Expression Analysis of Solute Carrier (SLC2A) Genes in Milk Derived Mammary Epithelial Cells during Different Stages of Lactation in Sahiwal (*Bos indicus*) Cows

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Abstract

Solute carriers (*SLC2A/ GLUT*) are one of the major types of transporter superfamily that have been predominantly involved in active transport of glucose across the plasma membrane. Glucose uptake by mammary epithelial cells (MECs) is an important step in the milk synthesis during lactation, and hence directly influences the milk yield. Use of MEC isolated from milk has been speculated to be a good alternative to mammary gland tissues in order to understand the expression profile of important genes associated with lactation , in particular large dairy animals where obtaining biopsies is sometimes difficult. The present study was therefore undertaken in milk purified MECs to assess relative mRNA expression of major solute carriers/glucose transporters (*SLC2A/GLUTs*) *viz.,* SLC2A1(GLUT1), SLC2A(GLUT4), SLC2A8(GLUT8), SLC2A12(GLUT12) and hexokinase (*HK2*) genes during; early(10-20 days), peak (30-50 days), mid (100-140 days) and late (215-245 days) lactation stages of Sahiwal cows . MECs were isolated from fresh milk, using Dyna Beads coated with anticytokeratin 18 antibodies. For normalization of qPCR expression data, 10 known housekeeping genes (*HKGs*) from different functional classes were evaluated. A panel of four best stable HKGs; eukaryotic translation elongation factor 1 alpha (*EEF1A1*), ribosomal protein *L4(RPL4)*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin- beta (*ACTB*) were identified through ge Norm, Norm Finder and Best Keeper analysis. Expression level of *SLC2A1* was significantly ($P<0.05$) higher during peak/mid lactation period as compared to late lactation period. Similarly, *SLC2A8* mRNA expression was relatively higher during mid-lactation stages than other stages of lactation. In contrast, *SLC2A4* expression level was relatively higher during the late lactation stages. The *SLC2A12* mRNA on the other hand was undetectable, indicating its expression to be either very low or absent in MEC of Sahiwal cows. Our expression data indicated *SLC2A1*, *SLC2A4* and SLC2A8 to be the major solute carriers in Sahiwal MEC. Further, *HK2* expression was significantly ($P<0.05$) higher during early and mid-lactation stages. The stage specific expression about of major *SLC2A/GLUT* and *HK2* genes indicate their functional role in regulating glucose uptake in *MECs* of Sahiwal cows.

Keywords: Solute carrier, Glucose transporter, mRNA expression, Lactation, Mammary epithelial cells, QPCR, Sahiwal cows

Introduction

In dairy animals, glucose is the most important nutrient that directly impacts the rate of lactose, protein and fat synthesis. Milk yield greatly depends on mammary lactose synthesis due to its osmoregulatory property for mammary uptake of water [1,2] and as the main precursor for lactose synthesis, large amounts of glucose are required by lactating dairy cows [3-5]. Compared with non-lactating dairy cows, the requirements of glucose are approximately fourfold greater in lactating cows [6]. Lactating mammary gland can consume up to 85% of the circulating glucose [7,8] and in a lactating cow, 72 g of glucose is essential to produce 1 kg of milk [9]. The metabolic products of glucose, such as ATP and NAPDH, are important factors in milk synthesis of dairy cows. Thus, the availability of glucose to the mammary gland could be a potential regulator of milk yield. Synthesis of glucose from other precursors is not possible in mammary gland because of the lack of glucose-6-phosphatase [10,11]. To supply glucose to the mammary gland, insulin-independent glucose uptake decreases in tissues, except for the mammary gland, and insulin resistance in the whole body increases at peak lactation [12].

ATP-binding cassette (ABC) superfamily and solute carriers/glucose transporters (*SLC/GLUT*) are the two major types of transporters that have been mostly involved in transport of milk constituents in dairy animals. While ABC transporters play an important role in regulating cellular cholesterol homeostasis and transfer of wide variety of substrate across cellular membrane, *SLC/GLUT* transporters are involved in glucose uptake across plasma membrane through facilitative diffusion. Each GLUT has a different transport efficiency, substrate affinity and tissue distribution, indicating that GLUTs have different biological functions in different tissues. Different solute carrier genes viz., SLC2A1 *(GLUT1)*, *SLC2A3 (GLUT3)*, *SLC2A4 (GLUT4)*, *SLC2A5 (GLUT5)*, *SLC2A8 (GLUT8),* and *SLC2A12 (GLUT12)* have been reported. Along with these glucose transporters, sodium dependent *SGLT1* and *SGLT2* responsible for basal glucose uptake were found to be expressed in the bovine lactating mammary gland at different levels. To meet the requirements for milk synthesis at the time of onset of lactation, glucose uptake in the mammary glands increases dramatically and this is accomplished by increases in the expression of glucose transporters (GLUTs) [13,14]. Various studies [15,16] have confirmed that glucose uptake by the mammary epithelial cells (MEC) is an important step in the milk synthesis during lactation and milk yield is directly related to the glucose uptake by the MEC. As *MECs* are responsible for converting most precursors into milk constituents and transporting them to the mammary lumen, these cells

isolated from the milk could be a good resource to unravel the expression profile of solute carrier genes and help in understanding the lactation biology of native cattle in Indian where mammary biopsy/slaughtering of cattle is not encouraged/permitted.

A number of studies have been carried out to study differential expression of some of these transporters during lactation and dry period in various species [17-19]. In contrast, no information is available about the expression profile of major solute carriers in mammary gland and mammary cells of Indian native cow. Any information pertaining to mammary gland biology of *Bos indicus* cows would be of great interest for the researchers. The present study was therefore aimed to determine relative mRNA expression pattern of important solute carrier genes in mammary epithelial cells of Sahiwal cows, a major Indian dairy type cattle breed.

Material and Methods

Animals and milk sampling

A total of 16 healthy multiparous Sahiwal cows maintained at cattle yard, National Dairy Research Institute, Karnal were included in the study. The animals were grouped on the basis of their stage of lactation; early (10-15 days), peak (30-50 days), mid (100-140 days) and late (>215 days) lactation. Milk samples (2-3 liters per animal) from selected animals were collected early morning (05:00-06:00 AM.) and brought to laboratory in sterile jars and stored at 4°C till further processing.

Isolation of mammary epithelial cells from milk somatic cells

Milk samples were filtered to remove dust particles, hairs, cow dung *etc.* with a sterile muslin cloth and defatted by centrifugation at 3000 rpm for 20 min at 4°C. The cell suspension was centrifuged and pellet was suspended in 1X PBS containing 1% BSA. Mammary epithelial cells (MEC) were isolated from somatic cells using Dynabeads (Pan Mouse IgG, Dynal Biotech and Invitrogen) coated with antimouse Cytokeratin 18(clone K8.13, Sigma-Aldrich Chimie). The protocol used for purification of MEC is described here under in brief. Firstly, dynabeads were coated with a primary mouse monoclonal antibody directed against cytokeratin 8 (clone K8.13, Sigma-Aldrich Chimie), which was specific to bovine epithelial cells. As per the cell count, a ratio of 1:4 beads was used for separation of MEC from somatic cells. 50 μl beads and 5μl antibodies were added in 1 ml 1X PBS containing 1% BSA and incubated it for 4 hours in 4°C temperature at orbital shaker. The cell suspension was poured in a 2 ml tube containing antibody activated beads and tube was placed on magnetic beads concentrator. Supernatant was discarded and beads were washed with 1X PBS to remove the unbound antibody. Incubation was done for 1 hour at 40°C in orbital shaker. The micro centrifuge tube was placed on magnetic bead concentrator where MECs got attached to the wall of tube. After discarding the supernatant the tube was washed twice with 1X PBS. Cells were trizolated by adding 1ml TRIzol and proceeded for RNA. The cells were trizolated and stored at -80°C before RNA isolation.

RNA Isolation and cDNA synthesis

Total RNA was extracted from MEC using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. After extraction, RNA was purified using RNeasy Mini kit (Qiagen, Germany) and followed by on-column digestion with the RNase-free DNase (Qiagen, Germany). RNA was quantified using Nano drop ND-1000 spectrophotometer (Nano Drop Technologies). RNA integrity was confirmed by denaturing agarose gel electrophoresis and experion bioanalyser (BioRad, USA,). From the purified RNA, cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA). First strand cDNA was synthesized using 2 μl RNA (200 ng), 1 μl Oligo dT 12- 18 primer (100 μM), 1 μl 10 mM dNTP mix, 1 μl random primers (100 μM), and 18 μl DNase/RNase free water. The mixture was incubated at 65°C for 5 min and subsequently snaps chilled on ice. A total of 18 μl of master mix consisting of 8 μl 5X First-Strand Buffer, 1 μl M-MuLV Reverse Transcriptase (200u/μl)), 0.5 μl RiboLockTM RNAse Inhibitor (20 u/μl) and 8.5 μl DNase/RNase free water was added. The reaction was performed in an Eppendorf Gradient cycler using the program: 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. cDNA was then diluted 1:4 (v:v) with DNase/RNase free water. cDNA was then diluted 1: 4 (v: v) with DNase/RNase-free water.

Real-time Quantitative PCR (qPCR)

qPCR was performed on a total of 52 MEC cDNA samples using Applied Biosystem Step one plus (Applied Biosystems, California). Each reaction in a 96 well plate (Thermo, USA) was comprised of 4 μL diluted cDNA and 6 μL of mixture which composed of 5 μL Maxima SYBER Green/ROX qPCR master mix (2x) (Fermentas Thermo, USA), 0.4 μl each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water. The reaction was performed with following amplification conditions: 95°C for 10 min followed by 40 cycles of 15 sec at 95°C (denaturation) and 1 min at 60°C (annealing + extension). A melting curve was produced after completion of the PCR program to assess the specificity of the amplified PCR product. Each reaction was performed in duplicate along with 6 point relative standard curve plus the non-template control. Further to normalize the expression data of target candidate genes, expression stability of 11 housekeeping (HKGs) genes from different functional classes were evaluated to select appropriate HKGs for MEC expression studies in Sahiwal cows.

Data analysis

In order to normalize the target gene expression data, geometric mean of *RPL4, EEF1A1, GAPDH,* and *ACTB* was used as *HKGs* (Figure 1). These four genes were selected as most appropriate from a set of 10 HKGs evaluated using excel-based visual basic macros tools *viz.,* geNorm, Normfinder and Bestkeeper (*unpublished data*). Several studies have recommended more than one *HKG* as an effective means for normalization of qPCR data to account for the experimental variations [20-22]. The C_T (cycle threshold) values of *SLC2A* and *HK2* mRNA were normalized with the geometric mean of C_T values of the 4 HKGs (*RPL4, EEF1A1, GAPDH,* and *ACTB*) to calculate ΔC_T. The primers details for each of the *SLC2A/GLUT* genes and *HKG*s utilized in the present study are given in Table 1. To compare the expression level of across different lactation stages, the ΔC_T values were analyzed using one way ANOVA followed by Tukey's multiple comparison tests. A p value of <0.05 was considered statistically significant.

Gene Symbol	Accession Number	Primers 5'-3' (Forward, Reverse)	Ta	PCR Efficiency (%)
SLC2A1/GLUT1	NM_174602.2	TCCACAAGCATCTTCGAGAAG AATAGCGACACGACAGTCAAC	600C	88.07
SLC2A4/GLUT4	D63150	GGACCGCGAATAGAAGAAAGAC CAACTTCATCATCGGCATGG	600C	118.89
SLC2A8/GLUT8	AY208940	GCCTCTGCCTTCTGCATCTT TCATGATGGAGCCGGCATGT	600C	98.65
SLC2A12/GLUT12	AY514443	ACTGAATACCGGATCGTCAC AGGCCAATGAGATCCGTCAC	600C	57.08
HK2	XM-002691189	AAGATGCTGCCCACCTACG TCGCTTCCCATTCCTCACA	600C	106.86
ACTB	AY141970	GCGTGGCTACAGCTTCACC TTGATGTCACGGACGATTTC	600C	106.08
EEF1A1	BC105315	CATCCCAGGCTGACTGTGC TGTAAGCCAAAAGGGCATGC	600C	102.39
GAPDH	BC102589	TGGAAAGGCCATCACCATCT CCCACTTGATGTTGGCAG	600C	102.91
RPL4	NM_001014894	TTGGAAACATGTGTCGTGGG GCAGATGGCGTATCGCTTCT	600C	99.97

Table 1: Gene symbol, GenBank accession numbers, primer sequences, annealing temperature (T_a), PCR efficiency of SLC2A/GLUT, HK2 and reference genes.
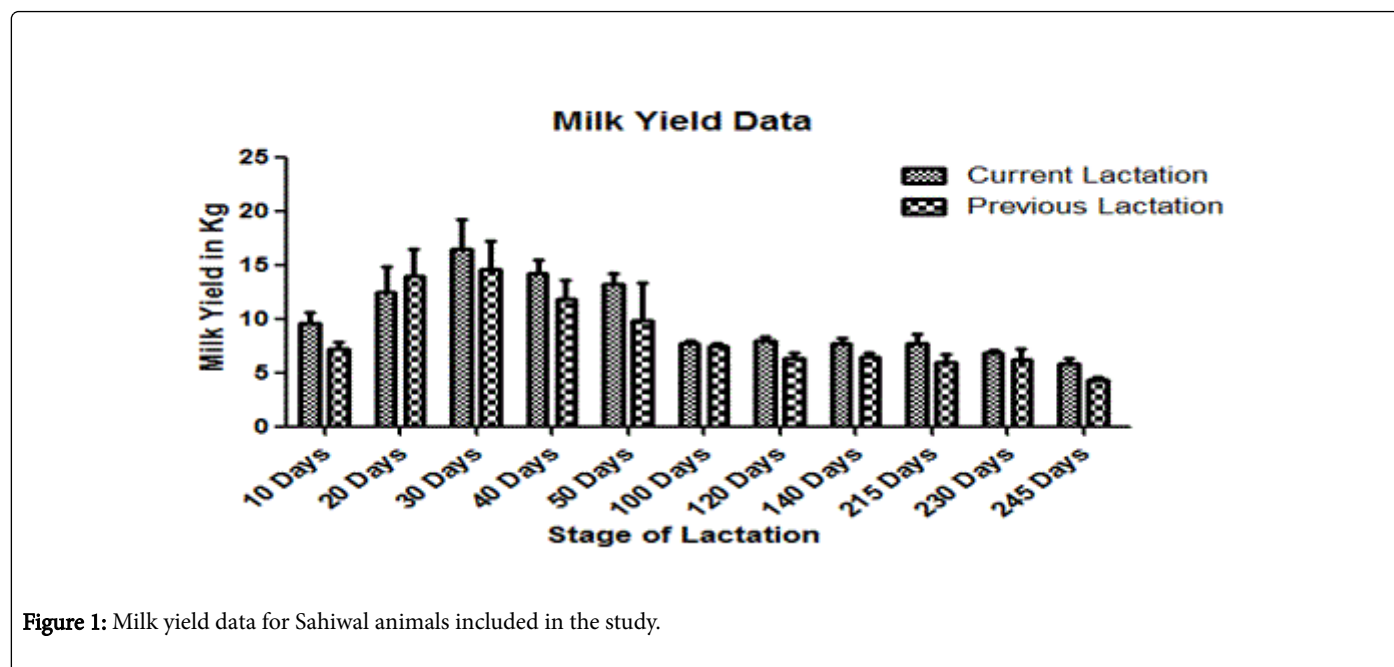

Figure 1: Milk yield data for Sahiwal animals included in the study.

Results and Discussion

Milk yield is reliant on amount of glucose uptake by the mammary epithelial cells (MEC), which is directly proportional to the number of glucose transporter molecules present on the MEC surface, indicating indirect correlation between milk yield and glucose transport. To understand the relationship between glucose transport and milk yield in indigenous cows (Sahiwal breed), expression pattern of important facilitative GLUT genes SLC2A1 (GLUT1), *SLC2A3 (GLUT3), SLC2A4 (GLUT4), SLC2A5 (GLUT5), SLC2A8 (GLUT8)*, and *SLC2A12 (GLUT12)* and a potential regulator of GLUT, Hexokinase 2 (*HK2*) gene were assessed in MEC during different lactation stages of Sahiwal cows.

Milk yield pattern across lactation cycle

Milk yield data from Sahiwal cows collected for two consecutive parities showed a constant increase after early lactation phase until a peak was observed at 30-50 days of lactation. This was followed by gradual decline in the milk yield all along the time points of lactation period included under the study. The typical lactation pattern observed during this study is presented as Figure 2.
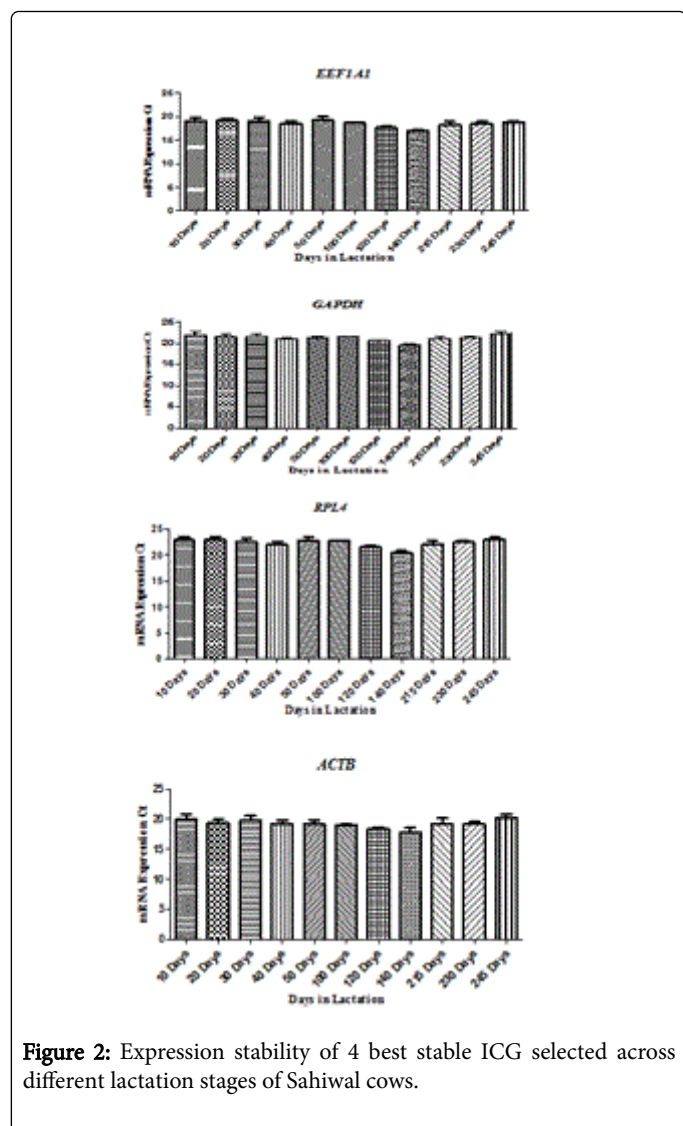

Figure 2: Expression stability of 4 best stable ICG selected across different lactation stages of Sahiwal cows.

Expression analysis of facilitative solute carrier genes

Glucose acts as prime precursor for lactose synthesis and its demand increases with increase in milk production. Glucose uptake in mammary epithelial cells is facilitated by glucose transporters (GLUTs). Our data showed that, among the different solute carrier genes, SLC2A1/GLUT1 gene transcript was upregulated within the first 3 weeks following initiation of lactation (10-20 days in lactation) (Figure 3) and remained elevated during peak/mid lactation period (30-140 days in lactation). However, the transcripts declined significantly (P<0.05) during the late lactation period (215-245 days in lactation) (Figure 3). The overall expression pattern of SLC2A/GLUT 1 coincided with lactation curve. The upregulated expression of SLC2A1/

GLUT1 gene could be attributed to increased demand for glucose during peak and mid lactation period. Komatsu et al. [23] also observed increased GLUT1 expression in mammary gland of peak- and late-lactating cows whereas in non-lactating cows expression of GLUT1 was barely detectable. Earlier, GLUT1 has been shown to be the major GLUT isoform expressed in the bovine mammary gland and its expression was found to be developmentally regulated [24,25]. GLUT1 expression in the bovine mammary gland has shown more than 100-fold increase at 7 days post calving in comparison to 40 days before parturition [26]. Because of the ubiquitous distribution and cellular localization, GLUT1 is considered to be the primary transporter responsible for basal glucose uptake in other tissues as well mammary epithelial cells [23,27].
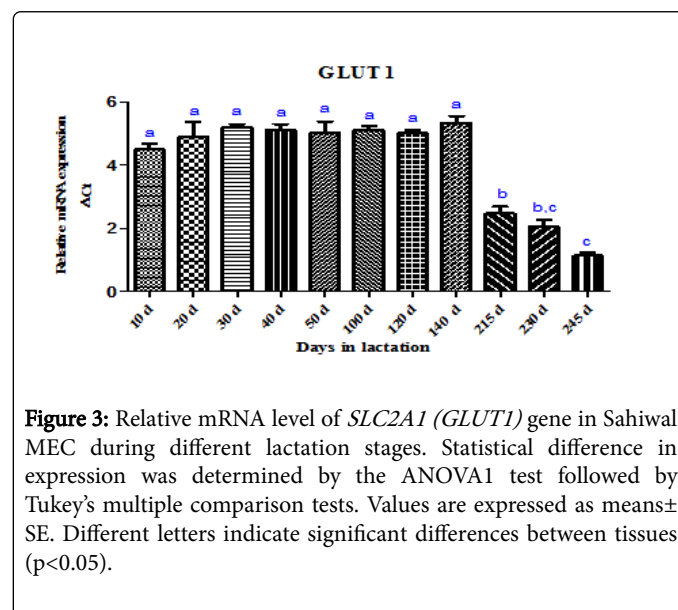

Figure 3: Relative mRNA level of *SLC2A1 (GLUT1)* gene in Sahiwal MEC during different lactation stages. Statistical difference in expression was determined by the ANOVA1 test followed by Tukey's multiple comparison tests. Values are expressed as means± SE. Different letters indicate significant differences between tissues (p<0.05).

Another major solute carrier SLC2A8 (*GLUT8*) that plays significant role in glucose transport in mammary gland indicated higher expression during mid-lactation period (100-140 days) in comparison to early and late lactation period (Figure 4). Unlike SLC2A1 (*GLUT1*), SLC2A8 (*GLUT8*) mRNA level was relatively high during the late lactation period. Zhao et al. [14] showed that GLUT8 mRNA in bovine mammary gland is developmentally regulated, with increased expression (>10-fold) during pregnancy and lactation in both mouse and cows.

Though the complete understanding of regulation of *GLUT8* expression is lacking, it is presumed that its expression in mammary gland is regulated by progesterone (mamogenic hormone) and prolactin (lactogenic hormone). Glucose starvation of differentiated 3T3-LI adipocytes resulted in relatively reduced *GLUT8* mRNA levels, that were restored by addition of glucose [28]. In human and bovine, *GLUT8* transcript was reported to be predominantly expressed in the testis, consistent with the glucose supply to mature spermatozoa [29]. The high level of expression of *GLUT8* in the present study indicated that *SLC2A8(GLUT8)* in conjunction with *SLC2A1* (*GLUT1*) has an important physiological role in mammary system to support milk production. Another transporter *SLC2A4* (*GLUT4*) mRNA expressed a little different pattern than *SLC2A1* (*GLUT1*) and *SLC2A8(GLUT8)* genes. *SLC2A4* expression was significantly (p<0.05) higher at 30 days of lactation and low during early and mid-stages of lactation period (Figure 5). Unlike *SLC2A1*, its expression was relatively high during

late lactation stages (>215 days). The higher *SLC2A4* mRNA abundance could be linked to its distinct physiological role during the late lactation stages. Its increased level during might influence mammary tissue making it more sensitive to increased level of insulin during the late lactation stages and allowing mammary gland to remodel towards involution [30-32].

Figure 4: Relative mRNA level of *SLC2A8* (*GLUT8*) gene in Sahiwal MEC during different lactation stages. Statistical difference in expression was determined by the ANOVA1 test followed by Tukey's multiple comparison tests. Values are expressed as means± SE. Different letters indicate significant differences between tissues (p<0.05).

The expression of *SLC2A12* (*GLUT12*) gene could not be analyzed as its expression was undetectable in several of the MEC samples, suggesting that *SLC2A12* is either not expressed or expressed at very low levels in Sahiwal MEC. The present observation was also supported by findings of Chandler, et al. [33] who reported low levels of *GLUT12* expression in normal breast tissue [33].

Further, expression of *HK2* that is considered to have a putative role in controlling the glucose transport rate in Sahiwal MEC was also evaluated. It was observed that this gene is expressed in MEC of Sahiwal cows all along different lactation stages. *HK2* expression was significantly (P<0.05) higher during early and mid-lactation stages as compared to late lactation stages (215 and 245 days) (Figure 6). During early period of lactation, its level increased continuously from 10 days till 30 days in lactation and this high level was maintained from 50 days to 140 days. Till now, four isozymes (*HK1* to *HK4*) of hexokinases have been reported. The major role of HKs is in phosphorylation of glucose to form glucose-6-phosphate for metabolic utilization. These HKs possess the tissue-specific expression pattern and differs in catalytic and regulatory properties [34]. Yamada *et al.* [35] has reported that there exists a positive relationship between HK activities and glucose uptake. Recently, Zhao et al. [36] have also shown that bovine MEC grown in higher glucose concentrations (5, 10 and 20 mM) had higher HK activities. Though, expression of *HK1* and *HK2* has been evaluated in lactating rat mammary gland [37] but their expression profile in lactating bovine mammary gland is still unclear. It is a known fact that GLUTs and HKs are involved in the control of glucose transport rate but their relationship in the regulation of glucose transport is still unclear. Recently Zhao, et al. [36] reported that mRNA of *HK1* is not detected in bovine MEC in cell culture condition, whereas *HK2* is expressed at a relatively high level. On the basis of this study we analyzed *HK2* mRNA in Sahiwal MECT he findings revealed that this gene is expressed in MEC of Sahiwal cows

all along the lactation period. Its level increased continuously from 10 days to 30 days in lactation and from 50 days to 140 days, its high level was maintained. Various roles have been assigned to *HK2*, *viz.,* role in the process of anabolism [38,39] and glucose transport [40-44]. Our study corroborated with the findings of Ong et al. [40] and Chehtane et al. [41] and indicated that over expression of *HK2* could induce the increase in glucose uptake.
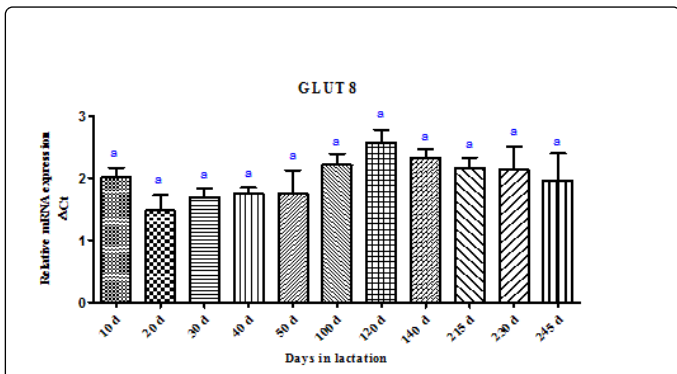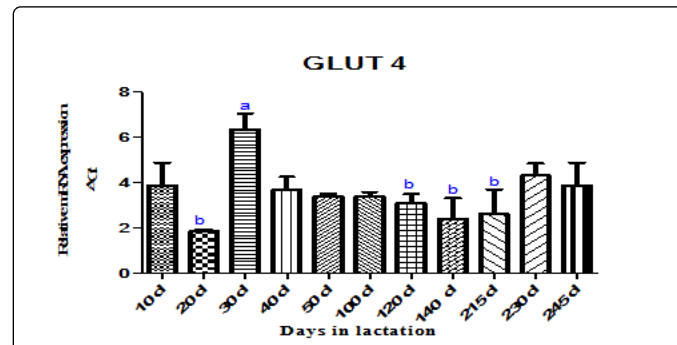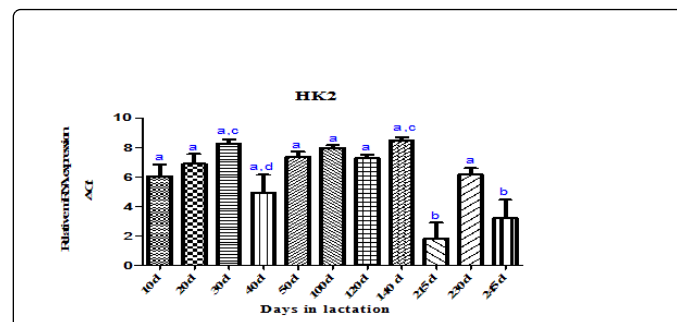

Figure 5: Relative mRNA level of *SLC2A4* (*GLUT4*) gene in Sahiwal MEC during different lactation stages. Statistical difference in expression was determined by the ANOVA1 test followed by Tukey's multiple comparison tests. Values are expressed as means± SE. Different letters indicate significant differences between tissues (p<0.05).

Figure 6: Relative mRNA level of *HK2* gene in Sahiwal MEC during different lactation stages. Statistical difference in expression was determined by the ANOVA1 test followed by Tukey's multiple comparison tests. Values are expressed as means ± SE. Different letters indicate significant differences between tissues (p<0.05).

Conclusion

The genesis of the present study stems from the fact that *SLC2A/GLUT* expression pattern was not understood in MEC of Indian native cows. As glucose uptake from the blood is a crucial step required for milk production, it was important to evaluate the expression of *SLC2A/GLUT* in MEC during the lactation cycle in Indian cows. The expression pattern of three of the four major facilitative glucose transporters and hexokinase 2 genes indicated their functional role in regulating glucose uptake in mammary epithelial cells of Sahiwal cows.

Acknowledgements

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