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Effect of Bifendate (DDB) On Normal and Diseased Liver of Adult Male Albino Rats: An Experimental Study.

Ehsan H.Hassan *, Nabil Abdel Mageed ** Nagwa AbdelWahaab *, Hoda Aref **** and Sameh Seif *****

Pathology Department*, Dermatology Department **, Biostatistics and Public Health Department ***, Biochemistry department****, Int. Medicine department*****
National Hepatology and Tropical Medicine Research Institute (NHTMRI)

Abstract

Background: Fructus Schizandrae Sinensis bail, a traditional Chinese medicine, has been shown to lower the elevated serum level of liver enzymes of patients suffering from chronic active hepatitis. A synthetic derivative compound of Schisandrian, Dimethyl Diphenyl Bicarboxylate (DDB) is now used widely in clinical fields as a hepatoprotective drug. Thus it is important to know whether DDB has a beneficial effect on damaged liver or not.

Objective: To evaluate the protective effect of DDB on induced liver tissue injury in rats.

Design: Experimental study.

Setting: National Hepatology and Tropical Medicine Research Institute. The study was conducted from October (2004) to February (2005).

Materials and methods: 120 male albino rats aged 6-8 weeks, weight 150-200g were grouped in six groups, 20 rats per group. Group 1 received food and water only, group 2 received food, water and DDB intragastric 6mg/kg daily for 12 weeks, group 3 received 20% ethanol instead of water, group 4 received 20% ethanol instead of water plus DDB, group 5 received thioacetamide (TAA) in a dose of 200mg/kg body weight intraperitoneal injection, group 6 received thioacetamide plus DDB at the same dose of the above group. At the end of the trial, blood samples were taken from all groups for biochemical analysis. Liver tissue excised from each rat was fixed in 10% neutral formalin, embedded in paraffin, and stained with Hematoxylin and Eosin, as well as Masson's trichome stain, for evaluation of hepatic injury and/or fibrosis.

Results: Statistical elevation of serum hepatic enzymes was noticed in rats received alcohol, Thioacetamide and alcohol + DDB (groups III, V and IV respectively) compared to the corresponding control (P= 0.000). On the other hand, administration of DDB to TAA treated group (group VI) induced significant improvement of liver function tests compared to other groups (P= 0.000). Histopathologically, the control livers showed normal lobular architecture without any pathological changes. Liver sections of animals administered alcohol, TAA respectively showed chronic inflammatory reaction, fat accumulation, hepatic parenchymal necrosis and/or hepatic fibrosis. Administration of DDB resulted in improvement of the pathological changes induced by TAA (group VI), but not that induced by alcohol (group IV).

Conclusion: Our results revealed that DDB has antitoxic effect against TAA and ameliorates the dangerous effect on the liver parenchyma, while it has no beneficial effect on alcoholic liver disease.

Introduction

Hepatic fibrosis is a consequence of sever liver damage which occurs in many chronic liver diseases forerunner to cirrh-osis, chronic fibrotic diseases can differ from each other in etiology, but in terms of pathogenesis they share common features (Tan *et al*., 1999 and Jun *et al.,* 2004). Bifendate, DDB (Dimethyle Diphenyl Bicarboxylate) is anexample of herbal medication, currently employed as a curative agent for patients with acute and chronic viral hepatitis in several countries, including China, Korea, Vietnam, Pakistan where the incidence rate of viral hepatitis is high (Kim *et al*., 2000a). Dimethyl-4, 4'-dimethoxy 5, 6, 5', 6' -dimethylene dioxybiphenyl1-2, 2'-dicarboxylate (DDB) is a synthetic hepatopr-otective agent drived from Schisandrin C present as a component of Fructus schisandrae,it is active against a variety of hepatotoxins and has been used as a curative agent for patients with acute and chronic viral hepatitis. Akbar *et al.* (1998), Joung (2000) found that treatment with DDB is effective to reduce liver impairment in acute and chronic liver diseases, as well as fatty liver, with no side effects, also they reported that DDB is a protective agent against chronic ethanol toxicity. Many authors considered DDB as a hepatoprotective against toxin induced liver injuries in animals. Also Lee *et al.* (1996) found that DDB lowered the elevated ALT and AST as well as improved the injured hepatic histology induced by intraperitoneal injection of thioacetamide in rats. IP *et al*. (1998) reported that pretre-ating mice with intragastric DDB 1.mmol-kg 1-d-1 for 3d protected against CCl4 hepatotoxicity. Kim *et al*. (2000 b) found that DDB has a protective effect against ethanol-induced toxicity in animals. On the contrary, Soon *et al*. (1999) reported that DDB did not significantly show histopat-hological improving effect on ballooning degeneration and necrosis due to CCl4 treatment. In agreement with this, Nan *et al*. (2000) reported that DDB did not improve any parameter in fibrotic liver in rats, and Kim *et al*. (2000b) stated that the anti-inflammatory effect of DDB is doubted. Also Huber *et al*. (2004) reported that the normalization of ALT during DDB treatment did not indicate therapeutic efficacy, and there was no beneficial effect of DDB on histopath-ological grade and stage of liver diseases, also they stated that the wide use of DDB in patients with chronic liver diseases who participate in clinical studies DDB use should be excluded.

Materials and Methods

1-<u>Animals and experimental design:</u>

The study was conducted from October (2004) to February (2005).The total study period was 12 weeks.120 adult male albino rats aged 6 – 8 weeks with average weight 150 to 200g were used in this study. The animals were housed in stainless steel wire cages. Animals were allowed to stabilize for a minimum of 6 days within our facility before treatment. Food was allowed ad libitum and they were housed at room temperature (23±2ºC) in a clean environment with a 12h-light-dark cycle. The animals were randomly divided into six groups, each comprised 20 rats:

Group 1: (control group) did not receive any medication, received only diet and water.
Group 2: received food, water and DDB intragastric 6mg/kg daily for 12 weeks, according to Kim *et al*. (2000a).
Group 3*:* given 20% ethanol instead of water according to Kim *et al*. (2000a).
Group 4*:* given 20% ethanol instead of water plus DDB
Group 5: received thioacetamide (TAA) in a dose of 200mg/kg b.wt.intraperitoneal injection dissolved in 0.9 NaCl twice a week for 12 weeks to induce liver cirrhosis according to Hori *et al*. (1993).
Group 6*:* received thioacetamide plus DDB.

Body weight was measured at the beginning of the study, weekly, and at the end of the study.

2-Chemicals:

Thioacetamide is a well-known hepatotoxin that has been used widely in studies of liver pathology. Acute and chronic administration of TAA leads to liver necrosis and cirrhosis in rats Martin *et al.* (1989). Dashti *et al.* (1989) reported that the liver damage caused in rats by chronic administration of TAA administration resembles human cirrhosis in both its biological and morphological aspects.

3-Biochemical analysis:

Serum levels of GOT/AST, GPT/ALT, bilirubin, total proteins, albumin and ALK. Phosphatase were estimated in all animals, first prior to study and finally by the end of study. Blood samples were drawn via an indwelling cannula in the tail artery.

4-Histopathological study:

At the end of the trial all animals were sacrificed to study the possible effects of Bifendate on the liver tissue. The liver biopsies were removed, fixed in 10% buffered formalin solution. Thin sections (3-4µ) were stained with Hematoxylin and Eosin as well as Masson–trichrome stain for assessment of fibrosis. Evaluation of hepatic fibrosis was determined by a semi-quantitative method to assess the degree of histologic injury and hepatic fibrosis applying the following score system according to Tsui. (1996) and Pilette et al. (1998), grading of the disease activity varied from 0 to 4 depending on the degree of portal and/or lobular inflammation and necrosis. Staging of fibrosis was also based on 0-4 scale according to the extent of portal and periportal fibrosis. 0 = absence of fibrosis; 1 = portal and/or pericellular fibrosis; 2 =occasional septal fibrosis; 3 = marked bridging fibrosis (incomplete cirrhosis); 4= complete cirrhosis. The degree of inflammation and fibrosis was expressed as the mean of different fields in each slide.

5-Statistical analysis:

An IBM compatible PC was used to store and analyze the data. Software package namely SPSS (Statistical Package for Social Science: SPSS Inc. Chicago, IL, USA) statical program release (11.01) was used for data management and calculation of means, standard errors and standard deviations to describe quantitative data.

For each measured data (the biochemical results as liver enzymes data, serum levels of bilirubin and total protein and albumin serum levels), tests of significance, to investigate whether the difference between the means of the six studied groups is significant or not, are conducted. The independent samples test is adopted and the statistical significance (P-value) is calculated for every two groups. A P-value of less than 0.05 indicates the difference between the means of the considered two groups is significant while bigger values suggest non significant differences between the sample means (Bryman and Cramer,1999).

Results

Before sacrifice, eight rats were died in the fifth group, 6 rats in the sixth group, within 12 weeks, so they were excluded from the final statistics.

Biochemical results:

Except the control group, all groups showed a statistical increase in liver enzymes (ALT and AST) except for group (3) which showed significant increase in serum ALT only, however such increase is variable from group to another. Also the serum levels of bilirubin were statistically elevated in group 4 , 6 and 3 (2 ± 0.75 , 2 ± 0.59 and 3.2 ± 1.1 mg/dl respectively). ($P = 0.000$). Markedly significant hypoalbuminemia (0.4 ± 0.09 g/dl) was reported in rats of group 5 ($P= 0.000$) . In chronically injured rats by ethanol, TAA, Liver functions were impaired as shown by highly significant increase in serum ALT ($P = 0.000$) and decreased serum albumin content compaired to that of normal control animals ($P= 0.000$) (Table 1). It was evident that alcohol administration produced statistically increase in serum level of ALT($P = 0.000$). Administration of thioacetamide produced marked statistical

increase in serum levels of both ALT and AST (P =0.000)(Table 1an2). Coadmin-istration of Bifendate to ethanol treated group did not improve any parameter except for bilirubin which is statistically decreased (P = 0.000) and total protein and albumin which are statistically increased (P = 0.000) , while coadministration of DBB to TAA treated group revealed marked and significant improvement of liver functions (ALT, AST, alkaline phosphatase total protein and albumin (P= 0.000). Such improvement was not reach the normal levels except for serum alkaline phosp-hatase and total protein as shown in table 1 and table 2.

Histopathological changes:

The control livers showed normal lobular architecture with central veins and radiating hepatic cords, as well as normal portal tracts. While examination of alcohol treated livers they showed pathological damages in the liver tissue in the form of chronic inflammation (20/20), fat accumulation (15/20), and parenchymal necrosis (5/20) (Fig 1& table 2). Co-administration of DDB did not improve the pathologic changes induced by alcohol. (Fig 2 & tables 3,4). Intraperitoneal injection of TAA for 12 weeks revealed presence of chronic inflammations (12/12), centrol-obular necrotic areas (12/12), bridging necrosis (12/12), bridging fibrosis (12/12), and cirrhotic pattern characterized by mixed sized cirrhotic nodules (10/12) (Fig 3). Co-administration of DDB and TAA for 12 weeks reduced the degree of hepatic injury in TAA+DDB group compared to the TAA alone group (fig 4,5&6 and table 4).

Table (1): Values of the liver function tests among the different groups:

Group	AST U/L		ALT U/L		Alk.phosphatase U/L		Bilirubin mg/dl		Total protein g/dl		Albumin g/dl	
	Mean± SD	SE	Mean± SD	SE	Mean ± SD	SE	Mean ± SD	SE	Mean ± SD	SE	Mean± SD	SE
Group (1) N = 20	12 ± 3.4	0.77	12 ± 3.5	0.78	87 ± 22.17	4.96	1.2 ± 0.32	0.07	7± 0.77	0.17	4.2 ± 0.7	0.16
Group (2) N = 20	58 ± 15.4	3.44	208 ± 72.8	16.28	192 ± 51	11.44	1.2 ± 0.57	0.13	7 ± 1.24	0.28	3.4 ± 1.07	0.24
Group (3) N = 20	12 ± 5.15	1.15	72 ± 24.9	5.58	128 ± 21.9	4.89	3.2 ± 1.1	0.25	4.4 ± 0.73	0.16	1.6 ± 0.44	0.10
Group (4) N = 20	38 ± 8.01	1.79	98 ± 26.4	5.9	120 ± 26	5.84	2 ± 0.75	0.17	6 ± 1.01	0.23	2.6 ± 0.82	0.18
Group (5) N = 12	230 ± 51.69	14.92	720 ± 122.25	35.29	66 ± 24.04	6.94	1.2 ± 0.41	0.12	1.8 ± 0.37	0.11	0.4 ± 0.09	0.03
Group (6) N = 16	44 ± 10.18	2.72	160 ± 34.33	9.18	20 ± 6.35	1.7	2 ± 0.59	0.16	7.2 ± 0.73	0.2	2.8 ± 0.73	0.2

SE= Standard Error.
SD= Standard Deviation.

Table (2): Comparison between different groups as regards liver function tests:

Groups	AST U/L		ALT U/L		ALK. PHOSPHATASE U/L		BILIRUBIN mg/dl		TOTAL PROTEIN g/dl		ALBUMIN g/ dL	
	t.test value	P Value (one tailed)	t.test value	P Value (one tailed)	t.test value	P Value (one tailed)	t.test value	P Value (one tailed)	t.test value	P Value (one tailed)	t.test value	P Value one tailed)
Group(1) vs (2)	-13	** 0.000	-12	** 0.000	-8.4	** 0.000	0.000	0.500	0.000	0.500	2.8	** 0.005
Group(1) vs (3)	0.000	0.500	-10.7	** 0.000	-5.89	** 0.000	-7.81	** 0.000	10.95	** 0.000	14.1	** 0.000
Group(1) vs (4)	-13.34	** 0.000	-14.45	** 0.000	-4.31	** 0.000	-4.38	** 0.000	3.51	** 0.0005	6.66	** 0.000
Group(1) vs (5)	-14.59	** 0.000	-20.06	** 0.000	2.5	** 0.009	0.000	0.500	25.5	** 0.000	23.98	** 0.000
Group(1) vs (6)	-11.3	** 0.000	-16.1	** 0.000	12.8	** 0.000	-4.6	** 0.000	-0.76	0.225	5.65	** 0.000
Group(2) vs (3)	12.67	** 0.000	7.9	** 0.000	5.15	** 0.000	-7.22	** 0.000	8.09	** 0.000	6.9	** 0.000
Group(2) vs (4)	5.15	** 0.000	6.35	** 0.000	5.6	** 0.000	-3.8	** 0.0005	2.8	** 0.004	2.7	** 0.006
Group(2) vs (5)	-11.2	** 0.000	-14.9	** 0.000	7.98	** 0.000	0.000	0.500	17.5	** 0.000	12.4	** 0.000
Group(2) vs (6)	2.97	** 0.003	2.57	** 0.008	14.9	** 0.000	-3.97	** 0.000	-0.54	0.295	1.94	* 0.03
Group(3) vs (4)	-12.2	** 0.000	-3.2	** 0.0015	1.05	0.15	4.03	** 0.000	-5.7	** 0.000	-4.8	** 0.000
Group(3) vs (5)	-14.6	** 0.000	-18.1	** 0.000	7.5	** 0.000	7.3	** 0.000	13.3	** 0.000	11.78	** 0.000
Group(3) vs (6)	-10.8	** 0.000	-8.7	** 0.000	20.9	** 0.000	4.1	** 0.000	-11.03	** 0.000	-5.5	** 0.000
Group(4) vs (5)	-12.8	** 0.000	-17.4	** 0.000	5.8	** 0.000	3.9	** 0.0005	16.8	** 0.000	11.9	** 0.000
Group(4) vs (6)	-1.9	* 0.03	-5.95	** 0.000	16.4	** 0.000	0.000	0.500	-3.8	** 0.0005	-0.7	0.23
Group(5) vs (6)	12.26	** 0.000	15.36	** 0.000	6.4	** 0.000	-3.95	** 0.0005	24.2	** 0.000	-12.2	** 0.000

**** :** Highly Significant at P <0.01 * : Significant at P <0.05.

Table (3): Showing effect of DDB on induced parenchymal hepatic injury:

Group	No	Degree of hepatic injury			
		Minimal	Mild	Moderate	Sever
Group 1	20	0	0	0	0
Group 2	20	7	13	0	0
Group 3	20	2	5	10	3
Group 4	20	3	3	11	3
Group 5	12	0	1	1	10
Group 6	14	1	2	2	9

Table (4) Showing effect of DDB on induced hepatic fibrosis:

group	No.	DEGREE OF HEPATIC FIBROSIS				
		0	1	2	3	4
Group 1	20	20	0	0	0	0
Group 2	20	20	0	0	0	0
Group 3	20	12	4	1	1	2
Group 4	20	11	6	1	1	1
Group 5	12	0	0	1	1	10
Group 6	14	0	0	2	3	9

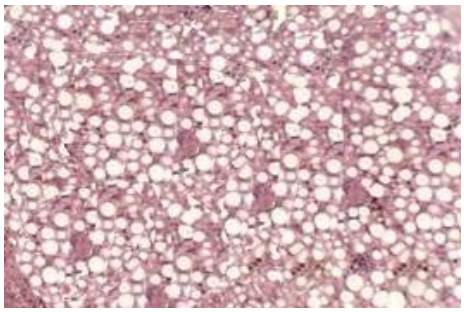

Fig. 1: liver section from a rat received alcohol for 12 weeks showing chronic hepatitis fatty changes and ballooning of hepatocytes (H&E, x100).

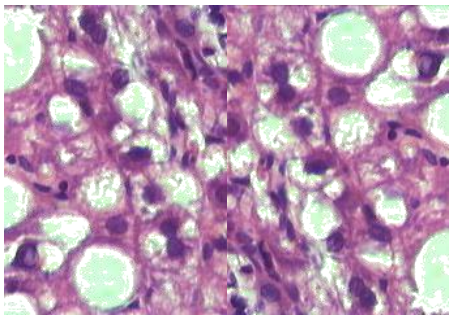

Fig 2: liver section from a rat received alcohol and DDB for 12 weeks showing ballooning degeneration of hepatocytes and areas of parenchymal necrosis. (H&E, x400).

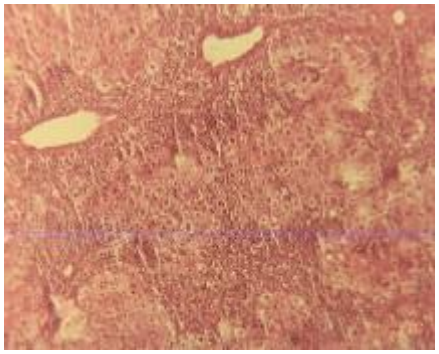

Fig 3: liver section from a rat received TAA for 12 weeks showing cirrhosis with mixed sized nodules and fibrotic septa (H&E, x100).

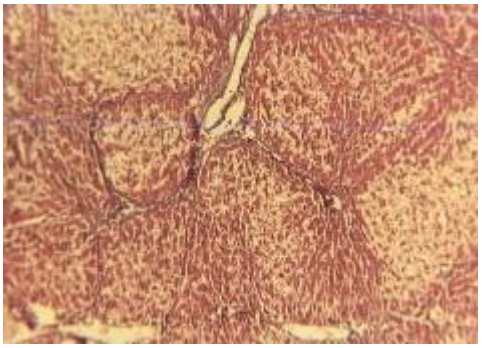

Fig 4 : liver section from a rat received TAA and DDB for 12 weeks , showing sever chronic hepatitis with bridging necrosis bridiging fibrosis (H&E, x100).

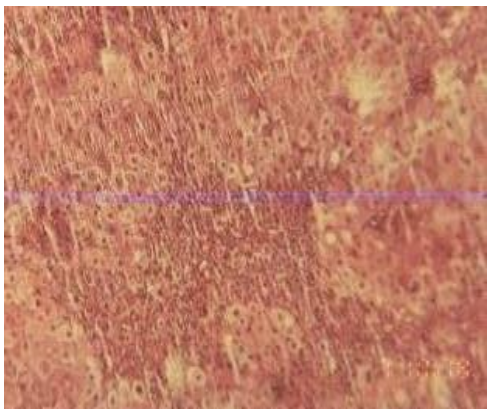

Fig 5: liver section from a rat received TAA + DDB for 12 weeks showing sever chronic hepatitis with areas of parenchymal necrosis (H&E, x100).

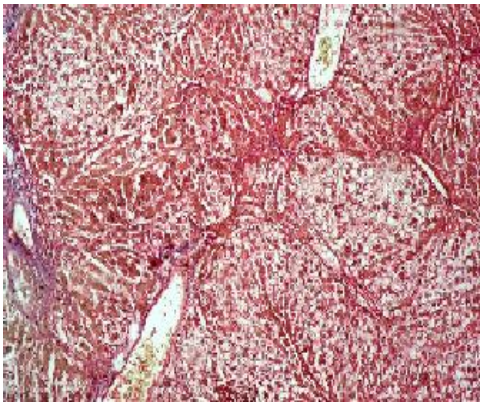

Fig 6: liver section from a rat received TAA + DDB for 12 weeks showing less nodular formation and fibrotic septa (H&E, x100).

Discussion

Hepatitis C virus (HCV) is considered the most common etiology of chronic liver disease (CLD) in Egypt, where prevalence of antibodies to HCV (anti-HCV) is approximately 10-fold greater than in the United States and Europe Strickland *et al*. (2002). Many therapeutic modalities have been advocated for the treatment of Hepatitis C without convincing results. Multiplicity of methods of treatment is always an indication that none of them is entirely satisfactory. Bifendate, DDB or (Dimethyle Diphenyle Bicarboxylate) is an example of herbal medication, currently employed as a curative agent for patients with acute and chronic viral hepatitis in several countries. Bifendate (BDD, biphenyl dimethyl dicarboxylate) is a traditional Chinese medicine from the fruit schisandra; a member of the magnolia family, (Latin name, *Schisandra Chinensis)*. Chinese medicine practioners prescribe it for a variety of disorders as varied as allergic skin reactions, insomnia and hepatitis. To investigate the anti-HBV efficacy of bifendate in treatment of chronic hepatitis B, Cui *et al*. (2002) concluded that higher doses of bifendate taken for a long term has remarkable anti-HBV efficacy in treatment of chronic hepatitis B. Lee et al. (1996), Akbar *et al*. (1998), Joung .(2000) declared that treatment with DDB is

effective to reduce liver impairment in acute and chronic liver diseases, as well as fatty liver, with no side effects. Also they reported that DDB is protective against chronic ethanol toxicity. The mode of action of DDB as a hepatoprotective agent was attributed to the enhancement of liver mitochondrial glutathione status Ip *et al.* (1998). Quite the opposite, Soon *et al.* (1999), Kim *et al*. (2000b) and Nan et al. (2000) reported that DDB did not significantly show histopathological improving effect on ballooning degeneration and necrosis due to CCl4 treatment, did not improve any parameter in fibrotic liver in rats, and the anti-inflammatory effect of DDB is doubted. This study aimed to evaluate the effect of Bifendate (DDB) on normal and diseased liver in adult male albino rats. It was evident that Alcohol and Thioacetamide produced a marked increase in the activity of serum hepatic enzymes. Co-administration of Bifendate and alcohol induced significant increase in enzyme levels compared to control. On contrary to this, co-administration of DBB showed slight improvement of hepatic enzyme in TAA+ DBB treated group. Consistent with this Cui and Co-workers. (2002), on their study performed to evaluate the anti-HBV efficacy of bifendate in treatment of chronic hepatitis B, found that the serum alanine aminotransferase (ALT) decreased to normal only one month later in 70.76% of patients in the treatment group and decreased to normal approximately 3 months in the control group. Activities of serum ALT, AST are the most frequently used indicator as parameters of liver diseases. In this study introduction of alcohol, Thioacetamide to normal rats caused a highly significant elevation in the level of these enzymes compared to normal control level, such elevation was explained by the occurrence of a hepatocellular injury leading to cell necrosis and discharge of the contents of the hepatocytes into the blood stream. Co-administration of DDB to alcohol treated group didn't improve any biochemical parameters or histopathological findings and these results are in agreement with those obtained by Nan *et al*. (2000), who found that DDB did not improve any parameters in fibrotic liver rats. Also administration of DDB to TAA treated group did not normalize the hepatic cell function and these results are irreconcilable with those obtained by Kim *et al*. (2000b), who reported that DDB has antitoxic protective effect for the hepatocytes against a variety of hepatotoxins such as CCl4, TAA, or galactosamine, leading to reduction of the enzymes but not reaching the normal levels. Lee and Co-workers. (1996), reported that DDB administration caused improvement of the histopathological findings of TAA treated group. These results are compatible with our histopathological findings, in which we noticed that there was a significant difference of the histopathological findings between the TAA treated group and TAA+ DDB-treated. In our study, we noticed marked pathological changes of livers in all groups (including positive control). The histopathological score was nearly the same in groups III &IV, i.e. DDB did not improve any parameter (biochemically or histopathologically) in diseased liver in rats, these results are in agreement with those obtained by Huber *et al* .,2004 who found that there was no beneficial effect on the histological grade and stage of liver diseases and the normalization of ALT during DDB treatment does not indicate therapeutic efficacy and the wide use of DDB in patients with chronic liver diseases who participate in clinical studies DDB use should be excluded.

Conclusion

Our results revealed that DDB has antitoxic effect against TAA and ameliorate the dangerous effect on the liver parenchyma, while it has no beneficial effect on alcoholic liver pathology.

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تأثير عقار ددب (الحبة الصفراء) على كبد جرذان التجارب المريضة والسليمة: دراسة تجريبية

إحسان حسن – نبيل عبد المجيد – نجوى عبد الوهاب – هدى عارف – سامح سيف
المعهد القومي للأمراض المتوطنة والكبد

يستخدم عقار الحبة الصفراء بكثرة ومنذ مدة في علاج مرضى الكبد في مصر وغيرها من الدول الأخرى. ومن ثم فقد تم تخطيط هذا البحث لإلقاء الضوء على الآثارالمفيدة أو الضارة المحتملة لهذا العقار على نسيج الكبد المريض والسليم باستخدام ذكور الجرذان البيضاء كحيوانات تجارب. وقد تم تقسيم الجرذان إلى ستة مجموعات, كل مجموعة تشمل عشرين فأرا, وهى المجموعة الضابطة {المجموعة الأولى }, المجموعة الثانية{ أعطيت الحبة الصفراء عن طريق الفم }, المجموعة الثالثة { أعطيت الكحول الأثيلى بدلا من الماء} المجموعة الرابعة (أعطيت الكحول الأثيلى بدلا من الماء+ الحبة الصفراء). وبالنسبة لجرذان كلا من المجموعة الخامسة و السادسة فقد تم حقنها بمادة ثيو أسيتامايد مع إعطاء المجموعة السادسة الحبة الصفراء. في نهاية التجربة تم الحصول على أنسجة الكبد الخاصة بالجرذان وحضرت منها عينات للفحص الهستوباثولوجى بالمجهر الضوئي العادي وذلك بعد التضحية بالجرذان. بالإضافة إلى ذلك أيضا تم تحديد مستوى وظائف الكبد في جرذان المجموعة الضابطة والمجموعات الأخرى وذلك قبل وبعد إجراء التجربة. ولقد بينت الدراسة أن عقار الحبة الصفراء لا ينتج عن استخدامه الفائدة المرجوة في علاج أمراض الكبد و تمثل ذلك في ارتفاع معدل إنزيمات الكبد وحدوث تغيرات مرضية هستوباثولوجية في الكبد في جميع المجموعات, سواء التي تم إعطائها عقار الحبة الصفراء أو التي لم يتم إعطاؤها العقار عدا المجموعة التي حقنت بثيوأسيتاميد حيث أظهرت النتائج أن الحبة الصفراء قللت من الأضرار التي يحدثها الثيو أسيتاميد علي كبد هذه المجموعة بالمقارنة بالمجموعة التي حقنت بثيوأسيتاميد فقط.