

## Ameliorated Effects of Verapamil on Hepatotoxicity Induced by Ethanol and Carbon Tetrachloride

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### Abstract

**Background:** Liver fibrosis progresses to cirrhosis in several settings for example, alcoholic hepatitis and produce hepatocellular dysfunction, which is also a risk factor for hepatocellular carcinoma.

**Aim:** This study was designated to investigate the potential toxicity of CCl<sub>4</sub> initiated by ethanol- as hepatotoxic agents on liver cellular structure glycogen, DNA contents and certain enzymes, and also to evaluate the preventive effect of a certain calcium ions antagonist like verapamil on carbon tetrachloride and ethyl alcohol-induced liver cellular injury in rats.

**Materials and methods:** Sixty healthy adult male albino rats weighing from 200-220 grams were used in this study. Animals were divided into 4 groups, each consisted of 15 rats: the first was considered the normal control, Group 2 was considered the model control which was given ethanol and CCl<sub>4</sub>. Group 3,4 were considered the model control which was given verapamil with 2 doses; 40 and 80 mg. Samples of the liver were subjected to histopathological, histochemical ultrastructure and biochemical examinations after the restricted times.

**Results:** Light microscopy revealed that rats given ethanol and CCl<sub>4</sub> combined together showed liver cell fibrosis and necrosis, nuclear fragmentation and inflammatory response. Glycogen disorder and DNA damage were also detected. On the other hand, animals that were given verapamil in two doses 40 and 80 reduced hepatocytes degeneration and necrosis, delayed formation of liver fibrosis was detected. There was marked improvement in hepatocytes architecture, glycogen and DNA contents. Electron microscopic and biochemical studies seem to confirm these findings.

**Conclusion:** The present findings suggested that treatment with Verapamil significantly reverses hepatotoxic dysfunctions induced by CCl<sub>4</sub> and ethanol combination and it seems to be dose dependent.

**Keywords:** Collagen; DNA; Glycogen; Liver; Rat; Verapamil-enzymes-electron microscope

### Introduction

Liver fibrosis is the main complication of chronic liver disease and leads eventually to cirrhosis. It is characterized by an excessive deposition of extra cellular matrix (ECM) components in the liver parenchyma. Certain collagen types deposit around the sinusoidal cell layer and re-organization of various ECM molecules results in alterations in the composition of the fibrotic matrix, with deterioration of hepatic functions [1]. The main causes of liver fibrosis are infection with hepatitis B or C, alcohol abuse, and non-alcohol steatohepatitis; leading to accumulation of ECM proteins, which distorts the hepatic architecture by forming a fibrous scar, and subsequent development of nodules of regenerating hepatocytes defines cirrhosis [2].

Alcoholic liver remains one of the most common causes of chronic liver diseases in the world [3]. It is well known that ethanol administration can elicit disturbances in the delicate balance between the pro and antioxidant system of the organism; leading to oxidative stress which is a reason of enhanced lipid per oxidation and change in structure and function of other important cellular components such as protein and DNA [4]. Moreover, ethanol induces hypoxia especially in the pericentral zone of the liver acinus [5].

Carbon tetrachloride (CCl<sub>4</sub>), which is a classic hepatotoxin, causes acute reversible liver injury characterized by centrilobular necrosis, followed by hepatic regeneration and repair. This liver injury is attributed to inflammatory responses originating from CCl<sub>4</sub>-derived

free radical formation in the liver and activation of non-parenchymal cells that release a variety of inflammatory cytokines in response to several radical species. Hepatic inflammation provoked by long-term treatment with CCl<sub>4</sub> is believed to induce hepatic fibrosis through ongoing hepatocytic necrosis and production of fibrogenic cytokines acting on fibroblasts, including activated hepatic stellate cells (HSCs) which normally exist in a quiescent state, but become activated following liver injury causing excess production of ECM [6]. Within the body, CCl<sub>4</sub> breaks down to highly toxic trichloromethyl (CCl<sub>3</sub>) and trichloromethyl peroxy (CCl<sub>3</sub>O<sub>2</sub>) free radicals by cytochrome P450 enzyme and causes damage to hepatocytes [7]. Thus, chemicals known to induce isoenzymes of cytochrome P450 such as ethanol have been shown to strengthen hepatotoxicity of CCl<sub>4</sub> [8] and its chronic ingestion is involved in fibrosis and cirrhosis, resulting in greater liver damage than with either agent alone [9], therefore ethanol was recommended to be used in this study to increase activation of

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CCl<sub>4</sub>. Verapamil is a calcium channel-blocker widely used as an anti-arrhythmic agent, treatment of hypertension, ischemic heart disease, and hypertrophic cardiomyopathy. Verapamil's mechanism in all cases is to block voltage-dependent calcium channels [10]. Verapamil is also used in cell biology as an inhibitor of drug efflux pump proteins such as P-glycoprotein. This is useful as many tumor cell lines over express drug efflux pumps, limiting the effectiveness of cytotoxic drugs or fluorescent tags. It is also used in fluorescent cell sorting for DNA content, as it blocks efflux of a variety of DNA-binding fluorophores such as Hoechst 33342. Radioactively-labelled verapamil and positron emission tomography can be used to measure P-glycoprotein function [11]. The present pathological and histochemical research aimed to study the effect of verapamil on the hepatotoxicity induced by CCl<sub>4</sub> and ethanol combined together.

## Materials and Methods

### Drugs, doses and route of administration

Drugs and chemicals used in this study were used according to Xu et al. [12].

A) 40% CCl<sub>4</sub> (98.8% purity) was prepared freshly (40.5 ml CCl<sub>4</sub> + 59.5 ml olive oil) and injected subcutaneously at a dose of 1 ml/kg body weight, three times a week for three weeks. CCl<sub>4</sub> was obtained from Sigma Company.

B) 10% Ethanol was freshly prepared in drinking water. Animals were given for 4 weeks. Ethanol was obtained from Mark Company.

C) Verapamil was prepared in two different doses; 40 mg/100 ml H<sub>2</sub>O and 80 mg/100 ml H<sub>2</sub>O and given in drinking water for 1 month. Verapamil hydrochloride tablets were purchased from Center Medicine Limited Company (Tianjing, China).

### Animals' maintenance and grouping

Sixty healthy adult male albino rats weighing from 200-220 gram were used in this study, all animal were housed under standard conditions and received human care in compliance with institutional guidelines, they were kept under good ventilation, fed on standard laboratory diet, and supplied with water *ad libitum*. They were divided into four groups, each consisted of 15 rats: Group I considered the normal control; Group II considered the model control which was given 10% ethanol alone in drinking water by using oral tube for a week, followed by subcutaneous injection of 40% CCl<sub>4</sub> combined with 10% ethanol in drinking water for 3 weeks; Group III was given combined ethanol-CCl<sub>4</sub> and then they were treated with 40 mg verapamil. 40 mg verapamil therapeutic were treated as group II, and then they were treated with verapamil in drinking water (40 mg/ 100 ml H<sub>2</sub>O for one month) by using oral tube and Group IV was given combined ethanol-CCl<sub>4</sub> and then they were treated with 40 mg verapamil. 40mg verapamil therapeutic treated as group II, and then they were treated with verapamil in drinking water (80 mg/ 100 ml H<sub>2</sub>O for one month) by using oral tube.

**Blood sampling:** Blood samples were collected from rats after dissection by cardiac puncture. All samples were divided into two parts. One of them was kept in heparinized tubes to measure plasma cholesterol. Whereas, other samples were centrifuged at 3000 rpm for 15 minutes to separate serum for determination of AST and ALT.

**Tissue sampling:** After dissection, the liver was divided into three parts; one of them was kept in carnoy fixative for 2-3 hours for

histopathological and histochemical purposes [13]. The second was kept in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2) for Transmission Electron Microscope [14]. The last part was kept in deep freezer (-20°C) for biochemical assays.

### Histopathology

Sections about 5 µm thick were exposed to two histological stains:

1) Haematoxyline and eosin (HE staining) for nucleus and cytoplasm examination.

2) Masson trichrome stain for identifying collagen by blue color and scoring fibrosis.

### Histochemistry

1) Glycogen granules in liver sections were detected using Periodic acid Schiff reagent (PAS). Tissue sections were oxidized with periodic acid for 5 minutes, stained with Schiff's reagent for 10-15 minutes then washed in distilled water for 2 minutes, counterstained in haematoxyline, washed in distilled water then differentiated in ammoniated alcohol until blue color appeared and washed in distilled water, followed by dehydration then purified by xylene and mounted. Glycogen granules located in the cytoplasm of the cell were stained with magenta color.

2) DNA contents in liver sections were detected using Feulgen reagent. Sections were treated with 1 N HCl at 60°C then stained with Schiff's reagent for 20 minutes, dehydrated, purified and mounted. DNA inside the nucleus was stained with purple to red color.

### Electron microscope preparation

Thin slices of liver tissue ranging in size from 0.5 mm to 1 mm were fixed in 2.5 % glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2) for 3 hours at room temperature, dehydrated in ascending grades of ethyl alcohol, cleared and then embedded in embedding medium (araldite). Tissue blocks were cut into thin section under LKB Ultra microtome at (800-900Ångstrom), Sections were then collected on 200 mesh copper grids and stained with 5% uranyl acetate and lead citrate. Finally, sections were examined and photographed with SEO transmission E.M

### Biochemical studies :

**AST and ALT assay:** AST and ALT were detected according to Reitman and Frankel [15].

**Malondialdehyde (MAD) assay:** MAD was detected according to Jena BS et al. [16].

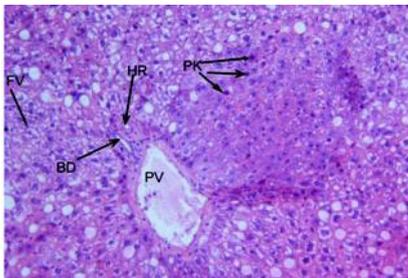
**Cholesterol Assay:** Plasma cholesterol is evaluated according to Beale RN [17]

## Results

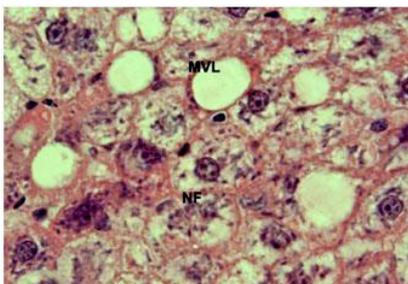
### Histopathology

The extent to which the liver was affected by the hepatotoxic factors and verapamil treatment was evaluated by two histological methods; HE staining and Masson's trichrome staining. Histological studies of livers from normal control rats indicated a normal architecture, while in contrast, group treated with CCl<sub>4</sub> – ethanol, show remarkable structural changes such as thickness of portal vein, pyknosis and coagulation of the cytoplasm with steatosis (Figure 1). Multifocal necrosis, macro-vacuolated lipidimia and nuclear fragmentation

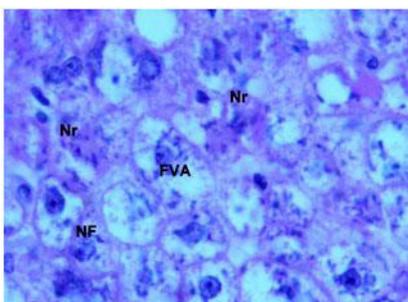
were seen in foamy vacuolated cytoplasm (Figure 2 and 3). Mild amelioration was remarkable in 40 mg verapamil treated group such as fatty degeneration and cytoplasmic granulation despite the wall thickness of the portal vein and vacuolization (Figure 4). The nuclei appeared to be in normal mitotic state; some were contracted and spherical with one or two permanent nucleoli while others showed hyper proliferation including abnormal nuclear division (tri and tetra-polar divisions) (Figure 5). Dead cell containing nuclear pyknosis was stained by brightly pink color (Figure 6) and stand out from other cells due to the degeneration of structural proteins which form a compact homogenous mass with progressively clumping heterochromatin. Much more improvement occurred in the 80 mg verapamil therapeutic group (Figures 7,8) including well organized lobular architecture with normal portal vein and rearrangement of hepatic acini that appeared as those in the normal hepatic architecture. A well-developed cell structure with granulated cytoplasm, rounded activated nucleus, and fatty changes can be detected.



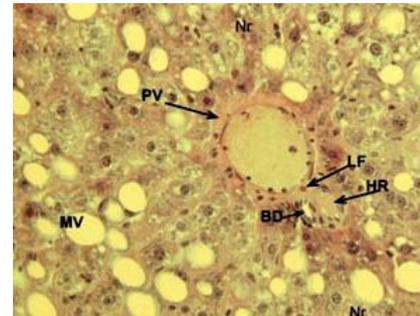
**Figure 1:** A photomicrograph of liver section from group 2 showing thickness of portal vein (PV), hepatic artery (HR) and bile ductule (BD), steatosis and foamy vacuoles (FV) with pyknotic stage (PK). (HE, 200X).



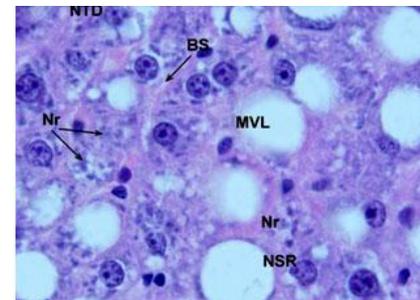
**Figure 2:** A photomicrograph of liver section from group 2 showing macro-vacuolated lipidmia (MVL) and nuclear fragmentation (NF). (HE, 1000X).



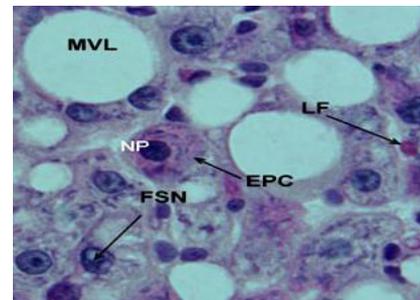
**Figure 3:** A photomicrograph of liver section from group 2 showing cytoplasm rich in necrotic cells (Nr), foamy vacuolated cytoplasm (FVA) and nuclear fragmentation (NF). (HE, 1000X).



**Figure 4:** A photomicrograph of liver section from group 3 showing portal vein (PV) filled with vacuoles, portal triad surrounded by connective tissue and containing hepatic artery (HR) and bile ductule (BD) with infiltrated lipofusin (LF), macro-vacuoles (MV) containing necrosis (Nr) (HE, 200X).



**Figure 5:** A photomicrograph of liver section from group 3 showing nuclear signet ring (NSR), nuclear tri-polar division (NTD) and necrosis (Nr). (HE, 1000X).



**Figure 6:** A photomicrograph of liver section from group 3 showing dead cell containing nuclear pyknosis (NP) (dark material) and homogenous cytoplasmic (Eosinophilic pink color) (EPC). Note lipofusin (LF) and shutting fat to nucleus (FSN). (HE, 1000X).

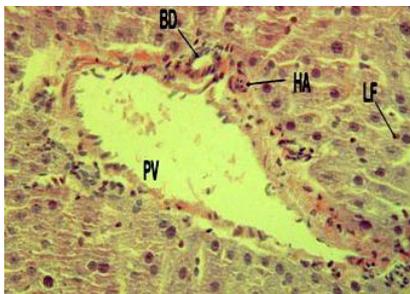
Prominent accumulation of collagen fibers around portal vein and portal triad were clearly detected in CCl<sub>4</sub>-ethanol-treated group causing thickness of the walls of both central and portal veins (Figure 9). Fibrous septa with hepatic nodules were observed (Figure 10). On the other hand, collagen fibers around the portal vein and blood capillaries were less condensed in the 40 mg verapamil group (Figure 11 and 12). These alterations were fully reduced in the 80 mg verapamil therapeutic group with no appearance of collagen fibers around the portal vein and portal trades (Figures 13 and 14).

### Histochemical study

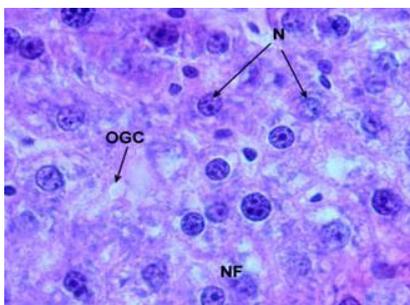
Methods used in this study were used to detect glycogen using Periodic acid Schiff technique and DNA content by Feulgen reaction.

Glycogen is found in the form of large amounts of aggregated clumps in cytoplasm of hepatic cells in normal livers, characterized by a deeply reddish stain. In the CCl<sub>4</sub>-ethanol-injected group, glycogen content of the hepatocytes revealed a marked depletion in glycogen content when compared with normal control liver cells (Figure 15). There was a gradual increase in glycogen content in the 40 mg verapamil group, but still less pronounced than in normal controls (Figure 16). Finally, glycogen seemed to return to its normal pattern in the 80 mg verapamil therapeutic group (Figure 17).

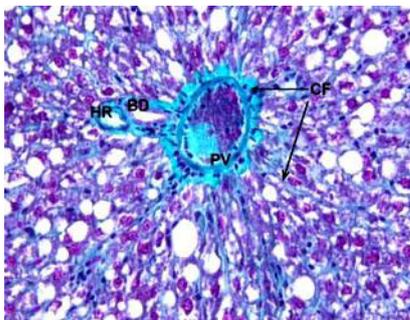
DNA content of the model group injected with CCl<sub>4</sub>-ethanol (Figure 18) showed marked increase and condensation. Slight improvement in DNA content can be recognized in the group treated with 40 mg verapamil (Figure 19). Treatment with 80 mg verapamil



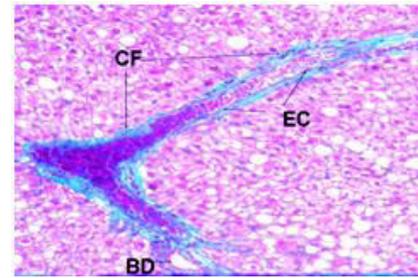
**Figure 7:** A photomicrograph of liver section from group 4 showing normal portal vein (PV) with bile ductule (BD) and hepatic artery (HA) with lipofusin (LF). (HE, 200X).



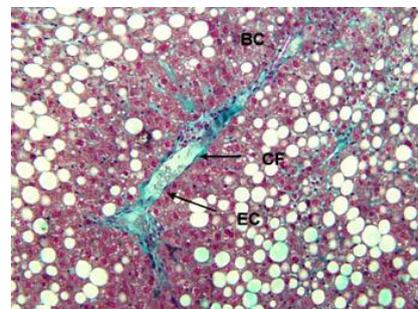
**Figure 8:** A photomicrograph of liver section from group 4 showing normal mitotic figure and rounded nucleus (N) with nuclear fragmentation (NF). (HE, 1000X).



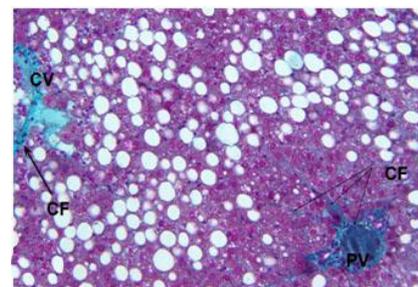
**Figure 9:** A photomicrograph of liver section from group 2 showing wall thickness of portal vein (PV) filled with condensed small particles. Collagen fibers (CF) surrounded the portal vein and extended in between the hepatocytes throughout the sinusoids. (Masson trichrome stain, 400X).



**Figure 10:** A photomicrograph of liver section from group 2 showing blood capillary surrounded with collagen fibers (CF). (Masson trichrome stain, 400X).



**Figure 11:** A photomicrograph of liver section from group 3 showing slightly normal blood capillary (BC) lined with endoepithelial cells (EC) and surrounded with collagen fibers. (Masson trichrome stain, 400X).



**Figure 12:** A photomicrograph of liver section from group 3 showing slight extension of collagen fibers from portal areas. (Masson trichrome stain, 400X).

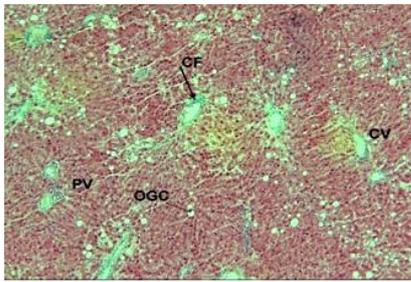
showed more obvious improvement in DNA content with increasing number of nuclei (Figure 20).

### Ultrastructure and biochemical studies

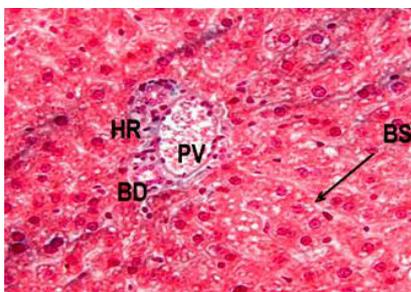
Electron micrographs of liver sections of group 2 show vacuolated cytoplasm (cy) (Figure 25-28) with abundance of collagen (CF) fiber, malformed mitochondria and increased amount of lipid particles (LD). Lipid can also be seen inside the destructive nucleus (Figures 21-24). On the other hand a gradual amelioration is seen as dose dependant in other treated groups with verapamil.

### Biochemical assay

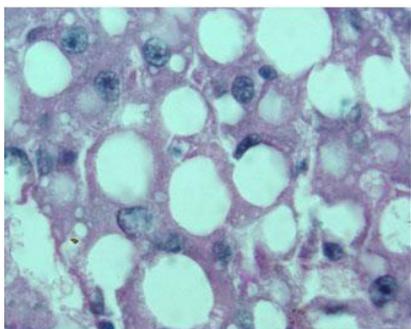
**Aspartate aminotransferase and Alanine aminotransferase ALT, AST levels:** Data in (Table 3) and (Figures 29, 30) show the effect of (40 and 80 mg / 100 ml H<sub>2</sub>O) verapamil on the activity of serum (ALT and AST per U/L). A highly significant increase of serum ALT and AST in



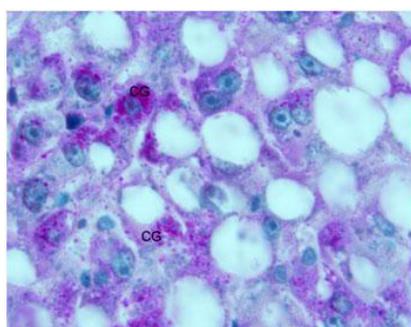
**Figure 13:** A photomicrograph of liver section from group 4 showing normal central vein (CV) with normal collagen fibers (CF). Hepatic acini rearranged with slightly narrow sinusoids and over granulated cytoplasm (GC). (Masson trichrome stain, 100X).



**Figure 14:** A photomicrograph liver section from group 4 showing normal portal vein (PV) with more improvement of portal triad and full reduction of collagen fibers. (Masson trichrome stain, 400X).



**Figure 15:** A photomicrograph of liver section from group 2 showing glycogen depletion. (PAS stain, 1000X).



**Figure 16:** A photomicrograph of liver section from group 3 showing glycogen (G) in normal distribution filling some hepatocytes to form clumps. (PAS stain, 1000X).

Groups Treat	AST (U/L)	ALT(U/L)
	Mean ± S.E	Mean ± S.E
Control	153 ± 8	49.8 ± 0.84
Ethanol and CCl <sub>4</sub>	430.11 <sup>***</sup> ± 5	159 <sup>***</sup> ± 4
Ethanol + CCl <sub>4</sub> +40 mg verapamil	299 <sup>a*</sup> ± 5	94.2 <sup>a*</sup> ± 7
Ethanol +CCl <sub>4</sub> +80 mg verapamil	197 <sup>a</sup> ± 9	69.8 <sup>a</sup> ± 2

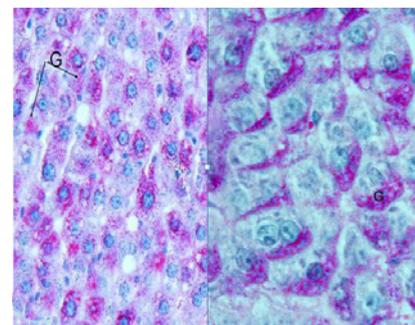
Each value is the mean of 5 records ±SE, <sup>\*\*\*</sup> P< 0.001 versus normal control <sup>a\*</sup> P<0.01 and a P< 0.05 v.s. ethanol +CCl<sub>4</sub>

**Table 1:** The effect of verapamil on serum AST and ALT (U/L) in adult albino rats treated by ethanol and CCl<sub>4</sub>.

Groups Treat	Hydroxyproline (µg/g)	MDA (nmol/g)
	Mean ± S.E	Mean ± S.E
Control	0.3083 ± 0.004	14.4 ± 0.3
Ethanol and CCl <sub>4</sub>	0.923 <sup>***</sup> ± 0.05	146.7 <sup>***</sup> ± 2.6
Ethanol + CCl <sub>4</sub> +40 mg verapamil	0.75 <sup>a*</sup> ± 0.023	103.6 <sup>a*</sup> ± 7.8
Ethanol +CCl <sub>4</sub> +80 mg verapamil	0.5143 <sup>a</sup> ± 0.001	56.2 ± 0.3

Each value is the mean of 5 records ±SE, <sup>a\*</sup> P< 0.01 and a P< 0.05 v.s. ethanol +CCl<sub>4</sub> **versus** normal control <sup>\*\*\*</sup> P<0.001

**Table 2:** The effect of verapamil on Hydroxyproline (µg/g) and Malondialdehyde MDA (nmol/g) level of liver homogenate in adult albino rats treated by ethanol and CCl<sub>4</sub>.



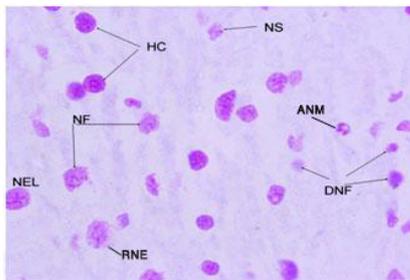
**Figure 17:** (a) A photomicrograph of liver section from group 4 showing glycogen granules (G) returning normal. (PAS stain, 400X). (b): higher magnification. (PAS stain, 1000X).

rats treated with ethanol and CCl<sub>4</sub> was obtained compared to other treated groups. Treatment with 40 and 80 mg verapamil ameliorate ALT and AST.

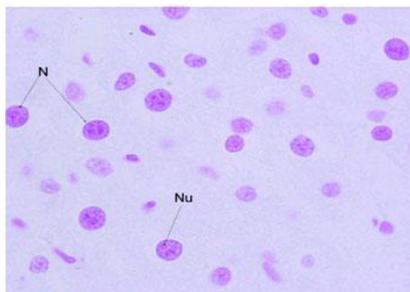
**Malondialdehyde and Hydroxyproline assay:** Data presented in (Table 2) and (Figure 31) illustrate the effect of (40 and 80 mg /100 ml H<sub>2</sub>O) verapamil on MDA levels of liver homogenate in adult albino rats. The result showed a highly significant increase of MDA level in a group treated with 10% ethanol and 40% CCl<sub>4</sub> (146.7 ± 2.6) (nmol/g) compared to control (14.4 ± 0.3) (nmol/g) or both (40 mg /100 ml H<sub>2</sub>O and 80 mg /100 ml H<sub>2</sub>O) verapamil as recorded in their mean values (103.6 ± 7.8) (nmol/g) and (56.2 ± 0.3) (nmol/g) respectively.

On the other hand data presented in table 2 and figure 32 explain the effect of different treatment on hydroxyproline of liver homogenate in adult albino rats. The results display a highly significant increase of hydroxyproline in group treated by 10% ethanol and 40% CCl<sub>4</sub> ( $0.923 \pm 0.05$ ) ( $\mu\text{g/g}$ ) compared to control ( $0.3083 \pm 0.004$ ) ( $\mu\text{g/g}$ ) or 40 and 80 mg verapamil group ( $0.75 \pm 0.023$ ) ( $\mu\text{g/g}$ ) and ( $0.5143 \pm 0.001$ ) ( $\mu\text{g/g}$ ).

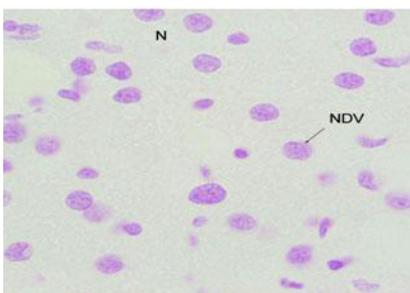
**Cholesterol level:** Data presented in (Table 3) and (Figure 33) represented the level of total plasma cholesterol according to the different treatments in adult albino rats. Highly significant increase in plasma cholesterol level was observed in group of animals treated with 10% ethanol and 40% CCl<sub>4</sub> ( $5.3 \pm 0.32$ ) (mg/ml) compared to (control) untreated group ( $1.2 \pm 0.1$ ) (mg/ml) and verapamil treated groups. More decrease in plasma cholesterol was detected when ethanol and CCl<sub>4</sub> group was treated by 80 mg/100ml H<sub>2</sub>O in drinking water for one



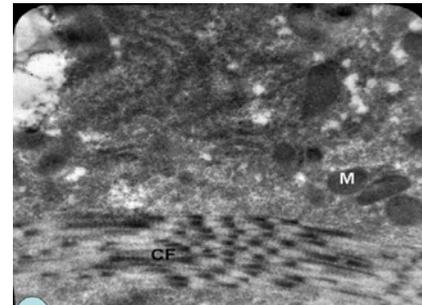
**Figure 18:** A photomicrograph of liver section from group 2 showing that most DNA in nuclei is hyperchromatism (HC), less nuclear integrity form nuclear shrinkage (NS), nuclear enlargement (NEL), nuclear fragmentation (NF) and disappearance of nuclear feature (DNF). (Feulgen reagent, 1000X).



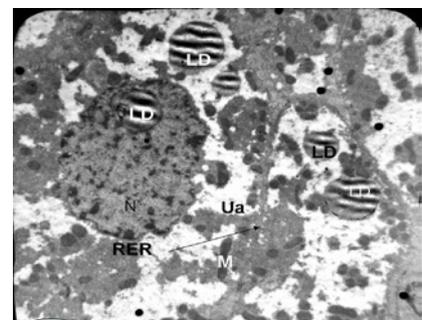
**Figure 19:** A photomicrograph of liver section from group 3 showing rounded or oval nucleus (N) containing one or two nucleoli (Nu) and improved pattern of DNA inside nucleus. (Feulgen reagent, 1000X).



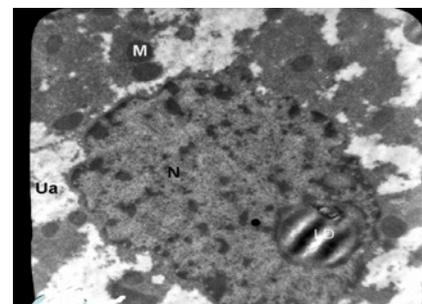
**Figure 20:** A photomicrograph of liver section from group 4 showing nuclei (N) in nuclear mitotic division (NDV). (Feulgen reagent, 1000X).



**Figure 21:** 10000x: Showing vacuolated cytoplasm with abundance of collagen(CF) fiber, and malformed mitochondria.



**Figure 22:** 6000x: Showing increased amount of lipid particle(LD) lipid can also seen inside the destructive nucleus.



**Figure 23:** 3000x: High magnification of Figure 22, note lipid particle inside the nucleus.

month ( $1.8 \pm 0.03$ ) (mg/ml) than those treated by 40 mg/ 100 ml H<sub>2</sub>O ( $3.5 \pm 0.2$ ) (mg/ml).

Finally it can be observed that a higher dose of verapamil (80 mg) proved to be much better than 40 mg.

## Discussion

### Histopathological discussion

CCl<sub>4</sub> is a hepatotoxin causing liver fibrosis, necrosis and cirrhosis sequentially when administrated. It also results in ballooning, inflammatory infiltration of lymphocytes and hydropic degeneration with a clear cytoplasm and vacuolization [18]. Ethanol alone causes chronic liver disease due to toxicity by generating free radicals [19] and apoptosis [20]. The combination between ethanol and CCl<sub>4</sub> result in liver damage greater than cirrhosis caused with either agents alone [9]. Major pathway for ethanol metabolism is alcohol dehydrogenase which

acts as a catalyst for the oxidation of ethanol to acetaldehyde, where hydrogen is transferred from ethanol to the cofactor nicotinamide adenine dinucleotide (NAD<sup>+</sup>); which is converted into the reduced form (NADH). Acetaldehyde is reduced to acetate, which is secreted to the blood stream [21].

Sections taken from control rats showed the normal structure of liver tissue composed of hepatocytes arranged in cords radiating from a central vein in a manner that forms a labyrinth and separated from each other by blood sinusoids, which are nearly equal in size. Polyhedral hepatocytes appear with acidophilic cytoplasm and rounded nuclei with one or two nucleoli.

Results obtained in the present study showed that fat droplets were accumulated in hepatocytes in rats treated with ethanol and CCl<sub>4</sub> [22]. This may be due to that CCl<sub>4</sub> alone caused accumulation of lipid

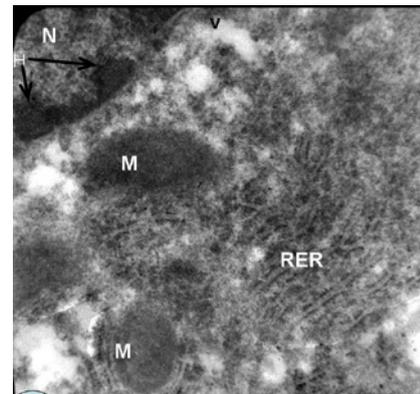


Figure 27: 8000x: Show normal cellular structure.

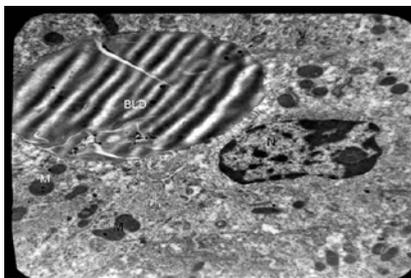


Figure 24: 8000x: Showing an electron micrographs of liver sections.

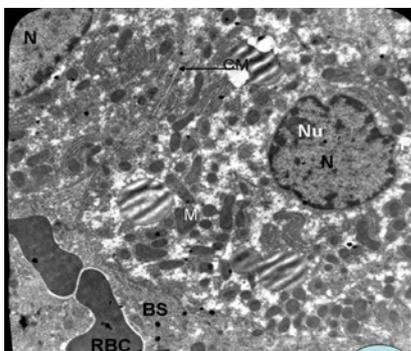


Figure 25: 4000x : Show vacuolated cytoplasm with nearly normal nucleus(N), the mitochondria retain its normality.

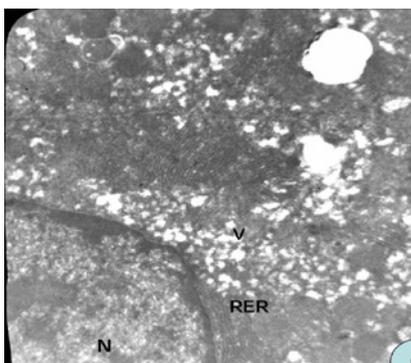


Figure 26: 6000x: Granulated cytoplasm.

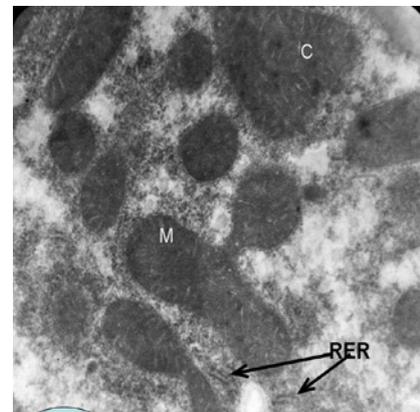


Figure 28: 10000x: Showing high magnification of Fig 27 show normal mitochondrial structure, note double membrane and short creaste.

droplets in cytoplasm of hepatocytes as well; consumption of ethanol alone caused the development of liver steatosis and swelling [23]. The histopathological feature of fat accumulation inside the liver is explained in steps; at the beginning the hepatocytes are present as small vacuoles around the nucleus (microvesicles) which are then filled with multiple fat droplets; later, the size of the vacuoles increases; pushing the nucleus to the periphery of the cell; giving a signet ring appearance (macrovesicular fatty change). These vesicles are well delineated and optically “empty” as fats dissolve during tissue processing. Large vacuoles coalesce, producing fatty cysts which are irreversible lesions [24]. Macro vesicular steatosis is the most common form in alcohol and CCl<sub>4</sub> liver disease. Each of ethanol and CCl<sub>4</sub> individually contribute in accumulation of fat droplets inside liver according to its metabolism. In alcohol metabolism, the increased NADH promotes the generation of building blocks of fat molecule and reduces the breakdown of fats in liver. Moreover, alcohol contributes to the release of fats from other tissue which are then transported to the liver and enhance the liver uptake of fats circulating in the blood [21]. CCl<sub>4</sub> is activated by cytochromes to form trichoromethyl radical (CCl<sub>3</sub>) which can bind to nucleic acid, protein and lipid, thus impairing crucial cellular processes such as lipid metabolism and resulting in steatosis [23].

Pathological alterations in hepatic architecture illustrated in disorganization of the hepatocytes, most hepatic acini were not arranged in definite patterns. The development of vacuoles might be

the starting point of cellular autolytic process [25] and “capillarization” which means a change in the phenotype of the sinusoidal endothelial cell to a vascular phenotype with loss of fenestration and formation of an organized basement membrane [26], thus, sinusoids impair the normal bidirectional exchange between the portal venous blood and hepatocytes, leading to substances that are normally metabolized by hepatocytes to bypass the liver and reach the systemic circulation, and prevent substances that are produced in the liver to reach the blood. This process further complicates problems primarily caused by portal hypertension and decreased hepatocellular synthetic function, such as hypo albuminuria and deficiency of coagulation factors [1].

Fibrosis is part of a dynamic process of continuous extracellular matrix (ECM) remodeling in chronic liver injury, which leads to an excessive accumulation of several extracellular proteins and carbohydrates [1]. In the present study, light microscope examination revealed liver fibrosis in the group treated with ethanol-CCl<sub>4</sub> as recorded before [27]. Ethanol contributes to increase in liver fibrosis by formation of acetaldehyde, resulting in the formation of reactive oxygen species (ROS) which activate hepatic stellate cells, thus promote fibrogenesis [28]. Beside fibrosis, the collagen fibers in the present study extend and destroy the architecture of liver samples. This was in coincidence with prior investigations CCl<sub>4</sub> [29]. Ethanol had a specific role to potentiate the toxicity of CCl<sub>4</sub>, causing greater liver fibrosis and more damage, where acetaldehyde is responsible for stimulation of collagen biosynthesis. Furthermore, increase in NADH levels result in formation of scar tissue that characterize fibrosis.

Ethanol and CCl<sub>4</sub> in the present work contributed in pushing the cell towards “apoptosis”, affecting only individual cells, leaving adjacent

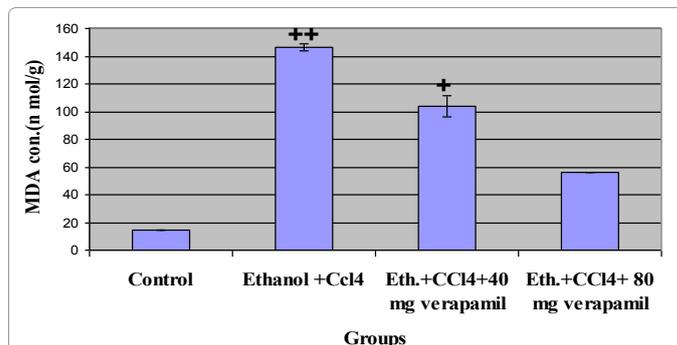


Figure 31: Concentration of Malondialdehyde (nmol/g) in liver homogenate of rats after different treatments.

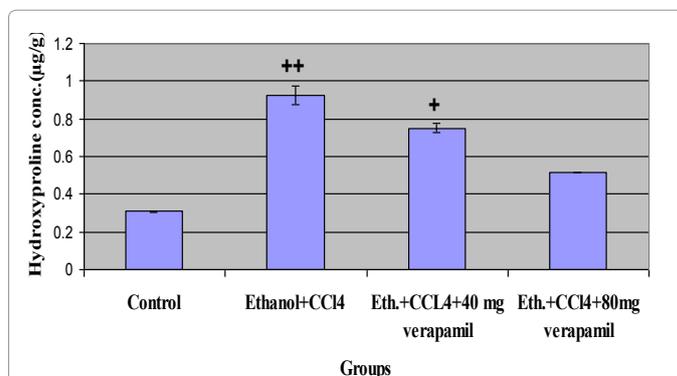


Figure 32: Concentration of Hydroxyproline (µg/g) in liver homogenate of rats after different treatments.

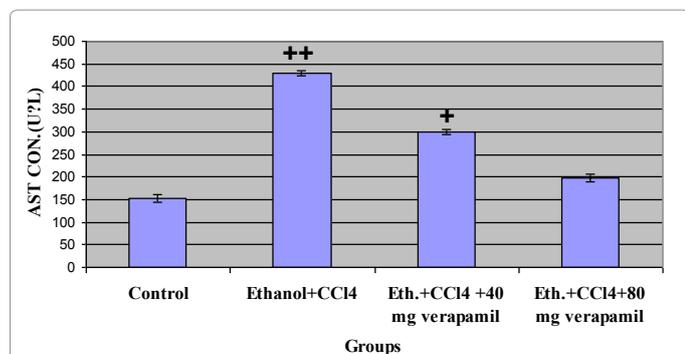


Figure 29: Concentration of ASTase (U/L) in serum of rats after 40mg and 80mg verapamil treatments.

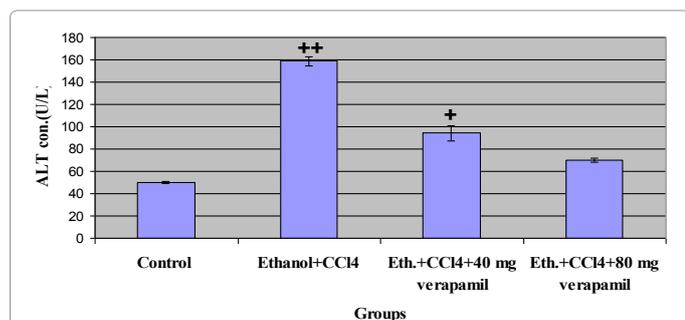


Figure 30: Concentration of ALTase (U/L) in serum of rats after different treatments.

cells intact. The condensed chromatin, fragmentation and shrinking of nucleus were seen as evidence for apoptosis. This histopathological feature showed morphological changes [30]; the cell body shrinks and loses contact to its neighboring cells, chromatin in nucleus condenses and migrates to the nuclear membrane, and finally the cell is fragmented into compact membrane-enclosed structures, called ‘apoptotic bodies’ which contain cytosol, condensed chromatin, and organelles. The apoptotic bodies are engulfed by macrophages (scavenging cells) and thus are removed from the tissue without causing an inflammatory response. Proteolytic enzymes mediate the cleavage of DNA into fragments as well as the cleavage of specific protein substrates which determine the integrity and shape of the cytoplasm or organelles. The mechanism of action of apoptosis is known to occur in mitochondria which release cytochrome c and the endoplasmic reticulum which release calcium [31]. CCl<sub>4</sub> causes accumulation of calcium in the nucleus and produce Ca<sup>+2</sup> endonuclease fragmentation of DNA.

The study revealed necrosis in liver sections of the group treated with ethanol and CCl<sub>4</sub> combined together. Cells undergoing necrosis swell and their organelles break down. They lose membrane integrity, rupture and spill debris that leads to local inflammation which then results in the death of adjacent cells. Necrosis is morphologically distinct from apoptosis and is defined as cell death accompanied by a rapid efflux of cell constituents to the extra cellular space due to a loss of cytoplasmic membrane integrity [32] and it takes place under extremely harmful environmental conditions such as exposure to toxic chemicals and microbial pathogens, and causes inflammation which gives rise to damage to surrounding cells [33].

Concerning the vacuolated cytoplasm (Figure 25), the increase in cell volume is due to the loss of regulating mechanism of the cell to pump out water from the inside. Energy required for this is given by the respiration which is suppressed by CCl<sub>4</sub>. Swelling of the cell in CCl<sub>4</sub> treatment is due to the changed permeability of the cell membrane, since CCl<sub>4</sub> is a nonpolar lipophilic substance and results in the changed characteristics of the lipid membrane of both cytoplasm and mitochondria [34].

Results showed increase in the chromatin content in the nuclei of most hepatocytes of rats which received ethanol-CCl<sub>4</sub>. Heterochromatin predominates over euchromatin and is peripherally emarginated along the inner nuclear membrane. Such situation of chromatin is observed as a change in the nucleus associated with irreversible injury leading to cell death. Moreover, the nuclei of hepatocytes treated with ethanol and CCl<sub>4</sub> showed enlargement and nuclear polymorphism which was suggested to have significance as a marker of carcinomous lesions [35].

In the present study, pyknosis was seen in the group treated with ethanol and CCl<sub>4</sub>, in harmony with the observations showing that CCl<sub>4</sub> caused pyknosis of nucleus and necrosis of the liver cells [36]. It is irreversible condensation of chromatin in the nucleus of a cell undergoing programmed cell death or apoptosis and it is followed by fragmentation of the nucleus [37]. Concerning DNA fragmentation, ethanol and CCl<sub>4</sub> together have role in increasing DNA degeneration in rat liver as it was induced by both ethanol and CCl<sub>4</sub> [20,38] individually.

In the present histopathological study, treatment with two concentration doses (40 and 80 mg) of calcium channel blocker Verapamil decreased the toxic effect of ethanol and CCl<sub>4</sub> in livers of rats. The 80 mg dose of verapamil reduced the collagen fibers, infiltrations, and steatosis, and improved the architecture of hepatocytes and shape of nucleus. Using 40 mg verapamil also showed improvement in liver tissue structure, in spite of the presence of slight thickening and fibrosis in blood vessels walls especially the central veins. Hypertrophy of kupffer cells in blood sinusoid can be explained by increased phagocytic activity in removing debris of dead cells in the stage of regeneration. Slight fibrosis remained surrounding the hepatocytes and extending between them towards neighboring areas and slight dilatation of blood sinusoid was noticed. The results are in accordance with observations which found that the two concentrations of verapamil reduced hepatocytes degeneration, necrosis and delayed the formation of liver fibrosis in rats [12] and confirmed that complex factors (ethanol, CCl<sub>4</sub>, and high fat diet) caused severe histopathological changes such as steatosis,

		Cholesterol(mg/l)
Groups	Treat	Mean ± S.E
Control		1.2 ± 0.1
Ethanol and CCl <sub>4</sub>		5.3 <sup>***</sup> ± 0.32
Ethanol + CCl <sub>4</sub> +40 mg verapamil		3.5 <sup>**</sup> ± 0.2
Ethanol +CCl <sub>4</sub> +80 mg verapamil		1.8 <sup>*</sup> ± 0.03

Each value is the mean of 5 records ±SE, <sup>\*\*\*</sup> P< 0.001 versus normal control <sup>\*\*</sup> P<0.01 and a P< 0.05 v.s. ethanol +CCl<sub>4</sub>

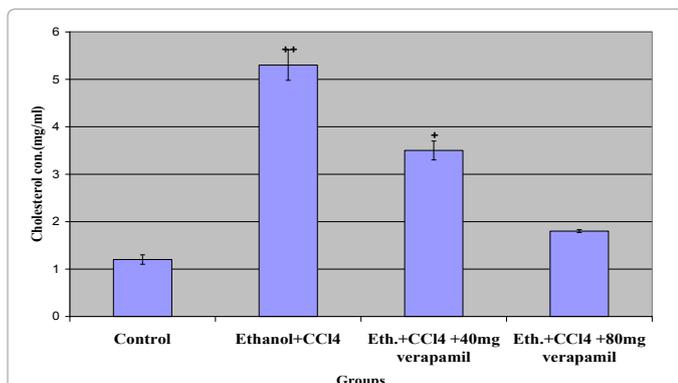
**Table 3:** The effect of verapamil on plasma cholesterol (mg/L) in adult albino rats treated by ethanol and CCl<sub>4</sub>.

macrophage infiltration, myofibroblast proliferation, and liver fibrosis which were evident in the disruption of tissue architecture, extension of fibers, large fibrous septa formation, pseudo-lobe separation and accumulation of collagen. These alterations were reduced markedly in the liver sections of rats that received verapamil at a dose of 80 mg/kg for 4 weeks. A single intravenous or oral dose of verapamil 5 mg/kg slightly decreased necrosis in rat liver. Fibrosis model is induced by high fat diet, alcohol and CCl<sub>4</sub> [39] through inhibition of Ca<sup>2+</sup> degeneration and DNA damage.

It is well known that calcium has an important role in a number of cell functions, such as differentiation, proliferation, contraction, migration, apoptosis, and protein synthesis [40]. When hepatocytes were impaired by chemical poisons as CCl<sub>4</sub> and ethanol, the [Ca<sup>2+</sup>] ions increased causing cell depolarization, augmentation of excitability, and release of large amounts of inflammatory factor, thus resulting in activation of Kupffer cells and HSCs [41]. Hepatocytes of plasma membrane contain Ca<sup>2+</sup> channels which admit Ca<sup>2+</sup> in response to receptor-operated channel agonists. The receptor-operated Ca<sup>2+</sup> channels allow various divalent cations to enter the hepatocyte [42]. Verapamil inhibit Ca<sup>2+</sup> entry into liver cells through its effect on receptor-operated channels [43]. The study demonstrated that verapamil blocks the initiating agent of hepatic fibrosis by inhibiting the activation of HSCs. Verapamil act as vasodilator and has effect on blood vessels in the liver as it prevents hypertension and hypoxia in liver cells, and as an angiotenic, it can increase the local blood circulation and oxygen supply, thus inhibiting lipid peroxidation which is a major cellular mechanism involved in CCl<sub>4</sub> hepatotoxicity, and thereby limiting hepatonecrosis and fibrogenesis [12,44]. This action could improve the oxygenation of the centrilobular region of the liver lobule. It also has the ability to scavenge free radicals [45].

### Histochemical discussion

Glycogen granules were observed by PAS stain depending on the intensity of the coloured compound formed which is directly proportional to the glycogen content of the cell. Periodic acid is an oxidizing agent which breaks the C-C bond between two adjacent hydroxyl groups. Tissues containing vicinal glycol groups or their amino or alkyl amino derivatives are oxidized by periodic acid to form dialdehydes, which combine with Schiff's reagent to form an insoluble magenta compound. In the present study, rats treated with ethanol-CCl<sub>4</sub> depleted glycogen granules. Transformation of a liver cell into a cancer cell occurs with characteristic depletion of the glycogen content.



**Figure 33:** Concentration of plasma cholesterol (mg/ml) of rats after different treatments.

Among toxic changes associated with the process of carcinogenesis is depletion of glycogen granules [46]. Centrilobular glycogen depletion would appear to play a definite role in the sequence of changes which result in liver damage, cirrhosis and possibly tumours. Histochemical images of glycogen granules showed improvement by filling the cytoplasm of hepatocytes in groups treated with verapamil. As a calcium ion influx inhibitor, verapamil acts on calcium channel causing calcium entry block and prevent the effect of calcium on phosphorylase kinase enzyme, thus it had the ability to ameliorate the glycogen granules in 40 and 80 mg doses gradually.

DNA histochemical technique established in the field of analytical cellular pathology is used as an important parameter providing significant information about the biological behaviour of cells [38]. DNA contents were classified as tetraploid, diploid and anaploid, based on the amount of DNA relative to the normal control where anaploidy increases with increasing stage of tumour [47]. DNA histochemical technique provides ploidy that result due to direct visualization and selection of cancer cells [38].

In the present study, DNA appeared to be increased in rats treated with ethanol-CCl<sub>4</sub> in comparison with the control group. The changes in the nuclear structure induced by CCl<sub>4</sub> involved a compensatory increase in nucleogenesis due to the decrease in the activity of the protein-synthesizing apparatus in hepatocytes, moreover, atypical hyper-chromatin was detected in hepatic cells and ethanol caused condensation of chromatin [48].

In this study, DNA appeared to show fragmentation. Ethanol induced apoptosis in liver cells and DNA fragmentation by intracellular Ca<sup>2+</sup> elevation in the cytoplasm. Also CCl<sub>4</sub> caused accumulation of Ca<sup>2+</sup> in the nucleus and produced Ca<sup>2+</sup> endonuclease fragmentation of DNA. The degree of DNA fragmentation correlates with the amount of Ca<sup>2+</sup> in the nucleus [49]. In confirmation to the above, the nuclear compartment is the site of Ca<sup>2+</sup> overload during toxic cell death and DNA is an important target undergoing lethal cleavage by Ca<sup>2+</sup> stimulated endonuclease [50]. Nuclear fragmentation subsequently breaks down into multiple small parts as apoptotic bodies. One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units [51]. The Present study showed that DNA contents seemed to be improved in the group treated with 40 mg verapamil, normal mitotic figures can be observed in group that treated with 80 mg verapamil. This may be due to the fact that calcium channel blocker verapamil protected the nuclear DNA against ethanol and CCl<sub>4</sub> by acting on voltage-sensitive channel which lead to decrease Ca<sup>2+</sup> entry into cells and prevention of DNA fragmentation by Ca- endonuclease activation [51].

Further studies were done in this work to evaluate and confirm the above finding. The serum activity of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were indicators of hepatotoxicity. AST levels fluctuated in response to the extent of cellular necrosis (cell death) and therefore may be temporarily and minimally elevated early in the disease process, and extremely elevated during the most acute phase. AST and ALT analyses showed high significant increase in CCl<sub>4</sub> treated groups. This explains the destruction and areas of necrosis found in sections of this group examined by electron microscope [52-54]. Gradual dose dependant amelioration were seen in other groups treated by verapamil and examined either by electron microscope or by biochemical analysis. Moreover, ultrastructure examination of the liver section from rats treated with CCl<sub>4</sub> and ethanol showed increased

amount of collagen fibers. This was clearly detected biochemically, hydroxyproline analysis showed a highly significant increase while in groups treated with 40 and 80 mg verapamil showed gradual decrease respectively [55]. This may be due to fact that hydroxyproline is significantly reduced by calcium channel blocker induced by CCl<sub>4</sub> [56]. It is also clearly observed that lipid droplets were increased in liver sections treated by CCl<sub>4</sub> and examined by electron microscope. These findings were in parallel to biochemical plasma cholesterol analyses which showed a high significant increase of plasma cholesterol [57]. Verapamil produced the most significant reduction in cholesterol level. This may be explained on the basis that verapamil has a strong ability to chelate multivalent metal ions especially zinc, calcium and iron. Alternatively its ability to chelate minerals has been reported to have some protective effect such as decreasing iron- mediated free radical formation and lowering serum cholesterol, lipid peroxide in experimental animals [58]. These statements were clearly observed in our electron and biochemical detection.

In conclusion it can be stated that in spite of the idea the verapamil had bad side effects, it has been found that it has an ameliorating effect on liver fibroses induced by alcohol and CCl<sub>4</sub> [59].

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