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Research Article

Real–Time PCR Based Expression Study of *Cyclin B* Gene in Buffalo Cumulus Oocyte Complex

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ABSTRACT

The success rate of *in vitro* fertilization (IVF) in buffalo is very low, though a research of more than a decade has been carried out. One of the reasons could be the lack of standardised protocol for *in vitro* maturation of oocytes. The present study was conducted to determine the temporal expression of *Cyclin B* gene in follicles of different size. A good quality cumulus oocyte complexes (COCs) were recovered from large (>6 mm) and medium (2–6 mm) follicles of buffalo ovaries by aspiration. The recovered COCs were matured in Tissue Culture Medium–199 medium supplemented with 10% foetal bovine serum (FBS), 0.4% bovine serum albumin (BSA) and hormones pregnant mare's serum gonadotropin (10 IU/mL) and human chorionic gonadotropins (10 IU/mL) and incubated for zero min, 30 min, 60 min, 90 min, 18 hr, 20 hr, 22 hr, 24 hr and 26 hr in a CO_2 incubator with 5% CO_2 under humid conditions at 38.5°C. Total RNA was isolated from COCs, reverse transcribed and real time PCR was done to study the expression pattern of *Cyclin B* gene in follicles of different sizes. Real time PCR assay revealed higher expression of *Cyclin B* in medium size follicles. However temporal expression of *Cyclin B* was highest at zero min in both large and medium follicles and decreases as the maturation time increases. These results suggest that the role of *Cyclin B* is more during early phases of maturation and less during later stages of maturation.

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INTRODUCTION

In vitro maturation (IVM) and *in vitro* fertilization (IVF) procedures performed on oocytes obtained from slaughter–house derived ovaries have recently provided a practical means for producing large number of bovine zygotes at low cost for research and commercial purpose (Hansen, 2006). The results for IVF in buffalo are very poor as compared to cattle because majority of IVF studies in buffalo use the protocols which are established for cattle (Yousaf and Chohan, 2003). The overall success rate of embryo production following IVF in buffalo is reported to be not more than 10–15 percent which is very low when compared to that of cattle (Chauhan et al., 1998; Nandi et al., 2001; Raghu et al., 2002). Nandi et al. (2002) have reported approximately 80 percent of buffalo oocytes fail to reach the blastocyst stage and most of blastocysts are not capable of further development. The poor success rate of *in vitro* fertilization and lower developmental competence of *in vitro* produced embryos present a principal barrier for the successful implementation of other assisted reproductive techniques like animal cloning, animal transgenesis, stem cell culture and nuclear transfer etc. in buffaloes. The maturation of oocytes under *in vitro* condition has significant influence on the number of embryos that develop to the blastocyst stage (Thompson et al., 1995; Jones, 2000; Rizos et al., 2002). The oocyte undergoes a series of modifications during maturation and these are necessary to acquire developmental competence. Therefore more advanced research for the development of suitable *in vitro* maturation (IVM) system is important.

Cyclins control the transition between the phases of the cell cycle as regulatory subunits of the cyclin–dependent kinases (CDKs) (Donjerkovic and Scott, 2000). The amount of *Cyclin B* (which binds to Cdk1) and the activity of the *Cyclin B*–Cdk complex rise through the cell cycle until mitosis (Ito, 2000), where they fall abruptly due to degradation of *Cyclin B* (Cdk1 is constitutively present). *Cyclin B* protein is degraded by ubiquitin pathway (Hershko, 1999). The complex of Cdk and *Cyclin B* is called maturation promoting factor or mitosis promoting factor (MPF; Maller, 1990). Activated *Cyclin B*–Cdk complex promotes several of the events of early mitosis. The active complex phosphorylates and activates 13S condensin, which helps to condense chromosomes (Kimura et al., 1998).

Maturation–inducing hormone (MIH) induces oocytes to synthesize *Cyclin B* (Hirai et al., 1992; Katsu et al., 1999). The rate of *Cyclin B* synthesis controls the length of the first meiotic M phase (Polanski et al., 1998). Human *Cyclin B* protein microinjected into cycloheximide–treated bovine oocytes triggered meiotic resumption. Thus, *Cyclin B* has a

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critical role in meiotic resumption in bovine oocytes (Lévesque and Sirard, 1996). The rates of *Cyclin B* synthesis and degradation determine the timing of the major events taking place during meiotic maturation of the mouse oocyte (Ledan et al., 2001). GVBD in mouse oocytes is sensitive to *Cyclin B* abundance and the changes in distribution of *Cyclin B* contribute to progression through Meiosis I stage (Marangos and Caroll, 2004). *Cyclin B* and cdc2 (cell division control) were expressed at the mRNA level in GV oocytes but cdc2 was the only pre–MPF protein detected at that stage, thus meiosis resumption seems to be regulated by the translation of *Cyclin B* mRNA. *Cyclin B* and CDC2 mRNA were high at 10 h of IVM and decreased gradually until 26 h, where most of oocytes reached the meiosis–II stage (Khalil et al., 2010).

Thus for improving reproductive performance of buffaloes, better understanding of expression level of different genes, which have a role in meiotic resumption, is important. The present study has been conducted to determine the expression pattern of *Cyclin B* gene in buffalo COCs from different size follicles at different time interval during *in vitro* maturation.

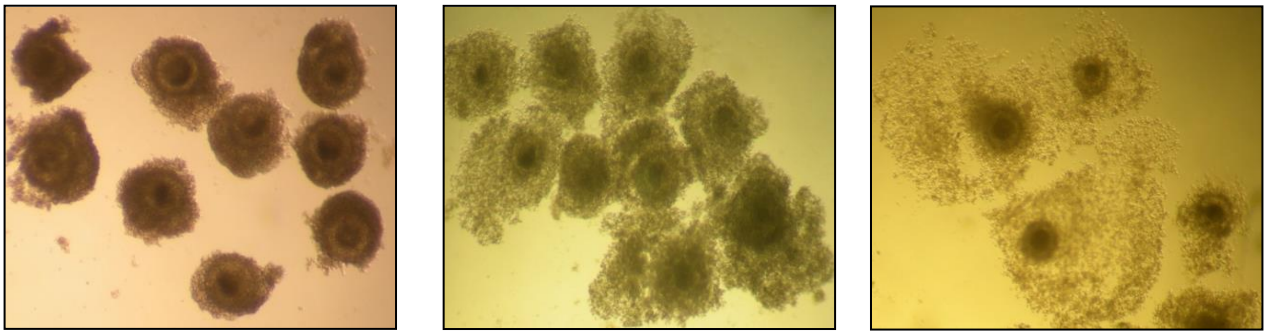
MATERIALS AND METHODS

Collection of Ovaries

Buffalo ovaries were collected from MCD slaughter house, Ghazipur, New Delhi. All ovaries were carried to the quality control laboratory of slaughter house within 15 minutes of slaughter in normal saline solution (NSS) (at 37°C) supplemented with antibiotics (Penicillin 100 IU mL and streptomycin sulphate 100 µg/mL). All the visible ovarian follicles were graded as medium follicles (2–6 mm) and large follicles (more than 6 mm) based on their diameter and oocytes were aspirated in the aspiration media containing TCM–199, Glucose, Sodium Pyruvate, Sodium Bicarbonate and antibiotics, using 18G needle. The aspirated contents were transferred to 60 mm petridish and searched under stereozoom microscope (Kyowa, Japan).

In Vitro Maturation of Oocytes

COCs were washed serially thrice in 35 mm petridish containing 100 µL drops of oocyte washing medium containing TCM–199, supplemented with 10% FBS and finally transferred into 100µL of washing drops prepared from maturation drops supplemented with hormones PMSG (10 IU/mL) and hCG (10 IU/mL). The COCs from graded follicles were matured in maturation media drops, TCM–199 supplemented with 10 percent FBS containing antibiotics (streptomycin 100 µg/mL, Penicillin 100 IU/mL) and hormones PMSG (10 IU/mL) and hCG (10 IU/mL). The maturation drops were covered with warm (35–37ºC) light weight mineral oil and kept for different time period (Zero minute, 30 min, 60 min, 90 min, 18 hr, 20 hr, 22 hr, 24 hr and 26 hr) in CO_2 incubator at 38.5ºC under 5% CO_2 pressure and with a relative humidity of 90 to 95%. Maturation of oocytes was evaluated by the cumulus cell expansion under stereo zoom microscope (Figure 1).



a b c

Figure 1: Morphology of the cumulus oocytes complexes after different time periods of incubation; zero hr (a); 20 hr (b); 26 hr (c)

Reverse Transcription (cDNA Synthesis); RNA Extraction and Preparation of cDNA

Total RNA was isolated from *in vitro* matured COCs by using TRIzol® LS reagent (Life technologies) as per the manufacturer's instruction. Total RNA was quantified using Qubit® 2.0 fluorometer (Invitrogen). To ascertain minimum variation among different groups, a pool of 66 COCs was used for RNA isolation. Reverse transcription was carried out with total reaction volume of 20 µL. Briefly, NFW (8.00 µL), 5X RT buffer (Fermentas) (5 µL), 10mM dNTPs (Fermentas) (0.5 µL), total RNA (4 µL), M–MuLV–RT 200 IU/µL (Promega) (1 µL), DMSO (1 µL), decamer primers (Ambion) (0.5 µL). The RT–PCR cyclic conditions were as: annealing at 25ºC for 10 min, reverse transcription at 42ºC for 1 hour, and denaturation at 90ºC for 5 min in thermal cycler (Eppendorf thermocycler). The cDNA was stored at –20ºC till further use.

Real Time PCR

For the analysis of temporal expression profile of gene, real time PCR was carried out using real time PCR Applied Biosystems 7500. For the real time PCR reaction, SYBER Green dye i.e. Quantitect SYBER Green PCR kit® (Qiagen) was used and all the instructions were followed as per the supplier. The reaction for the target gene, *Cyclin B* gene, and the endogenous control, *GAPDH* gene was carried out in triplicate along with NTC (Non–template Control) as a negative control for each sample.

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Table 1: Sequence of primers used in real time PCR experiments

Gene	Primers	Sequence (5'–3')	Product size
Cyclin B (Khalil et al. 2010)	Forward	GAG GGG ATC CAA ACC TTT GTA GTG A	318 bp
	Reverse	CAA TTT CTG GAG GGT ACA TTT CTT C	
GAPDH	Forward	GGC AAA GTG GAC ATC GTC GCC A	267 bp
	Reverse	ACC CTT CAA GTG AGC CCC AGC	

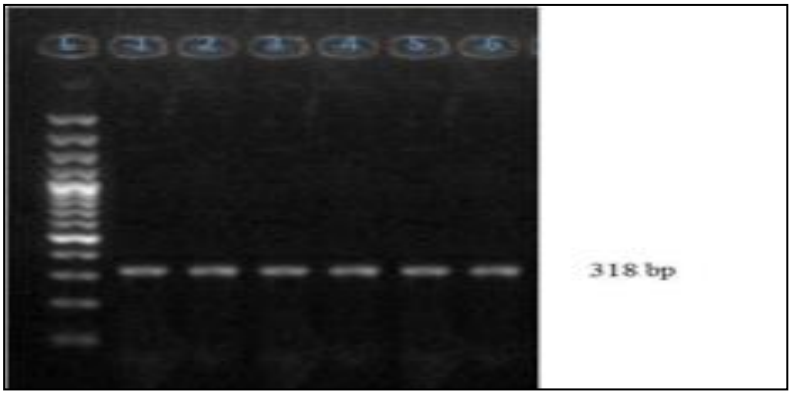

Figure 2: Agarose gel electropherogram of PCR product; Cyclin B (1.5%); Lane L: 100bp ladder (MBI Fermentas); Lane 1–6: PCR product of Cyclin B gene (318 bp)

The reaction mixture used to carry out the real time PCR reaction for *Cyclin B* and *GAPDH* gene a total reaction volume of 25 µL, was as: 2X Quantitect SYBER Green PCR Mix (Qiagen, 12.5 µL), primers (forward and reverse 0.3 M each), NFW (variable), 25mM $MgCl_2$ (Fermentas, 2 µL), and template (2 µL). The cyclic conditions used for amplification of *Cyclin B* and *GAPDH* transcript cDNA were according to the instructions of the supplier. The set of primers used for the real time PCR are as shown in Table 1.

Relative Quantification by Comparative C_T Method ($\Delta\Delta C_T$ Method)

The average C_T (Threshold cycle) value obtained for the *Cyclin B* (target) gene was normalized to *GAPDH* (endogenous control). The data obtained was subjected to comparative C_T method (Livak and Schmittgen, 2001) for the analysis of the expression levels of target (*Cyclin B*) gene and an endogenous control. The sample at 26 hour of incubation was selected as calibrator.

Cloning and Sequencing of PCR Products

In the present study the 318 bp PCR products of *Cyclin B* gene (Figure 2) of buffalo was used for cloning in CloneJET™ PCR Cloning Kit using pJET 1.2 blunt end cloning vector. The product was sequenced at Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences.

Nucleotide Sequence Analysis

The sequence obtained was analyzed by using BLASTn. The phylogenetic tree was constructed using Phylogeny.fr (http://www.phylogeny.fr/) server to show relatedness among the species with respect to *Cyclin B* cDNA.

RESULTS

Expression Profile of Cyclin B Gene

The differential expression level of *Cyclin B* gene transcripts in in vitro matured oocytes was studied by relative quantification method. The level of target mRNA in different gorups was determined by comparative CT method (ΔΔCT method). The level of *Cyclin B* mRNA was more in medium follicles than large follicles at any point of time. The expression level of Cyclin B mRNA decreases as the maturation time increases. The relative abundance of *Cyclin B* in COCs aspirated from graded follicles was higher just after aspiration (i.e. zero minute) as compared to maturation times of 24 and 26 hr. After 22 hrs the level of expression of *Cyclin B* remained low and almost constant (Figure 3 and Table 2.1, 2.2). The expression level of *Cyclin B* was highest at zero minutes in both large and medium sized follicles.

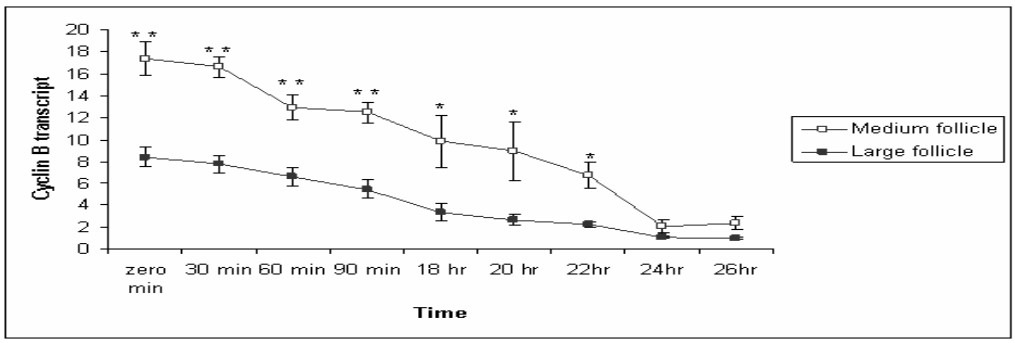

Figure 3: This figure shows the comparison of transcript levels of Cyclin B gene in medium and large sized follicles at different time points of maturation; **P<0.005 and *P <0.05 (T-test). Data is representative of three experiments

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Time	GAPDHC$_T$ (MF)	Cyclin B C$_T$ (MF)	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C}{}_T$
zero min	25	27.16	2.16	−4.12	17.38776
30 min	25	27.226	2.226	−4.054	16.61023
60 min	25	27.587	2.587	−3.693	12.93313
90 min	25	27.639	2.639	−3.641	12.47528
18 hr	25	27.986	2.986	−3.294	9.808279
20 hr	25	28.118	3.118	−3.162	8.950697
22hr	25	28.539	3.539	−2.741	6.685336
24hr	25	30.236	5.236	−1.044	2.061937
26hr	25	30.05	5.05	−1.23	2.34567

Table 2.1:$\Delta\Delta C_T$relative quantitation of Cyclin B transcript in medium follicle

Table 2.2: $\Delta\Delta C_T$ relative quantitation of Cyclin B transcript in large follicle

Time	GAPDHC$_T$ (LF)	Cyclin B C$_T$ (LF)	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C}{}_T$
zero min	25	28.21	3.21	−3.07	8.397733
30 min	25	28.326	3.326	−2.954	7.748946
60 min	25	28.567	3.567	−2.713	6.556837
90 min	25	28.834	3.834	−2.446	5.449032
18 hr	25	29.532	4.532	−1.748	3.358926
20 hr	25	29.863	4.863	−1.417	2.670297
22hr	25	30.115	5.115	−1.165	2.242332
24hr	25	31.137	6.137	−0.143	1.104199
26hr	25	31.28	6.28	0	1

Accession number	*Cyclin B* gene of specific species	%age similarity
XR_083650.1	*Bos taurus Cyclin B* mRNA	94%
NM_031966.3	*Homo sapiens Cyclin B* mRNA	92%
XM_845305.2	*Canis lupus familiaris* cyclin B, mRNA	92%
XM_001491280.2	*Equus caballus* cyclin B, mRNA	92%
NM_001170768.1	*Sus scrofa* cyclin B, mRNA	92%
NM_001261149.1	*Macaca mulatta* cyclin B, mRNA	92%
X60768.1	*Rattus norvegicus* cyclin B, mRNA	88%

Table 3: Maximum homology search using BLASTn

Phylogenetic Analysis of Nucleotide Sequences of Cyclin B

The identity analysis of the buffalo *Cyclin B* sequence with the other species GenBank sequence was done using BLASTn. The NCBI Genbank accession numbers of the sequences of *Cyclin B* of the various species considered were XR_083650.1 (*Bos taurus*), NM_031966.3 (*Homo sapiens*), XM_845305.2 (*Canis lupus familiaris*), XM_001491280.2 (*Equus caballus*), NM_001170768.1 (*Sus scrofa*), NM_001261149.1 (*Macaca mulatta*) and X60768.1 (*Rattus norvegicus*). The *Cyclin B* was found 94% identical to *Bos taurus*, 92% to *Homo sapiens*, 92% to *Canis lupus familiaris*, 92% to *Equus caballus*, 92% to *Sus scrofa*, 92% to *Macaca mulatta* and 88% to *Rattus norvegicus* (Table 3).

The phylogenetic tree was constructed using Phylogeny.fr server to show relatedness between the species with respect to *Cyclin B* fragment (Figure 4). In phylogenetic tree, *Cyclin B* of *Bubalus bubalis* were found more closely related to *Bos Taurus* than to *Sus scrofa*. From the phylogenetic tree it was observed that the *Rattus norvegicus Cyclin B* gene diverged much earlier in the evolution.

Sequence of Cyclin B (Partial mRNA)

318 bp product length was successfully sequenced using BigDye terminator chemistry and used as Query Sequence for constructing phylogenetic tree.

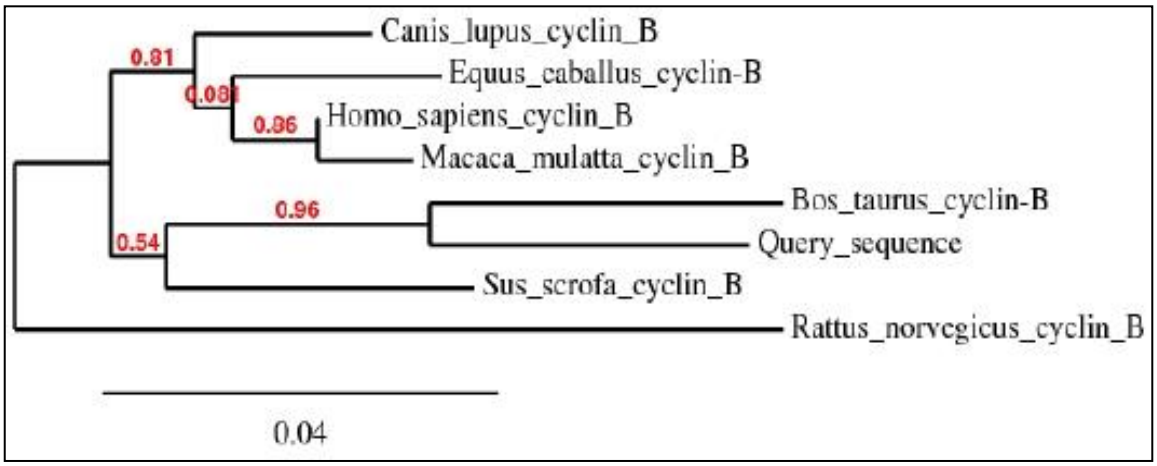

Figure 4: Phylogram of buffalo *Cyclin B* showing evolutionary relationship with other species

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DISCUSSION

Cell proliferation is an elementary cellular function and is tightly controlled by physiological signals as well as internal molecular mechanisms. In eukaryotic cells, the maturation promotion factor (MPF) is the master regulator of cell proliferation. MPF is a heterodimer of *Cyclin B* and cyclin dependent kinase. In this study, we report the partial sequence of buffalo *Cyclin B* cDNA. The identity analysis had shown that the *Cyclin B* gene of *Bubalus bubalis* is more closely related to *Bos Taurus* and lesser to *Rattus norvegicus.* Thus *Rattus norvegicus Cyclin B* gene has diverged much earlier in the evolution. But overall the sequence of Cyclin B has remained conserved throughout the evolution.

In present study the expression of mRNA for *Cyclin B* gene in *in vitro* matured COCs was studied by real time PCR. Our study revealed that the expression of *Cyclin B* gene was higher in the oocytes collected from medium sized follicles than large size follicle Majority of the oocytes completed maturation between 22 and 26 hr. These results were in agreement with those reported by Gasparrini et al. (2008), who found that the majority of buffalo oocytes accomplish nuclear maturation between 21 and 24 hr after the start of IVM. In another study, Nandi et al. (2002) found that cumulus expansion and extrusion of first polar body in buffalo oocytes commence at 6–17 hr post–maturation to reach the maximum levels at 22–24 hr. However, the results of the present study were in disagreement with the study of Neglia et al. (2001), in which the majority of buffalo oocytes reached the MII stage between 15 and 19 hr after the start of IVM and an increased incidence of degenerated oocytes was observed at later times. Large variations in the timing of the oocyte maturation process in vitro have also been reported in cattle (Ward et al., 2002; Park et al., 2005). The different oocyte maturation time–scale recorded among different buffalo studies may be accounted by different conditions of IVM and particularly by oocyte quality, which in this species, is also likely to be affected by seasonal factors.

The cytoplasmic maturation of the oocytes is a key parameter, which determines the success rate of in vitro production of embryos (Blondin and Sirard, 1995). Gaining knowledge about the variation/chronology of gene expression during oocyte maturation is crucial for optimization of IVF and other artificial reproduction technologies. Studies involving expression pattern of developmentally important genes in oocytes and embryos have not been widely investigated (Gaudette et al., 1993; Lechniak, 2002). Based on the *Cyclin B* mRNA transcripts in buffalo oocytes in our study, mRNA for genes that encode components of MPF were detected immediately after oocytes collection, at zero minute of IVM. Wu et al. (1997) reported that activity of MPF is low in GV–stage bovine oocytes and increases around GVBD to peak at the MI stage. In the current study, *Cyclin B* mRNA was high at zero minute of IVM and decreased gradually until 26 h, where most of oocytes reached the MII stage. This result correlates the resumption of meiosis with the abundance of MPF component mRNAs. The depletion of *Cyclin B* mRNAs could be associated with mRNA degradation or translation into MPF–component proteins.

This study is also in agreement with the results of Robert et al. (2000) who reported that bovine GV–stage oocytes, recovered from ovaries immediately after slaughter, possess a stockpile of maternal *Cyclin B* mRNA. Interestingly, it seems that *Cyclin B* protein can accumulate before oocyte maturation, when the ovary transportation time is long (Levesque and Sirard, 1996). *Cyclin B* mRNA probably is in a translationally inactive state (masked) in immature GV–stage bovine oocytes. Therefore, initiation of *Cyclin B* translation could be one of the early events leading to oocyte meiotic resumption. These studies clearly indicate that the role of *Cyclin B* is crucial during the early stages of folliculogenesis and its function and expression diminishes towards the later stages. Thus, the functions of *Cyclin B* is more conspicuous in the early phases of maturation and less during later stages of maturation of oocyte.

CONCLUSIONS

The mRNA expression of *Cyclin B* gene in buffalo cumulus oocyte complex was higher in medium follicles followed by large follicles. *Cyclin B* gene expression is found in all the IVM COCs irrespective of size of follicular source. The relative abundance of *Cyclin B* in COCs aspirated from graded follicles was higher just after collection i.e. at zero minute compared to those at 24 and 26 hrs of maturation. This expression pattern of *Cyclin B* reaffirms its role in germinal vesicle breakdown (GVBD) and early folliculogenesis and thereby proves that *Cyclin* B is an important factor regulating *in vitro* maturation of COCs.

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