Study of the Protective Effects of Benfotiamine Against CCl_4-Induced Hepatotoxicity in Rats

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Abstract

Liver is considered as the first target for the toxic effects of toxins and other xenobiotics, and this can be attributed to its role as a site which receive all absorbed xenobiotics from the gastrointestinal tract and its role as a major site for biotransformation of xenobiotics. The present study was designed to evaluate the possible hepatoprotective effect of benfotiamine against CCl_4-induced hepatotoxicity in rats. The study was conducted on 48 male albino rats; the animals were allocated into 8 groups (6 rats in each group) and treated as follow: 4 groups treated with oral doses of either normal saline, benfotiamine (100 mg/kg), thiamine (100 mg/kg), N-acetylcystein (400 mg/kg) only without induction of hepatic damage. The other 4 groups were treated as indicated previously with induction of hepatic damage with CCl_4; at the end of treatment period, rats were scarified, blood samples obtained and livers excised for the assessment of the oxidative stress parameters (MDA and GSH), cholesterol and triglycerides levels. Additionally, serum levels of total bilirubin, albumin, total protein and the activities of ALT, AST and ALP enzymes were evaluated before and after treatment with benfotiamine. Tissue sections were prepared for evaluation of histopathological changes. The results indicated that benfotiamine has the ability to protect hepatic tissue against the toxicity induced by CCl_4, revealed through reduction of serum levels of TSB and liver enzymes, decrease in the hepatic tissue MDA levels and elevation of GSH there. Histological evaluation of tissue sections prepared for this purpose confirmed the previous finding. In conclusion, benfotiamine is capable to protect liver tissue against CCl_4-induced toxicity in rats more than thiamine.

***Key words*: Benfotiamine, CCl_4, Hepatotoxicity**

الخلاصة

يعتبر الكبد الهدف الأول للتأثير السام للمركبات المختلفة لكونه الموقع الأول الذي يستلم جميع المركبات التي يتم امتصاصها عن طريق القناة الهضمية اضافة لكونه مركز التحولات الأيضية لهذه المركبات. تم تصميم هذه الدراسة لتقييم الحماية المتوقعة لمادة البنفوتيامين ضد التسمم المستحدث برباعي كلوريد الكربون في الجرذان. أجريت الدراسة على ٤٨ جرذا أبيض حيث تم تقسيمها الى ثمانية مجموعات (٦جرذان لكل مجموعة) تم علاج أربعة منها بجرع عن طريق الفم لواحد من المركبات التالية: محلول ملحي متوازن، بنفوتيامين ١٠٠ ملغم/كغم، ثيامين ١٠٠ ملغم/كغم، أسيتيل سستيين ٤٠٠ ملغم/كغم. أما المجموعات الأربعة الأخرى فتمت معالجتها بنفس الطريقة المذكورة آنفا مع استحداث تلف كبدي بواسطة رباعي كلوريد الكربون، وفي نهاية فترة العلاج تم قتل الحيوانات للحصول على أكبادها وعينات من دمها. تم قياس معايير فرط التأكسد (الكلوتاثيون والمالونداي الديهايد)، الكوليسترول والشحوم في خلاصة نسيج الكبد بالأضافة الى قياس مستوى مادة الصفراء، البروتين وفعالية الأنزيمات الكبدية (AST، ALT، ALP) في مصل الدم ومقارنة مستوى هذه المعايير قبل وبعد العلاج بالبنفوتيامين والمركبات الأخرى. تم تحضير مقاطع نسيجية للكبد ومتابعة التغييرات الحاصلة بواسطة المجهر. أثبتت نتائج الدراسة مقدرة البنفوتيامين على حماية نسيج الكبد ضد التسمم المستحدث بواسطة رباعي كلوريد الكربون من خلال خفض مستويات مادة الصفراء وفعالية الأنزيمات الكبدية في الدم، أضافة الى خفض مستويات الكوليسترول والشحوم والمالونداي الديهايد في نسيج الكبد مع رفع مستوى الكلوتاثيون هناك، كما أكدت نتائج الفحص النسيجي ماذكر آنفا. يمكن الأستنتاج بأن للبنفوتيامين المقدرة على وقاية نسيج الكبد ضد الضرر المستحدث بواسطة رباعي كلوريد الكربون في الجرذان اكثر من الثيامين.

Introduction

The liver plays a central role in carbohydrate, protein and fat metabolism and allows the detoxification of various xenobiotics. Additionally, it regulates the synthesis and secretion of bile. [(1)] Toxic injury occurs in the liver more often than other organs, because all ingested substances that are absorbed are first presented to the liver and that the liver is responsible for the metabolism and elimination of many substances.[(1)]Many xenobiotics such as acetaminophen, CCl_4 ,and yellow phosphorus produce liver damage in a predictable and dose-dependent manner; the most frequent mechanism of hepatocellular injury involves production of injurious metabolites by the cytochrome P450 system. [(2,3)] Preventive care can significantly reduce the progression of liver disease. [(4)]

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One of the drugs that used for this purpose is N-Acetylcysteine (NAC) which has been used clinically for the treatment of Acetaminophen poisoning [5]. Oral supplementation with (NAC) provides an alternate means of boosting intracellular glutathione via elevated intracellular cysteine, and this can scavenge peroxynitrite and hydroxyl radicals as well as convert hydrogen peroxide to water. [6] Benfotiamine (S-benzoyl thiamine-O-monophosphate) has been shown to reduce the formation of advanced glycation end products (AGE) byactivating transketolase. [7, 8] Benfotiamine has been noted to possess clinical efficacy in the treatment of diabetic cardiomyopathy, [9] diabetic nephropathy [10] and diabetic neuropathy. [11, 12] Moreover, benfotiamine has been shown to reduce the oxidative stress through a mechanism unrelated to AGE formation. [13] Activation of Akt (protein kinase B) has been demonstrated to stimulate eNOS activity, increase the bioavailability of NO and reduce the generation of ROS. [14] Benfotiamine has been reported to improve the function of endothelium by activating Akt and subsequently stimulating eNOS and inhibiting the generation of ROS. [15,16,17] Benfotiamine is thought to act by at least three different mechanisms. First, activation of the hexosamine pathway with subsequent decrease in the accumulation of deleterious glucose metabolites seems to be involved, second, normalization of PKC activity along with prevention of nuclear factor kappa B (NF-κB) activation has been found in retinas, and third, correction of imbalances in the polyol pathway by decreasing aldose reductase activity, sorbitol concentrations and intracellular glucose levels seems to play a role. [7] Absorption of benfotiamine was better than thiamine itself, and levels of thiamine and thiamine pyrophosphate (TPP) remain higher for longer period of time. [18] Absorption of thiamine in the form of benfotiamine is found to be five times greater than the absorption of the conventional thiamine supplements, and because of greatest intracellular access of benfotiamine, its tissue availability and effects are more impressive, especially in the brain and muscles. [19] The present study was designed to evaluate the possible protective effect of benfotiamine against CCL4-induced hepatotoxicity in rats.

Materials and Methods

Forty eight Sprague-Dawley rats of both sexes weighing 180-220 g were obtained from the animal house in the College of Pharmacy, University of Baghdad and used in the study. The animals were housed in the animal house of Collage of Pharmacy, University of Sulaimaniya under conditions of controlled temperature and allowed free access to water and food. The study protocol was approved by the Committee for Medical Research, College of Medicine, University of Sulaimaniya. The animals were allocated into eight groups, each contain 6 rats and treated as follow: Group I, received single oral daily dose of normal saline for 7 days. The group served as control; group II received single oral daily dose of normal saline for 7 days, at day 8 the animals received single dose of CCl4 (2 ml of a mixture of 1:1 v/v in a corn oil /kg/day) orally by oral needle to induce liver damage. [20] The animals were sacrificed 24 hr after CCl_4 administration; [21] group III received single oral daily dose of thiamine (70 mg/kg/day) for 7 days; group IV received single daily oral dose of thiamine (70 mg/kg/day) started 7 days prior to treatment with CCl_4 at day 7; group V received single oral daily dose of benfotiamine (70 mg/kg/day) for 7 days; group VI received single daily oral dose of benfotiamine (70 mg/kg/day) started 7 days prior to treatment with CCl_4 at day 7; Group VII received single oral daily dose of N-acetylcysteine (400 mg/kg/day) for 7 days; group VIII received single daily oral dose of N-acetylcysteine (400 mg/kg/day) started 7 days prior to treatment with CCl_4 at day 7. All animals were sacrificed on the day 8. After killing the animals by over dose of thiopental (Panpharma S.A. France) (100mg/kg), livers were obtained and utilized for preparation of tissue homogenate. The organ was quickly existed and placed in a chilled phosphate buffer solution (pH 7.4), blotted with filter paper, and 10% homogenate was prepared in the same solution utilizing metal head tissue homogenizer at 4^oC. All preparations were frozen (-18 ^0C) unless worked immediately. After killing the animals, blood was collected by intracardiac puncture. The clot was dispersed with glass rod and then centrifuged for 15-20 minutes at 2000 rpm and the supernatant was used for the estimation of ALT and AST, [22] ALP, [23] albumin and total protein [24] as parameters of liver function tests and total serum bilirubin [25] as excretory function test. Samples of liver tissue homogenates were used for determination the malondialdehyde (MDA), [26] reduced glutathione (GSH), [27] cholesterols and triglycerides levels. [28,29] Tissue sections were prepared for histological examination according to the method of Bauer, [15] using paraffin sections technique. The significance of differences between the mean values was calculated using unpaired Student's ***t*** -test. ***P***-

Values less than 0.05 were considered significant for all data shown in the study.

Results

Table 1 showed that serum activities of alanine-aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated in CCl_4-intoxicated animals (847.5% and 2381.6% respectively) compared to control group (P<0.05). Pre-treatment of rats with single oral doses of Thiamine, Benfotiamine or N-Acetylcysteine for 7 days prior to intoxication with CCl_4 showed a marked decline in the serum ALT and AST activities (36.6%, 32.8% and 88.6% respectively for ALT and 39.75%, 77.97% and 94.2% respectively for AST) compared to CCl_4-treated animals (P<0.05). Serum alkaline phosphatase (ALP) activity was significantly elevated in CCl_4-treated animals (53.4%) compared to control group (P<0.05, table 1), and pre-treatment with single oral doses of Thiamine, Benfotiamine or N-Acetylcysteine for 7 days, prior to induction of liver toxicity with CCl4, showed a marked decline in the level of serum ALP activity (17%, 37.9% and 32.2% respectively) compared to CCl_4-treated animals.

Table 1. Effects of Benfotiamine on serum levels of ALT, AST and ALP activities in experimental animal model of CCl_4-induced liver toxicity.

Type of Treatment	Serum ALT U/L	Serum AST U/L	Serum ALP U/L
Saline treated only	8.0 ± 1.06	6.0 ± 0.6	79.3 ± 18.3
Saline + CCl_4	75.8 ± 11.5 * [a]	148.9 ± 17.5 * [a]	121.7 ±26.6* [a]
Thiamine only	10.2 ± 1.5 [b]	6.5 ± 0.71 [b]	90.5 ± 19.1* [b]
Thiamine + CCl_4	48.0 ± 9.5 * [c]	89.7 ± 16.1 * [c]	101.0 ±20.2* [b]
Benfotiamine only	9.6 ± 1.2 [b]	7.1 ± 0.9 [b]	80.9 ± 8.1 [c]
Benfotiamine + CCl_4	50.9 ± 8.2 * [c]	32.8 ± 6.1 * [d]	75.5 ± 10.6 [c]
N-Acetylcysteine only	9.0 ± 1.2 [b]	7.2 ± 0.9 [e]	72.0 ± 9.0 [c]
N-Acetylcysteine + CCl_4	8.6 ± 1.4 [b]	8.6 ± 1.2 * [e]	82.5 ± 10.2 [c]

Each value represents mean ± SD; * significantly different compared to saline only treated group (P<0.05); values with non-identical superscripts (a,b,c,d,e) are considered significantly different within the same parameter (P<0.05).

Table 2 showed that animals intoxicated with CCl_4 presented with a highly significant increase in MDA (95.6%) and decrease in GSH (72.2%) contents of liver tissue homogenate compared to control (saline treated) group (P<0.05, table 2). Pre-treatment of rats orally with single doses of Thiamine, Benfotiamine or N-Acetylcysteine for 7-days before induction of hepatotoxicity with orally administered CCl_4 resulted in 32%, 31.1% and 51% decrease in hepatic MDA contents respectively, and significant increase in GSH (340%, 237% and 246% respectively) compared to CCl_4- treated only animals (P<0.05, table 2).

Table 2. Effect of Benfotiamine on liver tissue malondialdehyde (MDA) and reduced glutathione (GSH) levels in experimental animal model of CCl_4-induced liver toxicity

Type of Treatment	Liver tissue GSH µmol/g tissue	Liver tissue MDA nmol/g tissue
Saline	27.0 ± 6.75	230.0 ± 46.0
Saline + CCl_4	7.5 ± 0.81*[a]	450.0 ± 48.0*[a]
Thiamine	21.0 ± 4.2 [b]	246.0 ± 36.0 [b]
Thiamine + CCl_4	33.0 ± 6.0*[c]	306.0 ± 14.0*[c]
Benfotiamine	25.5 ± 5.2 [b]	218.0 ± 35.0 [d]
Benfotiamine + CCl_4	25.3 ± 2.6 [b]	310.0 ± 22.0*[c]
N-Acetylcysteine	31.2 ± 4.5 [c]	221.0 ± 26.0 [d]
N-Acetylcysteine + CCl_4	26.0 ± 5.3 [b]	220.0 ± 18.0 [d]

Each value represents mean ± SD; * significantly different compared to saline only treated group (P<0.05); values with non-identical superscripts (a,b,c,d) are considered significantly different within the same parameter (P<0.05).

In table 3, total serum bilirubin (TSB) levels were significantly elevated in CCl_4-treated animals (85.7%) compared to control group (P<0.05, table 3), and pre-treatment with single oral doses of Thiamine, Benfotiamine or N-Acetylcysteine for 7 days, prior to induction of liver toxicity with CCl4, showed a marked decline in the level of serum TSB (33.8%, 41.5% and 30.7% respectively) compared to CCl_4-treated animals. Serum albumin and total protein were shown to be significantly decreased in CCl_4-treated animals (31.8%) compared to control group (P<0.05), while total protein levels showed a non significant differences in CCl_4-treated animals (1.3%) compared to control group (Table 3). Pre-treatment of rats with single oral doses of Thiamine, Benfotiamine or N-Acetylcysteine for 7 days, prior to intoxication with CCl_4, showed increase in the level of serum albumin (45.4%, 36.36% and 13.6% respectively) compared to CCl_4-treated animals (P<0.05, table 3), while total protein levels were not significantly changed. However, rats treated with single oral dose of Thiamine or Benfotiamine for 7 days showed non-significant differences on both serum albumin and total protein (P > 0.05) compared to control group. Meanwhile, single oral dose of N-Acetylcysteine for 7 days showed a significant increase in both serum albumin and total protein levels (P < 0.05) compared to control group (Table 3; figure 4).Serum Triglyceride and Cholesterol levels in liver tissue homogenate were shown to be significantly increased in CCl_4-intoxicated animals (333% and 212.5% respectively) compared to control group (P< 0.05, table 3). However, Pre-treatment of rats with single oral doses of Thiamine, Benfotiamine or N-Acetylcysteine for 7 days, prior to induction of hepatotoxicity with CCl_4, showed a marked decline in the levels of triglycerides (20%, 57% and 58% respectively) and cholesterol(24.5%, 44.9% and 42.2% respectively) compared to CCl_4-treated animals (P<0.05, table 3). Histological examination of liver tissues of rats exposed to toxic dose of CCl_4 showed a marked hepatic damage revealed as zonal necrosis, extensive diffuse vacuolar degeneration of the hepatocytes, together with ballooning and fatty changes, dilatation and congestion of the central vein; the later showed focal hemorrhage with variable degree of inflammatory cell reaction were seen also (figure 1). Treatment of rats with Benfotiamine, thiamine or N-acetylcysteine before induction of toxicity clearly demonstrated protective effects against CCl4-induced toxicity, where Benfotiamine showed the greatest level of protection compared to that produced by N-acetylcysteine and thiamine respectively (figures 2, 3 and 4).

Table 3. Effect of Benfotiamine on serum levels of total serum bilirubin, albumin, total protein and Liver tissue Triglycerides and Cholesterol in experimental animal model of CCl_4-induced liver toxicity

Type of Treatment	Total Serum Bilirubin mg/dl	Serum albumin mg/dl	Liver tissue Triglycerides µmol/g tissue	Liver tissue Cholesterol µmol/g tissue	Serum total protein mg/dl
Saline treated only	0.35 ± 0.01	2.9 ± 0.08	12.0 ± 2.4	8.8 ± 0.3	6.17 ± 0.4
Saline + CCl_4	0.65 ± 0.14* [a]	2.2 ± 0.1* [a]	52.0 ± 7.2 *[a]	18.7 ± 2.1*[a]	6.3 ± 0.6 [a]
Thiamine only	0.31 ± 0.02 [b]	3.2 ± 0.2 *[b]	11.5 ± 2.1[b]	9.3 ± 1.8[b]	5.9 ± 0.38 [a]
Thiamine + CCl_4	0.43 ± 0.1* [c]	3.2 ± 0.1* [b]	41.5 ± 3.6 *[a]	14.1 ± 3.6*[c]	5.8 ± 0.3 [a]
Benfotiamine only	0.29 ± 0.03 [b]	2.9 ± 0.1 [b]	10.2 ± 1.3 [b]	8.5 ± 0.8 [b]	5.3 ± 0.7 [a]
Benfotiamine + CCl_4	0.38 ± 0.04 [d]	3.0 ± 0.1 [b]	22.3 ± 3.4 *[c]	10.3 ± 0.9 *[d]	5.9 ± 0.5 [a]
N-Acetylcysteine only	0.39±0.02 [d]	4.5 ± 0.4 * [c]	9.3 ± 1.6 [b]	8.3 ± 0.6 [b]	7.2 ± 1.2* [b]
N-Acetylcysteine + CCl_4	0.45 ± 0.02*[c]	2.5 ± 0.2 [b]	21.8 ± 2.6 *[c]	10.8 ± 0.5 *[d]	6.0 ± 1.1[a]

Each value represents mean ± SD; * significantly different compared to saline only treated group (P<0.05); values with non-identical superscripts (a,b,c,d) are considered significantly different within the same parameter (P<0.05).

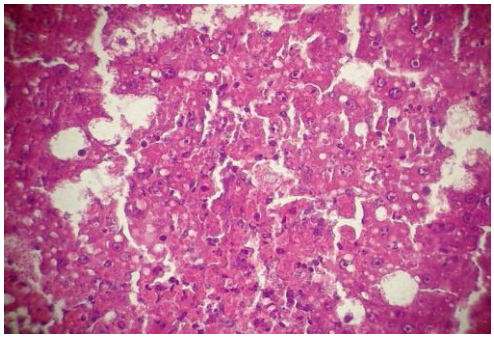

Figure 1. Section from liver tissue after CCl_4 intoxication. (X400, H and E stain)

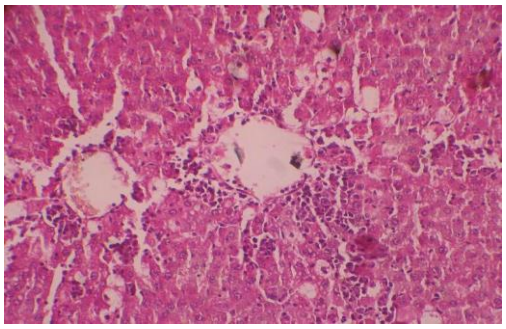

Figure 2. Section of liver tissue after intoxication with CCl_4 and protection with orally administered N-acetylcysteine. (X400; H and E stain)

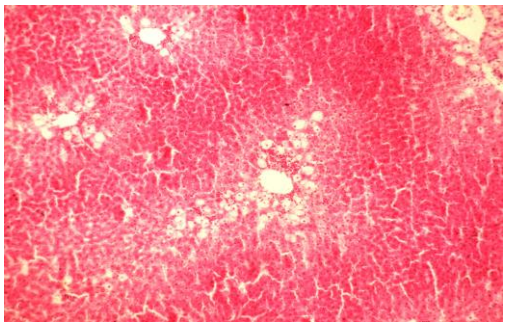

Figure 3. Section from liver tissue after CCl_4 intoxication and protection with orally administered thiamine (X 10; H and E stain)

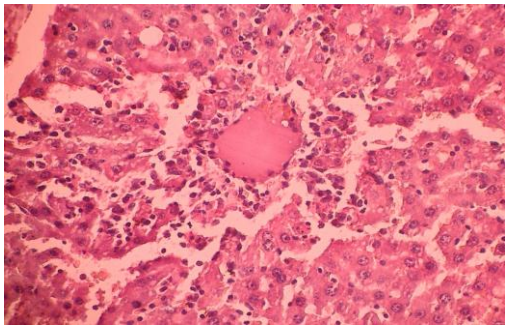

Figure 4. Section of liver tissue after intoxication with CCl_4 and protection with orally administered Benfotiamine. (X400; H and E stain)

Discussion

In animals acutely exposed to CCl_4 orally, the liver appears to be the primary target organ.[21] Numerous studies showed that metabolism of (CCl_4) is required for its toxicity; [30] it is known to be rapidly transformed by cytochrome P450-2E1 of the hepatocyte endoplasmic reticulum to $CCl_3^{\bullet}$ which is converted into $CClOO^{\bullet}$ in the presence of oxygen.[31] Peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids leads to the formation of lipid peroxides, these in turn give products like MDA that cause damage to the membrane [32] and alter cellular function.[33] The reduction in GSH is due to consumption for conjugation of metabolites, and then redox potential of the tissue will be impaired.[34] GSH depletion also results in lipid peroxidation and impaired antioxidant enzyme activities.[35] The data presented in this study clearly demonstrated the state of oxidative stress induced in hepatic tissues by CCl_4 treatment manifested by elevation of MDA content in tissue homogenate, which is associated with depletion of GSH content, these results are compatible with those obtained by others.[36] Administration of Benfotiamine results in increased intracellular thiamine diphosphate levels, a cofactor of transketolase enzyme; activation of this enzyme by thiamine may reduce superoxide overproduction through directing advanced glycation and lipoxidation end products substrates to the pentose phosphate pathway.[7,8] The data presented in this work showed a significant decline in MDA level and increase in GSH level in animals treated with Benfotiamine and thiamine prior to CCl_4 administration, this might be attributed to the up-regulation of transketolase but could be a positive side effect of Benfotiamine, which show an intrinsic antioxidative activity by itself.[37] It is important to mention that the effect of Benfotiamine was much better than thiamine concerning GSH levels. Lipid peroxidation and oxidative stress were attenuated by NAC administration to the rats prior to CCl_4, which may be due to enhancement of intracellular GSH biosynthesis. Lipid peroxidation has been proposed to disrupt cellular membrane, resulting in loss of membrane integrity, [38] and may lead to leakage of ALT and AST and increasing their activities in the plasma. The plasma transaminases (ALT and AST) are known to be increased significantly in rats after exposure to toxic doses of CCl_4.[39] The data presented in table 1, clearly support the

above explanation and seems to be consistent with those obtained by others. [40] The sharp elevation in serum activities of the enzymes that localized in bile ducts is a useful biochemical index for bile duct damage, particularly alkaline phosphatase (ALP). In the present work, serum activity of alkaline phosphatase that is present in the lining membrane of the hepatocytes was also increased in the CCl_4-treated rats compared to normal control animals, and these results are consistent with that reported by other investigators.[41] The major finding of the present study is that treatment of animals with Benfotiamine prior to CCl_4 elicits beneficial effects on the structure and functions of the liver; regarding the enzymatic activities, there is a significant decrease in AST level compared with that intoxicated with CCl_4, the reduction was three times more than that obtained through the use of thiamine prior to CCl_4 administration, but, still the protection was less than that observed in the animal group treated with NAC prior to CCl_4. The data also revealed attenuation of ALT activity induced by CCl_4 due to the use of Benfotiamine, which was approximately the same as that noted with thiamine; however, the protection was much less than that observed due to the use of NAC. Concerning serum ALP, the serum activity level of this enzyme almost normalized by Benfotiamine, again the protection was better than thiamine even it precede that of NAC. The data presented in this study regarding the effect of NAC was consistent with those observed by others.[38] The suggested mechanisms behind these effects of Benfotiamine may be due to indirect action of the lipophylic pro-drug through decreasing lipid peroxidation, which is the main factor affecting membrane permeability and integrity, and consequently preventing further leakage of cytosolic enzymes; [38] another possible explanation may be related to its negative action on protein kinase C activities.[7,8] It is important to mention that the impact of Benfotiamine was better than that of both thiamine and NAC when each one administered alone prior to CCl_4 in this respect. In conclusion, Benfotiamine protects hepatic tissue of rats against CCl_4-induced damage.

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