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Research Article

Understanding Na^+/K^+-ATPase alpha isoforms expression characteristics in heat stressed mammary epithelial cells of riverine buffaloes (*Bubalus Bubalis*)

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Abstract

Na^+/K^+-ATPase gene is known to maintain the electrochemical gradient of Na^+ and K^+ ions across the plasma membrane and regulates the transport of metabolites and nutrients. Recently, this gene has been linked with thermotolerance trait in dairy animals. Several anatomical and physiological evidences for adverse impact of summer stress on riverine buffaloes have been reported. In contrary, the information related to molecular and cellular mechanism involving heat stress is not well defined in riverine buffaloes. In the present study, an attempt was made to assess the expression pattern of four isoforms namely ATP1A1, ATP1A2, ATP1A3, and ATP1A4 of Na^+/K^+-ATPase gene in heat stressed buffalo mammary epithelial cells (MECs). The MECs were exposed to heat stress at 42°C for 1 hour. The cells were subsequently allowed to recover at 37°C and harvested at different time points. The qPCR data was normalized using RPL4, RPS23 and EEF1A1 reference genes that were identified previously for similar experimental condition. The buffalo MECs data showed induction of ATP1A1/ATP1A2 isoforms immediately post heat stress and reached to maximum level (4.65/1.84 folds) at 4 hr post stress. The expression pattern of ATP1A1/ATP1A2 mRNA in heat stressed buffalo MECs at different time points was also compared to that of HSP70, HSP90 and HSP60 transcripts. Among all, HSP70 gene was most responsive to heat stress, followed by ATP1A1 gene and the expression pattern of ATP1A1/ATP1A2 mRNA were positively correlated with HSPs expression data. The differential induction of ATP1A1/ATP1A2 mRNA post heat stress in MECs showed these isoforms to be heat responsive and could be utilized in future as an additional marker to understand the cellular tolerance of dairy animals to heat stress.

Keywords: *Na^+/K^+-ATPase, Electrochemical gradient etc.*

1. Introduction

Na^+/K^+-ATPase (sodium pump) is an integral plasma membrane protein complex associated with the active transport of Na^+ and K^+ ions across plasma membrane so as to maintain the transmembrane electrochemical potential (Lingrel and Kuntzweiler, 1994; Blom *et al*., 2011). This gene is composed of three subunits alpha (α), beta (β) and gamma (γ) subunits (Kaplan, 2002). The alpha subunit is the major functional protein and has four isoforms, α1, α2, α3, and α4, encoded by *ATP1A1*, *ATP1A2*, *ATP1A3*, and *ATP1A4* genes respectively (Shamraj and Lingrel, 1994; Underhill *et al*., 1999). Considering Na^+/K^+-ATPase an important protein for its role in maintaining the equilibria of ions, any altered activity of this protein under stress can have deleterious effects in the basal metabolism and cellular functioning (Arnaiz and Ordieres, 2014). Recent studies have reported the association of different isoforms of Na^+/K^+-ATPase with thermotolerance in dairy cattle (Bernabucci *et al*., 2002; Liu *et al*., 2011; Wang *et al*., 2012, Deb *et al*., 2015). Yang *et al.* (2007) have shown that Na^+/K^+-ATPase activity is associated with heat resistance ability with a high heritability of 0.53 in cattle. These studies have suggested the role of Na^+/K^+-ATPase as a potential candidate gene associated with heat tolerance trait in dairy animals.

To date, expression characteristics of important heat stress associated genes is not well understood in riverine buffaloes (*Bubalus bubalis*). Buffaloes in India is the major dairy species contributing more than 50% of country's total milk production. Though buffaloes have evolved in the hot arid region of India but due to its anatomical and physiological characteristics, both production and reproduction performance of this dairy

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animal suffers to a great extent due to summer stress. It has been shown in recent past that mammary epithelial cells (MECs) derived from mammary gland could serve as appropriate *in-vitro* model for evaluating the adverse impact of heat stress on mammary cells functioning (Collier *et al*., 2008; Kapila *et al*., 2013(a)). Taking into consideration the importance of MECs as major milk secretary cells and Na^+/K^+-ATPase gene as plausible marker for heat stress, the present study was planned to assess the temporal expression characteristics of different alpha isoforms of Na^+/K^+-ATPase genes in buffalo MECs exposed to heat stress *in-vitro*.

2. Materials and Methods

2.1 Buffalo mammary epithelial cells culturing and heat stress treatment

Mammary epithelial cells were isolated from approximately 5.0 gm of mammary gland tissue of a healthy adult buffalo from an abattoir at New Delhi. The primary MECs were cultured using DMEM/F12, supplements and growth conditions as described earlier (Kapila *et al*., 2013(a)). The purity of mammary epithelial cells was evaluated using immune fluorescence staining using cytokeratin 18 antibody. After 10th passages, 80% confluent buffalo MECs were distributed in collagen treated 12-well plates (Corning, USA) in two sets with one plate marked as control (kept at 37°C all the time) and other plate as treated. The plate marked as treated was exposed to 42°C for one hour. After 1hr, the cells were allowed to recover at 37°C, 5% CO_2 and harvested at different time points (30m, 2hr, 4hr, 8hr, 12hr, 24hr and 48hr). Cells viability was determined across all time points using MTT assay (Cayman). The cells were then trizolated in ice-cold Trizol (Invitrogen, Carlsbad, California) for RNA extraction.

2.2 Total RNA extraction and cDNA synthesis

Using ice-cold Trizol, total RNA was extracted from MECs harvested at various time points. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to measure the quality and concentration of extracted RNA and samples were stored at -80°C till further usage. cDNA was synthesized using oligo dT, random primer and Superscript III enzyme (Invitrogen Corp. CA) as described by Kapila *et al*., 2013(b). Primer express 3.0 software (Applied Biosystem) was used to design the primer sequences for various alpha isoforms of Na^+/K^+-ATPase gene. The primer details for ATPase isoforms, heat shock proteins (HSPs) and reference genes are given in Table 1.

2.3 Real- time quantitative PCR (qPCR)

The qPCR reaction was performed using 4 µL diluted cDNA combined with 6 µL of a mixture composed of 5 µL 2× Light Cylcer 480 SYBR Green I master mix (Roche, Germany), 0.4 µL each of 10µM forward and reverse primers and 0.2µL DNase/RNase free water in Light Cycler 480 instrument (Roche,Germany). Samples for each primer pair were run in duplicates along with 6 point relative standard curve and non-template control. The PCR conditions followed in the study was: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. A dissociation protocol with an incremental temperature of 95°C for 15 s plus 65°C for 15 s was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. The qPCR expression data for each target gene was extracted in the form of crossing point. The data was acquired using the "second derivative maximum" method as computed by the LightCycler Software 3.5 (Roche Diagnostics) and subjected for subsequent analysis.

2.4 Normalization and data analysis

For normalization of *ATPase* isoforms expression data in heat stressed and unstressed buffalo MECs; *RPL4*, *RPS23* and *EEF1A1* genes were utilized as panel of reference genes. These genes were identified as most stable reference genes in one of our earlier studies for similar experimental condition conducted in buffalo MECs (Kapila *et al*., 2013(b)). Expression fold change in the target genes was calculated using relative quantification $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). ΔCT was calculated by subtracting the geomean of the C_T values of the best reference genes from the C_T values of the target genes i.e. $\Delta CT = C_T$ (target)-C_T (reference genes) and expression fold was calculated using formula $\Delta\Delta CT = \Delta C_T$ (Test sample)-ΔC_T (Calibrator sample). The expression level of *ATPase* alpha isoforms were also compared for different time points post heat stress with previously generated mRNA expression data of heat shock proteins.

3. Results and Discussion

In the present study, the authors were successful in establishing the primary culture of buffalo MECs so as to use it as *in-vitro* model to undertake cellular and gene expression studies related to hyperthermia. The homogeneous and purified MECs was generated after selective trypsinization procedure. The microscopic evaluation showed normal characteristics of mammary epithelial cells (Fig. 1(a)). The buffalo MECs formed a monolayer and reached confluency at 5-6 days of seeding. The cells showed typical cobble stone morphology of MECs. The staining with anti-cytokeratin 18 antibody revealed strong signals indicating that high percentage of cells were of epithelial lineage (Fig. 1(b)). The immunostaining result showed that the primary culture established in the present study mainly consisted of mammary epithelial cells with no contamination of fibroblast cells. The cells were exposed to 42°C for one hour. After completion of incubation period, the cells were allowed to recover at 37°C, 5% CO_2 and harvested at different time points (30m, 2hr, 4hr, 8hr, 12hr, 24hr and 48hr).

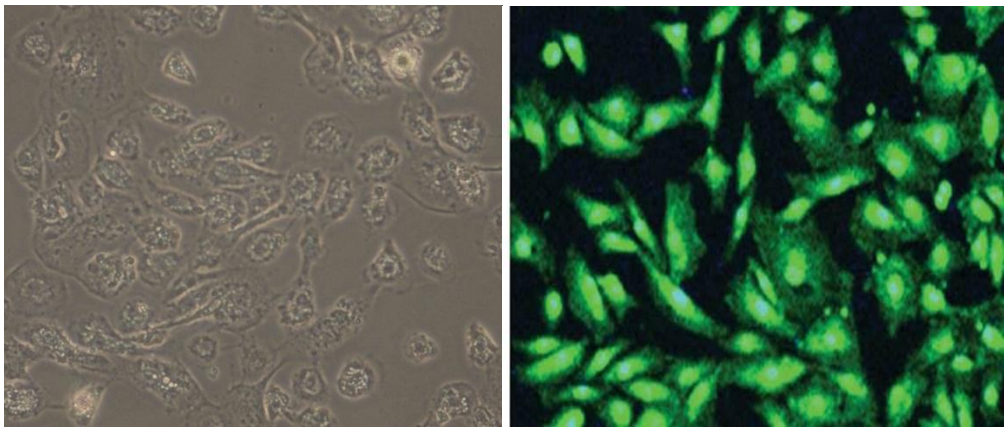

Fig.1: Mammary epithelial cells culture isolated from buffalo mammary gland. (a) Population of epithelial cells after 10th passage (b) Fluorescent image of MECs after staining for cytokeratin 18

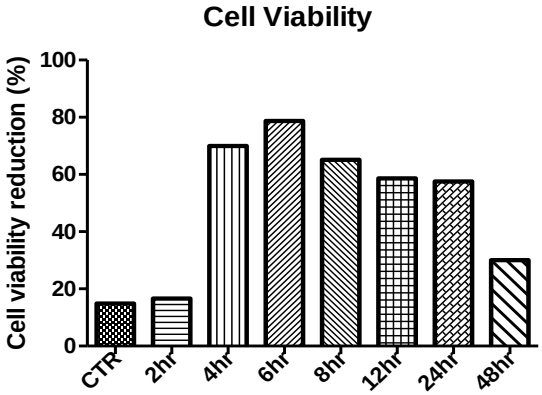

Fig. 2: Rate of cellular viability reduction in control and heat stressed MECs at different time points

Table 1: Gene name, Accession no., primer sequences, annealing temperature (Ta), Amplicon size, PCR efficiency, slope and regression coefficient for the target genes under present investigation.

Gene Symbol	Accession Number	Primers 5'-3' (Forward, Reverse)	T_a (°C)	Amplicon Size (bp)	PCR Efficiency (%)	Slope	R^2 Value
ATP1A1	NM_001076798.1	CAGCAGGGGATGAAGAACAAG GGAAGGCACAGAACCACCA	60	142	102.69	-3.259	0.997
ATP1A2	NM_001081524.1	TAGCTTGGGATGATTGCCCC GGTACCCCTCAGTTCCTTGG	60	130	107.22	-3.136	0.982
ATP1A3	XM_002695074.5	TCTTACTGCCCAGGCATGGATGT AAGGAGGAAAGAGACAGAGACG	60	NA*	-	-	-
ATP1A4	NM_001144103.2	GCCTTCGGCATCCAGTTGTATTTCAAT TTGGAGCTCTTGGCTTCCTG	60	NA*	-	-	-
HSP60	NM_001166610.1	CGACAACTTCTGCTGTTGTTA ATGATGCTATGCTTGGAGAT	60	109	97	-3.389	0.992
HSP70	JN604432.1	AACATGAAGAGCGCCGTGGAGG GTTACACACCTGCTCCAGCTCC	60	171	113	-3.03	0.993
HSP90	AB072368.1	CTGTCATCAGCAGTGGG ACATGCCAACAGGATCTAC	60	74	114	-3.009	0.992
RPL4	NM 001014894	TTGGAAACATGTGTCGTGGG GCAGATGGCGTATCGCTTCT	60	101	93.72	-3.482	0.992
RPS23	BC102049	CCCAATGATGGTTGCTTGAA CGGACTCCAGGAATGTCACC	60	101	115.5	-2.999	0.996
EEF1A1	BC105315	CATCCCAGGCTGACTGTGC TGTAAGCCAAAAGGGCATGC	60	101	115.5	-2.999	0.98

Before and after heat stress, the cells were evaluated for viability and cell proliferation rate and observed continuous decline in both the parameters post heat stress time points. (Fig. 2).

In order to assess the time dependent induction of different isoforms of Na^+/K^+-ATPase gene, the mRNA level of *ATP1A1*, *ATP1A2*, *ATP1A3* and *ATP1A4* isoforms were measured in heat stressed buffalo MECs. In buffaloes MECs, only *ATP1A1* and *ATP1A2* transcripts were found to be expressed at substantial level in unstressed as well as in stressed conditions while the expression of *ATP1A3* and *ATP1A4* isoforms

was negligible during pre-stress and post heat stress as *ATP1A3* is nervous specific and *ATP1A4* is testis specific isoform (Shamraj and Lingrel, 1994; Blanco and Mercer, 1998; Underhill *et al*., 1999; Edwards *et al*., 2013). Due to the reason, data related to *ATP1A3* and *ATP1A4* isoforms were not taken into consideration for further analysis. qPCR performance in terms of coefficient of determination (R^2) and efficiency of amplification ($E = 10^{-1/slope}$) on the basis of slope of six-point standard curve of the target as well as reference genes is illustrated in the Table 1 (Fig. 3 and 4).

Presence of single peak assured the specific amplification for each gene. For normalization of mRNA levels in buffalo MECs; *RPL4*, *RPS23* and *EEF1A1* were used (Kapila *et al*., 2013(b)).

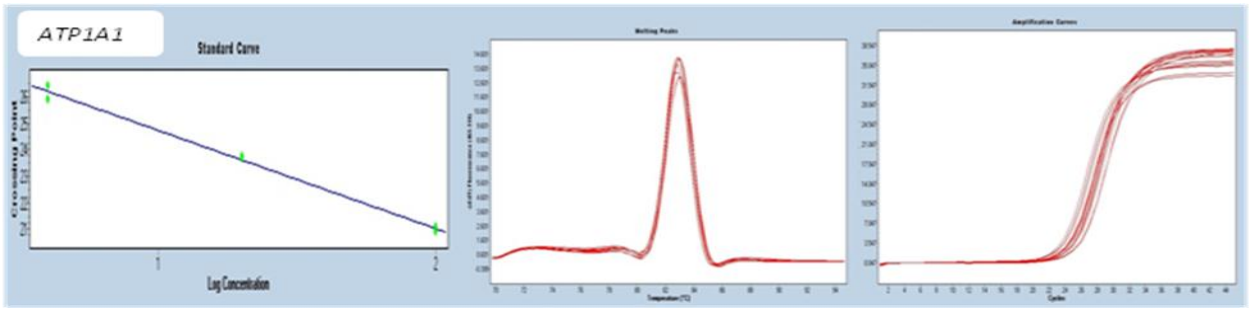

Fig. 3: Standard curve, melt curve and amplification plot of *ATP1A1* isoform

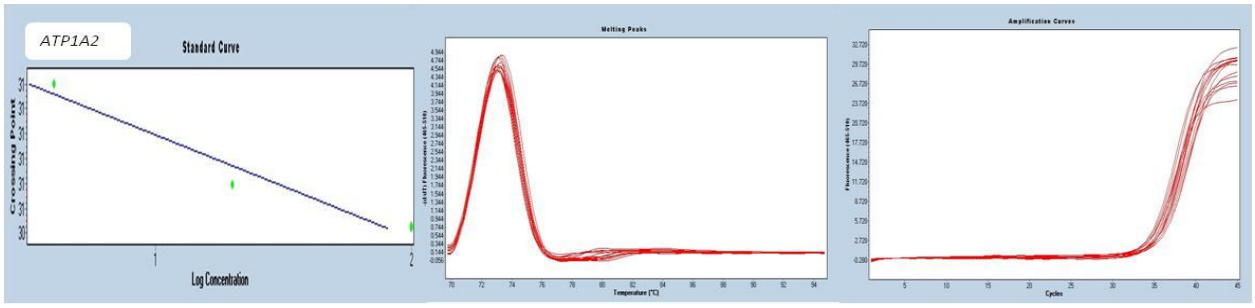

Fig. 4: Standard curve, melt curve and amplification plot of *ATP1A2* isoform

Our qPCR expression data showed immediate increase in *ATP1A1* and *ATP1A2* mRNA levels in buffalo MECs in response to heat stress (Fig.5). Both the isoforms showed substantial induction immediately after heat stress (30 m post heat stress), reached maximal level at 4 hr and declined gradually thereafter before reaching close to the basal level (Fig.5). The *ATP1A1* transcript showed 2.612, 3.219, 4.659, 3.102, 2.112, 1.982, fold of induction at different time points post heat stress *viz*., 30m, 2hr, 4hr, 8hr, 16hr time points, respectively and then gradually returning close to the basal level (Fig. 5). Similarly, *ATP1A2* mRNA also showed maximal increase in its expression at 4hr post heat stress and remained elevated till 16hr. The *ATP1A2* mRNA induced to 1.558, 1.742, 1.84, 1.327, 1.282, 1.199 folds at 30m, 2hr, 4hr, 8hr, 16hr time points, respectively (Fig. 5). As expected, the expression of both the transcripts were lower in unstressed samples in comparison to samples exposed to heat stress. At all-time points, *ATP1A1* transcript was expressed at higher level suggesting this isoform to be the dominant form in MECs of riverine buffaloes (Fig.5). Our results indicated the inducible nature of *ATP1A1* and *ATP1A2* transcripts under heat stress condition suggesting their plausible roles in heat stress response and cellular protection.

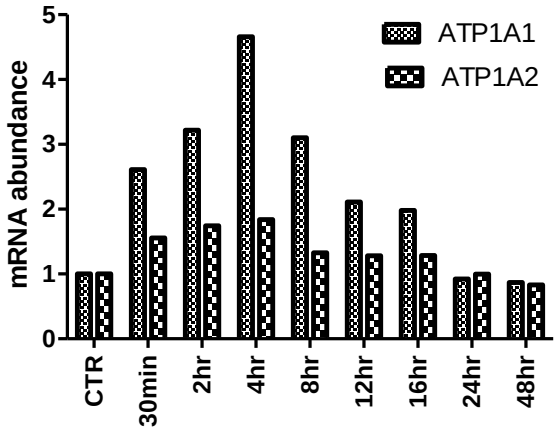

Fig 5: Expression profile of Na^+/K^+-ATPase alpha isoforms in heat stressed buffalo MECs at different time points. X-axis represents the time points and Y-axis represents the mRNA abundance

Considering the fact that two isoforms of Na^+/K^+-ATPase gene showed induction in response to the heat stress condition, we evaluated the expression characteristics of *ATP1A1* and *ATP1A2* transcripts with some of the major heat shock protein genes (HSPs).

Table 2: Comparative expression profile of *HSP70* and Na⁺/K⁺-ATPase alpha isoforms in heat stressed MECs at various time points

	HSP70	***ATP1A1***	***ATP1A2***
CTR	1.000	1.000	1.000
30min	33.94201	2.612	1.558
2hr	61.03931	3.219	1.743
4hr	72.50457	4.659	1.840
8hr	50.6796	3.102	1.327
12hr	4.901874	2.112	1.282
16hr	0.940609	1.982	1.285
24hr	0.581023	0.921	0.998
48hr	0.825496	0.870	0.831

Table 3: Degree of relationship (correlation coefficient) between Na⁺/K⁺-ATPase alpha isoforms and HSP genes

	ATP1A2	*ATP1A1*	*HSP90*	*HSP70*	*HSP60*
ATP1A2	1	.932**	.894**	.870**	.861**
ATP1A1	.932**	1	.904**	.927**	.949**
HSP90	.894**	.904**	1	.837**	.941**
HSP70	.870**	.927**	.837**	1	.892**
HSP60	.861**	.949**	.941**	.892**	1

**. Correlation is significant at the 0.01 level (2-tailed).

The expression data set for different HSPs (*HSP60*, *HSP70* and *HSP90*) in the same experimental conditions generated earlier (Kapila *et al*., 2013(a)) was utilized to correlate with expression behaviour of *ATP1A1* and *ATP1A2* isoforms. The combined data set of HSPs and Na⁺/K⁺-ATPase isoforms under heat shock conditions suggest that temporal pattern of all these genes get induced substantially, albeit at different levels (Fig 6 and Table 2).

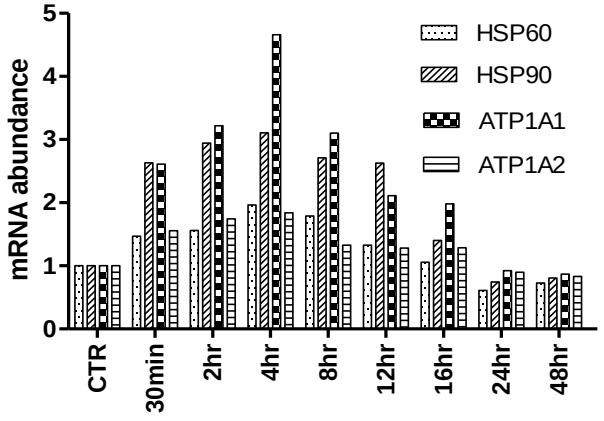

Fig. 6: Comparative expression pattern of heat shock proteins and Na⁺/K⁺-ATPase alpha isoforms in heat stressed buffalo MECs at various time points. X-axis represents the time point and Y-axis represents the mRNA abundance

Between the two gene class, *HSP70* transcript was most sensitive to heat stress as it showed the highest expression fold change followed by *ATP1A1* mRNA (Table 2). The relationship between Na⁺/K⁺-ATPase isoforms and HSP genes is described in terms of Pearson correlation coefficient (r) and the p value. $p<0.05$ was obtained for all genes indicating a positive correlation (Table 3). The observed correlation between Na⁺/K⁺-ATPase alpha isoforms and HSP genes suggest their common functional role in providing cellular protection during heat stress in buffalo MECs.

To our best knowledge, this is the first report to determine the expression kinetics of Na⁺/K⁺-ATPase alpha isoforms in buffalo MECs under heat stress. The transcriptional changes observed in alpha isoforms are in accordance with earlier studies wherein significant alterations in concentrations of Na⁺ and K⁺ ions and Na⁺/K⁺-ATPase activity under heat stress were reported (Srikandakumar and Johnson, 2004; Pearce *et al*., 2011; Deb *et al*., 2015). Recent studies have also suggested association between genetic variants of Na⁺/K⁺-ATPase (alpha and beta) subunits and heat tolerance traits in dairy cattle (Liu *et al*., 2010; Liu *et al*., 2011; Wang *et al*., 2011; Das *et al*., 2015). The increased synthesis of Na⁺/K⁺-ATPase alpha isoforms under heat stress observed in this study in conjunction with some of the recent studies strongly point towards the critical role that Na⁺/K⁺-ATPase gene might be playing in providing cellular protection to heat stress in dairy animals.

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