Hypocholesterolemic effect of camel milk on rats fed a high-cholesterol diet

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A B S T R A C T

The effects of fermented skim milk versus unfermented skim milk of camel on the levels of cholesterol in blood were investigated in rats. Levels of serum cholesterol and LDL-C/HDL-C ratio were decreased significantly in Wistar rats that fed with a cholesterol-enriched diet and administered fermented skim camel milk compared with rats administered unfermented milk (P<0.05). Furthermore, histopathological evaluation showed that liver tissue degeneration, apoptosis/necrosis, inflammation, and fatty changes (steatosis and fibrosis) decreased significantly at (P<0.05) in the rats that fed with fermented skim camel milk compared to the rats which fed unfermented skim camel milk. Based on these results it can suggest that fermented skim camel milk might reduce the risk of hypercholesterolemia development in rats. The hypocholesterolemic and hepatoprotective effects of fermented skim camel milk were evident.

Keywords: Hypocholesterolemic, Skim camel milk, Cholesterol, Hepatotoxicity.

INTRODUCTION

Coronary heart disease (CHD) and atherosclerosis is considered as the main causes of death in the western countries. Total cholesterol level and atherosclerosis are strongly associated (Glass and Witztum, 2001). Diet is one of the main preventative ways to manage CHD and atherosclerosis and may regulate the levels of cholesterol and triglyceride in blood through consumption of low-fat dairy products and drug treatments (El-Gawad et al., 2005; Pereira and Gibson, 2002). Nowadays, food proteins are consumed for health benefits when ingested (Roberta et al., 1999). Milk is important to prevent diseases and promote health (Meisel, 2005). Camel and bovine milk are important sources of essential nutrients and are consumed fresh or fermented in Saudi Arabia (Salmen et al., 2012). *Lactobacillus helveticus* ATCC 15009 strain requires all amino acids except four; cysteine, alanine, serine and glycine. Thus, *L. helveticus* considered as one of high proteolytic activity strain. This strain has a very effective proteolytic system, which contains each of endopeptidase-proteinase, X-prolyl-dipeptidyl aminopeptidase, and general aminopeptidas; in addition to other enzymes including proline iminopeptidase, dipeptidase, carboxypeptidase and tripeptidase (Alhaj and Kanekanian, 2014). The mechanisms of cholesterol removal by microorganisms are not clear yet. Nevertheless, the results of *in vitro* studies have suggested numerous proposed mechanisms. These including bile salts deconjugation, transformation of cholesterol to coprostanol by microorganisms, binding of cholesterol to bacterial cell walls, and assimilation of cholesterol by microorganisms (Kumar et al., 2011). Milk can be fermented with one or more bacterial strains such as *Lactobacillus helveticus* or in combination with *Streptococcus thermophilus* strains (Yamamoto et al., 1999; Sipola et al., 2002; Donkor et al., 2007). Fermented milk produced with two or more bacterial strains contains more functional substances compared to milk fermented with one strain (Gobbetti et al., 2000). Milk proteins contain bioactive peptides, which are considered health-promoting substances (Murray and Fitzgerad, 2007). A number of studies have documented that milk proteins, particularly whey proteins hydrolyzates or peptides, exert hypocholesterolaemic effects in different animal models. The aim of this study was to investigate the hypocholesterolemic effect of fermented and unfermented skim camel milk in rats.

MATERIALS AND METHODS

Materials

All strains were in freeze-dried form, *Lactobacillus helveticus* (LMG11445) strain purchased from Belgian Coordinated Collections of Microorganisms (BCCM-LMG). While, *Streptococcus thermophilus* (ATCC 19258) purchased form the American Type Culture Collection (ATCC). Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO, USA). The cholesterol kit, triglyceride kit, and HDL-C kit were purchased from UDI Company (Dammam, KSA). Lactase enzyme (*Aspergillus oryzae* 9000 FCC lactase units) was purchased from Webber Naturals Co. Canada.

Fermented and unfermented skim camel milk preparation

Camel milk samples were collected from a private farm in Riyadh - Saudi Arabia. The camel milk contains 2.1% fat, 2.4% proteins and 4.2% lactose. Fat was removed by centrifugal separation at 45°C. The lactose content in camel milk was 4.2%, however, the rate of lactase hydrolysis was 83% and the remaining lactose content in camel milk after adding lactose enzyme was 0.7%. Skimmed milk was subjected to pasteurization process at 85°C for 30 minutes. Later, skimmed, lactose-hydrolyzed camel milk separated into two parts: one without fermentation and another part subjected to fermentation by inoculation with 3% of activate (*L. helveticus* and *S. thermophilus*) and then incubated at 40°C until it reached a pH of 4.3. Then, samples were frozen at -80°C, freeze-dried, and refrigerated until experimental use.

Animal grouping, feeding, and blood sampling

Twenty Wistar male rats aged 11-12 weeks, weighing 220 ± 10 g were a gift from the Experimental Animal Care Center, Pharmacy faculty, King Saud University (KSU), Saudi Arabia. Rats were kept individually in stainless steel cages in controlled environmental conditions of 25°C, 12 h light/dark cycle, under relative humidity of 50% ± 5. During the experiment, rats were left over to have free access to water. Animals were maintained in accordance with the instruction of Health Guide for the Care and Use of Laboratory Animals of National Institute, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996). The experimental protocol of this study was approved by the Ethics Committee of the Experimental Animal Care Center, faculty of Pharmacy, King Saud University; Riyadh, Saudi Arabia (108-EACC-2015). Rats were separated in to four groups (five rats each) after one week of adaptation period: 1/negative control (NC) group which was only fed with a basal diet; 2/positive control (PC) group which was fed the basal diet in addition to 1% cholesterol; 3/UFCM group which was given a basal diet, 1% cholesterol, and 4% unfermented skim camel milk; 4/FCM group taken a basal diet, 1% cholesterol, and 4% fermented skim camel milk.

The basal diet for rat maintenance was prepared according to the American Institute of Nutrition guidelines (AIN). Basal diet is similar to AIN-93M Purified Diet (standard diet) and contains the following formula (g/kg): casein 140, L-Cystine 1.8, Corn starch 465.6, Maltodextrin 155, Sucrose 100, Soybean oil 40, Cellulose 50, Mineral Mix, AIN-93MX 35, Vitamin Mix, AIN-93-VX 10, Choline Bitartrate 2.5 and TBHQ, Antioxidant 0.008.

After 6 weeks of feeding, rats kept fastened for 12 h, and the blood was taken from the heart via cardiac puncture under mild anesthesia. Blood samples were collected in plane tubes without anticoagulant, and then centrifuged at 4°C for 10 min at 1200×*g* to obtain blood serum. The serum samples were kept at -80°C immediately until the date of analysis. Rat's livers tissues were extracted and immediately placed in 10% formalin solution and used for histopathological evaluation.

Monitoring rat body weight and liver weight

Rat body weight was measured weekly during the experiment, and by the end of experiment the weight of liver was measured by a special balance (Adam HCB 3001, UK).

Determination of serum lipid concentration

The serum levels of total cholesterol, triglyceride, and HDL-cholesterol samples were determined using the enzymatic colorimetric method of diagnostic kits (UDI: Cholesterol enzymatic reagent kit, No. Ref 024. K.S.A, UDI: HDL-Cholesterol Reagent Set Kit, No. Ref 041. K.S.A, UDI: Triglycerides (GPO) reagent set kit, No. Ref 059L. K.S.A). The VLDL-C, LDL-C, and atherogenic index were calculated as follows: VLDL-C = Triglycerides (TG)/5; LDL-C (mg/100 mL) = TC − (HDL-C+VLDL-C); Atherogenic index = (VLDL-C + LDL-C)/HDL-C.

Histopathological analysis

The animals were sacrificed and livers quickly removed under ethyl ether anesthesia and then placed in 10% neutral buffered formalin for 72 hrs. The fixed specimens of liver tissue were dehydrated overnight, cleaned then impregnated by an automatic tissue processor (Sakura, Japan). Tissue samples were fixed in paraffin blocks by using embedding station (Sakura, Japan) and slices of 4 micron thickness were cut with rotary microtome (Leica-RM2245, Germany) and Hematoxylin & Eosin staining was carried out by an Autostainer (Leica5020, Germany). The stained slices then were tested under light microscopy Eclipse BOI (Nikon, Japan) and required pictures were taken by digital microscopic camera (OMX1200C) (Nikon, Japan). Tissues were graded according to the following criteria: 1) degeneration, 2) apoptosis/necrosis, 3) inflammation, 4) fatty changes (steatosis) and 5) fibrosis. The observed changes were reported according to their intensity: mild, moderate or severe hepatotoxicity.

Statistical analysis

Differences between obtained values (means and standard deviation (SD)) were calculated, the results were analyzed by ANOVA one-way test, and the Duncan's Multiple test was sued to determine the significant differences at p≤0,05 by SPSS V,21 software package (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Body weight

As seen in Table 1, there were no significant differences (P>0.05) observed in body weights between experimental groups (NC, PC, UFCM, and FCM). Body weights of rats increased uniformly throughout the experimental period in all groups. These results agree with the results from a study by Xiao et al. (2003), who found no significant (P>0.05) difference in body weight in all groups fed sour milk and SL (*S. thermophilus* + *Lactobacillus delbrueckii*) milk. Similarly, no significant (P>0.05) difference in body weight gain were reported between hamsters take a high-cholesterol diet and fermented milk (Chiu et al., 2006).

Liver weight

The results in Table 1 show a significant (P<0.05) increase in final liver weight for all treatments compared with the NC group. Liver weights of FCM group rats had decreased significantly (P<0.05) in comparison with PC group rats. These results are similar to those reported by Lin et al. (2004) who observed increased relative liver weight of hamsters fed a high-cholesterol diet. However, the results of this study differed with those which reported by Chiu et al. (2006) who observed no significant difference (P>0.05) in liver weights of hamsters fed a high-cholesterol diet supplemented with sterilized milk and milk fermented by *Lactobacillus*. In the current study, the increased liver weight in rats fed a high-cholesterol (PC, UFCM) could be due to the accumulation of cholesterol and triglycerides in their livers during the treatment period. The lower increase in liver weights of rats fed skim milk of camel fermented by *L. helveticus* strain and *S. thermophilus* compared to PC group rats could be due to the effect of bacteria that facilitate cholesterol excretion in feces, thus reducing liver cholesterol accumulation (Nguyen et al., 2007).

Total cholesterol and triglycerides

The serum total cholesterol concentration of rats that fed with different diets (NC, PC, UFCM, and FCM) shown in Fig. 1. PC rats group exposed increasing significantly (P<0.05) in serum TC when compared with levels in NC group rats. FCM and UFCM group rats exhibited significantly (P<0.05) reduced serum cholesterol value in compare with PC group rats; however, these effects were not significantly different from those observed in NC group rats. These results are agree with findings from Hassanein

et al. (2013) who found that the TC levels in the plasma of rats fed yoghurt inoculated with *L. lactis* K F 147 was lower than the TC level of control group rats. Similarly, Guo and Li (2013) showed that rats fed high doses of *L. casei* F0822 had significantly (P<0.05) reduced serum TC concentration when compared with levels in control group of rats that fed a high-cholesterol diet. Schaarmann et al. (2001) found that the consumption of the probiotic yoghurt prepared by using each of *B. longum* and *L. acidophilus* strains reduced the concentration of TC and TG in women after 153 days. This effect is due to hydrolysis, which is necessary for the release of hypocholesterolemic peptides from the sequence of camel milk caseins. The changes in blood triglyceride content of rats fed FCM and UFCM shown in Fig. 2. The results demonstrated a significant (P<0.05) increase in TG levels in the PC group compared with levels in the NC group. FCM significantly (P<0.05) decreased serum TG levels in comparing with levels in the PC group. However, UFCM had no effects on TG levels in rats fed a PC diet. These results are in accordance with those of Xiao et al. (2003) who noticed that the TG levels in serum of the rats fed *bifidobacterium* milk were lower than levels in the control group and in rats fed SL milk. The current data confirmed that consumption of fermented skim camel milk in rats fed a cholesterol-enriched diet have cholesterol and triglycerides lowering effects. The results showed

Table 1: The effect of camel milk in body weight and liver weight of rats fed high cholesterol diet

Group	Body weight gain (g)	liver weight/body weight (g/100g)
NC	316.3±55.9[a]*	2.57±0.19[c]
PC	312.5±53.7[a]	4.03±0.56[a]
UFCM	312.5±55.1[a]	3.80±0.72[ab]
FCM	313.6±51.8[a]	3.33±0.35[b]

* Mean±SD (n=5). Means in column with different letters differ significantly (P<0.05). NC: negative control, PC: high cholesterol diet, UFCM: unfermented skim milk, FCM: fermented skim milk.
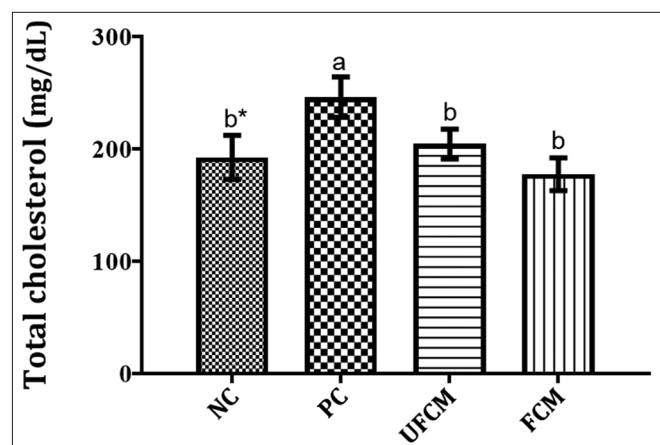

FIg 1. The effect of camel milk on total cholesterol levels in serum of rats. NC: negative control, PC: high cholesterol diet, UFCM: unfermented skim camel milk, FCM: fermented skim camel milk, * Mean ± SD (n=5). Bars with different letters differ significantly (P<0.05).

Table 2: Microscopic scoring of histopathological sections of liver tissue of rats

Microscopic description	Negative control group (NC)	High cholesterol diet group (PC)	Unfermented skim camel milk group (UFCM)	Fermented skim camel milk group (FCM)
Degeneration	0	+++	++	+
Apoptosis/necrosis	0	+++	++	+
Inflammation	0	++++	+++	++
Fatty changes (steatosis)	0	+++	++	+
Fibrosis	0	+++	++	+
Total score	0	+++	++	+

SCORING: NO HEPATOTOXICITY (0), MILD HEPATOTOXICITY (+), MODERATE HEPATOTOXICITY (++), SEVERE HEPATOTOXICITY (+++).

that animals in the FCM group were lower in serum total cholesterol and triglyceride concentration than animals in UFCM and PC groups. Skim camel milk fermented with *L. helveticus* and *S. thermophiles* may decrease cholesterol and triglyceride levels by many proposed mechanisms: 1) inhibition of liver cholesterol synthesis and distribution of cholesterol from the blood circulation to the liver, 2) intestinal bacteria may inhibit the absorption of cholesterol, and 3) milk fermented by lactic acid strains may inhibit cholesterol synthesis enzymes and decrease cholesterol level (Kimoto-Nira et al., 2007; Nguyen et al., 2007; Zhang, et al., 2008). Moreover, bioactive peptides derived from camel milk proteins may reduce cholesterol through the interaction between bioactive peptides and cholesterol (Li and Papadopoulos, 1998), and the presence of orotic acid in camel milk, which is thought to be responsible for lowering cholesterol level in rats (Rao et al., 1981; Ali et al., 2013).

Low- and very low-density lipoprotein- cholesterol
Animals in the PC group had significantly (P<0.05) higher VLDL-C levels than animals in the NC group. The VLDL-C level significantly decreased (P<0.05) in the FCM group compared with those in the PC group. Compared with NC animals, the FCM and UFCM group animals showed no significant differences in the levels of VLDL-C as in Fig. 3. LDL-C significantly (P<0.05) increased in the PC group in comparison with other groups (Fig. 4). FCM and UFCM groups exhibited significantly decreased (P<0.05) serum LDL-C level in rats fed these milks compared to rats fed PC; however, FCM was more effective than UFCM for reducing LDL-C. These results agree with results from Chiu et al. (2006) who observed that the fermented milk of *Lactobacillus* was very effective in reducing LDL-C in blood serum of hamsters. This is due to the presence of peptides generated during the fermentation process, peptide β-Lactotensin, obtained from chymotrypsin β-lactoglobulin hydrolysate, decreased total cholesterol, LDL, and VLDL cholesterol content in mice fed with a cholesterol enriched diet (Yamauchi et al., 2003). Furthermore, Ali et al. (2013) reported that rats fed fermented camel milk had significantly lower plasma VLDL-C and LDL-C levels compared to rats fed cow milk+Bb-12. Similarly, milk fermented with *Lactobacillus* and fed was also very active in reducing LDL-C in hamster serum [Chiu
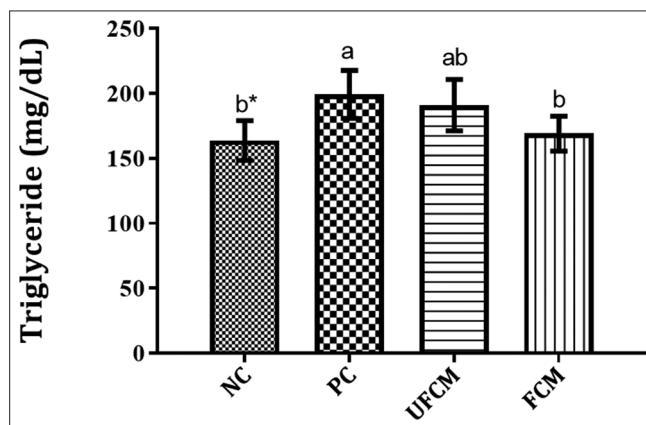

Fig 2. The effect of camel milk on triglyceride levels in serum of rats. NC: negative control, PC: high cholesterol diet, UFCM: unfermented skim camel milk, FCM: fermented skim camel milk, *Mean ± SD (n=5).
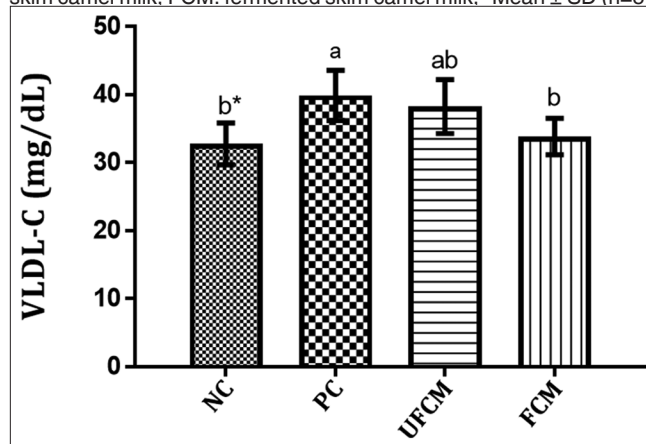

Fig 3. The effect of camel milk on VLDL-cholesterol levels in serum of rats. NC: negative control, PC: high cholesterol diet, UFCM: unfermented skim camel milk, FCM: fermented skim camel milk, *Mean ± SD (n=5). Bars with different letters differ significantly (P<0.05).

et al., 2006]. SHR rats fed milk fermented by *L. lactis* NRRL B-50571 and *L. lactis* NRRL B-50572 decreased LDL-C by 8.4 mg/dL and 21.9 mg/dL, respectively, compared to LDL-C of SHR rats given water (Rodriguez-Figueroa et al., 2013).

High-density lipoprotein (HDL-C)
Fig. 5 illustrate HDL-C concentrations in serum of rats fed different diets (NC, PC, UFCM, and FCM). The serum HDL-C concentration in rats fed fermented skim camel milk

(FCM) was higher significantly (P<0.05) than HDL-C levels in rats from PC and UFCM groups, and there was no significant difference between FCM and NC groups. These results are agreed with the results of Al-Sheraji et al. (2012) who found that rats fed yogurt cultured with *B. pseudocatenulatum* G4 and *B. longum* BB536 had elevated HDL-C levels. However, Guo and Li (2013) reported no effect on HDL-C rates of rats fed a high or low dose of *L. casei* F0822. In the present work results, HDL concentration was increased in serum of rats fed fermented skim camel milk in comparison with HDL concentration in serum of rats fed unfermented skim camel milk. The increase of HDL-C concentration and the decrease of LDL-C upon consumption of fermented skim camel milk may support the hypothesis that some lactic acid bacteria promote the excretion of cholesterol with bile acid.

Histopathology

The liver sections in rats from the negative control group (NC) showed liver tissue with preserved lobular architecture (Fig. 6 A and B). The portal tracts were not expanded by fibrosis (integrity sector 0), showed intact anatomical structures (hepatic artery branch, portal vein radical and interlobular bile duct). There was no evidence of portal or parenchymal inflammation (0), fatty changes (0), degeneration (0), nor apoptosis or necrosis (0). The parenchymatous fibrous interface was intact. The hepatic parenchyma was formed of hepatocyte cords arranged in one to two layers, separated by blood sinusoids and radiating towards normal appearing central veins. Finally, the rats in the negative control group exhibited total intensity levels of zero (0).

In the rats group that fed with high cholesterol diet (PC), the histopathological changes showed disturbed lobular architecture. The portal tracts were expanded by fibrosis with portoportal and portocentral fibrous septa (+++). There was remarkable portal and parenchymal inflammation (+++). Fatty changes (micro/macro-vesicular steatosis) were identified in about (80%) of the liver parenchyma (++++). There was evidence of parenchymal inflammation (cloudy swelling) (+++) and multifocal foci of apoptosis and necrosis in the perivenular areas (around the central veins) (++). Finally, the histopathological diagnosis revealed severe hepatotoxicity in high-cholesterol diet fed rats (+++). The present results are agreed with a previous report (Aleisa et al., 2013). Biomarkers in serum further confirmed the histopathological evaluation (Fig. 7 A and B).

In the rats fed unfermented skim camel milk (UFCM), liver sections showed disturbed lobular architecture in the form of portal expansion by fibrosis with fine incomplete septa (++). In addition, there was moderate portal inflammation (++). Fatty changes (micro/macrovesicular steatosis) was identified in about (65%) of the liver parenchyma (+++). There was evidence of parenchymal inflammation (cloudy swelling)
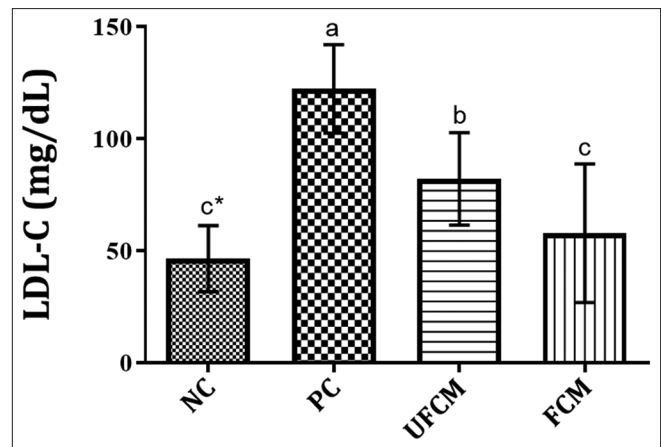

Fig 4. The influence of fermented skim milk on the LDL-cholesterol levels in serum of rats. NC: negative control, PC: high cholesterol diet, UFCM: unfermented skim camel milk, FCM: fermented skim camel milk. *Mean ± SD (n=5). Bars with different letters differ significantly (P<0.05).
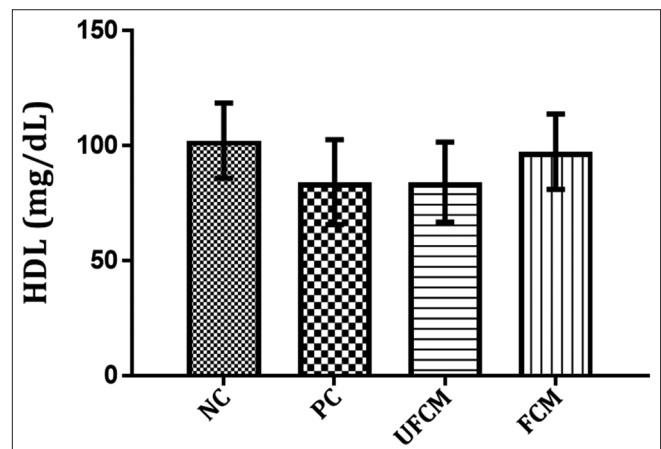

Fig 5. The effect of fermented skim camel milk on the HDL-cholesterol concentration in serum of rats. NC: negative control, PC: high cholesterol diet, UFCM: unfermented skim camel milk, FCM: fermented skim camel milk, * Mean ± SD (n=5). Bars with different letters mean that it differ significantly (P<0.05).
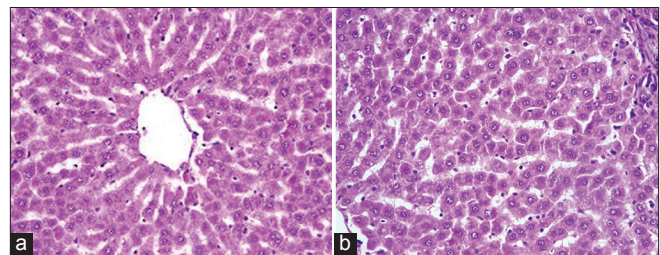

Fig 6. The hepatotoxicity in rats liver tissue of NC group. (a)A-Normal rat liver tissue showing liver parenchyma and normal central vein (X400).(b) B- Normal rat liver tissue showing parenchymal hepatocytes arranged in cords (X400).

(++) and multiple foci of apoptosis and necrosis in the perivenular areas (++). Finally, the histopathological diagnosis revealed moderate hepatotoxicity in rats fed unfermented skim camel milk along with a high cholesterol diet, as the total intensity levels were moderate (++) (Fig. 8 A and B).

The sections of liver tissue from rats fed fermented skim camel milk (FCM group) exhibited disturbed lobular architecture in the form of mild portal fibrosis (+). There was mild portal inflammation (+). Fatty changes (micro/macro-vesicular steatosis) were identified in about (45%) of liver parenchyma (++). There was evidence of parenchymal inflammation and a few foci of apoptosis (+). No necrosis was seen in the perivenular areas (+). The histopathological diagnosis revealed mild hepatotoxicity in rats fed fermented skim camel milk supplemented along with high cholesterol diet. The total intensity levels were mild (+), as shown in Fig. 9 (A, B).

CONCLUSION

Feeding fermented skim camel milk to Wistar rats fed high cholesterol diet resulted in a significant diminution in serum TC, TG, VLDL-C, LDL-C, HDL-C and Atherogenic
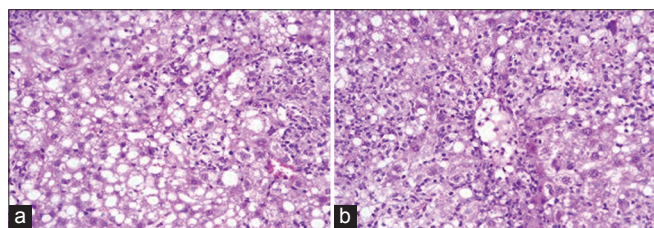

Fig 7. The hepatotoxicity in liver tissue of rats fed high cholesterol diet (PC group). (a)A-Rat liver tissue showing micro/ macrovesicular steatosis (about 80%) and parenchymal inflammation (X400).(b)B-Rat liver tissue showing inflammation and necrosis in the perivenular area around the central vein (X400).
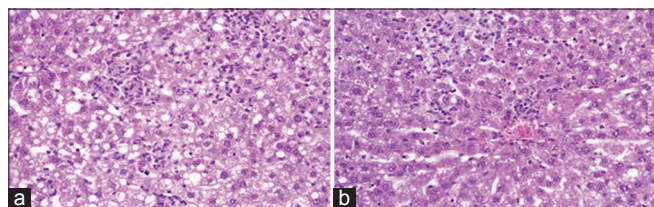

Fig 8. (A, B). The effect of unfermented skim camel milk on hepatotoxicity in liver tissue of rats fed high cholesterol diet (UFCM group). (a)A-Rat liver tissue showing micro/macrovesicular steatosis (about 65%) and evidence of parenchymal inflammation (X400).(b)B- Rat liver tissue showing multifocal areas of apoptosis and necrosis around the central vein and in the perivenular area (X400).
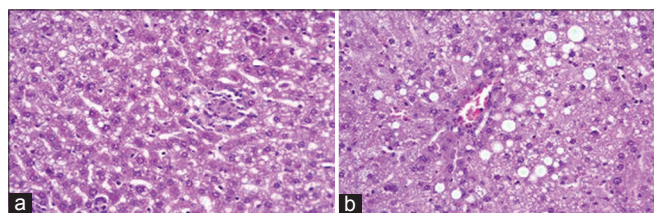

Fig 9. (A, B). The effect of fermented skim camel milk on hepatotoxicity in liver tissue of rats fed high cholesterol diet (FCM group). (a)A-Rat liver showing micro/macro-vesicular steatosis (about 45%) and mild parenchymal inflammation (X400).(b)B- Rat liver tissue showing parenchymal micro/ macrovesicular steatosis, without changes in the perivenular areas (X400).

index. Additionally, fermented skim camel milk reduced liver toxicity upon long-term administration.

Author's contributions

This work was carried out in collaboration between all authors. M. A. Yahya carried out the experiments, O.A. Alhaj and A. S. AL-khlifah Supervised, planning the study, wrote the protocol and did the statistical analyses. M. A. Yahya and A. T. Almnaizel carried out biochemical analyzes and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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294

Emir. J. Food Agric ● Vol 30 ● Issue 4 ● 2018