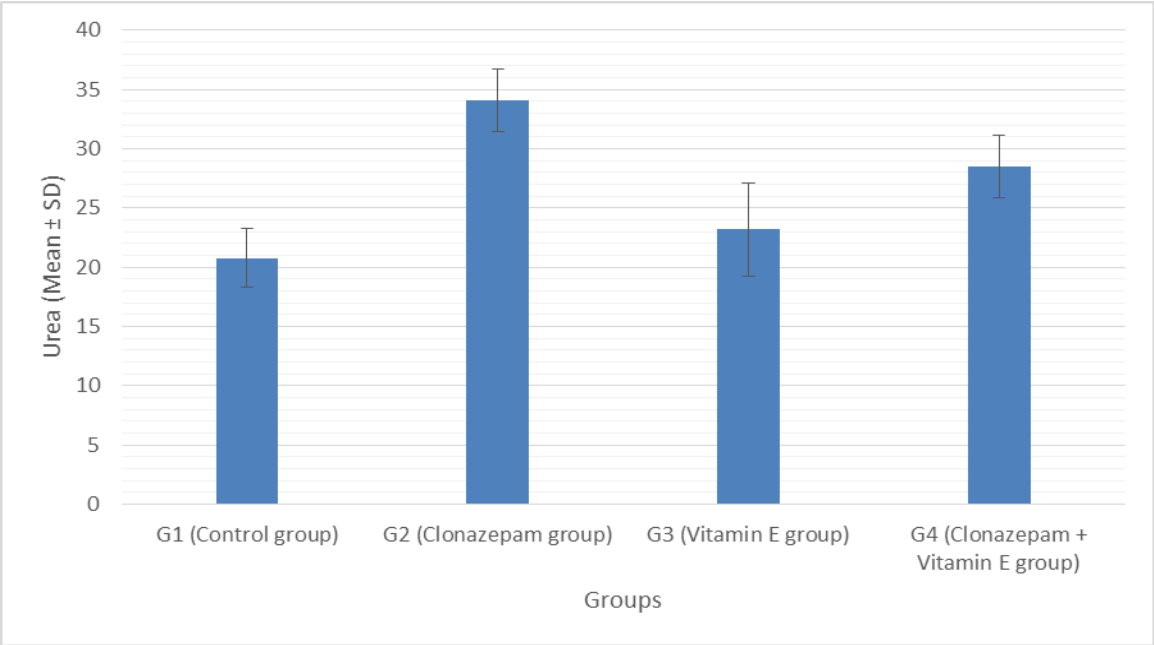

Figure (5): Effect of clonazepam and vitamin E on urea level.

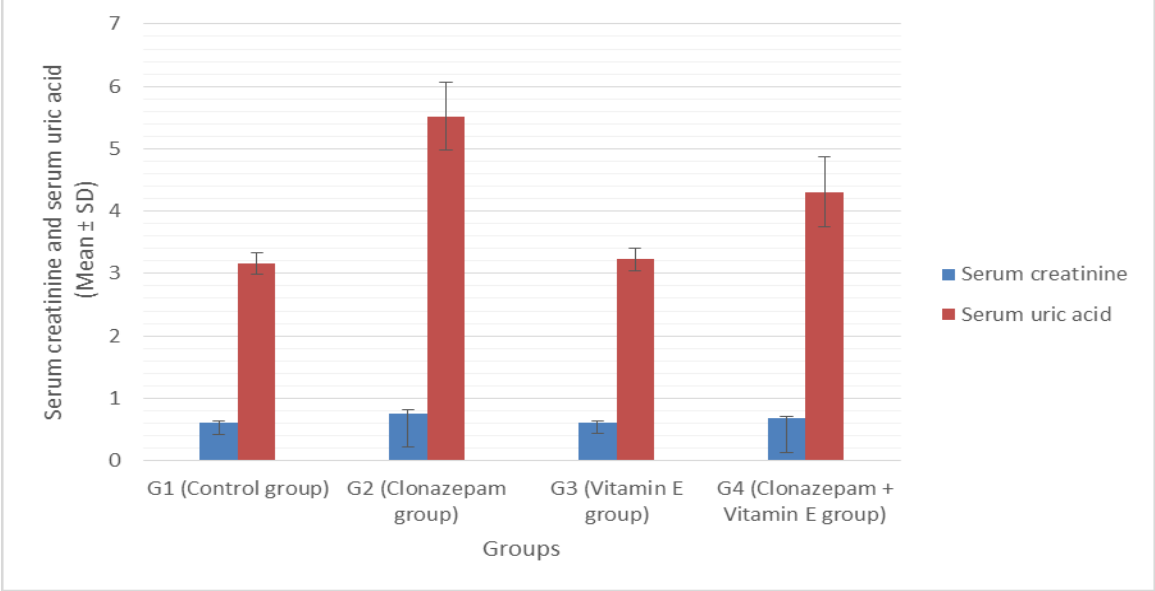

Figure (6): Effect of clonazepam and vitamin E on creatinine and uric acid levels.

Evaluation of histopathological changes:

I. Clonazepam-induced liver damage:

Liver sections obtained from control and vitamin E groups (G I, III) showed that hepatocytes were grouped in cords emanating from central veins, demonstrating the classical hepatic architecture. Hepatocytes were polyhedral with acidophilic granular cytoplasm and central vesicular spherical nuclei. Hepatic sinusoids appeared as narrow gaps between hepatic cords lined by flat endothelial cells and a few numbers of Kupffer cells. At the periphery of the hepatic lobules, portal tracts consisted of a branch of the portal vein and a bile duct (Figure7).

While sections from group (G II) (Clonazepam treated) demonstrated altered lobular shape with hydropic degenerated cells and nuclear degradation. Some hepatocytes show moderate fatty degeneration and dilated

blood sinusoids in between. There is an irregular enlargement of the hepatic central vein with lymphocytic infiltration. The portal vein appears congested with a thick wall and lymphocytic infiltration around a normal-appearing bile duct (Figure 8).

After sudden stoppage of clonazepam (withdrawal period) in the second group (G II), liver showed the typical arrangement of hepatocytes with very few lipid droplets. Several hepatic portal veins exhibited congestion. Several hepatocytes still exhibited signs of fatty degradation with nuclear degradation. Most hepatic regions revealed normal central and portal veins with intact endothelial walls. Intact bile ducts are also observed (Figure 9).

Treatment of rats with both clonazepam and vitamin E (G IV) showed potential protection against liver injury present in clonazepam group. The liver showed that majority of hepatic lobular architecture was intact with central veins that were rather normal, however, the central vein and blood sinusoids of some hepatic lobules remained dilated and/or congested. There is considerable congestion and a moderate fatty degeneration. Some hepatocytes exhibited a degree of degeneration, aberrant pale-stained vacuolated cytoplasm with either vesicular, condensed, dark-stained nuclei or lost their nuclei while some hepatocytes showed normal structure, retaining eosinophilic cytoplasm and vesicular nuclei. Some ballooned cells with fragments of cytoplasm and either heavily pigmented or missing nuclei (Figure 10).

Sections from rats during withdrawal period of (G II, IV) groups showed signs of improvement of hepatic tissue organization as compared to G II during active treatment with clonazepam. Both misuse groups (G II, IV) showed histological improvement after sudden stoppage of clonazepam with better result in group (G IV) during recovery period (table. 5).

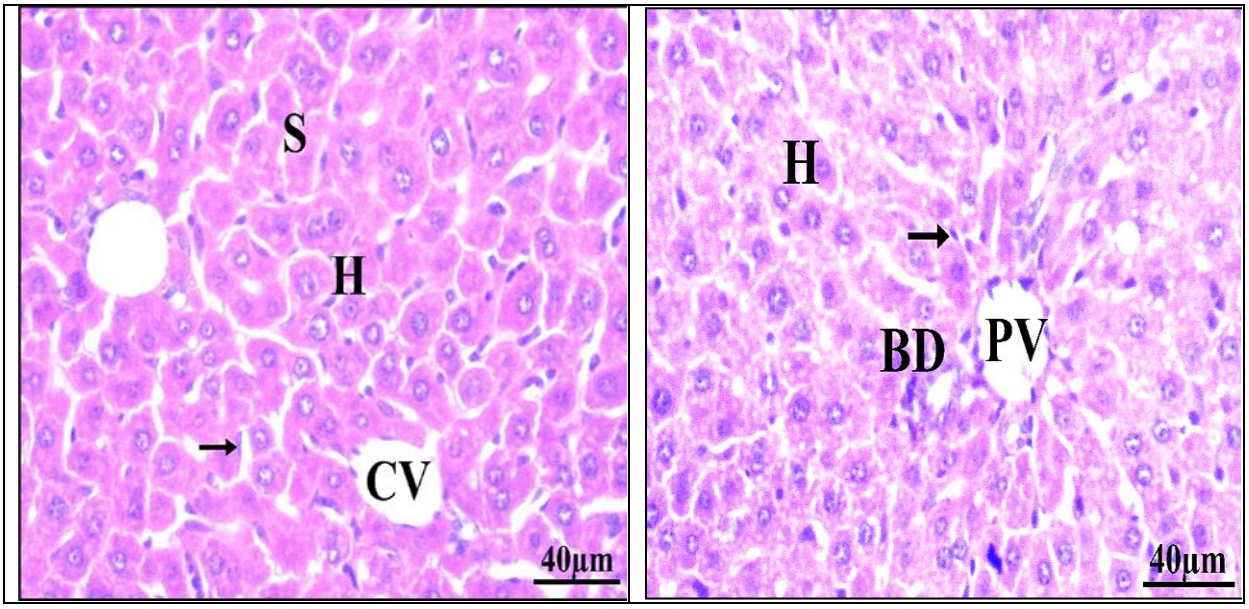

Figure (7): H&E-stained hepatic sections: Control and vitamin E (G I, III) groups revealed that hepatocytes were grouped in cords emanating from central veins (CV), demonstrating the classical hepatic architecture. Hepatocytes (H) were polyhedral with acidophilic granular cytoplasm and central vesicular spherical nuclei. Hepatic sinusoids (S) appeared as narrow gaps between hepatic cords lined by flat endothelial cells and a few numbers of Kupffer cells (Arrow). At the periphery of the hepatic lobules, portal tracts consisted of a branch of the portal vein (PV) and a bile duct (BD).

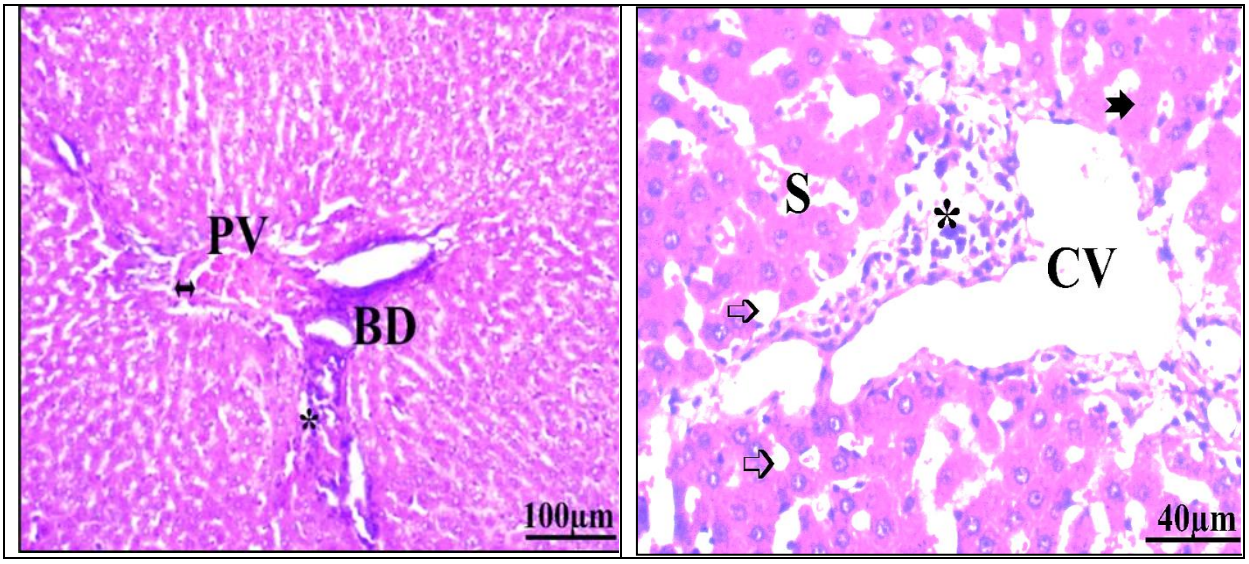

Figure (8): H&E-stained hepatic sections: from group (G II) (clonazepam treated) showed altered lobular shape with hydropic degenerated cells and nuclear degradation (notched arrow). Some hepatocytes showed moderate fatty degeneration (hollow arrow) and dilated blood sinusoids (S) in between. There is an irregular enlargement of the hepatic central vein (CV) with lymphocytic infiltration (*). The portal vein (PV) appears congested with a thick wall (double-headed arrow) and lymphocytic infiltration (*) around a normal-appearing bile duct (BD).

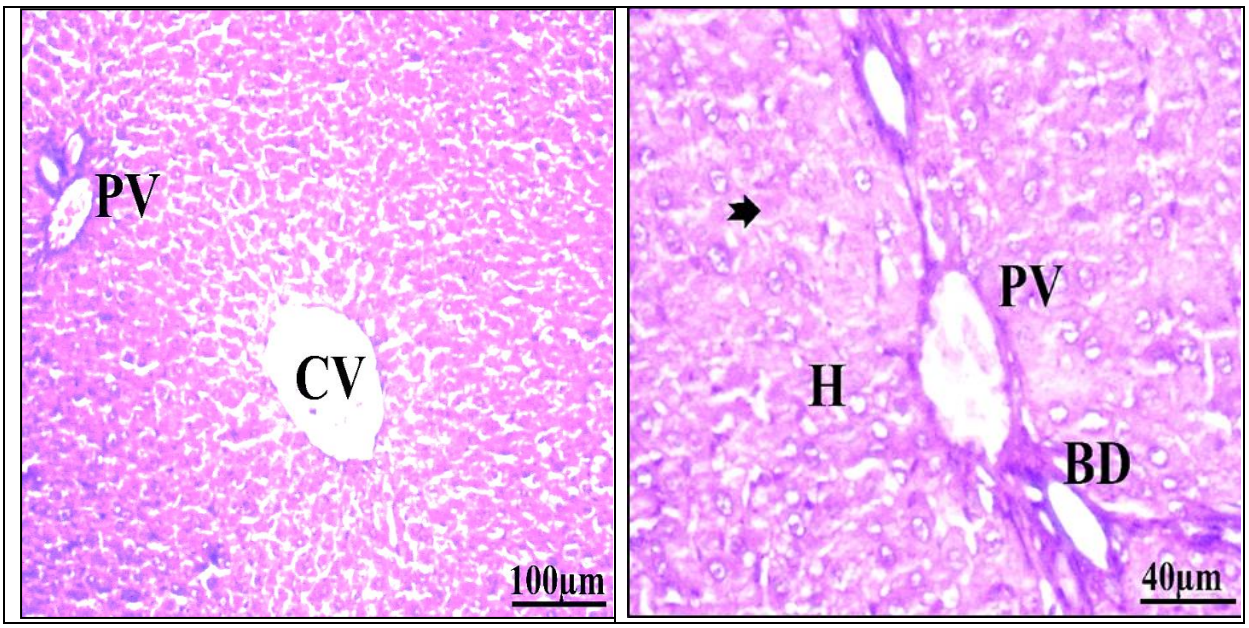

Figure (9): H&E-stained hepatic sections from group (G II) (clonazepam treated) after sudden stoppage of clonazepam (recovery or withdrawal period) showed the typical arrangement of hepatocytes with very few lipid droplets. A number of hepatic portal veins (PV) exhibited congestion. Several hepatocytes (H) still exhibited signs of fatty degradation with nuclear degradation (notched arrow). The majority of hepatic regions revealed normal central and portal veins with intact endothelial walls. Intact bile ducts (BD) are also observed.

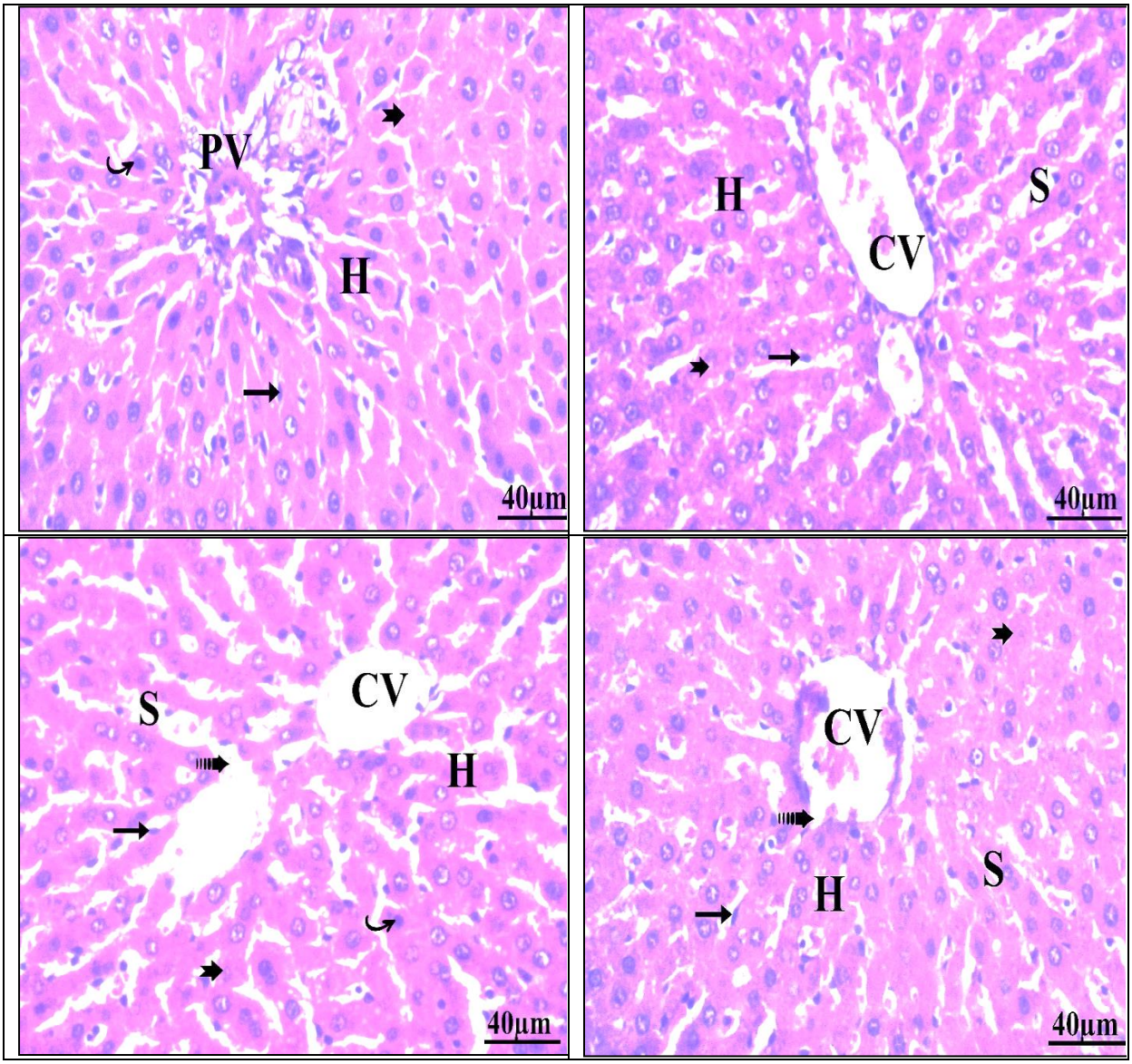

Figure (10): H&E-stained hepatic sections from group (G IV) (clonazepam + vit E) showed that majority of hepatic lobular architecture was intact with central veins that were rather normal, however, the central vein (CV) and blood sinusoids (S) of some hepatic lobules remained dilated and/or congested. There is considerable congestion and a moderate fatty degeneration. Some hepatocytes (H) exhibited a degree of degeneration (nuclear deterioration), aberrant pale-stained vacuolated cytoplasm with either vesicular, condensed, dark-stained nuclei (curved arrow) or lost their nuclei (notched arrow) while some hepatocytes showed normal structure, retaining eosinophilic cytoplasm and vesicular nuclei. Some ballooned cells (wavy arrow) with fragments of cytoplasm and either heavily pigmented or missing nuclei (notched arrow).

Table (5): Results of the scoring of the hepatic effects of the four groups.

	Group (I, III) Control & vit E	Group (II) Clonazepam		Group (IV) Clonazepam +Vitamin E
		First 40 days	Withdrawal Last 10 days	
Congestion blood vessel	-	+++	+	++
Lymphatic infiltration	-	++	-	++
Dilated portal vein	-	++	+	+
Dilated sinusoid	-	++	-	+
Necrosis	-	++	-	-
Fatty degeneration	-	++	-	++
Hepatocyte degeneration	-	++	+	+

II. Clonazepam-induced renal damage:

Light microscopy analysis of H&E-stained sections from control and vitamin E groups (G I, III) revealed the typical renal histological architecture. Renal corpuscles, glomerular capillaries, Bowman's capsules, and Bowman space filled the renal cortex. The proximal convoluted tubules make up the majority of the renal cortex and are situated close to the renal corpuscles. They were bordered with cuboidal epithelial cells and had limited lumens. The distal convoluted tubules had a broad lumen and were bordered with short cuboidal cells with an acidophilic cytoplasm that was less granular and rounded nuclei (Figure 11).

While sections from (G II) (clonazepam treated) showed injured irregular glomeruli with obliterated bowman capsules and degenerated cells. The majority of tubular cells exhibited vacuolated cytoplasm and nuclei with vesicles. Distal tubules appear with exfoliated epithelial cell lining, peritubular space dilation, capillary congestion and lymphocytic infiltration (Figure 12).

After sudden stoppage of clonazepam (withdrawal period) in the second group (G II), most kidney glomeruli and tubule's structure were restored. The majority of glomeruli, bowman space and tubules returned to normal. However, some renal tubular cells revealed hydropic degeneration while others showed thinning of their epithelial lining and degenerated nuclei (Figure 13).

Treatment of rats with both clonazepam and vitamin E (G IV) showed potential protection against kidney injury present in clonazepam group. Some convoluted tubule epithelial cells revealed considerable vacuolation and degenerative alterations. The glomeruli within Bowman's capsules have a typical size and volume of capsular space. Few glomeruli were atrophic. Some glomeruli were fragmented and lobulated. No evidence of lymphocytic infiltration was found. There was moderate rate of congestion (Figure 14).

Sections from rats during withdrawal period of (G II, IV) groups showed signs of improvement of renal tissue organization as compared to G II during active treatment with clonazepam. Both misuse groups (G II, IV) showed histological improvement after sudden stoppage of clonazepam with better result in group (G IV) during recovery period (table. 6).

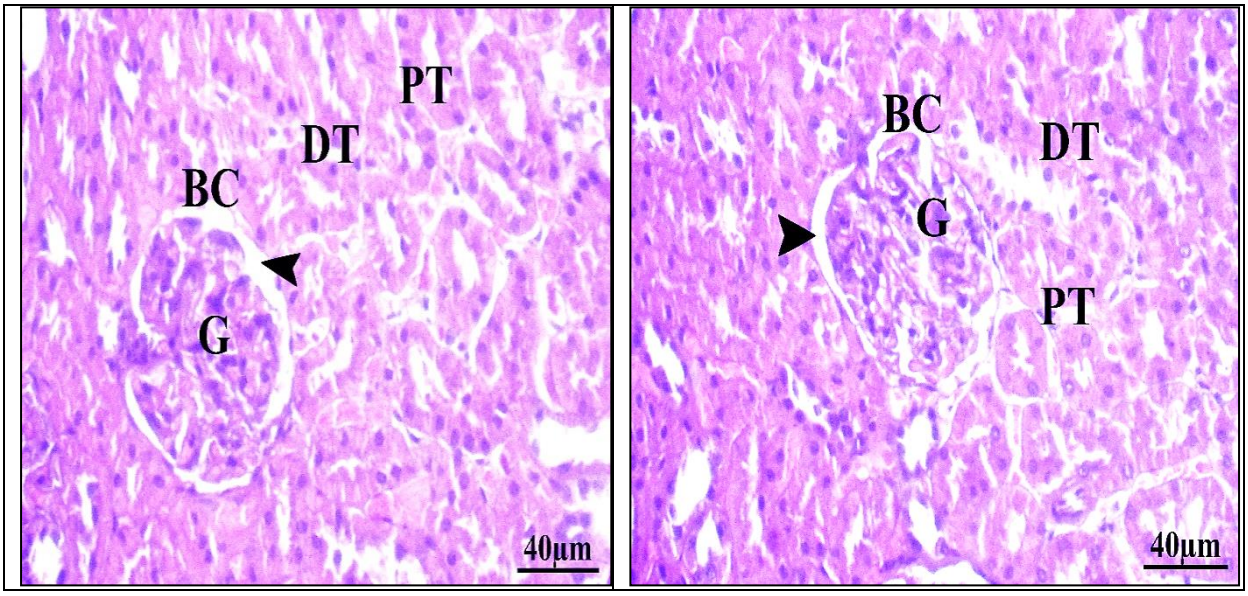

Figure (11): H&E-stained sections of control and vitamin E groups (G I, III) revealed typical renal histological architecture. Renal (Malpighian) corpuscles, glomerular capillaries (G), Bowman's capsules (BC), and Bowman space (arrowhead) filled the renal cortex. The proximal convoluted tubules (PT) make up the majority of the renal cortex and are situated close to the renal corpuscles. They were bordered with cuboidal epithelial cells and had limited lumens. The distal convoluted tubules (DT) had a broad lumen and were bordered with short cuboidal cells with an acidophilic cytoplasm that was less granular and rounded nuclei.

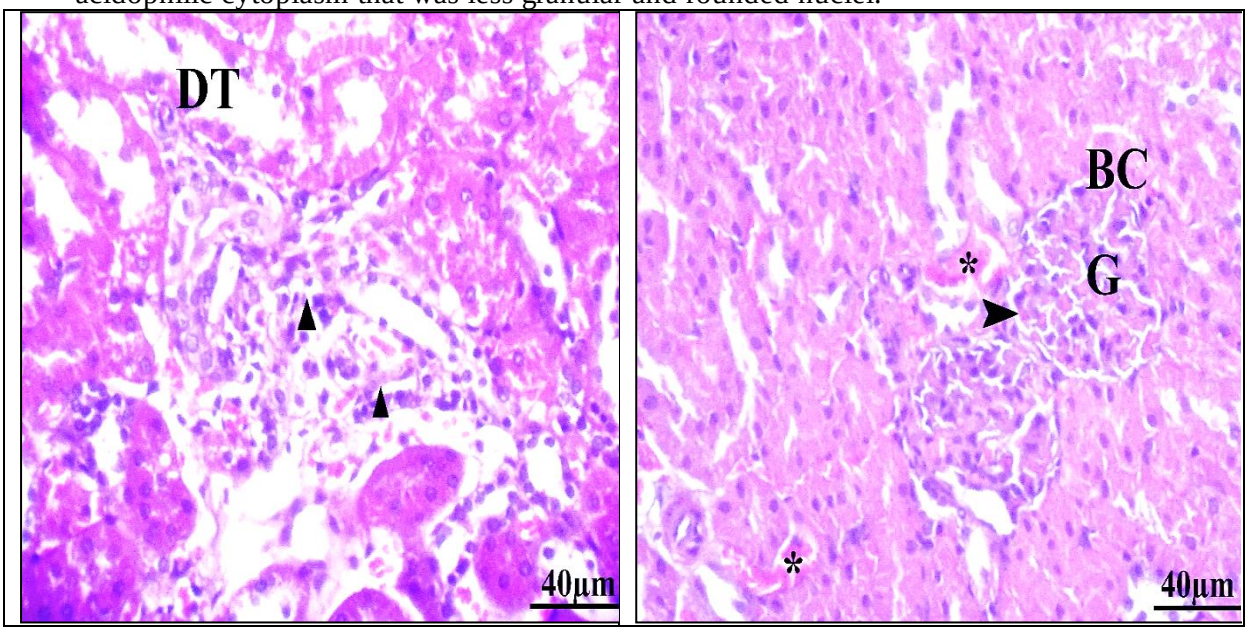

Figure (12): H&E-stained renal cortical sections from group (G II) (clonazepam treated) showed injured irregular glomeruli (G) with obliterated bowman capsules (BC) and degenerated cells (arrow head). The majority of tubular cells exhibited vacuolated cytoplasm (arrow) and nuclei with vesicles. Distal tubules appear with exfoliated epithelial cell lining (DT), peritubular space dilation, capillary congestion (*) and lymphocytic infiltration (triangle).

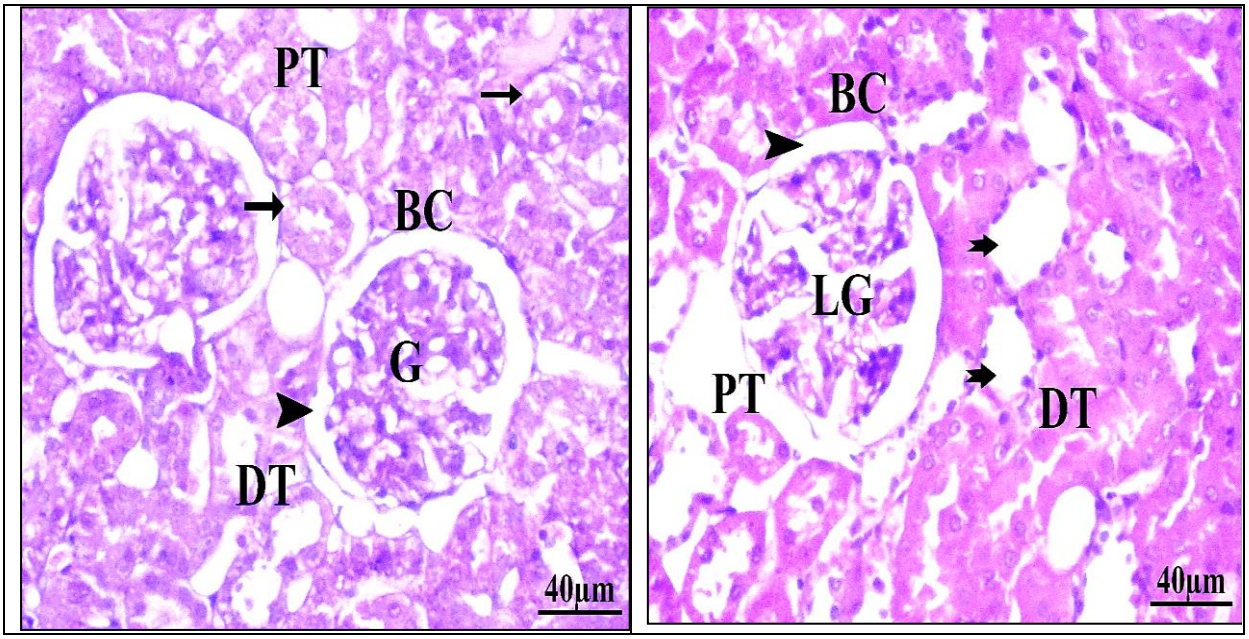

Figure (13): H&E-stained renal cortical sections from group (G II) (clonazepam treated) after sudden stoppage of clonazepam (recovery or withdrawal period) showed that most kidney glomeruli and tubule's structure were restored. The majority of glomeruli (G), bowman space (arrowhead) and tubules (PT & DT) returned to normal. However, some renal tubular cells reveal hydropic degeneration (arrow) while others show thinning of their epithelial lining and degenerated nuclei (notched arrow).

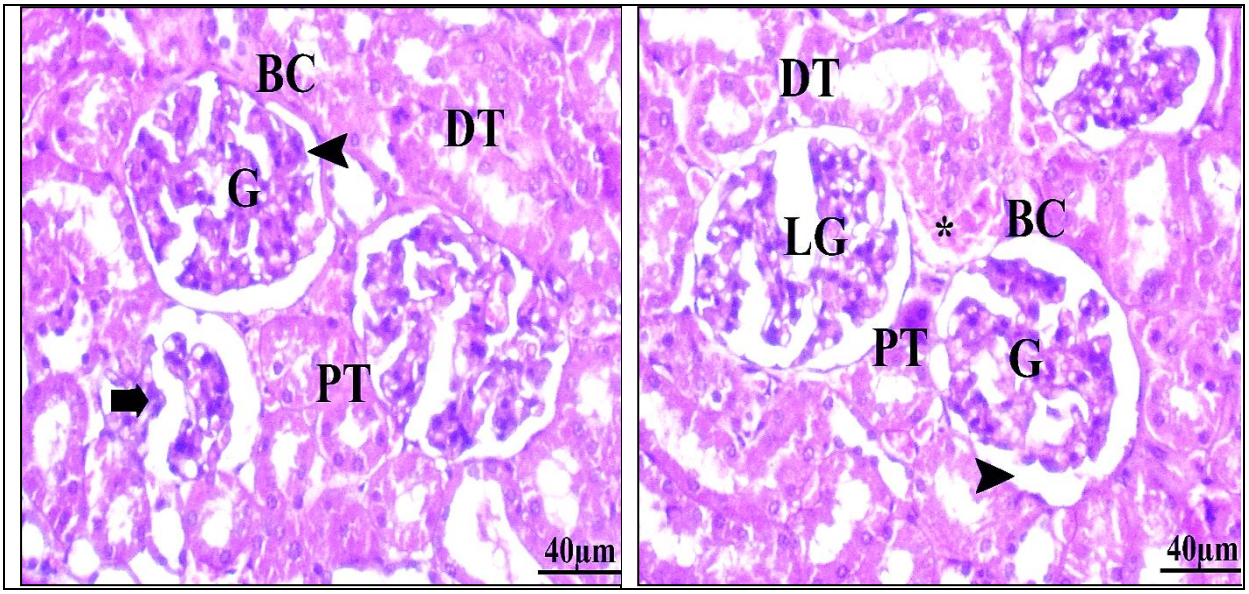

Figure (14): H&E-stained renal cortical sections from group (G IV) (clonazepam + vit E) showed that some renal convoluted tubule (PT & DT) epithelial cells revealed considerable vacuolation and degenerative alterations. The glomeruli (G) within Bowman's capsules (BC) have a typical size and volume of capsular space (arrowhead). Few glomeruli were atrophic (bold arrow). Some glomeruli were fragmented and lobulated (LG). No evidence of lymphocytic infiltration was found. There was moderate rate of congestion.

Table 6: Results of the scoring of the renal effects of the four groups.

	Group (I, III) Control & vit E	Group (II) Clonazepam		Group (IV) Clonazepam +Vitamin E
		First 40 days	Withdrawal last 10 days	
Congestion	-	+++	-	++
Lymphatic infiltration	-	+	-	-
Atrophied glomeruli	-	-	-	++
Tubular hydropic degeneration	-	+	+	+
Swollen glomeruli	-	++	+	-

DISCUSSION

Addiction is considered a prevalent and lethal disease all over the world that carries several social and economic impacts. Many evidence demonstrated increasing misuse and abuse of clonazepam among the adolescents **(Al-Husseini et al., 2018).**

Abusing clonazepam had become devastating especially it is purchased without prescription. The development of the euphoria as well as mood changes as adverse effects of clonazepam use had been suggested as the enforcing factors to take more frequent doses **(Larrey and Ripault, 2013).**

The present experiment studied the hepatotoxic and nephrotoxic effects of clonazepam sub chronic high dose exposure in albino rats as well as the important alpha-tocopherol role in ameliorating clonazepam induced hepatorenal toxicity.

The authors intended to produce an addiction module of clonazepam as regards the mode of administration as well as the dose and duration to be similar to what occurs with human addicts. This agrees with many previous studies that use this addiction module **(Taha et al., 2020; Elgazzar et al., 2021; Elsukary et al., 2022)** and case reports **(Ashwini et al., 2015; Carrus and Schifano, 2012; Yargic and Ozdemiroglu, 2011).**

In the current study, the long-term misuse of high doses of clonazepam was associated with significant weight gain. Animals in clonazepam treated groups (G II, IV) revealed significant weight gain compared to control (G I, III) groups at the end of the study. This might occur because clonazepam may increase appetite and may cause water retention. No significant results were detected as regarding liver and kidney weight changes in clonazepam treated groups (G II, IV) in comparison to other groups (G I, III).

Basińska-Szafrańska, 2021 stated that weight gain is the most common side effects associated with clonazepam. Weight changes related to clonazepam use are due to changes in energy levels and alertness. Clonazepam has been proven to slow down the metabolic process that converts food into energy, leading to more fat storage and subsequent weight gain. Clonazepam might also increase appetite, which can lead to weight gain over time.

Patient's metabolism usually and rapidly is altered by sleep disturbances. Poor sleep potentially has been linked to rapid weight gain and a slower metabolism. When a person takes clonazepam, they metabolize it in the liver. Patient suffering from previous liver damage may be unable to metabolize clonazepam, that usually impact clonazepam induced weight changes. People also found that it is very difficult to practice exercise while receiving clonazepam **(Blasi, 2000).**

Recently, **Buraniqi et al., 2022** systematically reviewed studies and stated that Anti-seizure medications (ASMs) had several adverse effects, and many of them may affect appetite, thus affecting weight gain and normal growth. In this research clobazam was included among the drugs which increase the appetite. In fact, clonazepam is roughly ten times stronger than clobazam, meaning it can have a very strong effect on weight gain especially in small children.

This current experiment showed that the sub chronic long-term misuse of clonazepam had resulted in numerous hepatorenal deleterious effects as indicated by biochemical and histopathological derangements. Furthermore, clonazepam withdrawal for 10

days provides great improvements in histopathological appearance. Also the use of alpha-tocopherol with clonazepam has inhibited the much more histopathological damage that occurs with clonazepam alone, emphasizing the protective role of alpha-tocopherol as antioxidant drug.

In the current work, clonazepam treated rats showed elevated levels of AST, ALT and ALP that indicate hepatic dysfunction. This is going in the same way with many previous studies and case reports that demonstrated high doses of clonazepam was associated with biochemical hepatic dysfunction **(Devarbhavi, 2012; Schumacher and Guo, 2015; Kishimoto et al., 2019).**

Nair et al., 2017 stated that serum ALT elevations are un-common with clonazepam therapy; also clinically evident acute liver injury is extremely rare. However, clonazepam as a member of benzodiazepines can lead to acute liver deterioration with recurrence or re-exposure and acute liver injury has been reported as many case reports. The latency period in benzodiazepine induced acute liver injury has ranged from few weeks to 6 months. The liver enzyme elevations patterns were usually mixed or cholestatic, but few reported cases were of hepatocellular patterns. On severity basis it is usually mild to moderate or self-limited. Autoantibody formation, rash or fever has not been described.

In the current research, we use long term high dose clonazepam however **Zimmerman, 1999** stated that the likelihood score of clonazepam induced hepatic injury is (D) (possible but rare cause of clinically apparent liver injury). The cause of benzodiazepines induced liver injury is probably a rarely produced toxic intermediate metabolite. The wide safety of these drugs is probably due to potentially low daily doses (from 5 to 10 mg).

Complete recovery, without evidence of residual or chronic injury is the usual outcome of many case reports of clonazepam induced liver injury. Neither chronic liver injury nor acute fulminant hepatic failure due to clonazepam has been described in the literatures **(Ahmed and Siddiqi, 2006).**

Our study is the first to detect clonazepam induced renal toxicity after long term high doses administration of clonazepam. Renal function tests had been elevated after sub chronic administration of clonazepam. Degenerative lesions and disintegrated nuclei in hepatic acini together with renal cortical hydropic degeneration, tubular cast, and glomerular atrophy as well as abnormal hepatic and renal function tests had been detected.

Previous studies however were minimal, they supported our finding. Authors stated that although it is a very rare adverse effect as compared to carbamazepine or valproic acid, long term use of clonazepam can induce acute kidney injury. They also advised prolonged follow-up for serum creatinine, urea, and uric acid results especially for those who use clonazepam for long term period or in addicts who take much higher doses **(Keränen and Sivenius, 1983).**

When vitamin E was used in parallel with clonazepam there were decreased serum levels of hepatic enzymes and renal function tests as well as reduction of their histopathological abnormalities. The current findings are in agreement with previous studies of vitamin E protective role as antioxidant against oxidative cell injury induced by different xenobiotics **(Santos, et al., 2016; Welson et al., 2021).**

The mechanisms of antioxidant properties of alpha tocopherol had been postulated in many previous research. These include that it acts as scavenger for reactive oxygen harmful radicals, enhancing glutathione formation, and inhibits oxidative lipid peroxidation. This emphasizing its important role in ameliorating the clonazepam induced oxidative hepatic and renal cell injuries **(Cruz-Álvarez et al. 2018; Welson et al., 2021).**

CONCLUSION

Based on the current findings, sub chronic long term high dose administration of clonazepam in male albino rat was associated with hepatotoxic and nephrotoxic effects as indicated by both biochemical and histopathological changes. Also the deleterious histopathological effects were less evidenced and greatly improved during withdrawal period after sudden stoppage of clonazepam for 10 days. Moreover, Alpha tocopherol administration protects against clonazepam induced impairment of hepatic and renal functions by improving histopathological regeneration and decreasing serum AST, ALT, ALP, urea, creatinine and uric acid levels emphasizing its protective role as antioxidant.

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Author contribution: This work was carried out in collaboration with all authors. The authors Mohammad El-Kattan and Ahmed Elshatory designed the study and wrote the protocol, the authors Mahmoud Ahmed Khattab, Fatma Abdel Wahab Abdel Maksoud, Maha Emad Eldien, Nada Elsayed Abdel-Roaf, managed the literature research and the authors Mohammad El-Kattan wrote and revised the final manuscript. All authors read and approved the final manuscript.

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Declarations

Competing Interests: The authors declare no competing interests.

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السمية الكبدية و الكلوية في الجرذان الناتجة عن التعرض الشبه المزمن للكلونازيبام والدور الوقائي لألفا توكوفيرول

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الخلاصة:

الكلونازيبام (CZP) هو دواء مضاد للصرع تمت الموافقة عليه في عام 1976 من قبل إدارة الغذاء والدواء (FDA) لعلاج أنواع مختلفة من النوبات الصرعية، الكلونازيبام لديه احتمالية عالية لسوء الاستخدام ، بالإضافة إلى الاعتماد الجسدي والإدمان في نهاية المطاف. على الرغم من أن العديد من الدراسات أكدت الآثار الضارة لمضادات الصرع الأخرى مثل حمض الفالبرويك والكاربامازيبين ، فإن الدرسات علي تأثيرات الكلونازيبام قليلة. الهدف من هذا البحث هو دراسة وتقييم التأثيرات السمية للكبد والكلي الناتجة عن تناول جرعات عالية شبه مزمنة من عقار الكلونازيبام والتأثير الوقائي لألفا توكوفيرول "فيتامين هـ". تم تضمين أربعين جرذان ألبينو ذكور أصحاء. تم تقسيمهم بشكل عشوائي إلى أربع مجموعات متساوية (10 جرذان لكل منهما): المجموعة الأولى (محلول ملحي عادي) ، المجموعة الثانية (إساءة استخدام CZP)، والمجموعة الثالثة (ألفاتوكوفيرول) والمجموعة الرابعة (ألفاتوكوفيرول + CZP) .تلقت جميع الفئران الأدوية التي بدأت لمدة 50 يومًا. تم قياس مستويات AST و ALT و ALP واليوريا والكرياتينين وحمض البوليك. تم أخذ أنسجة الكبد والكلى لتشريح الأنسجة. النتائج: يؤدي تناول الكلونازيبام بجرعات عالية إلى زيادة مستويات المؤشرات التحليلية في الكبد والكلى ، وتعطل الأنسجة الكبدية والكلوية وزيادة عدد الخلايا المتدهورة. خفف علاج ألفاتوكوفيرول بشكل كبير من الآثار الضارة التي يسببها كلونازيبام.

الكلمات المفتاحية: كلونازيبام ، سوء الاستخدام ، السمية الكبدية ، السمية الكلوية ، ألفا توكوفيرول ، الجرذان.