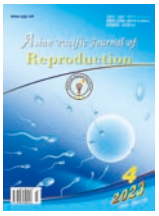

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305-0500.380986

Determination of the ovine ovarian reserve during the prenatal and neonatal periods

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ABSTRACT

Objective: To determine the ovine ovarian histomorphology and follicular staging at various age periods in Awassi breed.

Methods: Ovaries were collected from prenatal fetuses [gestational age (95±5) days], neonatal (day 0), and prepubertal ewe lambs (two and four months of age); each age group included six animals. Ovaries (n=12, each group) were dissected and processed for hematoxylin and eosin staining. Stained sections (n=24, each group) were imaged and utilized for histomorphology assessment, follicle measurement, and classification.

Results: Prenatal ovaries were mainly enriched with primordial follicles accompanied by a lower proportion of primary follicles. In addition to primordial and primary follicles, neonatal ovaries demonstrated a proportion of centrally located multilayered and antral follicles. In comparison with neonatal ovaries, the proportion of multilayered and antral follicles was significantly higher in the ovaries of two-month-old lambs; conversely, the proportion of peripherally situated primordial follicles dramatically declined compared to that of earlier age of lamb. Although there was no statistical variation in the sizes of primordial follicles across groups, the mean diameter of the primary follicle in the prenatal ovaries was substantially smaller than in postnatal ovaries. Compared to the neonatal ovaries, the size of the multilayered and antral follicles in the prepubertal ovaries was substantially larger.

Conclusions: The earliest follicular developmental stages were established prenatally whereas the advanced growth stages started in the neonatal period and greatly increased in the prepubertal period.

KEYWORDS: Prenatal ovine ovary; Prepubertal ovine ovary; Ovarian histomorphology; Immature ewe lamb ovary; Ovine ovarian reserve; Follicle staging; Ovarian follicular growth

1. Introduction

The ovarian reserve represents the entire accessible population of the non-growing primordial follicles that sustain fertility throughout the female reproductive lifespan[1,2]. Primordial follicles are quiescent, but periodically a limited number is activated and triggered to initiate the process of folliculogenesis. The activated follicles express both morphological and physiological events toward ovulation; however, the majority of these follicles endure atresia[3,4]. In all mammals, the ovarian reserve is established from the ovarian germ cells, although the folliculogenesis timepoint varies by species[1]. In ewes, folliculogenesis occurs prenatally; therefore, the construction of the ovarian reserve is directly affected either by maternal or external conditions, where both might interfere with the hypothalamic-pituitary-ovarian axis[5]. However, in rodents, the process is driving shortly after birth[6]. In ewe's fetuses, a massive germ cell (75%) is degenerated by apoptosis during a limited timeframe (days 75–90) of pregnancy[7]. Nevertheless, approximately after 75 days of pregnancy, the fetal lamb ovary contains around 805 000 germ cells[8]; though, between the days 90-100 of pregnancy, the population of the formed primordial follicles declines into 100 000 follicles[9]. In the ovary, there are two groups of primordial follicles with discrete physiological significance; the primordial follicles which originate at the earliest period of ovarian development are present within the medulla and undergo activation

Significance

The timeline of ovine ovarian follicle activation and development is poorly understood. This study revealed that the prenatal ovary is enriched with inactive primordial follicles and numerous primary follicles, and the neonatal ovaries have a proportion of secondary and antral follicles, providing new insights in terms of assisted reproductive technology.

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How to cite this article: Sharum IB. Determination of the ovine ovarian reserve during the prenatal and neonatal periods. *Asian Pac J Reprod* 2023; 12(4): 194-200.

Article history: Received: 29 June 2022; Revision: 21 September 2022; Accepted: 22 February 2023; Available online: 18 July 2023

earlier[10]. Again, until the postnatal day 60, more than 90% of these follicles are degenerate or principally function on the development of both the genital and endocrine systems. The second group develops in the ovarian cortical region, which determines the reproductive lifespan[11]. The earliest stages of follicle activation and development are controlled by complex intraovarian growth factors where the pituitary hormones are not required, gonadotropin-independent[12,13]. As the activated follicle advances into the multilayered preantral stage, although it is not required, such follicles can respond to gonadotropin hormones[14]. However, in addition to the local cell's interaction, the small and mature antral follicles are gonadotropin-dependent[15–17]. Interestingly, previous investigations indicated that the hypothalamic-pituitary-ovarian axis is established and transiently activated in postnatal and prepubertal ewe lambs[16–18].

The present study hypothesized that the postnatal activated reproductive axis might reflect an implication on ovarian development. The current study aimed to determine the ovine ovarian histoarchitecture and to characterize patterns of follicle growth during the prenatal, neonatal, and prepubertal periods in Awassi ewe fetuses/lambs.

2. Materials and methods

2.1. Animals and tissue collection

Ovaries from prenatal ovine fetuses, neonatal, and prepubertal ewe lambs were utilized in this work. Prenatal fetuses (n=6) at the gestational age of (95±5) days were collected from slaughtered pregnant ewes. The gestational age, in days, was estimated using the crown-rump length formula X=2.1(Y+17), where X represents the fetal age in days; Y is the fetal length from the top of the head to the tail base in centimeters[19]. Ovaries from full-term neonatal ewe lambs (n=6, day 0) were collected from newborn lambs delivered either by cesarean section or obstetrical maneuvers at the teaching hospital. The utilized neonatal lambs died shortly after operations due to exhausted dystocia. Ovaries from prepubertal ewe lambs aged almost two and four months (n=6 for each age group) were collected directly after being slaughtered at a local abattoir. All of the utilized fetuses, mothers, and ewe lambs were diagnosed free from any pathological disease.

2.2. Ovary collection and processing

Ovaries (n=12, each age group) were dissected free from the adjustment tissues and were immediately translocated in neutral buffered formalin 10% (v/v) for 72 h and then relocated into 70% ethanol (v/v) until being embedded in paraffin blocks. Ovarian blocks were sectioned at 5 μm using a manual microtome (Reichert-Jung). Ovary sections were stained with Gills hematoxylin Ⅱ stain and 1% aqueous eosin. Briefly, slides were double dewaxed in xylene (2×5 minutes each; AL Hanoof fact. Med. Lab Supp. Jordan, AF). For rehydration, slides were immersed in a series of decreasing ethanol changes (99%, 95%, and 70% for 5 min each). After washing under tap water, slides were submerged in Gills hematoxylin Ⅱ stain (AL Hanoof fact. Med. Lab Supp., AF1283) approximately for 120 s, followed by 3 min of washing with tap water. Sections were immersed in 1% aqueous eosin (Diagnostics) for 5 min followed by 30 s of rinsing in tap water. Slides were dehydrated in a series of ethanol (70% and 95% for 10 s each, and terminated by two steps in 99% ethanol for 30 s each). Ovary sections were cleared with a double run in xylene (3 min each). As a final step, sections were mounted with DPX (AL Hanoof fact. Med. Lab Supp. Jordan; c2201) and coverslips were fixed[20].

2.3. Ovarian histomorphology, follicle categorizing, and counting

Stained ovary sections (n=24, each group; two inconsecutive sections/ovary) were utilized to evaluate the ovarian histomorphology, estimation of follicle diameters, and population. Sections were imaged under low and high power with a digital camera (OMAX, A35180U3, China) fitted to an optical microscope (Kruuse, Primo phot 290205, Denmark). Only follicles with a clear architecture were included. Follicle diameters were estimated using ImageJ software (Fiji 1.46, 2012) and the final follicle size was calculated by averaging the obtained vertical and horizontal measurements. Follicles were measured from the follicular basement membrane, where the layer of thecal cells was excluded. Follicles were categorized according to their morphology and relative diameter. Follicles that comprised of small oocytes enclosed by several flattened pregranulosa cells were termed primordial. Oocytes surrounded by a layer of cuboidal granulosa cells were considered primary. Secondary follicles were recognized when the oocyte was enclosed with more than one layer of granulosa cells. Follicles acquired antrum were classified as antral[3,21]. The four months old prepubertal ewe lambs were not included because of the difficulty in distinguishing earlier stages of follicle development and incorporating a hemisphere of the large antral follicles. Atretic follicles were not included in the assessment and were recognized by the fragmented basement membrane and distorted oocytes.

2.4. Statistical analysis

Follicle diameter (μm) was assessed using ImageJ software (Fiji 1.46, 2012). One-way analysis of variance multiple comparison procedures (Duncan's method) was used to determine the statistical differences in the mean follicle diameter between the age groups. *Chi*–square test was used to compare the proportion of categorized follicles between groups. Data are expressed as mean±standard deviation (mean±SD) or percentage (%). Evaluations were achieved using Sigma Plot 12.5 software where differences at P<0.05 were considered significant.

2.5. Ethics statement

This study was approved by the Institutional Animal Care and Use Committee (approval number: UM.VET.2019.01) and by the Department's Scientific Committee. Tissues were processed following ethical standards, and ovaries (in all age groups) were obtained from dead animals.

3. Results

3.1. Ovarian histomorphology

Stained ovary sections were used to determine the ovarian morphology and the related follicular growth stages. Ovaries from the prenatal fetuses [gestational age (95±5) days] demonstrated the presence of both the primordial and primary follicles in the entire ovarian cortex. The majority of the primordial follicles were recognized in the peripheral region, just beneath the ovarian surface. Interestingly, most of the activated follicles (primary) were situated in the central region of the cortex. However, follicles with more than one layer of cuboidal granulosa cells were not exhibited in prenatal ovaries. High-power imaging revealed the presence of atretic/degenerated follicles. Ovary sections demonstrated the presence of only numerous medullary-situated small blood vessels (Figure 1).

Unexpectedly, in addition to the primary and secondary follicles, ovary sections from neonatal ewe lambs (day 0) revealed the presence of small to medium-sized antral follicles. Interestingly, the primordial follicles were exclusively situated in the marginal zone of the cortex. Unlike prenatal ovaries, the central region of the neonatal ovaries was recognized as a depleted area from follicles. Additionally, the neonatal ovaries demonstrated an extended stroma and the medulla contains larger and more blood vessels than the prenatal ovaries (Figure 2).

Interestingly, the ovaries obtained from the two months of postnatal ewe lambs revealed an increased proportion (12.3%) of varied-sized antral follicles, which mainly presented in the central region of the ovary section. However, the majority of these follicles demonstrated degenerative changes. Similar to the neonatal ovaries, the primordial follicles were determined in the peripheral ovarian zone. The majority of the growing follicles, primary and secondary, were situated beneath the primordial follicle zone (Figure 3).

To expand our knowledge about the ovarian histomorphological changes in older animals, ovaries were collected from prepubertal ewe lambs aged four months old and processed for assessment. Stained sections revealed the presence of large antral follicles (2 mm), but with a lower population than the two months old. In addition, the majority of the antral follicles were located near the ovarian surface. In comparison with previous ages, the population of the non-growing and growing follicles was largely reduced. The stroma is more extended, condensed with stromal cells, and with the presence of multiple enlarged blood vessels relative to other age groups. Similar to other age groups, no corpus luteum was presented (Figure 4).

3.2. Follicle proportion, sizes, and stage

To determine the follicle quantity, size, and growth stage, follicles were classified according to their morphological changes and diameters. The proportion of primordial follicles was significantly

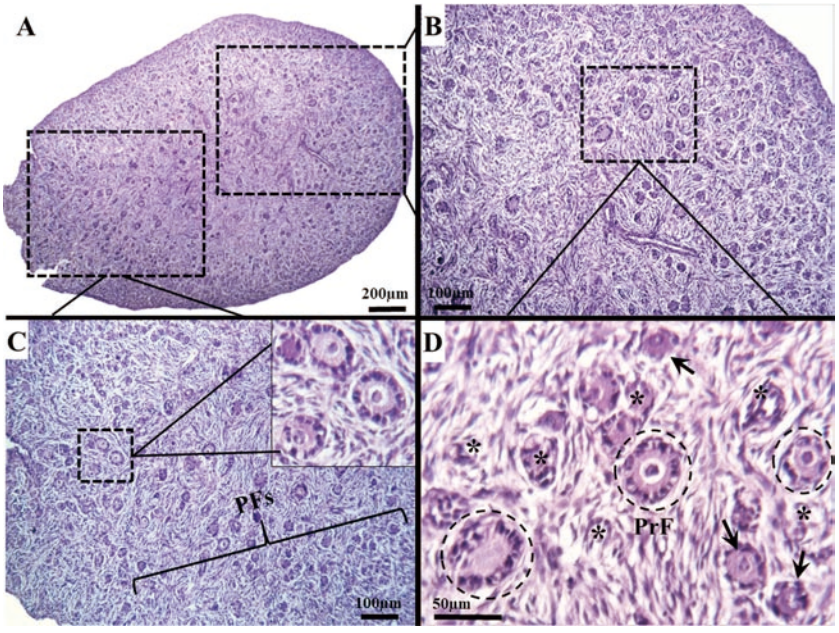

Figure 1. Ovarian histological section from the prenatal female fetus (gestational age: day 90). The hematoxylin and eosin stain reveals that the entire ovarian cortex is enriched with non-growing primordial follicles (PFs), mostly located beneath the surface epithelium (A, 4×). The activated primary follicles (PrF) are mainly presented centrally (B-C, 10×). The primordial follicles, small oocytes surrounded by 3-6 flattened pregranulosa cells, are further distinguished using a high-power image (D, arrows; 40×). Primary follicles, a larger oocyte enclosed with a single layer of cuboidal granulosa cells, are also presented (D; circled). The same section reveals the presence of numerous small atretic follicles (starred).

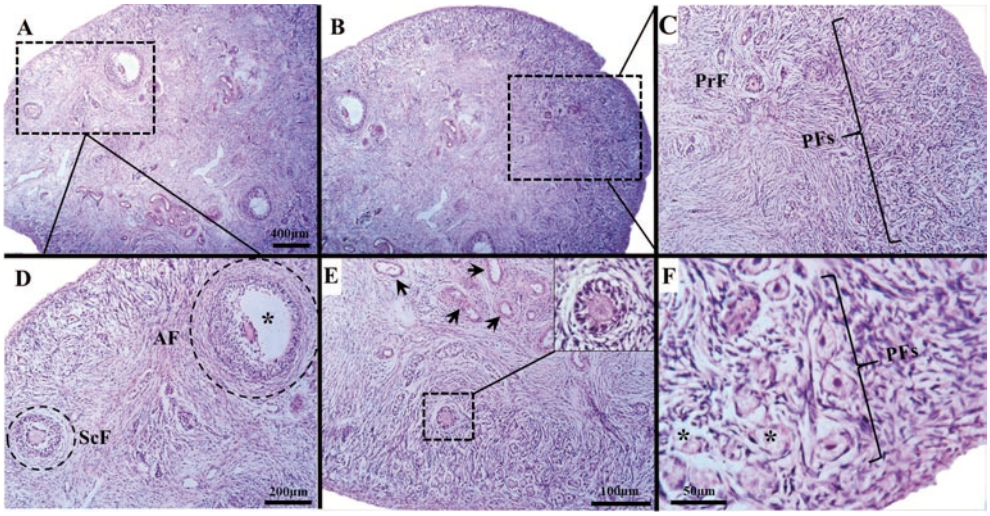

Figure 2. Ovarian histological section from a neonatal ewe lamb (day 0). Sections are stained with hematoxylin and eosin. The ovarian cortex is enriched with a zone of non-growing primordial follicles (PFs), (A-B, 4×; C-E, 10×). Next to this zone, the growing follicles [primary (PrF) and secondary follicles (ScF)] are located, where the ScF is surrounded by numerous layers of granulosa cells. Similarly, the antral follicle (AF) is located beneath the primordial follicle zone. Small theca cells enclosed the ScF, while the AF is surrounded by layers of developed theca cells (D). The blood vessels are presented in the stroma (E, arrows). High power image (F, 40×) shows primordial follicles just beneath the surface epithelium and several atretic follicles (starred).

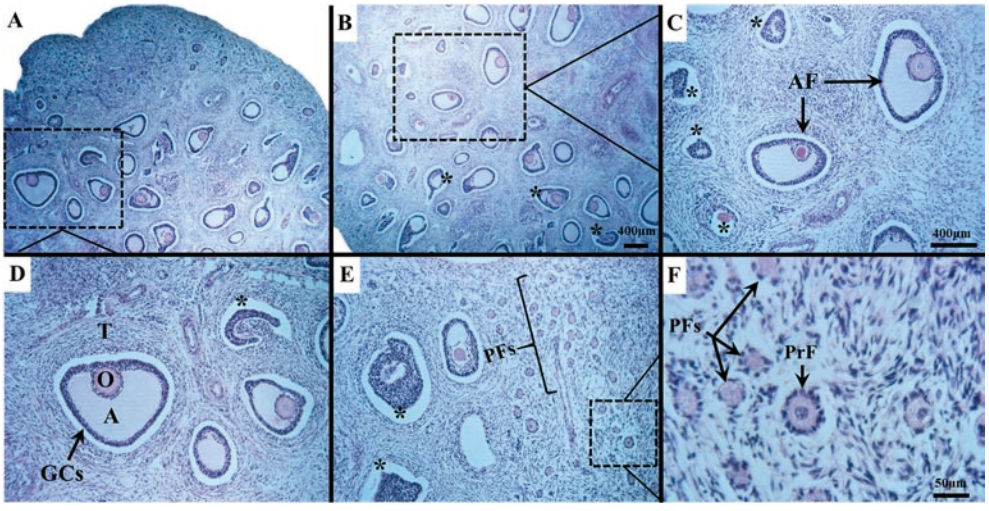

Figure 3. Ovarian histological section of postnatal ewe lamb (two months old). Sections are stained with hematoxylin and eosin. The ovarian cortex is enriched with a zone of non-growing primordial follicles (PFs). Underneath this zone, the growing follicles are located. The majority of the antral follicles (AF) are situated in the central area of the ovary section (A-B; 4×). The zoomed squares (C-D; 10×) reveal that antral follicles are enclosed with multiple layers of both the theca cells (T) and granulosa cells (GCs); oocytes (O) are surrounded by the cumulus oophorus, and a developed antrum (A). The blood vessels are presented in the stroma between follicles (C). Atretic follicles are labeled with stars (B-E). High power image (F, 40×) shows primordial follicles (PFs) adjacent to a primary follicle (Prf) just beneath the surface epithelium.

higher in the prenatal and neonatal ovaries compared to the two-month-old ovaries (P<0.001). In comparison with prenatal and the two months postnatal ovaries, neonatal ovaries exhibited a significantly lowered proportion of primary follicles (P<0.001). The proportions of both the secondary and antral follicles were expressively higher in the postnatal ovaries compared to the neonatal ovaries (P<0.001). However, neither the secondary nor antral follicles were recognized in the prenatal ovaries. There was no significant variation in the mean of the primordial follicle diameter between groups. However, the size of the primary follicles in prenatal ovaries was significantly smaller than in other age groups (P<0.05). The growth rate of both the secondary and antral follicles in the two months postnatal ovaries was detected as statistically higher than that in neonatal ovaries (P<0.05) (Table 1).

4. Discussion

The majority of ovarian researches have been predominantly conducted on rodents[11,20,22]; however, only a few pieces of literature are available on ewe fetuses and prepubertal ewe lamb's ovaries[16,23], particularly in the Awassi breed. Ovine is an

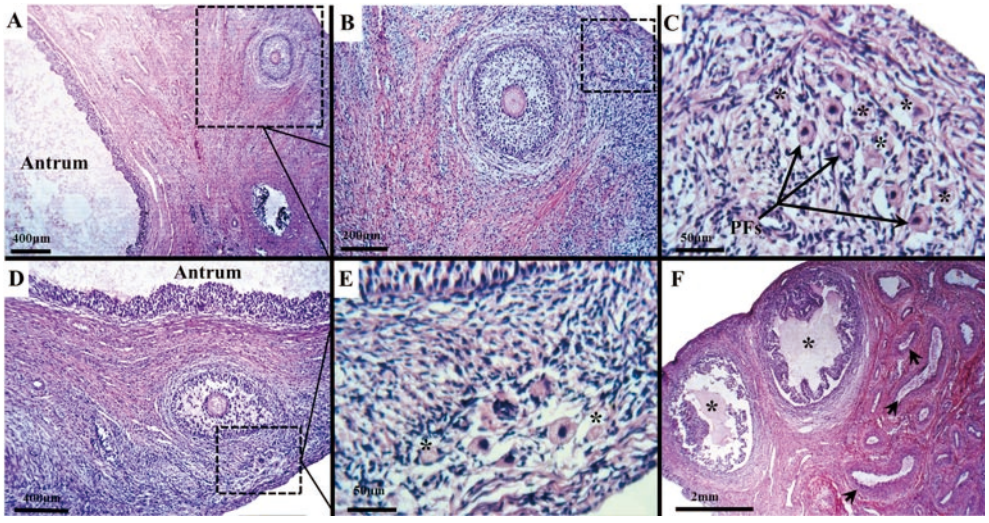

Figure 4. Ovarian histological section of prepubertal ewe lambs (four months old). The hematoxylin and eosin stain demonstrates marginally situated large antral follicle (A, 4×). The zoomed area (B, 10×) reveals a multi-layered secondary follicle, while high-power imaging (C, 40×) exposes a few peripherally located primordial (PFs) and atretic follicles (starred). Another ovary section (D-E) reveals a similar observation. The ovarian cortex (F, 4×) demonstrates a reduced number of primordial and growing follicles, with the presence of two atretic preantral follicles. The stroma is largely extended, and blood vessels are well-developed (arrows).

Table 1. The proportion and diameter of follicles in different growing stages.

Parameters	Primordial	Primary	Secondary	Antral
Proportion, *n*(%)				
Prenatal, (95±5) days (*n*=1 897)	1 697(89.5)[a]	200(10.5)[a]	-	-
Neonatal, day 0 (*n*=1306)	1 155(88.4)[a]	80(6.1)[b]	45(3.4)[a]	26(2.1)[a]
Postnatal, two months old (*n*=1 201)	795(66.2)[b]	140(11.7)[a]	118(9.8)[b]	148(12.3)[b]
Follicle diameter, μm (mean±SD)				
Prenatal, (95±5) days	24.38±2.75[a]	43.33±4.71[a]	-	-
Neonatal, day 0	24.39±2.27[a]	45.31±5.61[b]	80.14±18.84[a]	321.95±62.30[a]
Postnatal, two months old	24.37±2.60[a]	44.98±5.34[b]	101.02±17.53[b]	395.76±38.82[b]

Variation in follicle proportions among groups is assessed with *Chi*-square test. One-way analysis of variance multiple comparison procedures (Duncan's method) is used to detect the significant variation in follicle diameter between age groups. Different superscript letters (a, b) in the same column are significantly varied at $P<0.001$ for proportions, and $P<0.05$ for sizes.

appropriate animal model for studying ovarian follicle development since they are inexpensive, have a shorter gestational length, and reach puberty earlier than larger animals. Notably, in comparison with the standard rodent model, the ovine resembles humans in terms of antenatal ovarian programming and developmental conditions[18]. It has been suggested that dual periods of hypothalamic-pituitary-gonadal axis activation precede the onset of puberty; initially during the prenatal period and secondly throughout several postnatal months, which is termed mini puberty[24]. Thus, based on these statements, the present study hypothesized that the activated and growing follicles might be presented in the prenatal, neonatal, and prepubertal ovaries.

The outcome of the current work indicated a wide range of differences in ovarian histomorphology between the considered ages. The ovarian cortex of prenatal fetuses [(95±5) days gestational age] was particularly enriched with primordial follicles (89.5%) with the presence of several centrally-situated primary follicles (10.5%). However, no later follicular growth stages were recognized. The presence of the primary follicles indicates that primordial follicles are generated even earlier than day 90 of fetal age. These observations are consistent with a previous investigation on ovine fetal ovaries extended from the days 38-100 of gestational age[10]. Moreover, it has been reported that the construction of the first primordial follicle in ovine fetuses occurs approximately at day 75 of gestational age, while activation and progression into the primary follicle were postponed until the day 100 of pregnancy, reviewed by Monniaux[25]. It has been specified that the follicle growth at this developmental stage is entirely controlled by intraovarian secreted factors, gonadotrophin-independent[12,13]. Compatible with a previous investigation[11], prenatal ovarian sections revealed the presence of oocytes representing degenerative changes. It has been documented that during the process of primordial follicle formation approximately 75% of the oocytes are eliminated, either by apoptosis or programmed cell death[6]. At this stage of fetal age, the degeneration of oocytes is a crucial mechanism where the survived oocytes attain extra immature granulosa cells before the primordial follicle formation[10]. Interestingly, in addition to the multilayer follicles, the neonatal ovary sections presented a proportion (2.1%) of small-sized antral follicles. It is a fact that the advanced stages of follicle growth require pituitary gonadotropins to support their growth. In ewes, the receptor of the follicular stimulating hormone (FSH) was detectable in granulosa cells of follicles that developed the second layer of granulosa cells. However, to undergo mature, the reactivity to luteinizing hormone (LH) is postponed until the follicle

develops multilayers of granulosa cells[13,26]. It has been proposed that the fetal reproductive axis is mostly developed in the prenatal period; therefore, the uterine environment is potentially associated with ovarian follicle development[27]. Postnatal ovary sections (2 months old) demonstrated exciting ovarian morphological changes including: Firstly, the localization of primordial follicles in a zone-like area in the peripheral ovarian region. In comparison with the prenatal ovaries, although statistics did not detect differences in the mean primordial follicle diameter among groups, the proportion of primordial follicles was significantly declined in both the neonatal and postnatal ovaries. This reduction might be attributed to the continuous processes of follicle activation, programmed cell death, and atresia[3,4]. Secondly, in comparison with neonatal ovaries, there was a significant increase in both the proportion and sizes of growing and antral follicles, which were mainly located in the central region. These findings corroborated earlier research stating that ewe lamb ovaries demonstrated lower antral follicle counts and sizes in the first postnatal month, and these variables increased at the early prepubertal gonadotropin peak[23]. Mechanisms implicating the transient postnatal activation of the fetal pituitary hormones remain undefined. Even though mini puberty is common in ruminants[28], it has been proposed that the process is triggered immediately after birth by the sudden decreases of maternal placental estrogens, which in turn block the reproductive axis[18,24]. In other words, levels of FSH and LH in plasma are thought to be considerably raised in newborn animals until they reach the age of ten weeks. Later, the levels of these pituitary hormones are downregulated to a level that is greater than at birth but is equivalent to those of mature ewes[16]. Thus, at this postnatal age, the increased proportion and sizes of the antral follicles might be accountable for the elevated levels of gonadotropins. In contrast, a previous investigation indicated that ewe lambs with a high proportion of antral follicles demonstrated a decreased level of FSH, suggesting that these follicles produce a sufficient amount of estrogen and inhibin that in turn produces a negative feedback action on FSH secretion[29]. Similarly, another study conducted on ewe lambs (50 days old) declared that the proportion of antral follicles was significantly declined in ewe lambs that expressed intensely high levels of blood FSH, while ewe lambs with high levels of anti-Müllerian hormone (AMH) demonstrated a statistically higher antral follicle count and *vice versa*[18]. Consistently with the present finding, Graafian follicles were undetectable in postnatal goats' ovaries where the population of the medium-sized antral follicles was elevated at the age of two months and decreased at the age of 5 months[30]. In older prepubertal ovaries (4 months old), ovary sections exposed larger antral follicles (2 mm), but no ovulated follicles or corpora lutea were distinguished. This observation might explain that, postnatally, the increased FSH level enhances follicular growth to reach the stage of small/medium-sized antral follicles; however, the secreted FSH is inadequate to produce larger follicles[31]. Generally, ewe lambs express the first signs of estrus, mature Graafian follicle, ovulation, and development of the corpus luteum at the age of six months, accounting for the elevated levels of both FSH and LH[17]. Collectively, ovine reproduction is challenged by many issues, particularly the inadequate application of assisted reproductive biotechnology, where the availability of oocytes is one of the most pressing issues. Ovine ovaries collected from fetuses or slaughtered prepubertal ewe lambs can be exploited as a reliable resource for research in the context of either the initial follicle activation or the later growth stages, respectively.

The limitations of this study were taken into account because it was intended to be a preliminary investigative study, and they will be addressed in subsequent research. For instance, further investigation at various prenatal/postnatal ages is required to determine the expression of follicle growth-associated biomarkers (*e.g.* AMH) accompanied by estimation levels of numerous reproductive hormones (*e.g.* FSH, LH, and estrogen)[32]. Moreover, in the ovarian sections, it is essential to express factors that associate with vascular endothelial cells (CD31), cellular proliferation (Ki67), and/or programmed cell death (Caspase 3)[33]. Subsequently, the precise time points of folliculogenesis, initial follicular activation, and development/ atresia would be largely uncovered.

In conclusion, the current study investigates the ovine ovarian histomorphological changes and follicle staging in both the prenatal and postnatal periods. The earlier stages of follicle development are established during the fetal life, whereas the more advanced growth stages are detectable in neonatal and postnatal ewe lambs where their quantity and sizes are significantly increased in prepubertal ovaries.

Conflict of interest statement

The authors declare no conflicts of interest.

Funding

This study received no extramural funding.

Author's contributions

The author Isam B. Sharum confirms sole accountability for conducting all parts of this work including concept, design, literature, samples/data collection, lab work, statistical analysis, results interpretation, manuscript preparation, and editing.

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