Expression pattern of glucose metabolism genes correlate with development rate of buffalo oocytes and embryos in vitro under low oxygen condition

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Abstract

Purpose This study evaluates the effect of low oxygen conditions (5 Vs 20 %) on buffalo embryo development. Expression patterns of key glucose metabolism genes (*HK*, *PFK*, *LDH*, *PDH*, *G6PDH* and *Glut1*) were assessed in buffalo oocytes and embryos cultured at 5 and 20 % oxygen and correlated with development rate.

Methods Maturation rate was observed by determining MII stages by Aceto-orcein method and blastocyst formation was observed at 7 day post insemination (dpi). Expression levels

of genes were determined by real time PCR in oocytes / embryos at 5 and 20 % O₂.

Results Oocyte maturation and blastocyst formation rates were significantly higher at 5 % O₂ as compared to 20 % O₂ ($P<0.05$). The expression pattern of glycolytic genes (*HK*, *PFK* and *G6PDH*) indicated that oocytes and embryos under 5 % O₂ tend to follow anaerobic glycolysis and pentose phosphate pathways to support optimum embryo development. Under 20 % O₂, oocytes and embryos had high expression of *PDH* indicating higher oxidative phosphorylation. Further, less *G6PDH* expression at 20 % O₂ was indicative of lower pentose phosphate activity. Higher expression of *LDH* was observed in oocytes and embryos under 20 % O₂ indicating sub-optimal culture conditions. High *Glut1* activity was observed in the oocytes / embryos at 5 % O₂, indicative of high glucose uptake correlating with high expression of glycolytic genes.

Conclusion The expression patterns of glucose metabolism genes could be a valuable indicator of the development potential of oocytes and embryos. The study indicates the importance of reduced oxygen conditions for production of good quality embryos.

Capsule The expression pattern of glucose metabolism genes was found to be correlated with improved development of buffalo oocytes / embryos under low oxygen condition.

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Introduction

Studies in the past had shown that mammalian oocytes/embryos develop better under reduced oxygen tension compared to atmospheric oxygen in vitro resulting in higher blastocyst formation rates, less apoptotic cells and more inner cell mass cell numbers [1–3]. The oxidative stress on oocytes / embryos had been shown to be directly proportional to their

metabolism status where the aerobic metabolism results in the production of co-factors which impact the cellular redox status [4]. A reduced reactive oxygen species (ROS) content was observed in embryos cultured under the 5 % oxygen environment and was associated with a reduction in DNA fragmentation and significantly higher blastocyst formation rate [3, 5]. Aerobic metabolism (i.e. oxidative phosphorylation, OXPHOS) was inhibited by low oxygen tension and accordingly, ATP production shifted from the tricarboxylic acid (TCA) cycle to glycolysis [6, 7], which led to the decreased ROS production and increased embryonic development during hypoxia [5]. The decrease in ATP production on the other hand was compensated by an increase in expression of glucose transporters and glycolytic enzymes [8, 9]. Glucose was thought to be a major energy substrate for supporting the meiotic maturation of bovine oocytes under low oxygen tension [5]. Reduced oxygen tension increased the catabolic utilization of glucose by mouse morulae and blastocysts in vitro [10, 11]. Other studies in different species had also shown that the low O₂ concentration and high glucose were beneficial for in vitro maturation, fertilization, and pre-implantation embryonic development [5, 12, 13]. It had been observed that under reduced oxygen level, glucose metabolism pattern of in vitro embryos was similar to in vivo counterparts [4]. Also the global gene expression pattern was found to be different under 5 % O₂ than 20 % O₂ and the expression pattern under 5 % O₂ was found closer to their in vivo counterparts [14]. It reflects that the oxygen concentration in a culture environment had consequences on the glucose utilization pattern and a low oxygen environment could mimic better the in vivo conditions towards better developmental fate of oocytes/embryos.

Keeping in view the problems with the buffalo in vitro fertilization (IVF) system [15] and possible beneficial effect of reduced oxygen environment, the present study was aimed at investigating the effect of reduced oxygen level (5 % O₂) on glucose metabolism and development rate of buffalo oocytes and embryos. The associated expression patterns of glucose metabolism genes were studied to infer on the glucose metabolism behavior and correlated with development rate of oocytes/embryos.

Material and methods

All media and chemicals were procured from Sigma Aldrich, St. Louis, MO, USA unless otherwise indicated. Disposable plastic wares used were from Falcon NJ, USA and Nunc, Denmark. Fetal bovine serum used was from Hyclone, Canada.

Experimental design

The effect of reduced oxygen level (5 %) in combination with glucose levels during IVM (5.6 and 20 mM) was investigated

on in vitro maturation rate of the oocytes and subsequent blastocyst development rate of IVF produced buffalo embryos. Thus the study had 2 experimental groups w.r.t. IVM glucose level viz. 5.6 and 20 mM glucose under 5 % O₂ and 5.6 mM glucose under 20 % O₂ worked as a control. We have seen in our previous study that at 20 % O₂, 5.6 mM glucose yielded a highest blastocyst development rate in buffalo [16]. The 20 mM glucose group under 5 % O₂ was experimented during IVM to confirm whether a higher glucose under reduced O₂ has any positive effect on buffalo IVF also as noted earlier in other species [11, 12]. During IVC, 5.6 mM glucose was used under both oxygen levels. Expression levels of glycolytic genes (hexokinase, *HK*; phosphofructokinase, *PFK*; lactate dehydrogenase, *LDH*; pyruvate dehydrogenase, *PDH*; glucose-6-phosphate dehydrogenase, *G6PDH* and glucose transporter 1, *Glut1*) were measured at 4 development stages viz. matured oocytes, 2-cell, 16-cell, and morula. We considered 2- and 16-cell embryos as representatives of pre- and post-embryonic genome activation (EGA) stages respectively while morula were considered as post compaction stage embryos [17]. The cumulus oocyte complexes (COC) matured under different experimental conditions were used to observe MII % using Aceto-orcein staining method [18]. For real time PCR assay, matured COC and embryos at 2-cell, 16-cell and morula stages were collected at 36hpi, 84hpi, and 144hpi respectively. The cleavage and blastocyst development rate data were recorded as a percentage of COC (initially put for IVM). All experiments included four biological replicates.

Production of buffalo IVF embryos

Buffalo embryos were produced following the procedure described earlier [16]. Briefly, buffalo ovaries were collected from the slaughterhouse and oocytes were aspirated from visible follicles (3–8 mm) in modified synthetic oviduct fluid — in vitro maturation (mSOF-IVM) medium supplemented with 0.2 % BSA, 60 µg/ml penicillin, and 100 µg/ml streptomycin. Oocytes with completely surrounded the cumulus mass of 2–5 layers and homogenous ooplasm were picked up in the oocyte handling medium (mSOF supplemented with 10 % FBS, 1 mM glutamine, 0.33 mM sodium pyruvate, 0.1 mM taurine, 3.3 mM lactate, 0.5 mM citrate, 1X MEM essential amino acid, 1X non-essential amino acid, 1X BME vitamin solution and 50 µg/ml streptomycin). Following 3 washings, 25–30 oocytes were transferred to 100 µl drops of maturation medium composed of the oocyte handling medium supplemented with 5 µg/ml FSH, 10 µg/ml LH, 50 ng/ml EGF, 0.1 mM cysteamine, 1 % ITS and either concentration of glucose (5.6 or 20 mM). COC in maturation drops were incubated in a CO₂ incubator at either 20 or 5 % O₂ and 38.5 °C for 24 h. After maturation, oocytes were washed 3 times in IVF medium (mSOF-IVM medium supplemented with 1.9 mg/ml caffeine sodium benzoate, 0.1 mg/ml heparin

Table 1 Real time PCR Taqman primer / probe sequences used

Gene name	mRNA accession#	Primer / probe sequences
HK	GU324294	F 5' CGACGACAGTATCCTCGTGA 3' R 5'GCGGATCTTATCCACCACAG 3' P 5' GTGTGTGGGGTGGTGTCCAAGAG 3'
PFK	GU324297	F 5'GGGCCTGGTGTAAAGGAACG3' R 5'ACGTTCTTCCTGCTGTCGAAG3' P 5'TGCCCTTCCCTCCTCCGAGTACA3'
LDH	GU324295	F 5'TTGACAGTGCTTATGAGGTGATC3' R 5'TCATTATACTTTCTGCCAAATCGG3' P 5'CACTGACAGTCCAATGGCCCAGGA3'
PDH	GU324296	F 5'AGAAAGGCAAGGAACACACG3' R 5'TTTATCACCTCACATTCAATTCCC3' P 5'TGCTCATTCAAGACCTGTGGGCCA3'
Glut1	GU324293	F 5'TCCACAAGCATCTTCGAGAAGG3' R 5'AATAGCGACACGACAGTGAAGG3' P 5'AGCAGCCCGTGTATGCCACCATCG3'
G6PDH	GU324292	F 5'ATGACGTGCGCGATGAGAAG3' R 5'GGGGTTCCCCACATATTGGC3' P 5'CCACGTTGCTCGCCTGCACCTCTG3'
RPS15	NM_001024541.1	F 5'ACAACGGCAAGACCTTCAAC3' R 5'CAGGTTACTTGAGGGGGATG3' P 5'CTAGGCGAGTTCTCCATCACTTAC3'
GAPDH	GU324291	F 5'ACGTGTCTGTTGTGGATCTGAC3' R 5'CGTGTTGAAGTCGCAGGAG3' P 5'TGCCGCTGGAGAAACCTGCCAA 3'

F forward primer, R reverse primer, P probe sequence

and 0.14 mg/ml sodium pyruvate and 1 % BSA) and transferred to 50 µl fertilization drops in groups of 20–25. Frozen spermatozoa after thawing and washing in IVF medium were transferred to fertilization drops (1×10^6 sperms) containing matured oocytes and incubated in a CO₂ incubator for 12 h at 38.5 °C at either 20 or 5 % O₂. Following fertilization, oocytes were washed 5–6 times in the in vitro culture medium (mSOF-IVC) supplemented with 0.8 % bovine serum albumin (BSA), 5.6 / 20 mM glucose, 0.33 mM pyruvate, 3.3 mM lactate, 1 mM Glutamine, 1X MEM essential amino acid, 1X non-essential amino acid and 50 µg/ml gentamycin. Zygotes were transferred to 100 µl culture drops (IVC-I containing BSA) at either 20 or 5 % O₂. After 72 h, embryos were transferred to IVC-II medium (BSA in the IVC-I medium was replaced with 10 % fetal bovine serum (FBS)). Half of the culture medium was replaced every 48 h and the embryos were cultured till day 7.

RNA isolation, cDNA synthesis and real time PCR

RNA was isolated from 10 oocytes/embryos of each group using Trizol (Invitrogen, Cat# 15596–026) as per manufacturer's instructions. RNA was precipitated using 10 µg glycogen (Fermentas, Cat# R0551) and finally the RNA pellet was

washed with 70 % ethanol. RNA pellet was air-dried and dissolved in 10 µl nuclease free water and treated with DNase-I (Fermentas, Cat# EN0523) as per manufacturer's instruction. A constant amount of RNA (100 ng) from each sample was used for cDNA synthesis using Superscript III (Invitrogen, Cat # 18080–044) following manufacturer's instructions. Genes under study viz. HK, PFK, LDH, PDH, G6PDH and housekeeping genes viz. GAPDH and RPS15 were amplified using real time TaqMan assay. The buffalo specific TaqMan primers and probes for genes under study were designed from the deduced buffalo sequences (GenBank accession no GU324290 to 99) (Table 1). TaqMan probes were labeled with 5' FAM – 3' TAMRA and synthesized from Sigma-Aldrich (St. Louis, Missouri, USA). Respective primer and probe sequences are described in Table 1. All real time PCR reactions were of 10 µl consisted of 5 µl of 2X real-time TaqMan mix (Fermentas, Cat # K0221), 2 µl cDNA, 0.75 µl each of primers and probes (7.5 pM) and 0.75 µl water. A common thermal profile of 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 20 s and 60 °C for 60 s was used for all genes. Standard curves were generated by serial dilution of cDNA to validate the efficiencies of the probes and primers and amplification conditions. Standard curves of all genes had efficiency in the range of 96 to 107 % and slopes in the

range of -3.1 to -3.6 . Real time PCR reactions were set in triplicate using $2\ \mu\text{l}$ of diluted cDNA (3X) along with a negative control. All experiments included 4 biological replicates.

Analysis of data

The maturation rate and development rate data under different experimental groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. Real time amplification C_t value data obtained for genes under study were normalized using the geometric mean of RPS15 and GAPDH [19]. The relative abundance values for transcripts were calculated by $\Delta\Delta\text{CT}$ method [20] using immature oocytes as a calibrator for gene expression data of oocytes and matured oocytes under 5 % O_2 (20 mM glucose) as a calibrator for gene expression data of embryos. Relative abundance values were analyzed by two-way ANOVA followed by Bonferroni post hoc test to find out the significance of difference between different embryonic cell stages within a particular experimental group as well as for a particular cell stage between different experimental groups and control. The significance of differences was tested both at 5 and 1 % levels of significance.

Results

Effect of oxygen concentration on the developmental competence of oocytes and embryos

No significant difference was observed either in the maturation or blastocyst development rates at 20 and 5 % O_2 level when the oocytes were matured in IVM medium with 5.6 mM glucose. However, the oocytes matured at reduced O_2 level (5 %) and higher glucose level (20 mM) resulted in significantly more ($P<0.05$) number of oocytes reaching M-II stage and a higher number of blastocysts produced (Table 2).

Expression pattern of glucose metabolism genes in oocytes

The expression of glycolytic gene *HK* did not significantly differ between different experimental groups but the *PFK* was found to be expressed at significantly higher level under 5 % O_2 (20 mM glucose) as compared to 20 % O_2 (5.6 mM glucose) and 5 % O_2 (5.6 mM glucose) (Fig. 1). On the other hand the expression of TCA regulatory gene *PDH* was about 16 fold higher under 20 % O_2 and 5.6 mM glucose as compared to other two groups i.e. 5 % O_2 (20 and 5.6 mM glucose). Interestingly, the expression of *LDH* was also significantly higher in the same group (20 % O_2 , 5.6 mM glucose) (Fig. 1). The glucose transporter *Glut1* was expressed at much higher level under 5 % O_2 as compared to 20 % O_2 . The *G6PDH* expressed at moderately higher level under 5 % O_2 -5.6 mM glucose group over other two experimental groups (Fig. 1).

Expression pattern of glucose metabolism genes in embryos

Under control conditions (20 % O_2 and 5.6 mM glucose), *HK* didn't show significant variation in expression through different embryonic stages (2-cell, 16-cell and morula) while at 5 % O_2 and 5.6 mM glucose (during IVM as well IVC), *HK* expression was found to increase from 2-cell to 16 cell stage, but declined at morula stage. Interestingly however, when the glucose concentration was increased to 20 mM during IVM under 5 % O_2 , *HK* mRNA level was found declining gradually from 2-cell stage to morula, indicating a decline in glycolysis as the development of embryos progressed (Fig. 2a). Expression of *PFK* was found similar to *HK* except for the low O_2 and high glucose group (5 % O_2 and 20 mM glucose), where it showed a drastic increase at 16-cell stage (Fig. 2b). The expression of *LDH* revealed a declining trend from 2-cell to morula stage in high O_2 group and its expression was not significantly different across different cell stages in 5 % O_2 groups and were maintained at much lower levels than that in 20 % O_2 group (Fig. 2c). The most drastic effect of lowering O_2 concentration was seen in the *PDH* level (Fig. 2d). Sharp increase in *PDH* was observed from 2-cell to morula stage at

Table 2 Development rate of buffalo oocytes / embryos under different levels of oxygen (5 and 20 %) and glucose (5.6 and 20 mM)

Oxygen level (%)	Glucose level - IVM (mM)	# COCs	% M-II	% uncleaved oocyte	% 2 cell (blocked)	% 8–16 cell (blocked)	% Morula (blocked)	% Blastocyst
20	5.6	232	87.3 $\pm$ 2.06 ^a	33.3 $\pm$ 1.46 ^a	7.52 $\pm$ 0.29 ^a	8.32 $\pm$ 0.64 ^a	29.17 $\pm$ 1.22 ^a	21.66 $\pm$ 0.15 ^a
5	5.6	216	80.69 $\pm$ 0.1 ^a	30.45 $\pm$ 2.5 ^a	8.42 $\pm$ 0.87 ^a	14.5 $\pm$ 2.18 ^b	28.23 $\pm$ 1.38 ^a	18.32 $\pm$ 1.37 ^a
5	20	243	94.8 $\pm$ 2.6 ^b	25.3 $\pm$ 2.87 ^a	4.19 $\pm$ 0.93 ^b	5.74 $\pm$ 1.60 ^c	35.80 $\pm$ 4.52 ^b	28.96 $\pm$ 0.91 ^b

Values indicate Mean $\pm$ SEM. Different superscripts indicate significant difference along column ($p<0.05$). Glucose concentration at IVC-I and -II was kept at 5.6 mM both at 5 and 20 % O_2

Fig. 1 Expression pattern of glucose metabolism genes in the oocytes matured under different oxygen (5 or 20 %) and glucose (5.6 or 20 mM) levels. Oocytes were collected after 24 h of maturation and assayed for expression of glucose metabolism genes. Bars on respective columns

represent standard error of mean. Columns with different superscripts indicate significant differences between different experimental groups for respective genes ($p < 0.05$)

20 % O_2 , whereas its expression under lower O_2 level (5 %) was found reduced to more than 250 times (Fig. 2d). *G6PDH* expression, followed similar trend in high O_2 — low glucose and low O_2 — high glucose groups, although their magnitude of expressions were significantly different in these two groups

at all stages of embryonic development. However in low O_2 — low glucose group (5 % O_2 and 5.6 mM glucose), it revealed non-significant variation across cell stages and were maintained at much higher levels (Fig. 2e). An aberrant increase in the level of expression of *Glut1* between 2 and 16-cell stages

Fig. 2 Expression pattern of glucose metabolism genes in embryos at different cell stages of preimplantation development (2-cell, 16-cell and morula). a. HK, b. PFK, c. LDH, d. PDH, e. G6PDH, f. GLUT1. Bars on respective columns represent standard error of mean. The first superscript indicates level of difference between different cell stages within a

particular experimental group and the second superscript indicates difference for the same cell stage between different experimental groups. Columns with different superscripts indicate significant difference ($p < 0.05$)

under lower O₂ level (5 % O₂) was notable. Under 20 % O₂, it was maintained at a significantly lower level in all embryonic stages (Fig. 2f).

Discussion

We have observed that developmental competence of buffalo oocytes and embryos cultured under reduced oxygen level (higher glucose) was significantly higher than normal oxygen level. The expression levels of glucose metabolism genes were less under the reduced oxygen level (5 % O₂) as compared to atmospheric oxygen level, 20 % O₂. Glycolytic genes were found to be maintained at low level under 5 % O₂ than 20 % O₂ during early embryonic development. It had been reported earlier that glucose is metabolized more through the glycolytic pathway than oxidative phosphorylation (OXPHOS) during in vivo embryonic development in pig [21]. Oocytes matured in hypoxic conditions showed less metabolic activity, more closely resembling in vivo maturation and hence improved development competence [22]. In ruminant animals the embryos produced under 20 % O₂ differ in metabolism from in vivo embryos which included higher aerobic glycolysis, more lactate production and higher lactate oxidation [23, 24]. This may be a special requirement for fast proliferating growing embryos; something similar observed with cancer cells [25].

Culturing oocytes and embryos at below ambient O₂ levels have been shown to be favorable for embryo development in various mammalian species including human [2, 26, 27]. Moreover the glucose level has been found to be an important factor under different oxygen concentrations in the culture environment affecting oocyte and embryo development in vitro [5]. Glucose was not considered essential during early embryonic development, however high glucose concentration proved to be detrimental to development under normal oxygen level [5, 28]. But studies have shown that high glucose is more effective for oocyte maturation and further embryonic development [5, 12]. Our results indicated that 20 mM glucose at 5 % O₂ was better compared to 5.6 mM glucose for in vitro maturation of buffalo oocytes and development of resultant embryos. However, some studies have failed to demonstrate the beneficial effect of low O₂ on the development of pre-implantation embryos in-vitro [23, 29]. The reason for such contradictions could be differences in basic culture systems used by different workers while manipulating the O₂ levels as interplay between culture systems and O₂ concentrations is a crucial factor [30]. In the current study we used a chemically defined mSOF medium throughout the IVF protocol.

In the present study expression levels of *PFK* and *PDH* indicated that the oocytes prefer an anaerobic glycolytic

pathway of glucose metabolism to OXPHOS under 5 % O₂ (Fig. 1), which is thought to be the preferred pathway for embryos. OXPHOS is thought to be a major source of ROS production as inhibiting OXPHOS resulted in reduced ROS and improved embryo development in bovine and porcine [31, 32]. Low oxygen and high glucose have been shown to increase development competence of bovine oocytes also by decreasing ROS and increasing ATP production via glycolysis [33]. The high expression of *PDH* under 20 % O₂ (Fig. 2d) indicated that oocytes under high oxygen environment had higher OXPHOS activity affecting the developmental rate. The ‘Quiet Metabolism’ theory proposed for embryo development under in vivo conditions is thought to be an adaptation of the embryos to reduce ROS production through aerobic respiration [34, 35]. Reduced ROS level might help to reduce DNA damage and avoid aneuploidy that had been a major problem faced during human IVF [36, 37]. NADPH helps in counteracting the ROS by production of glutathione and thereby overcoming the deleterious effect of ROS [38, 39]. Lower expression of *G6PDH* under 20 % O₂ in buffalo oocytes/embryos (Fig. 2e) indicated that the PPP shunt would be less active in embryos produced under this condition, which might have resulted in the decreased NADPH production unable to combat ROS production by higher OXPHOS. Also a higher expression of *LDH* in the embryos produced under 20 % O₂ (Fig. 2c) was indicative of the stress conditions. High lactate production has been reported earlier as a result of stress response of the embryos under suboptimal culture conditions [40]. In the present study change in the culture environment from 20 to 5 % O₂ resulted in drastic reduction in *LDH* expression both in oocytes and embryos (Figs. 1 and 2c), which correlated with reduced lactate production in other species under reduced oxygen level resembling a more in vivo situation [4].

We observed that embryos had high expression of OXPHOS genes under 20 % O₂ which shifted to glycolytic genes under 5 % O₂ during later stages of embryonic development. OXPHOS is inhibited by low oxygen tension, and accordingly ATP production shifts from the TCA cycle to glycolysis [6, 7]. The decrease in ATP production due to decreased OXPHOS is compensated by an increase in expression of glucose transporter 1 and glycolytic enzymes [8, 9, 41]. Probably the high *GLUT1* expression under 5 % O₂ compensated for increased demand of glucose by embryos post maternal to zygotic transition (MZT) due to increased rate of cell proliferation. Studies in other species have also observed increased *GLUT1* expression under reduced oxygen level in oocytes/embryos [42, 43]. Interestingly, increase in *GLUT1* expression between 2-cell to 16-cell embryos (under 5 % O₂) overlaps with the recently reported EGA event in buffalo embryos from our laboratory [17]. Facilitated glucose transporters (viz. *GLUT1*, 2, 3 and 8) have been identified to be responsible for glucose uptake in oocytes/early embryonic

development. *GLUT1* is expressed throughout preimplantation development [44], *GLUT2* from 8-cell stage onward [45], while *GLUT3* and *GLUT8* (Insulin stimulated glucose transport) is restricted to morulla and blastocyst stage [46, 47].

We conclude that reduced oxygen level along with high glucose supplementation could improve the development rate of buffalo oocytes and embryos. Buffalo oocytes and embryos rely more on glucose under reduced oxygen level fulfilling the energy and anabolic demand of fast proliferating early embryos, a phenomenon quite similar to ‘Warburg Effect’ [25]. Further expression pattern of metabolism genes (such as *G6PDH* and *LDH*) correlated well with development competence of oocytes/embryos.

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