DOI : 10.5958/2277-8934.2020.00015.6 *Vol 27 No 1, p 105-110*

EFFECT OF HEAT TREATMENTS ON ANTIOXIDANT PROPERTIES AND INSULIN CONTENT OF CAMEL MILK

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ABSTRACT

The aim of this study was to evaluate the changes in antioxidant activity and insulin content of camel milk after thermal treatment. Camel milk samples were collected from dairy farm (ICAR-National Research Centre on Camel, Bikaner, India). The samples were processed with two heat treatment methods: High Temperature Short Time (HTST) and Low Temperature and Long Time (LTLT) and fresh camel milk (untreated) was used as control. The antioxidant activity of the milk was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) radical scavenging activity. Insulin concentration was determined using ELISA kits in the different milk samples. DPPH radical scavenging activity was significantly lower ($p<0.05$) in LTLT heat treated (pasteurised) milk compared to fresh milk (Control). However, no significant difference was found between HTST group and control. Similar results were observed for ABTS radical scavenging activity of the milk samples. There was also no significant ($p>0.05$) difference in the insulin content in heat treated groups when compared with control group. Thus, data suggested that the milk processing by HTST pasteurisation preserves the antioxidant activity as well as insulin content in camel milk.

Key words: ABTS, antioxidants, camel milk, DPPH, insulin, pasteurisation

Significant health benefits of camel milk in human disease conditions have been suggested by several researchers (Agrawal *et al,* 2004; Magjeed, 2005; Meena *et al,* 2016). The antioxidant and antimicrobial properties of pure and hydrolysed camel milk proteins is also reported (Salami *et al,* 2009; Kumar *et al,* 2016b, 2016c & 2017). The anti-diabetic effect of camel milk is the most studied area in the human health benefits. However, effect of heat treatment of camel milk regarding preservation of its antioxidant properties as well as insulin content and thus for the management of different disease conditions has not been explored.

Antioxidants protect the cells against over production of reactive oxygen species (ROS) and prevent oxidative tissue damage (Jackson *et al,* 2002). Though, oxidative metabolism is essential for the cell survival and various regulatory processes, its negative impact may lead to generation of free radicals or reactive oxygen species (ROS). Excess level of free radicals or ROS may have detrimental effect by oxidizing membrane lipids, proteins, DNA etc. which can further damage the normal cellular processes. Antioxidants in milk are known for preventing the damages caused by Reactive Oxygen Species (ROS) (Lindmark-Mansson and Akesson, 2000). Milk contains a number of enzymatic as well as non enzymatic antioxidant like glutathione peroxidase, superoxide dismutase, catalase, vitamin E, vitamin C, β- carotene, which may protect newborn calves against ROS at the early stage of life and during oxidative stress (L'Abbe and Friel, 2000; Scheibmeir *et al,* 2005). Very limited data is available regarding the comparative evaluation of antioxidant property of fresh and heat processed milk from different species.

Milk not only contains antioxidants but also contains an array of bioactive substances including insulin hormone (Read *et al,* 1984; Hamosh, 2001). Effect of heat treatment, pasteurisation on insulin concentration of camel milk has been studied (Wernery *et al,* 2006b).

Present study was therefore aimed to evaluate the effects of thermal treatment on antioxidant

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potential and changes in the level of insulin hormone in camel milk.

Materials and Methods

Chemical and Reagents

Insulin ELISA Kit was procured from RayBiotech (USA), and chemicals such as 2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were procured from SRL Pvt Ltd (India). Other required analytical chemicals were also procured. All solutions, prepared in Milli Q water, and were kept at 4°C for further use.

Collection and processing of camel milk samples

Fresh milk was collected from Camel Dairy Farm, ICAR-National Research Centre on Camel, Bikaner in sterile containers and chilled immediately to 5°C. The samples were defatted by cream separator and skimmed milk was used for different thermal treatments. Two thermal treatments: Low Temperature Long Time (LTLT, 63°C for 30 min.) and High Temperature Short Time (HTST, 72°C for 15 sec.) were given to milk. The untreated (control) and processed milk samples were stored at -20°C till further analysis.

Antioxidant activity

2-2-azinobis-3 ethylbenthiazoline-6-sulphonic acid (ABTS+) radical scavenging activity

ABTS assay is based on the reduction of ABTS+ radicals by antioxidants present in the milk. The ABTS+ radical scavenging activity of camel milk samples was determined by method as described by Salami *et al* (2009) with slight modifications. Briefly, ABTS stock solution was prepared by mixing equal volume of 2.45 mM potassium persulphate ($K_2S_2O_8$) to produce ABTS radical cation. Before use, this mixture was kept in the dark at room temperature (37°C) for 16 h. The working solution was obtained by diluting the stock solution of the ABTS radical cation with ethanol to obtain an absorbance of 0.70 ±0.005 at 734 nm. After that 1ml of ABTS+ working standard solution was mixed with 20 µl of milk samples. The reaction mixture was mixed gently and was passed through 0.2 µm syringe filter, absorbance was measured after 20 min (t_{20}) at 734nm using UV-VIS spectrophotometer (Smart Spec Plus Spectrophotometer, BioRad, USA). The activity of ABTS+ was calculated by using formula:

$$\textit{ABTS+ activity (\% inhibition)}=\left(\frac{0.7\text{-OD at t20}}{0.7}\right)X100$$

DPPH radical scavenging activity

The ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical of milk samples were determined using the method of Brand-Williams *et al* (1995) with a slight modifications. One ml of DPPH working solution (100µM) was mixed with 250 µL of Tris-HCl buffer (pH 7.4) and milk samples (25 µL) in the test tube for 30 min at room temperature (37°C). The reaction mixture was mixed gently and was passed through 0.2 µm, absorbance was measured at 517 nm immediately at time t=0 (t0) using UV-VIS spectrophotometer (SmartSpec Plus Spectrophotometer, BioRad, USA), the reaction mixtures were incubated for 30 min in dark at room temperature (37°C). The absorbance (t30) was again measured at 517 nm and the ethanol was used as blank. The activity of DPPH was calculated by using formula:

$$\textit{DPPH activity (\% inhibition)}= 100 - \left(\frac{\text{OD at t30}}{\text{OD at t0}}\right)X100$$

Determination of Insulin Concentration

Insulin concentrations in milk samples were analysed using an immunochemical insulin analysis with insulin ELISA Kit (Raybiotech, USA). The kit is based on Sandwich-based technique using colorimetric method of detection. The analysis was performed according to the manufacturer's instructions. Briefly, all the kit reagents were brought to room temperature (37°C) before use and dilutions were prepared as per manufacturer's instruction. 100 µL standard or samples was added to each well and incubated for 2.5 h at room temperature (37°C). The solution was discarded from the ELISA plate and washed 4 times with wash solution. 100 µL of biotin antibody was added to each well and incubated for 1 h. The solution was discarded and washed 4 times with wash solution. 100 µL of streptavidin solution was added and incubated for 45 min at room temperature (37°C). 100 µL of TMB one-step substrate reagent was added to each well and incubated for 30 min. 50 µL stop solution was added and immediately OD was recorded at 450 nm using plate reader (infinite 200 Pro Tecan, Switzerland).

Calculation

Standard curve (scattered chart) was prepared with insulin standard using Microsoft Office-EXCEL. The final insulin concentration of the respective samples was calculated from the standard curve.

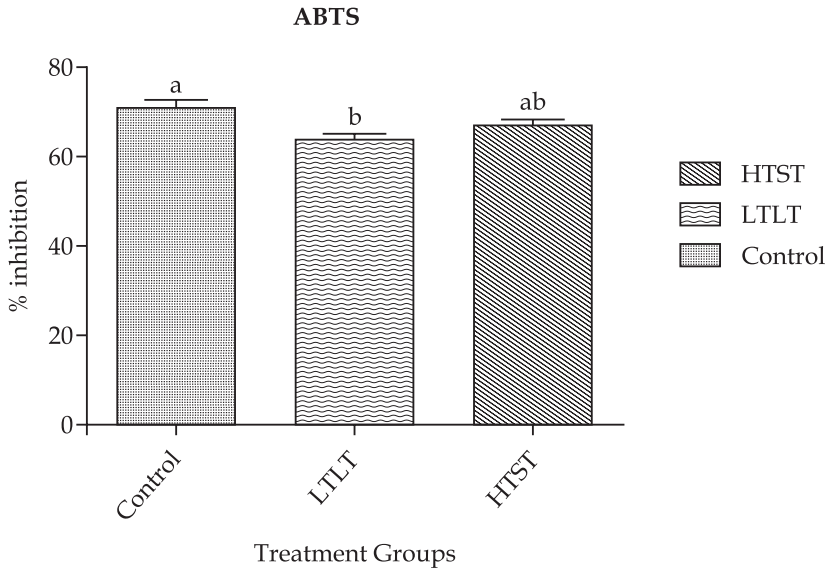

Fig 1. ABTS (% Inhibition) of raw and heat treated camel milk samples.

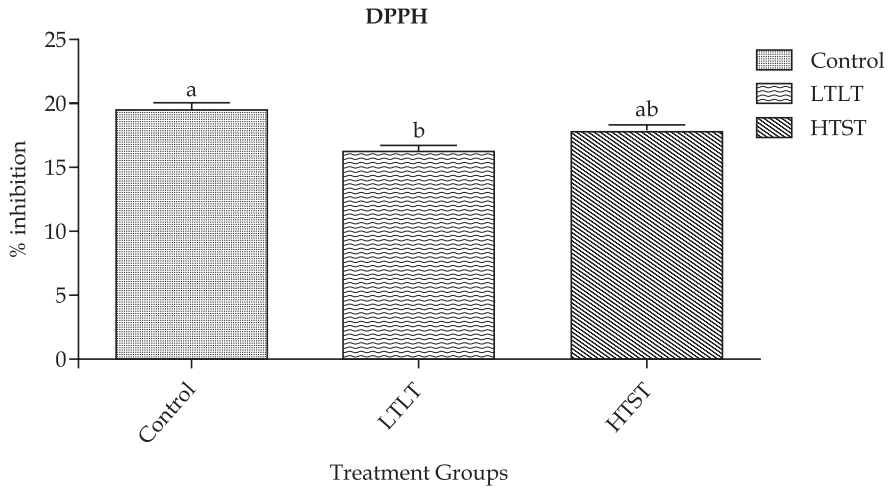

Fig 2. DPPH (% Inhibition) of raw and heat treated camel milk samples.

Statistical Analysis

All the experiments were repeated three times and parameters were analysed in duplicate (n=6). One-way analysis of variance (ANOVA) was done by comparing the means at 95% confidence level using GraphPad Prism software. Data were expressed as means with standard error.

Results and Discussion

Current study focused on the comparative evaluation of antioxidant activity/potential and insulin hormone of camel milk under different pasteurisation methods.

Antioxidant activity of thermal treated camel milk

Antioxidant activity of control (fresh camel milk) and thermally treated (LTLT and HTST) camel milk was determined by ABTS and DPPH assays, compared and discussed in this section.

The cationic radical scavenging activity of ABTS+ is mostly utilised to measure antioxidant activity of food ingredients and processed food products. Since, the reagents are dissolved well in both aqueous hydrophilic and organic solvent hydrophobic groups, this assay measures both the hydrophilic and lipophilic antioxidants. Its efficiency depends upon the number of aromatic rings, nature of hydroxyl groups and molecular weight (Hagerman *et al*, 1998). Hence, it is required to verify the antioxidant activity of food ingredients by conducting different assays, because the mechanism of action in one assay differs from another and also influence the end results.

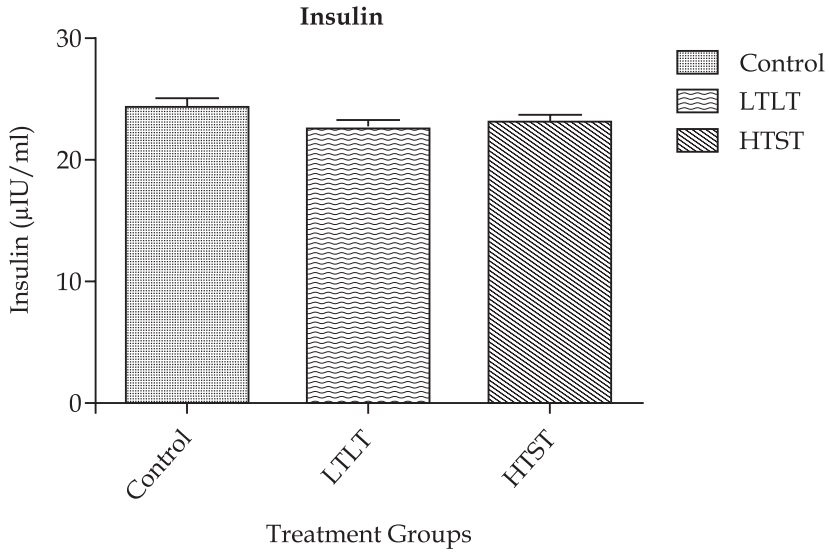

Fig 3. Insulin concentration of raw and heat treated camel milk samples.

A negative correlation was found between thermal treatment of camel milk and antioxidant activity. Both the assays (ABTS and DPPH) evidenced the lower antioxidant activity of pasteurised milk compared with control (fresh). The ABTS activity (% inhibition) of this experiment is presented in Fig 1. Very little information is available about the effect of heat on the antioxidant activity of camel milk. In this study the ABTS radical scavenging activities of raw camel milk and thermally treated camel milk was compared. The ABTS activity (% inhibition) of control, LTLT and HTST milk samples were 71.12±1.26, 63.81±1.22 and 66.88±1.207, respectively. The decrease in ABTS activity was significantly ($p \leq 0.05$) lower in LTLT milk samples; however, it was not statistically different from HTST samples. The ABTS activity of control samples were highest among three groups, however, it was comparable to that of HTST milk samples. The decrease in radical scavenging activity of thermally treated milk might be due to partial degradation of bioactive components responsible for antioxidant activity upon different level of heat application. In LTLT method, although the temperature applied is lower than the HTST method, but longer heat exposure might be responsible for reduced antioxidant activity. Yilmaz-Ersan *et al* (2018) also compared the ABTS activity of raw and pasteurised cow and ewe milk and reported reduction in antioxidant activity of both types of milk after pasteurisation. The significant reduction in antioxidant activity and reduction in total vitamin C content upon pasteurisation was also reported for human milk (Moltó-Puigmartí *et al,* 2011). The Holder pasteurisation (63°C for 30 min) induced a significant ($p < 0.05$) loss of total antioxidant capacity, while total antioxidant capacity was same in both the fresh milk samples and in the samples treated to HTST-pasteurisation (75°C for 15 sec) (Silvestre *et al,* 2008) similar results was found in the current study.

DPPH has been commonly used for the analysis of antioxidant activity for the primary characterisation of the scavenging potential of proteins/peptides. The DPPH scavenging activity was assayed as an additional measure for assessing antioxidant activity of camel milk. The DPPH activity (% inhibition) of fresh and heat treated samples is presented in Fig 2. The DPPH activity of fresh (control), LTLT and HTST milk samples were 19.47±0.4564, 16.14±0.49 and 17.70±0.55, respectively. The DPPH activity of LTLT samples were significantly lower ($p \leq 0.05$) as compared to control samples; however, it was comparable to HTST samples. The HTST samples had higher DPPH activity as compared to that of LTLT samples; however, the differences were non-significant ($p > 0.05$). Hilario *et al* (2010) reported that pasteurisation negatively affects the antioxidant components like total phenolic concentration in goat milk. Reduced DPPH scavenging activity of pasteurised and boiled milk in cow and buffalo has been reported by Khan *et al* (2017). Similar results were also reported by Yilmaz-Ersan *et al* (2018) for DPPH activity of raw and pasteurised cow and ewe milk.

Vitamins like with vitamin-C and reduced glutathione are some of the compounds which contribute to the antioxidant system of the milk. Camel milk is rich in with vitamin-C which is also an antioxidant and contributes its antioxidant activity in the milk (Farah *et al,* 1992). It is reported that the vitamins especially vitamin-C thermally degraded after thermal treatment (Munyaka *et al,* 2010). Lysozyme concentration is also high in camel milk than cow milk (Korhonen, 1977; Duhaiman, 1988; Singh *et al,* 2017) and may also contribute to the higher antioxidant capacity as it is rich in amino acid lysine which shows antioxidant activity (Ahmad *et al,* 1996). Casein is probably a major ABTS+ scavenger in the milk (Chen *et al,* 2003) as it has a high content of potential antioxidative amino

acids like tyrosine, tryptophan, histidine, lysine and methionine (Uchida and Kawakishi, 1992). Similar observation was also reported by Wernary *et al* (2005). They postulated that with vitamin-C and reduced glutathione are the most heat susceptible component in camel milk when pasteurised at 63°C. However, in another study Wernery *et al* (2003) reported that different components of camel milk are more heat resistant than those in cow milk by which it has tremendous advantage over other milk in relation to the commercial production and processing.

Changes in Insulin in camel milk

Insulin content of control (fresh) and heat treated (LTLT and HTST) camel milk was estimated with insulin ELISA kit. There are very few reports available regarding the effect of heat treatment on milk insulin content and as per best of our knowledge this the first with report which compares the effect of HTST and LTST on antioxidant activity with insulin content of camel milk. The insulin content in different groups is presented in Fig 3. In the present study the insulin content of raw milk and heat treated milk was compared. Concentration of insulin in control and both heat treated milk samples were 24.35±0.59, 22.51±0.633 and 23.01±0.61 µIU/ml, respectively. There was a non significant ($p>0.05$) decrease in insulin content was found after pasteurisation (Fig 3). In earlier reports, the mean concentration of insulin was reported in camel milk was 41.9±7.38 µIU/ml (Wernery *et al,* 2006a, 2006b), 45 to 128 µIU/ml (Singh *et al,* 2006), and 58.67±2.01 µIU/ml (Hamad *et al,* 2011). Different concentration of insulin was also reported in human milk (Young *et al,* 2017). In the present study the mean insulin concentration was 24.35 µIU/ml in fresh camel milk. This variable insulin content might be due to the difference in protocol/technique used in estimation and due to breed/region as well as lactation effects. In a study, Wernery *et al* (2006b) reported that pasteurizing camel milk at 72°C for 5 min and boiling at 98°C for 5 min resulted in significant reduction in milk insulin concentration. However, in another study Ollikainen (2013) found no changes in insulin concentration in bovine milk after pasteurisation at (63°C/ 30 min) and 72°C; though the losses in immunochemical activity were reported at higher temperatures.

Conclusion

The present study suggested that the fresh camel milk is having high antioxidant potential or free radical scavenging activity and pasteurisation by heat treatment (both by HTST and LTST) does not significantly affect the insulin content; though the antioxidants activity is lowered by LTLT treatment. Thus, HTST method of pasteurisation can be used for effective preservation of camel milk without much compromising on antioxidant activity and insulin content.

Acknowledgement

Authors are thankful to director(s) of ICAR-Indian Veterinary Research Institute, Izzatnagar and ICAR-National Research Centre on Camel, Bikaner, India for financial support for this study.

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