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Effect of roscovitine on developmental competence of small follicle-derived buffalo oocytes

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***Background & objectives*: The lower recovery of competent oocytes in buffalo species limits the commercialization of *in vitro* embryo production technology in field condition. In this context, pre-maturation of small follicle (SF)-derived oocytes with meiotic inhibition may be a promising alternative to obtain more number of competent oocytes. Thus, the present study was conducted with an objective to enhance the developmental potential of less competent SF-derived buffalo oocytes.**

***Methods*: All the visible follicles (used for aspiration) from buffalo ovaries were divided into two categories: large follicle (LF) (follicles having diameter ≥6 mm) and SF (follicles of diameter <6 mm). The competence of LF and SF oocytes was observed in terms of brilliant cresyl blue (BCB) staining, cleavage rate, blastocyst rate and relative gene expression of oocyte and blastocyst competence markers. Thereafter, less competent SF oocytes were treated with 0, 12.5, 25, 50 and 100 µM doses of roscovitine (cyclin-dependent kinase inhibitor) to enhance their developmental potential.**

***Results*: Based on parameters studied, LF oocytes were found to be more competent than SF oocytes. Pre-maturation incubation of SF oocytes with roscovitine reversibly arrested oocyte maturation for 24 h to ensure the proper maturation of less competent oocytes. A significantly higher number of BCB-positive oocytes were noted in roscovitine-treated group than SF group. Cleavage and blastocyst rates were also higher in roscovitine-treated group. The relative messenger RNA expression of oocyte (*GDF9*, *BMP15*, *GREM1*, *EGFR*, *PTGS2* and *HAS2*) as well as blastocyst (*INF-τ*, *GLUT1* and *POU5F1*) competence markers was significantly greater in roscovitine-treated group relative to SF group. Again, on comparison with LF group, these parameters depicted a lower value in the treatment group.**

***Interpretation & conclusions*: The findings of this study has revealed that pre-maturation incubation of SF-derived oocytes with 25 µM roscovitine can improve its developmental competence and thus can be utilized to get maximum number of competent oocytes for better commercialization of *in vitro* embryo production technology in buffalo.**

Key words Blastocysts - buffalo - cytoplasmic maturation - developmental competence - oocytes - roscovitine

Substantial research has been conducted for the improvement of culture requirements of buffalo *in vitro* embryo production (IVEP)[1]. The major concern is the lower oocytes recovery compared to other species[2] which is one of the major constraints in the commercialization of buffalo IVEP technology in field conditions[3]. The acquisition of developmental competence is a sequential process which occurs along with the follicular growth in ruminants. This developmental process includes both nuclear and cytoplasmic maturation[4]. Thus, the fully matured oocytes having complete follicular information in the form of messenger RNA (mRNA) or proteins must be collected for improvement in bubaline *in vitro* culture[5]. If oocytes are collected before the acquisition of adequate information, developmental potential of embryo decreases[6]. There are some important events occurring in the oocyte during the late follicular growth, which are essential to achieve full developmental competence[7]. This has already been proved as cumulus-oocyte complexes (COCs) derived from large follicle (LF, >6 mm) of adult cattle have better cytoplasmic maturation and show higher developmental competence, whereas small follicle (SF, <6 mm)-derived oocytes are less competent due to inadequate cytoplasmic maturation[8]. Along with bovine species, improved blastocyst rate through selection of competent oocytes from LF has also been reported in bubaline[9], caprine[10], ovine[11], porcine[7] and humans[12]. Hence, blastocyst rate can be improved either by selecting more competent LF oocytes or by ensuring cytoplasmic maturation of SF oocytes.

In most of the mammals, oocytes are maintained at germinal vesicle (GV) stage until pre-ovulatory luteinizing hormone (LH) surge. During this period of meiotic arrest, oocytes undergo morphological and biochemical changes to achieve developmental competence[13]. However, when oocytes are removed from follicle, they spontaneously resume nuclear maturation with impaired oocyte capacitation and result in lower rate of embryo development[13]. Competence of SF-derived oocytes may be enhanced by providing sufficient pre-maturation incubation for a specific period of time[14] in the presence of meiotic inhibitors such as roscovitine, cycloheximide, 6-dimethylaminopurine and butyrolactone. Amongst all meiotic inhibitors, roscovitine is an effective and reversible inhibitor of cyclin-dependent kinase 2[15], capable of arresting the cells in late G1 and G2/M cell cycle transition[16]. It has less detrimental effects on oocyte developmental competence than other inhibitors[14] and has been used effectively to reversibly block the nuclear maturation of oocytes for certain time period in bovine[14,16,17], equine[6], ovine[18] and porcine[19]. Moreover, porcine embryos obtained from oocytes pre-cultured with roscovitine developed to term, making its introduction desirable in assisted reproductive technology programmes[20].

These studies reported the reversible inhibitory effect of roscovitine in pooled oocytes derived from the visible ovarian follicles. The present study was carried out to investigate the effect of roscovitine on *in vitro* maturation of SF-derived buffalo oocytes and further development to the blastocyst stage.

Material & Methods

The study was conducted in the division of Physiology and Climatology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, India. On the basis of diameter, ovarian follicles were categorized as LF (≥6 mm) and SF (<6 mm)[21,22]. The study was undertaken as three individual experiments: (*i*) comparative analysis of developmental competence of LF and SF oocytes, (*ii*) determination of doses of roscovitine to improve the developmental competence of SF-derived oocytes, and (*iii*) comparison between the developmental correlates of SF, LF and roscovitine-treated SF group.

The best dose of roscovitine was decided based on the following criteria: (*i*) inhibitory effect of roscovitine (0, 12.5, 25, 50 and 100 µM) treatment for 24 h on nuclear maturation; (*ii*) reversibility of roscovitine on nuclear maturation after 24 h maturation in roscovitine-free medium; (*iii*) cleavage rate; and (*iv*) blastocyst rate. Thereafter, the developmental competence of LF, SF and 25 µM roscovitine-treated COCs was compared on the basis of brilliant cresyl blue (BCB) staining, cleavage rate, blastocyst rate, relative gene expression of oocyte competence markers in cumulus cells and denuded oocytes and blastocyst competence markers.

In vitro embryo production

Oocyte retrieval and grading: Buffalo (*Bubalus bubalis*) ovaries collected from local abattoir were transported to the laboratory in pre-warmed normal saline solution (NSS) supplemented with 50 µg/ml gentamycin sulphate (Sigma, USA) at 37-39°C, within 2-3 h of slaughter. Ovaries were washed with NSS to remove dirt, blood, extra tissues, *etc*. The follicular fluid was aspirated separately from LF (≥6 mm) and SF (<6 mm) using 18-gauge needle fitted with 5 ml syringe

containing oocyte collection medium [OCM consisting of TCM-199 HEPES modified (Sigma)]. Three mg/ml bovine serum albumin (BSA) (Sigma)[21,22]. All COCs with more than two layers of compact cumulus layers and homogeneous granular ooplasm were used for the study[21,22].

Treatment of oocytes with roscovitine: COCs derived from SF were rinsed with OCM and then with pre-maturation medium consisting of oocyte collection medium (TCM-199 HEPES) modified, 5 µg/ml luteinizing hormone (LH), 0.5 µg/ml follicle-stimulating hormone (FSH, Sigma), 1 µg/ml estradiol-17β, 20 ng/ml EGF, 0.25 mM sodium pyruvate, 0.68 mM L-glutamine, 10 µg/ml gentamicin sulphate, 3 mg/ml BSA and 10 per cent fetal bovine serum (FBS) and different concentrations of roscovitine (all from Sigma) (0, 12.5, 25, 50 and 100 µM)[16]. Oocytes were pre-incubated in the 50 µl drop (10-15 COCs each drop) of pre-maturation medium for 24 h at 38.5°C in a 5 per cent CO_2, humidified air atmosphere. After 24 h prematuration, one-third of the denuded oocytes (cumulus cells were removed by treating with 0.25% hyaluronidase) were treated with pronase for 30-45 sec and stained with 0.005 per cent ethidium bromide for 10 min at room temperature and washed in phosphate-buffered saline and observed under fluorescent microscope. Nuclear status seen was categorized as the GV, GV breakdown (GVBD), metaphase I (MI), ana-telophase (AT) and metaphase II (MII) (Fig. 1). The remaining two-thirds of the COCs were kept in inhibitor-free medium for additional 24 h period to resume the nuclear maturation.

Brilliant cresyl blue (BCB) staining to select competent oocyte: COCs derived from LF (n=97), SF (n=110) and 25 µM roscovitine-treated (n=107) groups were subjected for BCB staining as per the defined protocol[21,23]. The COCs were divided into two groups depending on the colour of cytoplasm; BCB-positive COCs were those with any degree of blue coloration, whereas BCB-negative COCs were without any blue coloration of the cytoplasm (Fig. 2). The percentage of BCB-positive and BCB-negative oocytes was recorded.

In vitro maturation: After 24 h of roscovitine pre-maturation incubation, COCs were rinsed 4-5 times in maturation medium to avoid the carry-over effect of roscovitine into the final drop of maturation medium. Pre-mature oocytes were subjected to *in vitro* maturation (IVM) to allow the resumption of meiosis, whereas oocytes derived from LF and control group were directly kept for IVM (no roscovitine treatment) in 50 µl droplets of maturation medium for 24 h at 38.5°C and 5 per cent CO_2 in the air with maximum relative humidity.

In vitro fertilization: *In vitro* fertilization (IVF) was done as per the modified protocol of Pandey *et al*[21]. *In vitro* matured COCs of different groups were subjected to IVF. The frozen buffalo bull semen straws

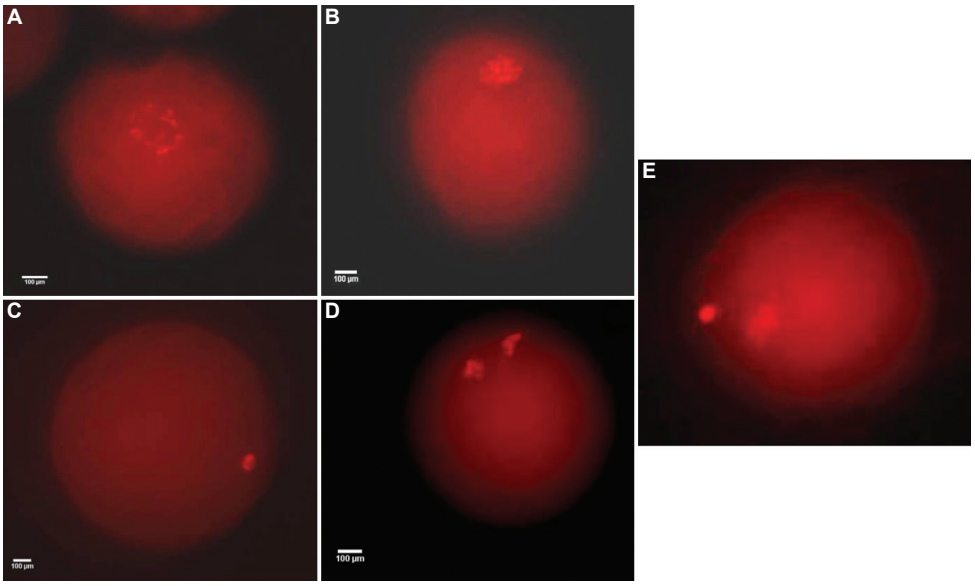

Fig. 1. Different nuclear stages observed after ethidium bromide (EtBr) staining of pre-matured and matured denuded oocyte: (**A**) germinal vesicle (GV); (**B**) germinal vesicle breakdown (GVBD); (**C**) metaphase I (MI); (**D**) ana-telophase (AT); (**E**) metaphase II (MII) (×400).

were procured from ICAR-National Dairy Research Institute (ICAR-NDRI), Karnal, India. The semen was washed twice in Tyrode's albumin lactate pyruvate (FerTALP) medium supplemented with 0.2 mM sodium pyruvate, 6 mg/ml BSA and 20 µg/ml heparin (Sigma) and centrifuged at 70 g for 10 min. The pellet formed was suspended in FerTALP, and progressively motile sperm from the supernatant solution was placed as 50 µl droplets having a final concentration of 2×10^6 spermatozoa/ml. The matured COCs (10-15) were washed, introduced to the semen droplets overlaid with the mineral oil and co-incubated for 18 h with 5 per cent CO_2 in air at 38.5°C with maximum relative humidity.

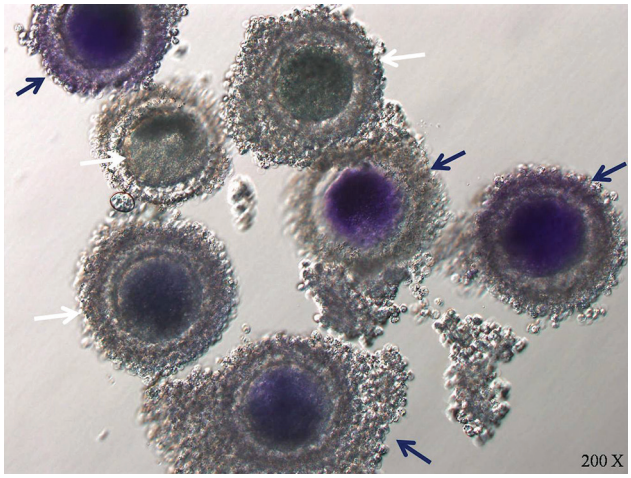

Fig. 2. Brilliant cresyl blue stained oocytes: Blue stained oocytes are designated as brilliant cresyl blue +ve (blue arrow) and colourless oocytes are designated as brilliant cresyl blue −ve (white arrow).

In vitro embryo culture : *In vitro* embryo culture was done as per the protocol described by Bhardwaj *et al*[23]. The presumptive zygotes were washed in modified synthetic oviductal fluid (mSOF) supplemented with 3 mg/ml BSA (fatty acid free), 0.25 mM sodium pyruvate with one per cent (v/v) essential (Sigma) and non-essential amino acids (Sigma), 100 ng/ml IGF1 (Sigma), 0.68 mM L-glutamine and 50 µg/ml gentamycin sulphate. The presumptive zygotes (10-15) were transferred in 50 µl drops of mSOF without FBS for an initial development of 48 h and further cultured in mSOF supplemented with 10 per cent FBS. Medium was replaced at alternate day till blastocyst development or day 8 post-IVF. Group of healthy blastocysts has been depicted in Fig. 3.

Relative gene expression of oocyte and blastocyst competence markers: The gene-specific primers (*RPS15A*, *HAS2*, *EGFR*, *GREM1*, *PTGS2*, *GDF9*, *BMP15*, *INF-τ*, *GLUT1* and *POU5F1*) along with specific annealing temperature and product length were obtained from published sources[21,24].

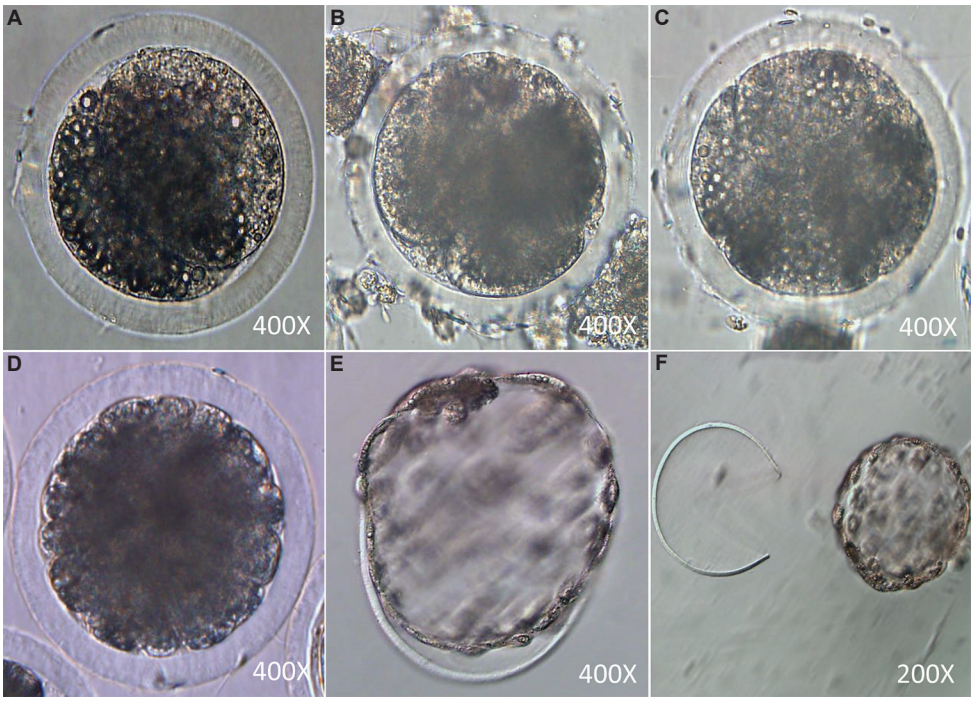

Fig. 3. Different embryonic developmental stages: (**A**) two-cell stage; (**B**) four-cell stage; (**C**) eight-cell stage; (**D**) Morula stage; (**E**) hatching blastocyst; (**F**) hatched blastocyst.

Total RNA extraction and cDNA synthesis: The total RNA was harvested in triplicate from denuded oocytes (n=100), cumulus cells isolated from COCs (n=100) and blastocysts (n=10) of respective groups using Trizol reagent (Ambion, USA). To assess the quality and integrity of the RNA, 5 µl of total RNA was subjected to denaturation in one per cent agarose gel electrophoresis. The purity and concentration of total RNA were checked using the NanoDrop Spectrophotometer (Thermo Scientific, USA) and samples with OD260: OD280 values between 1.8 and 2.0 were used for cDNA synthesis. cDNA synthesis was done using Verso cDNA synthesis kit (AB-1453/B; Thermo Scientific, California, USA) with total 20 µl reaction volume following the manufacturer's protocol. A total of 0.2 µg total RNA was used for reverse transcription as a template. The RNA was subsequently reverse transcribed by incubating at 42°C for 59 min followed by the final termination of the reaction by heating for two minutes at 95°C. The cDNA was properly labelled and stored at −20°C for later use. *RPS15A* was used as housekeeping gene.

Polymerase chain reaction (PCR) amplification: Polymerase chain reaction (PCR) amplification was done in Bioer XP cycler PCR machine (Hangzhou Bloer Technology Co., Ltd., Zhejiang, China) with the amplification reaction mixture of 20 µl, consisting of the 18 µl Platinum PCR mix (Life Technologies-11306-016, California, USA), 0.5 µl each forward and reverse primer of 0.5 µM concentration and 1 µl cDNA. PCR reaction was performed as the initial denaturation step at 94°C for two minutes followed by cDNA amplification cycles, including denaturation at 94°C for 30 sec, annealing at a specific annealing temperature of a primer for 30 sec and extension at 72°C for one minute. No template control (NTC) was maintained for each set of primers, and PCR products were analyzed by electrophoresis on 1.8 per cent agarose gel with LabSafe nucleic acid stain (G-Biosciences) and 50 bp ladder (Fermentas).

Real-time quantitative polymerase chain reaction (qPCR): Quantitative real-time PCR (qPCR) was performed with SYBR green master mix qPCR kit (Thermo Scientific, USA) and SmartCycler Real-Time qPCR (Cepheid, USA). NTC was placed with each reaction setup for checking any contamination in reaction components. Master mix total reaction volume of 20 µl was prepared by adding 10 µl 2X SYBR green mix, 0.5 µl each forward and reverse primer (0.5 µM each), 1 µl cDNA template and 8 µl NFW. *RPS15A* was taken as the housekeeping gene and cycle threshold (Ct) values, amplification plot and dissociation curve for all the required transcripts were acquired. The Pfaffl[25] method of relative quantification was used for calculation of relative gene expression.

Statistical analysis: The data for nuclear status were analyzed by Chi-square test using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) at 5 per cent level of significance. Data for cleavage rate and blastocyst rate were analyzed by one-way ANOVA with Duncan *post hoc* test. The change in relative expression of different genes in relation to *RPS15A* was analyzed by one-way ANOVA with Duncan *post hoc* test using GraphPad Prism V 5.0 software (GraphPad Software, CA, USA).

Results

The present study was conducted to analyze the effect of roscovitine on the developmental potential of SF-derived less competent oocytes, as during the initial experiments, the higher competence of LF oocytes was noted in comparison to SF oocytes.

Comparison of developmental competence between large follicle and small follicle (LF and SF): LF-derived oocytes were found to be developmentally more competent in terms of higher BCB-positive oocyte (LF-75.2% and SF-29.09%), cleavage rate and blastocyst rate than the SF-derived oocytes. The relative mRNA study also revealed higher developmental potential of LF oocytes than SF oocytes in terms of upregulated expression of the competence markers such as *GDF9* and *BMP15* in oocyte; *GREM1*, *PTGS2*, *HAS2* and *EGFR* in cumulus cells and interferon-t, *GLUT1* and *POU5F1* (Figs. 4-6).

Selection of best dose of roscovitine: Different concentrations of roscovitine were tested to select the best dose. First, the inhibition of cumulus expansion after pre-maturation incubation was determined. Roscovitine effectively arrested the cumulus cells' expansion in a dose-dependent manner. The number of COCs showing cumulus expansion was apparently lowest in 100 µM followed by 50 µM and 25 µM groups than 12.5 µM group. Thus, the maximum inhibition of cumulus expansion was observed in 100 µM roscovitine group, followed by 50 and 25 µM, and the least inhibitory effect was noted in 12.5 µM group (Fig. 7).

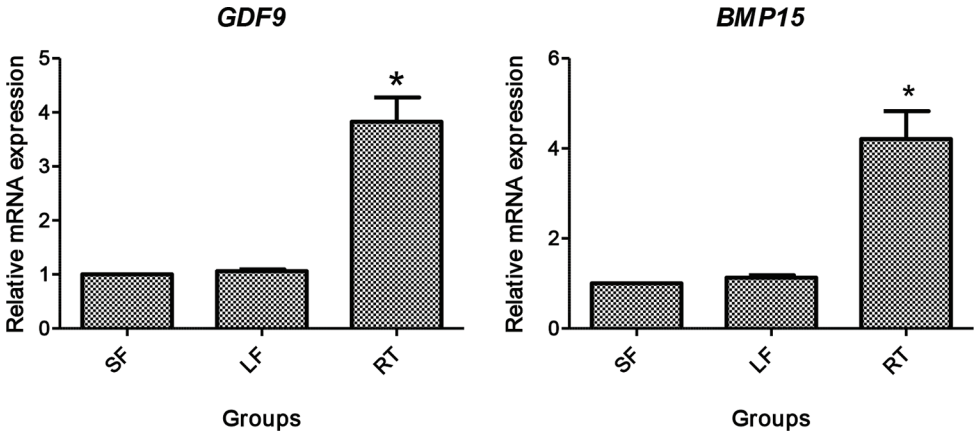

Fig. 4. Relative gene expression of *GDF9* and *BMP15* in denuded oocytes. SF, small follicle derived; LF, large follicle derived; RT, roscovitine treated. $^{*}P<0.05$ compared to SF and LF.

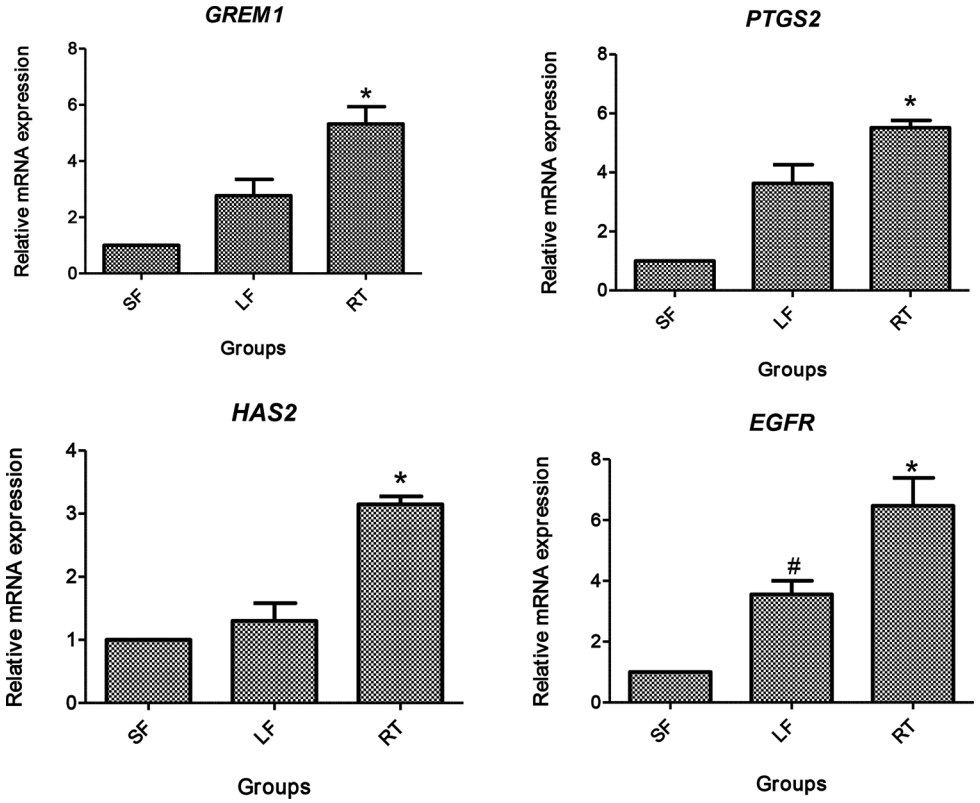

Fig. 5. Relative gene expression of *GREM1*, *PTGS2*, *HAS2* and epidermal growth factor receptor (*EGFR*) in cumulus cells. SF, small follicle derived; LF, large follicle derived; RT, roscovitine treated. $^{*}P<0.05$ compared to SF and LF; $^{\#}P<0.05$ compared to SF.

Second, data of the dose-dependent inhibitory effect of roscovitine were observed. Concentrations of 25, 50 and 100 μM roscovitine exhibited higher percentage of oocytes at GV and GVBD stages than control and 12.5 μM roscovitine (Table I). A comparatively smaller proportion of oocytes reached MII stage with these concentrations of roscovitine. Third, the reversible effect of roscovitine in the oocytes was observed through nuclear staining after 24 h of inhibitor-free maturation. All the groups depicted non-significant difference amongst themselves, but the concentration of 100 μM roscovitine revealed significantly ($P<0.05$) less number of MII oocytes in comparison to other groups (Table II). Finally, the cleavage and blastocyst rates of different groups were analyzed, and data are provided in Table III. The data depicted significantly ($P<0.05$)

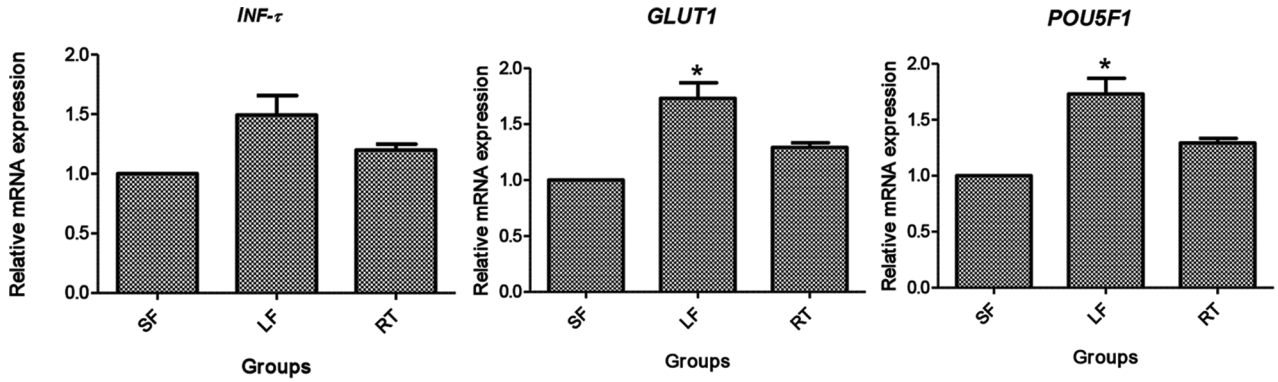

Fig. 6. Relative gene expression of *INF-τ*, *GLUT1* and *POU5F1* in blastocysts. SF, small follicle derived; LF, large follicle derived; RT, roscovitine treated. $^{*}P<0.05$ compared to SF and RT.

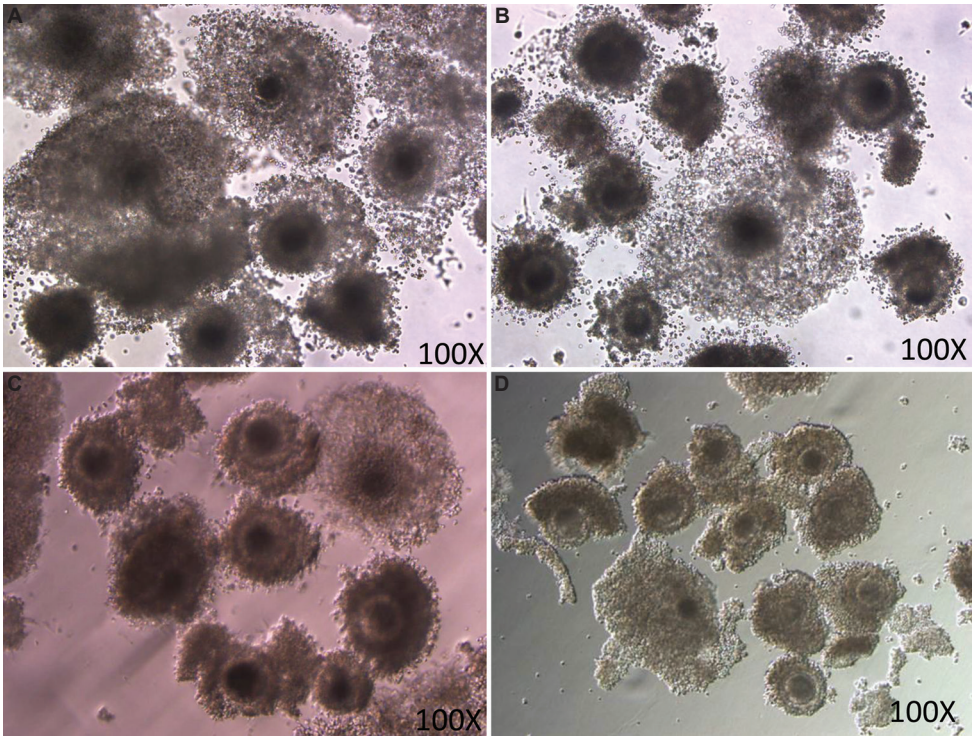

Fig. 7. Cumulus expansion in different groups. COCs treated with different doses of roscovitine- (**A**) 12.5 µM; (**B**) 25 µM; (**C**) 50 µM; and (**D**) 100 µM.

Table I. Nuclear status of oocytes cultured for 24 h in roscovitine-supplemented maturation medium

ROS (µM)	COCs, n[#]	GV, n (%)	GVBD, n (%)	MI, n (%)	AT, n (%)	MII, n (%)	Unstained, n (%)
0	97	1 (1.03)[a]	2 (2.06)[a]	5 (5.15)[a]	7 (7.22)[a]	72 (74.23)[a]	10 (10.31)[a]
12.5	85	20 (23.53)[b]	4 (4.7)[b]	19 (22.35)[b]	15 (17.65)[b]	20 (23.53)[b]	7 (8.24)[a]
25	89	67 (75.28)[c]	8 (8.99)[c]	3 (3.37)[a]	3 (3.37)[a]	2 (2.25)[c]	6 (6.74)[a]
50	97	77 (79.38)[c]	9 (9.28)[c]	2 (2.06)[a]	1 (1.03)[a]	1 (1.03)[c]	7 (7.22)[a]
100	78	65 (83.34)[c]	4 (5.13)[b,c]	1 (1.28)[a]	1 (1.28)[a]	1 (1.28)[c]	6 (7.69)[a]

Values (mean%) in the same column with different superscripts differ significantly ($P<0.05$). [#]COCs obtained from 5 replicates for each group performed on different days. ROS, roscovitine; COCs, cumulus-oocyte complexes; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, meiosis I; AT, ana-telophase; MII, meiosis II

Table II. Nuclear status of oocytes cultured for additional 24 h in inhibitor-free maturation medium

ROS (μM)	COCs n[#]	GV, n (%)	GVBD, n (%)	MI, n (%)	AT, n (%)	MII, n (%)	Unstained, n (%)
0	91	1 (1.1)[a]	3 (3.3)[a]	7 (7.69)[a]	10 (10.99)[a]	64 (70.33)[a]	6 (6.59)[a]
12.5	82	0 (0.0)[a]	4 (4.88)[a]	9 (10.98)[a,b]	10 (12.2)[a]	50 (60.98)[a]	9 (10.98)[a]
25	92	2 (2.17)[a]	2 (2.17)[a]	12 (13.04)[a,b]	14 (15.22)[a,b]	55 (59.78)[a]	7 (7.61)[a]
50	81	0 (0.0)[a]	3 (3.70)[a]	10 (12.35)[a,b]	11 (13.58)[a,b]	50 (61.72)[a]	7 (8.64)[a]
100	85	3 (3.53)[a]	7 (8.24)[a]	15 (17.65)[b]	4 (4.71)[b]	37 (43.53)[b]	19 (22.35)[b]

Values (mean%) in the column with different superscripts differ significantly ($P<0.05$)
[#]COCs obtained from 5 replicates for each group performed on different days.
Abbreviations as given in Table I

Table III. Effect of different concentrations of roscovitine on subsequent embryo development

Group	COCs, n[#]	Cleavage rate[*], n (%)	Blastocyst rate[$], n (%)
SF/0 μM RT	410	152 (37.07±1.4)	22 (14.47±0.9)[a]
12.5 μM RT	190	61 (38.27±1.4)[a]	19 (14.96±1.7)[a]
25 μM RT	228	108 (47.56±1.5)	31 (29.02±1.0)[b]
50 μM RT	274	136 (51.02±1.7)[b]	41 (30.56±1.4)[b]
100 μM RT	147	58 (40.83±2.2)[a]	9 (19.27±1.0)[c]
LF	248	153 (61.69±0.8)[c]	56 (36.6±1.4)[d]

[*,$]Values (mean±SE%) in the column with different superscripts differ significantly ($P<0.05$). [#]COCs obtained from five replicates for each group performed on different days; [*]On the basis of total COCs cultured; [$]On the basis of total oocytes cleaved. RT; roscovitine treated; COCs, cumulus-oocyte complexes; LF, large follicle; SF, small follicles

higher cleavage rate in 25 and 50 μM roscovitine-treated groups; however, no significant difference observed amongst control and 12.5 and 100 μM roscovitine-treated groups. A significantly ($P<0.05$) higher blastocyst rate was observed in 25 and 50 μM roscovitine-treated groups than control and 12.5 μM roscovitine-treated groups which reduced significantly ($P<0.05$) in 100 μM roscovitine-treated group though it was significantly ($P<0.05$) higher than the control and 12.5 μM roscovitine-treated group (Table III).

On the basis of the above findings, 25 μM roscovitine was selected as the minimum effective dose for the improvement of developmental competence of oocytes derived from the SF.

Comparison of best-selected dose of roscovitine with large follicle (LF)-derived oocytes: COCs pre-treated with 25 μM roscovitine were compared with LF-derived COCs in terms of the BCB staining, cleavage rate, blastocyst rate and relative mRNA expression of oocyte and blastocyst competence markers. BCB staining revealed that mean per cent of BCB-positive oocytes was significantly ($P<0.05$) higher in LF (75.26%) and 25 μM roscovitine-treated (64.49%) groups than SF (29.09%) group. However, no difference was noted between LF and treatment groups. The cleavage and blastocyst rate was significantly higher ($P<0.05$) in 25 μM roscovitine-treated group than the SF group but was significantly lower ($P<0.05$) than the LF group (data not shown).

PCR reaction and agarose gel electrophoresis confirmed predicted amplification of primers of respective genes. The relative mRNA expression of oocyte competence markers in denuded oocytes (*GDF9* and *BMP15*) and cumulus cells (*GREM1*, *EGFR*, *PTGS2* and *HAS2* are given in Figs. 4 and 5, respectively. The mRNA expression of *GDF9* and *BMP15* transcript was significantly higher ($P<0.05$) in denuded oocytes of roscovitine-treated group than LF and SF groups, whereas no significant difference was observed in *GDF9* and *BMP15* expression between the LF and SF groups (Fig. 4). The mRNA expression level of *GREM1*, *PTGS2*, *HAS2* and *EGFR* in cumulus cells was significantly higher ($P<0.05$) in roscovitine-treated group than LF and SF groups. Similarly, a significantly higher ($P<0.05$) expression of *GREM1*, *PTGS2* and *EGFR* was recorded in LF group than SF group, whereas no difference was noticed in the expression of HAS2 transcript (Fig. 5).

The relative mRNA expression of blastocyst competence markers has been provided in Fig. 6. *GLUT1* and *POU5F1* revealed a significantly higher ($P<0.05$) expression in LF group, followed by roscovitine-treated group than the SF group, whereas INF-τ expression was insignificant amongst the groups.

Discussion

Many studies[6,14,16-19] have already been conducted to enhance the developmental competence of oocytes

by the use of various nuclear inhibitors including roscovitine, but none conducted specifically to enhance the developmental competence of SF-derived oocytes. Hence, the present study was undertaken to enhance the developmental competence of SF-derived oocytes by pre-maturation incubation with roscovitine. During the study, the effect of the different concentrations of roscovitine on developmental competence of SF-derived buffalo oocytes was evaluated in terms of reversible nuclear maturation inhibition, embryo development and relative gene expression of oocyte and blastocyst competence markers, with its further comparison with LF-derived oocytes.

Cumulus expansion was better after 24 h of *in vitro* maturation in LF oocytes than SF oocytes; also, more number of BCB-positive oocytes was obtained from LF in comparison to the SF group. A similar result has been reported in ovine species[26]. BCB staining has already been employed as a non-invasive, indirect method for selection of more competent oocytes in various species[1,27,28]. The present study also reported a significantly higher cleavage and blastocyst rate in LF oocytes than those of SF-derived oocytes. A relationship between follicle size and oocyte quality has been demonstrated in several species. This is due to the greater storage of the transcripts and proteins in the cytoplasm of LF oocytes[29].

Results of roscovitine treatment revealed a dose-dependent inhibition of meiotic resumption, and this meiotic inhibition was reversible for all the doses of roscovitine. However, a concentration of 25 µM was found to be the minimum effective dose at which about 75 per cent of the oocytes were arrested at GV stage. The results of the present study were supported by previous studies[14,17] which reported that 25 µM roscovitine maintained the cattle oocytes at GV stage for 24 h. Albarracin *et al*[16] found 50 µM roscovitine as the best dose for calf oocytes. There are reports in equine[6] where oocyte nuclear maturation was inhibited with 50 µM roscovitine, whereas in swine, 80 µM roscovitine[30] and 50 µM roscovitine[19] were the best doses to inhibit the meiotic resumption. Further, Zhang *et al*[31] concluded that 25 µM roscovitine improved the developmental competence of porcine oocyte. In ovine, 75 µM roscovitine was found to be efficient to reversibly block the meiotic resumption without any detrimental effect on development and quality of *in vitro*-produced embryos[18].

BCB staining and embryo development rate was analyzed in roscovitine-treated group. The dose of 25 µM depicted better BCB staining and embryo development and thus selected as best roscovitine dose because it was the minimum dose which effectively arrested the oocyte meiotic division and significantly improved the *in vitro* developmental potential of SF-derived oocytes as well as embryos. It has been demonstrated that bovine oocytes arrested with roscovitine and butyrolactone I (BL-I) are competent to undergo normal early development and embryo derived from these oocytes developed into normal foetuses up to day 27[32]. Kaedi *et al*[33] found an enhanced blastocyst formation rate of reconstructed embryos derived from oocytes pre-cultured with roscovitine. In the present study, roscovitine pretreatment was given only to the SF-derived oocytes, which had a compromised developmental competence, and thus, during the pre-maturation incubation, these might have attained better cytoplasmic maturation and in turn revealed greater competence than untreated SF oocytes.

The enhanced relative expression of competence markers such as *GDF9* and *BMP15* in oocyte and *GREM1*, *PTGS2* and *EGFR* in cumulus cells depicted the role of 25 µM roscovitine in enhancing oocyte competence. Lequarre *et al*[34] also reported the increased mRNA expression in roscovitine-treated bovine oocytes. This may define the process of neotranscription, or the addition of poly(A) tails to transcripts not yet adenylated, or both in the GV-arrested oocytes. This effect of roscovitine is transient as it is no longer detectable after 24 h of maturation[31]. Hence, the significantly upregulated expression of the studied transcripts in roscovitine-treated group may be related to the neotranscription or increased polyadenylation.

Further, the blastocyst derived from LF and roscovitine-treated SF group showed significantly higher expression of blastocyst competence markers (*GLUT1* and *POU5F1*) than SF group, which suggested that roscovitine treatment enhanced the developmental potential of SF-derived oocytes in terms of blastocysts number as well as its quality.

Hence, it is concluded from the study that 25 µM roscovitine effectively improved the developmental potential of less competent SF oocytes as it enhanced the mRNA expression pattern of competence-related molecules. It provides a useful method for ensuring the availability of more number of competent oocytes for buffalo embryo transfer technology as well as an

efficient tool for transportation or manipulation of oocytes at the onset of maturation.

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