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Immuno expression pattern of proliferating cell nuclear antigen (PCNA) in normal and atretic follicles of buffalo ovary

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The river buffaloes (*Bubalus bubalis*) of the Indian subcontinent are maintained chiefly for milk production and contribute around 50% of total milk produced in India. Ovary is the primary dynamic reproductive organ having dual function of gametogenesis and steroidogenesis. Folliculogenesis is a dynamic event which finally leads to ovulation or atresia. An increased rate of follicular atresia has been implicated as one of the major factors for reproductive failure in buffalo (Rajesha *et al*. 2001). Proliferating cell nuclear antigen (PCNA) is known to serve as acofactor for DNA polymerase delta in S-phase and its synthesis correlates with the proliferation cells. PCNA expression has been immuno-localized in rat ovary in association with initiation of follicular growth (Oktay *et al.* 1995), in cows (Wandji *et al.* 1996) and during different stages of follicular development in buffaloes (Sharma *et al.* 2011). PCNA regulates primordial follicle assembly by promoting apoptosis of oocytes in fetal and neonatal mouse ovaries (Xu *et al.* 2016). The aim of the present study was to determine the expression pattern of PCNA by immunohistochemistry in normal and atretic follicles of buffalo ovary.

The ovary of buffaloes (48) were collected from local abattoir in different seasons (winter, spring, summer and rainy) and were fixed in 10% neutral buffered formalin and processed for paraffin sectioning. The immunohistochemistry was performed as described earlier by Choudhary *et al.* (2018) with certain modifications. Briefly, the sections after dewaxing and rehydration, heat induced antigen retrieval were done in citrate buffer. The endogenous peroxidase activity was blocked by immersing the sections in 3% H_2O_2 and then incubated with normal horse serum to prevent nonspecific binding. The sections were incubated with primary antibodies overnight at 4°C and then with secondary antibody for 30 min. The chromogen used was 3, 3'-diaminobenzidine tetra hydrochloride (DAB) and counterstained with Gill's III hematoxylin. Images were taken by Nikon 80i microscope. PCNA positive cells were counted using the Image J software and data were statistically analyzed.

In the present study, PCNA localization was used to differentiate proliferating cells from degenerating cells. Xu *et al.* (2011) demonstrated that PCNA regulates primordial follicle assembly by promoting apoptosis of oocytes. PCNA localization in ovary follicles has been used as a marker for the proliferating events in the follicles of the ovary of rat (Oktay *et al.* 1995) and mouse (Britt *et al.* 2004). It has also been used as tool to count the number of follicles in ovary of rat (Picut *et al.* 2008). PCNA was localized specifically in the nucleus of the different types of cells in the ovary of buffalo. It was localized in the follicular cells of primordial and primary follicles in cortical area of ovary (Fig. 1A). These follicles might form the group of healthy follicles in growth and differentiation. Some follicles appeared normal and healthy but PCNA localization was absent and these follicles might be the pool of reserve follicles not in cycle of growth and differentiation (Fig. 1B). The visibly atretic follicles revealed weak PCNA expression (Fig. 1C) while the negative control showed no reaction. Thus it can be concluded that degeneration in the preantral follicles might start from the oocyte and follicular cells might be normal in early stage of atresia. Strong immunohistochemical reactions were observed in the healthy follicles, stromal cells and surface epithelium in the ovary of fetal and neonatal guinea pigs (Sun *et al.* 2014).

Healthy secondary follicles revealed that almost all the granulosa cells were positive for PCNA protein (Fig. 1D). Similar findings were reported by Oktay *et al.* (1995) in rat ovaries and Wandji *et al.* (1996) in cow ovaries. In contrast to the findings of the present study, Tomanek and Chronowska (2006) reported no remarkable staining for PCNA either in granulosa cells or in the oocytes in primordial follicles of pig ovaries. In primary to secondary follicles, positive staining in oocytes and in some granulosa cells was reported by them. Authors observed PCNA labeling in nuclei of oocytes in preantral and small antral follicles. Seasonal changes were observed (Figs 1E, 2F)

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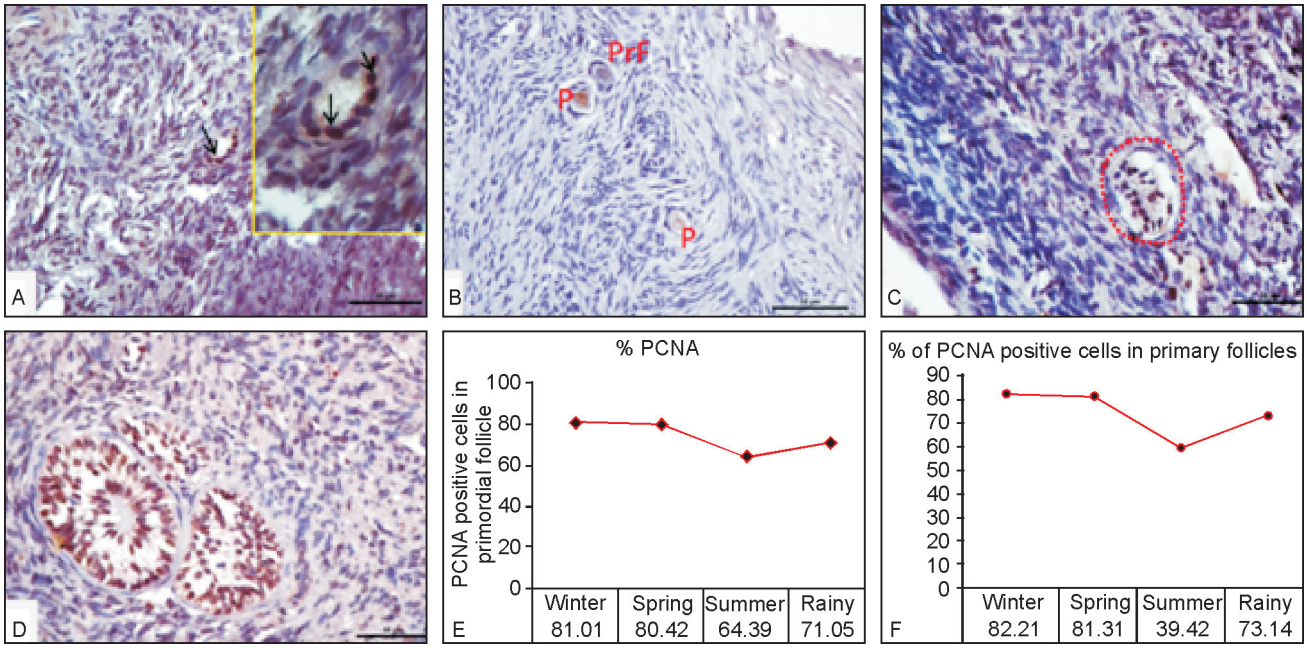

Fig. 1. Immunolocalization of PCNA in buffalo ovary. (**A**) Immune positive healthy follicular cells of primary follicles (arrow). Inset showing magnified view positive reaction (arrows). (**B**) No reaction in follicular cells of healthy primary (P) and primordial follicles (PrF). (**C**) Visibly atretic follicle (AF) with weak PCNA expression (in circle with dotted lines). (**D**) positive reaction in healthy secondary follicles with granulosa cells. Polymer HRP staining. Scale Bar=50µm (in original). Graph showing PCNA percentage positive cells in primordial follicles (E) and in primary follicles (B) in different seasons.

with highest expression in winter. PCNA was immunolocalized in the all the compartments of a healthy tertiary follicle. There was distinct nuclear reaction in granulosa cells of healthy follicles. More than 95% of granulosa cells were PCNA positive. Theca interna and theca externa cells were also PCNA positive. More than 50% of theca cells were positive for PCNA (Fig.2A). It showed that in a healthy follicle granulosa cells and theca cells were in a stage of proliferation. The corona radiata cells surrounding the oocyte and cumulus oophorus cells were also immunopositive for PCNA protein (Fig. 2B). Similar to these findings, Tomanek and Chronowska (2006) reported that the advanced preantral and particularly actively growing small to large antral follicles showed extensive PCNA labeling in the layers of granulosa and theca cells and in the cumulus cells encircling the oocyte. PCNA labeling was expressed in nuclei of oocytes in preantral and small antral follicles.

In early stage of atresia, granulosa cells were still positive for PCNA. As atresia progressed, revealed by large vacuolated spaces in between membrana granulosa cells. The number of immunopositive cells for PCNA were drastically reduced in membrana granulosa cells and theca cells (Fig. 2c). At later stage only few immunopositive cells were observed (Figs 2D and 2E). PCNA expression varied in different degrees of atresia (Fig. 2F). Tomanek and Chronowska (2006) studied PCNA expression in the ovary of pigs and observed that in atretic follicles, the level of PCNA protein expression was dependent on the stage of atresia. Follicles demonstrating advanced atresia showed only limited or no PCNA labeled granulosa and theca cells. They demonstrated that follicular growth and development in pig ovary might be effectively monitored by determining the granulosa cell expression of PCNA.

In a cystic type of atresia only few granulosa cells and theca cells were immunopositive for PCNA (Fig. 2G). In advanced stage of atresia, the theca cells which formed the boundary around the cyst of cystic atretic follicles were immunopositive for PCNA (Fig. 2H). In some of the atretic follicles, typical antral form of atresia was observed where the antral granulosa cells were negative for PCNA. The basal cells were immunopositive for PCNA (Fig. 2I). Feranil *et al.* (2004) in cattle and buffalo ovary and Feranil *et al.* (2005) in buffalo ovary observed significantly higher frequency of PCNA positive cells in healthy follicles than in the early and advanced atretic follicles. Reduced number of PCNA immunoreactive cells during atresia was also found in theca. Similar to our findings, Feranil *et al.* (2005) in buffalo ovary observed significantly fewer populations of the PCNA positive cells as atresia progressed from the early stage to the later stage.

SUMMARY

The present study was aimed to determine the expression pattern of PCNA in normal and atretic follicles of buffalo ovary. PCNA was localized specifically in the nucleus of the different types of cells in the ovary of buffalo. It was localized in the follicular cells of primordial and primary follicles, granulosa cells in secondary follicles and in all the compartments of a healthy tertiary follicle. The atretic

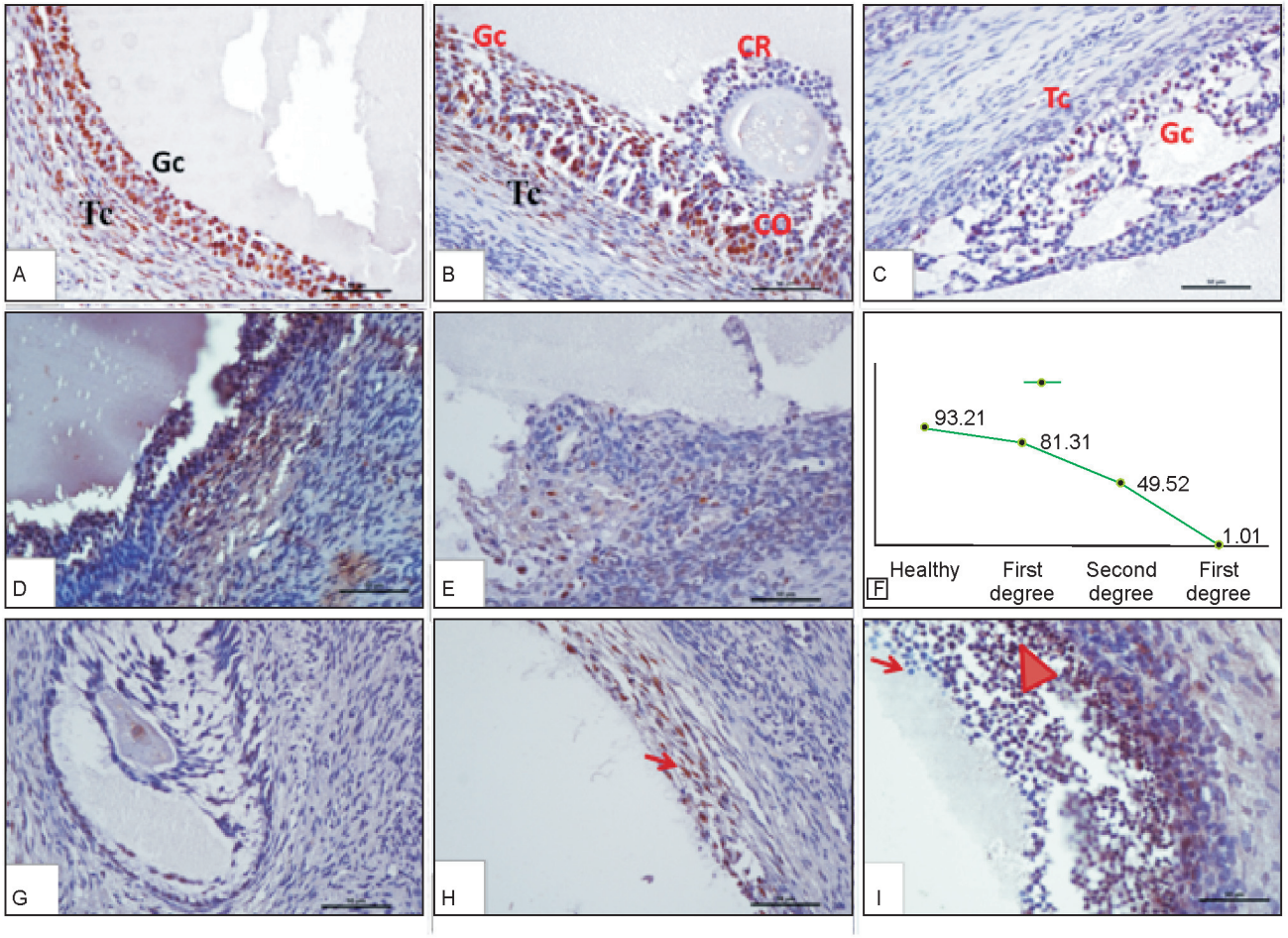

Fig. 2. Immunolocalization of PCNA in buffalo ovary showing (**A**) positive reaction in a healthy tertiary follicle in granulosa cells (Gc) and theca cells (Tc), (**B**) positive reaction in a healthy tertiary follicle in granulosa cells (Gc) and theca cells (Tc), corona radiata cells (CR) and cumulus oophorus cells (CO), (**C**) less number of positive cells in late stage of atresia; granulosa cells (Gc) and theca cells (Tc), (**D**) very few immunopositive cells for PCNA in membranagranulosa cells and theca cells**,** (**E**) very few immunopositive cells for PCNA in membranagranulosa cells and theca cells, (**F**) PCNA percentage positive cells in three degree of atresia in tertiary follicles, (**G**) very few immunopositive cells in a cystic type of atresia, (**H**) immunpositive theca cells formed the boundary around the cyst of cystic atretic follicles (arrow), (**I**) antral form of atresia was observed where the antral granulosa cells were negative for PCNA (arrow) and looked apoptotic in nature and the basal cells were immunopositive for PCNA (arrow head). Polymer HRP staining. Scale bar for all photographs =50µm (in oroginal).

follicles revealed weak PCNA expression. PCNA expression varied in different degrees of atresia and during different seasons.

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