

Full Length Research Paper

## Effect of camel milk on collagen abnormalities in streptozotocin-diabetic rats

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Non-enzymatic glycation of proteins, leading to chemical modification and cross-linking are of importance in the pathology of diabetic complications. In our early reported we showed that, camel milk possesses antihyperglycemic, antihyperlipidemic and exhibit beneficial role on membrane-bound ATPases in streptozotocin-diabetic rats after 45 days of treatment of camel milk at the optimum dose of 250 ml/day. In the present study, the effect of camel milk on the glycation and cross-linking of collagen from tail tendon of streptozotocin (STZ)-diabetic rats. Diabetes was induced in adult male albino rats of the Wistar strain, weighing 180 to 200 g, by administration of streptozotocin (STZ) (40 mg/kg) intraperitoneally. The animals were randomly divided into five groups of six animals each. Rats of groups II and IV were fed 250 ml of raw camel milk daily through watering bottle instead of water. Whereas animals in groups I, III and V were given tap water, and rats of group V were given 600 µg/kg body weight of glibenclamide orally, once in a day in the morning for 45 days. The levels of hydroxyproline and total collagen content elevated in the tail tendon of the diabetic control. The levels of extent of glycation and fluorescence of collagen increased while decreased levels of acid, neutral and pepsin soluble collagens were observed in the tail tendon of diabetic rats. These changes were alleviated by the simultaneous ingestion of camel milk. Our results demonstrate that intake of camel milk has a positive influence on tail tendon collagen glycation and other variables in STZ-diabetic rats and its effect was comparable with glibenclamide.

**Key words:** Camel milk, glycation, collagen, streptozotocin, diabetes.

### INTRODUCTION

Nonenzymatic glycation of proteins causes changes in the structural and functional properties, which are of importance in the etiology and pathology of secondary complications in diabetes (Vlassara et al., 1994). Collagen is a protein with slow turnover rate that contains several basic amino acids with free amino groups and is a strong candidate for extensive modification by glycation (Reiser et al., 1991). Advanced glycation end products (AGEs) have been shown to be associated with structural alterations of collagen. AGEs induce cross-linking between collagen fibers, resulting in increased mechanical

stiffness (Sims et al., 1996) and decreased susceptibility to enzymatic digestion (Sakata, 1995). Changes in collagen function have been documented to be the biochemical link between persistent hyperglycemia and diabetic microvascular disease (Brownlee, 1988).

Diabetic rats also have been shown to cause glycation and cross-linking of collagen that may contribute to diabetic complications (Brennan, 1989). Camel milk proteins have many characteristics similar to insulin (Beg et al., 1986). It is different from other ruminant milk, having low cholesterol, low sugar, high minerals (sodium, potassium, iron, copper, zinc and magnesium), high vitamin C (Knoess, 1979); low protein and large concentrations of insulin. There are no allergens, and it can be consumed by lactase deficient persons and those with weak immune systems. The milk is considered as having medicinal

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properties. It is claimed that the value of camel milk is to be found in the high concentrations of volatile acids especially, linoleic acid and polyunsaturated acids, which are essential for human nutrition. A series of metabolic and autoimmune diseases are successfully being treated with camel milk. Previous study (Agrawal et al., 2004) showed that administration of 250 ml camel milk to diabetic rat's resulted in decreased glucose level.

In our early study, we showed that the camel milk decreased the plasma glucose and increased the insulin levels, and regulated the carbohydrate metabolizing enzymes in streptozotocin-diabetic rats after 45 days of treatment at the optimum dose of 250 ml/day (Khalid et al., 2011) and the study also extended to explore the possibility of the hypolipidaemic action of camel milk in a STZ-diabetic state (Khalid et al., 2010), regulate the membrane-bound ATPases in STZ-induced diabetic rats (Khalid et al., 2010).

However, no experimental data are available on the effect of camel milk on collagen content and its properties which are abnormally altered due to diabetic complications and therefore, the present study aims to examine the influence of camel milk on collagen accumulation in the rat skin and on the variables such as glycation, fluorescence, hydroxyl proline, and solubility of collagen in STZ-diabetes in rats and also to compare the results with glibenclamide, a standard drug.

## MATERIALS AND METHODS

### Animals

Male albino rats of Wistar strain with body weight ranging from 180 to 200 g were procured from Central Animal House, King Saud University, and they were maintained in an air conditioned room (25±1°C) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum*. Procedures involving animals and their care were in accordance with the Policy of Research Centre, King Saud University.

### Chemicals

Streptozotocin was purchased from Sigma-Aldrich, St. Louis, USA. All other chemicals were of analytical grade.

### Experimental induction of diabetes

The animals were rendered diabetic by a single intraperitoneal injection of streptozotocin (40 mg/kg bodyweight) (Ramesh and Pugalendi, 2006) in freshly prepared citrate buffer (0.1 M, pH 4.5) after an overnight fast. Streptozotocin injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycemic mortality. Streptozotocin injected animals exhibited massive glycosuria (determined by Benedict's qualitative test) and diabetes in streptozotocin rats was confirmed by measuring the fasting plasma glucose concentration, 96 h after injection with streptozotocin. The animals with plasma glucose above 240 mg/dl were considered to be diabetic and used for the experiment.

### Experimental design

The animals were randomly divided into five groups of six animals each as given below. Rats of groups II and IV were fed with 250 ml (26 ml/rat/day) of raw camel milk daily for 45 days through watering bottle instead of water. Whereas animals in groups I, III and V were given tap water for 45 days, and rats of group V were given 600 µg/kg body weight of glibenclamide orally, once a day in the morning for 45 days.

Group I: Control (water)

Group II: Control + raw camel milk (250 ml/day)

Group III: Diabetic control

Group IV: Diabetic rats + raw camel milk (250 ml/day)

Group V: Diabetic rats + glibenclamide (600 µg/kg body weight/day) (Ramesh and Pugalendi, 2006).

Glibenclamide is a sulfonylurea antidiabetic agent, a class of drugs used to treat type II diabetes mellitus. This disease is a chronic metabolic illness characterized by a deficiency of insulin, a hormone produced by the pancreas which controls the sugar in the blood. For that, in this study we are using glibenclamide as standard drug for the comparison of efficacy with camel milk treated diabetic rats.

### Biochemical investigations

#### *Estimation of hydroxyproline and collagen content*

A weighed amount of the skin tissue (5 g) was defatted with chloroform/methanol (CM, 2:1) mixture and lyophilized. The lyophilized sample was hydrolysed with 6 N HCl for 18 h at 110°C. After hydrolysis, the digested sample was evaporated to dryness, dissolved in water, and the hydroxyproline content was measured by the method of Woessner, (1961). Oxidation of hydroxyproline by chloramine-T (N-chloro-p-toluene sulphonamide) results in a pyrrole carboxylic acid, which is further oxidized with p-dimethylamino benzaldehyde (PADB) to form purple color. The colour developed was read spectrophotometrically at 557 nm. Hydroxyproline was used as the standard. Collagen content of tissue samples was obtained by multiplying the hydroxyproline content by 7.46 (Woessner, 1961). The amount of hydroxyproline and collagen in the tail tendon was expressed as mg/100 mg of tissue.

#### *Extent of glycation*

The extent of glycation of collagen in the tail tendon was carried out by phenol-sulphuric acid method as described by Rao and Pattabiraman (1989). Saccharides with potential aldehydic and keto groups are dehydrated to furfural derivative on treating with phenol to give coloured complexes, which were detected at 480 nm.

#### *Analysis of fluorescence of collagen*

Fluorescence of collagen in the tail tendon was determined by the method described by Monnier et al. (1996a). AGEs exhibit a yellow-brown pigmentation and a characteristic fluorescence pattern, with excitation in the range at 370 and fluorescence emission at 440 nm.

#### *Analysis of collagen cross linking*

The solubility pattern was determined by the method of Miller and Rhodes (1982). Skin tissue (5 g) was extracted twice with neutral

**Table 1.** Effect of camel milk on body weight, plasma glucose and insulin in control and STZ-diabetic rats.

Groups	Body weight (g)	Glucose (mg/dl)	Insulin ( $\mu$ U/ml)
Control	202.79 $\pm$ 8.40 <sup>a</sup>	76.32 $\pm$ 07.59 <sup>a</sup>	15.39 $\pm$ 1.31 <sup>a</sup>
Control + camel milk (250 ml/day)	203.93 $\pm$ 7.00 <sup>a</sup>	75.45 $\pm$ 07.76 <sup>a</sup>	15.29 $\pm$ 1.30 <sup>a</sup>
Diabetic control	142.70 $\pm$ 9.92 <sup>b</sup>	292.38 $\pm$ 19.20 <sup>b</sup>	5.53 $\pm$ 0.41 <sup>b</sup>
Diabetic + camel milk (250 ml/day)	171.04 $\pm$ 5.50 <sup>c</sup>	141.57 $\pm$ 12.82 <sup>c</sup>	9.97 $\pm$ 0.80 <sup>c</sup>
Diabetic + glibenclamide (600 $\mu$ g/kg b.wt)	193.72 $\pm$ 7.57 <sup>a</sup>	106.22 $\pm$ 8.68 <sup>d</sup>	14.88 $\pm$ 1.26 <sup>a</sup>

Values are means  $\pm$  S.D for six rats. Values not sharing a common superscript differ significantly at  $p < 0.05$  (DMRT).

**Table 2.** Effect of camel milk on hydroxyproline and total collagen content in the tail tendon of control and STZ- diabetic rats.

Name of the group	Hydroxyproline (mg/250 ml tissue)	Total collagen (mg/250 ml tissue)
Control	8.19 $\pm$ 0.54 <sup>a</sup>	54.63 $\pm$ 4.06 <sup>a</sup>
Control + camel milk (250 ml/day)	8.03 $\pm$ 0.66 <sup>a</sup>	54.46 $\pm$ 4.94 <sup>a</sup>
Diabetic control	12.10 $\pm$ 0.95 <sup>b</sup>	84.80 $\pm$ 7.10 <sup>b</sup>
Diabetic + camel milk (250 ml/day)	10.83 $\pm$ 0.65 <sup>c</sup>	75.35 $\pm$ 4.15 <sup>c</sup>
Diabetic + glibenclamide (600 $\mu$ g/kg b.wt )	9.23 $\pm$ 0.55 <sup>a</sup>	63.42 $\pm$ 4.18 <sup>a</sup>

Values are means  $\pm$  S.D for six rats. Values not sharing a common superscript differ significantly at  $p < 0.05$  (DMRT).

salt solvent containing 20 m Methylene diamine tetra acetic acid (EDTA) and 2 mM N-ethyl maleimide. The supernatants were pooled. The residue obtained from neutral salt extraction was again extracted with 0.5 M acetic acid twice. The supernatants containing acid-soluble collagen were pooled. The residue obtained was extracted with pepsin (100 mg/g wet tissue) to obtain pepsin-soluble collagen. The collagen contents in the pooled fraction were determined by performing the hydroxyproline assay (Woessner, 1961).

### Statistical analysis

Values were given as means  $\pm$  SD for six rats in each group. Data were analyzed by one-way analysis of variance followed by Duncan's Multiple Range Test (DMRT) using SPSS version 11 (SPSS, Chicago, IL). The limit of statistical significance was set at  $P < 0.05$ .

## RESULTS

Table 1 shows the effect of administration of camel milk for 45 days on body weight and plasma glucose in control and streptozotocin diabetic rats. Body weight and plasma insulin significantly decreased and plasma glucose significantly increased in diabetic rats. Both, camel milk or glibenclamide significantly improved the body weight and plasma insulin and brought down the plasma glucose towards normal level.

The levels of hydroxyproline and total collagen content

in the tail tendon of control and diabetic rats are given in Table 2. The levels of hydroxyproline and total collagen content elevated in the tail tendon of diabetic control and treatment with camel milk reversed the levels of hydroxyproline and total collagen content towards normalcy. Table 3 represents the levels of extent of glycation and fluorescence of collagen in the tail tendon of control and diabetic rats. Increased levels of the extent of glycation and fluorescence of collagen were observed in the tail tendon of diabetic rats and treatment with camel milk brought the extent of glycation and fluorescence of collagen towards normalcy. The levels of acid, neutral and pepsin soluble collagens of tail tendon of control and diabetic rats are represented in Table 4. In diabetic rats, decreased levels of acid, neutral and pepsin soluble collagens were observed in the tail tendon and treatment with camel milk reversed the levels of soluble collagens towards normalcy.

## DISCUSSION

Diabetes induced by streptozotocin caused a significant increase in the total collagen content and more markedly enhanced the AGE-related fluorescence of collagen. Alterations in physicochemical properties indicate increased cross-linking and maturation of collagen STZ-rats. In our study, the levels of hydroxyproline and total collagen are elevated in the tail tendons of diabetic rats,

**Table 3.** Effect of Camel milk on extent of glycation and fluorescence of collagen in the tail tendon of control and STZ-diabetic rats.

Name of the group	Extent of glycation ( $\mu\text{g}$ of glucose/mg of collagen)	Fluorescence (AU/ $\mu\text{mol}$ of hydroxyproline)
Control	12.21 $\pm$ 0.94 <sup>a</sup>	29.34 $\pm$ 2.58 <sup>a</sup>
Control + camel milk (250 ml/day)	11.96 $\pm$ 0.95 <sup>a</sup>	27.07 $\pm$ 2.37 <sup>a</sup>
Diabetic control	24.83 $\pm$ 1.86 <sup>b</sup>	52.07 $\pm$ 4.65 <sup>b</sup>
Diabetic + camel milk (250 ml/day)	20.48 $\pm$ 1.63 <sup>c</sup>	41.87 $\pm$ 3.72 <sup>c</sup>
Diabetic + glibenclamide (600 $\mu\text{g}$ /kg b.wt)	15.65 $\pm$ 1.19 <sup>d</sup>	35.00 $\pm$ 3.10 <sup>d</sup>

Values are means  $\pm$  S.D for six rats. Values not sharing a common superscript differ significantly at  $p < 0.05$  (DMRT). AU- Arbitrary Units.

**Table 4.** Effect of camel milk on neutral soluble, acid soluble and pepsin soluble Collagen in the tail tendon of control and STZ-diabetic rats.

Name of the group	Neutral soluble collagen ( $\mu\text{g}/250$ ml)	Acid soluble collagen (mg/250 ml)	Pepsin soluble collagen (mg/250 ml)
Control	138.07 $\pm$ 9.34 <sup>a</sup>	3.89 $\pm$ 0.26 <sup>a</sup>	4.20 $\pm$ 0.27 <sup>a</sup>
Control + camel milk (250 ml/day)	141.43 $\pm$ 12.71 <sup>a</sup>	3.95 $\pm$ 0.21 <sup>a</sup>	4.35 $\pm$ 0.26 <sup>a</sup>
Diabetic control	82.48 $\pm$ 7.34 <sup>b</sup>	2.61 $\pm$ 0.12 <sup>b</sup>	2.58 $\pm$ 0.14 <sup>b</sup>
Diabetic + camel milk (250 ml/day)	105.16 $\pm$ 9.40 <sup>c</sup>	3.26 $\pm$ 0.21 <sup>c</sup>	3.15 $\pm$ 0.19 <sup>c</sup>
Diabetic + glibenclamide (600 $\mu\text{g}$ /kg b.wt)	123.30 $\pm$ 11.06 <sup>d</sup>	3.60 $\pm$ 0.15 <sup>d</sup>	3.83 $\pm$ 0.25 <sup>d</sup>

Values are means  $\pm$  S.D for six rats. Values not sharing a common superscript differ significantly at  $p < 0.05$  (DMRT).

which could be due to increased blood glucose and non-enzymatic glycation. In addition, prolyl hydroxylase, an ascorbic acid dependent enzyme, is required to maintain the normal properties of collagen. The activity of prolyl hydroxylase has been reported to change in diabetic rats (McLennan et al., 1988). Camel milk abolished the STZ-induced accumulation of collagen and alterations in its properties. Collagen from camel milk-administered STZ-rats displayed decreased glycation, AGE formation, aldehyde, and peroxide content in skin collagen, together with a decline in total collagen content, as compared with untreated STZ-diabetic rats. The relative increase in neutral salt- and acid-soluble collagen. These changes indicate the reduction in cross-linking of collagen in camel milk-treated rats.

The positive effects of camel milk on collagen could be explained by its effects on glucose levels and insulin action. Camel milk has been shown to normalize insulin action and improve insulin sensitivity (Agrawal et al., 2004) in diabetic rats. The hypoglycemic activity of camel milk may be because of high concentrations of insulin like protein in camel milk which contains about 45 to 128 units/liter (Singh, 2001) and also it contains high amount of zinc (Mohamed, 1995). Zinc is playing a major role for insulin secretory activity in pancreatic beta cells. Previous reports show that zinc supplementation attenuates insulin secretory activity in pancreatic islets of the ob/ob mouse

(Begin-Heick et al., 1985) and also Clintoria et al. (2008) reported that, extracellular ATP and zinc are co-secreted with insulin and activate multiple P2X purinergic receptor channels expressed by islet beta-cells to potentiate insulin secretion. Ming-Der Chen et al. (1998) reported that, zinc supplementation alleviated the hyperglycemia of ob/ob mice, which may be related to its effect on the enhancement of insulin activity. Present study shows that, the increased insulin levels may be due to the insulin like protein and high amount of zinc present in camel milk. Our findings are in agreement with those reported by Agrawal et al. (2004), camel milk can increase body weight and decrease plasma glucose level in streptozotocin- diabetic rats after receiving 250 ml of camel milk daily for 22 days; also it is confirmed from our early study which showed that the camel milk decreased the plasma glucose and increased the insulin levels, and regulated the carbohydrate metabolizing enzymes in streptozotocin-diabetic rats after 45 days of treatment at the optimum dose of 250 ml/day (Khalid et al., 2011). Oral insulin has been known since many years but the critical draw back is its coagulum formation in acidic media in stomach, which neutralizes its potency. One property of camel milk is that, it does not form the coagulum in the stomach or acidic media; thereby it prevents degradation of insulin in the stomach. Beg et al. (1986) reported that amino acid sequence of some of the

camel milk protein is rich in half cystine, which has superficial similarity with insulin family of peptides.

Treatment with camel milk had reversed the levels of hydroxyproline to near normal, this could also be due to an increased level of ascorbic acid. In our study, the elevated level of the extent of glycation was observed in the tail tendons of diabetic rats which could be due to increased exposure of tissues to glucose. This is in line with the result of Bensusan who reported that, collagen glycation is increased during exposure to high glucose levels *in vitro* and *in vivo* (Bensusan, 1965). Treatment with camel milk has reversed the extent of glycation to near normal which could be due to improved glycaemic control.

AGEs exhibit a yellow-brown pigmentation and a characteristic fluorescence pattern, with excitation in the range 350 to 390 and fluorescence emission at 440 to 470. AGEs have been measured by the use of fluorescence spectroscopy in different kind of tissues (Soulis-Liparota, 1991). In our study, collagen from the tail tendons of diabetic rats showed increased fluorescence which could be due to increased formation of AGEs. This is in agreement with previous report (Sakata et al., 1995), and treatment with camel had reversed fluorescence which might be due to decreased formation of AGEs as evidenced by improved glycaemic control.

The covalent reaction between glucose and collagen may not be the only factor involved in the formation of AGEs and cross-linking observed in diabetes may play a significant role. It has been proposed that both free glucose (Wolff, 1987) and protein-glucose adduct (Ahmed et al., 1986) undergo oxidation in the presence of trace amounts of metal ions, generating free radicals and reactive carbonyls. These reactive oxygen species and carbonyls may contribute significantly to the increased cross-linking of collagen. Elgawish et al. (1996) demonstrated that, hydrogen peroxide is directly involved in glucose-induced cross-linking of collagen. The cross linking of tail tendon collagen was assessed by the solubility of collagen. The percentage of acid, neutral and pepsin soluble collagens decreased in the tail tendons of diabetic rats. As cross-linking proceeds, the solubility of collagen in neutral buffer and acid solution also changes. Highly cross-linked collagen becomes less soluble in the aforementioned solutions and can be released only by limited pepsin digestion (Wolff, 1987). Treatment with camel milk had increased the solubility of collagen in neutral, acid and pepsin digestion, which could be associated with decreased cross-linking of collagen which is evidenced by improved glycemic control and decreased extent of glycation.

In conclusion, the present study suggests that camel milk could prevent collagen changes during hyperglycemia and provide evidence for the potential utility of camel milk in the treatment of diabetic complications. Further detailed investigation is necessary to find out its

mechanism of action and to establish its therapeutic potential in the treatment of diabetes and diabetic complications.

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