

Changes in number of granulosa cells during folliculogenesis in prepubertal goat ovary

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ABSTRACT

Histomorphological study in prepubertal goat ovary revealed the presence of primordial, intermediate, primary, secondary, and tertiary follicles. The primordial follicles were in abundance and occurred in series of 9 to 26 and even up to 40 follicles in a row. Initiation of follicle growth consisted of two distinct consecutive phases. The first phase was characterized by transformation of granulosa cells from a flattened to a cuboidal shape and by their proliferation. In the second phase an increase in the number of granulosa cells was accompanied by a rapid increase in the size of the oocyte. Oocyte commenced growth when there were at least 15 granulosa cells in the largest cross section. The oocyte diameter increased from $25.47 \pm 1.14 \mu\text{m}$ (mean $\pm$ SE) in primordial follicles to $102.01 \pm 2.15 \mu\text{m}$ in large antral follicles. The zona pellucida first appeared as small patches in primary follicles but formed a complete ring around the oocyte at the early antral stage. The average size of the secondary follicle was $138.07 \pm 32.80 \mu\text{m}$ and tertiary follicle was $482.0 \pm 148.67 \mu\text{m}$. Large antral follicles may reach up to a diameter of $1537 \mu\text{m}$ in prepubertal goat ovary. It is concluded that some interrelationship exists between the number of granulosa cells around the follicle and the oocyte diameter in all types of follicles.

Key words: Prepubertal goat, Ovary, Granulosa Cells, Follicles, Oocyte.

In the prepubertal animal the ovary contain both growing preantral follicles and early antral follicles at various stages of development and atresia (Fortune 2003, Hamaidi 2006). Initiation of follicle growth involves the passage of primordial follicles from quiescent to growth phase and is characterized by three main events, change in shape of the granulosa cells from squamous to cuboidal, proliferation of granulosa cells and enlargement of the oocyte (Hirshfield 1991, Lundy *et al.* 1999, Fortune 2003). In several species, changes in the granulosa cells precede oocyte growth. Earlier studies have demonstrated that the oocyte starts to grow when there are about 10 cuboidal granulosa cells in mice and rats (Lintern-Moore and Moore 1979, Andersoen de Wolf-Exalto and Groen-Klevant 1980), 15 in sheep (Cahill and Mauleon 1981) and about 40 granulosa cells in bovine ovary (Braw-Tal and Yossefi 1997). Mhawi *et al.* (1991) reported that in new born calves transition of flattened granulosa cells to cuboidal is followed by ultrastructural changes in the oocytes. However the exact stage at which the oocyte enters the growth phase in goat ovary is unknown.

The aim of the present study was to carry out the detailed

micromorphometric study of folliculogenesis in the prepubertal goat ovary and to examine the interrelationships among the number of granulosa cells in the section through the oocyte nucleolus and the diameter of the oocyte. This information will be of value for *in vitro* manipulation of germ cells.

MATERIALS AND METHODS

Goat genitalia were collected from local slaughterhouse from apparently healthy goats of unknown age, physiological status and were transported into the laboratory under sterile conditions. The genitalia were categorized into prepubertal stage depending upon the condition of the uterus, oviduct, size and appearance of the ovary. Very small genitalia with light pink smooth uterus having no tonicity of muscles, small and soft light pink colored oviduct and small ovaries (<0.6cm in size), without corpus luteum or corpora albicans were considered to be from prepubertal animals. These ovaries were excised from the genitalia, adhering connective tissue and fixed in Alcoholic-Bouin's fixative. After dehydration and paraffin embedding, one ovary per animal was randomly selected, serially sectioned at a thickness of $6 \mu\text{m}$, mounted and stained with haematoxylin eosin and observed microscopically.

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The follicles were classified according to the method described by Braw-Tal and Yossefi (1997) for bovine with some modifications as follows: (i) primordial follicles-one layer of flattened granulosa cells; (ii) transitory follicles-one layer of a mixture of flattened and cuboidal granulosa cells; (iii) primary follicles-from two to complete three layers of cuboidal granulosa cells; (iv) secondary follicles-more than three layers of granulosa cells; (v) Tertiary follicles as small antral follicles-more than five layers of cuboidal granulosa cells and patches of antrum; (vi) antral follicle-more than five layers of cuboidal granulosa cells and a fully formed antrum and (vii) large antral follicle-with large antrum.

Follicle, oocyte and nuclear diameter was measured at right angles in the section through the oocyte nucleolus and the mean diameters were calculated. Only non-atretic follicles devoid of pycnotic granulosa cells and oocyte degeneration were considered in the study. Granulosa cell number was counted from the largest cross section of the follicle. Data

were presented by mean and standard error.

RESULTS AND DISCUSSION

The primordial follicles mostly occupied the peripheral area of the ovarian cortex, usually close to the tunica albuginea (Fig. A) as also reported by Dellmann and Brown (1971) in domestic animals. In immature ovary, the primordial follicles are in large numbers and occur in variable groups of 9 to 26 and even up to 41 follicles in a series, sometimes in a clutch of 13, which formed a continuous sheet below the tunica albuginea (Fig. B). Primordial follicles often arranged in nest formations have also been observed in sheep ovary (Lundy *et al.* 1999). The micrometrical data of primordial follicles is shown in Table 1. The oocyte of primordial follicles is usually rounded with a large round, oval and eccentrically placed nucleus (Fig. C). Comparable diameter of follicle, oocyte and oocyte nucleus has been observed in Assam goats (Kalita *et al.* 2000), although the

Table 1. Micrometrical data of different types of ovarian follicles (mean $\pm$ SE) in prepubertal goat ovary (μ m)

Follicle structure/ type of follicle	Follicular diameter	Oocyte diameter	Nuclear (oocyte) diameter	Zona pellucida	Basement membrane	Theca interna	Theca externa
Primordial follicle	32.24 $\pm$ 1.04	25.47 $\pm$ 1.14	14.62 $\pm$ 0.34	—	1.625 $\pm$ 0.22	—	—
Intermediate	36.03 $\pm$ 1.66	25.83 $\pm$ 1.58	13.75 $\pm$ 0.64	—	1.87 $\pm$ 0.19	—	—
Primary	40.53 $\pm$ 2.43	27.92 $\pm$ 1.47	16.10 $\pm$ 0.54	Small patches	2.21 $\pm$ 0.15	7.60 $\pm$ 1.25 (Undifferentiated)	—
Secondary	138.07 $\pm$ 32.80	53.54 $\pm$ 6.22	23.60 $\pm$ 0.89	Incomplete	2.47 $\pm$ 0.38	22.25 $\pm$ 1.80	12.25 $\pm$ 1.15
Small antral	300.69 $\pm$ 38.82	89.72 $\pm$ 5.78	24.11 $\pm$ 1.53	3.89 $\pm$ 0.68	2.57 $\pm$ 0.25	28.75 $\pm$ 1.25	16.50 $\pm$ 2.50
Antral	482.0 $\pm$ 148.67	90.25 $\pm$ 7.13	26.25 $\pm$ 1.10	4.13 $\pm$ 0.58	3.12 $\pm$ 0.64	30.25 $\pm$ 2.30	57.50 $\pm$ 4.71
Large Antral/Graafian	1287.25 $\pm$ 159.82	102.01 $\pm$ 2.15	25.89 $\pm$ 1.76	5.03 $\pm$ 0.78	3.43 $\pm$ 0.57	44.17 $\pm$ 1.97	51.50 $\pm$ 3.88

Table 2. Variation in granulosa cell size, number compactness and granulations in different categories of growing follicles in prepubertal goat ovary

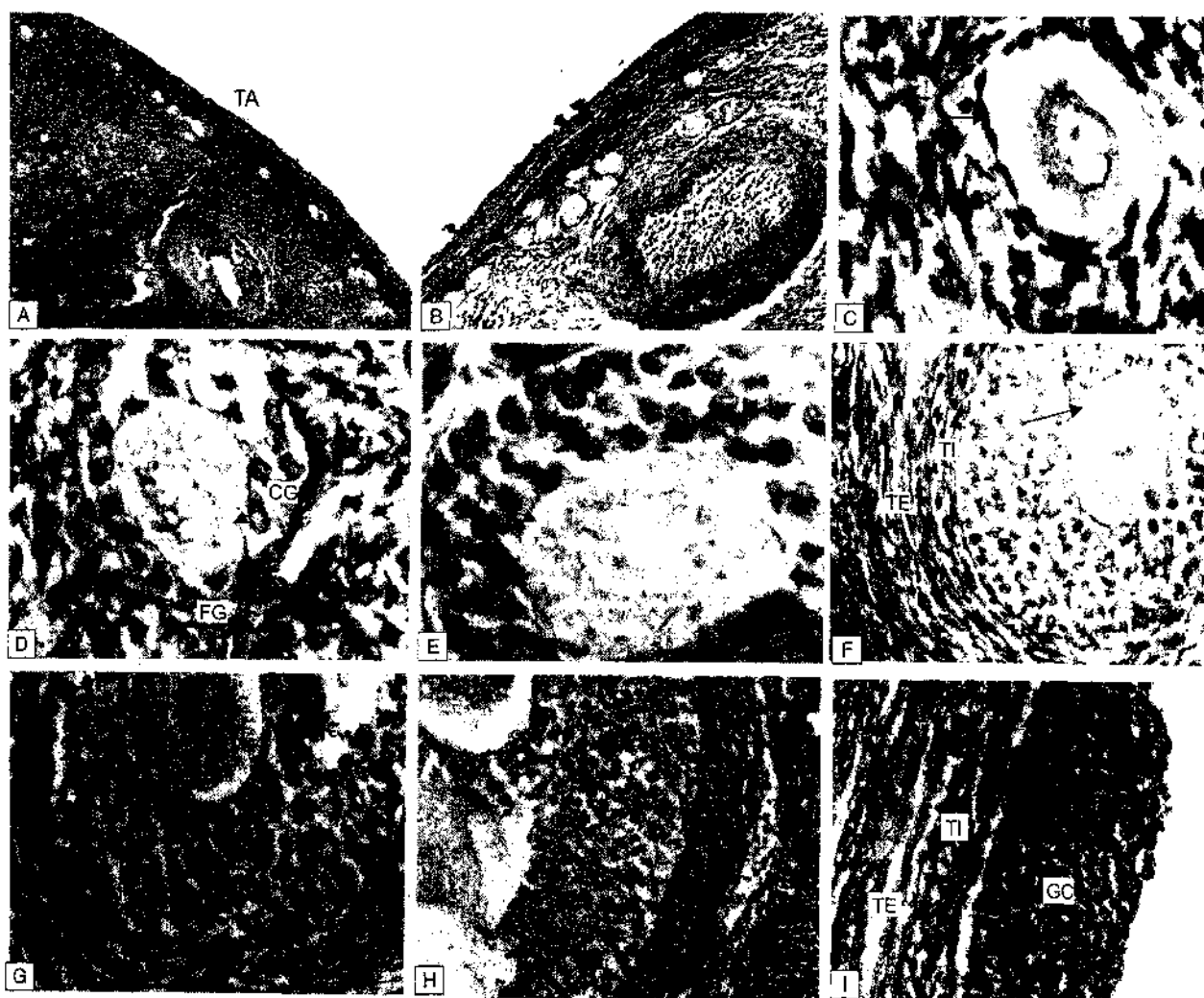
Type of follicle	Layers of granulosa cells (range)	Number of granulosa cells (range)	Shape of granulosa cells	Size of granulosa cells mean $\pm$ SE (range)	Compactness of granulosa cells	Granulations
Primordial follicle	1	4-7 (6.0 $\pm$ 1.08)	Flattened	4.60 $\pm$ 0.82 (2.50-6.25)	Scattered	Absent
Intermediate	1-1.5 (10.0 $\pm$ 2.61)	4-24 Flat:Cuboidal	1:1 (2.50-6.25)	4.94 $\pm$ 1.04 1-1.5 layers	Loose forming	Absent
Primary	2-3	10-55 (16.25 $\pm$ 12.01)	All cuboidal	5.16 $\pm$ 1.07 (3.13-7.19)	Loose to moderate	Scarce
Secondary	>3 layers	150-250 (167 $\pm$ 65.50)	Oval, Round few polyhedral	6.80 $\pm$ 1.36 (3.75-8.75)	Basal-Compact Middle-loose	Mild
Small antral	>6 layers patches of antrum	>300 polyhedral	Oval, round (4.38-8.75)	6.70 $\pm$ 1.73	Basal- Compact Middle & antral-loose	Moderate
Antral	4-5 layers around antrum	Uncountable	Oval, round, polyhedral	7.04 $\pm$ 1.34 (5.0-9.38)	Basal- Compact Middle & antral- loose	Moderate to high
Large antral/ Graffian	4-5 layers around antrum	Uncountable	Oval, round, polyhedral	7.27 $\pm$ 1.01 (5.0-10.50)	Basal- Compact Middle & antral- loose	High

primordial follicles of sheep and prepubertal bovines have significantly less diameters (Paramasivan and Sharma 2003, Bhardwaj and Roy 2004).

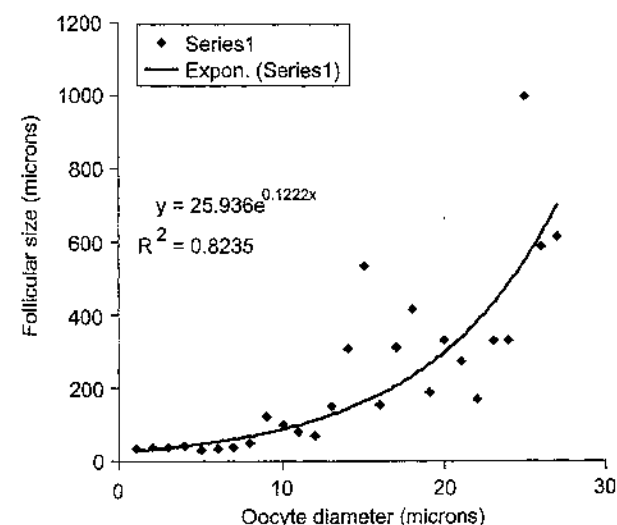
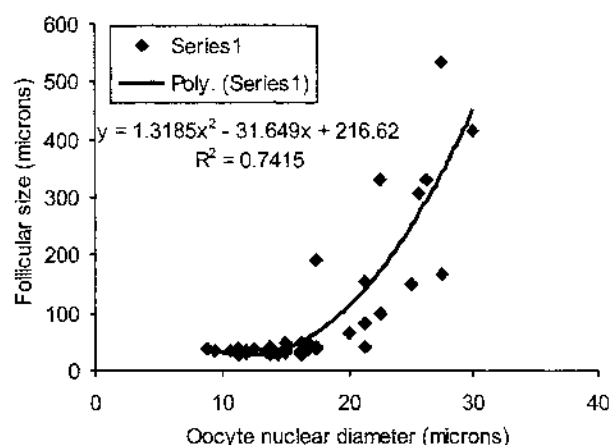
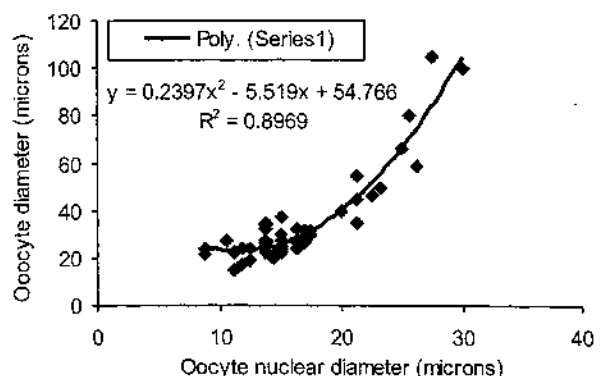
The stromal cells adjoining the primordial follicles known as pregranulosa cells had irregularly shaped nuclei with chromatin condensed at the nuclear membrane (Fig. C). Primordial follicles contained an average (mean $\pm$ S E) of 6.0 ± 1.08 flattened granulosa cells varying 4–7 in number (Table 2). The mean diameter of the granulosa cells was $4.60 \pm 0.82 \mu\text{m}$ (Table 2). Similar range was observed in the number of granulosa cells in cows (2–12), bovines (24), humans (1–14), sheep (2–12), mice (2–10), and rats (2–8) (Himelstein-Braw *et al.* 1976, Lintern-Moore and Moore 1979, Gougeon and Chainy 1987, Hirshfield 1991, van Wezel and Rodgers 1996, Braw-Tal and Yossefi 1997, Lundy *et al.*

1999). The present study showed that in prepubertal ovary oocyte begins to grow when there are at least granulosa cells in the largest cross section. In mice and rats the oocyte starts to grow when there are about 10 granulosa cells in the largest cross-section (Lintern-Moore and Moore 1979, Anderson de Wolf-Exalto and Groen-Klevant 1981). For sheep the critical point is about 15 cells (Cahill and Mauleon 1981). In the bovine ovary oocytes start to grow later and the critical point is about 40 granulosa cells (Braw-Tal and Yossefi 1997).

Intermediate/transitory follicles contained a mixture of flattened and cuboidal cells in the ratio of 1:1 forming 1–2 layers (Fig.D). Proliferation of granulosa cells involves change in shape and number, which increased up to 24 and then decreased to an average number 10 ± 2.61 (Table 2). The diameter of



Figs 1A–H. A. Primordial follicles (arrows) in series below the tunica albuginea (TA); B. Primordial follicles forming a clutch follicles (arrow); C. Magnified view of primordial follicles surrounded by flattened (FG) and cuboidal granulosa (CG) cells; D. Intermediate follicle; E. Primary follicle with 3 layers of granulosa cells around the oocyte; F. Secondary follicle—note the differentiation of theca into theca externa (TE) and theca interna (TI), Zona pellucida is in the form of patches (arrows); G. Portion of early antral follicle—zona pellucida forms a complete ring (arrow); H. Antral follicle; I. Follicular wall of an antral follicle showing different layers of granulosa cells (GC) and theca externa (TE) and interna (TI).



Figs 2-4. 2. Relation between oocyte nuclear diameter and oocyte diameter. 3. Relation between oocyte nuclear diameter and follicular size. 4. Relation between oocyte diameter and follicular size.

follicle did not increase significantly and that of the oocyte and its nucleus remained unchanged in the intermediate stage (Table 1). Transitory follicles have also been identified in sheep ovary (Lundy *et al.* 1999).

Primary follicles were growing follicles and contained

16.25 ± 12.01 (15-55) cuboidal granulosa cells that formed 2-3 layers of cells around the oocyte (Table 2; Fig. E). Oocyte had also initiated growth and diameter increased to $27.92 \pm 1.47 \mu\text{m}$ with nuclear diameter to $16.10 \pm 0.54 \mu\text{m}$ (Table 1). The primary follicle in its later stage showed the formation of patches of zona pellucida. Theca layer remained undifferentiated in the primary follicles (Fig. E). In the secondary follicles of goat ovary granulosa cells exhibited cellular heterogeneity in size and shape enclosed within a basement membrane (Table 2, Fig. F). There was a gradual increase of size of follicle and oocyte in the ovary of prepubertal goat ovary (Table 1). These observations are in close conformity with the observations of Guraya (1985) in mammals, in rat by Kaur and Guraya (1987), Priedkalns (1987) in cows and Bhardwaj and Roy (1998, 2004) in prenatal and prepubertal buffalo calves respectively.

In the late stages of secondary follicles the beginning of an antrum was observed in follicles with at least 300 granulosa cells in the largest cross section. Zona pellucida first appeared as small patches in primary follicles but only in early antral follicles formed a complete ring around the oocyte (Fig. G). In mice and humans, the zona pellucida already forms a complete ring around the oocyte in the primary follicles (Oakberg, 1979). However this occurs much later, when the follicle reaches the late preantral stage in bovines (Braw-Tal and Yossefi, 1997). A clear theca interna layer was formed in some of the large preantral and all small antral follicles (Fig. F,G). Distinct theca layer is present in rodent follicles as early as the late primary/early secondary stage (Fortune and Eppig 1979) but in ovine, bovine and primate follicle it cannot be observed until the mid or late preantral stage (Gougeon 1996, Braw-Tal and Yossefi 1997, Lundy *et al.* 1999).

Tertiary (antral) follicles are also noticed in the prepubertal goat ovary. The developing tertiary follicles were in two phases. In the first phase of development, the follicles from primary to tertiary follicles end with the formation of an antrum. At this stage, the follicle diameter was $300.69 \pm 38.82 \mu\text{m}$ and the oocyte $89.72 \pm 5.78 \mu\text{m}$. After first phase of development, the follicle size increased abruptly to $535 \mu\text{m}$ and then to $>1535 \mu\text{m}$ in the second phase of development. The mean size of large antral follicles measured up to $1287.25 \pm 159.82 \mu\text{m}$ (Table 1). In the second phase of development, the size of the oocyte increased slowly in diameter from 89.72 ± 5.78 to $102.01 \pm 2.15 \mu\text{m}$ (Table 1). The relationships between the oocyte nuclear diameter and follicle size and oocyte size are represented in Figs 2-4. The analysis of the correlation between oocyte diameter, oocyte nuclear diameter and follicular size during follicle growth showed two distinct consecutive phases. Phase 1 was characterized by a change in the shape of the granulosa cells from flattened to cuboidal and an increase in their number without a significant increase in the oocyte diameter and oocyte nuclear diameter. In phase II, a linear and positive correlation between oocyte nuclear

diameter, oocyte diameter and follicular size was found. The tertiary follicles in all age groups of goat were almost identical (Gupta *et al.* 2007). In gilts, the average diameter of oocyte was maximum when developed up to 120–160 mm³ of the follicle volume remained its size during later expansion (Chiou *et al.* 2004). In bovines also, the expansion in diameter of the oocyte was accompanied by an increase in follicle diameter reached beyond 4 mm. There were no further changes in the diameter of the oocytes (Arlotto *et al.* 1996, Fair *et al.* 1995). The present investigation shows the presence of tertiary follicles in the ovaries of prepubertal goat. These follicles do not rupture due to insufficiency of FSH and LH. Hence these follicles might have undergone atresia later on. Further insufficient or excess of hormones leads to failure in follicular growth and result into atresia of follicles (McNatty *et al.* 1984). Granulosa cells became stratified (Figs H,I). Cellular heterogeneity has earlier been observed in developing antral follicle of mature goat ovary (Sangha *et al.* 2004).

In summary, the present study provides an interrelationship between the size and number of granulosa cells around the follicle and oocyte diameter indicating that in all types of follicles, changes in the number of granulosa cells are dependent of oocyte diameter.

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