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Comparative analysis of developmental and molecular correlates of developmental competence of buffalo oocytes derived from small and large follicles

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ABSTRACT

The present study was conducted to correlate the developmental competence of large follicle (LF, >6 mm) and small follicle (SF, <6 mm) derived buffalo oocytes in terms of morphological assessment and molecular characteristics. A pool of culture grade cumulus oocytes complexes (COCs) from both the groups separately were subjected to brilliant cresyl blue (BCB) staining. LF derived oocytes had significantly higher percentage of BCB^{+ve} oocytes and were larger in size and maturation status was also visibly superior. Cleavage and blastocysts rate was also significantly higher in LF group than SF group. Relative mRNA expression analysis revealed that expression of *GDF9* was significantly higher in LF group than SF group while expression of *BMP15* increased insignificantly. Expression of *GREM1, EGFR* and *PTGS2* was significantly higher in LF group than SF group while expression of *HAS2* was insignificantly higher. Similarly, expression of *GLUT1, OCT4* and *IFN*-τ in *in vitro* produced blastocyts was significantly higher in LF group than SF group. Thus, it can be concluded that LF derived oocytes are larger in size and express higher level of competence markers and resulted in better quality embryos. This information may be further utilized for the enhancement of developmental competence of small follicle derived oocytes.

Key words: Buffalo, Developmental competence, *In vitro* embryo production, Molecular correlates

In vitro developmental competence of buffalo oocytes depends on the follicular size, follicular morphology, and oocyte diameter (Raghu *et al.* 2002). Also, the follicle size directly influences the oocyte competence in buffalo (Raghu *et al.* 2002), bovine (Shabankareh *et al.* 2015), goat (Majeed *et al.* 2012), pig (Bagg *et al.* 2007) and sheep (Wani *et al.* 2013). Attainment of oocyte developmental competence is generally assessed on the basis of morphology of cumulus cells and homogeneity of the ooplasm (Torner *et al.* 2008). However, these morphological evaluations are not reliable enough to act as the sole criteria for the evaluation of oocyte competence. Therefore, the novel “TRANSCRIPTOMICS” technologies which define molecular signatures of oocyte competence along with morphological parameters would be useful to predict the developmental competence of oocytes *in vitro* and *in vivo* and that could allow the development of more appropriate oocyte maturation and embryo culture regimens (Calder *et al.* 2001). Several molecular markers have been investigated for oocyte developmental competence (Bhardwaj *et al.* 2016a). Still the mechanism of acquisition of buffalo oocyte developmental competence either *in vivo* or *in vitro* is not clearly known. Thus, the present study was designed with the aim to investigate the effect of follicle size on the buffalo oocyte developmental competence in terms of BCB differential staining, cleavage and blastocyst rate and relative mRNA expression of competence markers in oocytes, cumulus cells and blastocysts.

MATERIALS AND METHODS

To assess the effect of follicle size on oocyte development competence, the oocytes were divided in 2 groups: LF (large follicle derived oocytes; ≥6 mm) and SF group (small follicle derived oocytes; <6 mm). The oocyte developmental competence was assessed on the basis of BCB staining; relative mRNA expression of different transcripts related to developmental competence in denuded oocytes and cumulus cells; cleavage rate; blastocyst rate; and relative mRNA expression of blastocyst competent markers. All the media, chemicals, growth factors, molecular biology reagents, kits and enzymes used in the present study were procured from reputed firms described

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Fig.1. BCB stained oocytes. Blue stained oocytes are designated as BCB $^{+ve}$ (blue arrow) and colorless oocytes are designated as BCB $^{-ve}$ (white arrow).

where mentioned.

In vitro *embryo production: In vitro* maturation, fertilization and embryo culture was done as per our laboratory protocol (Bhardwaj *et al.* 2016b).

Briefly, buffalo ovaries were collected from local abattoir and transported to the laboratory in pre-warmed normal saline solution (NSS) supplemented with 50 µg/ml gentamycin sulphate (G1272, Sigma) at 37–39°C, within 2–3 h of slaughter. These ovaries were washed and follicular fluid was aspirated separately from LF and SF. Grade A and B COCs were selected for the study.

COCs derived from LF and SF group were subjected to BCB staining (Bhardwaj *et al.* 2016b). The COCs were divided into two groups depending upon the colour of cytoplasm; BCB^{+ve} COCs as those with any degree of blue colouration while BCB^{-ve} COCs were without any blue colouration of the cytoplasm (Fig. 1). The percentage of BCB^{+ve} and BCB^{-ve} oocytes was recorded.

Cumulus cells and oocytes were separated from immature COCs of LF and SF group by treating with 0.25% hyaluronidase (H3506, Sigma) for 50–60 min and intermittent pipetting in 5–10 min interval and at last vortexed for 1 min. All the denuded oocytes were confirmed under inverted microscope at 40× (71X Olympus), collected in sterile eppendorf tube and suspended CCs in DPBS were transferred with pipette in another sterile eppendorf tube. Collected cumulus cells were then centrifuged @1500 rpm for 10 min followed by washing in DPBS twice. Pellet of cumulus cells and denuded oocytes were processed for RNA isolation.

COCs derived from LF and SF were matured *in vitro* for 24 h at 38.5°C by using the protocol of Bhardwaj *et al*. (2016b). Nuclear status of immature and matured denuded oocytes was evaluated by Hoechst staining. Denuded oocytes recovered were fixed with acetic acid:methanol (1:3 v/v) for 24 h at 4°C. After fixation, denuded oocytes were again washed in 1% PBS-PVP to remove fixative and then stained with Hoechst stain (Sigma) for 10 min at room temperature. Extra stain was washed with 1% PBS-PVP and stained oocytes observed under fluorescence microscope. Oocytes showing metaphase-II stage were considered as fully matured.

In vitro matured COCs of different groups were subjected to IVF for 18 h at 38.5°C as per protocol used by Bhardwaj *et al*. (2016b). After 18 h post co-incubation, the

Table 1. Gene specific oligonucleotide primers for PCR amplification

Gene	Primer pairs	Annealing temp. (°C)	Amplicon size (bp)	References
GAPDH	F- CGACCACTTTGTCAAGCTCA R- GGACCTTACTCCTTGGAGGC	60	101	Sharma *et al.* (2010)
RPS15A	F- AATGGTGCGCATGAATGTC R- GACTTTGGAGCACGGCCTAA	60	100	Dangi *et al.* (2014)
HAS2	F- ATAATGTGGCAGGCCCAAGAAGG R- GTCTTTGTTCAAGTCCCAGCAGCA	61	183	Assidi *et al.* (2008)
EGFR	F- CCAGGAGGTTGCCGGCTATGT R- GCAGCTCCCTCAGTCCGGTTTT	60	161	HM749883.1
GREM1	F- GCATGAGCCGCACAGCCTACA R- CCCCGGTTCCTGGAGCCA	61	177	XM006048186.1
PTGS2	F- CAGCGGTGCAGCAAATCCTT R- TCGGGAGTGGGTTTCAGGAGT	57	173	EF028069.1
GDF9	F- CCATCAGTGGATCTGCTGTTTA R- GGGAAGGGAAAAGAAATGGA	58	107	Nath *et al.* (2013)
BMP15	F- TGGTTTACCGCCATCAACTT R- AAGGCTTTGTGGAGGCTCTT	58	120	Nath *et al.* (2013)
IFN-τ	F- GATGGCCCTGGTGCTGGTCA R- GTCGCCCTCCACCATCTCCTG	59	184	JX481992.1
GLUT1	F- GCCGGTAGGGAGGAGCAAGTC R- GGCACCTCACACTTGGGAATC	61	85	NM001290870.1
OCT4	F- GAGCCGAACCCTGAGGAG R-AGGGTAAGCCCCACATCG	60	125	Sreekumar *et al.* (2014)

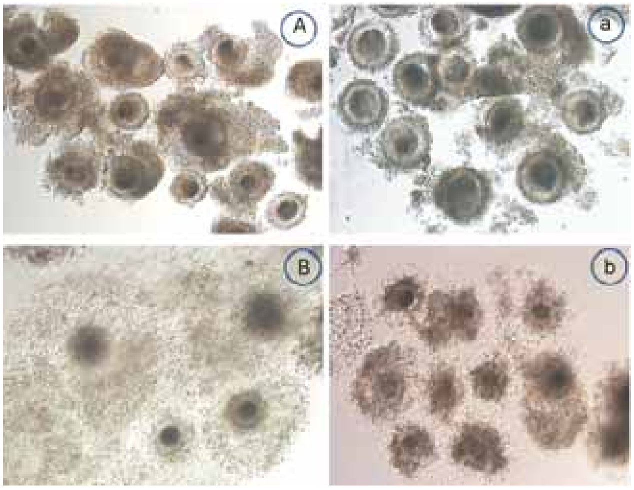

Fig. 2. Morphological evaluation of maturation. More cumulus investment is seen in Immature oocytes derived from LF (A) than SF (a) and *in vitro* matured oocytes showed better cumulus expansion in LF (B) than SF (b) group.

presumptive zygotes were cultured in modified synthetic oviductal fluid till day 08 post IVF. Blastocyst rate was evaluated on day 08 post IVF and then these blastocysts were further used for RNA isolation.

Relative gene expression of oocyte and blastocyst competent markers: The gene specific primers were taken from different published research papers or designed from sequences available in the GenBank (www.ncbi.nlm.nih.gov) using Gene Tool Software and custom synthesized from GCC Biotech. Details of all the primers as sequences of primer pairs, specific annealing temperature, product length and reference are mentioned in Table 1.

Total RNA extraction and cDNA synthesis: The total RNA was harvested from denuded oocytes (50 each group), cumulus cells (20 oocytes each group), and blastocysts (10 each group) of respective groups by using Trizol reagent (Ambion, USA). Similar sampling was done thrice for each group. To assess the quality and integrity of the RNA, 5 µl of total RNA was subjected to denaturing in 1% agarose gel electrophoresis. The purity and concentration of total RNA were checked using the Nanodrop Spectrophotometer (Thermo Scientific, USA) reading at OD_{260} and OD_{280} taken against 1 µl nuclease free water (Ambion) as blank. Samples with OD_{260}:OD_{280} values between 1.8 and 2.0 were used for cDNA synthesis. cDNA synthesis was done using Verso cDNA synthesis kit (Thermo Scientific, USA) with total 20 µl reaction volume following the manufacturer’s protocol. Denuded oocytes and cumulus cells total RNA (0.2 µg each) and blastocysts total RNA (0.5 µg) was used in the reverse transcription as a template. The RNA was subsequently reverse-transcribed in Bioer XP cycler PCR machine (Biotron Healthcare Pvt. Ltd.) by incubating at 42°C for 59 min followed by the final termination of the reaction by heating for 2 min at 95°C. The cDNA were properly labeled and stored at –20°C for later use. The quality of cDNA was assessed by an amplification reaction for housekeeping genes, viz. GAPDH and RPS15A.

PCR amplification: PCR amplification was done in Bioer XP cycler PCR machine with the amplification reaction mixture of total 20 µl volume consisting of the 18 µl Platinum PCR mix (Life Technologies, USA), 0.5 µl each forward and reverse primer of 0.5 µM concentration and 1 µl cDNA. PCR reaction was performed as per manufacturer’s protocol. No template control (NTC) was maintained for each set of primers and PCR products were analyzed by electrophoresis on 1.8% agarose TBE gel with LabSafe nucleic acid stain (G-Biosciences) and 50 bp ladder (Fermentas).

Real Time PCR (qPCR): Quantitative Real Time PCR was performed with SYBR green master mix qPCR kit (Thermo Scientific, USA) and Smart Cycler Real Time qPCR (Cepheid, USA). NTC was placed with each reaction set up for checking any contamination in reaction components. Master mix of total 20 µl reaction volume was prepared by adding 10 µl 2× SYBR green mix, 0.5 µl each forward and reverse primer of 0.5 µM concentration, 1 µl cDNA template and 8 µl NFW. Samples were run in duplicates and mean Ct value was used for calculation of relative expression. For each group, sampling was done thrice. GAPDH and RPS15A were taken as the housekeeping genes and cycle threshold (Ct) values, amplification plot and dissociation curve for all the required transcripts were acquired. Average Ct value of GAPDH and RPS15A was used for analysis of relative gene expression. The Pfaffl (2001) method of relative quantification was used for calculation of relative gene expression.

Statistical analysis: Data for BCB differential staining, size of oocytes, cleavage rate, blastocyst rate and relative expression of different genes were analyzed by paired t-test using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) at 5% level of significance.

RESULTS AND DISCUSSION

In the present study, the developmental competence of LF and SF derived oocytes was analyzed and morphological, biochemical, developmental and relative abundance of transcripts related with developmental competence were correlated.

In the study, a distinctly higher cumulus expansion was observed in *in vitro* matured COCS derived from LF than in SF group (Fig. 2). Although cumulus expansion was noticeably higher in LF derived COCs but nuclear maturation did not depict a significant change. LF derived oocytes have greater developmental competence than those derived from SF due to its completed nuclear and cytoplasmic maturation. The transcripts and proteins stored in the cytoplasm of the oocyte are involved both in maturation and subsequent cellular events such as fertilization, pronucleus formation and early embryogenesis (Meirelles *et al.* 2004).BCB staining (Fig. 1) revealed that mean per cent of BCB^{+ve} oocytes was significantly ($P<0.05$) higher in LF than SF group and mean diameter of LF oocytes was also significantly ($P<0.05$) higher in LF than SF group (Table 2). The oocytes that stain positive with

Table 2. Comparison of LF and SF oocytes in terms of BCB^{+ve} (%), mean diameter, cumulus expansion and nuclear maturation status

Group	BCB^{+ve} COCs n (%)	Mean diameter of oocytes (µm) n (size ± SEM)	Cumulus expansion after *in vitro* maturation (Scale +1 to +5)	Nuclear maturation$^{\$}$ n (%)
LF	74 (76.69)^{a}	153±4.59^{a}	+ + + + +	72 (80.23)^{a}
SF	34 (30.79)^{b}	110.72±1.35^{b}	+ + +	75 (74.14)^{a}

Values (mean% or mean±SE) in the same column with different superscripts differ significantly ($P<0.05$). Five replicates (cultures) were used for each group. $^{\$}$Number of metaphase-II oocytes.

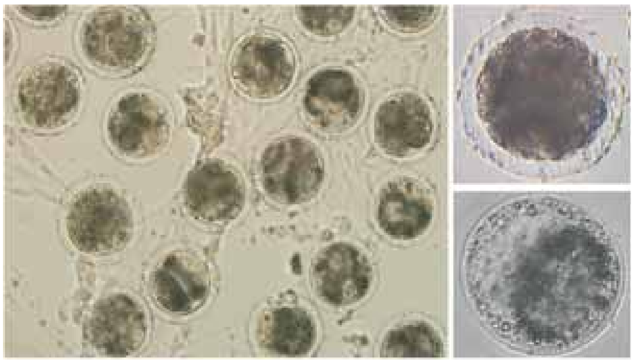

Fig. 3. *In vitro* produced embryos. Embryos at different developmental stages (a), morula (b) and blastocyst (c).

Table 3. Cleavage and blastocyst rate in different groups

Group	COCs cultured	Cleavage rate$^{\#}$ n (%)	Blastocyst rate$^{\$}$ n (%)
LF	178	113 (62.95±0.85)^{a}	41 (35.73±1.35)^{a}
SF	340	124 (36.69±1.4)^{b}	17 (13.55±0.97)^{b}

Values (mean%±SE) in the same column with different superscripts differ significantly ($P<0.05$). Five replicates (cultures) were used for each group. $^{\#}$On the basis of total COCs cultured. $^{\$}$On the basis of total oocytes cleaved.

BCB have a relatively higher developmental competence (Catala *et al.* 2011, Shabankareh *et al.* 2015, Castaneda *et al.* 2013, Bhardwaj *et al.* 2016a).

Again the higher developmental potential of LF oocytes was noted in terms of embryo development. The cleavage and blastocyst rate are provided in Table 3, Fig. 3. The data depicted a significantly ($P<0.05$) higher cleavage and blastocyst rate in LF than the SF group. Similar results were reported in various species and a clear relationship between follicle size and oocyte quality had been demonstrated in buffalo (Raghu *et al.* 2002), cattle (Shabankareh *et al.* 2015), goat (Majeed *et al.* 2012), sheep (Wani *et al.* 2013) and pig (Marchal *et al.* 2000). Further, the developmental competence of LF and SF derived oocytes was compared based on the mRNA expression of the transcripts present in denuded oocytes, cumulus cells and blastocysts. Relative mRNA expression of *GDF9* was significantly higher in denuded oocytes of LF than SF group while *BMP15* expression did not change significantly (Fig. 4.1). *GDF-9* proprotein and mature *GDF-9* protein increased during maturation and has important role in cumulus expansion (Bayne *et al.* 2015). *GDF9* and *BMP-15* are potent stimulator of granulosa cell mitosis and proliferation (de Castro *et al.* 2016). The transcripts studied in the cumulus cells revealed significantly higher relative mRNA expression of *GREM1*, *EGFR* and *PTGS2* in LF than the SF group while *HAS2* depicted no significant change (Fig. 4.2). *HAS2* and *PTGS2* are essential for normal cumulus expansion. *HAS2* is a crucial enzyme for the synthesis of hyaluronic acid (HA), the chief constituent of the extracellular matrix, from products of the cellular metabolism such as hexosamines, glucosamines and glucose (Schoenfelder and Einspanier 2003). *PTGS2* is an enzyme that catalyzes the conversion of arachidonic acid into prostaglandin. The expression of *PTGS2* and *HAS2* was 6-fold higher, and that of *GREM1* was 15-fold higher in cumulus yielding higher grade embryos versus lower grade embryos, hence these genes are considered as a marker of oocyte competence (McKenzie *et al.* 2004). As *GREM1* is considered to inhibit *BMP15*, an oocyte derived factor, and permits EGF expression which exerts a control on gene expression in the cumulus cells (Pangas *et al.* 2004). EGF

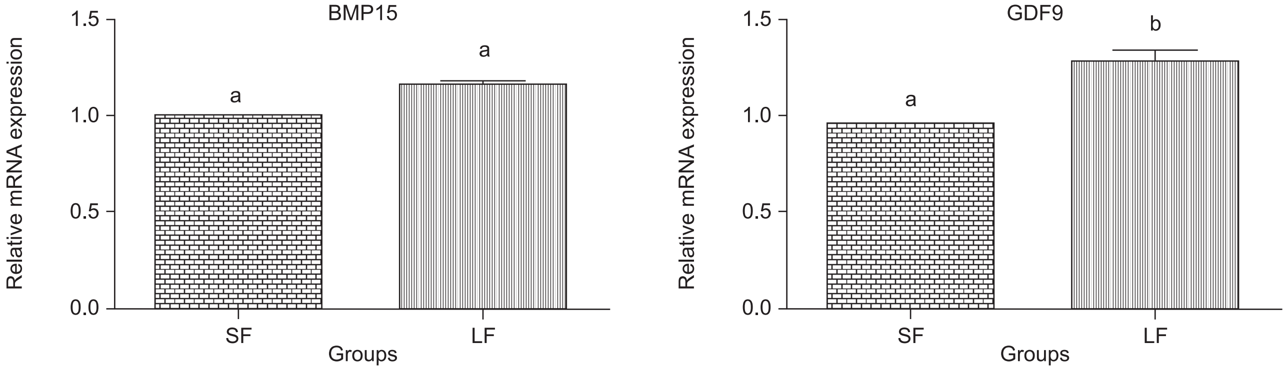

Fig. 4.1. Relative gene expression of oocyte competence markers (*BMP15* and *GDF9*) in denuded oocytes. Data are presented as mean ± SE and groups with different superscripts differ significantly ($P<0.05$).

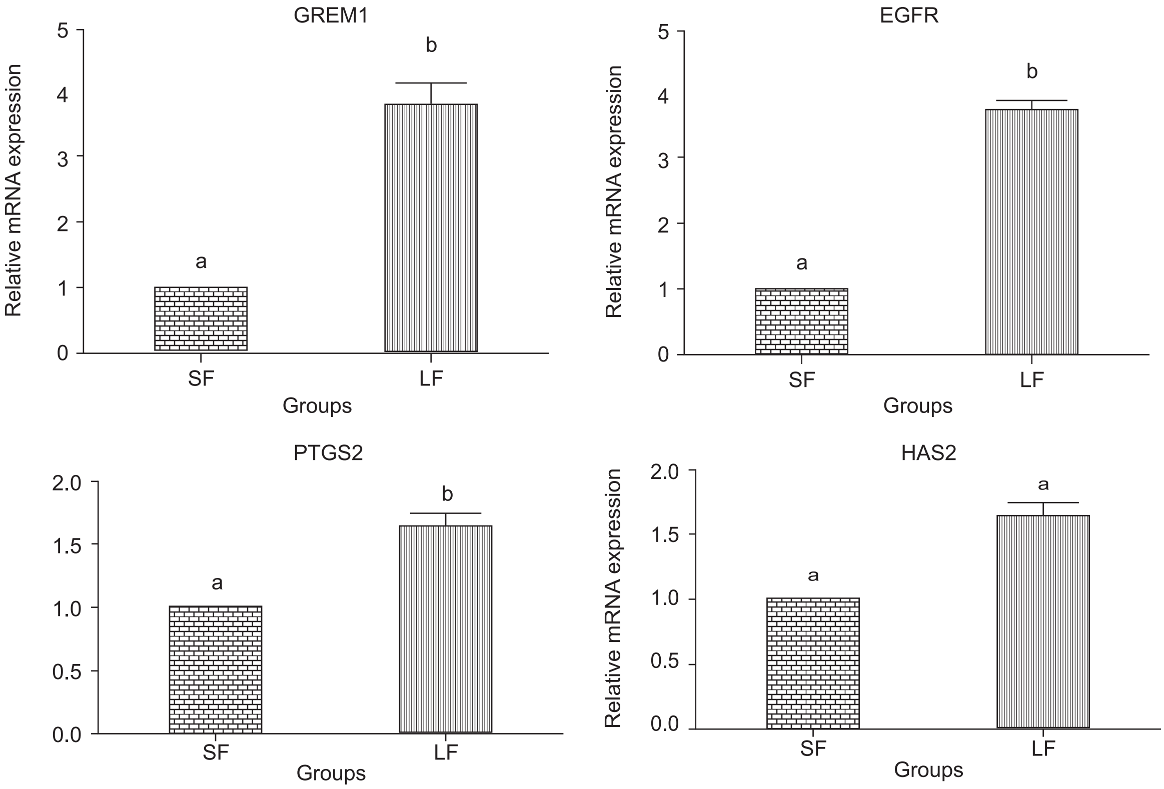

Fig. 4.2. Relative gene expression of oocyte competence markers (*GREM1, EGFR*, *PTGS2* and *HAS2*) in cumulus cells. Data are presented as mean ± SE and groups with different superscripts differ significantly (P<0.05).

and EFG-like factors affect cumulus expansion by inducing cumulus enabling factors; namely, *HAS2, PTGS2* and *TNFAIP6* mRNA (Park *et al.* 2004). Bhardwaj *et al.* (2016a) reported a significant increase in the expression of such markers in BCB^{+ve} oocytes than in BCB^{-ve} oocytes.

The blastocyst competent markers studied, viz. *INF-τ*, *GLUT1* and *OCT4* depicted significantly higher expression in LF than in SF group (Fig. 4.3). Interferon-tau (IFN-τ) is a conceptus secreted protein which plays a critical role in the establishment of ruminant pregnancy by its antiluteolytic effects (Bazer 2013). A positive relationship between IFN-τ production and *in vitro* development of quality embryos has been observed (Neira *et al.* 2007). *GLUT1* is necessary for successful blastocyst development, energy metabolism and subsequent implantation (Carayannopoulos *et al.* 2000) and its expression along with G6PDH were affected by quality and stage of development (Lopesa *et al.* 2007). OCT4, a transcription factor protein, is the earliest expressed transcription factor that is known to be crucial in pre-implantation development (Dode *et al.* 2006) and it binds to DNA and activate or repress transcription of several of these genes expressed during early embryonic development (Smith *et al.* 2007). Thus, in present study, the higher expression of these markers in blastocyst indicates better quality embryo production and higher developmental potential of blastocysts derived from LF group.

It may be concluded from the present study that during follicular growth, oocyte gradually attains developmental competence mainly by increasing its size, cumulus investment and increased expression of oocyte secreted

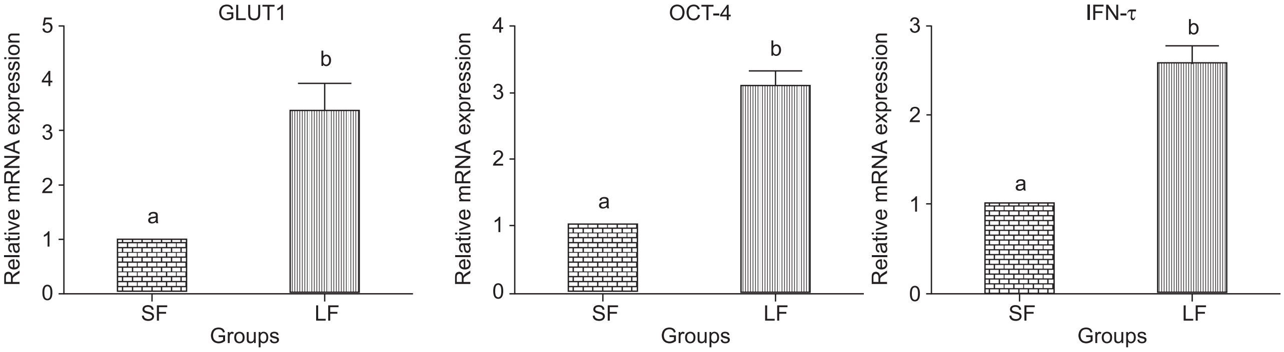

Fig. 4.3. Relative gene expression of blastocyst competence markers (*GLUT1, OCT4* and *IFN-τ*). Data are presented as mean ± SE and groups with different superscripts differ significantly (P<0.05).

factors (*GDF9*/*BMP15*). The OSFs inturn enhance the expression of cumulus expansion enabling factors (*GREM1*, *EGFR*, *HAS2* and *PTGS2*) through autocrine and paracrine mechanisms which act on the oocyte to enhance its further cytoplasmic maturation. These findings may be utilized for improvement of buffalo IVEP to enable the widespread use of embryo transfer technology in buffalo species.

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