

HISTOPATHOLOGICAL AND BIOCHEMICAL ASSESSMENT OF LIVER FIBROSIS INDUCED BY CARBON TETRACHLORIDE ADMINISTRATION IN RAT

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ABSTRACT

This study investigated liver fibrosis and hepatotoxicity caused by carbon tetrachloride (CCL₄) in albino rats. Two groups of twenty-two adult male rats, each weighing 150 to 170 gms, were created. Ten rats from the CCL₄-administered group (I) received subcutaneous injections of CCL₄ in olive oil at a dose of 2 ml/kg twice weekly for 12 consecutive weeks. The control group (II) contained twelve rats and was divided into two groups of six rats each. One group served as the standard control, and the other group only got olive oil via the same route and dosage as group (I). All rats were sacrificed 12 weeks post dosing, and tissue specimens from livers were collected for histopathological examination. Additionally, serum samples were collected in order to measure various biochemical factors, including (alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), total antioxidant capacity (TAC) and lipid peroxidation (LPO). The histopathological examination of group I showed hepatic degenerative, necrotic and angiopathic alterations, as well as pronounced hepatic fibrosis. The histopathological examination of group II showed normal hepatic appearance with no pathological changes. The biochemical results detected a significant upregulation in serum AST, ALT and LPO in group I compared to the control group, while TP and TAC were significantly downregulated. In conclusion, the administered dose of CCL₄ in rats caused variable degenerative and necrotic hepatic changes, besides activation of the proliferative potential of collagen fibers, and changed the biochemical parameters compared to normal control rats.

Keywords: Carbon tetrachloride, liver fibrosis, Histopathological examination, Biochemical parameters.

INTRODUCTION

Carbon tetrachloride (CCl₄) is an artificial chemical that does not naturally occur in the environment. It is a colorless, volatile, non-flammable liquid created when light-activated chloroform and chlorine are combined.

Carbon tetrachloride (CCl₄) is structurally a polychlorinated hydrocarbon chemical that harms several organs, primarily the liver, kidneys and lungs when exposed to it (Guo *et al.*, 2000).

A known hepatotoxic substance, CCl₄, is processed by the cytochrome P450 oxygenase system of the endoplasmic reticulum and the superfamily of monooxygenases (CYP family) in the liver to produce reactive trichloromethyl radicals (CCl₃) and trichloromethyl peroxy radicals

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(CCl_3O_2). Subsequently, this radical reacts with major cellular biomolecules including nucleic acid (DNA), proteins, lipids and carbohydrates, leading to impaired essential physiological functions, altered lipid metabolism and decreased protein levels (Lee *et al.*, 2005). CCl_4 causes injury to the liver through different mechanisms, such as oxidative stress, inflammation and programmed cell death, leading to degenerative changes, such as fatty and hydropic degenerations, as well as apoptosis and necrosis (Chen *et al.*, 2015)

Hepatic fibrosis is regarded as a major health problem that happens as an end result of chronic liver illness. It's marked by an increase in collagen fiber synthesis and deposition of glycoproteins and proteoglycans, which all form an extracellular matrix (Albillos *et al.*, 2014).

Serum liver enzymes biomarkers (ALT, AST, alkaline phosphatase and lactate dehydrogenase), nitric oxide, tumor necrosis factor alpha, liver malondialdehyde content and the percentage of collagen fibers in the liver were all elevated in adult male albino rats with liver fibrosis brought on by CCl_4 . However, it down-regulated the reduced liver glutathione concentration as an endogenous antioxidant (Ahmed *et al.*, 2011).

The Masson trichrome stained sections of rat's liver induced by CCl_4 revealed extensive collagen fibers deposition, forming bridging fibrosis in the portal areas and around central veins (Alshawsh *et al.*, 2011).

Cirrhosis, which is characterized by the loss of the traditional hepatic architecture and the subsequent emergence of newly regenerating nodules, develops as hepatic fibrosis proceeds. Cirrhosis produces a series of complicating diseases; like portal, gastrointestinal bleeding and hepatic

encephalopathy (Schuppan and Afdhal, 2008).

The present study determined the hepatotoxic and fibrotic effect of the administration of CCl_4 to rats for 12 weeks. This was done by histopathological examination of sections of liver tissue, measuring of oxidative indices (TAC & LPO) and also detecting liver enzyme seromarkers (AST& ALT) and TP.

MATERIALS AND METHODS

MATERIALS:

1. Reagents and chemicals:

Carbon tetrachloride (CCl_4): was purchased from ADVENT Company, India.

Olive oil: was procured from local markets in Egypt.

Liver seromarkers AST, ALT, and TP kit was obtained from Biodiagnostic Company, Egypt.

Oxidative stress markers (TAC& LPO) kit was obtained from Biodiagnostic Company, Egypt.

2. Experimental Animals

Apparently healthy twenty two male albino rats weighting about 150-170 gms were purchased from Animal House of Faculty of Veterinary Medicine, Assiut University. The animals were kept on cages under regulated temperature (25°C) and humidity. All animals were allowed to access both food and tap water ad libitum. They were kept in the laboratory for one week to help them adjust and got all necessary human care. The animals were assigned into two groups:

Group 1: CCl_4 -administrated group: Ten male albino rats were administered CCl_4 in a dose of 2 ml/kg (Li *et al.*, 2016). CCl_4 was dissolved in olive oil (1:1) and injected s/c twice weekly. All rats were sacrificed by cervical dislocation after 12 weeks.

Group 2: Control group: Twelve rats were subdivided into two subgroups:

Subgroup A (Normal control rats): Six rats were kept at normal conditions of laboratory temperature and humidity. They were used as control negative group.

Subgroup B (Rats administered olive oil only): Six rats were injected only CCl₄ vehicle (olive oil) at the same dose and route of the CCl₄-administered group.

METHODS:

1. Histopathological and histochemical examinations:

All rats were sacrificed at the end of the experiment, and their livers were instantly taken out. Specimens from all livers were taken, washed with saline, and fixed by immersion in 10% neutral-buffered formalin solution. After proper fixation, all liver specimens were routinely prepared for traditional histological examination (Bancroft and Gamble 2008).

The histopathological lesions observed in different groups were presented in a table to demonstrate their incidence.

In addition, Masson's trichrome stain was conducted for demonstrating collagen fiber in liver tissue sections (Suvik and Effendy, 2012). Thereafter, all stained sections were examined by light microscopy (Olympus CX-31 microscopy) and then photographed with (Sc30 Olympus camera).

2. Fibrosis grading:

Semiquantitative grading of fibrosis in the examined cases was carried out on the trichrome stained sections, and according to the histological METAVIR fibrosis grading system (Mohamadnejad *et al.*, 2010) as shown in Table (1).

Table 1: Histological METAVIR fibrosis grading system.

Fibrosis expansion stage	Fibrosis grade
Normal histological liver or no fibrosis	F0
Fibrosis expansion of some portal areas $\pm$ short fibrous septa	F1
Fibrosis expansion of most portal areas $\pm$ short fibrous septa.	F2
Fibrosis expansion of most portal areas with occasional portal to portal bridging (P-P).	F3
Marked bridging with occasional nodules as incomplete cirrhosis.	F4
Cirrhosis	F4

3. Biochemical investigation:

Blood samples were sucked from the medial canthus of the eye and collected in sterile plain tubes (without anticoagulant) from all experimental animals before sacrificing. Blood samples were centrifuged, and the separated sera were pipetted into Eppendorf tubes and stored at -20 °C until the biochemical parameters were estimated. Biochemical parameters were done at the Central Laboratory of Pathology and Clinical Pathology Department, Faculty of Veterinary Medicine, Assiut University by using of 6705 UV |Vis Spectrophotometer (JENWAY) as the following:

- 1- Liver seromarkers AST, ALT were determined using colorimetric assay kit according to (Reitman and Frankel, 1957) and TP kit (Gornal *et al.*, 1949).
- 2- Total antioxidants capacity (TAC) was measured using a colorimetric assay kit (Koracevic *et al.*, 2001).

3- Malondialdehyde (MDA) was estimated using a colorimetric assay kit (Ohkawa *et al.*, 1979).

4. Statistical analysis:

The obtained data were analyzed using the Statistical Package for Social Science program SPSS (version 16) software. For comparison across different experimental groups, one-way analysis of variance (one-way ANOVA) was utilized, followed by the Duncan test as a Post Hoc test. The graphs were done using the Prism program, version 5.01 (GraphPad Prism). The acceptance level for statistical significance was $P < 0.05$. All data were reported as mean $\pm$ S.E.

RESULTS

Histopathological findings:

Microscopic examination of H&E stained liver tissues of the sacrificed rats given CCl₄ for 12 weeks showed marked hydropic and fatty degeneration, as well as coagulative necrosis, angiopathic changes and fibrosis in all 10 rats.

Vacuolar degenerated hepatocytes appeared swollen, with a foamy appearance of the cytoplasm and vesicular nuclei (Fig. 1A). Fatty degeneration revealed variable sized fat globules replacing most of the cell cytoplasm and pushed the nucleus towards the periphery (Fig. 1B). The necrotic hepatocytes were manifested by increased eosinophilia of the cytoplasm with karyolysis of their nuclei (Fig. 1C).

Regarding the angiopathic changes, all 10 examined rats of this group showed severe congestion of blood vessels and hemorrhages (Fig. 1D). The angiopathic changes in the portal areas were expressed by severe congestion and dilatation of the portal blood vessels, accompanied by damage to the lining endothelial cells and perivascular proliferation of fibrous connective tissue (Fig. 1E).

Hepatic fibrosis was recorded in all 10 examined rats, which appeared as thick fibrous connective tissue bands invaded the

parenchyma and surrounded groups of hepatic cells suffering from degenerative changes (Fig. 2A). Atypical hepatic lobules were formed in which extensive fibrous connective tissue proliferated and outlined groups of hepatic cells forming a pseudolobular pattern (Fig. 2B). The proliferated connective tissue was confirmed by Masson trichrome stain as green colored bundles (2C).

Additionally, there were noticeable thick bands of connective tissue proliferating in the portal area, a condition known as portal fibrosis (Fig. 2D). Masson trichrome highlighted the proliferated connective tissue with green color (Fig. 2E). Thick fibrous connective tissue bands in Glissonian capsule with pressure atrophy of the underneath hepatic cells that suffered from was also noticed (Fig. 2F).

Careful microscopic examination of rats of this group showed an advanced lesion in the form of hepatic cirrhosis in 4 rats out of 10 where extensive fibrous connective tissue proliferated and replaced most of the hepatic lobular tissue (Fig. 3A). Such proliferated connective tissue was confirmed by Masson trichrome stain (Fig. 3B). Bile duct hyperplasia with dilatation of the newly formed ductules was detected (Fig. 3C). Oval cells proliferation was observed as oval shaped cells with pale blue round to oval nuclei surrounded by scanty basophilic cytoplasm appeared in groups within the portal area (Fig. 3D).

Group II: Control group:

Careful histopathological examination of the liver sections of the control rats (normal control rats and rats administered olive oil only) showed normal histological structure (Fig. 3E).

The incidences of different histopathological lesions in the CCL₄-administered group and control group were demonstrated in Table (2).

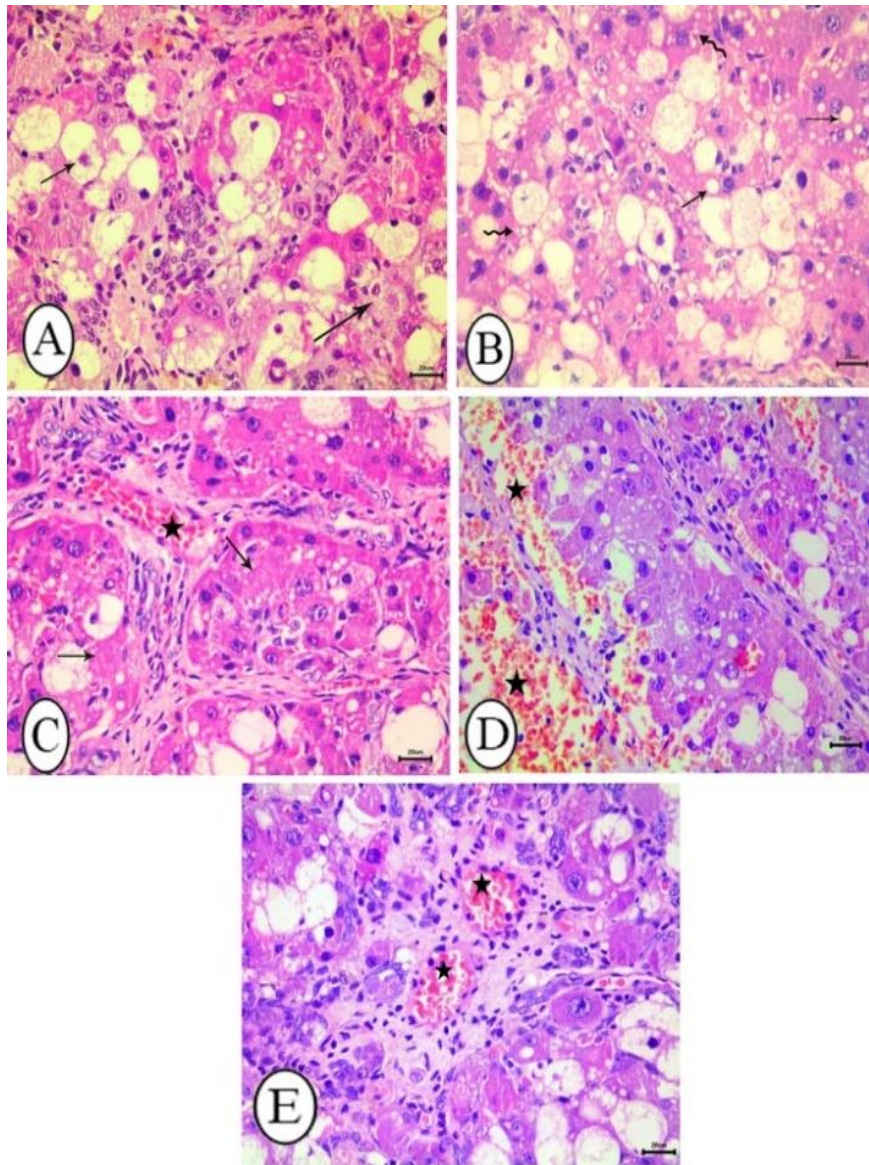


Fig.1: Liver tissue sections of CCl₄-administered rats stained by H&E showing vacuolar degeneration (arrow) of hepatocytes (A), macrovesicular (arrow) and microvesicular (twisted arrow) fat globules in degenerated hepatic cells (B), necrosis of hepatic cells (arrow) and congested blood vessel (star) (C), severe hemorrhage (star) (D) and congested portal blood vessels associated with endothelial damage and perivascular connective tissue proliferation (star) (E) (bar= 20 µm).

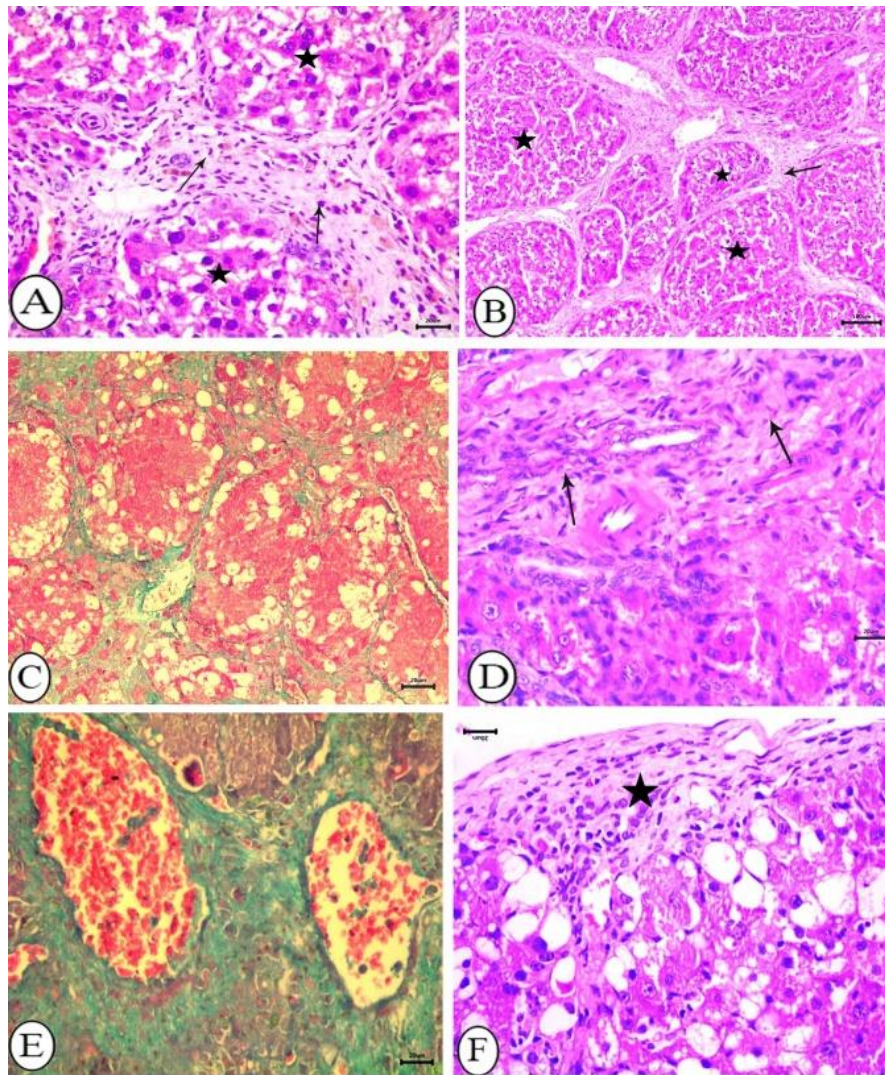


Fig.2: Liver tissue sections of CCl₄-administered rats showing thick band of collagenous fiber disrupted the hepatic parenchyma (arrow) and surround groups of degenerated hepatocytes (star) (H&E, bar= 20um) (A), connective tissue proliferation (arrow) surrounded hepatic parenchymal cells forming pseudolobules (star) (H&E, bar= 100 um) (B), proliferated collagenous fibers with green color (Masson trichrome, bar= 100 um) (C), massive connective tissue proliferation in the portal area (arrow) (H&E, bar= 20 um) (D), green-colored connective tissue proliferation in portal area (Masson trichrome, bar= 20 um) (E) and thick connective tissue proliferation in Glisson capsule (star) (H&E, bar= 20 um) (F).

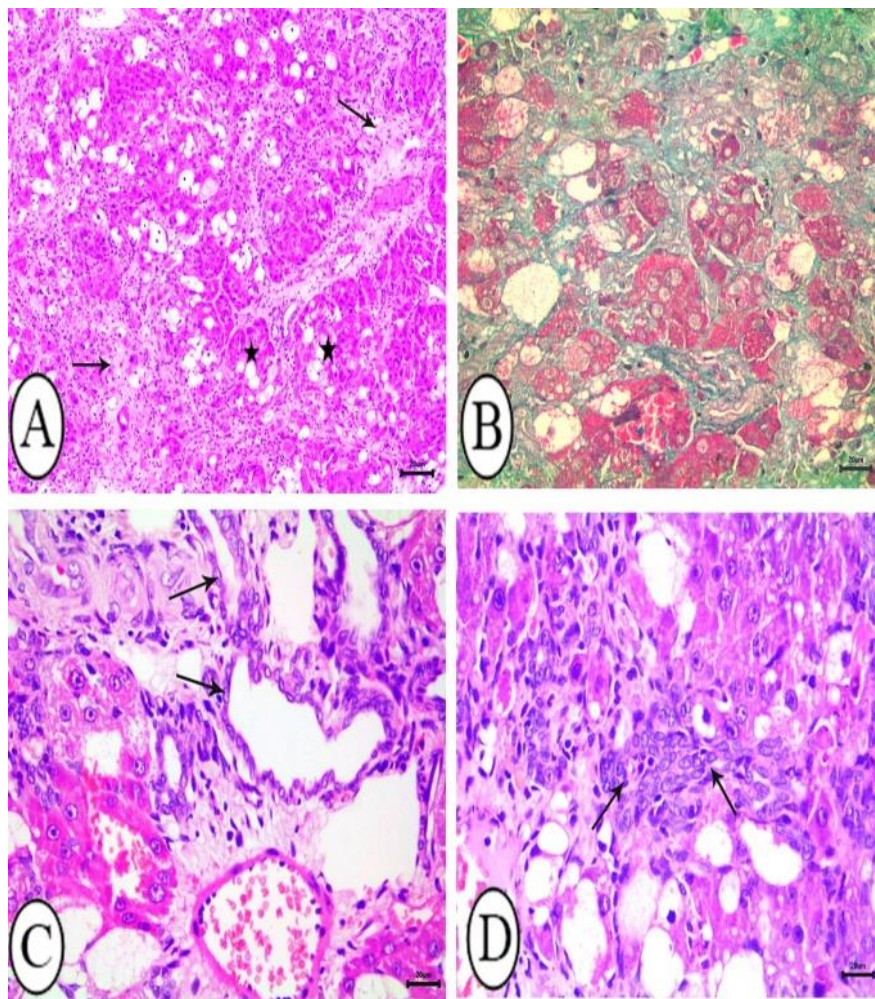


Fig. 3: Liver tissue sections of CCL₄-administered rats showing cirrhotic liver in which thick c.t extensively proliferate and replace most of the hepatic parenchyma (arrow) (H&E, bar= 100 µm) (A), proliferated connective tissue appeared green-colored (Masson trichrome, bar= 100 µm) (B) and hyperplastic and dilated bile ductules (arrow) (H&E, bar= 20 Groups of hyperplastic oval cells (arrow) (H&E, bar= 20 µm) (D).

Table 2: Incidence of histopathological lesions in different experimental groups:

Lesions	Control group		CCL ₄ -administered group
	Normal	Olive oil	
Degenerative changes	0(0%)	0(0%)	10(100%)
Necrotic changes	0(0%)	0(0%)	10(100%)
Angiopathic changes	0(0%)	0(0%)	10(100%)
Fibrosis	0(0%)	0(0%)	10(100%)

No (%) of animals with lesion.

Fibrosis grading:

Histopathological fibrosis scoring was carried out using Masson trichrome stained sections from the livers of rats administered CCL₄ and the control rats. Fibrosis grading of livers of rats sacrificed 12 weeks post CCL₄ showed a highly significant increase compared to the control values. The histopathological grading of liver fibrosis in different groups is reported in Table (3) and Fig. (4).

Table 3: Fibrosis grading in the livers of CCL₄-administered group and control group.

Grades	Control group		CCL ₄ -administered group
	Normal control	Olive oil	
F1	0(0%)	0(0%)	1(10%)
F2	0(0%)	0(0%)	2(20%)
F3	0(0%)	0(0%)	3(30%)
F4	0(0%)	0(0%)	4(40%)
Mean±SE	0.00± 0.00	0.00±0.00	3.80±0.200**

No. (%) of rats with lesions.

F1: Fibrosis expansion in some portal area.

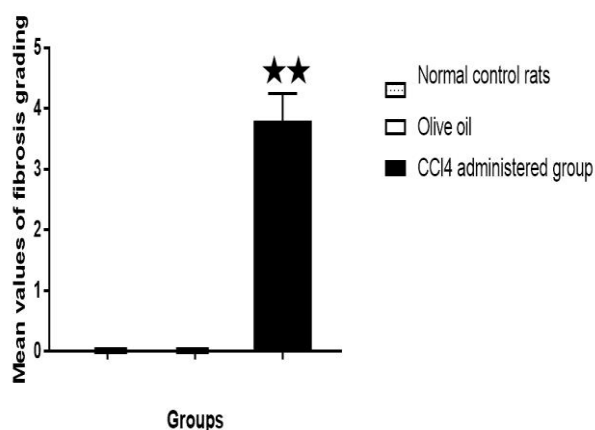
F2: Fibrosis expansion in most portal areas with portal-to-portal bridging.

F3: Fibrosis bridging portal to portal and portal to central (incomplete cirrhosis).

F4: cirrhosis.

Data were expressed as the mean ± S.E.

** = Significant levels (P<0.01) versus normal control rats.

**Fig. 4:** Fibrosis grading in CCL₄-administered group and control group. Data were expressed as the mean ± SE. ** (P<0.01) versus normal control rats.

Biochemical results:

Liver seromarkers (AST, ALT and TP):

CCL₄-administered group showed a highly significant upregulation in the level of AST and ALT compared to control rats. However, the CCL₄-administered group showed highly significant downregulation

of the TP serum levels compared to control rats. Serum levels of AST, ALT and TP in rats of different groups are presented in Table (4) and Fig. (5 A, B & C).

Oxidative stress indices (LPO and TAC):

Mean values of the serum levels of LPO showed a significant elevation in the CCL₄-administered group after 12 weeks compared to the control one, while the serum level of TAC was significantly decreased in comparison with the control group. Serum levels of LPO and TAC of rats of different groups are reported in Table (5) and Fig. (5 D & E).

Table 4: Values of liver seromarker enzymes (AST & ALT) and TP in rats of different groups.

Groups	Control group		CCL ₄ administered group
	Normal control	Olive oil	
AST	140.5± 0.866	118.67± 16.836	1405± 217.04**
ALT	84.40 ± 2.80	78.33 ± 16.19	1210.0 ± 63.51**
TP	10.30± 0.31	10.30± 0.23	6.23± 0.09**

Data were expressed as the mean ± S.E.

** = Significant levels (P<0.01) versus normal control rats.

Table 5: Values of oxidative stress indices (LPO & TAC) of rats in different groups.

Groups	Control group		CCL ₄ administered group
	Normal control	Olive oil	
TAC	1.87± 0.08	1.84± 0.16	1.24± 0.09*
LPO	8.85± 1.05	10.55± 2.24	38.54± 4.87*

Data were expressed as the mean ± S.E.

* = Significant levels (P<0.05) versus normal control rats.

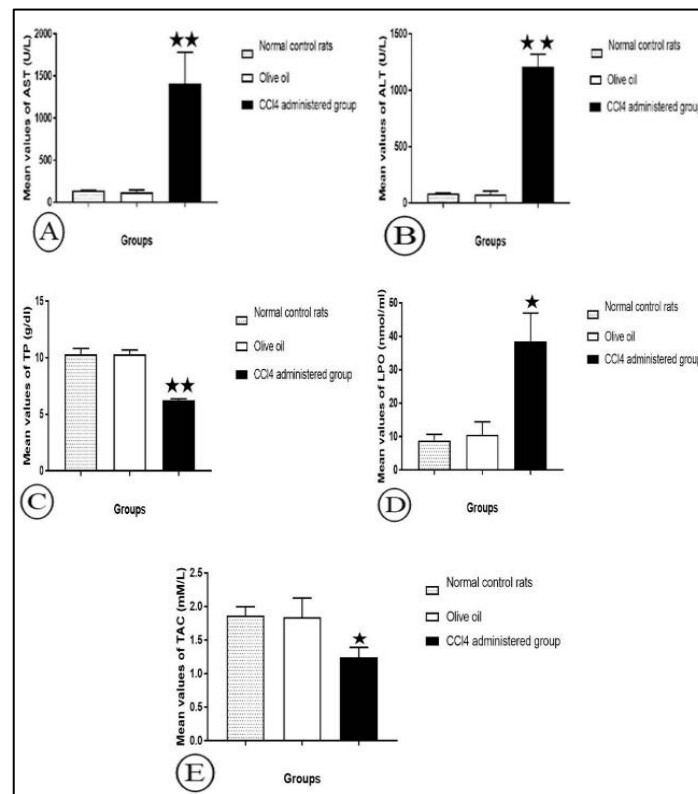


Fig. 5: Mean values of different biochemical parameters in CCL₄-administered group and control group. A) Values of AST (U/L). B) Values of ALT (U/L). C) Values of TP (g/dl). D) Values of LPO (nmol/ml). E) Values of TAC (mM/L). Data were explained as the mean $\pm$ SE. * ($P < 0.05$), ** ($P < 0.01$) versus normal control rats.

DISCUSSION

In our experiment, we investigated the effect of CCL₄ on the hepatic tissue in rats at a dose of 2 mg/kg twice weekly by S/c injection for 12 weeks (Li *et al.*, 2016). The rats in both CCL₄-administered group and control group were sacrificed 12 weeks post dosing. The livers' tissue samples were removed and subjected to a histological analysis. For biochemical examination, serum samples from both the CCL₄-administered group and the control group were taken. Histopathological findings of tissue sections from the livers of the sacrificed rats administered CCL₄ for 12 weeks revealed marked hepatic lesions. These lesions were marked degenerative changes, such as hydropic degeneration, fatty degeneration, as well as coagulative necrosis in all these 10 rats. Similar lesions were described by many authors

(Weber *et al.*, 2003; Valko *et al.*, 2004; Höhme *et al.*, 2007), who went on to explain that these changes were brought on by lipid peroxidation and the inhibition of oxidative enzymes, which resulted in accumulation of O₂ and H₂O₂ which is a cascade phenomenon of free radical production that damaged the liver.

In our work, there were angiopathic changes, where all 10 examined rats of this group showed severe congestion of blood vessels and hemorrhage in between hepatic cords. Moreover, the blood vessels in the portal areas were also affected. The injury was brought on by the free radicals' oxidative stress, which encourages lipid peroxidation and damages the membrane of all blood vessels. These results were in agreement with Zhao *et al.*, (2013), who studied the liver damage of CCL₄ in Sprague Dawley rats.

After a chronic liver injury, a number of predetermined molecular alterations occur. Hepatic stellate cells (HSCs) get activated and develop into a myofibroblast phenotype, which gains the capacity to express and deposit enormous amounts of collagen (Tamayo *et al.*, 2018; Tacke & Weiskirchen, 2012 and Urtasun *et al.*, 2008).

At the current study, hepatic fibrosis was detected in all 10 examined rats, which appeared as thick fibrous connective tissue bands invaded the parenchymal tissue and atypical hepatic lobules were formed. Moreover, portal fibrosis and newly formed bile ducts were also seen. These confirmed morphometrically by significant increase of area percentage of collagen fibers in case of CCL₄ hepatotoxicity (Olsson *et al.*, 2000; Morio *et al.*, 2001; Alshawsh *et al.*, 2011; Dooley *et al.*, 2011 and Tsuchida & Friedman, 2017).

In addition, Glissonian capsule fibrosis with pressure atrophy and degeneration of the underneath hepatic cells was noticed. Similar observations were recorded by Hefnawy and Ramadan, (2013) and Ahmed *et al.*, (2011).

There was hepatic cirrhosis detected in 4 rats, where extensive fibrous connective tissue proliferated and replaced most of the hepatic lobular tissue. Yang *et al.*, (2010) suggested that the increased collagen fibers deposition was due to oxidative stress produced by CCL₄.

In the current investigation, it was discovered that CCL₄ increased ALT and AST levels. According to Yadav *et al.* (2019), the disruption of hepatocytic transport function after hepatic injury results in altered membrane permeability, which allows for the leakage of enzymes from the cells, lowering ALT and AST

levels in the hepatic cells and raising their levels in the serum. Additionally, Rajeh and Latha (2004) have demonstrated that elevated liver enzyme levels are associated with cellular leakage and liver cell membrane integrity.

By contrast, CCL₄ was found to decrease the TP levels in the CCL₄-administered group in comparison with the control group. This is indicative of hepatic injuries caused by oxidative stress (Manna *et al.*, 2006). Zhao *et al.* (2012) also demonstrated that this decrease of TP occurred due to liver inflammation with subsequent edema of the organelles and structural damage.

Serum measurements of LPO and TAC in the current investigation revealed a substantial increase in LPO levels in the CCL₄-administered group compared to the control group. Malondialdehyde, an end product of LPO, in the liver tissue acts as an indicator of LPO, which is known to occur in hepatic toxicity due to the formation of reactive oxygen species (ROS) (Dalton *et al.*, 2009). On the other hand, the serum level of TAC in the CCL₄-administrated group was significantly decreased in comparison with the control group. Numerous authors came to the same conclusion that CCL₄ modifies oxidative stress indicators, citing data that were similar to their own (Tang *et al.*, 2022; Yang *et al.*, 2019; Bardi *et al.*, 2014; Hefnawy and Ramadan, 2013).

It could be concluded that administration of CCL₄ for 12 weeks causes various forms of hepatic lesions as pronounced degenerative, angiopathic, and necrotic changes in addition to fibrosis. Also, biochemical indices including liver seromarkers and oxidative stress indices were altered.

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التقييم الهستوباثولوجي والبيوكيميائي للتليف الكبدي المحدث بواسطة رابع كلوريد الكربون في الجرذان

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تم إجراء هذا البحث لتقييم التأثير السمي لرابع كلوريد الكربون على كبد ذكور الجرذان البيضاء البالغة. باستخدام التصوير المورفومتري لتقييم درجة التليف المرتبط بتلف خلايا الكبد المتني ومراقبة إنزيمات مصل الكبد المتغيرة ألانين، الاسبرتات، ومؤشرات الإجهاد التأكسدي مثل المالونديالدهيد والقدرة الكلية المضادة للأكسدة. تم تقسيم اثني وعشرين من ذكور الجرذان البيضاء إلى مجموعتين. تم إعطاء رابع كلوريد الكربون مجموعة (I) المشتملة على عشرة جرذان بجرعة ٢ مل / كجم من وزن الجسم عن طريق الحقن تحت الجلد مرتين أسبوعياً لمدة ١٢ أسبوعاً. تنقسم المجموعة الضابطة (II) إلى مجموعتين فرعيتين؛ واحدة من الجرذان كانت ضابطة ضمت ٦ فئران والمجموعة الأخرى التي حصلت على زيت الزيتون وتضمنت ٦ فئران. تم ذبح جميع الفئران بعد ١٢ أسبوعاً. جمعت عينات أنسجة الكبد من جميع المجموعات للفحص الهستوباثولوجي وكذلك تم الحصول على مصل الدم لتحديد المعايير البيوكيميائية. أظهر الفحص الهستوباثولوجي للمجموعة الأولى تغيرات تنكسية كبدي مثل التنكس المائي والتنكس الدهني والاستماتة. تم الكشف عن تغيرات اعتلال الأوعية الدموية وكشفت عن احتقان ونزيف حاد في الأوعية الدموية بين الحبال (صفوف الخلايا) الكبدية. تم تسجيل التليف الكبدي في جميع الفئران العشرة التي تم فحصها وظهر على شكل عصابات/حزم ليفية ضامة سميكة غزت النسيج المتني مكونة فصيصات كبدية غير نمطية. أفة متقدمة على شكل تليف كبدي في ٤ جرذان حيث تكاثر النسيج الضام الليفي الواسع واستبدل معظم النسيج المفصص الكبدي. أظهرت النتائج البيوكيميائية أن الحيوانات في المجموعة الأولى أظهرت زيادة معنوية في مصل ألانين (ALT) والاسبرتات (AST) والمالونديالدهيد (LPO) مقارنة بالمجموعة الضابطة، بينما انخفض البروتين الكلى (TP) و القدرة الكلية المضادة للأكسدة (TAC). ومن هذه الدراسة يمكن استنتاج ان رابع كلوريد الكربون تسبب في تليف أنسجة الكبد بالإضافة إلى تغيير المعايير البيوكيميائية.