

Original Research Article

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## Immunohistochemical Localization of Proliferating Cell Nuclear Antigen (PCNA) in the Ovaries of Sheep and Goat

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The present study was to determine the expression of proliferating cell nuclear antigen protein (PCNA) in the sheep and goat ovaries. The localization of PCNA was demonstrated in 5µm thick formalin fixed, paraffin embedded tissue sections of sheep and goat ovarian tissue using primary mouse monoclonal anti-PCNA antibody. Staining for PCNA was not observed in pregranulosa cells but observed in the oocytes of primordial follicles. In primary to secondary follicles, positive staining in oocytes and in some granulosa cells was detected. The preantral follicles and actively growing small to large antral follicles showed strong PCNA labelling in the layers of granulosa, and theca cells. PCNA staining was expressed in nuclei of oocytes in preantral and small antral follicles. In atretic follicles, the level of PCNA protein expression was negatively correlated on the stage of atresia. The follicles demonstrating advanced atresia showed mild or no PCNA labelled granulosa and theca cells. The results of the study demonstrate that follicular growth and development in ovaries may be effectively monitored by determining the rate of granulosa cell proliferation of PCNA.

### Introduction

Proliferating cell nuclear antigen (PCNA) is a well conserved 36 KDa protein that plays a crucial role in regulating cellular functions of eukaryotic organisms (Strzalka *et al.*, 2015). It was clearly established that PCNA was linked with DNA synthesis, repair, damage avoidance, cell cycle control and cell survival (Prakash *et al.*, 2005; Helleday *et al.*, 2007;

Mirkin and Mirkin, 2007; Stoimenov and Helleday, 2009). PCNA contains an auxiliary protein of DNA polymerase-delta and epsilon enzymes that are essential in DNA synthesis (Wood and Shivji, 1997; Muskhelishvili *et al.*, 2005; Strzalka and Ziemienowicz, 2007; Sun *et al.*, 2012); hence it is an ideal marker of cell proliferation. PCNA was used to demonstrate the ovarian follicle counts in different laboratory and farm animal species

(Muskhelishvili *et al.*, 2003; Machodo *et al.*, 2017). Immunohistochemical labelling of follicles and oocytes with antibody directed against proliferating cell nuclear antigen (PCNA) was reported in ovaries of rat (Oktay *et al.*, 1995), mouse (Kerr *et al.*, 2006), rabbit (Hutt *et al.*, 2006), pig (Tomanek and Chronowska., 2006) and sheep (Patel *et al.*, 2018). To our knowledge, there was no detailed study of proliferation process throughout the follicular development in goat and sheep ovaries. Hence, the aim of the present study is immunostaining of PCNA in order to identify the distinct pattern of follicular cell proliferation in different follicular stages.

## Materials and Methods

### Animals and tissue handling

The ovaries of sheep and goat were obtained from a local abattoir and transported to laboratory in a thermo-container filled with PBS. Ovarian tissue was cut into small pieces and fixed in 10% neutral buffered formalin solution. After fixation, the specimens were dehydrated by ascending grades of ethanol (70-80-90-100%) and following the xylene clearing they were embedded in paraffin (Merck, Germany). The pieces of ovarian tissue were serially sectioned at 4 $\mu$ m with the help of microtome (Leica RMZ 2125) and mounted on APES (Amino Propyl triethoxy Sialine) coated slides and incubated overnight at 37°C. These slides were subjected to the following immunohistochemistry protocol (Lillie RD, 1999; Luna LG, 1968; Lynch *et al.*, 1969).

### Immunohistochemistry

Prior to PCNA localization, sections were deparaffinised by passed through two changes of xylene 15 minutes each and rehydrated in decreasing concentrations of ethanol and

PBS. The slides were kept in Tris EDTA buffer (pH-9.0) and hot water bath treatment was given for 20 minutes at 100°C to retrieve the antigenic sites and then cooled to the room temperature and then kept in the distilled water for 5 minutes and in Tris buffer saline for 5 minutes.

In order to block the endogenous peroxidase the slides were kept in the peroxidase block solution (3% hydrogen peroxide in methanol) for 10 minutes. Then slides were washed in Tris buffer saline for thrice, five minutes each time. The power block solution using 1.5% bovine serum albumin (BSA) was poured on tissue section and kept for 30 minutes. Monoclonal mouse anti-PCNA (DAKO, carpinteria, CA, USA: 1:200 dilution) was added on the sections and slides were kept at 4°C overnight. The next day, sections were washed in Tris buffer saline for five minutes each in three changes. The secondary antibody with horse radish peroxidase (HRP) was added and kept for half hour at room temperature. Then sections were washed in Tris buffer saline for five minutes each in three changes. The binding of primary antibody was visualized using diaminobenzidine (DAB, Sigma, Germany) for 5-8 min. After washing with distilled water, sections were counterstained with Harris haematoxylin for 1 minute. The slides were washed in tap water for 5 minutes. For negative control, PBS was used instead of primary antibody. Then air dried and mounted with DPX. The PCNA labelling was examined using Leica DMLB microscope and the images were recorded by Leica DC 200 digital camera.

## Results and Discussion

Follicles were classified according to the stage of development, considering the shape, and layers of the granulosa cells (GC), as follows: primordial (oocyte surrounded by a

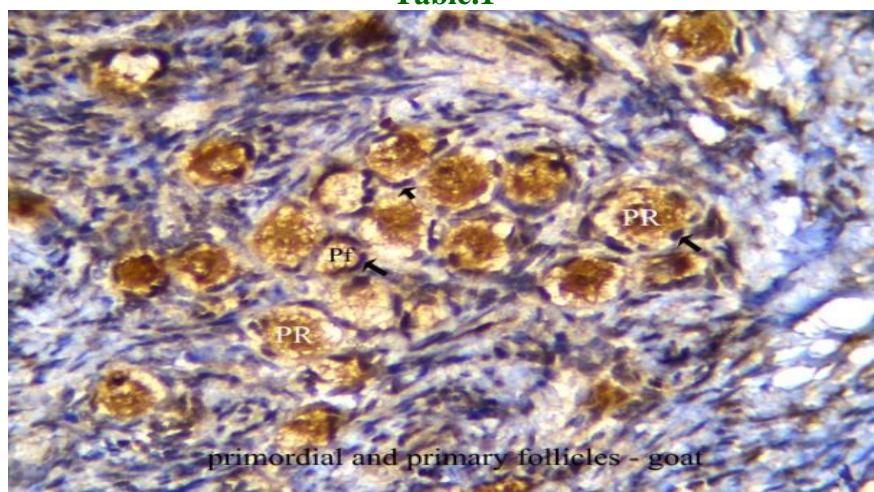
flat granulosa cell layer); primary (one layer of cuboidal granulosa cells) and secondary (two or more layers of cuboidal cells but without formation of vesicles), antral (three or more layers of cuboidal granulosa cells and the presence of antrum) and large antral follicles (Silva-Buttkus *et al.*, 2008).

In primordial and primary follicles ovum and nucleus showed strong positive reaction (Fig: 1, 2, 3). The pre granulosa cells unveiled no immunoreactivity in both the species under study (arrow in Fig: 1, 2, 3). In preantral and secondary follicular stage the ovum showed positive reaction and few granulosa cells are mildly showing reactivity in both sheep and goat ovaries (Fig: 4). In early antral follicle ovum showed strong reactivity, granulosa cells took the stain at moderate to high degree

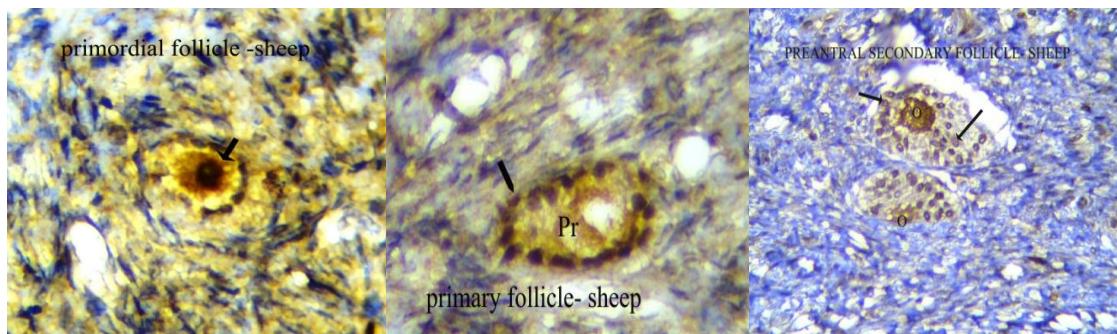
in both goat and sheep ovaries. The thecal cells of early antral follicles showed mild to moderate staining in goat but in sheep only few cells showed mild reaction (Fig: 5, 6).

In antral follicles granulosa layer, cumulus cells showed strong positive reactivity and theca interna showed moderate to high reactivity in both the species under study (Fig:7,8). The blood vessels present in the theca interna of large antral follicles showed reactivity in goat (Fig: 7). The ovum of antral follicle of both the species showed moderate reactivity. The early stages of atretic follicles showed reaction in granulosa cells as atresia progresses the staining regressed. Medulla showed immunoreactivity for PCNA at their blood vessels in both goat and sheep ovaries (Fig: 9, 10).

**Table.1**



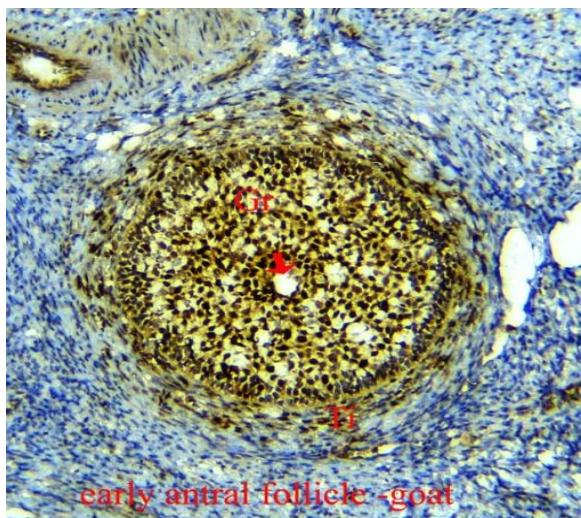
**Fig.1** primordial and primary follicles of goat ovary



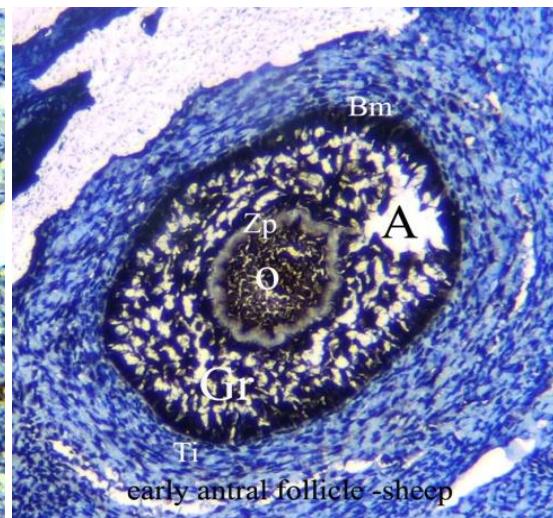
**Fig.2** Primordial follicle-sheep

**Fig.3** Primary follicle

**Fig.4** Preantral secondary follicle



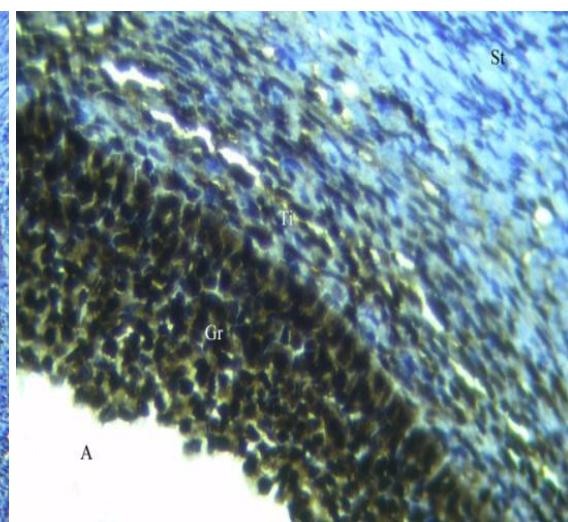
**Fig.5** Early antral follicle of goat



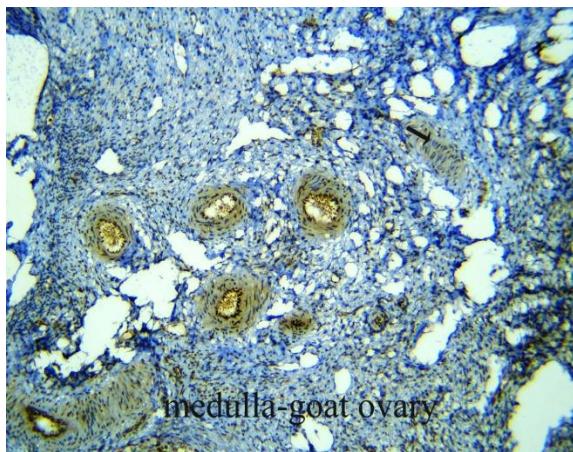
**Fig.6** Early antral follicle of sheep



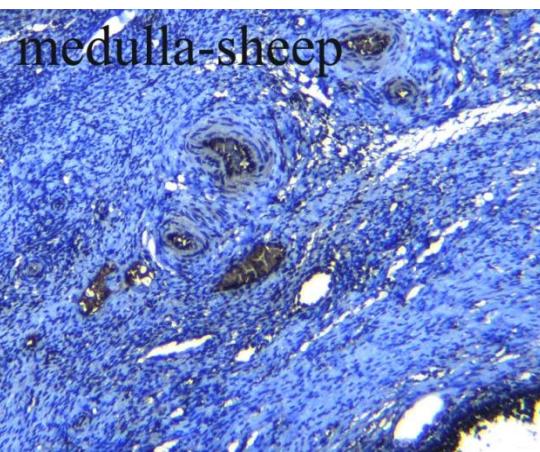
**Fig.7** Antral follicle of goat



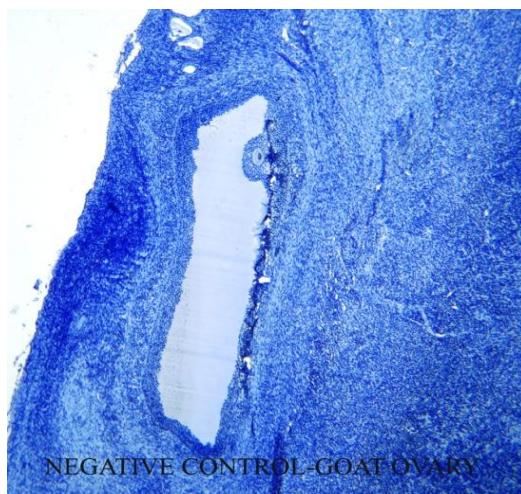
**Fig.8** Wall of large antral follicle of sheep



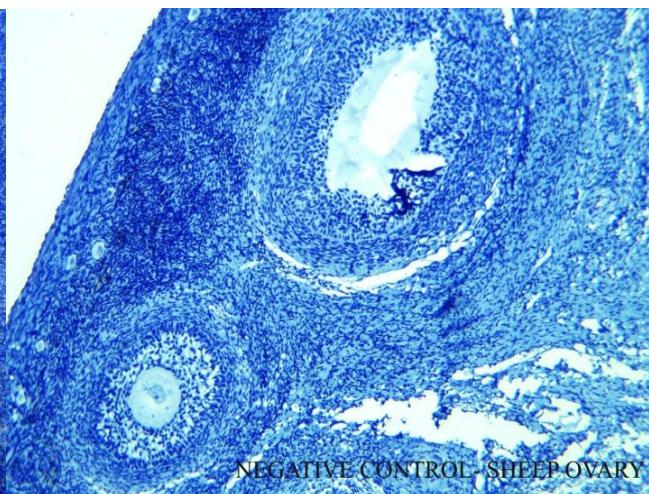
**Fig.9** Medulla of goat ovary



**Fig.10** Medulla of sheep ovary



**Fig.11** Negative control–Goat ovary



**Fig.12** Negative control –Sheep ovary

In the present study the sheep and goat ovaries were fixed in 10% neutral buffered formalin and heat induced epitope retrieval (HIER) was applied to aid immunostaining of proliferating cells (Foley *et al.*, 1993; Muskhelishvili *et al.*, 2003). In our study it appears that PCNA expression in the oocytes of primordial follicles in both species under study is lower than the mature stages (Hutt *et al.*, 2006; Kerr *et al.*, 2006; Picut *et al.*, 2008) but complete absence of immunostaining for PCNA in the primordial follicles was also reported earlier (Wandji *et al.*, 1996; Wandji *et al.*, 1997; Tomanek and Chronowska, 2006; Rodrigues *et al.*, 2009).

It could be due to use of Bouin's solution as fixative which makes immunohistochemical staining hard in the previous studies (Tomanek and Chrowska *et al.*, 2006), or use of HIER technique in the current study that has been demonstrated to enhance the visualization of stained primordial follicles in ovaries of rat (Muskhelishvili *et al.*, 2005), pig (Tomanek and Chronowska, 2006), buffalo (Machodo *et al.*, 2017) and sheep (Patel *et al.*, 2018). However, immunopresence of PCNA in the oocytes of primordial follicles could not be ascribed to the cell proliferation as the oocytes in this stage are in meiotic arrest phase (Hirshfield,

1991). So it is hypothesised that PCNA protein has some role even in the early stages of follicular development probably being an auxiliary protein of DNA polymerase it takes part in mechanism of DNA repair during transcription process (Wandji *et al.*, 1996; Wandji *et al.*, 1997).

Therefore, importance of immunolabelling of PCNA in the oocytes of primordial follicles needs to be elucidated in future studies. On the other hand we observed that proliferative activity as detected by PCNA localization is higher in the later stages of folliculogenesis which is in accordance with previous reports (Oktay *et al.*, 1995; Wandji *et al.*, 1996; Tomanek and Chrowska *et al.*, 2006; Myoung *et al.*, 2006).

Interestingly we noted that PCNA expression was detected consistently in the primary/secondary to early antral follicles in both the species studied which is in accordance with earlier observations (Oktay *et al.*, 1995; Wandji *et al.*, 1996; ). The rate of granulosa and theca cell proliferation is rapid in the antral and large antral follicular stage (Hirshfield, 1981) which is reflected by extensive immunoreactivity of PCNA in this study. Also we observed that higher concentration of PCNA labelling in the

healthy follicles than that of atretic follicles which is parallel to the findings of Feranil *et al.* (2004 and 2005). In the follicles with signs of early atresia, PCNA localization is reduced in the granulosa cells but completely absent in theca cells and this observation may be crucial in view of *in vitro* granulosa cell studies. Altogether, PCNA detection using Immunohistochemistry serves as a tool to identify the rate of follicular cell proliferation in various stages of folliculogenesis in ovaries of sheep and goat.

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### References

- Helleday, T., Lo, J., van Gent, D. C & Engelward, B. P. (2007). DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)*, 6: 923–935.
- Hutt, K. J., McLaughlin, E. A & Holland, M. K. (2006). Primordial follicle activation and follicular development in the juvenile rabbit ovary. *Cell Tissue and Research*, 326: 809–822.
- Kerr, J.B., Duckett, R., Myers, M., Britt, K. L & Mladenovska, T. (2006). Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. *Reproduction*, 132: 95–109.
- Mirkin, E. V. & Mirkin, S. M. (2007). Replication fork stalling at natural impediments. *Microbiology and Molecular Biology Reviews*, 71: 13–35.
- Muskhelishvili, L., Freeman, L. D., Latendresse, J. R. & Bucci, T. J. (2002). An immuno-histochemical label to facilitate counting of ovarian follicles. *Toxicologic Pathology*, 30(3): 400–402.
- Myoung, H., Kim, M. J., Lee, J. H., Ok, Y. J., Paeng, J. Y. & Yun, P. Y. (2006). Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma: a clinical and histopathological analysis of 113 patients. *International Journal of Oral Maxillofacial Surgery*, 35:1005-1010.
- Oktay, K., Schenken, R. S. & Nelson, J. F. (1995). Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biology of Reproduction*, 53: 295–301.
- Patel, H., Bhartiya, D. & Parte, S. Further characterization of adult sheep ovarian stem cells and their involvement in neo-oogenesis and follicle assembly. *Journal of ovarian research*, 11(1):3.
- Picut, C. A., Swanson, C. L., Scully, K. L., Roseman, V. C. & Parker, R. F. (2008). Ovarian follicle counts using proliferating cell nuclear antigen (PCNA) and semiautomated image analysis in rats. *Toxicologic Pathology*, 36: 674–679.
- Prakash, S., Johnson, R. E. & Prakash, L. (2005). Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annual Review of Biochemistry*, 74: 317–353.
- Rodrigues, P., Limback, D., McGinnis, L. K., Plancha, C. E. & Albertini, D. F. (2009). Multiple mechanisms of germ cell loss in the perinatal mouse ovary. *Reproduction*, 137: 709–720.
- Silva Buttkus, P., Jayasooriya, G. S., Mora, J. M., Mobberley, M., Ryder, T. A., Baithun, M., Stark, J., Franks, S. &

- Hardy, K. (2008). Effect of cell shape and packing density on granulosa cell proliferation and formation of multiple layers during early follicle development in the ovary. *Journal of Cell Science*, 121:3890-3900.
- Stoimenov, I. & Helleday, T. (2009). PCNA on the crossroad of cancer. *Biochemical Society Transactions*, 37: 605–613.
- Strzalka, W. & Ziemienowicz, A. (2007). Molecular cloning of Phaseolus vulgaris cDNA encoding proliferating cell nuclear antigen. *Journal of Plant Physiology*, 164:209-213.
- Strzalka, W. & Ziemienowicz, A. (2011). Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. *Annals of Botany*, 107:1127-1140.
- Strzalka, W. K., Aggarwal, C., Krzeszowiec, W., Jakubowska, A., Sztatelman, O. & Banas, A. K. (2015). Arabidopsis PCNAs form complexes with selected D-type cyclins. *Frontiers in Plant Science*, 6:1-11.
- Sun, Y. L., Zhang, J., Ping, Z. G., Wang, C. Q., Sun, Y. F., Chen, L., Li, X. Y., Li, C. J., Zhu, X. L., Liu, Z., Zhang, W. & Zhou, X. 2012. Relationship between apoptosis and proliferation in granulosa and theca cells of cystic follicles in sows. *Reproduction in Domestic Animals*, 47:601-608.
- Tománek, M and Chronowska, E. (2006). Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the pig ovary. *Folia histochemica et cytobiologica*, 44:269-274.

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