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A canine model of carbon tetrachloride (CCl_4) induced liver cirrhosis: II.Ultrasonographic and histopathological study

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ABSTRACT

Liver cirrhosis is a diffuse state with replacement of the normal lobular architecture by abnormal nodules and fibrous septa. The Laennec staging system subdivides cirrhosis into 3 groups (4A,4B,4C) based on the thickness of fibrous septa and the size of nodules.

This study aimed to assess the degree of liver cirrhosis through ultrasonographical and histopathological study after the administration of CCl_4 in order to establish a canine model of liver cirrhosis for further research work. Experimental induction of liver cirrhosis was carried on 18 skeletally mature mongrel dogs using carbon tetrachloride (CCl_4) for 16 weeks; dogs were evaluated for cirrhosis using ultrasonographical examination with portal vein (PV) and hepatic vein (HV) diameter, histopathological changes every 4 weeks and finally postmortem examination. Liver fibrosis was subdivided through liver biopsy under the guidance of ultrasonography using the Laennec staging system. Liver cirrhosis was confirmed by abnormal liver ultrasononography with portal hypertension, liver cirrhosis on biopsy sample was scored 4A,4B,4Cbased on the thickness of the fibrous septa and the size of nodules according to Laennec staging system. The degree of liver cirrhosis was recorded through ultasonographical and histopathological examination.

Keywords: dogs, CCl_4, liver, cirrhosis, fibrosis staging.

INTRODUCTION

Liver cirrhosis is a diffused condition in which the normal lobular architecture of the liver is replaced by abnormal nodules and fibrous septa (Anthony et al., 1978).

Damage to the liver was induced by carbon tetrachloride (CCl_4).There is an excessive lipid peroxidation of hepatocytes membrane leading to functional and structural disruption (Muriel et al., 2001) resulting in fatty degeneration,centrolobular necrosis(Adebayo et al., 2011 and Teli et al., 2015) and mononuclear cell infiltration (Natsume et al., 1999).Furthermore, there is excessive accumulation of extracellular matrix into the liver parenchyma(fibrosis). Chronic fibrosis progresses from fibrosis to cirrhosis that characterized by septa formation and rings of scar tissue surrounding nodules of surviving hepatocytes (Friedman, 2003).The toxic effect of $CCl_4$is attributed to trichloromethyl radical produced during oxidative stress (Stoyanovsky and Cederbaum, 1999) followed by a series of cascades of cellular events such as the massive release of inflammatory mediators or cytokines, which eventually lead to liver injuries and fibrosis (Canbay et al., 2004,Ramadori et al., 2004 and Saile and Ramadori., 2007).

Ultrasonography is an essential diagnostic imaging technique for the assessment of the stages of liver fibrosis and measuring the diameter of portal and hepatic veins.

Liver biopsy is the gold standard for the diagnosis of most liver diseases and also assesses the severity of liver fibrosis and necro-inflammation (Stockhaus et al.,2004).The degree of liver fibrosis on biopsy can be evaluated through the Laennecstaging system based on the thickness of the fibrous septa and the size of nodules(Kutami, et al., 2000, Wanless et al., 2002 and Kim et al., 2011).

The objective of this study was to determine the degree of CCl_4 induced liver cirrhosis through ultrasonographical,histopathological and postmortum examination in order to establish a canine model of liver cirrhosis for stem cells treatment.

MATERIALS AND METHODS

All study procedures were approved by the Institutional Animal Care and Use ethical Committee (IACUC) of Faculty of Veterinary Medicine-Cairo University, Egypt (Cu F Vet/F/SUR/2013/16).

Eighteen dogs aging 2 – 2½ years old weighing 15-20 Kg and of both sex (11 male and 7 female) were used in this research work. Before commencing the study, dogs were acclimated to the kennel environment for at least 2 weeks. Complete clinical, laboratory and ultrasonographic examinations were conducted on each dog to exclude those with evidences of systemic and/or hepatic disease.During the study, dogs were fed twice daily on a standard dry food with free access to drinking water. liver cirrhosis was induced by weekly oral administration of CCl4 1 ml/kg (98%) using orogastric tube 16 weeks. Liver cirrhosis was diagnosed through ultrasonography, histopathologic examination obtained by liver biopsyand finally postmortem examination at the end of the study period. Liver biopsy was obtained using a single-handed automatic biopsy needle (16 gauge ×150 mm) under ultrasound-guidance.

Ultrasonography:

Liver ultrasonography was applied four weeks intervals till the end of the study period (16 week) according to (Kantrowitz, et al.,1989, Nyland and Fisher, 1990 and Lamb and Mahoney, 1994).

Surgical procedure for obtaining of liver biopsy sample using CCl_4:

The food was withdrawn from the dogs overnight prior to the examinations. Suitable regiem en anaethesia was used for dogs (atropine sulphate 0.05 mg/kg B.W S/C (Atropine®, ADWIA), xylazine HCl 1mg/kg B.W. I/M (Xyla-ject® 2%, ADWIA Co., A.R.E and ketamine HCl 50mg/kg B.W. I/V (Ketamine®, Protexmedia, Trittan, Germany)).

The operation site wasunder complete aseptic condition. Dogs were placed in right lateral recumbency. Under ultrasound guidance,liver specimen (Core biopsy) was obtained by withdrawing the handle of the tru cut needle; the needle was introducing at 45° into the left lateral lobe of the liver till the tip of the needle was seen on the screen, then a cutting cannula at the end of the automatic needle was fired to obtain the sample. Coagulant drugs were given to animals with careful observation for haemorrhage over the following 4–6 hours. From every dog, three biopsy samples were obtained each time (Stockhaus et al., 2004 and Mannion, 2006). Liver biopsy specimens were formalin fixed and paraffin embedded. Then 4-µm thickness sections were stained with haematoxyline and eosine and Masson's trichrome stains).

The degree of liver fibrosis was evaluated semi quantitavely according to Laennec staging system (Wanless et al., 2002). Fibrosis was first scored as follows: 0, no definite fibrosis; 1, minimal fibrosis (no septa or rare thin septum may have portal expansion or mild sinusoidal fibrosis); 2, mild fibrosis (occasional thin septa); 3, moderate fibrosis (moderate thin septa; up to incomplete cirrhosis); and 4, cirrhosis. Stage 4 was distinguished from stage 3 by more numerous septa per unit length of biopsy sufficient to display nodules with rounded contours. Then, stage 4 was sub-classified into three groups: 4A, mild cirrhosis, definite or probable; 4B, moderate cirrhosis (at least two broad septa); and 4C, severe cirrhosis (at least one very broad septum or many minute nodules). The terms 'broad septum' and 'very broad septum' were defined by comparing the relative thickness of fibrous septa and sizes of nodules (Kim et al., 2011).

Postmortem evaluation:

At the end of the 16 week, all dogs were humanely sacrificed using T61 (Bertol et al., 1993). Necropsies were performed. The excised livers were washed in sterile saline then photographed. Representative sections from all lobes of the liver were collected for morphological (cut section, borders, color and lobes consistency) and histopathological evaluation.

Histopathological evaluation:

Liver biopsy specimens were formalin fixed and paraffin embedded. Then 4-µm thickness sections were stained with haematoxyline and eosine and Masson's trichrome stain for collagen fibers.

Statistical analysis:

Data of average diameter of PV and HV were expressed as mean ± SD. Independent-samples T test (SPSS version 17.0 computer Software) was used to determine the statistically significant differences between the values at the base line (0 day) and 16 week. Results were considered significant at $P<0.05$.

RESULTS

Ultrasonographic evaluation:

Ultrasonographic examinations of the liver showed marked difference from the baseline (0 day)as the liver had a irregular margin, blunt edges, thickened capsule and blurred hyperechoic areas with uneven distributions (Fig 1a,b,c and d), portal vein (PV) and hepatic vein (HV) were dilated (0.44±0.16 cm and 0.44±0.13 cm; respectively) at 16 week ($P< 0.05$). The changes in PV and HV diameters are recorded in (table 1).

Biopsy results:

Biopsy specimens of liver showed diffused areas of vacuolar degeneration of hepatocytes individual cell necrosis (apoptosis) at the 4 week (Fig 2 a), mild to moderate portal fibrosis with multiple areas of vacuolar degeneration of hepatocytes, individual cell necrosis at the 8 week (Fig 2 b and c) while portal and early septal fibrosis (incomplete cirrhosis) with severe damage of hepatocytes at the 12 week(Fig2d) and finally proliferation of fibrous connective tissue in the portal area with septal fibrosis (centrol-obular necrosis) associated with severe vacuolar degeneration of hepatocytes at the 16 week (Fig 2e and f).

Postmortem examination of the liver:

Liver was icteric with mottled appearance, hard in texture and blunt edges (Fig 3 and b).

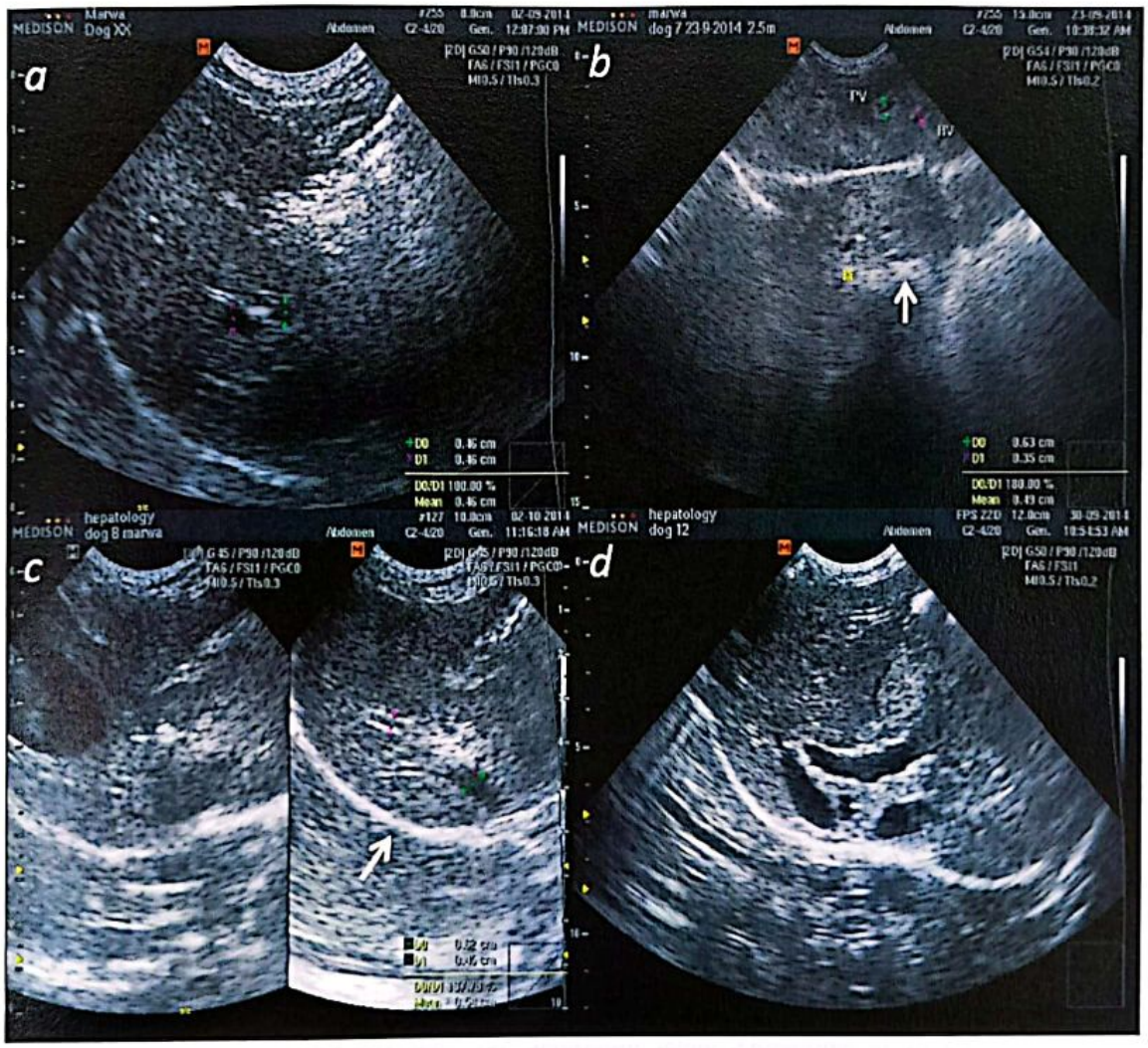

Fig (1): Two dimensional ultrasound scan at:
(a) The 4th week showing a slight thickening at the liver capsule
(b) The 8th week showing granular hypo-echogenic pattern in the liver parenchyma with thickening at the liver capsule (arrow)
(c) The12th week showing different coarse hyper-echogenic regions with marked uneven thickened capsule (arrow)
(d) The16th week showing marked hyperechoic areas of the liver with severe uneven thickened margin.
Table (1): the mean value of the portal and hepatic vein during the study.

(c)The12th week showing different coarse hyper-echogenic regions with marked uneven thickened capsule (arrow)
(d)The16th week showing marked hyperechoic areas of the liver with severe uneven thickened margin.

Table (1): the mean value of the portal and hepatic vein during the study.

week	PVØ	HVØ
0	0.32*	0.26*
4	0.4	0.39
8	0.63	0.43
12	0.64	0.47
16	0.73*	0.67*

*Statistically significan difference at P< 0.05.

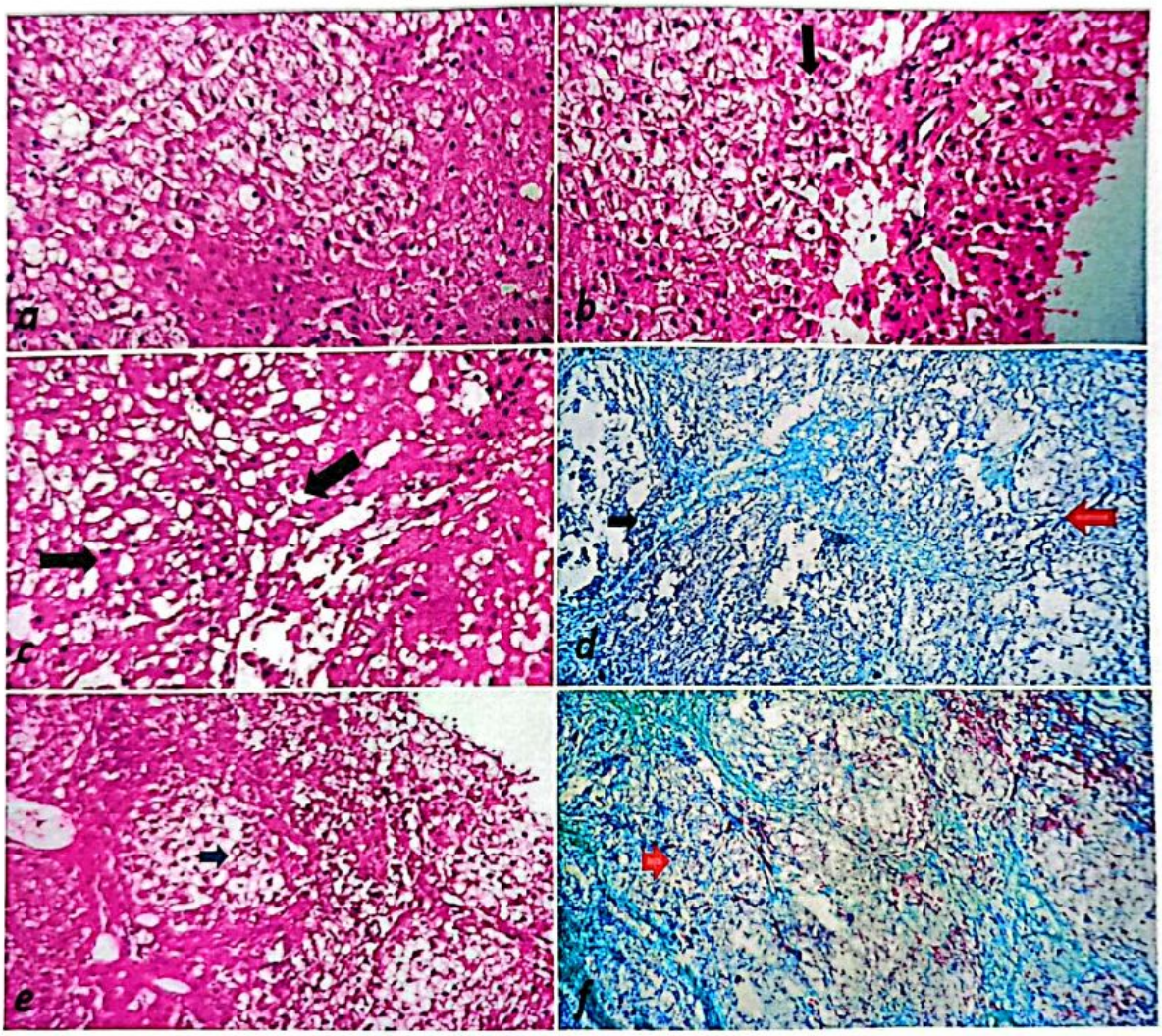

Fig (2): photomicrograph of liver sections at:
(a)The 4th week showing diffused areas of vacuolar degeneration of hepatocytes (arrow) with few infiltrations of inflammatory cells, individual cell necrosis (apoptosis) (H&E×400). Fibrosis grading F1
(b)The8th week showing mild portal fibrosis with multiple areas of vacuolar degeneration of hepatic cells (red arrows) with few infiltration of inflammatory cells (black arrows), sinusoidal dilatation and individual cell necrosis (H&E×400). Fibrosis grading F2
(c)The8th week showing moderate fibrosis with multiple areas of vacuolar degeneration of hepatic cells with few infiltration of inflammatory cells, bile pigment and individual cell necrosis (H&E×400). Fibrosis grading F3

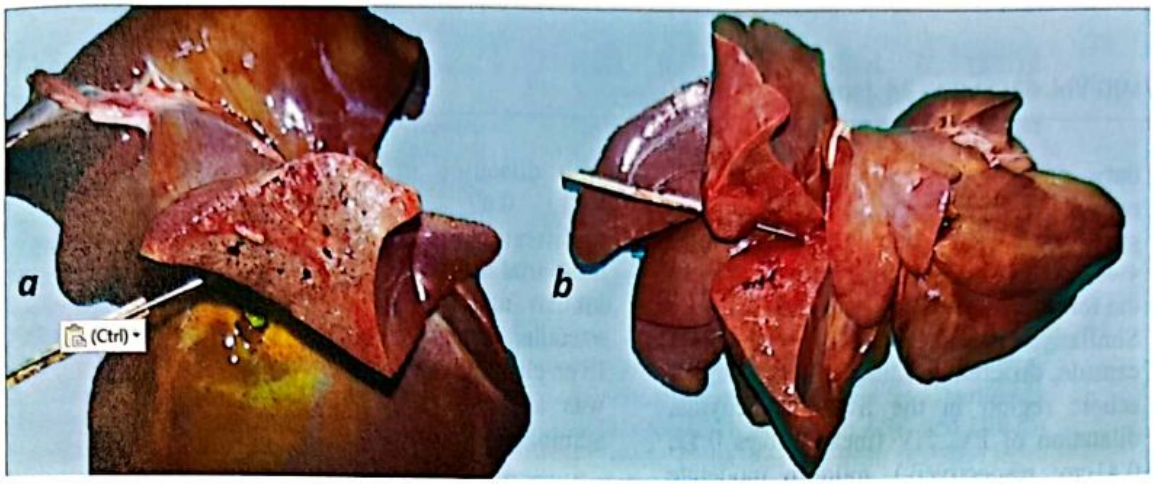

Fig (3): post mortem lesions of dog's liver at 16th week after CCl_4 administration showing **(a)** icteric color in some parts with **(b)** mottled appearance in cut section, blunt edges.

DISCUSSION

End-stage liver cirrhosis is an irreversible disease, characterized by loss of hepatocytes and increased deposition of the scar tissue in the liver parenchyma(Bigoniya et al., 2009).

Ultrasonography is a quick and a noninvasive method which could be used for the diagnosis of liver cirrhosis Liver.Oral administration of CCl_4 is known to produce hepatocellular damage of variant degrees. After 16 week from the CCl_4 administration,the liver appeared hyperechoic, non-homogenous with severe thickened capsule on ultrasonography similar to (Partington and Biller, 1995).This can be interpreted as a result of the replacement of the hepatocytes with fibrous connective tissue. Results showed various but progressive degrees of portal and hepatic vein dilatation similar to (Kuroda et al., 2006).This started to be evident at the 8th week and continued to increase till the 16th week. This could be explained by the fact, that during chronic liver failure, Endothelien-1 (ET-1) is produced which is the most important inducers of portal hypertension. ET-1 catalyzes the phosphorylation of amino acid residues in many kinases through the G-protein complex-phospholipase C-protein kinase C signaling pathway, regulates gene expression, the synthesis of collagen and matrix proteins. After ET-1 binds to endothelin receptor, it activates voltage-dependent calcium channels, promoting calcium influx and leading to vasoconstriction leading to increased portal and hepatic veins diameter (Kuroda et al., 2006).

Histopathological findings of the liver due to CCl_4 administration are in agreement with previous studies(Adebayo et al., 2011, Teocharis et al., 2001and Teli et al., 2015). The changes mostly include apoptosis, fatty accumulation or vacuolar degeneration, inflammatory cells infiltration and centro-lobular necrosis.

This refinement in the clinical assessment of cirrhosis has been matched by the recent introduction of the Laennec staging system that subdivides cirrhosis into three degrees of histological severity. The Laennec staging sub-classified cirrhosis into 3 stages 4A, 4B, and 4C based on thickness of septa and size of nodules (Kim et al., 2011).

The post mortem showed that, the liver at 16 weeks was firm in consistency as a result of deposition of the fibrous connective tissue between the lobes of the liver. The developed icterus could be attributed to the toxic damage of CCl_4 on hepatocytes affecting its capability of metabolizing the bilirubin.

From the obtained results, it was possible to establish a canine model of different degrees of liver cirrhosis. Grading of these stages was possible with the help of ultrasound and histopathological examinations according to the Laennec staging system.A slightly thickening at the liver capsule sonographically and dilatation of PV, HV (mean valuesof0.32 and0.26cm; respectively), in addition to

fatty degeneration with individual cell necrosis (apoptosis)indicated liver fibrosis grade one. This was achievable after 4weeks of oral administration of CCl_4 in the fore mentioned dose.

Similarly, marked thickening of the liver capsule, different granular to coarse hyper echoic region in the liver parenchyma, dilatation of PV, HV (mean values 0.52, 0.41cm; respectively), mild to moderate portal fibrosis with multiple areas of vacuolar degeneration of hepatocytes and individual cell necrosis, indicated liver fibrosis grades two and three. This was achieved after 8 weeks of oral administration of CCl_4.

While portal and early septal fibrosis (incomplete cirrhosis) with severe damage of hepatocytes, dilation of PV, HV (mean value 0.64, 0.47 cm; respectively), liver cirrhosis has reached grade 4A. This was achievable after 12 weeks of oral administration of CCl_4.

Finally, when severe uneven thickened margin, blunt edges, hyperechogenic liver with dilatation of PV, HV(mean value 0.73, 0.67 cm; respectively) and proliferation of fibrous connective tissue in the portal area with septal fibrosis (centro-lobular necrosis) associated with severe vacuolar degeneration of hepatic cells, the liver cirrhosis has reached grade 4B. This was achievable after 16 weeks of oral administration of CCl_4.

CONCLUSION

It can be concluded that the CCl_4 is hepatotoxic substance that can induce hepatocellular damage using the specified dose. The different echogenic pattern with portal hypertension sonographically in addition to different vacuolar degeneration and centrilobular necrosis histopathologically can be taken as a staging indicator of liver cirrhosis. A canine model of different stages (4A and 4B) of reversible liver cirrhosis can be established after 16 weeks of administration of CCl_4.

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الملخص بالعربى

استحداث نموذج للتليف الكبد في الكلاب باستخدام رابع كلوريد الكربون: 2:دراسة الاشعة فوق الصوتية و التشريحية المرضية

تليف الكبد هو حالة استبدال خلايا الكبد الطبيعية الي خلايا غير طبيعية وانشاءحواجز ليفية .وقد قسم نظام الليفيك تليف الكبد إلى 3 مجموعات (4A، 4B، 4C) وذلك على أساس سمك الحاجز ليفي وحجم العقيدات التي تكونت اثناء التليف وهدفت هذه الدراسة إلى تقييم درجة تليف الكبد من خلال الفحص باستخدامالسونارو الفحص النسيجي وذلك بعداعطاء رابع كلوريد الكربون CCl4 من أجل وضع نموذج الكلاب لتليف الكبد لإجراء مزيد من البحوث .وقد تم الحث التجريبي لتليف الكبد في 18 كلاب هجينة ناضجة باستخدام رابع كلوريد الكربون (CCl4) لمدة 16 أسبوعا .تم تقييم الكلاب لتليف الكبد باستخدام السونار مع قياس قطر الوريد البابي (PV) والوريد الكبدي (HV) ، والتغيرات التشريحية المرضية والفحص بعد الوفاة .تم تقسيم تليف الكبد عن طريق اخذ عينة من الكبد بتوجيه من الموجات فوق الصوتية 4 مرات / 4 أسابيع باستخدام نظام الليفيك .وأكد تليف الكبد عن طريقسونار الكبد الغير طبيعية مع ارتفاع ضغط الدم البابي، وسجل تليف الكبد على عينة خزعة 4A، 4B، 4Cعلى أساس سمك الحاجز ليفي وحجم العقيدات .وفي النهاية سجلت درجة تليف الكبد من خلال الفحص بالسونار والأنسجة.