

Camel milk and bee honey regulate profibrotic cytokine gene transcripts in liver cirrhosis induced by carbon tetrachloride

Kadry Sadek, Doha Beltagy, Ebeed Saleh, and Reham Abouelkhair

Abstract: The lack of studies regarding the mechanism of the protective effects of camel milk and bee honey against hepatotoxic compounds led us to perform this study. Thirty-six male rats were divided into two main groups. The first group ($n = 9$) comprised control non-cirrhotic rats. The rats of the second group ($n = 27$) were administered carbon tetrachloride (CCl_4) by intraperitoneal injection to induce liver cirrhosis. The cirrhotic rats were then divided into three equal subgroups, each comprising nine animals, as follows: (i) cirrhotic rats, (ii) cirrhotic rats treated with camel milk, and (iii) cirrhotic rats treated with camel milk and bee honey. The present findings revealed that CCl_4 elevated the activities of liver enzymes, blood glucose levels, non-esterified fatty acids (NEFA) in the serum and glycogen content in the liver. On the other hand, CCl_4 significantly decreased phosphorylase activity in the liver tissue and significantly increased carbohydrate intolerance and insulin resistance index (HOMA-IR). Moreover, CCl_4 induced a significant increase in oxidative stress, along with increased expression of the profibrotic cytokine genes TNF- α and TGF- β . However, camel milk either alone or in combination with bee honey ameliorated these toxic actions. The antioxidant properties of these protective agents and their effects of downregulating certain procirrhotic cytokine gene transcripts underlie this protection.

Key words: camel milk, bee honey, profibrotic cytokines, gene expression, liver cirrhosis, oxidative stress.

Résumé : Nous avons effectué ces travaux en vue de combler le manque d'études portant sur les mécanismes sous-jacents des effets protecteurs du lait de chameau et du miel d'abeille contre des substances hépatotoxiques. Nous avons réparti 36 rats mâles en deux principaux groupes. Le premier groupe ($n = 9$) était constitué de rats non cirrhotiques. Chez les rats du second groupe ($n = 27$), nous avons injecté du tétrachlorure de carbone (CCl_4) par voie intrapéritonéale en vue de produire une cirrhose hépatique. Nous avons réparti les rats cirrhotiques dans trois sous-groupes égaux comprenant chacun neuf animaux de la manière qui suit : (i) rats cirrhotiques, (ii) rats cirrhotiques traités par du lait de chameau et (iii) rats cirrhotiques traités par du lait de chameau et du miel d'abeille. Les présents résultats ont révélé que l'administration de CCl_4 entraînait une augmentation de l'activité des enzymes hépatiques, de la glycémie, des taux d'acides gras non estérifiés (NEFA) dans le sérum et des taux de glycogène dans le foie. Par ailleurs, le CCl_4 a entraîné une diminution marquée de l'activité des phosphorylases dans le tissu hépatique ainsi qu'une augmentation de l'intolérance aux hydrates de carbone et de l'indice de résistance à l'insuline (HOMA-IR). De plus, le CCl_4 a entraîné une augmentation marquée du stress oxydatif ainsi qu'une augmentation de l'expression des gènes des cytokines profibrosantes TNF- α et TGF- β . Cependant, le lait de chameau, seul ou en association avec du miel d'abeille, a permis d'atténuer ces effets toxiques. Les propriétés antioxydantes de ces agents protecteurs et leurs effets de régulation à la baisse de certains transcrits de gènes de cytokines procirrhotiques sous-tendent cette protection. [Traduit par la Rédaction]

Mots-clés : lait de chameau, miel d'abeille, cytokines profibrosantes, expression génique, cirrhose hépatique, stress oxydatif.

Introduction

The liver is a key organ that regulates many important metabolic, detoxification, and secretory functions in the body (Núñez and Soriano 2005). Hepatic injury is associated with disruptions of these metabolic functions (Gupta 2004). Carbon tetrachloride (CCl_4) is a highly toxic chemical agent and is the most commonly used drug to experimentally induce liver damage. Histopathological examination of liver tissue sections indicated that CCl_4 induces fibrosis, cirrhosis, and hepatocarcinoma (Karakus et al. 2011). The toxic effect of CCl_4 is attributed to the production of trichloromethyl radicals during oxidative stress (Stoyanovsky and Cederbaum 1999). The numbers of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes, and natural killer cells are significantly increased after liver injury induced by hepatotoxins such as CCl_4 .

CCl_4 induces the activation of resident liver macrophages and (or) the chemoattraction of extrahepatic cells (e.g., neutrophils and lymphocytes) (Ramadori and Saile 2004). The activated macrophages are then released and contribute to liver fibrosis, inflammation, and injury (Canbay et al. 2004). Once the liver is injured, the efficiency of treatment with common drugs is limited (Lee et al. 2007). Therefore, interest in using alternative medicines for the treatment of hepatic disease has arisen. The camel is among the animals mentioned in the Quran as a miracle of God. Camel milk has been shown to have medicinal effects; thus, Islamic populations have been encouraged and permitted to drink camel milk in cases in which medical treatment is necessary. Milk of a specific humped camel (*Camelus dromedaries*) has been medically used for centuries in different regions of Arab countries. Alhaider

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et al. (2013) reported that over 200 proteins were identified by two-dimensional gel electrophoresis and peptide mass mapping and liquid chromatography – tandem mass spectrometry (LC-MS/MS) following one-dimensional polyacrylamide gel electrophoresis. The same authors found some known camel milk proteins, including heavy-chain immunoglobulins, antioxidative peptides, lactoperoxidase, and lactoferrin protein and others exhibiting regions of exact homology with proteins from other species. The peptides and proteins present in camel milk exert biological activities that have beneficial effects on many bioprocesses such as digestion, absorption, growth, and immunity (Korhonen and Pihlanto 2003). Camel milk is different from the milk of other ruminant animals; it is low in cholesterol and sugar but high in minerals (sodium, potassium, iron, copper, zinc, and magnesium) and vitamins (A, B₂, C, and E), and it contains a high concentration of insulin (Yousef 2004). Furthermore, camel milk can be stored at room temperature for a longer period than milk from other animals (Omer and Eltinay 2009). The most commonly described uses for camel milk are as a drug against autoimmune diseases, dropsy, jaundice, splenomegaly, tuberculosis, asthma, anemia, piles, and diabetes (Rao et al. 1970). In addition, camel milk has antitoxic effects against cadmium chloride (Al-Hashem et al. 2009), CCl₄ (Khan and Alzohairy 2011), cisplatin (Afifi 2010), paracetamol (Al-Fartosi et al. 2011), and aluminum chloride (Al-Hashem 2009). Patients who suffer from chronic hepatitis show improved liver function after drinking camel milk (AL-Ayadhi and Elamin 2014). Although Khan and Alzohairy (2011) have studied the protective effect of camel milk against CCl₄-induced hepatotoxicity, the mechanisms of this protective effect have not been fully investigated. Natural honey is widely used worldwide as a complementary and alternative medicine for various disorders, including gastrointestinal lesions. Although honey is a high-carbohydrate food, its glycemic index varies within a wide range from 32% to 85%, depending on the botanical source. Honey contains small amounts of proteins, enzymes, amino acids, minerals, trace elements, vitamins, aromatic compounds, and polyphenols (Bogdanov et al. 2008). The effective use of honey in treating hepatic toxicity could be due to its other constituents, especially the various abundant antioxidants (Fasanmade and Alabi 2008). The therapeutic properties of honey, which was once considered a form of folk or preventive medicine, have acquired importance for the treatment of acute and chronic free radical mediated diseases such as atherosclerosis, diabetes, and cancer (Beretta et al. 2007). Therefore, in the present study, we investigated the mechanism that underlies the protective effects of camel milk and bee honey against CCl₄-induced hepatotoxicity in rats by assaying the liver functions, carbohydrate tolerance, insulin resistance, oxidative status, and gene expression of certain profibrotic cytokines.

Materials and methods

Chemicals and kits

Diagnostic kits for total serum proteins, albumin, alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), γ -glutamyl transferase (GGT), lactate dehydrogenase (LDH), total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), non-esterified fatty acids (NEFA), insulin, and glucose were purchased from Vitro Scient Co., Germany. TNF- α and TGF- β primers were purchased from Alex Biotechnology Co., Egypt. Paraffin oil, CCl₄, agarose, ethidium bromide, chloroform, and isopropanol were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). QuantiFast SYBR Green PCR Master Mix kit was supplied by Qiagen GmbH, Hilden, Germany. All of the other reagents were of analytical grade, high-performance liquid chromatography (HPLC) grade, or the best available pharmaceutical grade.

Table 1. Chemical composition of camel milk and bee honey.

Chemical composition	Camel milk	Bee honey
Moisture (%)	87.07 $\pm$ 1.54	15.83 $\pm$ 0.58
Total solid (%)	12.93 $\pm$ 1.50	84.17 $\pm$ 1.13
Fat (%)	4.48 $\pm$ 0.81	0.20 $\pm$ 0.10
Protein (%)	3.94 $\pm$ 0.77	0.50 $\pm$ 0.10
Sugar (%)	3.98 $\pm$ 0.61	83.09 $\pm$ 0.54
Ash (%)	0.75 $\pm$ 0.12	0.37 $\pm$ 0.008
Energy (kcal/L)	759 $\pm$ 4.51	1407.11 $\pm$ 10.94
Mg (mg/100 mL)	14 $\pm$ 0.70	23.38 $\pm$ 9.21
Zn (mg/100 mL)	0.19 $\pm$ 0.018	1.08 $\pm$ 0.84
Mn (mg/100 mL)	0.06 $\pm$ 0.003	0.33 $\pm$ 0.15
Cu (mg/100 mL)	0.19 $\pm$ 0.006	0.19 $\pm$ 0.12
Vitamin C (mg/100 mL)	4.6 $\pm$ 0.54	20.68 $\pm$ 2.83
Total phenol (mg/100 g)	67.89 $\pm$ 3.94	72.6 $\pm$ 4.99

Camel milk and bee honey

Camel milk samples were collected daily, early in the morning, by hand milking camels that were maintained on a free range in Matrooh, Egypt. The samples were collected in sterile screw-top bottles and kept in cool boxes until transported to the laboratory. The rats were given the fresh milk (5 mL/kg body mass (BM) by stomach tube; Al-Fartosi et al. 2012) as is, without any further treatment. Pure bee honey was also obtained from Matrooh, Egypt. The rats were given the pure honey (10 mL honey diluted with 5 mL distilled water/kg BM by stomach tube; Hassan and Bayoumi 2010). The administration of camel milk and bee honey was continued for 8 weeks after induction of liver cirrhosis.

Sample authentication

The camel milk and bee honey were authenticated at the Department of Nutrition, Faculty of Veterinary Medicine, University of Elsadat City, Egypt (Table 1).

Animals and treatments

A total of 36 albino rats (aged 7 weeks and weighting 130–150 g) were obtained from the College of Science at Tanta University and were acclimated for 10 days before the experiment was started. All of the animals were housed in standard cages (9 rats/cage) and were provided a standard laboratory diet and tap water ad libitum. The experimental animals were housed in air-conditioned rooms at 22 °C and 50%–55% relative humidity and were maintained on a light cycle of 12 h light – 12 h dark. All of the animals received humane care according to the guidelines outlined by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA 2003) in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA).

Induction of hepatic cirrhosis by CCl₄

Liver cirrhosis was induced by intraperitoneal (i.p.) injection of CCl₄ (1 mL/kg BM), diluted 1:1 with paraffin oil, two times per week for six successive weeks (Khan and Alzohairy 2011).

Experimental groups and protocol

The rats were randomly divided into four groups comprising nine rats each, and they were all fed the same diet throughout the experimental period. The experimental design is described as follows.

- Group I: Rats are fed only a basal diet and tap water and are injected i.p. with paraffin oil throughout the entire experimental period (6 + 8 weeks); this group served as the control group.
- Group II: Rats are fed a basal diet and tap water and intoxicated with CCl₄ (1 mL/kg BM), diluted 1:1 with paraffin oil, two times

per week for six successive weeks and complete the experimental period (8 weeks) by injection of paraffin oil only.

- Group III: Rats are fed a basal diet and intoxicated with CCl_4 (1 mL/kg BM), diluted 1:1 with paraffin oil, two times per week for six successive weeks and then treated with camel milk (5 mL/kg BM) by a gastric tube along with injection of paraffin oil for 8 weeks.
- Group IV: Rats are fed a basal diet and intoxicated with CCl_4 (1 mL/kg BM), diluted 1:1 with paraffin oil, two times per week for six successive weeks and then treated with camel milk (5 mL/kg BM) and bee honey (10 mL honey diluted with 5 mL distilled water diluted/kg BM) through an gastric tube along with an injection of paraffin oil for 8 weeks.

Blood and tissue collection

At the end of the experiment, the overnight-fasted animals (control and experimental animals) were sacrificed under light ether anesthesia. Blood samples were collected by cardiac puncture before an abdominal incision was made; 3 mL blood samples were collected in plain tubes, and the serum was collected and frozen at -20°C until the time of analysis. Rat liver tissue specimens for biochemical analysis were rinsed with ice-cold saline and then immediately stored at -80°C . Tissue samples were weighed for each rat and then homogenized in 50 mmol/L phosphate-buffered saline

(PBS), pH 7.0. The samples were then centrifuged at 2300g at 4°C for 15 min, and the supernatants were utilized for subsequent analyses.

Biochemical analysis

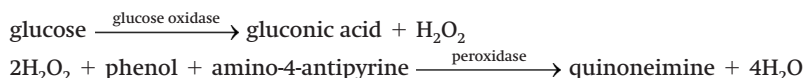
Using commercially available diagnostic kits (Vitro Scient Co., Germany), the activities of the serum AST, ALT, ALP, LDH, and GGT, as well as the serum protein, albumin, NEFA, glucose, and total antioxidant capacity (TAC), were spectrophotometrically determined following the manufacturer's instructions.

Oral glucose tolerance test (OGTT)

Blood samples were obtained from the lateral tail vein of overnight fasted rats (10–12 h). Successive blood samples were then taken at 30, 60, 90, and 120 min following the administration of a glucose solution (3 g/kg BM) orally by gastric intubation. Blood samples were centrifuged and serum was obtained for determination of glucose concentration according to the method of [Trinder \(1969\)](#).

Determination of serum glucose concentration

Serum glucose concentration was determined according to the method of [Trinder \(1969\)](#). Glucose present in the sample is determined according to the following reaction:



Quinoneimine has a faint pink colour and measured photometrically at 505 nm. The density of the colour produced depends on the amount of glucose in the sample and compared with that obtained from a standard glucose solution treated similarly.

Determination of liver glycogen content

Liver glycogen content was determined according to the method of [Seifter et al. \(1950\)](#). To extract glycogen, first, a definite mass of fresh liver tissue was boiled in a known volume of 30% KOH solution. Then glycogen was precipitated from the extract by 95% ethyl alcohol. After separation by centrifugation, the glycogen precipitate was treated with 95% sulfuric acid containing 0.2% anthrone to give a green colour that was compared colourimetrically with that produced with a standard glucose concentration at 620 nm. Glycogen concentration was calculated by application of the following equation:

$$\begin{aligned} \text{Concentration} &= \frac{\text{OD of unknown}}{\text{OD of standard}} \\ &\times \text{concentration of standard} \times \frac{\text{volume of extract}}{\text{g of tissue}} \\ &\times 100 \times 0.9 \text{ (mg/100 g fresh tissue)} \end{aligned}$$

where OD represents optical density and 0.9 was used as the factor for converting the glucose value to a glycogen value.

Estimation of glycogen phosphorylase activity

Glycogen phosphorylase (EC 2.4.1.1) activity in liver homogenate was determined according to the method of [Stalmans and Hers \(1975\)](#) using reagents prepared in the laboratory. The method is based on the incubation of specific substrate with the enzyme found in liver homogenate and determination of the liberated orthophosphate.

Determination of insulin concentration

Serum insulin was assayed by radioimmunoassay kits (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, California, USA) according to the method of [Marschner et al. \(1974\)](#). In the Coat-A-Count insulin procedure, ^{125}I -labelled insulin competes with insulin in the sample for sites on insulin-specific antibodies immobilized to the wall of a polypropylene tube. After incubation, separation of the antibody-bound fraction is achieved simply by decanting the supernatant. The tubes are then counted in a gamma counter; the counts are inversely related to the amount of insulin present in the sample. The quantity of insulin in the sample was then determined by comparing the counts to a standard curve.

Determination of insulin resistance

Insulin resistance was evaluated by homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated by using the following formula: $\text{HOMA-IR} = [\text{fasting plasma insulin (mU/L)} \times \text{fasting plasma glucose (mmol/L)}] / 22.5$ ([Matthews et al. 1985](#)).

Determination of NEFA

Non-esterified fatty acid (NEFA) was analyzed according to [Duncombe \(1964\)](#). The concentration of fatty acids (C8 and longer) is determined by a coupled enzyme assay, which results in a colorimetric (570 nm) – fluorometric ($\lambda_{\text{ex}} = 535\text{nm}$ / $\lambda_{\text{em}} = 587\text{nm}$) product, proportional to the fatty acids present. The values obtained from the appropriate palmitic acid standards are used to plot a standard curve. The concentration of fatty acids (C) present in the samples may be determined from the standard curve:

$$C = \text{Sa/Sv}$$

where Sa is the amount (nmoles) of fatty acids in an unknown sample from the standard curve and Sv is the sample volume (mL) added to reaction well.

Indices of antioxidant pathways

Aliquots of the supernatant were utilized to spectrophotometrically assess the levels of the following parameters: lipid peroxidation (LPO) was assessed as the production of the thiobarbituric acid reactive substances (TBARS) in the presence of BHT (Buege and Aust 1978); reduced glutathione was assessed by using Ellman's reagent (Sedlak and Lindsay 1968); glutathione peroxidase (GPx) levels were chemically determined using cumene hydroperoxide as a substrate (Chiu et al. 1976); superoxide dismutase (SOD) activity was assessed as the liver fraction was reacted with an adrenaline solution; the rate of inhibition of adenochrome formation from the auto-oxidation of adrenaline was spectrophotometrically measured at 480 nm (Misra and Fridovich 1972); and catalase (CAT; EC 1.11.1.6) activity was assessed as the rate of disintegration of hydrogen peroxide (Aebi 1974). The protein content was spectrophotometrically estimated using Folin reagent and bovine serum albumin as the standard.

Total RNA isolation and RT-PCR analysis of hepatic TNF- α and TGF- β

Frozen liver samples (approximately 1 g per sample) were immediately added to lysis buffer (Qiagen GmbH, Germany) and homogenized using a homogenizer (Tissue Ruptor, Qiagen GmbH, Germany). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a Qiagen One-Step RT-PCR kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. The purity of RNA at a 260/280 OD ratio and the RNA integrity were evaluated using a multi-mode microplate reader (Synergy Mx, BioTek, Winooski, Vermont, USA). Only high-purity samples (OD 260/280 > 1.8) were subjected to further manipulation. The master mix was prepared according to the manufacturer's instruction. The whole volume of the reaction was 25 μ L for each gene of interest, including 5 μ L of 5 $\times$ buffer, 5 μ L of Q-buffer, 1 μ L of dTNPs, 1 μ L of forward primer, 1 μ L of the reverse primer, 1 μ L of enzyme mix (reverse transcriptase and Taq polymerase), 6 μ L of nuclease-free water, and 5 μ L of RNA. The reaction was performed in a thermal cycler (MyCycler, Bio-Rad, Germany). The RT-PCR conditions were as follows: (i) reverse transcription, 30 min, 50 $^{\circ}$ C; (ii) initial PCR activation step, 15 min, 95 $^{\circ}$ C; and (iii) three-step cycling for 40 cycles, with each cycle consisting of denaturation for 30 s at 94 $^{\circ}$ C, followed by annealing for 30 s at 55 $^{\circ}$ C and extension for 1 min at 72 $^{\circ}$ C. The template concentration and cycle number were optimized to ensure the linearity of the response and to avoid the saturation of the reaction (40 cycles was better). The PCR products were then resolved on 1.5% agarose gels. The bands were identified based on the product size using a 5000 bp DNA ladder; they were documented using a gel documentation system (Gel DocTMXR System, Bio-Rad), and the prints were scanned. The scanned images were densitometrically quantified with the aid of an NIH image program (<http://rsb.info.nih.gov/ni-image/>). The results were normalized to the levels obtained for the β -actin gene by taking a ratio of the value obtained for the gene of interest to that of β -actin and then relative to the control. We used the β -actin gene as a house-keeping gene. The primers used are listed in Table 2.

Histopathological examinations

Liver tissues were cut into small pieces and immersed in neutral buffered formalin for 24 h. The fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinized, and rehydrated using the standard techniques (Bancroft and Gamble 2002). The extent of CCl₄-induced cirrhosis was evaluated by assessing the morphological changes in the liver sections stained with hematoxylin and eosin (H&E), using standard techniques.

Statistical analysis

The values were recorded as the mean $\pm$ standard error of the mean. Significant differences between the means were deter-

Table 2. Nucleotide sequences of the primers used in RT-PCR.

Primer	Forward	Reverse
TNF- α	CCACGTCGTAGCAAACCAC	TGGGTGAGGAGCACGTAGT
TGF- β	CATCAGCAACAACATAAGCGTCA	CTCCTTTCCGCTTCCTGA
β -actin	GGACCTGACAGACTACC	GGCATAGAGGTCTTTACGG

mined by ANOVA, followed by Duncan's post hoc test. $P < 0.05$ was accepted as statistically significant.

Results

Biochemical findings

Effects of treatments on liver-specific enzymes

The activities of AST, ALT, GGT, LDH, and ALP were estimated in serum samples as biomarkers of liver function. These results are provided in Table 3. The CCl₄ treatment markedly affected the liver-specific enzyme activities. A significant ($P < 0.05$) increase in serum AST (127.6 $\pm$ 9.2 U/L), ALT (166.2 $\pm$ 11.8 U/L), GGT (68.1 $\pm$ 3.41 U/L), LDH (1073.41 U/L), and ALP (291.2 $\pm$ 13.7 U/L) activities was observed in the CCl₄-treated rats compared with those of the control rats (66.5 $\pm$ 6.5 U/L, 81.6 $\pm$ 8.3 U/L, 31.6 $\pm$ 2.19 U/L, 649.5 $\pm$ 28.7 U/L, and 146.8 $\pm$ 11.2 U/L, respectively). These results suggest that these hepatic biomarkers were elevated in the serum due to a release of enzymes from the damaged liver. However, a significant decrease ($P < 0.05$) was observed in the respective serum activities of the rats that were treated with camel milk and bee honey in combination (89.4 $\pm$ 4.3 U/L, 129.9 $\pm$ 5.6 U/L, 32.4 $\pm$ 2.36 U/L, 759.7 $\pm$ 19.8 U/L, and 181.6 $\pm$ 13 U/L, respectively) compared with those of the CCl₄-treated rats, but values did not return to normal control group levels.

Effects of the treatments on the serum protein pattern

The serum protein profiles are presented in Table 4. Significant decreases ($P < 0.05$) in total serum proteins (4.1 $\pm$ 0.03 g/dL), albumin (2.7 $\pm$ 0.1 g/dL), and globulin (1.4 $\pm$ 0.01 g/dL) were observed in the CCl₄-treated rats compared with the control rats (6.8 $\pm$ 0.3 g/dL, 3.9 $\pm$ 0.3 g/dL, and 2.9 $\pm$ 0.04 g/dL, respectively). However, the total protein values in the rats intoxicated with CCl₄ and treated with camel milk and bee honey in combination were significantly increased ($P < 0.05$; 5.1 $\pm$ 0.02 g/dL) compared with the untreated CCl₄-intoxicated rats. On the other hand, significant changes in the albumin (3.1 $\pm$ 0.2 g/dL) and globulin (2.0 $\pm$ 0.02 g/dL) were absent ($P < 0.05$) in rats that were intoxicated with CCl₄ and treated with camel milk and bee honey in combination compared with those treated with CCl₄ alone and significantly decreased compared with control group.

Effect of treatments on hepatic lipid peroxidation

Table 4 shows that CCl₄-induced oxidative stress in the rat livers was reflected as significantly increased ($P < 0.05$) hepatic MDA levels (269.68 $\pm$ 12.18 nmol/g) compared with the control rats (91.98 $\pm$ 3.57 nmol/g). In contrast, the administration of camel milk either alone or in combination with bee honey significantly decreased ($P < 0.05$) the hepatic MDA levels (171.62 $\pm$ 9.11 nmol/g and 139.71 $\pm$ 8.70 nmol/g, respectively) compared with the group of CCl₄ alone and significantly increased compared with control group.

Effect of treatments on TAC, GSH, and hepatic antioxidant enzyme activities

TAC levels, GSH concentrations, and antioxidant enzyme activities (GPx, CAT, and SOD) are shown in Table 5. TAC levels, GSH concentrations, GPx, CAT, and SOD activities were significantly decreased ($P < 0.05$) in the CCl₄-intoxicated rats (0.78 $\pm$ 0.01 mmol/L, 17.16 $\pm$ 1.21 μ mol/g, 19.31 $\pm$ 2.11 IU/g, 38.27 $\pm$ 2.57 U/mg protein, and 7.16 $\pm$ 0.81 U/mg protein) compared with the control rats (2.79 $\pm$

Table 3. Effects of carbon tetrachloride (CCl₄), camel milk (CM), and bee honey (BH) on serum enzyme activities of liver in rats.

Group	ALT (IU/L)	AST (IU/L)	GGT (IU/L)	LDH (IU/L)	ALP (IU/L)
Control	81.6±8.3c	66.5±6.5d	31.6±2.19c	649.5±28.7c	146.8±11.2d
CCl ₄	166.2±11.8a	127.6±9.2a	68.1±3.41a	1073.41±39.6a	291.2±13.7a
CCl ₄ +CM	135.9±7.9b	108.8±6.7b	47.6±2.68b	771.7±21.3b	229.1±9.4b
CCl ₄ +CM+BH	129.9±5.6b	89.4±4.3c	32.4±2.36c	759.7±19.8b	181.6±13.6c

Note: Means within the same column followed by different letters are significantly different ($P < 0.05$).

Table 4. Effects of carbon tetrachloride (CCl₄), camel milk (CM), and bee honey (BH) on protein patterns and GSH in rats.

Group	Total protein (g/dL)	Albumin (A) (g/dL)	Globulin (G) (g/dL)	Ratio of A to G	MDA (nmol/g)
Control	6.8±0.3a	3.9±0.3a	2.9±0.04a	1.34±0.002	91.98±3.57d
CCl ₄	4.1±0.03c	2.7±0.1bc	1.4±0.01c	1.92±0.003	269.68±12.18a
CCl ₄ +CM	5.3±0.1b	3.0±0.3b	2.3±0.02ab	1.30±0.001	171.62±9.11b
CCl ₄ +CM+BH	5.1±0.02b	3.1±0.2b	2.0±0.02bc	1.55±0.001	139.71±8.70c

Note: Means within the same column followed by different letters are significantly different ($P < 0.05$).

Table 5. Effects of carbon tetrachloride (CCl₄), camel milk (CM), and bee honey (BH) on oxidant-antioxidant status in rats.

Group	GSH (μmol/g)	GPx (IU/g)	CAT (U/mg protein)	SOD (U/mg protein)	TAC (mmol/L)
Control	69.27±2.32a	39.79±1.36a	61.61±3.71b	14.31±0.78a	2.79±0.13a
CCl ₄	17.16±1.21d	19.31±2.11c	38.27±2.57c	7.16±0.81b	0.78±0.01c
CCl ₄ +CM	41.56±2.52c	27.84±1.35b	62.11±3.13b	12.99±0.63a	1.64±0.23b
CCl ₄ +CM+BH	54.26±1.30b	37.96±2.73a	78.67±2.88a	13.12±0.83a	2.68±0.19a

Note: Means within the same column followed by different letters are significantly different ($P < 0.05$).

Table 6. Effects of carbon tetrachloride (CCl₄), camel milk (CM), and bee honey (BH) on blood glucose during an oral glucose tolerance test (OGTT) in rats.

Group	Time of blood sample				
	0 min	30 min	60 min	90 min	120 min
Control	5.82±0.78c	7.87±0.90c	8.90±1.78c	7.15±0.44c	6.69±0.21c
CCl ₄	14.37±2.68a	16.74±2.87a	18.97±3.67a	18.67±2.89a	17.89±3.45a
CCl ₄ +CM	9.35±1.57b	13.21±1.76b	11.23±2.31b	12.56±1.23b	10.47±2.37b
CCl ₄ +CM+BH	8.94±2.78b	10.56±1.68b	10.16±1.88bc	11.87±2.23b	7.81±2.29bc

Note: Means within the same column followed by different letters are significantly different ($P < 0.05$).

Table 7. Effects of carbon tetrachloride (CCl₄), camel milk (CM), and bee honey (BH) on fasting plasma glucose (FPG), fasting plasma insulin (FPI), index of insulin resistance (HOMA-IR), liver glycogen content, glycogen phosphorylase activity, and non-esterified fatty acid (NEFA) in rats.

Group	FPG (mmol/L)	FPI (mU/L)	HOMA-IR	Liver glycogen (mg/g liver)	Glycogen phosphorylase (mg P _i ·h ⁻¹ ·g ⁻¹ liver)	NEFA (mmol/L)
Control	5.82±0.78c	11.23±2.32	2.90±0.47c	17.89±2.34c	32.86±2.88a	0.588±0.89b
CCl ₄	14.37±2.68a	10.92±2.34	6.97±0.89a	33.78±3.45a	19.35±2.23b	1.890±0.64a
CCl ₄ +CM	9.35±1.57b	10.34±1.67	4.29±0.46b	24.96±2.05b	29.26±2.34a	0.591±0.16b
CCl ₄ +CM+BH	8.94±2.78b	10.89±1.97	4.32±0.71b	23.80±1.09b	31.12±3.45a	0.345±0.09c

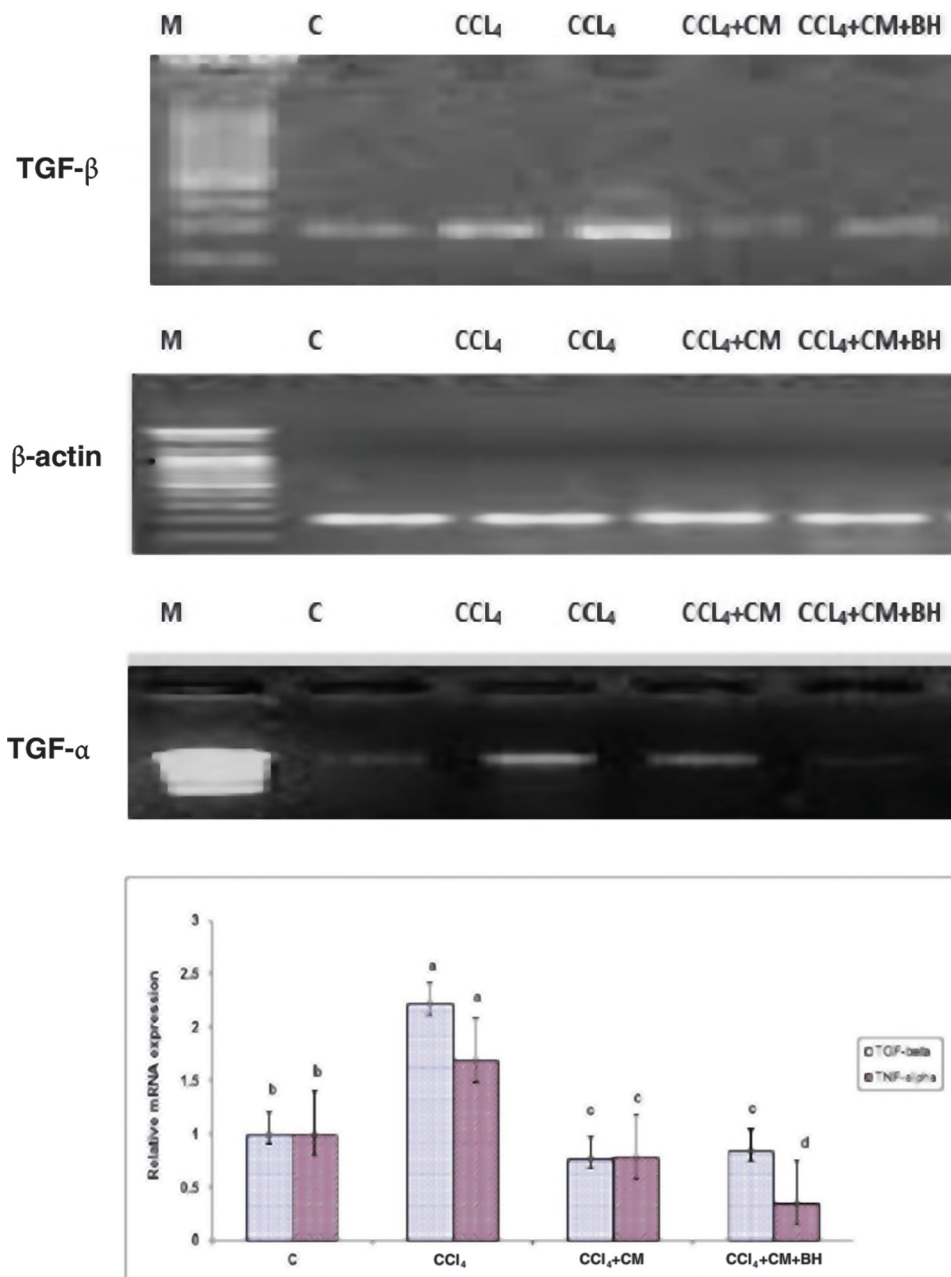
Note: Means within the same column followed by different letters are significantly different ($P < 0.05$).

0.13 mmol/L, 69.27 ± 2.32 μmol/g, 39.79 ± 1.36 IU/g, 61.61 ± 3.71 U/mg protein, and 14.31 ± 0.78 U/mg protein, respectively). In contrast, the administration of camel milk either alone or in combination with bee honey significantly increased ($P < 0.05$) the TAC level (1.64 ± 0.23 mmol/L and 2.68 ± 0.19 mol/L), the GSH concentration (41.56 ± 2.52 μmol/g and 54.26 ± 1.30 μmol/g), the GPx activity (27.84 ± 1.35 IU/g and 37.96 ± 2.73 IU/g), the CAT activity (62.11 ± 3.13 U/mg protein and 78.67 ± 2.88 U/mg protein), and the SOD activity (12.99 ± 0.63 U/mg protein and 13.12 ± 0.83 U/mg protein) in the rat livers compared with those in the CCl₄-intoxicated rats, and most values returned to normal compared with the control group.

Effects of the treatments on the serum glucose level, liver glycogen content, NEFA concentration, liver phosphorylase activity, and HOMA-IR

The data presented in Tables 6 and 7 revealed a significant increase ($P < 0.05$) in fasting blood glucose (14.37 ± 2.68 mmol/L), liver glycogen (33.78 ± 3.45 mg/g liver), NEFA (1.890 ± 0.64 mmol/L), and HOMA-IR (6.97 ± 0.89) and a significant decrease in liver phosphorylase activity (19.35 ± 2.23 mg P_i·h⁻¹·g⁻¹ liver) in the CCl₄-treated rats compared with the control rats (5.82 ± 0.78 mmol/L, 17.89 ± 2.34 mg/g liver, 0.588 ± 0.89 mmol/L, 2.90 ± 0.47, and 32.86 ± 2.88 mg P_i·h⁻¹·g⁻¹ liver, respectively). However, fasting blood glucose, liver glycogen, NEFA, and HOMA-IR values in the

Fig. 1. RT-PCR analysis of TGF- β and TNF- α in liver tissues of control noncirrhotic (C), experimentally cirrhotic (CCl₄), camel milk (CM), and camel milk plus bee honey (CM+BH) treated cirrhotic rats. RT-PCR was performed with Qiagen One-Step. The densitometric analysis of the expressed bands (lower columns) was normalized with that of β -actin and then calculated relative to the control group. Values are expressed as mean $\pm$ SEM for nine rats. Values are significantly different ($P < 0.05$) compared with the control noncirrhotic group. [Colour online.]



rats intoxicated with CCl₄ and treated with camel milk and bee honey in combination were significantly decreased ($P < 0.05$; 8.94 ± 2.78 mmol/L, 23.80 ± 1.09 mg/g liver, 0.345 ± 0.09 mmol/L, and 4.32 ± 0.71), and liver phosphorylase activity was significantly increased ($P < 0.05$; 31.12 ± 3.45 mg $P_i \cdot h^{-1} \cdot g^{-1}$ liver) compared with the untreated CCl₄-intoxicated rats, and most values did not return to normal compared with the control group.

Molecular findings: effects of the treatments on the gene expression of TNF- α and TGF- β

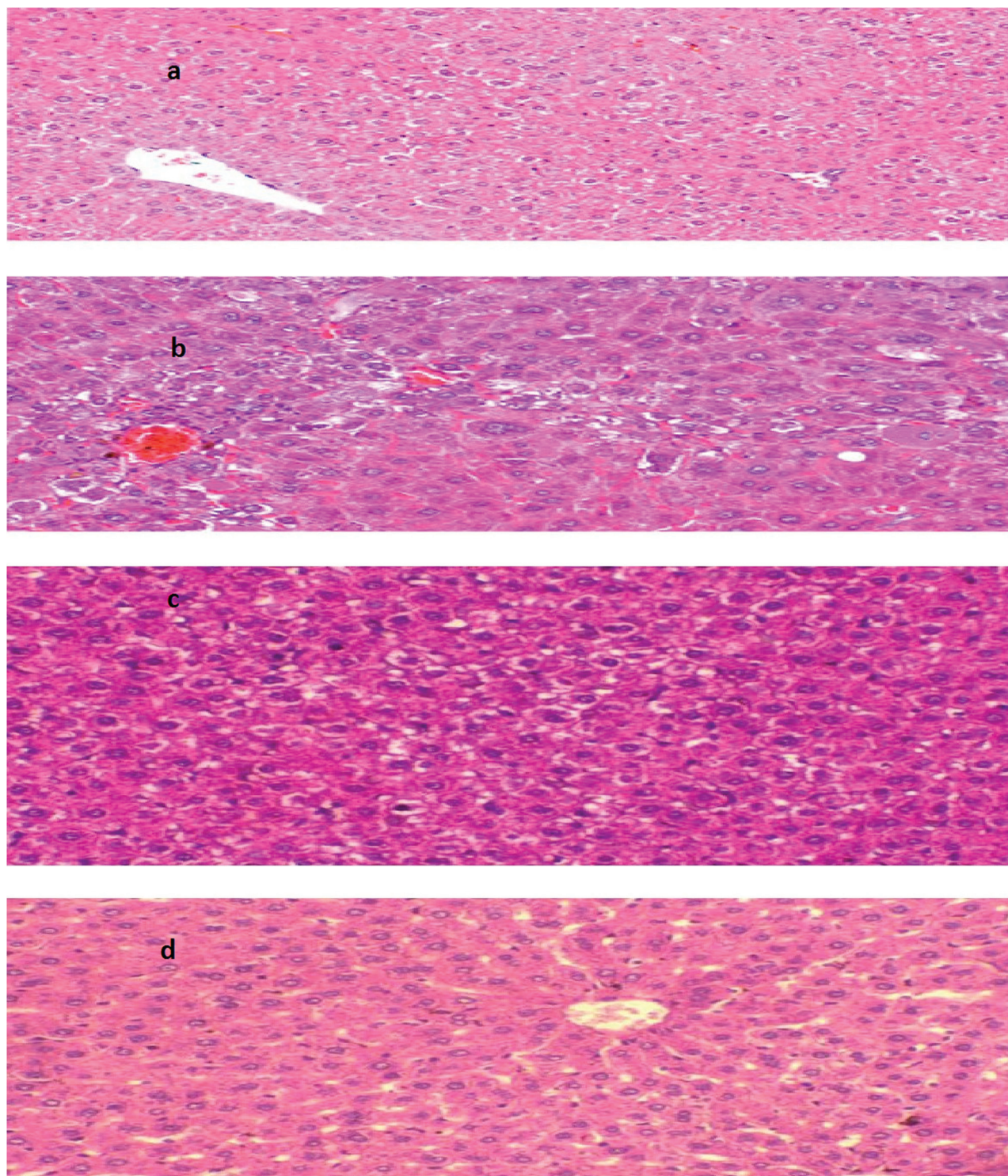
The expressions of TNF- α and TGF- β were significantly increased ($P < 0.05$) in the livers of the CCl₄-treated rats compared with the control rats. However, treatment with camel milk either alone or in combination with bee honey significantly decreased

($P < 0.05$) the expression of these pro-inflammatory cytokines compared with the control group and the untreated CCl₄-intoxicated group (Fig. 1).

Histopathological findings

Liver damage in rats was evaluated by histological examination after staining with H&E; liver tissue from each group was examined. Normal architecture with a central vein is observed in the normal (control) rats (Fig. 2a). Marked fatty acid degeneration, portal inflammation, necrosis, and hepatocyte loosening are observed in rats with CCl₄-induced hepatic cirrhosis (Fig. 2b). In rats treated with camel milk and camel milk plus bee honey, the liver exhibited clear hepatic recovery characterized by a complete re-

Fig. 2. H&E staining for liver tissue of albino rats: (a) normal (control); (b) CCl₄-treated albino rats; (c) camel milk treated albino rats, and (d) camel milk + bee honey treated albino rats. H&E ×400. [Colour online.]



generation of hepatocytes and the hepatic tissue appeared more or less normal in most cases (Figs. 2c and 2d).

Discussion

The present study aimed to clarify the possible beneficial effects of camel milk and bee honey against CCl₄-induced liver cirrhosis using biochemical, molecular, and histopathological assays.

First of all, five separate specific liver enzymes were used to evaluate the liver functions: AST and ALT, which are together known as transaminases, ALP, which is a cholestatic liver enzyme, GGT, and LDH are all reliable indicators of liver function. In the present study, the activities of these enzymes were greatly increased in the rats treated with CCl₄ compared with control rats.

The liver damage induced by CCl₄ was reflected in increased serum ALT, AST, LDH, GGT, and ALP activities (Arıcı and Çetin

2011; Khan and Alzohairy 2011; Pispirigos et al. 1993; Whitfield 2001).

From a biochemical point of view, these disturbances can be considered a direct reflex of marked liver damage caused by the selectively destructive cytotoxic effect of CCl_4 on liver cells (Mehmetcik et al. 2008).

Also, CCl_4 induces lipid peroxidation and subsequently leads to a loss of membrane fluidity, changes in the membrane potential, increases in the membrane permeability, and enzymatic leakage (Nehru and Anand 2005).

However, treatment with camel milk and bee honey was found to suppress the increase in serum enzymatic activities induced by CCl_4 treatment in rats. The present findings indicate that there are protective effects of camel milk on the structural integrity of hepatic cell membranes or on the regeneration of damaged liver cells (Palanivel et al. 2008). The efficacy of any hepatoprotective drug is dependent on its capacity for either reducing the harmful effect of a hepatotoxin or restoring the normal hepatic physiology that has been disturbed by the toxin. The reversal of the increased serum enzyme levels in CCl_4 -induced liver damage by camel milk might be due to the prevention of intracellular enzyme leakage by its antioxidant and membrane-stabilizing activities. This finding is in agreement with the commonly accepted view that serum transaminase levels return to normal as the hepatic parenchyma heals and hepatocytes regenerate (Thabrew et al. 1987).

Albumin is the most abundant protein in human plasma, representing 55%–65% of the total protein. The rate at which albumin is synthesized in the liver depends on the protein intake, which is regulated by the plasma albumin level. Most of the albumin that is filtered through the kidney glomeruli is reabsorbed by proximal tubule cells, in which lysosomal enzymes degrade the albumin into fragments that are then returned to the circulation. The decreased serum albumin in the rats treated with CCl_4 compared with the control rats indicates poor liver function or impaired synthesis due to either primary causes such as liver cell damage or secondary causes such as decreased protein intake, reduced amino acid absorption caused by malabsorption syndrome or malnutrition, or a loss of protein in the urine due to nephritic syndrome or chronic glomerulonephritis (Al-Fartosi et al. 2012). A significant increase in the serum albumin concentration was observed in the rats that received camel milk either alone or with bee honey compared with the CCl_4 -treated group. The increased albumin concentration after the camel milk and bee honey treatment could be attributed either to their nutritional values or to decreased lipid peroxidation and increased plasma protein thiol activities (Al-Fartosi et al. 2012; Al-Hashem et al. 2009).

Early reports indicate that the hepatotoxic effects of CCl_4 depend on lipid peroxidation and are largely due to its active metabolite, CCl_3 (this metabolite can remove hydrogen molecules from fatty acids, which initiates lipid peroxidation), leading to cell injury and eventually liver damage (Park et al. 2005). Lipid peroxidation of biological membranes leads to a loss of membrane fluidity, changes in the membrane potential, increases in the membrane permeability, and alterations in the receptor functions (Nehru and Anand 2005). The results of the present study, which revealed increased hepatic MDA levels, support this point. The elevation in MDA could be due to the loss of balance between prooxidation and antioxidant, which results in energy depletion and accelerated aging in target organs such as the liver, heart, kidney, and brain (Al-Fartosi et al. 2012). The increased lipid peroxidation is due to the inhibition of or a change in the activities of nonenzymatic and enzymatic components of the oxidative system, i.e., GSH, SOD, CAT, and GPx, in the liver.

Glutathione is considered to be the first line of cellular defense against oxidative damage. GSH functions by detoxifying various xenobiotics and scavenging free radicals, and it is consequently converted to its oxidized form, glutathione disulfide (GSSG). GSH, an essential oxidative system component, serves as a cofactor for glu-

tathione transferase, which helps remove certain drugs and chemicals, as well as other reactive molecules, from cells (Wu and Cederbaum 2003). Moreover, GSH can directly interact with and detoxify certain reactive oxygen species (ROS), i.e., hydroxyl radicals, and can also perform other critical cellular activities. Thus, GSH is likely the most important antioxidant that is present in cells (Wu and Cederbaum 2003). In the present study, CCl_4 significantly decreased GSH content, whereas supplementation of camel milk and bee honey significantly increased its content. The amino acid sequences of some camel milk proteins are rich in half-cystine residues, which are important during GSH peptide synthesis (Beg et al. 1986).

To prevent biological macromolecules from oxidative damage, antioxidant enzymes are considered to be the second line of cellular defense. The enzymatic antioxidant defense system, which includes SOD, GPx, and CAT, can decompose superoxide and hydrogen peroxide in cells and represents the main defense against oxidative injuries. SOD catalyzes the rapid removal of superoxide radicals, thereby generating H_2O_2 . Therefore, SOD works in collaboration with H_2O_2 -removing enzymes. In the present study, CCl_4 exposure induced free radical formation; CCl_4 can also inhibit the enzymes that are involved in antioxidant defense, specifically SOD and GPx (Nehru and Anand 2005). The reduction of SOD activity in the liver of the CCl_4 -treated animals could be due to an accumulation of superoxide radical anions. CCl_4 intoxication also decreased the CAT activity, an effect that is potentially explained by the influence of CAT on hydrogen peroxide (a product of SOD) as a substrate (formed in excess in the process of the dismutation of the superoxide radical anion). The significant decrease in GPx activity in the CCl_4 -treated rats may have been a response to the increased oxidative stress due to high SOD activity, which converts O_2 into H_2O_2 . Ho et al. (1997) reported that GPx induction was increased by increased levels of both organic and inorganic peroxides. The oral administration of camel milk and bee honey with CCl_4 significantly increased and thereby normalized the activity levels of enzymatic and nonenzymatic antioxidant system components (SOD, GPx, CAT, and GSH) in the liver and TAC levels in the serum of treated rats.

The protective effect of camel milk could be attributed to its antioxidant activity. It has been reported that camel milk contains high levels of vitamins A, B₂, C, and E, and it is very rich in magnesium (Mg), manganese, zinc (Zn), copper, and other trace elements (Knoess 1979). These vitamins are antioxidants that are useful in preventing tissue injury caused by toxic agents (Yousef 2004). In fact, Mg deficiency has been associated with ROS production (Martin et al. 2003). Additionally, Mg protects cells against oxyradical damage and assists in the absorption and metabolism of vitamins B, C, and E (Barbagallo et al. 1999), which play a large role in protecting cells from free radicals via their antioxidant functions. Previous evidence suggests that vitamin E enhances glutathione levels and could play a protective role in cardiac lesions induced by Mg deficiency (Barbagallo et al. 1999). Additionally, it has been reported that Mg is essential for glutathione biosynthesis because the enzyme glutathione synthetase requires γ -glutamyl cysteine, glycine, ATP, and Mg ions to form glutathione (Minnich et al. 1971). Additionally, camel milk is rich in Zn (Knoess 1979), which is a trace element that is essential for living organisms. More than 300 enzymes require Zn for their activities. Zn also plays an important role in DNA replication, transcription, and protein synthesis, thereby influencing cell division and differentiation (Frederickson 1989). Notably, Zn has a relationship with many enzymes in the body and can prevent cell damage through antioxidant system activation (Ozdemir and Inanc 2005). Zinc is an essential component of the oxidant defense system and functions at many levels (Sato and Bremner 1993). Furthermore, Zn deficiency increases lipid peroxidation in various rat tissues, whereas Zn supplementation corrects this increase (Ozdemir and Inanc 2005).

Honey is composed of minerals such as magnesium, potassium, calcium, sodium, chlorine, sulfur, copper, iodine, zinc, iron, and phosphate. It also contains vitamins B₁, B₂, C, B₆, B₅, and B₃ (Gheldof et al. 2002), some of which have antioxidant properties, as previously discussed.

Liver cirrhosis induced by CCl₄ had a deleterious effect on metabolism reflected in significant increases in blood glucose level, liver glycogen content, NEFA level, and insulin resistance along with significant decreases in liver phosphorylase activity and glucose intolerance. The liver is a key organ that regulates many important metabolic, detoxification, and secretory functions in the body (Núñez and Soriano 2005). Hence, hepatic injury induced by chemicals such as CCl₄ is associated with disruptions of these metabolic functions (Gupta 2004). According to the literature, 50%–80% of cirrhotic patients and even up to 96% have impaired glucose tolerance (García-Compeán et al. 2012). Similarly, glucose intolerance characterized by postprandial hyperglycemia, and hyperinsulinemia is commonly seen in patients with liver cirrhosis (Taguchi et al. 2014). The same authors revealed that liver parenchymal damage and insulin resistance in both liver and peripheral tissues are the early etiology of metabolic abnormality in the patients with liver cirrhosis. Fasting patients with cirrhosis have high plasma nonesterified fatty acids, indicating increased lipolysis (Kaye et al. 1994). The same authors attributed this increase in NEFA to insulin resistance, which is frequently associated with liver cirrhosis.

On the other hand, camel milk and bee honey ameliorate and mitigate these adverse effects. Camel milk contains “insulin-like” small-molecule substances that not only mimic insulin interaction with its receptor but also have the regulatory and immunomodulatory functions of β cells (Malik et al. 2012), and it does not form coagulum in acidic environment, thus, safeguarding the viability of its components and making it available for absorption in the intestines and subsequently improved overall metabolism. The mechanism(s) by which CCl₄ and camel milk affected the liver phosphorylase enzyme is unclear and needs further studies at the molecular level. We speculate that the decrease of inorganic phosphate associated with liver diseases might be one of the real causes of decreased liver phosphorylase activity and subsequently increased liver glycogen content.

The transcription of TGF- β and TNF- α were upregulated by CCl₄. TNF- α is a major endogenous mediator of hepatotoxicity in several experimental liver injury models (Gantner et al. 1995). TGF- β is primarily secreted by T cells and macrophages (Gattoni et al. 2006). Moreover, TGF- β is a Th1-type pro-inflammatory cytokine that is actively involved in almost all phases of immune and inflammatory responses, including macrophage activation, antibacterial immunity, antigen presentation, innate immune system activation, lymphocyte–endothelial interactions, Th1/Th2 balance, and cellular proliferation and apoptosis (Gazzinelli et al. 1992). TGF- β enhances certain macrophage functions such as microbicidal and tumoricidal activities and reactive oxygen and reactive nitrogen intermediate production (Ohmori and Hamilton 1994).

The increased ROS production consequently increases oxidative stress, which promotes the increase of other inflammatory factors (Guo et al. 2009).

Camel milk combined with bee honey downregulate the transcription of these pro-inflammatory cytokines, which might be due to the antioxidant properties of the camel milk and bee honey, which decrease ROS and subsequently decrease inflammatory cytokines (El-Said et al. 2010; Gheldof et al. 2002; Guo et al. 2009).

The absence of significant differences between the group taking camel milk alone and the group taking camel milk in combination with bee honey at the level of the most studied parameters indicates obviously that the most of beneficial effect was attributed mainly to camel milk, and to a large extent, there is no additional or synergistic effect of bee honey.

The biochemical findings were also confirmed by histological observations. The changes mostly include hepatocellular necrosis or apoptosis, fatty acid accumulation, inflammatory cells infiltration, and other histological manifestations, which were also consistent with the findings of other authors (Khan and Alzohairy 2011).

Conclusions

In conclusion, camel milk had protective effects against CCl₄-induced liver damage and improved the biochemical parameters related to liver damage. The mechanism that underlies this protective effect might involve the transcriptional regulation of profibrotic cytokines, which are involved in inducing hepatic cirrhosis, and the improvement of the oxidative status, which is involved in the pathogenesis of hepatic fibrosis. The most of beneficial effect was attributed mainly to camel milk, and there is no additional or synergistic effect of bee honey.

Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

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