

CAMEL MILK MODULATES LIPID METABOLISM, EXPRESSION OF ENZYMATIC ANTIOXIDANTS GENES AND PARAOXONASE ACTIVITY IN RATS FED HIGH CHOLESTEROL DIET

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Abstract: Current findings aimed to find out camel milk's effects on lipid metabolism, expression of the genes for antioxidant enzymes and paraoxonase-1 (PON-1) activity in rats fed diet-containing cholesterol 1%. Therefore, 30 rats were divided into three groups (10 rats per each). Rats of the first group, which acted as a control group, fed a basic diet. Rats of the second group fed a basal diet that included 1% cholesterol however, rats of the third group fed cholesterol 1% accompanied by oral administration of camel's milk (100mL/24h/cage/5 rats) as the only source of water for them. Diet of cholesterol 1% induced significant increase in serum total cholesterol, triacylglycerol (TAG), low-density lipoprotein cholesterol (LDL-c), alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities whereas lower serum PON-1 activity was observed when compared to control. Diet of cholesterol 1% induced significant increase in hepatic Thiobarbituric acid reactive substance (TBARS) however, compared to the control, a significant decrease in the activity and gene expression of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione-S transferase (GST), and reduced glutathione (GSH) were detected. These detrimental effects were ameliorated into accepted range in camel milk treated group. Fatty degeneration and fatty cysts in liver tissues were detected in rats fed cholesterol diet but the affected liver showed acceptable degree of recovery in camel milk treated group. Conclusively, camel milk was potential for the treatment of hyperlipidemia and hypercholesterolemia in rats.

Key words: camel milk; biochemistry; paraoxonase; antioxidants; lipids

Introduction

Hyperlipidemia and hypercholesterolemia are the main risk factors for cardiovascular illnesses (1). The most well-known barriers to employing synthetic medications are their high cost and known negative effects (2). The best options are natural products because they have few side effects and are reasonably priced. An essential source of protein in tropical and subtropical regions is camel milk (3). Low carbohydrate, low cholesterol and higher level of vitamin C minerals are the key distinctions between camel milk

and milk from other ruminants (4, 5). The most frequent applications for camel's milk are anti-autoimmune (6, 7), antimicrobial (8), antitoxic (9-14), antioxidant (14) and antidiabetic (15, 16). Camel milk has been claimed to be useful in the treatment of cardiovascular disorders because it contains high concentrations of essential fatty acids, which are known to have a hypolipidemic impact (17). Additionally, camel milk's hypocholesterolemic impact has been attributed to its high ascorbic acid concentration, which is important for bile acids production from cholesterol (18). There are conflicting reports on the hypolipidemic and hypocholesterolemic effects of camel milk, though. Although some studies (15, 19, 20) demonstrated the insignificant influence

of camel milk on the plasma lipid profiles, other studies (21) showed that it can lower cholesterol and triglycerides. An antioxidant enzyme called paraoxonase guards lipoproteins against oxidative damage (22). The main prognosis if lipoprotein oxidation has taken place is arteriosclerosis (23). Hypercholesterolemia has been associated with low serum paraoxonase activity (24). The literatures regarding the control of higher level of TG and lower HDL-c level by camel milk still requires additional research. Furthermore, there is a lack of evidence reported the influence of camel milk on paraoxonase activity. The oxidative stress of high cholesterol diet and its protection by camel milk still not fully studied. The objective of the current work was to find out the influence of camel milk on lipid metabolism, expression of the genes for antioxidant enzymes and paraoxonase-1 (PON-1) activity in rats fed diet containing cholesterol 1%.

Materials and methods

Reagents of the experiment

The commercial kits for the detection of blood total proteins, albumin, total lipid, triglyceride, total cholesterol, HDL-c, LDL-c, and VLDL-c were given by ELIPSE, United Diagnostic Industry, UDI, Dammam, Saudi Arabia. Sigma-Aldrich, USA, sold pure cholesterol (Cat# C3045). The common reagents employed in the current investigation were of the highest degree and were readily accessible from retailers. According to the instructions, an ELISA kit (USCN, Life Science Inc; Cat #. E90243Ra) was used to measure the activity of paraoxonase. The following items were purchased from Sigma Chemical Co.: EGTA, EDTA, butanol, EDTA, and H₂O₂ (St. Louis, MO, USA). The remaining compounds were all of an analytical grade.

Camel's milk samples

Daily samples of camel milk were taken at morning from the camel farm at the King Faisal University in Al-Ahsa, Saudi Arabia. Manual labor was used to obtain camel milk. Prior to being delivered to the lab, sterile screw-top vials were used to collect the samples, which were then kept in cool boxes for storage. Without any additional

care, fresh milk was offered to the rats (100 ml/24 hours/cage) (25).

Animals and treatment

Prior to the experiment, 30 albino rats (230±20 g) were adapted for 10 days in the laboratory of the King Faisal University's College of Veterinary Medicine in Al-Ahsa, Saudi Arabia. All animals were kept in typical cages (5 rats per cage), given a regular laboratory meal, and given access to unlimited amounts of tap water. The experimental animals were kept on a 12-hour light/12-hour dark cycle in air-conditioned rooms that ranged in temperature from 21 to 23°C and relative humidity levels between 60 and 65°C. The King Faisal University in Saudi Arabia's ethics of scientific research committee published the instructions for use and care of Laboratory Animals, and the animals were handled humanely in compliance with its guidelines (permission # AN000456; GRANT1486).

Experimental hypercholesterolemia

A total of 1g of cholesterol powder should be added to each 99 grams of basal diet (1%), with the exception of the control, in order to generate hypercholesterolemia (26, 27, 28).

Experimental groups and protocol

After receiving a standard diet, rats were randomized into one of three groups, with ten rats in each. Group 1: Rats used as a control were fed a basic meal without any extra ingredients. Rats receiving a 1% cholesterol diet (1g/99g of a basal diet; Sharma et al., 1984) (27) comprise the second group. Group 3 includes rats who are fed a high-cholesterol diet and receive their only water from camel's milk (100 mL/24 hours/cage/5 rats (14, 25).

Samples collection

After two weeks of treatment, blood samples were taken to verify the development of hypercholesterolemia. After overnight fasting, all experimental animals were slaughtered at the end of the experiment while being lightly sedated with diethyl ether. Before making an abdominal incision, blood samples were taken by cardiac

puncture. The collected serum stored frozen at 30°C to be analyzed for liver function enzymes and paraoxonase activities by commercial kits in accordance with manufacturer's instructions. For a molecular and biochemical investigation of antioxidant enzymes, tissues of liver were excised, and liver pieces were quickly frozen by liquid nitrogen and preserved at -80°C. For a histological investigation, a portion of liver tissues was cut into small pieces and placed in neutral buffered formalin for 24 hours.

Analysis of biochemical parameters

Commercial diagnostic kits (United Diagnostic Industry, UDI, Dammam, Saudi (Rome, Italy) were used to determine serum total proteins, albumin, glucose, ALT, AST, CK, BUN, uric acid, creatinine, total cholesterol, TAG, HDL-c. The production instructions were followed while calculating the concentration of the biological ingredients. LDL-c and VLDL-c were calculated as described earlier (29). Paraoxonase activity was estimated by using commercial ELISA enzyme immunoassay (Cat #. E90243Ra).

Detection of antioxidant enzymes activities, TBARS and GSH concentrations in liver tissue

The process of homogenization of liver tissues and estimation of TBARS concentrations and antioxidant enzymes activities were discussed in our previous published work (14). The commercial ELISA kits (Cayman Chemical Company, USA) were used for detection CAT, GPX, SOD, GST and concentrations of GSH and TBARS by using The ELISA reader (BioTek®, USA).

Analysis of RNA expression for enzymatic antioxidants

The processes started from homogenization of liver tissues until RNA expression of antioxidants enzymes are illustrated previously (14). Table 1 showed primer sequences.

Histopathology of liver tissues

After fixation, liver tissues underwent regular processing, including paraffin embedding, sectioning, deparaffinizing, and rehydrating (30). H & E stains were used to examine the impact of diet containing cholesterol 1% and camel milk.

Statistical analysis

Using one way analysis of variance, the data were showed as mean standard error of mean (ANOVA). The statistical analysis system's computer was used to conduct each test (31).

Results

Determination of biochemical parameters and paraoxonase activity

TAG, total cholesterol and LDL-c were increased in rat fed diet containing cholesterol 1% compared to control (Table 2). Administration of oral camel milk to rats fed a diet containing cholesterol 1% significantly lowered the values of serum cholesterol compared to rats fed a diet containing cholesterol 1% alone but still higher than that of the control. Insignificant changes were observed in all groups' biochemical measurements of remaining biochemical parameters (Table 2).

Table 1: The sequences of primers used in the current study (14)

Gene	Forward primer sequence	Reverse primer sequence
β-actin	5/-AGC CAT GTA CGT AGC CAT CC-3/	5/- CTC TCA GCT GTG GTG GTG AA-3/
SOD	5/- AGG ATT AAC TGA AGG CGA GCA T-3/	5/- TCT ACA GTT AGC AGG CCA GCA G-3/
CAT	5/-ACG AGA TGG CAC ACT TTG ACA G -3/	5/-TGG GTT TCT CTT CTG GCT ATG G-3/
GPx	5/-AAG GTG CTG CTC ATT GAG AAT G-3/	5/-CGT CTG GAC CTA CCA GGA ACT T-3/
GST	5/- GCT GGA GTG GAG TTT GAA GAA-3/	5/- GTC CTG ACC ACG TCA ACA TAG-3/

SOD: Superoxide dismutase; CAT: Catalase; GPX: Glutathione peroxidase; GST: Glutathione-S transferase

Table 2: Protein and lipid profile and glucose concentration in rats fed diet-containing cholesterol 1% (group 2) and treated with camel milk (group 3) compared to the control (group 1).

Parameters	Group 1	Group 2	Group 3
Glucose (mg/dl)	130.2 ± 2.01	135.1 ± 4.02	131.0 ± 4.02
Total proteins (g/dl)	5.0 ± 0.34	5.1 ± 0.40	5.2 ± 0.51
Albumin (g/dl)	4.3 ± 0.29	4.4 ± 0.49	4.3 ± 0.19
Globulins (g/dl)	1.2 ± 0.09	1.1 ± 0.19	1.1 ± 0.19
Triglycerides (mg/dl)	35.9 ± 2.60	60.1 ± 2.40*	40.0 ± 2.22*
Total cholesterol (mg/dl)	25.2 ± 0.09	45.0 ± 0.19*	31.0 ± 0.21*
HDL-c (mg/dl)	12.4 ± 0.77	11.87 ± 0.90	12.0 ± 0.91
LDL-c (mg/dl)	5.4 ± 0.99	24.0 ± 0.99*	11.1 ± 0.89*
VLDL-c (mg/dl)	7.1 ± 0.9	9.1 ± 0.90	6.4 ± 0.90

Every number corresponds to the mean ± standard deviation of 10 rats. HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol; VLDL-c: Very low density lipoprotein cholesterol. *When compared to the control, mean values are significantly (P<0.05) different.

Table 3: Liver and kidney function Biomarkers in rats fed diet-containing cholesterol 1% (group 2) and treated with camel milk (group 3) compared to the control (group 1).

Parameters	Group 1	Group 2	Group 3
ALT (IU/L)	22.14 ± 0.39	40.11 ± 0.59*	30.0 ± 0.68**
AST (IU/L)	80.13 ± 0.70	110.11 ± 0.91*	90.1 ± 0.81**
CK (IU/L)	509.98 ± 5.12	493.20 ± 8.21	500.30 ± 7.11
BUN (mg/dl)	7.99 ± 0.29	6.98 ± 0.95	7.97 ± 0.96
Creatinine (mg/dl)	0.19 ± 0.94	0.18 ± 0.89	0.17 ± 0.76
Uric acid (mg/dl)	1.00 ± 0.09	1.10 ± 0.08	1.10 ± 0.09

Every number corresponds to the mean ± standard deviation of 10 rats. ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; ACP: Acid phosphatase; CK: Creatine kinase; BUN: Blood urea nitrogen* When compared to the control, mean values are significantly (P<0.05) different. ** When compared to the cholesterol treated rat (group 2), mean values are significantly (P<0.05) different.

ALT and AST activities were increased significantly in rat a diet containing cholesterol 1% compared to control groups (Table 3). However, administration of camel milk to rats fed a diet containing cholesterol 1% corrected the serum levels of both ALT and AST activities towards the normal control values (Table 3). Activities of CK and the values of BUN, uric acid, and creatinine remained unaltered (Table 3).

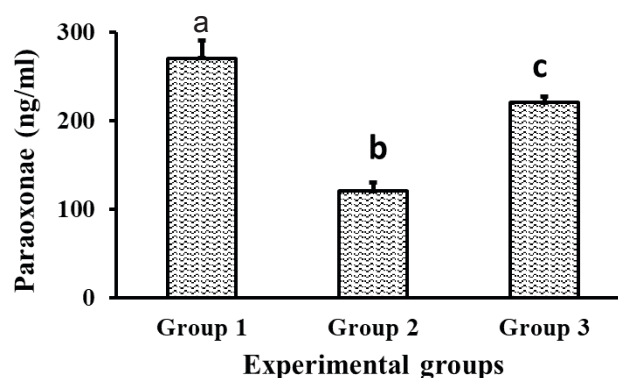
Rats fed diets containing 1% cholesterol had lower paraoxonase activity than the controls (Figure 1). When these rats were given camel milk, the paraoxonase activity was almost fully recovered.

Analysis of hepatic lipid peroxidation

Rats fed diet containing 1% cholesterol induced a significant (P<0.05) higher levels of hepatic TBARS than the control group (Fig. 2A). After administration of camel milk, liver tissue TBARS levels significantly (P<0.05) reduced; nevertheless, this decline did not reach the normal control value (Fig.2A).

Determination of GSH concentration and activities enzymatic antioxidants in liver tissues

Significant (P<0.05) reduction of GSH concentration was observed in rats fed diets containing

**Figure 1:** Paraoxonase activities of rats fed diet-containing cholesterol 1% (group 2) and treated with camel milk (group 3; 100ml/24h/cage) compared to the control (group 1).

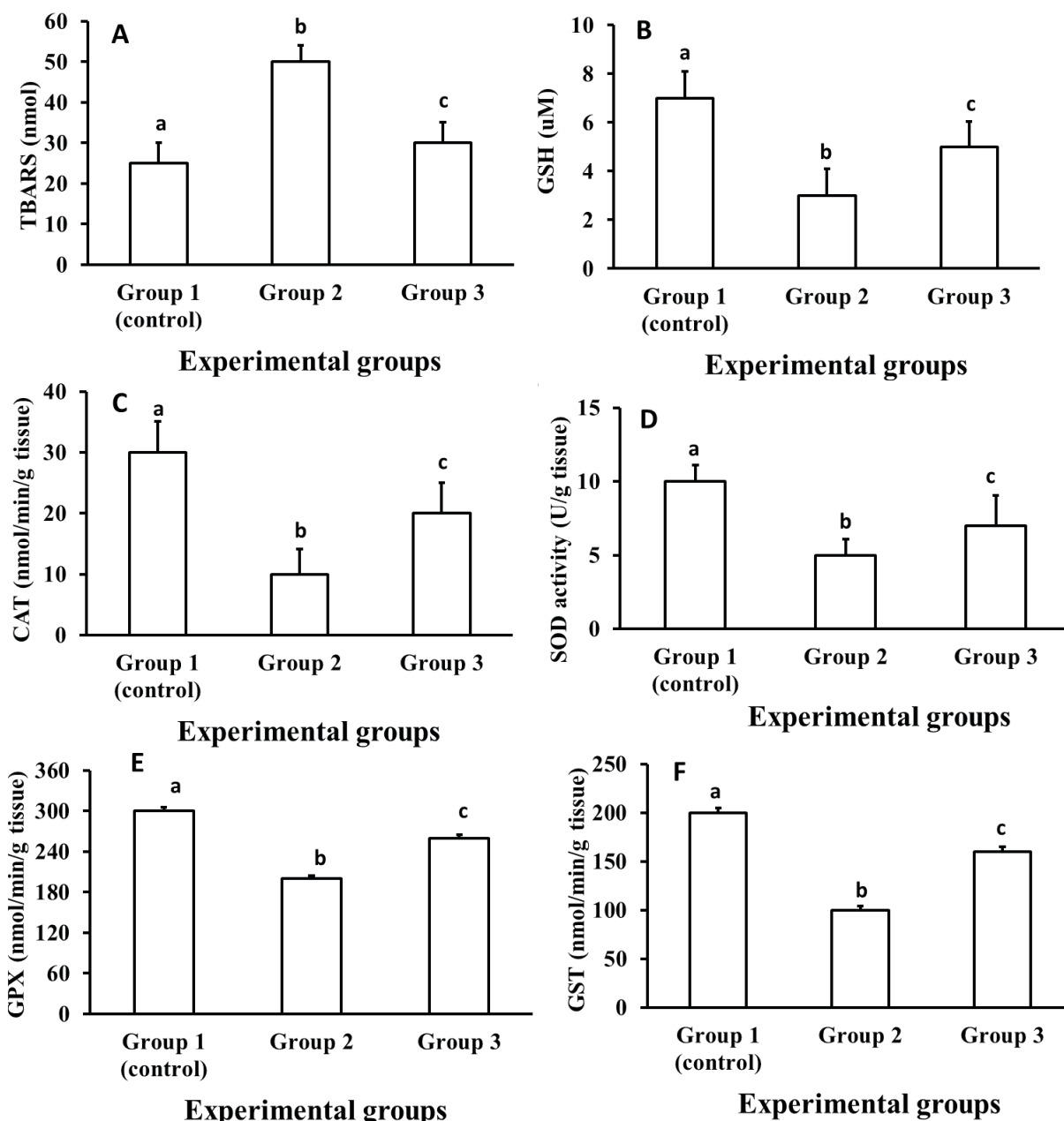


Figure 2: Colorimetric estimation of (A) TBARS, (B) GSH, (C) CAT, (D) SOD, (E) GPX, (F) GST liver of rats received diet containing cholesterol 1% (Group2) and treated with camel milk (group 3) compared to the control (group 1)

cholesterol 1% compared to the control (Fig. 2B). Although, providing of camel milk restored GSH concentration in these rats, the normal control values have not been achieved (Fig. 2B). Significant reduction ($P < 0.05$) of CAT (Fig. 2C), SOD (Fig. 2D), GPX (Fig. 2E) and GST (Fig. 2F) activities were observed in rats fed cholesterol diet 1% in comparison to the control. While camel milk delivery induced a considerable ($P < 0.05$) recovery in the activity of these enzymes, the normal control values have not been reached (Fig. 2C, D, E, F).

Determination of expression of the genes for antioxidant enzymes

The amount of mRNA expressed was measured using real-time PCR (Fig. 3). According to the current research, rats fed a 1% cholesterol diet had significantly ($P < 0.05$) lower levels of CAT (Fig. 3A), SOD (Fig. 3B), GPX (Fig. 3C), and GST (Fig. 3D) expression than control rats. Although camel milk delivery resulted in a considerable ($P < 0.05$) up-regulation of these enzymes' activity, the normal control values have not been reached (Fig. 3).

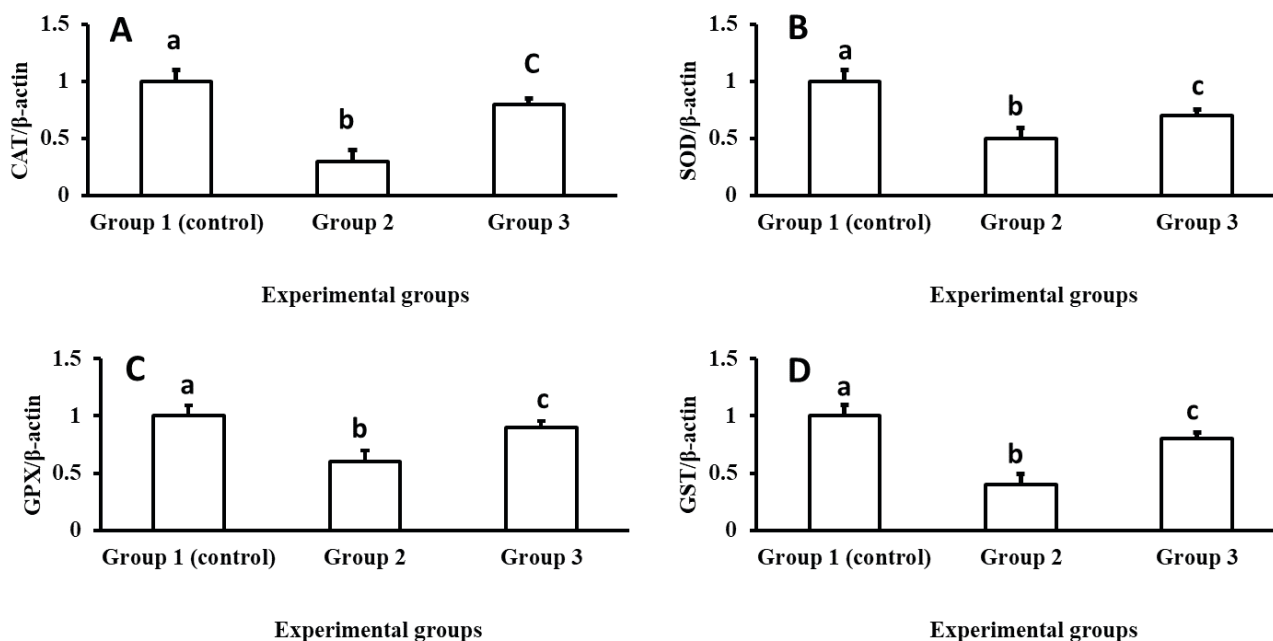


Figure 3: mRNA gene expression of enzymatic antioxidants, CAT (A), SOD (B), GPX (C) and GST (D) in liver tissues of all studied group.

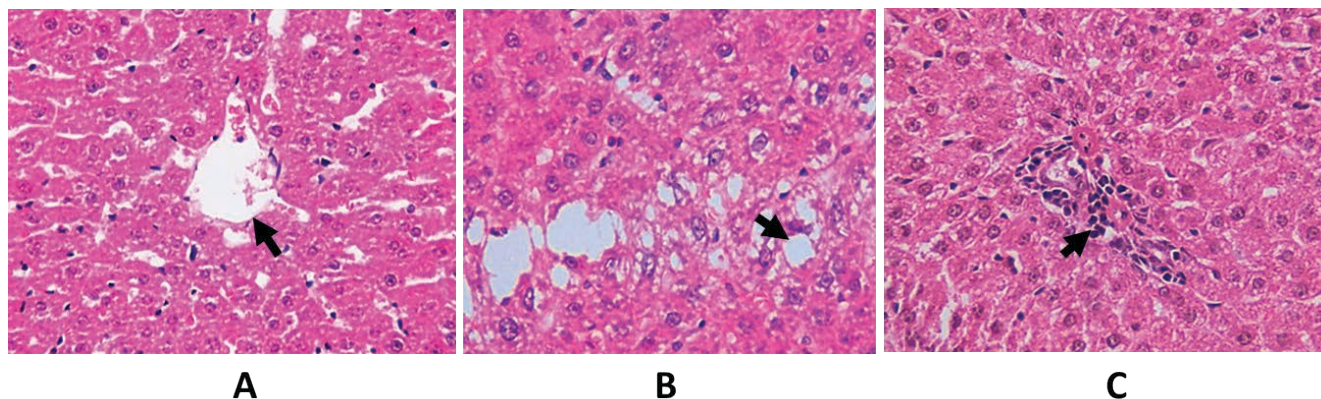


Figure 4: Histopathology of liver of (A) control rats (group 1) showed normal central vein and hepatic cords (ar-row), (B) liver of cholesterol treated rats (group 2) showed fatty changes (arrowhead) and cysts of fats (arrow), (C) liver of camel milk treated rats (group 3) showed normal architecture (arrowhead) of portal and hepatic cords (ar-row). HE bar= 40.00

Histopathological examination

The control rats' livers had sinusoids separating the principal veins from the polygonal cells grouped in regular cords (Fig. 4A). Rats given cholesterol revealed different levels of vacuolar degeneration and variations in sizes, shapes, and staining preferences in their liver (Fig. 4B). Rats fed a high-cholesterol diet and given camel milk showed complete degrees of healing in their livers. The liver cell plates simultaneously recovered their usual structure (Fig. 4C).

Discussion

Hypercholesterolemia is a significant risk factor for cardiac diseases which diagnosed by rise of LDL-c and decrease of HDL-c levels in serum of affected patients or animals (32). Chemical antihyperlipidemic and antihypercholesterolemic medications are widely utilized, but their main drawbacks are their high costs and side effects (16). There is increasing interest concerning the role of natural products in health and medicine (33). Camel milk is gaining great importance in

control and prevention of many diseases as steatohepatitis, insulin resistance and lipid peroxidation (33). However, results of hypolipidemic and hypolcholesterolemic effect of camel milk still contradictory (15, 19, 20, 21, 28, 33, 34). The data concerning the management of high Triglyceride (TG) levels and low HDL cholesterol levels by camel milk remains inconclusive. Furthermore, there is a lack of evidences reported the impact of camel milk on paraoxonase activity. There is still much to learn about the oxidative stress caused by a high cholesterol diet and the involvement of camel milk to protect against it. Therefore, the present study aimed to find out camel milk's effects on lipid metabolism, expression of the genes for antioxidant enzymes and paraoxonase-1 (PON-1) activity in rats fed a diet containing cholesterol 1%. According to the current research, there were insignificant changes in any of the experimental groups' serum total proteins, albumin, globulins, or glucose levels. The current study supports earlier research (34) that showed that treatment of camel milk had insignificant effect on the level of glucose in female albino rats. The current results, however, do not correlate with other studies (33, 35), which showed that rats fed a high fat and high-cholesterol diet showed a considerable rise in serum glucose concentration. The results of the current investigation showed that rats' TAG concentration was increased by a diet high in cholesterol. These results support earlier studies (33, 35, 36, 37) showing that a high-fat, high-cholesterol diet causes hyperlipidemia, which is linked to increased de-esterification of the ample free fatty acids and lower lipoprotein levels (38). The significant reduction of TAG made by administration of camel milk come in concurrent of previous work (34) in female rats. The current study's observation of a significant rise in total cholesterol in rats fed a diet containing cholesterol 1% compared to the control group is consistent with earlier studies (33, 35, 36, 37, 39) in rats fed a high fat and high-cholesterol diet. Higher de-esterification of ample free fatty acids and the reduced lipoproteins may be responsible for this hyperlipidemia (38). Parallel to the current study, earlier investigations (33, 35, 37) showed that rats fed a high fat and cholesterol diet experienced large increases in serum LDL-c and significant decreases in HDL-c levels in comparison to control. The significant effect of camel milk on

total cholesterol and LDL-c come in harmony with previous reports in rats (15, 19, 20) and disagree with other reports (21) that indicated the non-significant effect of camel milk the same parameters. The presence of vitamin C, an antioxidant, is thought to be responsible for camel milk's impact on cholesterol. Stress was induced on liver function through biochemical changes and changes in enzyme activity. Rats fed a diet containing cholesterol 1% in the current research had higher ALT and AST activity than the control group. These findings agree with early study in rats fed high fat and cholesterol diet (35, 37). The treatment of rats fed a diet containing cholesterol 1% with camel milk induced a significant decrease in these enzyme activities, which suggests a sort of membrane stabilizing activity of camel milk (40). The current study might claim that camel milk may have a hepatoprotective effect based on the current results. Several reports (12, 13, 14, 25, 41) demonstrated the protection of hepatocytes by camel milk treatment. Previous report (34) demonstrated that camel milk did not affect ALT and AST activities of healthy female albino rats. Current histopathological observations that showed fatty alterations in the liver of rats fed diet containing cholesterol 1% and a considerable recovery after camel milk administration provided evidence of the hepatoprotective action of camel milk. Current histopathological findings about the impact of a diet containing cholesterol 1% on liver tissues are consistent with earlier research (33) in humans. As far as the author is aware, the current work provided the first histological report demonstrating the preventive impact of camel milk on hepatic fatty alterations brought on by administration of a high cholesterol diet to rats. However, camel milk has been shown to protect rats' livers against alcohol-induced hepatotoxicity (42). Cholesterol and camel milk were safe to rats at the level of renal and mineral metabolism. This was demonstrated by BUN, creatinine, and uric acid levels that were unchanged when compared to control in all experimental groups. Current results reported the reduced paraoxonase activity in rats fed a diet containing cholesterol 1% come in accordance with previous report (43) demonstrating that, hyperlipidemia and hypercholesterolemia are best media for lipid peroxidation and subsequent inhibition of paraoxonase activity. The current study's demonstration of the stimulation of paraoxonase activity in rats may have validated

the antioxidant effect of camel milk that was recently identified at the gene expression level as proven earlier (14) and confirmed in the current investigation. Rats fed a diet containing cholesterol 1% had significantly higher levels of TBARS and significantly lower levels of enzymatic antioxidants like CAT, SOD, GST, and GSH in their livers compared to the control group, indicating that the high-cholesterol diet caused lipid peroxidation and oxidative stress (28). SOD protects cells from oxidative damage by converting free radical superoxide to H_2O_2 and O_2 . The H_2O_2 produced will decompose enzymatically by CAT (44). Serum from rats fed a diet containing cholesterol 1% showed a significant increase in lipid peroxidation product and significant decreases in CAT, SOD, and GST (28). Camel milk may have a protective effect against cholesterol-induced oxidative stress, as demonstrated by the significant reduction in TBARS in the liver of rats given 1% cholesterol and treated with the milk. Due to the presence of vital vitamins and minerals, camel milk has a protective impact (45). The body's antioxidant system depends heavily on GSH. Through redox and detoxifying reactions, it keeps the cells' natural structure and function. In the present investigation, rats fed a diet containing 1% cholesterol showed a significant decline in GSH values. The reduction in GSH may be caused by GPX's urgent need for GSH to scavenge free radicals. The potential of camel milk to remove free radicals and restore the antioxidant state may be responsible for the ameliorative effect of camel milk as evidenced by the lowering of GSH levels. As shown in the current study, this mechanism was strengthened by an increase in the activity and gene expression of antioxidant enzymes. After intoxication with alcohol (42), cadmium chloride (9), aluminum chloride (9), and CCL4 (14), camel milk increased the GSH levels in rat liver. The present findings demonstrated that all studied antioxidant enzymes (SOD, CAT, GPX, and GST) had their gene expression downregulated by a diet containing 1% cholesterol. The current research revealed that camel milk increased the state of antioxidant by increasing genes expression of CAT, SOD, GPX, and GST. Similar findings (10, 14) showed that camel milk controlled gene expression of these enzymes in rats given cisplatin or CCL4 intoxication, respectively. The current study demonstrated that rats fed a diet containing 1% cholesterol had lower paraoxonase activity and

lower levels of antioxidant enzymes. These indicators both increased when camel milk was received. This suggests that these values could be employed as hyperlipidemia and hypercholesterolemia diagnostic biomarkers.

Conclusion

Diet of cholesterol 1% induced significant increases in serum values of total cholesterol, TAG, LDL-c, ALT, AST and TBARS. The same diet induced significant reduction in activities and expression of CAT, SOD, GPX and GST compared to control. The activity of PON-1 in serum of these rats was significantly reduced as well. Fatty degeneration and fatty cysts in liver tissues were observed in histopathological picture of rats fed cholesterol diet. All these detrimental effects were ameliorated into accepted range in camel milk treated group. Conclusively, camel milk was potential for the treatment of hyperlipidemia and hypercholesterolemia in rats.

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