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Prenatal ovarian development in local sheep of Kashmir Valley

MIR SHABIR AHMAD[1]✉, MASSARAT KHAN[2], S A SHAH[3],
A R COUDHURY[4], Z A PAMPORI[5] and S H DAR[6]

Sher-e-Kashmir University of Agricultural Sciences and Technology, Srinagar-190 006 (J&K) India

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ABSTRACT

Morphological, histological and histochemical studies were conducted on prenatal ovary at different stages of gestation of local sheep of Kashmir. The genital ridge was observed as a minor thickening on the ventro-medial aspect of mesonephros which contained mesenchymal cells, primordial germ cells (PGCs), differentiating erythrocytes and spindle shaped fibroblasts upto 50 days of gestational age. Later on small, elongated to almond shaped ovaries caudal to respective kidneys were observed. The ovaries were covered by germinal epithelium. The sex cords were observed along with primordial and primary follicles in 50-100 days of prenatal life. Secondary follicles were first observed from 100 days of prenatal ovary onwards. Tertiary follicles were first observed at the inner part of the cortex near the full term (140-149 days). Development of the tunica albuginea was initiated from 100 days of gestation onward. Regarding connective tissue fibres, reticular fibres were evident from 50 days onward and collagen fibres appeared from 100 days onward of the gestation period.

Keywords: Age, Development, Sheep, Morphology, Pre-natal ovary

Sheep, with a population of 32 lakh in Jammu and Kashmir (4.3% of India's total livestock; 20th Livestock Census, 2019), are efficient converters of grass into meat, milk, and wool (Banerjee *et al.* 2010). Ovary, a key reproductive organ, supports oocyte development and produces hormones crucial for the hypothalamo-pituitary-ovarian axis and secondary sex characteristics. The prenatal formation of a fixed germ cell reserve determines future follicular stock and fertility. Any disruption in ovarian development or folliculogenesis may lead to infertility or premature ovarian failure (Guigon and Magre, 2006). The ewe serves as an excellent model for studying folliculogenesis due to gene–environment influences on ovulation. Understanding ovarian morphology and histology is essential for evaluating reproductive status and improving fertility. The ovarian surface epithelium, of heterogeneous origin, contributes to granulosa cells and may be linked to ovarian pathologies. Detailed insights into follicular development aid reproductive biotechnologies such as hormonal therapies, genetic testing, oocyte/embryo analysis, and cryopreservation (Pawshe and Totey, 2003). Since ovarian differentiation begins before the fetal period, studying prenatal development provides crucial anatomical and functional understanding of fertility. However, data on the prenatal ovarian development of local Kashmiri sheep is scarce, highlighting the need for this investigation.

Present address: [1]Animal Scientist KVK, Shopian. [2]Retd. Prof. and Head, Division of Veterinary Anatomy. [3]Associate Prof. Div. of Pathology, [4]Prof. and Head, Div. of Veterinary Anatomy, [5]Prof., Division of Physiology, [6]Associate Prof. Div. of Surgery. Faculty of Veterinary Sciences and Animal Husbandry SKUAST Kashmir, Shuhama. ✉Corresponding author email: shbr.ahmad@gmail.com

MATERIALS AND METHODS

The study was conducted in the Division of Veterinary Anatomy and Histology, F.V.Sc and A.H, SKUAST-K, Shuhama, to investigate the prenatal development of the ovary in sheep across different gestational age groups. The study was conducted on 30 embryos/foetuses, which were grouped according to age: Group I (up to 50 days), Group II (51–100 days), and Group III (101–150 days), with 10 specimens in each group. Embryos/foetuses and ovaries were collected from local slaughterhouses in the Kashmir Valley and preserved using specific fixatives, including neutral buffered formalin**,** and Bouin's fluid**.** The approximate age of embryos/fetuses was estimated by Richardson's formula (Richardson, 1980) i.e X= 2.1(Y+17), where X denoted the developmental age in days where Y denoted Crown Rump Length (CRL) in cm. The determination of sex in Group-I was done by examining the position of genital tubercle which remains near the base of tail.

Gross observation: The ovaries in Group-II and III were dissected out from rest of the genitalia immediately after collection. Various biometrical parameters of both left and right ovaries like length (distance between two extremities), breadth (distance between two borders) and thickness (between two surfaces) was recorded with the help of Vernier Calliper, scale and non-stretchable thread. Common weighing balance was used to record weight of

Table 1. Histological and Histochemical Techniques Used for Study

S.No.	Method	Purpose	Reference
1	Haematoxylin & Eosin method (H&E)	Histomorphology	Luna,1968
2	Gomori's method	Reticular fibres	Luna,1968
3	Weigert's method	Elastic fibres	Luna,1968
4	Masson's Trichrome method	Collagen tissue fibres	Luna,1968
5	Periodic Acid Schiff reaction (PAS)	Neutral mucopolysaccharide (NMPS)	Bancroft & Stevens, 1996
6	Alcian Blue (AB) method (at pH 2.5)	Acid mucopolysaccharide (AMPS)	Bancroft & Stevens, 1996
7	Bromophenol blue	Basic proteins	Chayen *et al.*1969
8	Best Carmine	Glycogen	Bancroft & Stevens, 1996
9	Oil Red O	Lipids	Bancroft & Stevens, 1996

each ovary and data was expressed in gram (gm).

Histological and histochemical Studies: All tissues collected were processed by the routine Alcohol-Benzene schedule and paraffin blocks were made (Luna, 1968). Sections of 3-5 µm thickness were cut for histomorphological, quantitative and micrometrical studies. Sections of ovary were stained with standard procedures as indicated in Table 1.

Micrometry: The diameter of all developmental stages of follicles was recorded (at least 10 follicles of each kind from different samples) with the help of Filar and Ocular Micrometer duly calibrated with stage micrometer. For each follicle, the diameter of oocyte (ovum), diameter of nucleus, thickness of tunica albugenia, granulosa layer, theca interna, theca externa and zona pellucida were also recorded.

Statistical analysis: All the gross and micromorphometrical values recorded were averaged age and group wise. The data was then subjected to statistical analysis as per standard procedure of Snedecor and Cochran, (1994) using Analysis of Variance (ANOVA) and the differences between means were tested using the Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Histomorphology: In Group-I, the genital ridge appeared as a minor thickening on the ventromedial aspect of the mesonephros, extending from the mid-thoracic to sacral vertebral region at CRL = 2.0 cm (39.9 days) (Fig. 1). Similar gonadal ridge formation has been reported at 28, 26, and 24 days post-conception in bovine, pig, and dog embryos respectively, (Gier and Marion, 1970), and between 17–23 days in goats (Farooqui *et al.* 2012), while in African elephant embryos, it was noted at 76 days (Stansfield *et al.* 2012).

The genital ridge contained mesenchymal cells**,** primordial germ cells (PGCs)**,** immature erythrocytes**,** and spindle-shaped cells. The mesenchymal cells were irregular with small intermingling processes having granulated nuclear chromatin, while the spindle-shaped cells showed eosinophilic cytoplasm and dark nuclei, likely indicating future fibroblast cells (Fig. 2) as reported earlier in 23-day-old goat embryos (Farooqui *et al.* 2012).

The PGCs appeared larger, with acidophilic cytoplasm**,** dark nuclei**,** and concentrated chromatin. Some were undergoing mitotic division and migrating toward the germinal epithelium. Occasional binucleated PGCs suggested transitional developmental stages (Fig. 3), similar to findings in camel (Zolain and Osman, 2021) and African elephant (Stansfield *et al.* 2012). PGC proliferation, along with somatic cell activity, is known to promote gonadal ridge enlargement and differentiation into testis or ovary (Guigon and Magre, 2006). However, at this early stage, sex determination was not possible histologically, marking it as an indifferent stage**.** Later in this group (CRL = 4.4

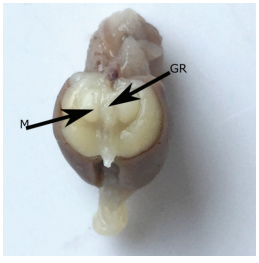

Fig. 1 2.0 cm CRL sheep embryo showing genital ridge (GR) mesonephros (M).

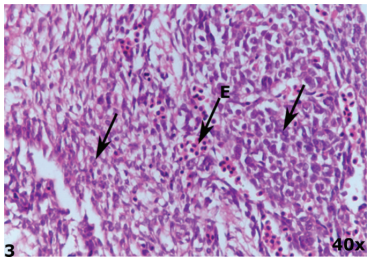

Fig 2. Photomicrograph of sheep embryo (2.0cm CRL) showing mesenchymal cells (arrow), differentiating erythrocytes (E). H &E. 400

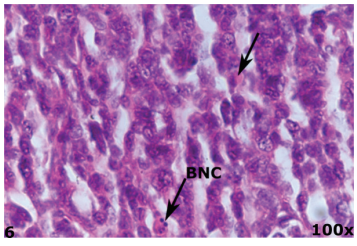

Fig. 3 Photomicrograph of sheep embryo (2.0cm CRL) showing primordial germ cells (arrow), Binucleated cells (BNC). H&E. 1000

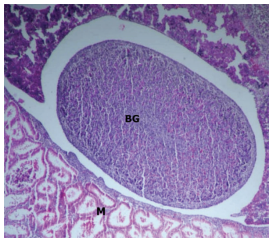

Fig. 4 Photomicrograph of 45 days old sheep embryo showing mesonephros (M) and bipotential gonad (BG). H&E. 40

Table 2. Length (mm), breadth (mm), thickness (mm) and weight (gm) of left and right ovaries of prenatal groups of sheep (Mean ± SE)

Prenatal Groups	Length			Breadth			Thickness			Weight		
	Left ovary	Right ovary	Average	Left ovary	Right ovary	Average	Left ovary	Right ovary	Average	Left ovary	Right ovary	Average
Group-II (50-100 Days)	3.88 ± 0.20[a]	4.38 ± 0.21[a]	4.13 ± 0.20[a]	2.2 ± 0.09[a]	2.5 ± 0.08[a]	2.35 ± 0.08[a]	1.65 ± 0.14[a]	1.75 ± 0.15[a]	1.7 ± 0.14[a]	0.011 ± 0.001[a]	0.014 ± 0.001[a]	0.0125 ± 0.002[a]
Group-III (100-150 days)	5.1 ± 0.14[b]	5.6 ± 0.14[b]	5.35 ± 0.02[b]	2.53 ± 0.13[a]	3.00 ± 0.13[a]	2.76 ± 0.13[a]	1.72 ± 0.09[a]	2.00 ± 0.12[a]	1.86 ± 0.02[a]	0.022 ± 0.001[a]	0.023 ± 0.001[a]	0.0225± 0.001[a]

Mean value with same superscript, within column, do not differ significantly (P > 0.05)

cm , 45 days), the gonadal ridge developed into bipotential gonads, protruding into the coelomic cavity as paired, globular structures on either side of the dorsal mesentery**,** medial to the mesonephros (Fig. 4). These were attached by a thin stalk, likely a precursor of the mesovarium, as also noted in elephant embryos (Stansfield *et al.* 2012). While the bipotential gonads and metanephros increased in size, the mesonephros showed signs of degeneration**,** similar to findings in buffalo foetuses (Kaur *et al.* 2010). Histologically, the bipotential gonads were lined by a simple cuboidal germinal epithelium with interspersed PGCs. The underlying tissue consisted of undifferentiated sex cords**,** mesenchymal cells**,** and germ cells.

In Group-II, both the ovaries were situated on the ventro-medial aspect of the mesonephric remnant at the cranial pole. The biometrical parameters of the right and left ovary are summarized in Table 2. Their shape varied from elongated to oval. The left ovary was located just caudal to the left kidney and related ventro-medially to the coils of intestine while the right one was related to the dorsal part of the liver (Fig. 5). The right ovary was found slightly away from the right kidney in the later stage (80-100 days). The morphological features and location of ovaries were in close correlation with Harshan and Singh (1992) in prenatal goats and they had also reported that the ovaries of goat foetuses at 75-90 days of age moved a little away from the caudal extremity of ipsilareral kidney. According to Harshan and Singh (1992), the goat ovaries were bean shaped and closely related to caudal extremity of respective metanephros at 55-70 days.

The germinal epithelium was a single layer of low cuboidal cells, except at the mesovarium**,** where it appeared simple squamous. Similar observations were made in sheep foetuses by Sawyer *et al.* (2002)**.** In early stages (50–60 days), a few large primordial germ cells with vesicular nuclei were present, which disappeared in later stages (80–100 days). Reticular fibres began developing early in this group. The ovarian parenchyma mainly consisted of ovigerous cords, invaginations from the germinal epithelium extending centrally. These cords contained primordial germ cells and mesenchymal cells, with spindle-shaped fibroblasts between them. The cords formed cell clusters surrounded by fibroblasts, indicating the onset of primordial follicle formation, which was consistent with findings by Stansfield *et al*. (2012) and Guraya (1985). Primordial and primary follicles were composed of an oogonium surrounded by flattened to low cuboidal cells, as reported by Hasanzadeh and Asghari (2005) in Makuii sheep at 71 to 100 days. The cytoplasm of primordial germ cells was more eosinophilic, and their nuclei stained faintly with haematoxylin (Fig. 6). Similar cords and germ cells were seen in the medullary region, with a higher density of reticular fibres, though they were scarce in the basement membrane.

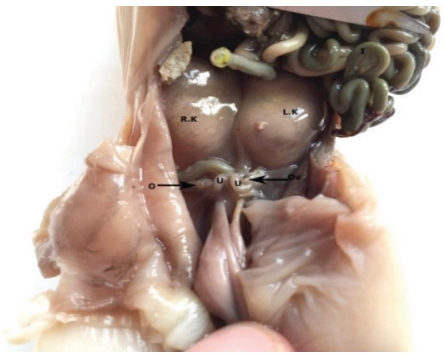

Fig. 5 Photograph of 70 days old sheep embryo showing ovaries (Ov), kidney (K), Oviduct (O)uterus (U) , Intestines (I).

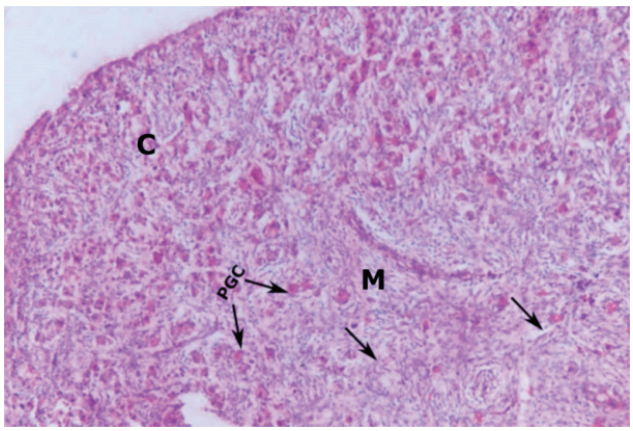

Fig. 6 Photomicrograph of 80 days old ovary of sheep foetus showing medullary cord (arrow) with primordial germ cells (PGC), in medulla(M) inside cortex (C) H&E.100

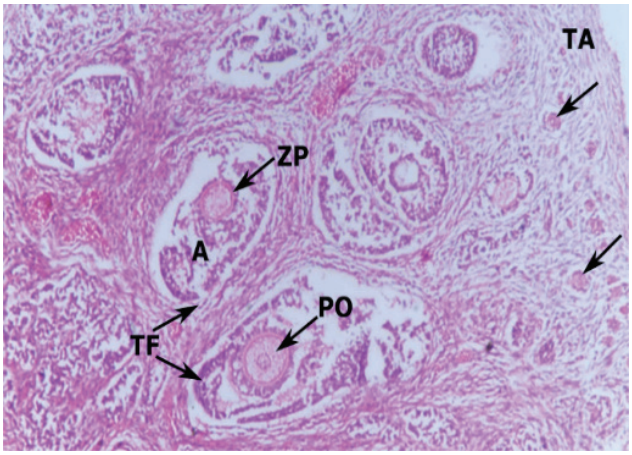

Fig. 7 Photomicrogrph of 140 days old ovary of sheep foetus showing tunica albuginea (TA), primordial & primary follicle (arrow), primary oocyte (PO), tertiary follicle (TF) with Antrum(A), zona pellucida(ZP) . H&E.100

In Group-III, the ovaries were almond shaped and situated in the pelvic cavity away from the respective kidneys. At 95-120 days there was a gradual caudal shift and both ovaries reached their adult position as reported by Harshan and Singh (1992) in goats. The length of ovary increased significantly ($p < 0.05$) from Group-II to III while breadth, thickness and weight increased non-significantly ($p > 0.05$). The various dimensions of the right ovary were slightly higher than the left one (Table 2).

The average thickness of the germinal epithelium was 7.12 ± 0.636 µm, slightly lower than in Group II. Just beneath it, the tunica albuginea appeared (Fig. 7), composed of dense fibrous connective tissue, reticular fibres and spindle-shaped fibroblasts. Its average thickness was 23.52 ± 2.265 µm, in agreement with Bhardwaj (1996) and El-Ghannam & El-Naggar (1974) in prenatal buffalo ovaries.

The ovarian parenchyma showed numerous primordial and primary follicles, predominantly in the outer cortex, along with connective tissue and blood vessels. Primary follicles consisted of an oogonium surrounded by squamous or cuboidal cells, while secondary follicles in the medulla comprised a primary oocyte encircled by multiple cuboidal layers and a basement membrane, consistent with Bhardwaj and Roy (2000). According to Zolain and Osman (2021), antral follicles appeared only during the third trimester in foetal camel ovaries. In the present study, at 145 days, a few tertiary follicles were observed in the inner cortex near the medulla, showing eccentric oocytes, multiple granulosa cell layers, a distinct antrum, vascularized theca interna, and reticular-rich theca externa. The medulla also exhibited mesenchymal cells, fibroblasts and blood vessels. Reticular fibres were prominent in the medulla, tunica albuginea, basement membrane of the germinal epithelium, and theca externa, while elastic fibres were noted in vessel walls.

Contrastingly, Hasanzadeh and Asghari Azar (2005) did not report any tertiary follicles in Makuii sheep foetuses. However, Aruna *et al.* (2017) observed primordial, preantral, and antral follicles in goat foetal ovaries at gestation day (GD) 121–145, with antral follicle size of 346.33 ± 8.8 µm and oocyte size of 87.33 ± 2.2 µm. Baishya (1991) documented collagen fibres at 62 days and elastic

Table 3. Micrometrical data (Mean ± SE in µm) of primary, secondary and tertiary follicles in various stages of ovarian development.

	Parameters	Group - II	Group – III
	Germinal epithelium	8.53 ± 0.26	7.12 ± 0.64
	Tunica albuginea	-	23.52 ± 2.27
Primary follicle	Diameter of follicle	23.67 ± 1.43	29.05±1.80
	Diameter of oogonium	17.35 ± 1.17	20.55 ±1.75
	Diameter of nucleus	4.67 ± 0.70	4.92 ± 1.01
Secondary follicle	Diameter of follicle	-	75.99 ± 5.17
	Diameter of oocyte	-	38.69±2.36
	Diameter of nucleus	-	13.53 ± 0.77
	Thickness of zona pellucida	-	1.90 ± 0.21
	Membrana granulosa	-	21.19±2.48
Tertiary follicle	Diameter of follicle	-	238.66±13.22
	Diameter of oocyte	-	53.49 ± 2.24
	Diameter of nucleus	-	16.18 ± 1.90
	Thickness of zona pellucida	-	2.38 ± 0.27
	Membrana granulosa	-	19.01 ± 1.76
	Thickness of theca interna	-	21.20 ± 3.25
	Thickness of theca externa	-	15.23 ± 1.66
	Thickness of antrum	-	89.42 ± 9.86

Table 4. Histochemical observations on ovaries of sheep foetus (Group II and III)

Histochemical moieties	Surface epithelium	Tunica albuginea	Ovigerous/Sex cords	Ovarian follicles	Medulla
Neutral mucopolysacharides (NMPS)	±/+	±	±/++	±/++	+
Acid mucopolysacharides AMPS	±/+	+	++	+/++	±/+
Glycogen	±/+	±	+/++	±/++	+/++
Lipids (Oil Red O)	±/++	±	±/++	±/++	±
Basic Proteins	++/+++	±/++	++/+++	±/++	±/++

- = Negative, ± = In traces, + = Mild Reaction, ++=Moderate reaction, +++= Intense reaction

fibres in the blood vessels at 191 days in prenatal buffalo ovaries. Oyelowo *et al.* (2021) reported indistinct cortical and medullary zones in the first trimester camel foetus, which became progressively clearer in later trimesters. The rete ovarii was also found in the medulla**,** consistent with Singh *et al.* (2019). Micrometrical data of different follicular stages are provided in Table 3.

Histochemistry: The surface epithelium exhibited weak to mild activity for neutral (NMPS) and acid mucopolysaccharides (AMPS), while the underlying basement membrane showed a mild PAS/AB reaction (Table 4). The tunica albuginea demonstrated a weak reaction, which could be attributed to early connective tissue differentiation. The ovarian cortex showed weak to moderate PAS positivity along with weak AMPS reaction. Both ovigerous cords and primordial follicles showed weak to moderate reactivity for NMPS and AMPS, with the basement membrane of primordial follicles exhibiting weak to moderate PAS positivity (Fig. 8). The cytoplasm of primordial germ cells reacted intensely for both NMPS and AMPS, while mesenchymal cells showed moderate PAS activity. The cytoplasm of the oogonium showed stronger reactivity for AMPS and PAS than that of the mesenchymal cells. The zona pellucida of secondary and tertiary follicles was strongly AB positive (Fig. 9). The cytoplasm of fibroblasts, mesenchymal cells**,** and theca interna cells showed moderate AMPS reaction**,** whereas follicular cells showed a relatively more intense AMPS reaction. Reticular fibres were moderately positive for AMPS (Fig. 10). Similar histochemical patterns have earlier been reported in bovines and buffaloes by Talukdar *et al.* (1991)**,** Bhardwaj (1996**)** and Singh *et al*. (2016)**.**

Lipid droplets of weak to moderate intensity were observed in the surface epithelium**,** ovigerous cords, and ovarian follicles, while tunica albuginea and medulla showed weak lipid activity. Fine to medium lipid droplets were present in the basement membrane and oocyte of primordial follicles. Guraya and Uppal (1978) described ovarian lipids in field rats as including triglyceride**s,** phospholipids**,** cholesterol, and esters**,** while Patel *et al*. (1991) emphasized the role of phospholipids in membrane formation. Sawyer *et al*. (2002) observed lipid droplets and smooth endoplasmic reticulum in oogonia and pregranulosa cells. A moderate to strong reaction for basic proteins was seen in the surface epithelium and ovigerous cords, with

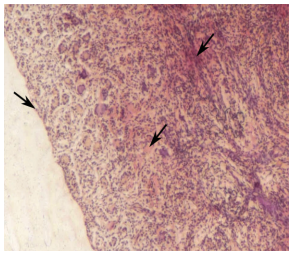

Fig. 8 Photomicrograph of 110 days old ovary of sheep foetus showing PAS reaction in surface epithelium (arrow), tunica albuginea (TA), follicles (F), and different reaction in cortex (C). PAS stain.2 00

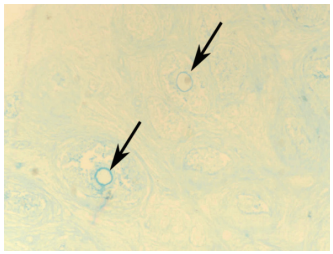

Fig. 9 Photomicrograph of 140 days old ovary of sheep foetus showing strong AB reaction in zona pellucida (arrow). Alcian blue stain. 200

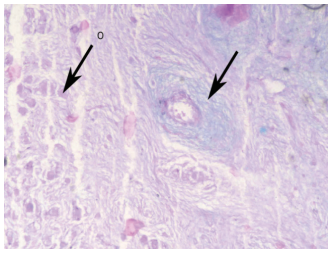

Fig. 10 Photomicrograph of 60 days old ovary of sheep foetus showing PAS-AB reaction in fibres (arrow), strong reaction in oogonium (O). PAS-AB stain. 200

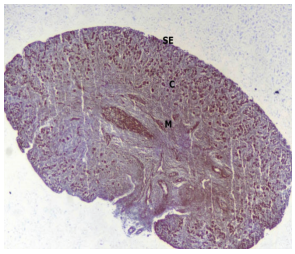

Fig. 11 Photomicrograph of 75 days old ovary of sheep foetus showing bromophenol reation in surface epithelium (SE), Cortex(C) and Medulla (M)). Bromophenol Blue stain. 40

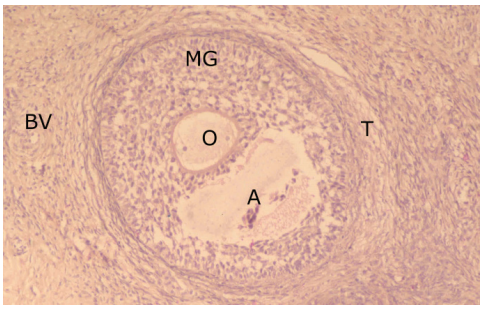

Fig. 12 Photomicrograph of 140 days ovary showing ovum (O), antrum (A), membrana granulosa (MG), Blood vessel (BV) and mild content of glycogen in theca follicluli (T) . Best carmine. 200

weak to moderate activity in the tunica albuginea**,** follicles, and medulla (Fig. 11), consistent with findings by Singh *et al*. (2016). Mild to moderate amount of glycogen was found in ovarian cortex especially PGCs, ovigerous cords, granulosa cells (Fig. 12).

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