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Hepatoprotective effects of natural *Calculus Bovis* against diethylnitrosamine induced hepatic injury in rats

Tien-Chun Wan[1], Chih-Ming Chen[2] and Liang-Chuan Lin[3*]

[1]Animal Products Processing Division, Livestock Research Institute, Council of Agriculture, Tainan, Taiwan.
[2]Department of Animal Science, National Pingtung University of Science and Technology, Taiwan.
[3]Department of Animal Science, National Chung Hsing University, Taichung, Taiwan.

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Natural *Calculus Bovis* (NCB) is an important Chinese medical material commonly referred to as pigment gallstones of cows, which has been applied in many effective traditionally preparations of Chinese medicine for many years. The purpose of the study was to investigate effects of NCB on diethylnitrosamine induced hepatic injury in rats. Hepatoprotective and antioxidant effect of NCB on liver injury were also analyzed. Male, six-week old Wistar rats were treated with a single dose of diethylnitrosamine (200 mg/kg b.w., i.p.) and left for four weeks. For hepatoprotective and antioxidant studies, the NCB group was treated daily for four weeks. Hepatoprotective markers of aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase were determined in serum of rats. All the experimental animals administered with diethylnitrosamine were obviously elevated serum hepatoprotective markers. The treatment of the NCB prevented the diethylnitrosamine induced hepatic damage and oxidative stress. The experimental rats administered with the NCB protected the liver against diethylnitrosamine induced hepatic injury.

Key words: *Calculus bovis,* diethylnitrosamine, hepatic injury, hepatoprotective, rats.

INTRODUCTION

Calculus Bovis, an animal byproduct in slaughterhouses, is an important Chinese medical material commonly referred to as cow bezoar. *C. Bovis* known an effective traditionally Chinese medicine has been applied in many preparations of Chinese medicine for a long time (Wan et al., 2008). The components of NCB were rich in bilirubin and biliverdin and had higher content of essential amino acids (Wan et al., 2009). NCB has also been used as an important component of many effective Chinese medical preparations (Tian et al., 2005). These *C. bovis* products are shown to have many beneficial effects in treating convulsions, epilepsy and mental illness, according to the practical utilities of traditional Chinese medicine (Yen, 1984). NCB also has sedative, fever reduction and anti-inflammatory effects (Hu et al., 2006).

Diethylnitrosamine is a well known hepatotoxin and hepatocarcinogen (Shu and Hollenberg, 1997). A possible involvement of oxidative stress induced hepatocarcinogenesis of rats is shown as follows. The hepatic metabolism of diethylnitrosamine produces reactive oxygen species resulting in oxidative stress and liver cellular injury (Nishimura et al., 2008). The aspartate aminotransferase values of rats intoxicated with diethylnitrosamine are evaluated in a preventive and curative model. The aspartate aminotransferase, cytoplasmic in nature, enters into the circulatory system because of the altered membrane permeability of liver injury (Wills et al., 2006). The evaluation of traditional

*Corresponding author. E-mail: tcwan52@kimo.com. Tel: +886-6-5911211. Fax: +886-6-5912474.

Table 1. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum aspartate aminotransferase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	108.40	±6.47^{a,x}	114.80	±26.78^{a,z}	111.40	±16.99^{a,x}
1	115.20	±13.50^{b,x}	136.25	±12.50^{a,yz}	132.50	±8.66^{a,x}
2	113.40	±20.13^{a,x}	145.25	±28.06^{a,xy}	133.75	±21.22^{a,x}
3	112.80	±15.12^{b,x}	163.00	±19.37^{a,x}	126.50	±12.61^{b,x}
4	117.50	±18.50^{b,x}	166.75	±16.40^{a,x}	128.75	±10.59^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

drugs has revealed a chemopreventive function of hepatoprotection (Sultana et al., 2008). Natural occurrence substances in ruminant products might have important physiological effects, including anticarcinogenic effects (Tanaka, 2005). However, the literature describing the formation of hepatoprotection of the NCB against experimental animals induced by diethylnitrosamine is limited.

These experimental animals were treated with NCB to increase the antioxidant function and to withstand diethylnitrosamine induced oxidative injury in the study. Thus, the objectives of the present study were to investigate protective roles of the NCB treatments on diethylnitrosamine induced serum liver enzymes in a time-dependent manner over the study period.

MATERIALS AND METHODS

Experimental animals

The animal model of the study was according to the method of Pradeep et al. (2007). Male six-week old Wistar rats purchased from Taiwan BioLASCO Co. (Taipei, Taiwan) were used in this study. The experimental rats were housed in polypropylene cages with 12 h light and dark cycle in an environment controlled room (25°C, 85% humidity). The experimental animals were fed standard pellet feed and tap water ad libitum. These animals were balanced for one week prior to the start of the experiment. Procedures involving experimental animals and their care were executed in conformity with the institutional guideline of Council of Agriculture (Taiwan) for the care and use of laboratory animals.

Experimental design and materials

Wistar rats were divided into three groups with five animals in each group. The experimental design was as follows: Group I rats were served as the control group and were treated with saline water orally for four weeks; Group II rats were administered a single dose of diethylnitrosamine (200 mg/kg b.w., i.p.) at the initial start of the experiment and left for four weeks. The diethylnitrosamine was purchased from Sigma-Aldrich (St. Louis, MO, USA); Group III rats were treated with diethylnitrosamine (200 mg/kg b.w., i.p.) at the initial start of the experiment followed by NCB (100 mg/kg b.w., p.o.) from day 1 till day 28. The doses of NCB of the experimental animals were selected by performing an effective dose according to Yu (2004). The NCB samples of the study were purchased from local drugstores.

Biological assays

Animals were subjected to ether anesthesia over the experimental period. Blood samples were removed from the lateral tail vein with a 26-gauge needle at week 0, 1, 2, 3 and 4, respectively. The blood samples were collected and kept for 30 min at 4°C. Then, samples after clotting were centrifuged at 2,500 rpm at 4°C for 15 min. The supernatant serum of the blood samples was immediately isolated to avoid hemolytic and then frozen at –80°C for further analysis.

Serum liver enzymes, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase activities were carried out by the usual routine clinical methods with a Roche automatic biochemical analyzer (Roche, Mannheim, Germany) using commercially available kits.

Statistical analysis

The study was executed in triplicate. Data were analyzed using a general linear model in the SAS system (SAS, 2006). The significant differences among samples were analyzed by using Duncan's multiple range tests. Significance is reported at the $p<0.05$ level.

RESULTS AND DISCUSSION

Aspartate aminotransferase

The aspartate aminotransferase values of the control group (Group I) were not significantly different during the study period (Table 1). The experimental animals were administrated with a single dose of diethylnitrosamine to have higher values in the activities of the aspartate aminotransferase, the increase values of the diethylnitrosamine treatment being near 45% for aspartate aminotransferase when compared with the initial group. The groups (Group II and III) showed an increase of aspartate aminotransferase after the treatment with diethylnitrosamine.

The aspartate aminotransferase values of the initial

Table 2. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum alanine aminotransferase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	38.60	±5.18^{a,y}	38.60	±2.88^{a,y}	41.40	±7.70^{a,y}
1	43.60	±6.40^{b,xy}	69.00	±23.82^{a,x}	55.75	±7.59^{ab,x}
2	46.20	±6.38^{b,x}	78.00	±6.73^{a,x}	53.50	±14.25^{b,x}
3	44.00	±6.28^{c,xy}	82.00	±8.49^{a,x}	56.75	±11.56^{b,x}
4	41.50	±7.14^{c,xy}	80.00	±6.22^{a,x}	57.00	±10.71^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

groups did not show significant difference. The diethylnitrosamine group significantly increased the activities of aspartate aminotransferase after two weeks compared to the initial group ($p<0.05$). The treatment of NCB significantly decreased the activities of aspartate aminotransferase at weeks 3 and 4 during the study period compared to the diethylnitrosamine group ($p<0.05$).

Levels of aspartate aminotransferase were increased in the diethylnitrosamine treated rats. The liver function was restored to its normal activity by the hepatoprotective action of NCB. The elevated levels of aspartate aminotransferase are known to illustrate liver damage (Balamurugan et al., 2008). The increased activities of aspartate aminotransferase in the rats administered with diethylnitrosamine were due to extensive liver damage. The treatment with NCB decreased the serum aspartate aminotransferase activities. The results indicated the stabilization of plasma membrane and the repairing of the hepatic tissue damaged by diethylnitrosamine. The observation correlated with the results of Wills et al. (2006) who indicated that aspartate aminotransferase activities were significantly increased in the study because of diethylnitrosamine induced hepatotoxicity in Wistar rats. The aspartate aminotransferase values of Wistar rats are increased by the diethylnitrosamine treatment (Pradeep et al. 2007).

The experimental rats showed lower values of aspartate aminotransferase showing the protective role of NCB. The antioxidative capacity of NCB might have potent antioxidative capacities. Therefore, the natural *C. Bovis* might be counteracting the diethylnitrosamine induced liver injury although diethylnitrosamine is a hepatotoxin and hepatocarcinogen (Shu and Hollenberg, 1997). There have been reports on the effect of natural substances on serum biochemical parameters related to hepatic functions (ALP, AST, ALT and γ-GT) (Olorunnisola et al., 2012). The NCB components, strong antioxidants, are rich in bilirubin and biliverdin which might be correlated to the hepatoprotection in the study (Wan et al., 2009). A possible involvement of oxidative stress induces hepatocarcinogenesis of rats. The hepatic metabolism of diethylnitrosamine produces reactive oxygen species resulting in oxidative stress and liver cellular injury (Nishimura et al., 2008). The aspartate aminotransferase values of rats intoxicate with diethylnitrosamine in a preventive and curative model. The enzyme is cytoplasmic in nature. The aspartate aminotransferase enters into the circulatory system because of the altered membrane permeability of liver injury (Wills et al., 2006).

Alanine aminotransferase

The alanine aminotransferase values of the control group varied in a narrow range during the study period (Table 2). The results of rats administered with diethylnitrosamine showed a significant increase in the activities of the alanine aminotransferase after one week ($p<0.05$). The increased values of the diethylnitrosamine treatment at week 4 were over two-fold for alanine aminotransferase when compared to the initial group. To clarify the direct effect of *C. Bovis* on serum alanine aminotransferase, the experimental rats were fed with natural *C. Bovis*. The rats were given an injection of diethylnitrosamine and fed with natural *C. Bovis.*

The results of the various initial groups of alanine aminotransferase were not significantly different. The alanine aminotransferase values of the diethylnitrosamine treated group were higher than those of other groups until at the end of the study. The values of the natural *C. Bovis* group were significantly lower than those of the diethylnitrosamine treatment after two weeks ($p<0.05$).

The initial activities of alanine aminotransferase were not shown to be significantly different among treatments. The activities of alanine aminotransferase of rats treated with diethylnitrosamine were significantly higher than those of other groups after two weeks. The values of alanine aminotransferase of the diethylnitrosamine group at the end of the study were shown over two-fold when compared to the control group. The alanine aminotransferase enzyme of liver injury is indicated. The

Table 3. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum γ-glutamyl transferase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	3.60	±1.14^{a,x}	4.00	±1.22^{a,y}	5.00	±2.55^{a,x}
1	4.00	±0.70^{b,x}	7.84	±1.85^{a,x}	5.25	±2.22^{b,x}
2	3.40	±1.34^{b,x}	7.26	±2.03^{a,x}	5.00	±0.82^{b,x}
3	3.66	±1.30^{b,x}	7.38	±2.02^{a,x}	5.13	±0.70^{b,x}
4	3.98	±0.94^{b,x}	7.95	±2.15^{a,x}	5.18	±0.74^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

alanine aminotransferase catalyzes the conversion of alanine to pyruvate and glutamate. The enzyme, an indicator of liver injury, could be released from injured liver cells. The alanine aminotransferase levels conversely are related to the function of the hepatic cells (Balamurugan et al., 2008). A significant increase of alanine aminotransferase levels is illustrated due to diethylnitrosamine induced hepatotoxicity in experimental rats (Wills et al., 2006; Pradeep et al., 2007). The antioxidant of natural *C. Bovis* might be bile pigments, amino acids and bile salts. The bilirubin content is rich in the natural *C. Bovis*. The bile pigment might play an important role as an antioxidant substance (Lin et al., 2007). The antioxidative activities of bilirubin and biliverdin are studied. The bilirubin and its metabolic precursor biliverdin are strong antioxidant agents (Farhan et al., 2001). The bilirubin of natural *C. Bovis* is an effective antioxidant of peroxynitrite mediated protein oxidation. The bilirubin is a bile pigment having an important role as an antioxidant. Thus, the potent antioxidant might decrease the injury of the liver induced by diethylnitrosamine (Minetti et al., 1998). Antioxidants are essential for intracellaur free radical scavenging. The bilirubin of natural *C. Bovis* manifested as a prooxidant showing its cytopathic effect (Rao et al., 2006).

Gamma glutamyl transferase

The rats treated with diethylnitrosamine with or without NCB showed an increase in γ-glutamyl transferase activities at weeks 1, 2, 3 and 4 as compared to control animals (Table 3). The γ-glutamyl transferase activities of rats administered with diethylnitrosamine after one week were significantly higher than those of the initial group ($p<0.05$). The values of γ-glutamyl transferase of the diethylnitrosamine treatment at week 4 were near two-fold higher than those of the initial group. The activities of γ-glutamyl transferase of the rats treated with NCB were not significantly different during the study period. The activities of γ-glutamyl transferase were significantly increased in the diethylnitrosamine group.

The results of γ-glutamyl transferase of experimental animals did not appear significantly different among the various initial groups. The γ-glutamyl transferase values were significantly increased in diethylnitrosamine treated rats as compared to the other groups at week 1 ($p<0.05$). The experimental rats treated with the NCB group for one week significantly reduced the values of the γ-glutamyl transferase. These results showed natural *C. Bovis* tended to prevent liver damage by suppressing the leakage of enzymes through cellular membranes by preserving the integrity of the plasma membranes.

The hepatoprotective and antioxidant effects of rats treated with diethylnitrosamine are studied. The serum γ-glutamyl transferase values of rats were elevated after the administration of diethylnitrosamine (Pradeep et al., 2007). The antioxidants might have the protective effects in diethylnitrosamine induced oxidative stress. Several compounds, which possessed antioxidant potential, such as ellagic acid, curcumin and garlic powder, are shown to counteract diethylnitrosamine induced oxidative damage (Bansal et al., 2005). Natural *C. Bovis* might have a higher content of bile acids and ursodeoxycholic acid (Lin et al., 2007). The reducing power of carboxyl acids, ursodeoxycholic acid, is shown. The antioxidant mechanism of carboxyl acids is due to its carboxylic groups (Campo et al., 2004). NCB has higher content of bilirubin which might be related to the better reducing capacity because bilirubin might be proton donors. NCB has higher content of bile acids which has a carboxyl group and are good sources of proton donors. The proton donors might stop the reaction chain of free radicals to form stable products (Lin et al., 2007). The function of ursodeoxycholic acid having protective effects causes by oxidative stress and apoptosis (Perez et al., 2006).

Alkaline phosphatase

The results of alkaline phosphatase of the control group varied in a narrow range during the study period (Table 4). The alkaline phosphatase activities of the rats treated with diethylnitrosamine showed a significant increase of

Table 4. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum alkaline phosphatase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	141.00	±24.58^{a,x}	144.60	±28.69^{a,z}	159.80	±6.14^{a,z}
1	142.80	±19.10^{b,x}	175.00	±7.85^{a,y}	166.75	±9.25^{a,yz}
2	140.20	±17.34^{b,x}	180.75	±7.63^{a,xy}	168.25	±7.80^{a,xyz}
3	133.80	±12.32^{c,x}	190.75	±7.54^{a,xy}	173.25	±8.22^{b,xy}
4	137.20	±11.90^{c,x}	199.00	±9.63^{a,x}	177.75	±8.38^{b,x}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

Table 5. Effect of diethylnitrosamine (DEN) of natural *Calculus Bovis* (NCB) on serum lactate dehydrogenase activities of experimental animals.

Week	Treatment					
	Group I		Group II		Group III	
0	140.74	±20.16^{a,y}	144.40	±17.21^{a,z}	148.60	±3.78^{a,z}
1	143.40	±18.60^{b,xy}	174.75	±13.77^{a,y}	167.25	±6.34^{a,y}
2	145.40	±16.53^{c,xy}	199.00	±11.46^{a,x}	166.50	±6.54^{b,y}
3	144.60	±12.12^{c,xy}	208.25	±15.78^{a,wx}	177.25	±4.03^{b,x}
4	148.75	±3.50^{c,x}	216.25	±14.66^{a,w}	187.50	±4.04^{b,w}

Values are expressed as arithmetic mean ± standard deviations, unit: U/L. $^{a-c}$: Means in the same row with different superscripts are significantly different ($p<0.05$). $^{x-z}$: Means in the same column with different superscripts are significantly different ($p<0.05$). Group I: control rats; Group II: rats administered with DEN alone; Group III: rats administered with DEN + NCB (100 mg/kg b.w., p.o.).

alkaline phosphatase after one wk when compared to the control group ($p<0.05$). The values of the diethylnitrosamine administrated rats were significantly higher than those of the initial group at week 1, 2, 3 and 4. The alkaline phosphatase activities of rats treated with the diethylnitrosamine groups were significantly higher than those of the NCB group at week 3 and 4 ($p<0.05$).

The initial alkaline phosphatase values of various groups were not significantly different. The alkaline phosphatase values of the control group were significantly lower than those of the diethylnitrosamine treatment after one week ($p<0.05$). The alkaline phosphatase activities of the diethylnitrosamine with the NCB group were significantly lower than those of the diethylnitrosamine treatment along after three weeks ($p<0.05$). An increase in the activities of the alkaline phosphatase was observed in the treated rats after administration of diethylnitrosamine in the study period. The formation of reactive oxygen species of the diethylnitrosamine treatment might alter the antioxidant system, while the presence of NCB might counteract diethylnitrosamine induced oxidative stress.

Lactate dehydrogenase

The lactate dehydrogenase values of the control group are shown in Table 5. An administration of a single dose of diethylnitrosamine to rats produced a significant increase in lactate dehydrogenase after one week ($p<0.05$) compared to the initial group. The values of lactate dehydrogenase of the diethylnitrosamine treated groups were increased throughout the study period. The lactate dehydrogenase activities of the diethylnitrosamine and the NCB groups treated rats were increased after one week.

The values of lactate dehydrogenase of the initial groups with various treatments were not shown to be significantly different. The lactate dehydrogenase values of the NCB group were significantly lower than those of the diethylnitrosamine treated group after two weeks ($p<0.05$). The treatment of NCB decreased the lactate dehydrogenase values in the study period compared to the diethylnitrosamine treatment. The lactate dehydrogenase enzyme might be released from the cytoplasm into the blood circulation immediately after

rupture of the plasma membrane and cellular injury. Reactive oxygen species released by the metabolism of diethylnitrosamine might have caused damage to the hepatocellular membranes. The reactive oxygen species might cause the oxidative stress and cellular damage. The cytosolic contents were released into the systemic circulation.

The inhibition of hepatocellular carcinoma by natural antioxidants in experimental animals administrated with diethylnitrosamine is studied. The alkaline phosphatase activities are significantly increased after the treatment of diethylnitrosamine (Shiota et al., 1999). The protective role of an antioxidant treatment (vitamin E) on diethylnitrosamine induced oxidative stress in rats is illustrated. There is evidence of the formation of reactive oxygen species resulting in oxidative stress which might be one of the factors in the etiology of cancer. The alkaline phosphatase activities are significantly increased following the diethylnitrosamine treatment to rats (Bansal et al., 2005).

The increased lactate dehydrogenase activities are caused by the hepatocellular necrosis. The enzyme of the rats is significantly increased after the administration of diethylnitrosamine (Arai et al., 2002). The lactate dehydrogenase values of experimental animals treat with diethylnitrosamine showed a significant increase over the study period (Bansal et al., 2005). The utilities of ursodeoxycholic acid in liver diseases are studied. Ursodeoxycholic acid having cytoprotective, membrane stabilizing and antioxidative effects is currently the only drug for the treatment of chronic liver diseases (Kumar and Tandon, 2001).

CONCLUSION

The effects of natural *C. Bovis* on diethylnitrosamine induced hepatic injury in rats were analyzed in the study. The results of the present study showed natural *C. Bovis* to be effective in reducing the aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase activities. In conclusion, natural *C. Bovis* exhibited good hepatoprotective properties by reversing the oxidant-antioxidant imbalance during rats treated diethylnitrosamine induced oxidative stress in the study.

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