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Effect of oxytocin on milk proteins and fatty acid profile in Sahiwal cows during lactation periods

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Abstract: Bovine milk, a rich source of proteins and fatty acids, has a beneficial impact on physicochemical and organoleptic properties. The current study was planned to identify oxytocin's effects on protein and fatty acid profiles of cow milk (the Sahiwal breed) at various lactation stages (early, middle, and late) under a controlled atmosphere and feeding inputs. Examination of milk protein and fatty acid profiles by urea polyacrylamide gel electrophoresis and gas chromatography-mass spectrometry respectively revealed the significant effect ($P < 0.01$) of oxytocin during the cow's lactation stages. In electrophoretic patterns, casein (αs1-CN, αs2-CN, β-CN, and k-CN) and whey protein fractions (Ig, BSA, β-Lg, and α-La) appeared as low-intensity bands in oxytocin-injected milk when compared to the control. Reductions in concentrations of fatty acids C16:0 (25.00%) and C18:1 (21.60%) were observed in oxytocin-treated milk as compared to the control group (29.30% and 29.10%, respectively) at late lactation.

Key words: Oxytocin, Sahiwal cow, milk proteins, fatty acids, lactation

1. Introduction

Milk and milk products are important nutritional sources in the human diet. The proteins, fat, and fatty acid composition of bovine milk are part of a balanced diet and responsible for physicochemical, sensory, and manufacturing properties in milk and milk products, and they also present potential health benefits for consumers (1). Bovine milk fat typically contains 70% saturated fatty acids, 25% monounsaturated fatty acids, and 5% polyunsaturated fatty acids (2). The protein-containing major portions of caseins are relatively large aggregates of four molecules: αs_1-casein (αs_1-CN), αs_2-casein (αs_2-CN), β-casein (β-CN), and k-casein (*k*-CN) (3). After casein, other parts of proteins are whey or serum proteins that are composed of α-lactalbumin (α-La), β-lactoglobulin (β-Lg), immunoglobulin (Ig), bovine serum albumin (BSA), proteoses, and peptones (4). The prime biological function of β-Lg is to bind fatty acids and thereby stimulate lipase. It also binds several hydrophobic molecules as it binds and protects retinol (5).

Generally, the milk composition varies due to species and breed differences, lactation stages, nutritional status, health, and milking intervals (6). The Sahiwal cow breed originated from the Sahiwal region of Punjab Province, Pakistan, and is considered as one of the best dairy animals existing in the Indo-Pak region (7). Similarly, production of milk is also affected by a number of factors, including the secretion of oxytocin. Oxytocin is a hormone released into the blood by neural stimulation that causes the contraction of myoepithelial cells, leading to the expulsion of milk from the alveoli. The mechanism of fast and complete milk removal from the udder is a complex one, normally not considered as one of the main factors of milk yield (8).

In dairy practices, the use of exogenous oxytocin in cows before milking is intended to treat the disturbed milk ejection caused by reduced endogenous oxytocin release and also for mastitis cure, as well. The use of oxytocin injections before milking on a regular basis reduces the release and sensitivity of endogenous oxytocin in the udder, resulting in reduced spontaneous milk ejection after withdrawal of oxytocin (9). In underdeveloped countries, indiscriminate use of oxytocin on a regular basis before milking has a negative effect on milk enzyme activities (10) and is a common practice in resource-limited countries only with intentions to increase the milk yield, without knowing the physiological effects. Therefore,

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farmers use external oxytocin before each milking due to unawareness and lack of education. Oxytocin's effects on milk composition, i.e. milk fatty acids and proteins, are not clear yet. According to some scientists, oxytocin affects milk components in diverse ways, while some are of the view that milk composition is not affected.

The current study was formulated keeping in view the oxytocin mechanism, stage of lactation, and supply chain situation of milk from cows to consumers. The core objective of this study was to evaluate whether the main constituents of milk, proteins and fatty acids, within bovine mammary secretions are affected by external oxytocin treatment during the lactation period.

2. Materials and methods

2.1. Selection and management of animals

Lactating Sahiwal cows (n = 48) were selected from the herd at the Livestock Experiment Station, Livestock Production Research Institute, Bahadurnagar, Okara, Pakistan, during May–August 2011. All cows were in their 2nd to 5th lactation having an average 1552 kg of milk production per lactation (267 days) for each. These animals were kept under the same atmospheric conditions and fed with similar inputs and seasonal fodder/feed. In addition to green fodder, cows were offered concentrate mixture (cotton seed cake 10%, maize 10%, rape seed cake 5%, wheat bran 34%, maize gluten 24%, molasses 15%, and mineral mixture 2%) at approximately 3 kg per animal before the start of milking and had free access to clean drinking water. Milking was performed daily at 0500 hours and 1700 hours.

2.2. Treatment and milk sampling

Forty-eight cows were divided into three groups comprising sixteen animals each according to their lactation stage, i.e. early lactation, middle lactation, and late lactation.

- Early lactation: when the milk production was on the upswing after calving. This lasts from calving to 60–80 days.
- Middle lactation: when the milk production was relatively stable, or more properly dropping off slowly and lasting a few months. This stage lasts for 6–8 months after calving.
- Late lactation: when the milk production starts dropping off faster, basically after 8 months.

In each lactation stage (early, middle, late) animals were divided into two subgroups (A and B), comprising eight animals in each group. In each lactation, group A was treated intramuscularly with a 20 IU dose of oxytocin (Lawrence Pharma, Pvt. Ltd.) daily before the start of milking for a period of 50 days, while group B was given no treatment and was considered as the control. Milking was carried out under hygienic conditions and milk samples were collected from all cows during the 50-day experiment. Procured milk samples were transferred into plastic bottles of 100 mL and stored at 4 °C until further use for analysis.

2.3. Protein profiling using Urea-PAGE

Skim milk was prepared by centrifugation at 5000 × *g* for 15 min at 4 °C. The pH of skim milk was adjusted to 4.6 with 1 M HCl and kept at 4 °C for 30 min and then at 37 °C for 30 min for easy separation of casein and whey. Casein and whey proteins were filtered through filter paper (Whatman No. 1). The casein was washed 2 or 3 times with distilled water and then stored at –20 °C until further analysis. The whey collected was dialyzed at 4 °C. Dialysis membranes with 12–14 kDa molecular weight were used. The dialysis was performed for 24 h by changing the distilled water several times and the whey sample was stored at –20 °C for further use for electrophoresis (11).

The characterization of proteins by urea-polyacrylamide gel electrophoresis (Urea-PAGE) (Electrophoresis Mini-PROTEAN, Bio-Rad) was carried out using the method described by Andrews (12). Five microliters of protein marker (Fermentas Page Ruler Prestained Protein Ladder) and sodium caseinate were loaded as the reference for evaluation of whey and casein protein in various samples, respectively. Four major bands of Na-caseinate (Lane 7, Figure 1), i.e. αs_1-CN, αs_2-CN, β-CN, and *k*-CN, were separated. The molecular weight of the standard marker loaded for whey protein ranged from 11 to 170 kDa (Lane 7, Figure 2).

2.4. Fatty acid profiles using gas chromatography-mass spectrometry (GC-MS)

Evaluation of short- and long-chain fatty acids, i.e. butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1), was performed with an appropriate method of milk extraction. The separated lipid (40 mg) was transesterified by a transmethylation procedure (13). The fatty acid methyl esters were quantified using a Shimadzu Gas Chromatograph Model 14-A (Shimadzu Co., Japan) fitted with a methyl lignocerate-coated (film thickness = 0.25 μm) polar capillary column SP-2330 (30 mm × 0.32 mm, Supelco). Quantification was done by using a standard mixture of known fatty acids.

2.5. Statistical evaluation

Analysis of variance under a completely randomized design with two-factor factorial design was used for the data obtained (14). Means and standard errors of means were calculated. To find the difference between means, Duncan's multiple range test was performed.

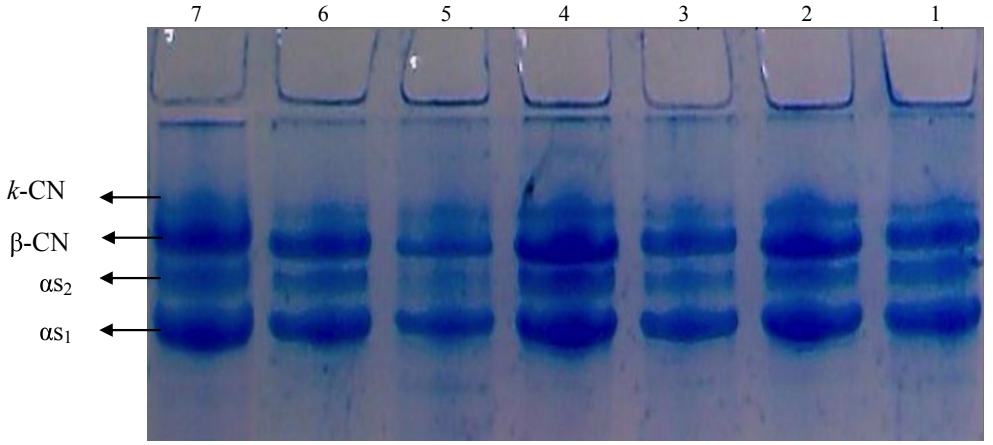

Figure 1. Electrophoretic pattern of cow milk casein at different lactation stages on Urea-PAGE representing differences in band patterns: 1- oxytocin-treated milk at early lactation stage; 2- control milk at early lactation stage; 3- oxytocin-treated milk at middle lactation stage; 4- control milk at middle lactation stage; 5- oxytocin-treated milk at late lactation stage; 6- control milk at late lactation stage; 7- indicates sodium caseinate used to identify bands of milk caseins at different lactation stages.

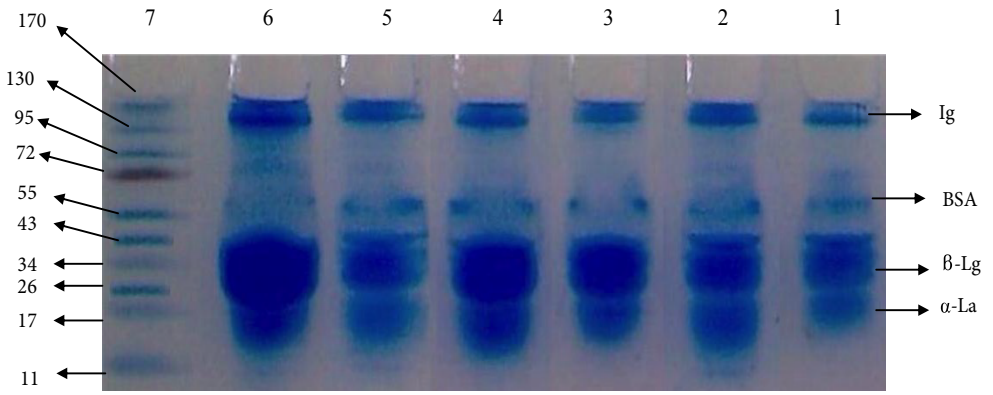

Figure 2. Electrophoretic pattern of cow milk whey proteins at different lactation stages on Urea-PAGE representing differences in band patterns: 1- oxytocin-treated milk at early lactation stage; 2- control milk at early lactation stage; 3-oxytocin-treated milk at middle lactation stage; 4- control milk at middle lactation stage; 5- oxytocin-treated milk at late lactation stage; 6- control milk at late lactation stage; 7- indicates marker (Fermentas, 11–170 kDa) used to identify molecular weight of milk proteins at different lactation stages.

3. Results

3.1. Protein profiles: Urea-PAGE

Separation of various casein fractions was observed in the control and oxytocin-treated milk samples on Urea-PAGE (Figure 1). The bands of αs_1-CN and β-CN in the control and oxytocin-treated milks were observed to be denser as compared to αs_2-CN and *k*-CN.

Darker bands of β-CN were noticed at middle lactation in the control milk (Lane 4) as compared to early and late lactation. The β-CN bands in oxytocin-injected milk samples were almost the same in intensity at the early and middle lactation stages as compared to late lactation. Lactational response in αs-CN in oxytocin-treated milk showed a declining trend with respect to lactation stages (Figure 1). The proportions of casein fractions varied with respect to lactation stages and decreased casein content was observed at the end production stage.

In the electrophoretic profile of whey proteins (Figure 2), four separated bands based on charge to mass ratio, i.e. Ig, BSA (69 kDa), β-Lg (18 kDa), and α-La (14.1 kDa), were visible both in control and oxytocin-injected milk.

The appearance of a darker band of β-Lg (Lane 6, Figure 2) was observed at late lactation as compared to other lactation stages for control milk samples. The intensity of β-Lg was found low in oxytocin-treated milk at early and late lactation as compared to control milk samples.

The concentration of α-La was found low in treated milk samples throughout the lactation stages in the current study. It was also confirmed that the concentration of α-La decreased while β-Lg increased throughout lactation stages (Figure 2).

3.2. Fatty acid profiles: GC-MS

The results regarding short- and medium-chain fatty acids, namely butyric acid C4:0, caproic acid C6:0, caprylic acid C8:0, and capric acid C10:0, and long-chain fatty acids lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) of group A (oxytocin) and group B (control) cows at the three lactation stages are presented in the Table. A nonsignificant difference was observed for C4:0 and C10:0 both in groups A and B, while significant variations in C4:0 with a highest concentration of 4.00% were observed at the middle lactation stage. At the late lactation stage, significant differences in concentrations of C6:0 and C8:0 were detected in both groups.

The parabola trend for change in concentrations of C12:0 and C14:0 was similar in both groups across lactation stages (Table). The highest C12:0 contents were observed in middle lactation milk samples at 4.30% and 4.40% in group A and group B cows, respectively. The highest C14:0 contents were also noticed at 13.80% and 13.90% in middle lactation milk samples of group A and group B cows, respectively. However, no significant differences of these fatty acid contents were found in either group.

In the control group of cows (group B), palmitic acid contents had an inclined trend, but in oxytocin-injected cows (group A), they had a parabola trend across lactation stages (Table). The significantly lowest palmitic acid contents (25.00%) were found in late lactation of group A cows as compared with the palmitic acid contents (29.30%) in milk tested at late lactation of group B cows (Table).

The stearic acid contents did not differ significantly between oxytocin-treated and control milk samples. However, lactation stages had a significant influence on stearic acid contents and these contents showed a decreasing trend with the progression of lactation stages. A declining trend was also observed for C18:1 contents in the milk of both groups A and B through the lactation stages. A significant variation was present in milk between control and oxytocin-treated cows over the three lactation stages for C18:1 expression. A major decrease in C18:1 from 29.10% (group B) to 21.60% (group A) was observed at the late lactation stage of milking.

4. Discussion

The findings of the current study, with denser profiles of αs_1-CN and β-CN in control and oxytocin-treated milk as compared to αs_2-CN and *k*-CN, were similar to those described by Van Hekken and Thompson (15). The lighter band of β-CN, observed at the late lactation stage in the present study, was consistent with higher milk proteinase activity seen in late lactation (16). The concentration of αs-CN in milk falls quickly in the first month after calving (17), supporting the reduction of this protein at the early lactation stage seen in the present study. Similar results were found by Lucey and Fox (18), who reported

Table. Short- and long-chain fatty acid concentrations (%) in milk of Sahiwal cows treated with or without oxytocin during different stages of lactation.

Fatty acids	Early lactation		Middle lactation		Late lactation	
	Group A (n = 8)	Group B (n = 8)	Group A (n = 8)	Group B (n = 8)	Group A (n = 8)	Group B (n = 8)
Butyric acid (C4:0)	3.80 ± 0.04^{b}	3.83 ± 0.02^{b}	3.90 ± 0.07^{a}	4.00 ± 0.08^{a}	3.45 ± 0.04^{c}	3.50 ± 0.04^{c}
Caproic acid (C6:0)	1.90 ± 0.00^{c}	2.00 ± 0.00^{c}	2.50 ± 0.00^{b}	2.50 ± 0.00^{b}	1.50 ± 0.00^{d}	3.10 ± 0.00^{a}
Caprylic acid (C8:0)	1.60 ± 0.00^{c}	1.50 ± 0.00^{c}	2.00 ± 0.00^{a}	1.90 ± 0.00^{ab}	1.50 ± 0.00^{c}	1.80 ± 0.00^{b}
Capric acid (C10:0)	3.67 ± 0.004^{b}	3.66 ± 0.09^{b}	3.88 ± 0.13^{a}	3.86 ± 0.02^{a}	3.36 ± 0.01^{c}	3.36 ± 0.003^{c}
Lauric acid (C12:0)	3.81 ± 0.01^{b}	4.30 ± 0.04^{ab}	4.30 ± 0.01^{ab}	4.40 ± 0.24^{a}	3.88 ± 0.04^{b}	4.11 ± 0.24^{ab}
Myristic acid (C14:0)	13.20 ± 0.04^{c}	13.09 ± 0.04^{c}	13.80 ± 0.20^{a}	13.90 ± 0.18^{a}	13.50 ± 0.12^{b}	13.60 ± 0.04^{b}
Palmitic acid (C16:0)	25.80 ± 0.04^{c}	25.10 ± 0.41^{c}	26.80 ± 0.35^{b}	27.30 ± 0.33^{b}	25.00 ± 0.08^{c}	29.30 ± 0.25^{a}
Stearic acid (C18:0)	12.20 ± 0.33^{a}	12.50 ± 0.41^{a}	10.73 ± 0.41^{b}	10.83 ± 0.40^{b}	9.80 ± 0.04^{c}	9.70 ± 0.08^{c}
Oleic acid (C18:1)	30.40 ± 0.33^{ab}	31.20 ± 0.63^{a}	28.13 ± 0.41^{d}	29.99 ± 0.40^{bc}	21.60 ± 0.04^{e}	29.10 ± 0.08^{cd}

Means with the same letters in a column or row are not significantly different from each other.
n = Number of animals; ± = SD; Group A = oxytocin-injected cows; Group B = control group of cows.

that late lactation milk could have markedly lower casein concentrations than middle lactation milk. In casein protein, αs-CN contributing a major portion of total casein and being maximum at middle lactation in the control milk is supported by the findings of Ostersen et al. (19), who found that the proportion of αs-CN in total casein decreased at end lactation. Barry and Donnelly (20) reported that αs-CN increased in early lactation and then fell for the rest of lactation. Bands of lower intensity were observed in oxytocin-treated cow milk as compared to milk of control cows in all milk lactation stages.

The varied proportions of casein fractions with respect to lactation stages might be due to increase in plasmin activity at the late lactation stage because more plasmin enters the mammary glands in late lactation (21). The decrease in plasmin activity observed in normal milk during the middle lactation stage was associated with a reduction in pH that probably inhibited the enzyme expression, which is why a maximum intensity of casein in milk was observed at the middle lactation stage (22).

In the electrophoretic profile of whey proteins, the finding of four visible bands, i.e. Ig, BSA, β-Lg, and α-La, in control and oxytocin-injected cows was in agreement with the findings of Van Hekken and Thompson (15). In another study, Sheldrake et al. (23) reported that whey protein was also affected by season, stage of lactation, age of cow, and somatic cell count. The inclined trend of Ig (Figure 2) with respect to lactation stage was also reported by Kroeker et al. (17). Results of the present study indicated no proper detection of BSA because the milk had very low contents (1.2% of total protein), as also reported by Bylund (4).

The darker band of β-Lg observed at late lactation for control milk samples as compared to other lactation stages was in agreement with the study of Kroeker et al. (24). Lighter bands of α-La appeared in the current study as bovine α-La represents about 20% of bovine whey protein (25).

Across lactation stages, C4:0 and C6:0 followed the same trend as that observed in buffalo milk (26). In another study, similar results were observed for C4:0 and C6:0 in milk of Canadian Holstein cows at early, late, and middle lactation (27). Experimental results of the present study demonstrated that the total concentrations of medium-chain fatty acids (C8:0 and C10:0) at early lactation (5.27% in group A, 5.16% in group B) in Sahiwal cows were slight lower than in Holstein cow milk (5.56%) and in buffalo milk (5.5%). Another study regarding cow milk showed 3.15% fatty acids (C8:0 and C10:0) at 16 weeks of lactation (28). These fatty acids (5.88% in group A and 5.76% in group B) at middle lactation were in close relation to cow and buffalo milk (5.9% and 5.1%, respectively). At the late lactation stage, 4.86% fatty acids in group A and 5.16% fatty acids in group B (C8:0 and C10:0) were noted, which was between the values of 5.65% and 3.8% reported for cow and buffalo milk by Kgwatalala et al. (27).

In the present study, at the early lactation stage, the lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0) of Sahiwal cows were found lower than the values reported in Holstein cows at 5.10%, 13.85%, and 26.96%, respectively (27). However, stearic acid (C18:0) and oleic acid (C18:1) were higher than the previously reported values of 8.94% and 20.87%, respectively. Myristic acid (C14:0) was substantiated with a reported value (13.5%) from buffalo milk at early lactation (26). At middle lactation, fatty acids C12:0, C14:0, and C16:0 were lower than the reported values of 5.61%, 15.15%, and 27.89%, respectively, and at late lactation C12:0 and C14:0 were also observed lower than the reported 5.48% and 14.96%, respectively, while C16:0 were higher than the reported value (26.98%) (27). In another study, values of 13.5%, 13.7%, and 13.2% for C14:0 at early, middle, and late lactation, respectively, are in line with observations in the current study (26). Trends followed by C12:0 and C14:0 across lactation stages are similar to the findings described above. Means for C18:0 and C18:1 across lactation stages were higher than previously reported (27), but C18:0 showed similar results to those found by Arumughan and Narayanan (26).

The fatty acids containing from 4 to 14 carbon atoms are synthesized de novo in the mammary glands. Parts of palmitic acid (C16:0) are also synthesized de novo (6). Variations in the fatty acid profile throughout lactation may be linked to the energy balance of the cows. During early lactation, dairy cows in negative energy balance mobilize adipose tissues for secretion of palmitoleic acid, oleic acid, and other long-chain fatty acids into milk (29), and the increased concentrations of these fatty acids might therefore explain the relatively lower concentrations of de novo synthesized C10:0, C12:0, and C14:0 compared with the later lactation stages (30). Accomplishment of a positive energy balance during middle lactation is probable to reverse the inhibitory effects of C18:1 fatty acid on de novo fatty acid synthesis and consequently lead to an increase in the concentrations of C4:0, C6:0, C8:0, C10:0, C12:0, and C14:0 during middle lactation as compared to early lactation.

Sufficient literature is not available on the effect of oxytocin on protein and fatty acid fractions. Conclusively, Urea-PAGE showed significant differences in bands of protein in control and oxytocin-injected Sahiwal cows across lactation stages. Excessive use of oxytocin injections in cows for milking may disturb cell mechanisms for the synthesis of protein within mammary glands. The lighter band of β-CN observed at the late lactation stage in the present study was consistent with the higher milk proteinase activity seen in late lactation. The amount of

whey protein was high at the late lactation stage with higher whey nitrogen in the late than early lactation stage. Variable concentrations of fatty acids were observed in Sahiwal cattle milk associated with lactation stages. Reduction in oleic acid concentration was observed throughout lactation in the milk of oxytocin-treated cows as compared to the control, but a major decrease of 4.3% in palmitic acid (C16:0) and 7.5% in oleic acid (C18:1) was noticed at late lactation in the milk of oxytocin-injected cows as compared to the control. The current study concluded that oxytocin-injected milk has variations in long-chain fatty acids, which may affect milk fat composition and eventually milk quality. Indiscriminate and inappropriate use of oxytocin injections before milking should be prohibited by authorities and farmers should be educated through seminars and workshops. Further research is necessary to evaluate oxytocin's effects on complete milk fatty acid profiles.

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