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FULL PAPER

Ultrasonography, Biochemical and Hematological Profiles in Liver Disease Caused by Intravenous Administration of Dimethylnitrosamine in Dogs.

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Abstract

The results of liver function tests and ultrasonographical findings were analysed in 7 dogs that were intravenously injected dimethylnitrosamine (DMNA 2 mg/kg body weight) on 2 consecutive days each week for 10 weeks. Typical clinical signs and similar changes in liver enzyme concentrations that develop in dogs with natural cirrhosis were observed in this canine model. Severe anaemia and a significant reduction in the platelet numbers occurred in the dogs that died in the 5th week, while in all the other dogs these parameters decreased slightly. Serum total protein and the albumin/ globulin ratio decreased gradually while the alkaline phosphatase, alanine amino transferase, aspartate amino transferase and gamma glutamyl transpeptidase activities increased significantly ($p<0.05$) in all dogs after beginning the administration of DMNA. Ultrasound findings of a coarsened and heterogeneous echo pattern with increased echogenicity that are characteristic of canine cirrhosis were noticed at the same time when the changes in liver enzymes became evident. Present results suggest that ultrasonography in conjunction with liver function tests may be useful in the evaluation of experimentally induced liver cirrhosis.

Key words: cirrhosis, dimethylnitrosamine, dog, liver enzymes, ultrasonography

Introduction

Liver diseases are quite common in both dogs and cats. They can result from direct damage to the liver by toxins or other infectious agents as well as metabolic, immune-mediated, or neoplastic problems[10]. Extra hepatic obstruction due to cholelithiasis is a major cause of liver disease in the dog. The most common cause of obstruction is mechanical due to neoplastic diseases of the liver, gallbladder and bile ducts, pancreas, gastrointestinal tract, and lymph tissue. The final common pathway of chronic liver diseases regardless of the causes is liver cirrhosis.

Liver cirrhosis has been described as the presence of extensive fibrosis and regenerative nodules replacing the normal liver parenchyma. Parenchyma necrosis is followed by connective tissue proliferation and distortion of lobular and vascular hepatic architecture[6].

This distortion of the liver architecture

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interferes with blood flow leading to the inability of the liver to perform its many different functions. Abnormal biochemical function may lead to a drop in protein concentration, especially albumin. The production of clotting factors may also decrease. Concentrations of enzymes and other metabolic products in the blood change[9,17,23]. Cirrhosis of the liver can also cause problems in other organ systems such as the brain and kidneys due to the accumulation of catabolic products like ammonia in the blood stream. Diagnosis can then be established considering the history, physical findings and results of laboratory examinations. A number of chemical laboratory tests are usually used. The main parameters routinely utilised are the determination of liver enzyme concentrations and the determination of the synthesis and excretion ability of the liver[7,14].

Other tests like liver biopsies and laparotomies may be contraindicated in patients with compromised liver function. In such cases, a non-invasive diagnostic tool is likely to be more useful. Radiology can be used to characterize the morphological manifestations of cirrhosis and to evaluate the hepatic and extra hepatic vessels[6,18]. Ultrasound as a non-invasive imaging modality can be useful in the evaluation of parenchymal abnormality, hepatic vessel flow characteristics and flow velocities and patterns[5].

Dimethylnitrosamine (N-nitrosodimethylamine ; DMNA) is a N-alkyl-N-nitroso compound that is chemically stable and non-toxic under normal conditions. It must be metabolically activated before it can produce specific toxicity and carcinogenic potency for the liver[4]. Enzymatic transformation by the hepatic cytochrome P-450 drug metabolizing system results in the production of several metabolites, including an active carbonium ion[15]. It has been used by many researchers to produce models of cirrhosis in the dog and rat because of its liver specificity. The changes of liver functions have been studied in dogs orally administered with the toxin to induce acute toxicity specific for the liver[3,4,8,17,21,22]. There are, however, no reports describing the relationship between ultrasound, biochemical and hematological findings after intravenously injecting DMNA in dogs.

The aims of this study were, therefore, to make a contribution to the ultrasound study of cirrhosis caused by dimethylnitrosamine and to examine the pathophysiology of its intravenous administration in normal dogs.

Materials and Methods

Experimental Animals.

Experiments were performed on 7 adult mongrel dogs (5 males and 2 females) between the ages of 1 and 3 years and weighing between 8 and 15 kg (average 11.8 kg) before and after DMNA administration. The dogs were housed in individual cages in the experimental animal quarters of the Laboratory of Veterinary Surgery and were fed a complete commercial standard dry food diet. Food and water intake as well elimination were continuously monitored.

Toxin administration.

DMNA (Wako Pure Chemicals Co. Ltd., Tokyo, Japan) was administered at a dosage of 2 mg/kg body weight intravenously on 2 consecutive days each week for 10 weeks. DMNA was dissolved in physiological saline solution and injected via indwelling intravenous catheters to avoid human contact with the toxin.

Clinical examination.

Routine physical examination, thoracic radiography and clinical laboratory tests were done before the commencement of the administration of the toxin to screen for liver disease and to establish baseline values for each dog. Physical examinations including the determination of body weight, temperature, heart rate and respiration rate were performed throughout the study period. Heart rate, respiratory rate and

temperature were also measured during the toxin administration.

Laboratory examination.

Laboratory examinations including a complete blood cell count (CBC) and blood chemical profiles were done before and after the toxin administrations. Blood was collected from the external jugular vein. The samples for a CBC were immediately analysed after collection using a hematology cell counter (Serono Diagnostics System 9000, Baker Instruments Corporation. PA, USA). Serum was separated by centrifugation after the blood had coagulated. The samples were immediately frozen and stored at –20°C until analysis. The serum samples were analysed for total protein (TP), albumin/globulin (A/G) ratio, glucose (Glc), blood urea nitrogen (BUN), alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST), gamma glutamyl transpeptidase (GGT), total bilirubin (TBil), cholesterol (Cho) and total fasting serum bile acids (FSBA). The A/G ratio was calculated from the results of cellulose acetate membrane electrophoresis. FSBA concentrations were determined by an enzymatic assay for 3-alpha hydroxylated bile acids (Nycomed, Oslo, Norway) purchased from Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan, while the other tests were performed on an automatic analyzer (Cobas Mira, Nippon Roche, Tokyo, Japan).

Ultrasound examination.

Ultrasound examinations were performed using a ultrasound system (HITACHI EUB-565A, Hitachi Med. Co., Tokyo, Japan), according to the method previously described[18,25]. The ultrasound examination was done on the first day of toxin administration each week. The dogs were fasted overnight before the examination because food and gas in the gastrointestinal tract disturb the propagation of the ultrasound waves. Sedation of the dogs was often not necessary. Manual restraint was usually enough. If needed flunitrazepam (0.03 mg/kg, iv.) and atropine sulphate (0.03 mg/kg, sc.) were administered to overcome operator difficulties. The cranial ventral abdomen up to the rib cage was clipped. An ultrasonic coupling gel was applied to the transducer. Transducer selection depended on the animal size. A 3.5 MHz electronic sector transducer was used for most of the animals while a 5.0 MHz or 6.5 MHz electronic transducer was used for the smaller animals.

With the animal laying on its right side, the transducer was placed caudal of the xiphoid and the ultrasound beam directed cranially. The resulting picture showed a longitudinal view through the liver and gallbladder with the diaphragm appearing as an echogenic cranial border. Then the transducer was moved long the rib cage until all of the left side had been examined. Starting again at the xiphoid but with the transducer turned around 90° transverse scans were done. The procedure was repeated with the animal on its left side, starting in the 5th intercostal space progressing cranially in order to determine the best locations for visualizing the gallbladder, the portal and hepatic veins and the caudal vena cava. Changes in echogenicity, hepatic vessels, appearance of the gallbladder (size, shape, wall) were evaluated.

Gross pathological examination.

Gross pathological examinations were done on the liver of each dog after euthanasia at the end of the experiment. The dogs were euthanized with an intravenous overdose injection of pentobarbital sodium.

Statistical analysis.

The paired *t*-test was used to compare the baseline data and the data during the toxin administration. The value of $p<0.05$ was considered to be significant.

Results

Clinical findings.

The dogs became depressed and lost appe-

tite in the 2nd week of the toxin administration. By the 3rd week they had all regained appetite and became active again. Two dogs died in the 5th week after showing signs of icterus, fever, weight loss, ascites, vomiting and gastrointestinal haemorrhage. The ascitic fluid had a total protein concentration of <1.0 g/dl.

Hematological findings (Table 1).

All the 7 dogs had normal CBC values at the beginning of the experiment. A significant ($p<0.05$) decrease in the platelet number occurred starting in the 3rd week and maintained the low values until the 10th week. The lowest value of $70\times10^3/\mu l$ was reached by only one dog. A significant decrease in the RBC in all dogs was observed in the 5th and 6th week. The hemoglobin concentration also significantly dropped starting in the 8th week. There was no significant change in the WBC count throughout the examination period.

Biochemical findings (Table 2).

There was a significant decrease in the TP concentration beginning in week 4 and the lowest value reached was 4.5 ± 0.6 g/dl in the 8th week. The decrease in the plasma protein content was accompanied by a reduction in the A/G ratio from a preinduction average of 1.24 ± 0.40 to 0.65 ± 0.16 in the 7th week with a drop in the albumin

Table 1. Hematological changes before and after the intravenous administration of dimethylnitrosamine.

	Weeks after beginning the administration of dimethylnitrosamine.										
Parameter	0	1	2	3	4	5	6	7	8	9	10
RBC$\times10^6/\mu l$	7.59± 0.40	7.04± 2.00	7.07± 0.10	6.58± 1.40	6.67± 1.00	6.20± 1.20*	6.8 ± 0.10*	7.04± 0.70	7.13± 0.50	7.20± 0.60	7.24± 0.50
Hb g/dl	16.6 ± 1.2	17.0 ± 1.8	15.7 ± 1.0	15.0 ± 2.0	16.3 ± 3.0	14.8 ± 1.0	15.0 ± 1.2	14.6 ± 2.0	14.6 ± 1.5*	14.5 ± 1.4*	15.2 ± 2.0*
WBC$\times10^3/\mu l$	15.0 ± 4.0	16.9 ± 2.9	17.8 ± 3.6	17.0 ± 5.0	15.9 ± 4.6	16.2 ± 5.0	15.5 ± 4.7	16.7 ± 6.4	15.8 ± 4.9	16.0 ± 3.8	16.0 ± 3.2
Plt$\times10^3/\mu l$	323 ±90	241 ±62	223 ±71	233 ±69*	198 ±40*	182 ±12*	154 ±99*	121 ±75*	109 ±40*	195 ±55*	159 ±51*

(Mean±SD)
Week 0–5: n=7
Week 7–10: n=5
*=$p<0.05$

RBC: Red blood cell.
Hb: Hemoglobin.
WBC: White blood cell.
Plt: Platelets.

Table 2. Biochemical changes before and after the intravenous administration of dimethylnitrosamine.

	Weeks after beginning the administration of dimethylnitrosamine.										
Parameter	0	1	2	3	4	5	6	7	8	9	10
TP g/dl	6.2 ± 0.6	6.3 ± 0.6	6.2 ± 0.6	5.9 ± 0.2*	5.9 ± 0.2*	5.6 ± 0.6*	5.0 ± 0.8*	4.9 ± 0.8*	4.5 ± 0.6*	4.9 ± 0.5	5.2 ± 0.9*
A/G ratio	1.24± 0.40	1.15± 0.44	1.05± 0.26	0.97± 0.22	0.86± 0.28	0.84± 0.21	0.85± 0.30	0.65± 0.16*	0.75± 0.16*	0.66± 0.21*	0.81± 0.11
Glc mg/dl	108 ± 9	102 ± 9	116 ± 13	108 ± 14	107 ± 10	113 ± 11	113 ± 7	127 ± 9	108 ± 12	105 ± 7	112 ± 12
BUN mmol/l	13.3 ± 6.0	13.1 ± 4.0	14.1 ± 5.0	13.8 ± 7.2	16.9 ± 8.0	13.6 ± 8.0	10.2 ± 4.2	9.7 ± 3.0	8.8 ± 2.0	9.0 ± 2.0	9.0 ± 2.4
ALP IU/l	27 ± 8	61 ± 48	146 ± 66*	840 ±193*	566 ±205*	472 ±186*	378 ± 64*	412 ±136*	307 ±103*	271 ±132*	221 ±131*
ALT IU/l	49 ± 23	114 ± 34*	218 ± 45*	283 ±150	402 ±216*	346 ±230	394 ± 23	203 ±159	207 ±141	217 ± 16	119 ± 92
AST IU/l	40 ± 11	68 ± 29	105 ± 58	186 ±107	204 ±127*	211 ±127	191 ± 91	331 ±237	216 ±132*	142 ± 85	174 ±111
GGT IU/l	5.9 ± 1.0	12.3 ± 8.0	16.7 ± 23.0*	19.2 ± 2.0	29.4 ± 11.0*	44.0 ± 4.0	17.9 ± 11	18.6 ± 7.0	16.8 ± 14*	14.5 ± 2.0	16.4 ± 15.0
TBil mg/dl	0.24± 0.20	0.57± 0.40*	0.39± 0.20	0.75± 0.80	0.67± 0.50	0.68± 0.80	0.43± 0.20	0.87± 0.50	0.89± 0.90	1.22± 1.00	1.32± 0.40*
Cho mmol/l	143 ± 56	148 ± 50	156 ± 97	160 ±114	162 ± 86	149 ± 95	137 ± 35	114 ± 24	104 ± 24	111 ± 25	106 ± 30
FSBA μmol/l	6 ± 4	14 ± 18	53 ± 36*	121 ± 62*	79 ± 24*	111 ± 51*	137 ± 61*	190 ±118*	156 ± 39*	197 ± 51*	205 ±118*

(Mean±SD)
Week 0–5: n=7
Week 6–10: n=5
*=$p<0.05$

TP: Total protein
A/G ratio: Albumin/ globulin ratio
Glc: Glucose
BUN: Blood urea nitrogen
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
GGT: Gamma glutamyl transpeptidase
TBil: Total bilirubin
Cho: Cholesterol
FSBA: Fasting serum bile acids

fraction. In all the dogs, the administration of DMNA led to increase of serum enzyme activities. The serum enzyme levels started to rise remarkably starting in the 2nd week. Highest levels were reached between the 4th and 8th week. The ALP activity increase was the highest followed by ALT and AST. ALP increased from an average baseline value of 27±8 IU/l to 840±193 IU/l in the 3rd week. The ALT rose from 49±23 IU/l to 402±216 IU/l in the 4th week and AST from 40±11 IU/l to 331±237 IU/l in the 7th week. The GGT rose from a preinduction average of 5.9±1.0 IU/l to a maximum of 44.0±4.0 IU/l in the 5th week. TBil increased starting in the 3rd week and maintained high levels until the 10th week. The FSBA values reached the highest concentration of 205±118 μmol/l in the 10th week. Only ALP and GGT maintained significant higher values up to the end of the examination period. The other parameters were still insignificantly higher than the pre induction levels except for Cho and Glc which did not show significant changes during the 10 weeks.

Ultrasonographic findings.

The two dogs that died started showing changes in the echogenicity and texture of the liver in the 5th week, while the others started at 6–8 weeks after beginning the administration of DMNA. The livers of the dogs showed an increased diffuse echogenic pattern. The liver was hyperechoic "bright" when compared to the adjacent renal parenchyma (Fig. 1). At the same time, a characteristically coarsened and heterogeneous echo patterns, and nodularity of the surface were seen (Fig. 2). Increased echogenicity and bile stasis seen as diffusely echogenic sludge was observed in all the dogs. By the 8th week the gallbladder neck could be seen in most scans and the wall was thickened and highly echogenic (Figs. 3a and 3b). Dilatation of the gallbladder and enlargement of intrahepatic ducts were also a common feature. Towards the end of the experimental period, there was a noticeable loss of detail of liver vessel

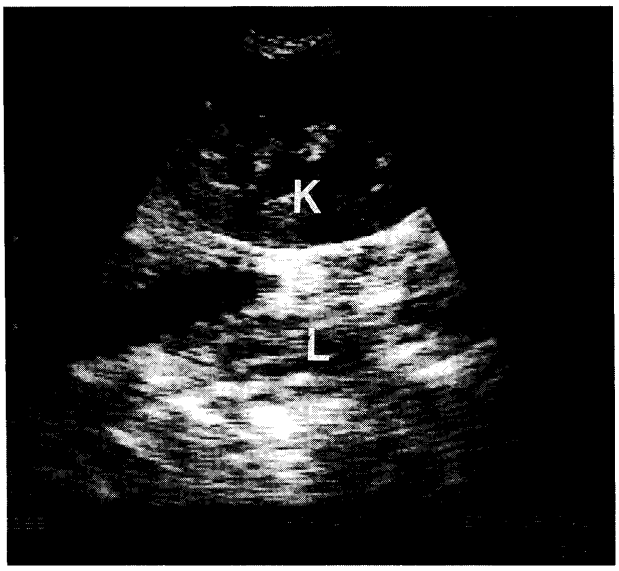

Fig. 1. Scan of the liver showing heterogeneous diffusely increased echogenic areas at 6–8 weeks.

K=Kidney.
L=Liver.

Fig. 2. A sagittal scan through the liver from the right side showing a diffusely coarse echo pattern at the end of 8 weeks.

D=Diaphragm.
GB=Gallbladder.
L=Liver.

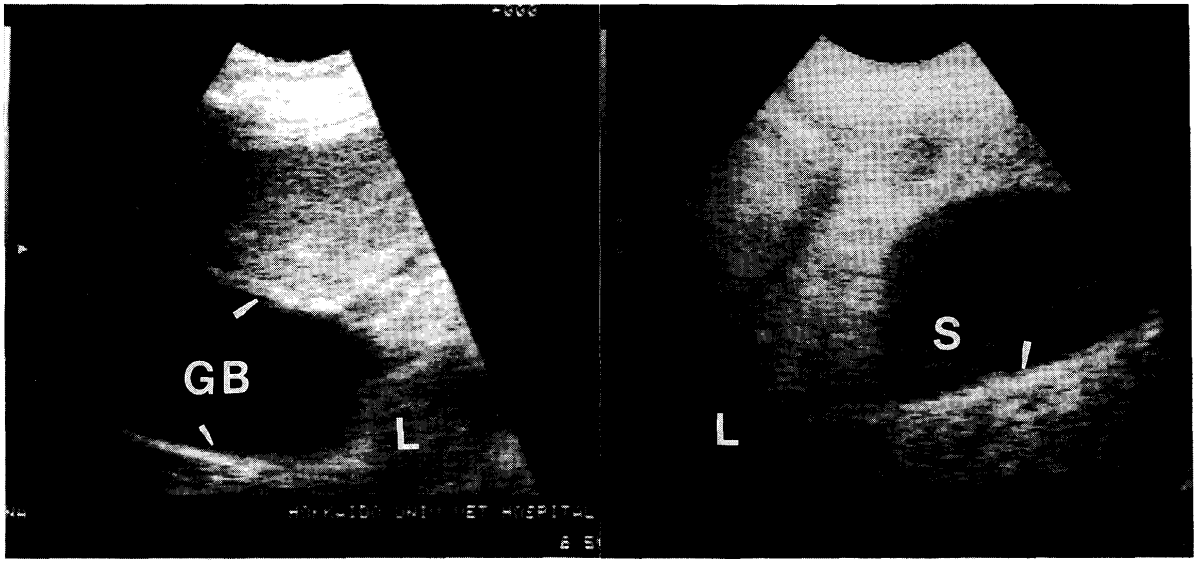

Fig. 3. Transverse sonogram of the liver. Note the hyperechoic thickening of the gallbladder wall (a) at 8 weeks on the left and the hyperechoic bile sludge within the gallbladder lumen (b) at 10 weeks.

GB=Gallbladder.
L=Liver.
S=Sludge.
Arrow=Echogenic gallbladder wall.

echo and color Doppler flow signal. Since the size of the gallbladder varied with the feeding status of the dogs, it was not possible to accurately measure its size.

Gross pathological findings.

At postmortem examination, the two dogs that died in the 5th week had slightly enlarged livers with rounded margins, a texture friable than normal and pale to brownish discolouration. The external surfaces were smooth. Gross lesions in the dogs that were euthanized at the end of the experiment consisted of congestion and red discolouration. The livers were small, firm and fibrotic. The capsule was in all the livers wrinkled and depressed.

Discussion

In this study, intravenous administration of DMNA produced hematological, biochemical, and ultrasonographic changes like those typically seen in naturally occurring liver cirrhosis[1,17,20]. Clinical signs that develop in dogs with natural cirrhosis as described by Boothe et al.[2] and Madden et al.[17], were produced in this model. Extremes in the A/G ratio were seen in the dogs which had the lowest protein content values. This decrease in the A/G ratio could have indicated that a decrease in both the albumin and globulin fraction occurred. The decrease in albumin provides information on the presence of hepatic disease in terms of the synthesizing ability of the liver[2,12]. Serum ALP, ALT, AST and GGT activities increased significantly in all dogs after beginning the administration of DMNA. Increased activity of liver-specific enzymes early during the toxin administration indicates acute and active liver disease. Hepatic parenchyma cell damage is reflected by increased serum ALT and AST levels, while high increases in GGT and ALP activities reflect a dysfunction of the biliary system[11]. In the hematological examination, the major finding was the non-responsive anemia especially in the 2 dogs that died in the 5th week. This could have been the

result of gastrointestinal bleeding which was evident at postmortem autopsy. This further lead to a reduction in the platelet count, a result of over consumption of clotting factors[21,26].

The enlarged livers in the dogs that died in the 5th week occurred in the acute phase. Degeneration of hepatocytes leads to swelling and cytoplasmic vacuolization. Necrosis of hepatocytes in a particular zone of the lobule results in dilatation and congestion of sinusoids so that the affected zones appear brownish pale. If the process is focal or multifocal, the liver typically is small with a wrinkled capsule, and depressed, dark reddish areas that coincide with areas of parenchyma necrosis. This was indicated by the small, firm livers at 12 weeks suggesting chronicity. Histopathological examination would have helped in determining which zones of the lobules were affected[16].

The most severe histopathological lesions described by Madden et al.[17] and Boothe et al.[4] in such dogs were hepatocyte necrosis, degeneration, hepatocyte regeneration and proliferation of connective tissue. Since liver cirrhosis is a progressive disease, such changes appear gradually. Ultrasonographically, these changes were noticed starting in the 6th week as increased surface nodularity and echogenicity, parenchyma heterogeneity and coarsening of the liver surface. These ultrasonographic changes became more evident at this time when the disease was turning from acute to chronic. Both fat and fibrosis in the liver had been shown to produce a loss of detail of vascular echoes and an increase in the brightness of the ultrasound image[1,20]. The increase in echogenicity may represent an increase in the amount of collagen within the liver[24]. Conditions that commonly increase the echogenicity are : lipidosis, fibrosis/cirrhosis, steroid induced hepatopathy and rarely, multi centric lymphoma[20]. Increased echogenicity is easier to evaluate in real time images than on static images.

On ultrasonography, the normal canine gallbladder wall either is not visualized or it appears as a smooth, thin line separating the bile from the hepatic parenchyma. Cholestasis, when it occurs, leads to the enlargement of the intrahepatic bile ducts, which are not ultrasonographically detectable in normal cases. As the liver attempts to heal itself by regeneration, nodules of different sizes may appear causing irregularities in the outline of the liver surface. This surface nodularity is a more specific sonographic sign of cirrhosis, especially in the presence of ascites[1,27]. It is also readily visualized in real-time examination than on static images.

Due to the difference in response to DMNA, the mean values of the liver enzymes measured varied greatly among the dogs. Two dogs which showed a much higher response in the liver enzymes and died in the 5th week were young adult dogs (about 1 year old). The older dogs tolerated the toxin up to the end of the examination. Starting in the 7th week, there was a gradual decline in the activity of the liver enzymes measured. Normal enzymes usually occur in the very advanced stages of liver disease when there is still very little inflammatory reaction[12].

Clinical signs and biochemical test results are good indicators for the interpretation of ultrasound images. The ultrasound examination can then be done repeatedly and in a non-invasive manner even in cases where liver biopsy is contra-indicated. This ability to acquire information through a totally non-invasive and safe procedure makes ultrasound a very desirable imaging modality. It is also a very valuable tool in the monitoring of the progression of the disease process, even though few hepatic diseases, diffuse or focal have specific sonographic features[1,20]. Although the number of animals used in this study was limited, the results obtained indicate that intravenous administration of DMNA resulted in clinical, hematological and biochemical findings of liver disease similar to

those caused by oral feeding of the toxin[4]. Intravenous injection of DMNA at the dosage used in this study was sufficient to make this model of liver cirrhosis and did not cause any unexpected local or systemic adverse reactions at the time of administration.

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