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Localization and expression of proliferating cell nuclear antigen (PCNA) and cyclin B1 in buffalo (*Bubalus bubalis*) ovary during different stages of follicular development

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ABSTRACT

The present study was designed to determine the expression of proliferating cell nuclear antigen protein (PCNA) and cyclin B1 in buffalo during ovarian follicular development. PCNA and cyclin B1 were localized immunohistochemically in paraffin embedded ovarian sections whereas the expression of mRNA was done through semi-quantitative RT-PCR. Immunohistochemical studies demonstrated presence of PCNA and Cyclin B1 immunoreactivity throughout the follicular development. Positive immunoreactions of PCNA in preantral and actively growing small to large antral follicles showed extensive labeling in the layer of granulosa and theca cells. In contrast, cyclin B1 immunoreactivity was localized to the granulosa cells and it increased from small, medium to large follicles. mRNA transcript of PCNA (496 bp) and cyclin B1 (293 bp) was detected in ovarian stromal tissue, granulosa cells, preantral and antral follicles respectively. Follicles demonstrating advanced atresia showed only limited or no PCNA and cyclin B1 labeled granulosa and theca cells. In conclusion, follicular growth and development in buffalo ovary may be effectively monitored by determining the expression of PCNA and Cyclin B1 in granulosa cells.

Key words: Antral follicle, Buffalo, Cyclin B1, PCNA, Preantra follicle

Growth and development of ovarian follicles is characterized by marked processes of granulosa cell proliferation and differentiation. Proliferating cell nuclear antigen (PCNA), an essential regulator of the cell cycle, is 36 kDa molecule is highly conserved amongst species. PCNA serves as a co-factor for DNA polymerase delta in S-phase and is involved in DNA damage repair during DNA synthesis (Bravo *et al.* 1987). The expression of PCNA was detected in ovary in association with the studies of initiation and early events of follicular growth in pig (Tománek and Chronowska 2006), cow (Isobela and Yoshimura 2000) and baboon (Wandji *et al.* 1997). During S phase, PCNA pairs with cyclin D, another important regulator of cell proliferation. This complex is modulated by various growth factors and other growth stimuli (Xiong *et al.* 1991). If appropriate stimuli are received during G, cells become committed to S phase, and PCNA expression increases through G/S-phase interface, reaching a plateau during G2 (Liu *et al.* 1989). PCNA expression sharply declines in M phase and in quiescence. These characteristics make PCNA a useful marker for proliferation and determination of leading follicles from reserve pool of ovarian follicles *in vivo*.

Once the oocyte is extracted from the follicular environment, resumption of meiosis is occurred. During resumption, the M-phase promoting factor (MPF), a protein complex composed of subunits cyclin B1 and p34cdc2 (Robert *et al.* 2002), is activated which regulate the germinal vesicle breakdown (GVBD) (Taieb *et al.* 1997). MPF activity was described in many mammalian oocytes: it appears just before GVBD and increases until metaphase I stage, then its activity decreases in anaphase–telophase and increases again, reaching its maximum level in metaphase II in goat (Anguita *et al.* 2008), sheep (Ledda *et al.* 2001) and cow (Mihm *et al.* 2008). The candidate genes (PCNA and Cyclin B1) has merit and approaches that could be utilized to identify all key factors which regulate cell differentiation during dominant ovarian follicle development. However, in best of our knowledge there is no information available about the distribution and role of PCNA and cyclin B1 in buffalo ovarian follicles. Therefore, present study was designed to determine the presence of PCNA and cyclin B1 during follicular growth throughout follicular development leading to dominant follicles in buffalo.

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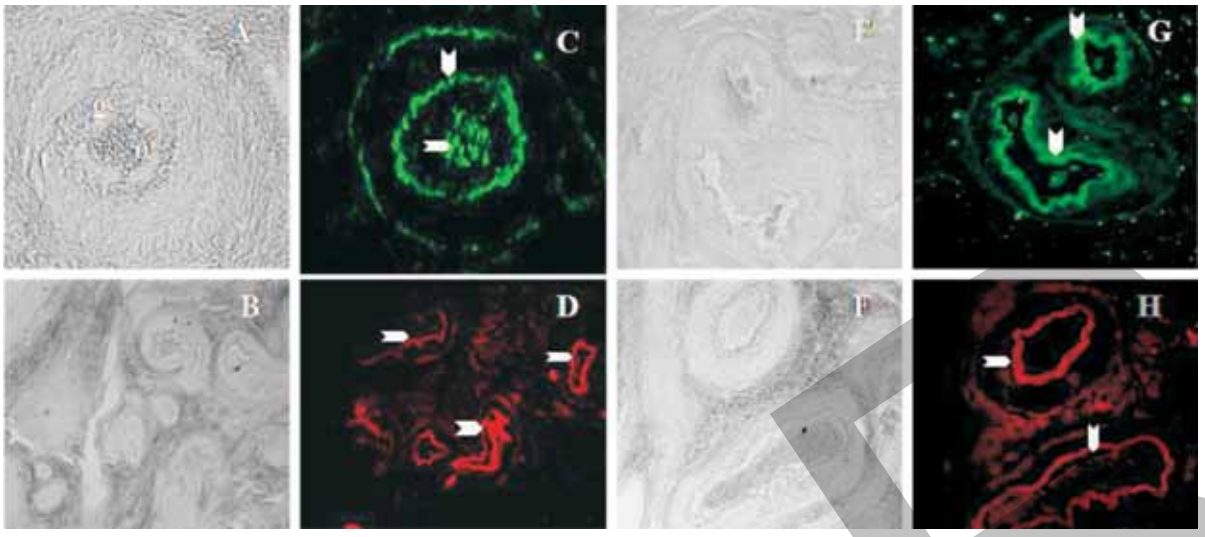

Fig. 1. Immunofluroscence detection of PCNA and cyclin B1 in buffalo ovarian follicles. Light micrographs of the buffalo preantral (A&B) and antral (E&F) follicles. Protein of PCNA and cyclin B1 was localized in oocytes and granulosa layers of preantral (C&D, green and red fluroscence, arrow mark) and antral (G & H, green and red fluroscence, arrow mark) follicles respectively. Magnification at 200×; Symbols: g-granulosa, t-theta, o-oocyte.

MATERIALS AND METHODS

Follicles and tissue sampling: Ovaries from random stages of the estrous cycle were collected from local abattoir and transported to the laboratory at 25–30°C in 0.9% normal saline within 2 h after slaughter. Preantral follicles (PFs; 200–250 µM) were isolated by micro dissection (Sharma *et al.* 2009a). Medium size (1–3 mm) and large size (3–5 mm) antral follicles (AFs) were isolated by cutting the ovary into 2 half and removing the connective tissue around AFs with surgical blade and a pair of forceps. The follicular granulosa cells (GCs) were isolated by aspiration of follicular fluid from large antral follicle with the help of 22 gauge needle fitted into 5 ml syringe. The isolated GCs were washed in DMEM supplemented with 10% fetal bovine serum (FBS). The isolated stromal tissues, PFs, AFs and GCs were placed at –70°C until they were used for mRNA expression. The ovarian cortical pieces were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for immunolocalization.

Immunohistochemistry: Paraffin embedded sections (5 µm) of buffalo ovarian cortical pieces were deparaffinized and treated with citrate buffer (0.01 M, pH 6.0) for 5 min. After 3 washes in phosphate-buffered saline (PBS), the sections were treated with normal goat serum equilibrated (1:100 v/v) in 0.05 M phosphate buffer to 0.15 M NaCl (PBS (pH 7.4) for 30 min to reduce background staining. Tissue sections were incubated with PCNA (1:300), and cyclin B1 (1:200) primary antibodies in PBS in a humid chamber at 37°C for 1 h. After brief PBS wash, slides were incubated with secondary antibody (goat anti-rabbit IgG conjugated with FITC or Texas red) diluted 1:500 in PBS at room temperature for 1 h. Sections were mounted in Vectashield after washing and observed under an epifluorescence microscope. Negative controls were processed in the same manner except the primary antibody was omitted.

RNA extraction and reverse transcription: Total RNA was isolated from ovarian tissue, GCs, PFs and AFs using Trizol reagent and quantified through Nano-drop 1000 UV visible spectrophotometer. Total RNA (500 ng) was used for cDNA synthesis using a first strand cDNA synthesis kit for RT-PCR. The RT reaction was carried out at 25°C for 5 min, 42°C for 60 min, 70°C for 5 min and then subsequent cooling at 4°C in thermal cycler for cDNA synthesis.

Detection of PCNA and Cyclin B1 transcript by PCR: The PCR reaction was carried out using 3.0 µl of cDNA, 3.0

Table 1. List of specific primers used in this study

Gene	Primer Sequence	Product size(bp)	Annealing temperature (°C)
PCNA-F	5'- ACTCGTCTCATGTCTCCTTG -3'	496	53
PCNA-R	5'- TCATTCATCTCTATGGCAACAG -3'		
Cyclin B1–F	5' ATTGGAGAGGTTGATGTTGAG -3'	293	56
Cyclin B1–R	5'- GTGGCATACTTGTTCTTGATAG -3'		

Table 2. Immunohistochemical localization of PCNA and cyclin B1 in the ovaries of buffalo. The intensity of staining is indicated by up to (+++), for maximal staining

Tissue/cell type	PCNA
Cyclin B1	
Surface epithelium -	-
Intersitium stroma +	+
Preantral follicle oocyte ++ Granulosa cells ++++ +++ Theca cells +++ +++	++
Antral follicle oocyte ++ Granulosa cells ++++ +++ Theca cells +++ +++	+

µl $MgCl_2$ (25 mM), 0.75 µl dNTP mix (10 mM), 0.75 µl (20 pmol) of PCNA and Cyclin B1 specific forward and reverse primers (Table 1) and 5.0 µl PCR reaction buffer. Finally 0.5 µl of Taq DNA polymerase (5 units/µl) was added and the final volume of 25 µl was made with nuclease free water.

The PCR program consisted an initial heating at 94°C for 3 min and samples were amplified for 35 cycles (94°C for 1 min; 53°C PCNA and 56°C for cyclin B1 for 45 sec and 72°C for 30 sec). The final extension at 72°C incubation was continued for further 7 min. PCR products were seprated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The image of each gel was recorded using a gel documentation system equipped with CCD camera and the intensity of each band was assessed by densitometry using the scion program.

RESULTS AND DISCUSSION

Localization of PCNA and cyclin B1 proteins in ovarian follicles: Immunohistochemical assessment showed the distribution of PCNA and cyclin B1 protein in various stages of follicles (Table 2). The localization of PCNA protein varied according to the follicle size. PCNA was detected mainly in the granulosa and theca cells of PFs and AFs. However, weak immunoreactivity of PCNA was localized in the stromal tissue of preantral and antral follicles (Fig. 2A, B and C). PCNA displayed intense staining in the granulosa cells of all sizes of follicles and found that immunoreactivity increases with the advancement of follicle stages (Fig. 2D, E and F) suggesting that dominant (healthy) follicles possess more proliferating capacity than early and atretic follicles. During the follicular development, more than 99% of follicles undergo the proccess of atresia while only a few reach the final size and ovulate. Using PCNA as a marker, in buffalo and cattle ovary Feranil *et al.* (2005) found that significantly higher frequency of PCNA labeled cells in healthy follicles than in the early and advanced atretic follicles. Reduced numbers of PCNA immunoreactive cells during atresia were also found in theca. Cystic follicles showed an extremely low PCNA-positive cell frequency in the granulosa layer at all regions. The frequency in the cystic follicles tended to be

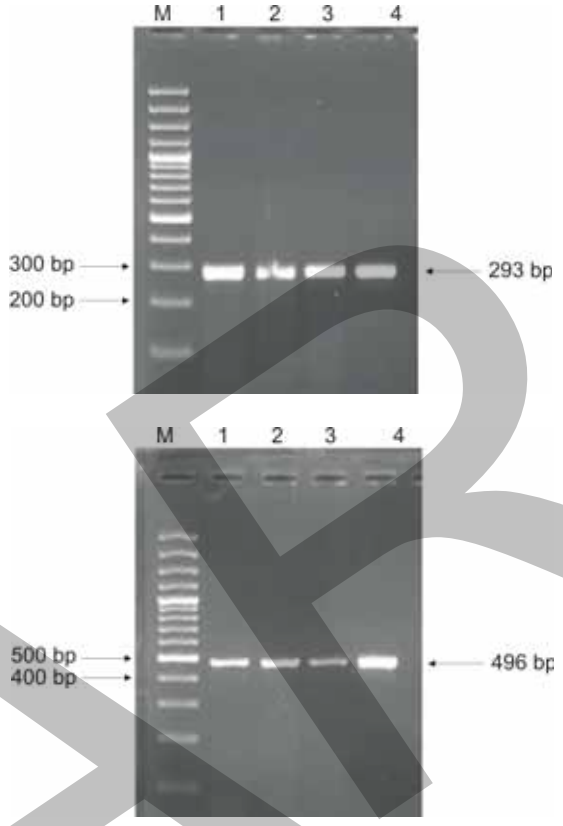

Fig. 2. mRNA expression of PCNA (A) and cyclin B1 (B) in buffalo ovarin tissue, preantral, antral follicles and granulosa cells. Lane M: 100 bp DNA ladder; Lane 1: Ovarian stromal tissue; Lane 2: Preantral follicles; Lane 3: Antral follicles; Lane 4: Granulosa cells.

lower than atretic follicles, mainly near the basal region of a follicle. These differences in positive cell frequency may appear where population is higher in the healthy follicles.

The immunoreactivity of cyclin B1 was found in granulosa and theca cells of all stages of ovarian follicles. Besides the granulosa cells, also showed less intense staining found in surface epithelium and stroma. Cyclin B1 showed a similar distribution to that of PCNA, with positive staining of granulosa and theca cells within all follicle categories (Fig. 2 G, H and I). Presence of cyclin B1 in buffalo ovarian follicular cells suggested that maturation promoting factor (MPF) was actively involved in the proliferation and growth of follicular cells. Likewise in rat and pig studies, in preantral follicles PCNA and Cyclin B1 positive staining was observed in a number of granulosa cells. According to Hirshfield (1981), the most rapid granulosa cell proliferation occurs in large preantral follicles just before the antrum formation. At advance stage of development, follicles start to enlarge rapidly and extensive PCNA immunoreactivity reflects the high proliferation rates in granulosa and theca cells of healthy preantral and antral follicles. These results suggested that further decrease in granulosa cell proliferation occurs in the cystic follicle as compared with the atretic one, which may

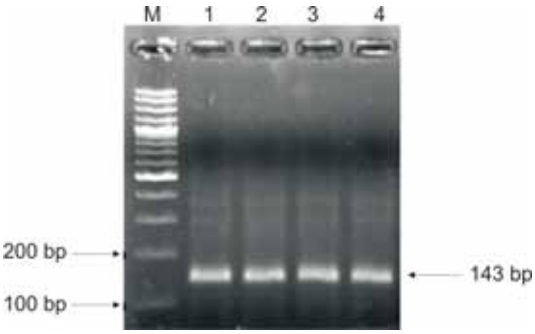

Fig. 3. mRNA expression of b-actin gene in buffalo ovarian tissue, preantral, antral follicles and granulosa cells. Lane M: 100 bp ladder; Lane 1: Ovarian stromal tissue; Lane 2: Preantral follicles; Lane 3: Antral follicles; Lane 4: Granulosa cells.

be related to cystic follicle formation. *PCNA and cyclin B1 mRNA RT-PCR analysis*: Nevertheless, the PCR analysis revealed that cyclin B1 mRNA was present in ovarian stromal tissue, GCs, PFs and AFs, indicating that transcript of these marker genes accumulated very early during follicular development. *In situ* hybridization revealed cyclin B1 mRNA in fetal ovaries from 135 to 265 dpc, showing that the accumulation of the transcript occurs extremely early in ovarian development (Ohashi *et al*. 2003). The cyclin B family is composed of 2 members, cyclin B1 and cyclin B2, and both types are reported to be part of the MPF. The activation mechanisms of the MPF have been studied in several species of mammalian oocyte, and several species-specific differences have been identified. The mouse oocyte is dependent on the synthesis of p34cdc2 (Yamashita *et al*. 2000) to resume meiosis, whereas in the pig the mechanism must be controlled at a different level because both MPF components are present (Kanatsu-Shinohara *et al.* 2000). In goat (Anguita *et al*. 2008) and porcine (Kuroda *et al.* 2004) oocytes, cyclin B1 and p34cdc2 were detected in both meiotically competent and incompetent oocytes suggesting that additional protein synthesis and/or protein modifications may be necessary to allow MPF formation and activation. The mRNA expression of cyclin B1 in buffalo ovarian tissue and follicles suggested that cyclin B1 associated with the MPF which actively involved in the cell proliferation and one of the responsible factors for growth and development of buffalo ovarian follicles.

The observation of proliferative activity in apparently morphologically homogeneous layers of granulosa cells during ovarian follicles development may be important from the point of view of *in vitro* granulosa cell studies. In summary, the expression studies of PCNA and cyclin B1 may serve as a reliable tool to detect the proliferative activity of granulosa and theca cells in the buffalo ovary.

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