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Research Article

Chemically induced low oxygen condition leads to significant induction of hypoxia-related genes in mammary epithelial cells of riverine buffaloes (*Bubalus bubalis*)

Preeti Verma[1#], Umesh K Shandilya[1], Monika Sodhi[1], Ankita Sharma[1], Pawan K Tripathi[#], and Manishi Mukesh[1*]

[1]ICAR-National Bureau of Animal Genetic Resources, Karnal, Haryana, India
[#]Singhania University, Jhunjhunu, Rajasthan, India

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Abstract

Hypoxic condition is known to influence the cell survivability by inhibiting its metabolism and growth. Till date, no information is available on how the mammary epithelial cells (MECs) of dairy species respond to chemically induced hypoxia. This study was therefore planned to assess the transcriptional responsiveness of mammary epithelial cells (MECs) of riverine buffaloes- the major dairy species of India to chemically induced hypoxic condition. Initially, the primary MECs culture from buffalo mammary gland tissue was established and thereafter different doses of $CoCl_2$ (50 µM, 100 µM and 250 µM) were evaluated for inducing hypoxia in MECs under culture condition. The 250 µM of $CoCl_2$ was selected and cells were grown for 48 h before assessing the expression of HIF-1α and its target genes in treated (hypoxic) as well as in control (normoxic) cells. The mRNA expression of all studied genes viz; HIF-1α, GLUT-1, GLUT-8, HK2, JAK2 and STAT5 was found to be significantly induced in hypoxic condition in comparison to control condition. The present investigation suggests that $CoCl_2$ induced hypoxia significantly alters the gene expression of hypoxia related genes in buffalo MECs. Such model can be utilized in future for studying the impact of hypoxia stress in mammary gland development and function of dairy species.

Keywords: *Hypoxia, Mammary epithelial cells, Gene expression, Riverine buffaloes*

1. Introduction

Oxygen concentration is an important physiological and pathological regulator that influences the living organisms by affecting their cellular metabolism and growth. Reduction in O_2 supply leads to hypoxia that alters the normal metabolic and cellular responses by concomitant depletion of intracellular ATP (Li *et al.,* 2007). The persistent and prolonged disruption of energy production might lead to growth arrest and/or apoptosis (Di Carlo *et al.,* 2004). As a consequence, to counteract O_2 deficiency, cells activate a variety of adaptive mechanisms such as reduced energy consumption, cell cycle arrest and secretion of survival and pro-angiogenic factors (Majmundar *et al.,* 2010). In addition, hypoxia also triggers changes in gene expression mediated by *HIF-1* (hypoxia-inducible factor-1); master regulator of the hypoxia response that plays an important role in maintaining O_2 homeostasis by facilitating O_2 delivery to the respiring tissues (Semenza *et al.,* 2010; Wenger *et al.,* 2002; Lendahl *et al.,* 2009). The active *HIF-1* also induces expression of various genes involved in angiogenesis and glucose transport (Semenza, 2000). The *HIF-1* mediated induction of *GLUT-1*, *GLUT-8* and *HK-2* has been suggested to play an important role in ATP synthesis required to carry out the cellular metabolic processes during hypoxia (Sarkar *et al.,* 2003).

Several livestock species like yak, cattle, dzo (a yak-cow cross breed), sheep, goat, donkey, horse, double hump camel are well adapted at high elevation of Leh and Ladakh region of India since centuries. These animal genetic resources have developed certain anatomical, physiological and genetic capabilities that help them to survive and produce under high-altitude hypoxia environments. These livestock inhabiting the inhospitable terrains play a major role in providing food and nutritional security to the human population living in Leh and Ladakh. Interestingly, this particular ecosystem (cold arid zone) does not harbour riverine buffaloes (*Bubalus bubalis*), the major dairy species of India, for reasons unknown till date. In the present study, an effort was made to observe the effect of *in vitro* induced hypoxia on buffalo mammary epithelial cells (MECs) that are major cell types regulating milk

*Corresponding Author: Dr ManishiMukesh, Principal Scientist and ICAR-National Fellow, Department of Animal Biotechnology, ICAR-National Bureau of Animal Genetic Resources, Karnal

production and mammary gland function The MECs being able to synthesize and secrete milk are always considered to be unique cell types in ruminants (Canovas *et al.*, 2014) and MECs derived from mammary gland of various livestock species have been widely used to understand the mechanism involved in mammary gland development and functions (Li *et al.,* 2009; Hu *et al.,* 2009; Zhao *et al.,* 2010; Anand *et al.,* 2012; Shandilya *et al.,* 2016). In recent past, our group has successfully utilized buffalo mammary gland derived MECs to understand the impact of heat stress on its cellular and transcriptomic adaptation (Kapila *et al.*, 2013a; Kaur *et al.,* 2016; Kapila *et al.*, 2016). However, its response towards hypoxia is poorly understood. The aim of the present study was to assess the impact of chemically induced hypoxia on expression pattern of buffalo specific MECs. The experimental plan of present study demanded the creation of hypoxic condition *in vitro*. Several metals such as cobalt, nickel, cadmium, zinc *etc*. have been reported to induce hypoxia and therefore are known as hypoxia-mimetic agents (Bansal *et al.,* 2009).

Amongst all these agents, the present study utilized cobalt chloride ($CoCl_2$) to induce hypoxic environment *in vitro* considering the fact that, it is one of the classic examples of a hypoxia mimetic agent and has been used widely to induce hypoxia like condition (Bansal *et al.,* 2009; Wu and Yotnda, 2011). $CoCl_2$ has been reported to be a strong chemical inducer of hypoxia-inducible factor-1 (Piret *et al.*, 2002). Here we report for the first time the responsiveness of bubaline mammary epithelial cells (BuMECs) to $CoCl_2$ induced hypoxic condition in terms of transcriptional changes of hypoxia associated gene transcripts *viz;* hypoxia inducing factor-1 (*HIF-1*), glucose transporter-1 (*GLUT-1*), glucose transporter-8 (*GLUT-8*), hexokinase2 (*HK2*), signal transducer & activator of transcription 5 (*STAT-5*) and janus kinase 2 (*JAK2*) .

2. Materials and Methods

2.1. Culturing of mammary epithelial cells and hypoxic stress treatment

Mammary tissue of adult riverine buffalo was bought from slaughter house at Gajipur, New Delhi in Dulbecco's Modified Eagle's Medium/Ham's F12 media (Hyclone, Logan, UT, USA) containing 100 U/ml penicillin-streptomycin antibiotics (Hyclone, USA). The tissue sample was washed 2-3 times with phosphate buffer saline (PBS, Ca^{2+}, Mg^{2+} free) (Hyclone, USA) and was cut into fine pieces and washed again with PBS. The smaller pieces were then cultured in collagen-coated cell culture dishes (Corning, USA), containing growth media DMEM/F12 (Hyclone, USA) supplemented with 10% FBS, antibiotic–antimycotic solution (1X), 5 µL/ml insulin, 50 µM hydrocortisone, 1 µg/ml beta-estradiol, 5 µg/ml holotransferrin, and 1 µg/ml progesterone (Sigma, USA). Initially, the media was replaced after every 12h and then again after every 48h until cells visibly spread across the bottom of the culture dish. At about 75–80% confluency, the cells were detached with 0.25% trypsin–0.02% EDTA (Sigma–Aldrich, USA), and transferred to T25 and T75 culture flasks (Corning, USA). Using selective trypsinization steps, MECs were enriched and fibroblast cells were removed from the culture. The cells were grown and morphological assessment of typical mammary epithelial cells was carried out.

To induce hypoxia, pure culture of MECs was trypsinized and seeded at concentration of 10^5 cell/ml in a 12 well culture plate having different concentration of Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$, MW=237.9) (50 µM, 100 µM and, 250 µM) for 48h. Cells were then detached with 0.25% trypsin containing 0.02% EDTA (Sigma-Aldrich) and pelleted down by centrifugation for RNA extraction.

2.2. RNA extraction and cDNA synthesis

Using ice-cold Trizol, total RNA was extracted from Buffalo MECs according to the manufacturer's instructions (Invitrogen, Corp., CA). The traces of genomic DNA were removed by RNase free DNase treatment (Qiagen, Germany) using RNeasy Mini kit columns (Qiagen, Germany). The quality and concentration of extracted RNA was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized using oligodT, random primer and Superscript III enzyme (Invitrogen Corp. CA) as described by Kapila *et al.* (2013b). The reaction was performed in an Eppendorf Gradient cycler using the program: 25^0C for 5 min, 50^0C for 60 min and 70^0C for 15 min. cDNA was then diluted 1 : 4 (v/v) with nuclease-free water. The qPCR primers details for various genes are provided in table 1.

2.3. Real time quantitative PCR (q-PCR)

The qPCR reactions were carried out in 96 well transparent plate (Thermo, USA) using Applied Biosystems StepOne plus Real-Time PCR (ABI, California). The qPCR mixture comprised of 4 µl diluted cDNA, 0.4 µl each of 10 µM forward and reverse primers, 0.2 µl nuclease free water and 5 µl Maxima SYBR Green/ROX qPCR master mix (2x) (Fermentas Thermo, USA). The PCR conditions were as follow: 10 min at 95^0C, 40 cycles of 15s at 95^0C (denaturation) and 1 min at 60^0C (annealing+ extension). A dissociation protocol with an incremental temperature of 95^0C for 15s plus 65^0C for 15s was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. The qPCR data of target genes was normalized using geometric mean of stably expressed *GAPDH* and *β-actin* genes. The qPCR performance of each gene in terms of PCR efficiency of amplification ($E = 10^{-1}$/slope) was determined on the basis of slope of standard curve as illustrated in table 1.

2.4. Statistical analysis

To compare differences in the expression levels of different genes in hypoxic and normoxic conditions, the delta CT values were analysed using one-way ANOVA test followed by Tukey's test, and P-value of ≤ 0.05 was considered statistically significant. All data are presented as the mean ±SEM.

Table 1: Gene symbol, GeneBank accession numbers, primer sequences, annealing temperature (Ta), amplicon size and PCR efficiency of target genes

Gene Symbol	NCBI Accession no	Primers sequence (5' Forward, 3'Reverse)	Ta PCR (^{0}C)	Amplicon size (bp)	PCR Efficiency (%)
HIF-1α	NM_174339.3	F:5'TGAAGGCACAGATGAATTGC3' R:3'GTTCAAACTGAGTTAATCCC5'	60	129	102
GLUT-1	NM_174602.2	F:5'TCCACAAGCATCTTCGAGAA3' R:3'AATAGCGACACGACAGTGAA5'	60	98	89.8
GLUT-8	AY208940	F:5'GCCTCTGCCTTCTGCATCTT3' R:3'TCATGATGGAGCCGGCATGT5'	60	101	98.6
HK2	XM_002691189	F:5'AAGATGCTGCCCACCTACG3' R:3'TCGCTTCCCATTCCTCACA5'	60	123	108
STAT5	NM_001012673.1	F:5'GTGAAGCCACAGATCAAGCA3' R:3' TCGAATTCTCCATCCTGGTC5'	60	176	96.8
JAK2	XM_006068205.1	F:5'TCTGGTATCCACCCAACCATG3' R:3'AATCATGCCGCCACTGAGCAA5'	60	201	102.6

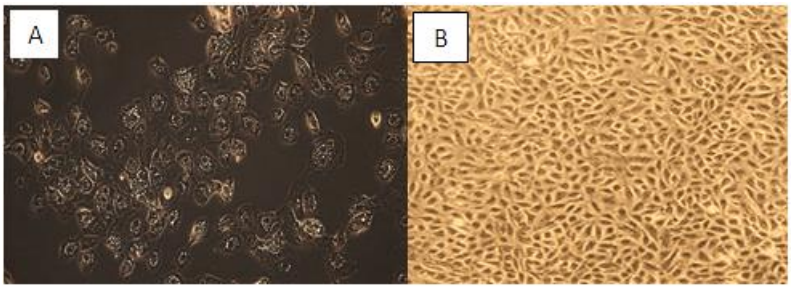

Fig 1: Images showing morphological changes of buffalo mammary epithelial cells (BuMECs) grown in chemically induced hypoxic environment (A) in comparison to cells grown in normal cell culture condition (B)

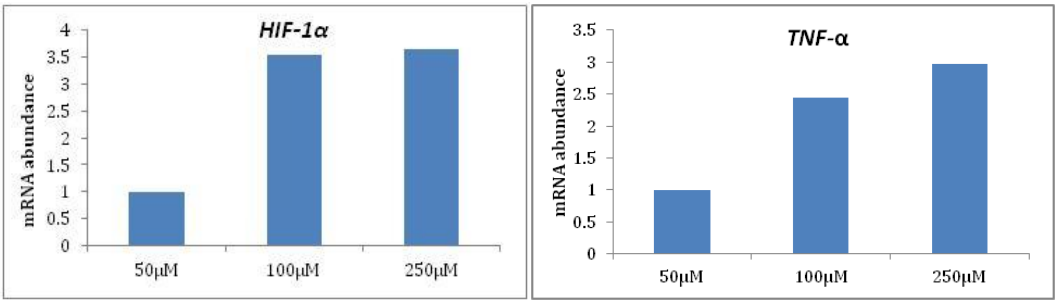

Fig 2: Evaluation of different concentration of cobalt chloride on mRNA expression of *HIF-1α* and *TNFα* genes in bubaline mammary epithelial cells (BuMECs)

3. Results and Discussion

3.1. Evaluation of $CoCl_2$ concentration in inducing hypoxia like condition in BuMECs

The main aim of this study was to assess whether low-oxygen condition generated by $CoCl_2$ affects the mRNA expression of hypoxia related genes in buffalo mammary epithelial cells (MECs). The primary culture derived from buffalo mammary gland (BuMECs) was successfully established. The heterogeneous populations of epithelial and fibroblast-like cells derived from mammary tissue were subjected to selective trypsinization procedure, fibroblast cells, being more sensitive to trypsin treatment were removed and subsequently purified homogeneous population of MECs was obtained. Under microscope, these cells showed typical characteristics of mammary epithelial cells. The monolayer culture of buffalo MECs grown under normoxic and hypoxic conditions is shown in figure 1.

The cells became confluent on 5th day of seeding and formed a monolayer revealing typical cobble stone morphology of MECs. The purity of MECs was also confirmed using immunostaining with cytokeratin-18 and vimentin antibodies (Kapila *et al.*, 2016). When cells were cultured in the presence of $CoCl_2$, the MECs showed distinct changes in their morphology in comparison to cell frown under normal condition. The treated cells become relatively larger in size and round in shape and after 48h none of the cells in culture showed characteristics epithelial cell morphology (Fig. 1).

To assess the ability of MECs to respond to chemically induced hypoxic condition, the cells were initially exposed to different concentrations (50 µM, 100 µM, 250 µM) of Cobalt (II) chloride hexahydrate. Amongst several agents (cobalt, nickel, cadmium and zinc), known to create hypoxia like condition $CoCl_2$ has commonly been used as a chemical inducer to create hypoxia like condition *in vitro* (Wu and Yotanda, 2011; Piret *et al.*, 2002). Hypoxia like condition generated *in vitro* was verified by examining the changes in expression of hypoxia inducible factor -1 (*HIF-1*) gene. Amongst the 3 doses of $CoCl_2$, maximum induction in expression of *HIF-1*α gene was obtained at 250 µM followed by 100 µM and 50 µM concentration (Fig. 2).

Hence, concentration of 250 µM was selected for treating the cells to achieve maximal response. Further mRNA expression of tissue necrotic factor- α (*TNF-α*)-a pro-inflammatory cytokine was also examined to assess the inflammatory response of BuMECs under hypoxic condition. Similar to *HIF-1α*, the expression of *TNF-α* mRNA was maximally induced in samples treated with highest concentration of $CoCl_2$ (Fig. 2).

3.2. Comparative mRNA expression of hypoxia associated genes in BuMECs grown under hypoxic and normoxic environments

On finalizing the dose, one part of BuMECs was grown for 48h in the presence of $CoCl_2$ at a final concentration of 250 µM to obtain the maximal response. The second part of BuMECs was grown under normal culture condition (untreated; without $CoCl_2$). The untreated BuMECs were used to simulate the normoxia culture condition. The cells were subsequently harvested to assess the expression pattern of *HIF-1α* mRNA and its target genes in treated as well as untreated (control) BuMECs. On comparing the expression pattern, *HIF-1α* mRNA was observed to be elevated (~3.5 folds) in treated BuMECs over the BuMECs taken as normoxic control (Fig. 3). The induction of HIF-*1α* in treated BuMECs was as expected as it is the major transcription factor known to be elevated during hypoxic condition. This gene mediates the expression of several other genes during low oxygen concentration and plays important role in maintaining O_2 homeostasis by facilitating both O_2 uptake and delivery (Semenza, 1999; Lendahl *et al.,* 2009). During hypoxia, *HIF-1α* gets stabilized and is translocated to the nucleus, where it binds to *HIF-1β* (Ivan *et al.,* 2001; Jaakkola *et al.,* 2001). The active *HIF-1α* induces the expression of various hypoxia-responsive genes including vascular endothelial growth factor (*VEGF*), glucose transporter-1 (*GLUT-1*) and several glycolytic enzymes (Sememza *et al.,* 1999). In our study as well, several of the *HIF-1α* target genes like glucose transporters 1 and 8 (*GLUT-1* and *GLUT-8*), hexokinase 2 (*HK2*) showed upregulation in $CoCl_2$ treated BuMECs in comparison to untreated (normoxic control) cells (Fig. 3).

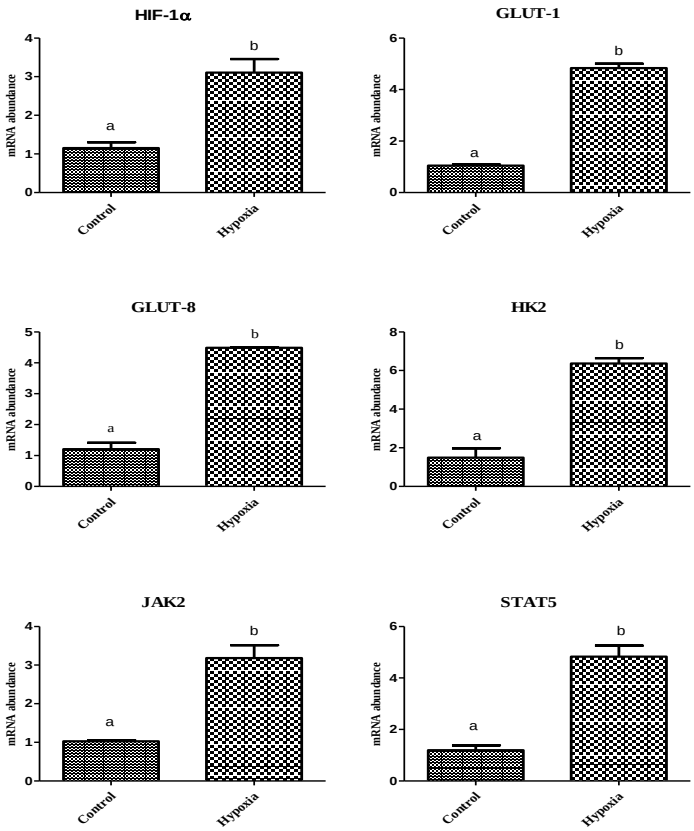

Fig 3: Comparative mRNA expression of hypoxia-related genes under chemically induced hypoxic condition and normoxic condition in buffalo mammary epithelial cells (BuMECs). A significant increase in the expression of all genes was observed in hypoxic condition in comparison to control; error bars with different letters are significantly different at $p<0.05$

Similar to present study, Hayashi *et al.* (2004) also reported higher expression of *HIF-1α* and *GLUT-1* in placental cell line treated with 250 μM of $CoCl_2$ suggesting that upregulation of *GLUT-1* in presence of $CoCl_2$ is mediated by *HIF-1α.* Our results were also consistent with the study by Esterman *et al.* (1997) wherein, under hypoxic condition 3 fold increased expression of *GLUT-1* mRNA in human placental tissue was reported. Further, hypoxia has also been shown to significantly induce the expression of *GLUT1* in rat fibroblast cells (Behrooz and Ismail-Beigi, 1997, Chen *et al.,* 2001). Similar to *HIF-1α* and *GLUT-1*, the present study also showed increased expression of *GLUT-8* mRNA in BuMECs maintained under hypoxic condition (Fig. 3). The induced expression of both the glucose transporters under limited oxygen concentration could be explained by the fact that for generating ATP under hypoxic condition, the cells normally switch their oxidative phosphorylation based glucose metabolism pathway to oxygen-independent glycolysis (Seagroves *et al.,* 2001). For this switch, the increased requirement of glucose by the cellular machinery is achieved by enhanced expression of glucose transporters and glycolytic enzymes (Chen *et al.,* 2001; Sarkar *et al.,* 2003). The glucose transporters are activated in order to enhance the glucose uptake required for glycolysis process to take place. Interestingly, the inhibition of oxidative phosphorylation has shown to activate and stabilize *GLUT-1* mRNA via *HIF-1α* mediated process (Behrooz and Ismail Beigi, 1999).

The hexokinase2 (*HK2*), mRNA was also found to be upregulated in $CoCl_2$ treated BuMECs as compared to control BuMECs. *HK2* mediates glucose metabolism by catalysing the conversion of glucose to glucose-6-phosphate for metabolic utilization (Zhao *et al.,* 2007). The upregulation of all the studied hypoxia associated genes under chemically induced low oxygen condition indicated changes in cellular metabolism of BuMECs. Our results thus suggest that BuMECs can be utilized as an efficient *in vitro* model to understand cellular response to hypoxic stress conditions.

Our data also showed increased expression of *JAK2* and *STAT5* genes in BuMECs under hypoxic condition. *JAK2/STAT5A* pathway is suggested to provide resistance to cellular/ tissue injury under stress condition (Dudley *et al.,* 2005). *STAT5* activation by hypoxia is suggested to be mediated by *JAK2*. *In vivo* model of hypoxia has implicated *STAT5* as a player in responses to cellular stress, inflammatory and immune responses (Dudley *et al.,* 2005; Joung *et al.,* 2005; Yamaura *et al.,* 2003). Similar to our results, Yamaura *et al.* (2003) reported that *STAT5* has protective role against cardiac ischemia/reperfusion injury under hypoxia by activating anti-apoptotic signals. Hypoxia activates signal transducers and activators of *STAT5* and increases its binding activity to the GAS element in mammary epithelial cells promoting cell survival and growth (Joung *et al.,* 2003). *JAK2/STAT5* pathway has also shown to be essential for endothelial cell survival during tissue hypoxia for maintaining retinal neovascularization common in diabetic retinopathy (Dudley *et al.,* 2005). Liu *et al.* (2005) reported that hypoxia mediated *HIF-1α* expression stimulates the expression of *JAK2*, *STAT5* and *NF-κB* in mouse neuronal cells. For inflammatory response of BuMEC, mRNA expression of *TNF-α* was checked and an increase was observed in our study. In line with our finding, previous studies reported that stimulation of *TNF-α* accumulates *HIF-1α* gene expression in primary inflammatory cells (Albina *et al.,* 2001). Similarly, Thornton *et al.* (2000) also reported increased *HIF-1α* mRNA expression mediated by *TNF-α* in human synovial fibroblasts indicating the important role of *TNF-α* in hypoxia and inflammatory response. It is also important to consider that *HIF-1α* signalling pathway is regarded as a key signalling pathway involved in hypoxia-induced responses. Many studies suggest that *HIF-1α* up-regulates the downstream target genes involved in cell proliferation and differentiation, and plays a key role in the process of hypoxia signal transduction and overall affects physiology and pathophysiology of animals.

In conclusion, the buffalo MECs have shown the transcriptional responsiveness to chemically induced hypoxia stress *in vitro*. The induction of *HIF-1α* and its target genes clearly suggested the suitability of buffalo MECs as a suitable model to understand the mammary gland expression signature in response to hypoxic conditions. In future, such studies could be extended in evaluating the impact of hypoxia on mammary cell proliferation, differentiation and cell damage in livestock species and related gene regulation to understand buffalo mammary gland functions.

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