Light microscopical and ultrastructural characterization of goat preantral follicles

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Abstract

Goat ovarian preantral follicles were morphologically and ultrastructurally described in this work. Primordial follicles are oocytes surrounded by one layer of squamous or squamous-cuboidal granulosa cells; primary follicles have a single layer of cuboidal granulosa cells, and secondary follicles are oocytes surrounded by two or more layers of cuboidal granulosa cells. At all developmental stages a thick layer of glycoproteins, the basement membrane, surrounded the preantral follicles. The quiescent oocyte is spherical or oval and it has a large eccentrically located nucleus with a conspicuous nucleolus. The organelles were uniformly distributed in the cytoplasm. A large number of vesicles were spread throughout the cytoplasm in all the oocytes. The cytoplasm of oocytes also contains numerous rounded mitochondria besides the usual organelles. As the follicle develops, the mitochondria become elongated. The communication between the oocyte and the granulosa cells is apparently mediated through endocytosis as indicated by the abundant coated pits and vesicles noted in the cortical cytoplasm of the oocyte. The oocyte plasma membrane presented projections that penetrated between adjacent granulosa cells and a few short microvilli lying parallel to the oocyte surface. In secondary follicles, patches of zona pellucida material were observed. Overall, the results indicate that the morphological and ultrastructural organization of caprine preantral follicles resembles that of other mammals. However, some particularities were observed, and that may indicate species specific differences.

Keywords: Goat; Oocyte; Preantral follicle; Ultrastructure

1. Introduction

At birth, the mammalian ovary is endowed with hundreds of thousands of preantral follicles, which represent the resting stockpile of germ cells (Saumande, 1991). However, very little is known about the biology of these follicles and the events surrounding their growth or atresia. Advances in this field will depend on in vitro systems which study preantral follicles.

During the past few years the possible exploitation of preantral follicles from domestic animals, by isolation and culture, has received much attention (Figueiredo et al., 1994, Hulshof et al., 1994, Jewgenow and Goritz, 1995, Nuttinck et al., 1996, Wandji et al., 1996, Lucci et al., 1999 and Cecconi et al., 1999). However, in some studies, the results were not promising possibly due to follicle damage induced by the isolation procedures (Van den Hurk et al., 1997 and Katska and Rynska, 1998). In general, the quality of the follicles was assessed by light

microscopic evaluation, but there is a great need for better viability analysis. With this perspective in mind, the ultrastructural evaluation, after isolation, may provide more information about the quality of the follicles and their oocytes, as it is capable of fine discrimination of damage to membranes and organelles. In this way, a description of the standard ultrastructure of preantral follicles in situ is very important as a model. Moreover, the need for cell biological knowledge of preantral follicles is accentuated in order to develop suitable culture systems.

Ultrastructural studies have been carried out on follicles and oocytes from farm animals such as cows (Hyttel et al., 1986, Assey et al., 1994 and Van Wezel and Rodgers, 1996; Fair et al., 1996, Fair et al., 1997a and Fair et al., 1997b) and ewes (Brand and Jong, 1973 and Cran et al., 1980), mainly in tertiary and preovulatory follicles, and some differences among species were demonstrated. However, little information about the ultrastructural aspects of goat preantral follicles is available in the literature (Sharma et al., 1994). In order to increase our knowledge of preantral follicle development in goat ovaries, the aim of the present study is to describe the morphological and ultrastructural aspects of follicular development during the preantral phases in the adult goat ovary. Oocyte and granulosa cells of primordial, primary and secondary follicles were studied using light and transmission electron microscopy.

2. Materials and methods

Ovaries (n=6) from adult (1–3 years old) and non-pregnant goats of mixed breed were collected at a slaughterhouse. The ovaries were removed, stripped of surrounding fat tissue and ligaments, washed three times in phosphate buffered saline (PBS) and processed for light and transmission electron microscopy. Primordial (n=162 and 6, respectively for light and TEM), primary (n=162 and 5) and secondary (n=48 and 4) follicles were evaluated.

2.1. Light microscopy

The ovarian cortex was fixed by immersion for 24–48 h in 10% formaldehyde, dehydrated in ethanol, and embedded in paraffin. Sections of 7 μm were stained with periodic acid Schiff (PAS) and haematoxylin. Preantral follicles were classified by their morphological appearance, according to Hulshof et al. (1994). The diameters of the follicles and the oocytes were determined when the nucleolus of the oocyte was observed (equatorial section), using an ocular micrometer and considering the smaller diameters. The granulosa cells in the

equatorial section were counted. Sections were examined and photographed using a Zeiss Axiophot light microscope.

2.2. Transmission electron microscopy (TEM)

Small pieces of ovarian cortex were fixed during 3 h at room temperature in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2. After fixation, the specimens were rinsed in buffer and post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM CaCl2 in 0.1 M sodium cacodylate buffer for 1 h, followed by block staining in 0.5% uranyl acetate. Subsequently, the samples were dehydrated in a series of ascending acetone concentrations (30–100%) and then embedded in Spurr epoxy resin. Thin sections (70 nm) were contrasted with uranyl acetate and lead citrate, and examined using a Jeol 100 C and a Zeiss 912 transmission electron microscopes. The basement membrane thickness was estimated in all observed follicles (n=15) by measurement in electron photomicrographs (10 000× final magnification) in three different regions (all of them in the equatorial section).

2.3. Statistical analysis

The diameters of follicles and oocytes and the number of granulosa cells in the equatorial section, as well as the basement membrane thickness, were compared between primordial, primary and secondary follicles by Scheffe's test (Stat View for Macintosh). Differences were considered statistically significant at 0.05 level.

3. Results

In this work, the preantral follicles were classified in three categories: primordial follicles (oocytes surrounded by one layer of squamous or squamous-cuboidal granulosa cells — Fig. 1a); primary follicles (with a single layer of cuboidal granulosa cells around the oocyte — Fig. 1b), and secondary follicles (oocytes surrounded by two or more layers of cuboidal granulosa cells — Fig. 1c). The mean (±S.E.M.) diameter of follicles and oocytes and the number of granulosa cells at the equatorial section are presented in Table 1. At all developmental stages a thick layer of glycoproteins, the basement membrane, surrounded the preantral follicles. The basement membrane was strongly stained by Schiff's reagent in

histological sections. Its thickness in different follicles varied from 231 to 727 nm, but no statistical differences were observed between follicular classes. The mean thickness of basement membrane was 513±38 nm in preantral follicles in general.

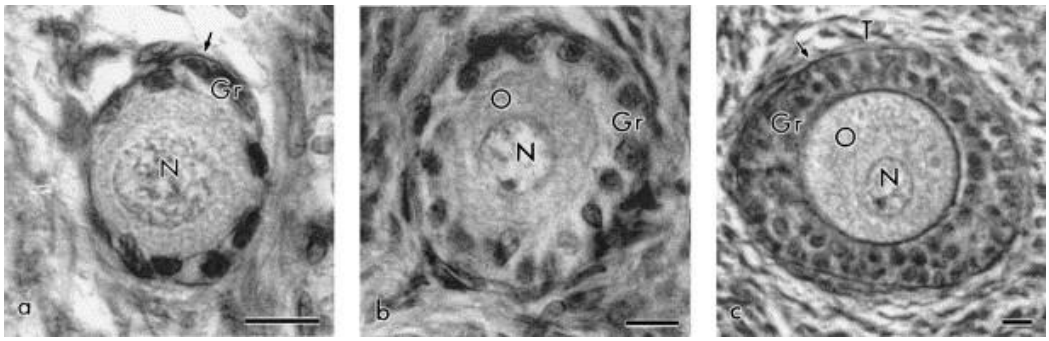

Fig. 1. Light micrographs showing (a) primordial, (b) primary, and (c) secondary goat follicles. O: oocyte, N: nucleus of oocyte, Gr: granulosa cell, T: theca cells (arrow marks the basement membrane). Bar=10 µm.

Table 1.
Diameter of primordial, primary and secondary follicles and their oocytes and number of granulosa cells at the equatorial section (mean±S.E.M.)[a]

Follicular class	*n*	Diameter (µm)		Number of granulosa cells
		Follicle	Oocyte	
Primordial	162	20.05 ± 0.31 a	15.91 ± 0.23 a	5.73 ± 0.17 a
Primary	162	24.42 ± 0.32 b	17.38 ± 0.26 b	11.39 ± 0.30 b
Secondary	48	44.24 ± 3.61 c	24.49 ± 1.59 c	31.04 ± 2.66 c

[a] Values with different letters in the same column are significantly different (Scheffe's test; P<0.005).

3.1. Primordial follicles oocytes

The quiescent oocytes in primordial follicles were spherical or oval and were completely surrounded by 1–14 squamous or squamous-cuboidal granulosa cells at the equatorial section. A large oocyte nucleus occupied a central or eccentric position showing a conspicuous nucleolus (Fig. 2). The organelles were uniformly distributed in the cytoplasm or, sometimes, more closely to the nucleus. The mitochondria were the most evident organelle and were predominantly round. These round mitochondria normally present few cristae often arranged parallel close to the surface of the outer mitochondria membrane, leaving a very large central area of moderately electron-dense inner matrix (Fig. 3). A small number of elongated mitochondria were also observed in some cases. A few cistern of smooth endoplasmic reticulum and Golgi apparatus were observed and a variable number of vesicles were spread throughout the cytoplasm. The zona pellucida (ZP) was always absent at this

stage, and the oocyte and granulosa cells appeared only juxtaposed, without any specific junctions.

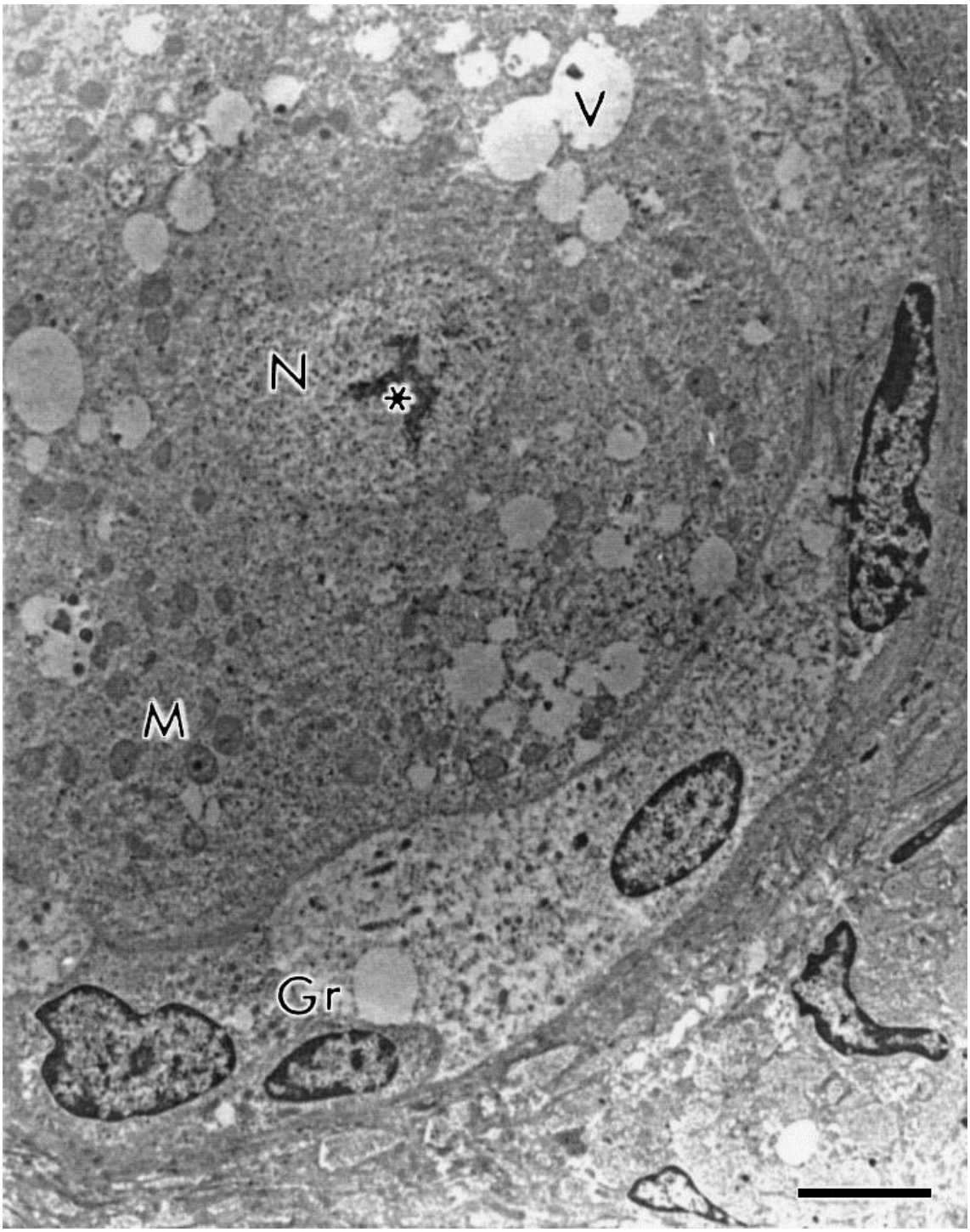

Fig. 2. Primordial follicle in a general TEM sight. N: nucleus of oocyte (∗ nucleolus), Gr: granulosa cell, V: vesicles, M: mitochondria. Bar=5 μm.

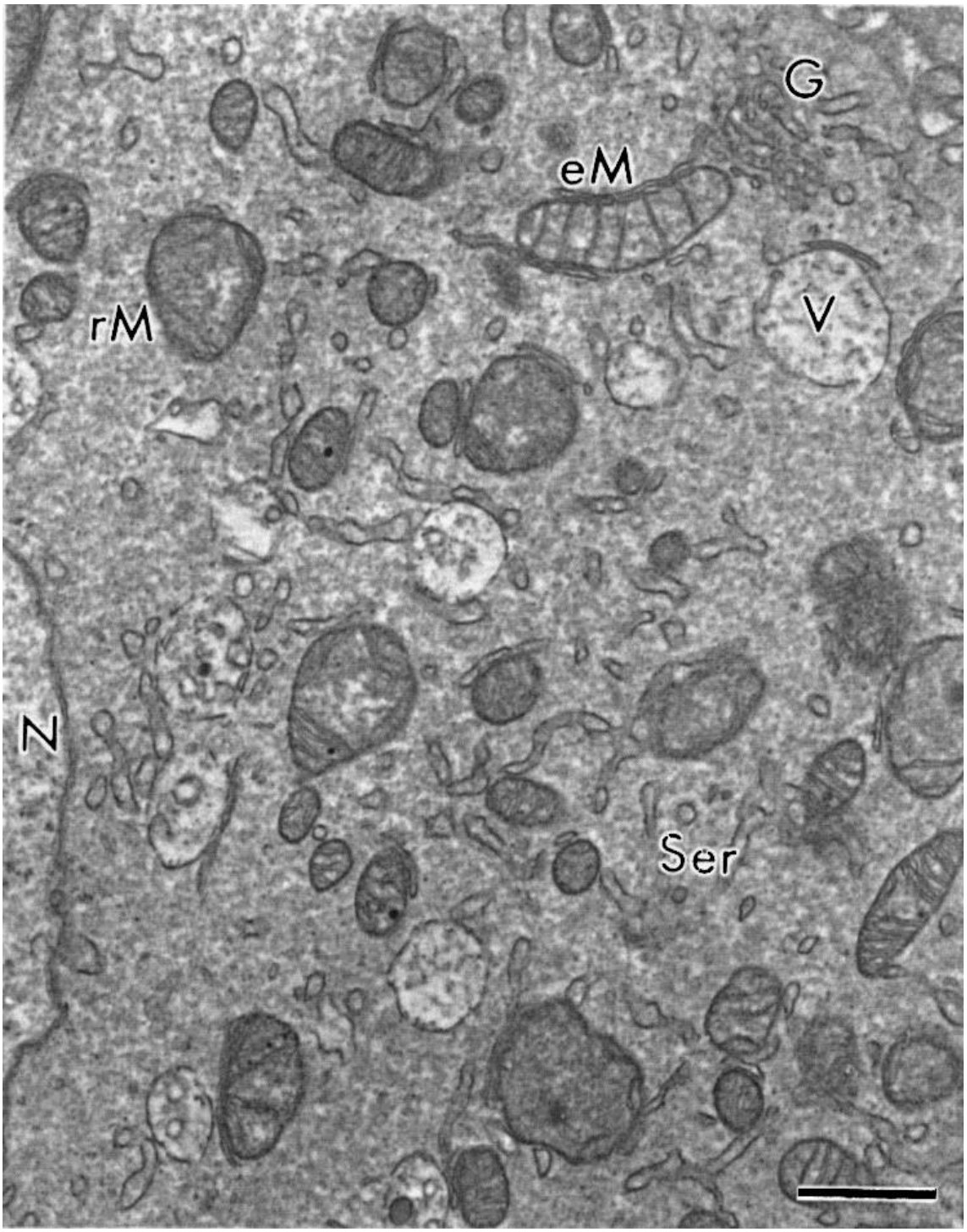

Fig. 3. Detail of cytoplasmic structures. N: nucleus of oocyte, V: vesicles, rM/eM: round or elongated mitochondria, G: Golgi apparatus, Ser: smooth endoplasmic reticulum. Bar=1 µm.

3.2. Primary follicles oocytes

The oocytes in primary follicles appeared not to be quiescent any more. They were surrounded by 5–26 cuboidal granulosa cells disposed in a single layer at the equatorial section, with which they kept a narrow contact. The communication between the oocyte and the granulosa cells is mediated through endocytosis as indicated by the abundant coated pits and vesicles noted in the cortical cytoplasm. The plasma membrane of the oocyte presented

projections that penetrated between adjacent granulosa cells and a few short bent microvilli lying parallel to the oocyte surface (Fig. 4). As well as the primordial follicles oocytes, the cytoplasm of oocytes in primary follicles also contains numerous rounded mitochondria beyond the usual organelles. As the follicle develops the mitochondria take the elongated shape (Fig. 3).

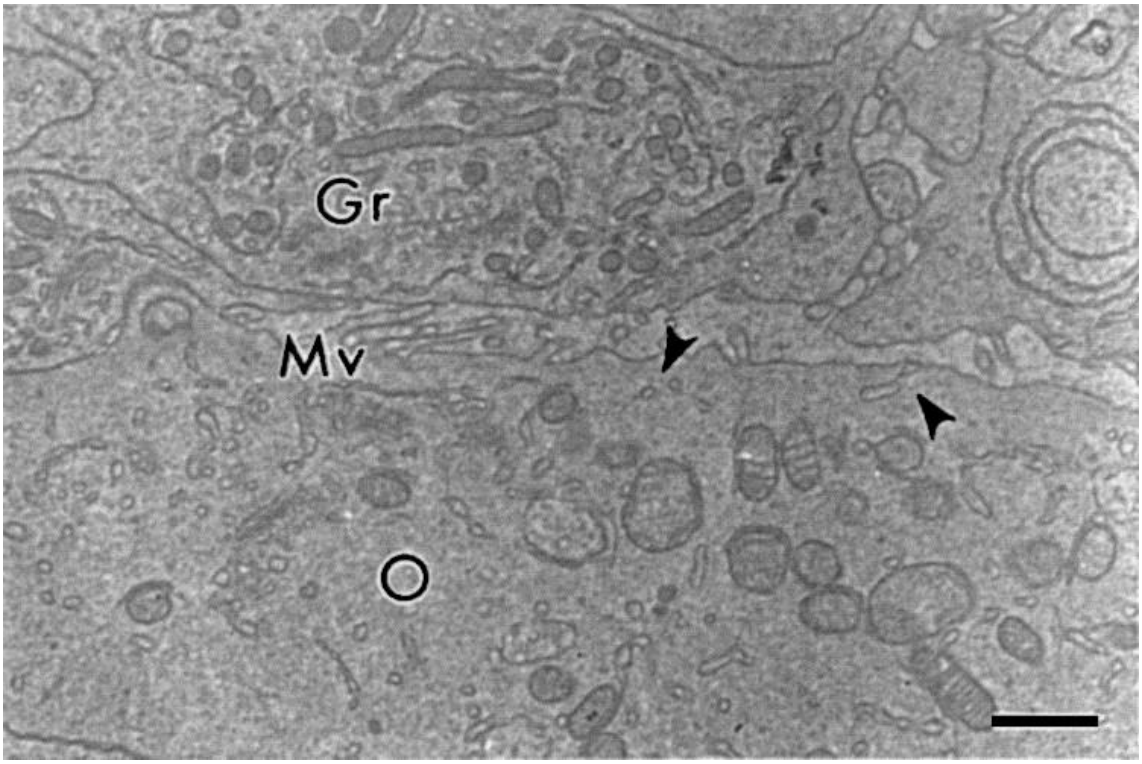

Fig. 4. Communication between oocyte and granulosa cells in a primary follicle. Gr: granulosa cell, O: oocyte, Mv: microvilli, arrowheads: coated pits. Bar=1 μm.

3.3. Secondary follicles oocytes

In these follicles, the oocytes were surrounded by 13–114 cuboidal granulosa cells forming two or more layers at the equatorial section. The nucleus of the oocyte assumed an eccentric position and the organelles started to move to the periphery. The number of smooth endoplasmic reticulum increased and the vast majority of mitochondria were elongated. With the development of the follicle, there appeared to be a slight increase in the number of microvilli. In secondary follicles, patches of ZP material were observed and were usually associated with erect oocyte microvilli (Fig. 5). Also in these follicles, electron-dense droplets were commonly observed in the cytoplasm of both the oocyte and granulosa cells (Fig. 6). These droplets could often be seen in histological sections and were strongly dyed by the Schiff's reagent (Fig. 7). At this stage, we could identify, at the light microscopy, the first theca

cells surrounding the follicle (Fig. 1c), outside the basement membrane. However, the theca interna could only be clearly defined in follicles with four or more layers of granulosa cells.

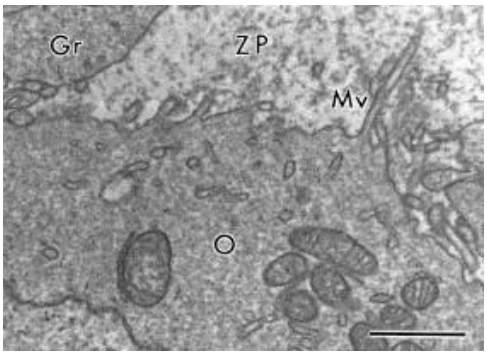

Fig. 5. Forming zona pellucida in a secondary follicle. O: oocyte, Gr: granulosa cell, Mv: microvilli, ZP: zona pellucida. Bar=1 μm.

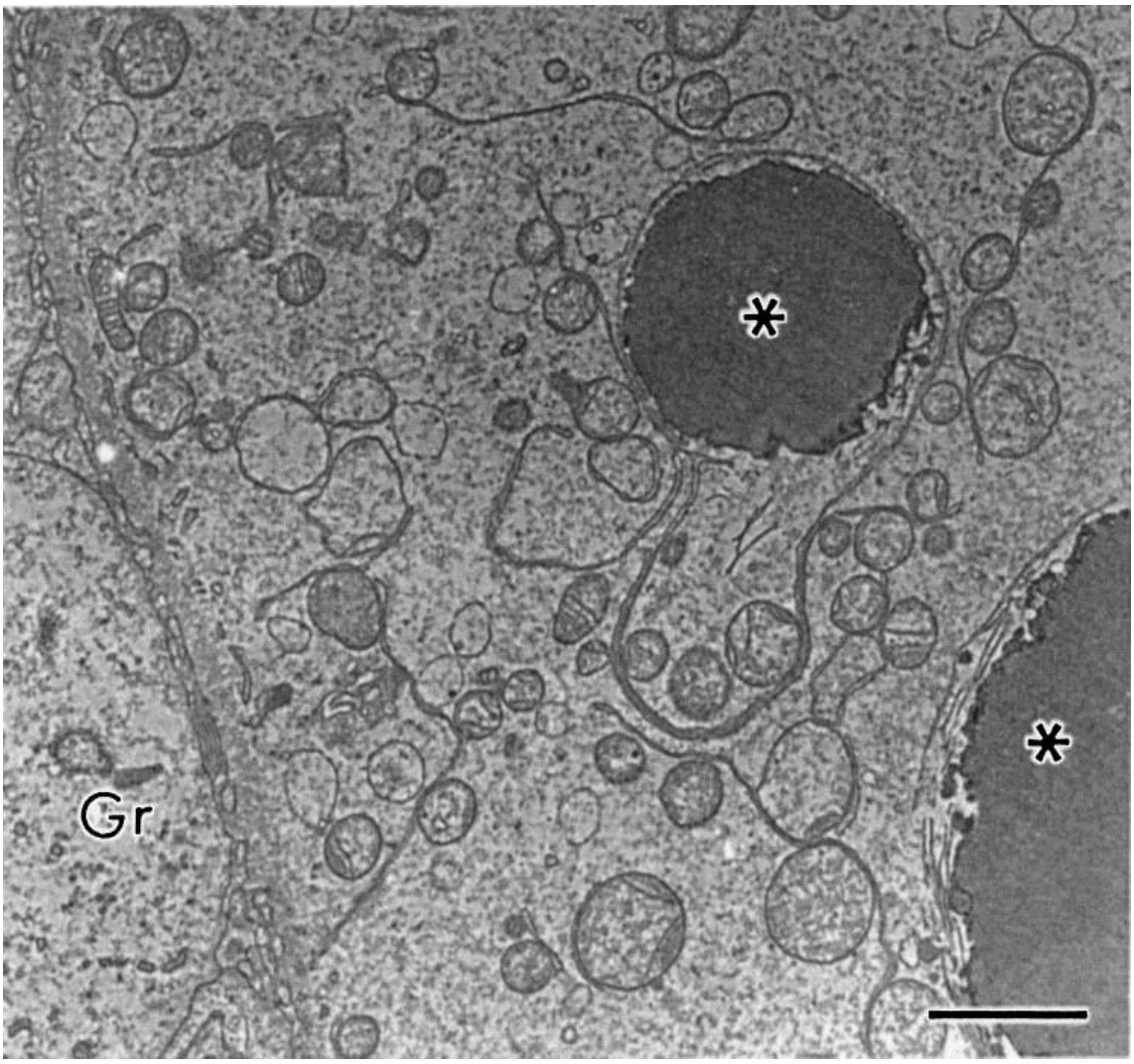

Fig. 6. Secondary follicle showing electron-dense droplets (∗) in the cytoplasm of the oocyte. Gr: granulosa cell. Bar=2 μm.

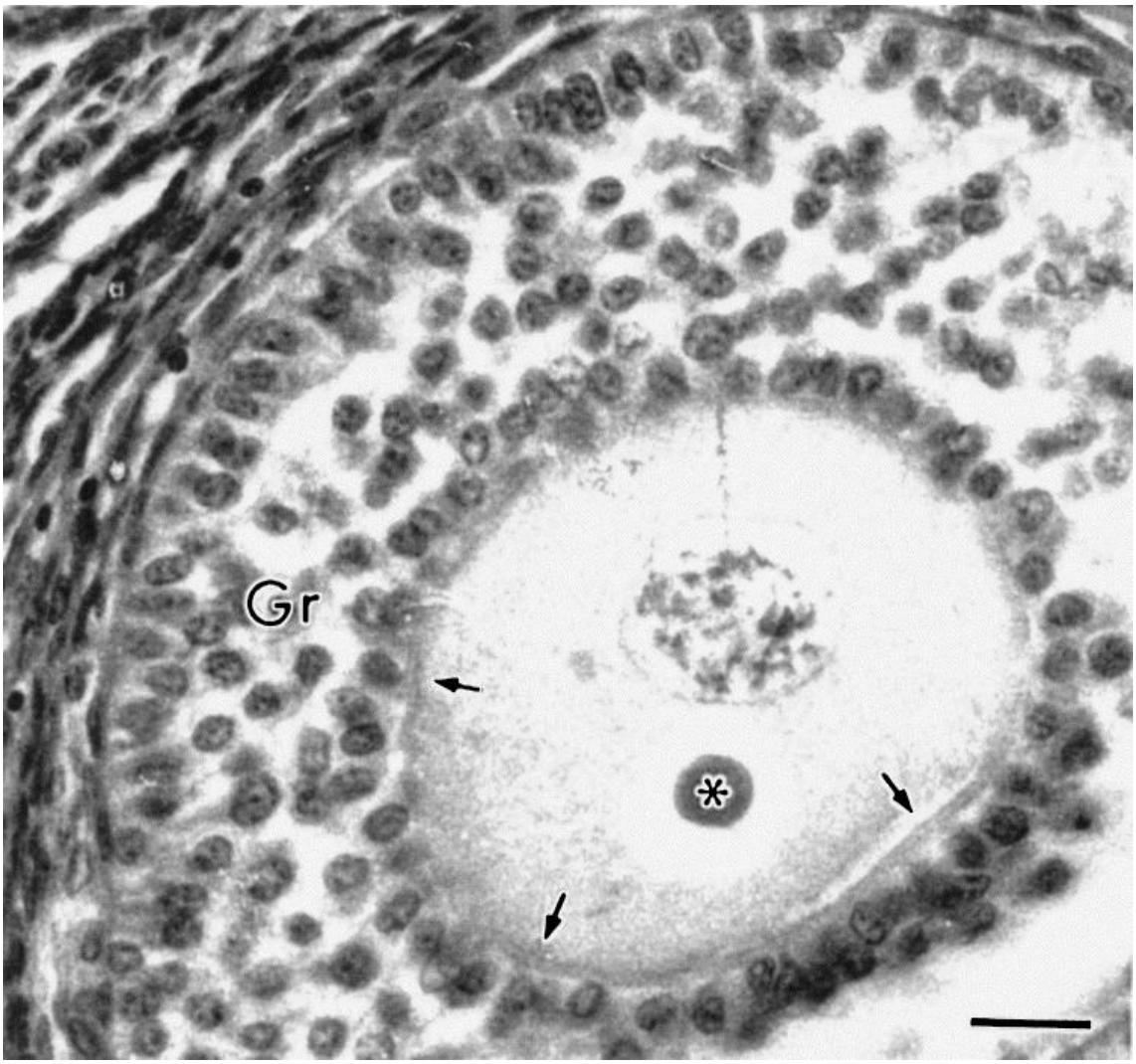

Fig. 7. Glycoprotein droplet (dyed by the Schiff's reagent) in a histological section of a secondary follicle (∗). Arrows indicate the zona pellucida forming a complete ring around the oocyte. Gr: granulosa cell. Bar=20 μm.

3.4. Granulosa cells

Granulosa cells were small, with high nuclear:cytoplasmic ratio. Squamous granulosa cells had an elongated nucleus and few organelles in general. Cuboidal granulosa cells, on the other hand, showed many elongated mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus and some electron dense vesicles. Their nucleus showed an irregular shape with loose chromatin and little peripheral agglomerates of dense chromatin (Fig. 8a and b). In secondary follicles, the granulosa cells began to separate, forming wide irregular intercellular spaces. At all follicular stages, granulosa cells rested on a basement membrane (Fig. 8b) which was tightly attached to the ovarian stroma.

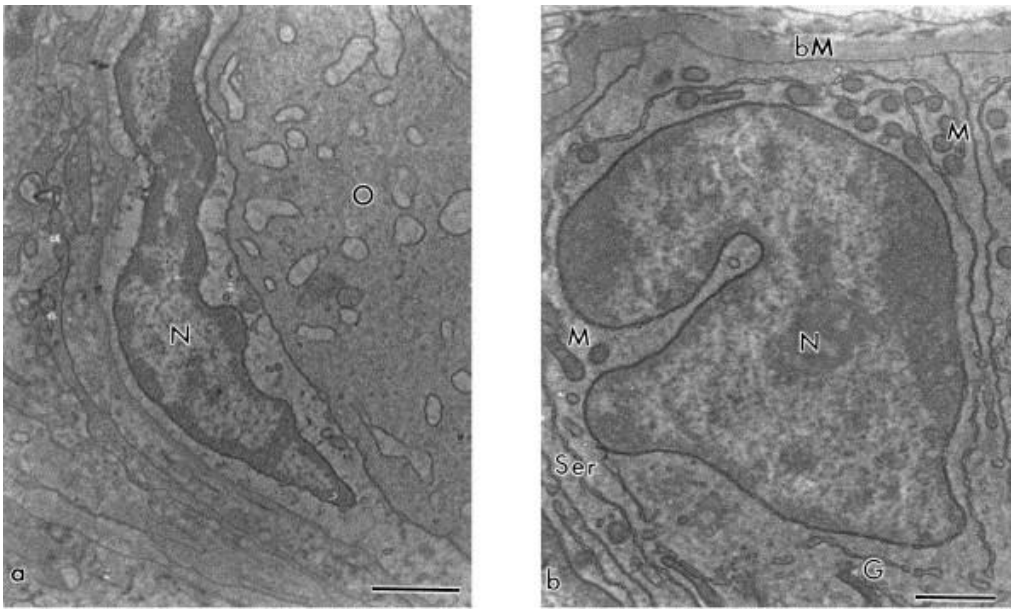

Fig. 8. (a) Squamous and (b) cuboidal granulosa cells. N: nucleus, M: mitochondria, Ser: smooth endoplasmic reticulum, bM: basement membrane, G: Golgi apparatus, O: oocyte. Bar=1 μm.

4. Discussion

The results of this study highlight much information about the cell biology of ovarian preantral follicles in the goat, which can be useful as a model to studies of integrity of follicles/oocytes handled in vitro. Concerning the histological aspects, the mean diameter of oocytes and follicles, in all the three developmental stages (i.e. primordial, primary and secondary), was smaller than those observed in ewes (Lundy et al., 1999) and cows (Van Wezel and Rodgers, 1996, Braw-Tal and Yossefi, 1997 and Hyttel et al., 1997), but was similar to those observed in humans (Lintern-Moore et al., 1974). However, the mean and range in the number of granulosa cells at the equatorial section were compatible to those described for ewes (Lundy et al., 1999), cows (Braw-Tal and Yossefi, 1997) and humans (Lintern-Moore et al., 1974), even when the diameter of oocytes and follicles was smaller. These differences may be species-specific or due to differences in measurement techniques.

In general, the ultrastructure of goat preantral follicles was similar to that of other mammalian species (cattle — Van Wezel and Rodgers, 1996, Fair et al., 1997b and Hyttel et al., 1997; pig — Greenwald and Moor, 1989; feline — Jewgenow and Stolte, 1996; human — Hertig and Adams, 1967 and Oktay et al., 1997), but some differences were observed, which may indicate either species specific differences or different phases of follicular development. For example, the concentration of cytoplasmic components in the juxta-nuclear region, commonly observed in oocytes of other species, was not observed in goats.

In addition, in bovine secondary follicles, gap junctions have developed between the oocyte and granulosa cells and the first small clusters of cortical granules were seen (Fair et al., 1997b and Hyttel et al., 1997). Immature cortical granules were also present in feline primary follicles (Jewgenow and Stolte, 1996). Neither of these two structures were observed in goat follicles analyzed in our study. Moreover, in bovine, adherens junctions were detected between granulosa cells and oocyte since primordial follicles, and gap and zonula adherens-like junctions were also observed between adjacent granulosa cells (Fair et al., 1997b). Desmosomes were observed joining follicular cells to each other and/or to the oocyte in cats (Jewgenow and Stolte, 1996), monkeys (Zamboni, 1974) and rabbits (Nicosia et al., 1975). However, in goats, special types of junctions (i.e. desmosomes, adherens or tight junctions) were not observed among granulosa cells or between granulosa cells and oocyte. In this last case, only the endocytotic contact was clearly visible. Specifically, regarding to gap junctions, the reason why they were not observed in this study may be that none of the follicles evaluated by TEM had the oocyte completely surrounded by ZP. Thus, it can be inferred that while the ZP is not fully developed around the whole oocyte, gap junctions are not indispensable, and therefore, not present yet. At this stage, substances required for the oocyte metabolic needs could gain access to its cytoplasm by diffusion through the closely opposed membranes of oocyte and granulosa cells in the areas of intercellular contact, or they could be incorporated by endocytosis, as suggested by the presence of numerous coated vesicles at the cortical cytoplasm. Anyway, whichever the way of contact between oocyte and granulosa cells, this association is extremely important to oocyte growth. The granulosa cells cooperate in oocyte metabolic processes and this cooperation is probably essential for normal oocyte development. Isolated oocytes normally grow in vitro only when their association with the companion granulosa cells is maintained and the rate of oocyte growth in vitro is directly correlated with the number of granulosa cells coupled to it (Eppig, 1991).

Concerning cytoplasmic organelles, the observations indicate that the changes undergone by the follicle during sequential phases of growth are directed mainly to providing the oocyte with the environment needed for its maturation and metabolism. All the characteristics observed show that oocytes in goat preantral follicles are equipped for the absorption, utilization and intracellular transport of material delivered to their surface membrane, as well as for the biosynthesis of stored material. The observed pleiomorphic variations in mitochondrial shape possibly represent their role in the metabolism of the developing oocyte. The round shape of the mitochondria, indicate immaturity (Perkins and Frey, 2000), in accordance with the quiescent stage of primordial follicle oocytes. In these follicles the round mitochondria were the vast majority in the oocyte cytoplasm, being

gradually replaced by elongated mitochondria in the oocytes of primary and secondary follicles.

In the present study, the ontogeny of formation of the ZP was similar to that in cows (Braw-Tal and Yossefi, 1997 and Fair et al., 1997b) and ewes (Lundy et al., 1999), in which it appeared as islands of zonal material in secondary follicles with two to three layers of granulosa cells and as a complete PAS-positive ring in follicles with more than three layers of granulosa cells (Fig. 7). However, it differed from that in mice (Oakberg, 1979), guinea pigs (Adams and Hertig, 1964), rabbits (Nicosia et al., 1975), cats (Jewgenow and Stolte, 1996), monkeys (Zamboni, 1974) and humans (Himelstein-Braw et al., 1976) in which the ZP forms around the oocyte in primary follicles. Electron-dense droplets in the cytoplasm of both the oocyte and granulosa cells of secondary follicles were detected in this study, and these droplets were strongly dyed by the Schiff's reagent in histological sections confirming their glycoproteic content. This is a sign that these droplets are secreting vesicles carrying material for the ZP formation. Previous works indicate that the ZP is made of glycoproteins synthesized by the oocyte and the granulosa cells, and that the ZP glycoproteins can be localized in the cytoplasm of follicular cells in multilayer follicles (rabbit — Wolgemuth et al., 1984).

Since the primordial follicular stage, oocytes were always completely surrounded by granulosa cells and were never in direct contact with the follicular basal lamina. In contrast, in monkey primordial follicles oocytes a discontinuous investment of squamous follicle cells is not unusual (Zamboni, 1974). Oocytes are large cells having a low plasma membrane area to cytoplasmic volume ratio. According to Eppig (1991), the plasma membrane of oocytes is probably unable to maintain an adequate inflow of nutrients and outflow of metabolic products to support optimal oocyte growth. Therefore, the plasma membrane of the granulosa cells may act as an extension of the oocyte plasma membrane providing for the uptake of nutrients, which diffuse into the oocyte through the narrow connection between granulosa cells and oocyte.

The ultrastructural characteristics of granulosa cells in this study are very similar to that described for monkeys, even to squamous and cuboidal cells (Zamboni, 1974). Cuboidal granulosa cells appeared engaged in active steroidogenesis as shown by abundant smooth endoplasmic reticulum and mitochondria. The predominance of these organelles was also observed in rat granulosa cells cultured in vitro (Sanbuissho et al., 1993). In secondary follicles, the granulosa cells were separated by irregular spaces. According to Zamboni (1974), these lacunae will be filled with liquor folliculi. Progressive accumulation of fluid will induce distension and coalescence of the lacunae and result in formation of the antrum in antral

follicles. In addition, in goat, the formation of the theca appears to be similar to that in cattle (Braw-Tal and Yossefi, 1997) and sheep (Lundy et al., 1999).

The basement membrane surrounding preantral follicles of goat ovaries was strongly stained by the Schiff's reagent in paraffin sections. In the TEM analysis, it appeared to be composed of a compact and well organized lamina in the inner part, surrounded by a thick layer of collagen fibers, according to the description of Figueiredo et al. (1995) for bovine preantral follicles. The ovarian follicles surrounded by a thick basement membrane are well known, but this is the first time the thickness was assessed. Also, in the present study, the basement membrane did not change in thickness or appearance as the follicles grew in size suggesting that the basement membrane is continuously being remodeled. Recent studies suggest that the extracellular matrix can be actively rearranged by granulosa cells in vitro (Richardson et al., 2000). Since basement membranes are specialized extracellular matrices, it is possible that the basement membrane around the ovarian follicles is rearranged by granulosa cells in vivo too. However, further studies are needed to support this statement. Several potential functions of basement membranes have been proposed including the creation and maintenance of tissue integrity, transport and filtration of various substances (Hessle et al., 1984) and attachment of some growth factors (Ruoslahti and Yamaguchi, 1991 and Feige and Baird, 1992). In the case of isolated preantral follicles handled in vitro, the integrity of the basement membrane is a very important characteristic.

5. Conclusion

In conclusion, this work shows, for the first time, an ultrastructural description of goat preantral follicles. In general, the results indicate that the morphology and the ultrastructure of caprine preantral follicles resemble that of other mammals. However, some differences were observed, and that may indicate species specific differences. An insight into the fine structural organization of goat follicles would be of great help in explaining cellular and molecular aspects of follicle growth and atresia in this species. Moreover, in the future, basic knowledge on the ultrastructure of preantral follicles may facilitate the understanding of their in vitro development.

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