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Preantral follicle morphometry and ultrastructure of antral follicles in Anatolian Water Buffalo

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Abstract: This study aimed to evaluate quantitative and morphometric analyses of preantral follicles and the ultrastructural characteristics of antral follicles in different oestrous cycle stages in Anatolian water buffaloes. Twenty-four ovaries collected from twelve slaughtered Anatolian water buffaloes were classified macroscopically as luteal or follicular stages. The ovaries were prepared for histological examination (Hematoxylin-eosin staining), and primordial, primary, and secondary follicle numbers were calculated, and the diameters of oocytes, follicles, and nuclei were measured under a light microscope with a micrometre. The theca and granulosa cells of antral follicles were observed under a transmission electron microscope. The mean number of preantral follicles was 18584 ± 4855, and there was a significant difference in the number of primordial follicles ($p < 0.0001$) and primary follicles ($p < 0.001$) between buffaloes. The number of primordial follicles was 10,636, that of primary follicles was 6514, and that of secondary follicles was 1434; the statistical difference was found between primordial, primary, and secondary follicle and oocyte diameters ($p < 0.001$) in Anatolian water buffaloes. In this study, the ultrastructural evaluation of antral follicles showed that the theca cells were active in the luteal stage with their functional organelles and higher lipid droplets. The granulosa cells were still inactive in the luteal stage. In the follicular stage of the oestrous cycle, the theca cells were found inactive, although granulosa cells showed moderate or high activity. It was found that the serum progesterone concentration and cycle stage directly affected the theca and granulosa cell ultrastructural activity in Anatolian water buffalo. In this research, information from light and electron microscopic analyses of preantral and antral follicles has been obtained for the first time for Anatolian water buffaloes. The result of our study suggests that detailed molecular research is needed to evaluate the ultrastructural activity of antral follicles in different oestrous cycle stages and steroidogenic circumstances.

Key words: Anatolian water buffalo, preantral follicle, morphometry, ultrastructure

1. Introduction

The reproductive physiology of mammalians is complicated with different reproductive organs, hormones, molecules, and biochemical interactions [1]. The ovaries of mammalians are one of the primary reproductive organs that control gametogenesis and steroidogenesis. Folliculogenesis and oogenesis on the ovaries continue during the reproductive life of mammalians [2,3]. Primordial follicles are generated from primordial germ cells during foetal life, and primary and secondary follicle development persist during folliculogenesis. These follicles are termed preantral follicles [4-6]. Preantral follicles are functional pools of female mammalians and limit the lifelong fertility of individuals. Recent studies focused on the potential of preantral follicles following ovarian tissue cryopreservation [7-9]. The preantral follicle population varies between species. This variation is significant between cattle and buffalo (almost ten times lower in buffaloes) [10-12]. Buffaloes have some reproductive disadvantages compared to cattle, not only for the preantral follicle population but also for late puberty, longer gestation period, longer calving interval, and shorter oestrous cycle [13]. The reproductive efficiency of buffalo is affected by environmental factors such as geographical

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location, management systems, photoperiod, relative air humidity, and temperature [14]. Thus, researchers are still studying how to enhance buffaloes' fertility and reproductive efficiency [13,15,16]. Studies have revealed that the environment has an important role in regulating buffaloes' reproductive functions [17]. However, most studies on ovarian morphology and follicle in buffaloes were carried out in tropical and subtropical locations [9,11,18,19]. Information on the ovarian morphology, morphometry, and preantral population of buffaloes breeding in continental climates is insufficient. There are only a few data about reproductive efficiency, antral follicle population, quality of oocytes, and composition of follicular fluid on Anatolian water buffaloes [20-22].

There is lack of studies on the preantral follicle population and ultrastructural changes concerning the oestrous cycle stage in the antral follicles of Anatolian water buffaloes. Therefore, the present study aimed to evaluate quantitative and morphometric analyses of preantral follicles and the ultrastructural characteristics of antral follicles in Anatolian water buffaloes.

2. Materials and methods

2.1. Collection and evaluation of ovaries

This research was carried out in accordance with the directions of Guide for the Care and Use of Animal in Research (AKUHADYEK - 48 - 10). The study was performed in Afyonkarahisar (38°45′2″N, 30°32′3″E; altitude: 1015 m, Turkey). A total of 24 ovaries from 12 healthy and nonpregnant slaughtered Anatolian water buffaloes were collected in this study. The buffaloes were 2–5 years old and had clinically normal uterus, oviducts, and ovaries without any pathologic findings on the macroscopic examination. Inactive ovaries without corpus luteum (CL) or ovaries containing cystic follicles more than 20 mm in diameter were not included in this study. Both ovaries of each animal were quickly delivered to the laboratory in an ice container.

The diameter of ovaries was measured and classified macroscopically as follicular (orange, yellow, or white, regressing CL, with a dominant follicle up to preovulatory size) or luteal (red to pink in colour, new, active CL, with dominant and subordinate follicles present) stages according to Acar et al. [20] and Nandi et al. [23] to assess the ultrastructural differences in antral follicles.

2.2. Blood collection and progesterone analysis

Following the slaughter of the animals, approximately 10 mL of jugular blood was collected into plain tubes (Vacutainer® SST™ II Advance Serum Tubes) for serum collection. The collected blood samples were centrifuged at 3000 rpm for 15 min, and the sera were stored at –20 °C until progesterone analysis was performed. The oestrous cycle of the buffalo was classified with serum progesterone levels (<1 ng/mL as follicular and >1 ng/mL as luteal stage) and supported the ovarian macroscopic classification.

The serum progesterone concentrations were detected using an electrochemiluminescence immunoassay (ECLIA) analyser (Roche E170; Roche Diagnostics GmbH, Mannheim, Germany).

2.3. Quantitative and morphometric analyses of preantral follicles

2.3.1. Light microscopy

Ovaries were fixed initially in 10% neutral formalin (Sigma Chemical Co., Taufkirchen, Germany). After the fixation, the ovaries were bisected along the longitudinal axis and embedded in paraffin. Samples were cut into 5-µm serial sections, and every 120th was stained with haematoxylin and eosin for preantral follicle and oocyte evaluation [18]. The sections were examined under a Nikon Eclipse E600 (Japan) light microscope and photographed with an Olympus DP70 (Japan) camera. Preantral follicle numbers with oocyte nuclei in different development stages were evaluated according to Mondadory et al. [11]. The atretic follicles were eliminated based on the integrity of the basement membrane, pyknotic bodies, and cellular density.

2.3.2. Follicular quantification

Follicular quantification was carried out according to Gougeon and Chainy [24]. The primordial, primary, and secondary follicle numbers were calculated based on the formula.

$$Nt = (No \times St \times ts) / (So \times do)$$

Nt: Total calculated number of follicles
No: Number of follicles observed in the ovary
St: Total number of sections obtained per ovary
ts: Section thickness (µm)
So: Number of sections analysed
do: Mean oocyte nucleus diameter of follicle class analysed

Follicular diameters were measured from the follicle's basement membrane under an inverted microscope (magnification: 400×; Nikon Eclipse E600, Japan) using a micrometre at the oocyte equatorial region with the presence of oocyte nucleus. Oocyte diameters were measured together with its zona pellucida, and the oocyte nucleus was measured from its outer border.

2.3.3. Transmission electron microscopy

Intact antral follicles (3 to 8 mm in diameter) were excised with a scalpel and placed into Karnovsky's solution (2.5% glutaraldehyde, 2% paraformaldehyde, 3% sucrose in 0.1 M sodium cacodylate buffer, pH 7.2) immediately after the slaughtering of Anatolian water buffaloes. The samples were fixed in Karnovsky's solution for 24 h at + 4

°C and postfixed with 1% osmium tetroxide. The samples were washed in 0.1 M phosphate buffer, kept in 3% uranyl acetate, dehydrated in graded ethanol solutions, and embedded in Araldite CY 212. Semithin sections (2 µm) were treated with citrate to ensure ultrathin sections (90 nm) for follicles with theca and granulosa cells were observed under light microscopy. The ultrathin sections were contrasted with lead citrate and uranyl acetate and examined under a transmission electron microscope (JEM 1200EX, Jeol, Tokyo, Japan). The comparison between the luteal and follicular stage antral follicles was evaluated.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 8.0 in the present study. Data on the preantral follicle and oocyte numbers obtained after morphological evaluations were analysed. The variables were expressed as mean ± standard error of means (SEM) and average rank. The Tukey test was used to determine the significance of differences among the mean values. Values were considered statistically significant when $p < 0.05$.

3. Results

The size of the ovaries was varied from 25 × 17 × 11 mm to 30 × 32 × 20 mm. The macroscopical evaluation of ovaries revealed that seven buffaloes were at the luteal and five were at the follicular stage. The progesterone concentrations (6.5 ± 1.8 ng/mL in luteal-stage buffaloes and 0.3 ± 0.02 ng/mL in follicular-stage buffaloes) confirmed the oestrous cycles of buffaloes.

3.1. Morphometry and numbers of preantral follicles and oocytes

Follicles that consist of an oocyte surrounded by one layer of flattened or flattened-cuboidal granulosa cells (intermediary/transitory) were classified as primordial follicles. In primary follicles, the oocyte was surrounded by a single layer of cuboidal granulosa cells. In secondary follicles, the oocyte was surrounded by two or more layers of cuboidal granulosa cells, and great variations were detected in the number of cell layers (Figure 1).

The numbers of each preantral follicle class are presented in Table 1. Despite the high variability in the

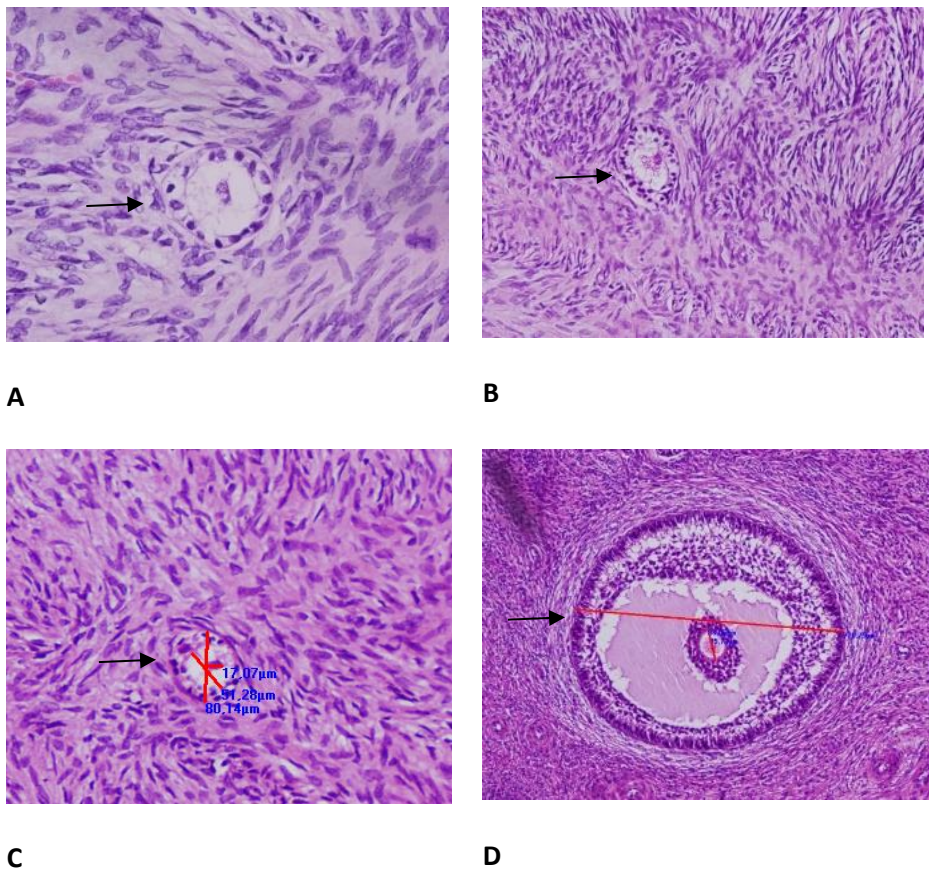

Figure 1. Different preantral follicles (A, 400×), (B, 200×), and measurement of preantral and antral follicle, oocyte, and nucleus diameters (C, 200×), (D, 100×) in the ovaries of Anatolian water buffaloes under a light microscope (Nikon Eclipse E600, Japan).

Table 1. Preantral follicle numbers in Anatolian water buffalo.

Follicular stages	Mean number (± SEM)	Minimum	Maximum
Primordial	10,636.08 ± 3682.77[a]	1636	38,296
Primary	6514.33 ± 1955.51[b]	950	22,588
Secondary	1434.08 ± 433.81[c]	213	5607
Total	18,584.50 ± 4855.87	3905	47,412

*Values with different letters differ significantly within the column ($p < 0.001$).

preantral follicle numbers among individuals, the preantral follicles' mean number was 18,584 ± 4855. There was a significant difference in the number of primordial follicles ($p < 0.0001$) and primary follicles ($p < 0.001$) between buffaloes (Figures 2 and 3). The primordial follicle number for each buffalo ranged from 1636 to 38,296, the primary follicle number ranged from 950 to 22,588, and secondary follicle number ranged from 213 to 5607 in Anatolian water buffaloes. There was a significant difference between primordial, primary, and secondary follicle numbers ($p < 0.001$). The number of antral follicles, including oocytes in the sections, was lower than that of preantral follicles ($p < 0.0001$). The mean follicle, oocyte, and nucleus diameters of follicles are shown in Table 2. A total of 895 preantral and antral follicles were measured, most of which were primordial or primary.

3.2. Ultrastructure of antral follicles in accordance with luteal and follicular stages

Theca and granulosa cells of antral follicles were analysed using TEM in luteal and follicular stages of Anatolian water buffaloes. A total of 24 antral follicle samples (3- to 8-mm diameter) were evaluated for ultrastructural analyses. No difference was detected between the right and left ovarian antral follicles. Therefore, the results were presented according to individual buffaloes.

In the samples of luteal-stage antral follicles, the theca cells have shown numerous mitochondria and a few rough endoplasmic reticulum cisternae; free ribosomes, Golgi apparatus, and partly smooth endoplasmic reticulum cisternae. In the luteal stage, theca cells had lipid droplets, and these cells showed high activity. A basement membrane seemed to separate granulosa cells from theca cells. Granulosa cells presented less dense elongated mitochondria with cristae and distinct nucleolus. Rough endoplasmic reticulum cisternae, free ribosomes, Golgi apparatus, lipid droplets, and a few smooth endoplasmic reticula were detected. Granulosa cells were less active in the luteal-stage antral follicles (Figure 4).

In the follicular-stage antral follicles, theca cells have shown mitochondria with dense transverse cristae, rough endoplasmic reticulum, free ribosomes, Golgi apparatus, and a few smooth endoplasmic reticula. The lipid droplets in the cells were rare. The theca interna cells were noticed to be inactive in relation of lipid droplet density which controls the steroidogenic activity. Desmosomes and zona adherens were observed between the granulosa cell junctions. Elongated mitochondria and apparent nucleolus were present in the granulosa cells. Rough endoplasmic reticulum, free ribosomes, Golgi apparatus, and a few smooth endoplasmic reticula were detected. It was observed that the lipid droplets appeared in almost every cell. Granulosa cells showed moderate or high activity with rich lipid droplet existence in the follicular stage of the oestrous cycle in Anatolian water buffaloes (Figure 5).

4. Discussion

To our knowledge, this is the first report of the preantral follicle population and morphometry and ultrastructural comparison of antral follicles between the luteal and follicular stage of the oestrous cycle in Anatolian water buffalo (Bubalus bubalis). The present study provides new knowledge regarding the reproductive physiology of Anatolian water buffalo bred in continental climate conditions in Afyonkarahisar Province. It was observed that there was a significant variation in each class of preantral follicle population between animals. The Anatolian water buffalo included in the present study had an average of 18,584 preantral follicle population. The results of our study are in accordance with those of Mondadori et al. [18], who found great variation in the preantral follicle population between buffaloes and found a total number of 19,819 preantral follicles. As stated previously, other ruminant species have higher preantral follicle numbers than buffalo, and our study supported those data [10,25,26].

Primordial, primary, and secondary follicle numbers also showed great variations in Anatolian water buffalo in the present study. The mean number of primordial follicles was higher than that of primary and secondary follicles. In the study by Mondadori et al. [18], the number of primary follicles was higher than the number of those in other follicle stages. The results of our study

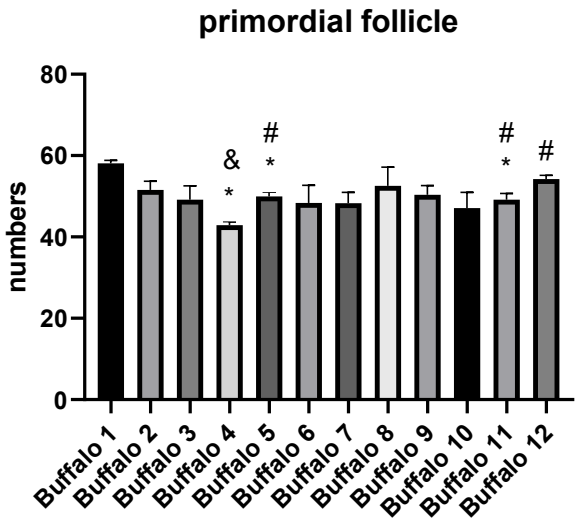

Figure 2. The number of primordial follicles in Anatolian water buffaloes. *: Significantly different from Buffalo 1, 5, and 11; &: significantly different from Buffalo 2; #: significantly different form Buffalo 4 ($p < 0.0001$). Values are expressed as mean ± SEM.

Table 2. Preantral and antral follicle, oocyte, and nucleus diameters in Anatolian water buffalo.

Follicular stages	**Follicle diameter (µm) (± SEM)**	**Oocyte diameter (µm) (± SEM)**	**Oocyte nucleus diameter (µm) (± SEM)**
Primordial	49.88 ± 0.42^{a}	26.72 ± 0.39^{a}	11.36 ± 0.16^{a}
Primary	69.52 ± 0.58^{b}	35.89 ± 0.62^{b}	14.04 ± 0.27^{b}
Secondary	113.39 ± 3.16^{c}	48.18 ± 1.77^{c}	16.30 ± 0.51^{c}
Antral	834.95 ± 154.35^{d}	99.23 ± 10.53^{d}	39.11 ± 5.32^{d}

*Values with different letters differ significantly within the column ($p < 0.0001$).

were similar to those described by Amorim et al. [27] in mixed breed ewes, Lucci et al. [10] in Zebu cows, Rondina et al. [26] in Massese lambs, Santos et al. [19] in buffalo foetuses, and Alves et al. [28] in mares. Considering all the studies stated above, the differences between numbers could be due to the age of the animals. Mondadori et al. [18] collected ovarian samples form buffaloes aged 7–10 years. In the present study, we used the ovarian samples of buffaloes aged 2–5 years. Except for Mondadori et al. [18], the studies mentioned above compared the number of preantral follicles between foetal and adult ovarian samples in different age groups. The results revealed that the number and density of preantral follicles were directly affected by age. Gastal et al. [12] reported the adverse effect of aging on the preantral follicle population, quality, and morphology. Studies performed on women reported that there was an age-related decrease in primordial follicle counts by follicular dynamics and follicle atresia [29,30].

Each preantral follicle and oocyte was evaluated as the oocyte nuclei surrounded by follicular cells, and each follicle was sampled by a single nucleus. Diameters of primordial follicles and oocytes were slightly larger, but those of primary and secondary follicles and those of their oocytes were similar in the postpubertal Zebu cattle [31]. The preantral follicle and oocyte diameters were slightly larger in Anatolian water buffalo than those of the adult buffaloes in the study of Mondadori et al. [11]. The primordial, primary, and secondary follicle diameters were significantly higher in our study than those of foetal buffalo follicles reported by Santos et al. [19]. No oocyte nuclei diameter comparison between preantral follicles was found in the literature. Therefore, we could not compare the results of oocyte nucleus diameters in the Anatolian water buffalo with any other study.

The ultrastructure of the theca and granulosa cells of antral follicles was analysed concerning the oestrous cycle

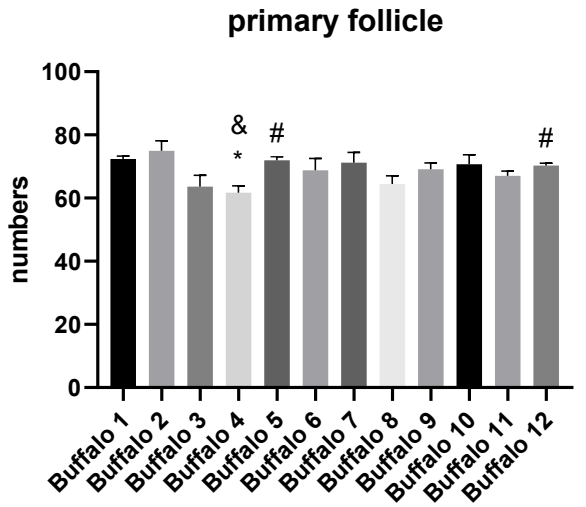

Figure 3. The number of primary follicles in Anatolian water buffaloes. *: Significantly different from Buffalo 1; &: significantly different from Buffalo 2; #: significantly different form Buffalo 4 ($p < 0.001$). Values are expressed as mean ± SEM.

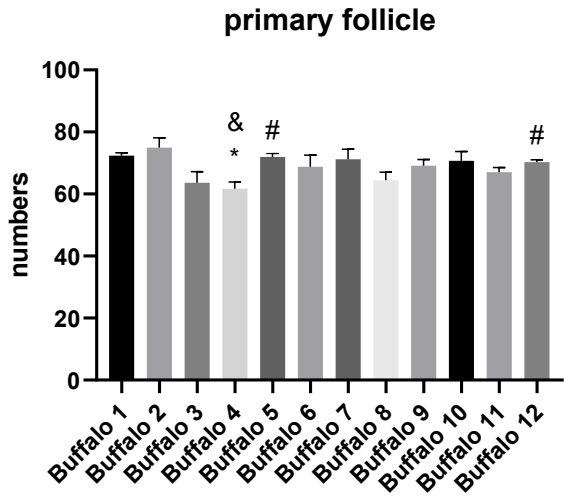

Figure 4. Ultrastructure of antral follicles in the luteal stage (JEM 1200EX, Jeol, Tokyo, Japan). Black arrow: granulosa cell; White arrow: lipid droplets; Star: mitochondria.

in Anatolian water buffalo. The ultrastructural feature of preantral and antral follicles was investigated in detail in previous studies [8,11,32,33]. However, the comparison of theca and granulosa cell structure between the luteal and follicular stages of the oestrous cycle is insufficient. In the present study, the theca cells were active in the luteal stage with their functional organelles and higher lipid droplets. The granulosa cells were still inactive in the luteal stage. In the follicular stage of the oestrous cycle, the theca cells were found inactive, although granulosa cells showed moderate or high activity in the follicular stage of the oestrous cycle. It was found that the serum progesterone concentration and cycle stage directly affected the theca and granulosa cell ultrastructural activity in Anatolian water buffalo. Theca and granulosa cells play an active role in mammalians' folliculogenesis and preovulatory process. These cells control steroidogenesis, follicle growth, and maturation synchronously with follicle-stimulating

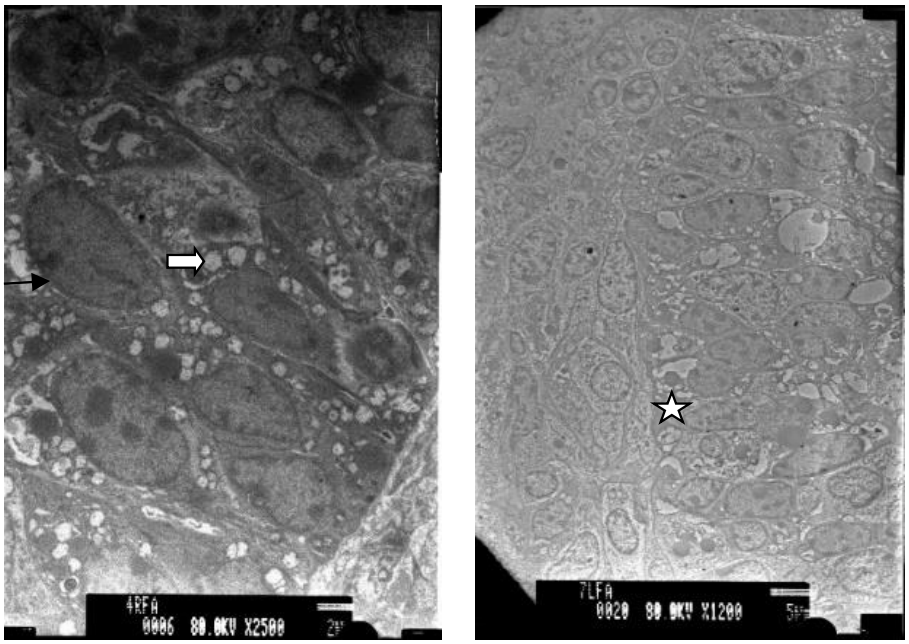

Figure 5. Ultrastructure of antral follicles in the follicular stage (JEM 1200EX, Jeol, Tokyo, Japan). Black arrow: granulosa cell; White arrow: lipid droplets; Star: mitochondria.

hormone (FSH) and luteinizing hormone (LH), and this interaction is explained with the two cells-two gonadotropins theory. FSH stimulates granulosa cells of early antral follicles, and LH receptor formation begins in granulosa cells. LH activates the production of cAMP. LH also stimulates theca cells by cAMP-mediated mechanism and androstenedione synthesis from cholesterol. Androstenedione diffuses across the basal lamina into the granulosa cells, and FSH stimulates the metabolization of androstenedione to estradiol [34,35]. The theca and granulosa cells manage this process with active organelles such as numerous mitochondria with vesicular cristae and lipid droplets. Lipid droplets synthesize cAMP, store cholesterol, and play an essential role in steroidogenesis and antral follicle development [36]. Therefore, the cell lipid droplet density also suggests ovarian cell activity [37]. In the present study, the ultrastructural view of the theca and granulosa cells was examined in the antral follicles of Anatolian water buffalo. However, more complicated molecular investigations are needed to reveal the interactions between the oestrous cycle and theca and granulosa cell ultrastructure in buffaloes.

5. Conclusion

The reproductive performance and parameters of Anatolian water buffaloes have been studied in general terms up to the present. However, in this research, the information on the morphometric and ultrastructural analysis of preantral and antral follicles was detected for the first time in these animals. The preantral follicle population and morphometry were similar to that of other buffalo breeds. There was a significant difference in the preantral follicle numbers between individuals and the primordial, primary, secondary, and antral follicle and oocyte diameters in the Anatolian water buffaloes. The lipid droplets play a crucial role in steroidogenesis by storing cholesterol esters and triglycerides for steroid biosynthesis. Our study showed that the theca and granulosa cells of antral follicles displayed different ultrastructural activities in luteal and follicular stages of oestrous cycle in accordance with lipid droplet density. The results of our study suggest that detailed molecular research is needed to evaluate the ultrastructural activity of antral follicles in different oestrous cycle stages and steroidogenic circumstances.

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Conflict of interest

The authors have no conflicts of interest in this study.

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